REGULATION OF PROTEIN SYNTHESIS AND INITIATION FACTOR-2 IN TRANSLATING WHEAT GERM LYSATES

Thesis Submitted for the Degree of

DOCTOR OF PHILOSOPHY

By

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1996

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To my parents

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This is to certify that I, N. Janaki, have carried out the research work embodied in the present thesis in the Department of Biochemistry under the supervision of **Dr.** K. **V. A. Ramaiah,** for the full period prescribed under the Ph. D ordinance of the University.

I declare that no part of this thesis was earlier submitted for the award of research degree of any University.

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ACKNOWLEDGEMENTS

I do not have adequate words to express my gratitude to my supervisor, Dr. Kolluru V. A. Ramaiah, who impressed upon me the beauty of protein synthesis In general, and in particular, the role of eIF-2. The fact that this thesis has been completed is mainly because of his constant encouragement and close supervision of my work. He has been unfailingly courteous, helpful and a tremendous motivator. It was because of him that the Herculean task of research work never turned somber.

I place on record my gratitude to Prof. A. R. Reddy (Dean, School of Life Sciences), Prof. K. Subba Rao, FMS, (Head, Dept. of Biochemistry), Prof. N. C. Subrahmanyam, FNA, (former Dean) and Prof. T. Suryanarayana (former Head) for providing me with the necessary central facilities to carry out my research work. I also thank Prof. A. R. Reddy for tolerating my frequent visits to his lab.

I thank Dr. M. Ramanadham and Dr. N. Sivakumar for their constant encouragement

I wish to thank Prof. I. M London and Dr. Jane-Jane Chen from MIT, Cambridge, USA for generously providing me with purified HRI.

The help and encouragement that I received from Mr. V. M. Krishna with whom I have been associated for long, is ineffable. His 'helpful deeds' for my research work will be remembered by me with reverence.

I thank my labmates Mr. Naresh Babu, Ms. Thanuja, Mr. Sudhakar, Ms. Neelima and especially Mr. Kumar for the help extended to me and making my stay here cordial.

I wish to thank my friends Ms. Karuna, Ms. Madhuri, Mr. Bhaskar, Mr. Rehman and others, with whom I have spent many happy moments, for their constant support and insight that helped me tide over the tough moments.

It is beyond words to thank my parents. The love, affection, encouragement and admonition extended by them provided me succor and solace, and made me strong in my commitment to carry out my research work. I dedicate this thesis to my father, who till the last day he could speak, waited eagerly to see the thesis completed and my mother who has been ever available with everything and anything that I needed. But for the support from my parents, brother, sisters, brothers-in-law and other members of the family, I would not have succeeded in reaching this stage of life. I thank them all.

Finally, I acknowledge CSIR for having financially supported me for 5 years in the form of JRF and SRF.

janaki..

ABBREVIATIONS

ADP	-	Adenosine 5' diphosphate
Ala	-	Alanine
cAMP	-	Adenosine 3' 5' monophosphate
AMP	-	Adenosine monophosphate
AMV RNA	-	Alpha alpha mosaic virus mRNA
APS	-	Ammonium per sulfate
ATP	-	Adenosine 5' triphosphate
Bis-acrylamide	-	N, N'-methylene-bis-acrylamide
Ci	-	Curie
CHO	-	Chinese hamster ovary cells
CK-II	-	Casein Kinase II
CM-S	-	Carboxy methyl sephadex
CP	-	Creatine phosphate
CPK	-	Creatine phospho kinase
cpm	-	counts per minute
DMSO	-	Dimethyl sulphoxide
DNA	-	Deoxy ribonucleic acid
cDNA	-	Complementary deoxy ribonucleic acid
DAI/dsI/PKR	-	double stranded RNA activated inhibitor
DTT	-	dithiothreitol
EDTA	-	Ethylene diamine tetra acetic acid
EF	-	Elongation factor
eIF	-	Eukaryotic initiation factor
eIF-2	-	Eukaryotic initiation factor 2
eIF-2a	-	Alpha subunit of eukaryotic initiation factor 2
eIF-2(aP)	-	Phosphorylated alpha subunit in eIF-2

eRF	-	Eukaryotic releasing factor or termination factor
Fig.	-	Figure
Fru-l,6-BisP	-	Fructose 1, 6-diphosphate
GCN	-	General control non derepressable
GlcNAc	-	N-acetyl glucosomine
GDP	-	Guanosine 5' diphosphate
GEF/eIF-2B/RF	-	Guanine nucleotide exchange factor ofeIF-2 or reversing factor
GMP	-	Guanosine 5'monophosphate
GNE	-	Guanine nucleotide exchange
GSH	-	Glutathione (Reduced)
GSSG	-	Oxidized glutathione
GTP	-	Guanosine 5' triphosphate
HCR/HRI	-	Heme regulated inhibitor
HEPES	-	N-[2-hydroxyethyI] piperzine-N'-[2-ethane-sulfonic acid]
HIV	-	Human immuno deficiency virus
HSP	-	Heat shock protein
I-2	-	Inhibitor 2
H_2O_2	-	hydrogen peroxide
U	-	International Units
kDa	-	Kilodaltons
min	-	minutes
Mol. wt.	-	Molecular weight
NADH	-	Nicotinamide adenine dinucleotide, reduced
NADPH	-	Nicotinamide adenine dinucleotide phosphate, reduced
NDK	-	Nucleotide diphosphate kinase
NEM	-	N-ethylmaleimide
PAGE	-	Polyacrylamide gel electrophoresis
P11	-	phosphocellulose
pmol	-	pico moles
PP	-	Protein phosphatase
PQQ	-	Pyrroline quinoline quinone

$PQQH_2$	-	Pyrroline quinoline quinone, reduced
RF	-	Reversing factor; see GEF
RNA	-	ribonucleic acid
dsRNA	-	double stranded ribonucleic acid
mRNA	-	messenger RNA
rRNA	-	ribosomal RNA
tRNA	-	transfer RNA
BMV RNA	-	Brome mosaic virus RNA
Met-tRNAi	-	Initiator transfer RNA
RNasin	-	Ribonuclease inhibitor
S	-	Svedberg
-SH	-	Protein sulfydryl groups
SDS	-	Sodium dodecyl sulphate
Ser	-	Serine
TCA	-	Trichloro acetic acid
TEMED	-	N',N N,N', N'-tetramethyl ethyl-ethylene diamine
TLC	-	Thin layer chromatography
Tris	-	Tris (hydroxymethyl) amino methane
µ	-	micro

CHAPTER 1

INTRODUCTION

Almost everything that happens inside the cell involves a role for one or more proteins. Proteins provide structure, catalyze cellular reactions, and carry out a myriad of other tasks. Their central role in cells is reflected by the fact that most of the genetic information is ultimately expressed as protein. Each protein is specified by a segment of DNA called a gene that codes information specifying the sequence of amino acids. The regulation of gene expression is a critical component in regulating cellular metabolism, orchestrating and maintaining the structural and functional differences that exists in cells during development. Given the high energetic cost of protein synthesis, regulation of gene expression is essential if the cell is to make optimal use of the available energy.

Not all proteins are required always in the cells. For example, proteins involved in cellular differentiation or DNA repair are present only for a brief time while enzymes involved in central metabolic pathways are constantly required. Therefore gene expression is regulated in such a manner to suit the requirement of their products for the cell.

The various processes involved in making of a protein from the gene is shown in Fig. 1. As can be seen, the concentration of the protein in the cell can be controlled at any of the 6 major points indicated viz., synthesis of primary RNA transcript (transcription), maturation of RNA or post-transcriptional modification of mRNA, mRNA degradation, translation, post-translational modification of the proteins, or protein degradation. The major sites of regulation are transcription and translation. The present work basically deals with the regulation of gene expression in eukaryotes at the level of translation with emphasis on initiation involving eukaryotic initiation factor-2 (eIF-2).

Translation is the process in which the genetic information stored in the form of a chain of nucleotides (messenger RNA) is converted into a functional form, the protein. Translation by itself is a very elaborate process and requires a translational machinery consisting of numerous protein factors, enzymes, ribosomes, nucleotides (ATP and GTP), and RNA (tRNA and mRNA). The process, for convenience, is divided into three steps initiation, elongation and termination. The factors involved in the translation of eukaryotic systems at each step are designated as eukaryotic initiation factors (eIF's), elongation

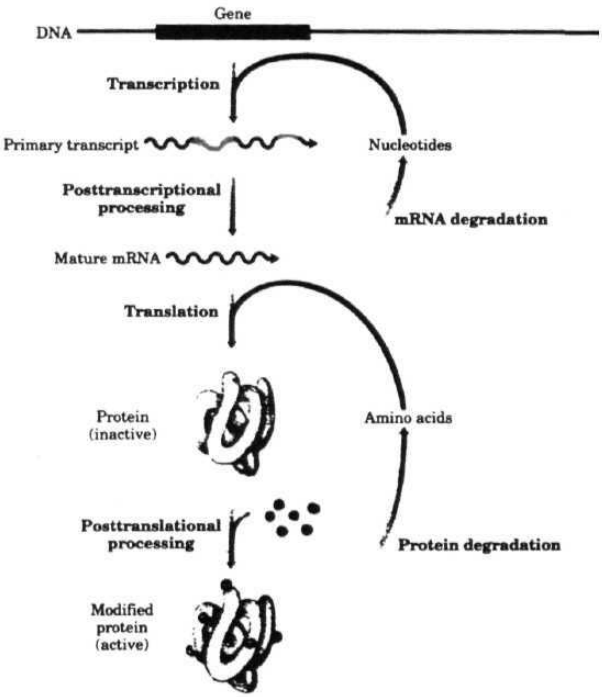


Fig. 1 **Six processes that affect the steady-state concentration of a protein. Each of these processes is a potential point of regulation.**

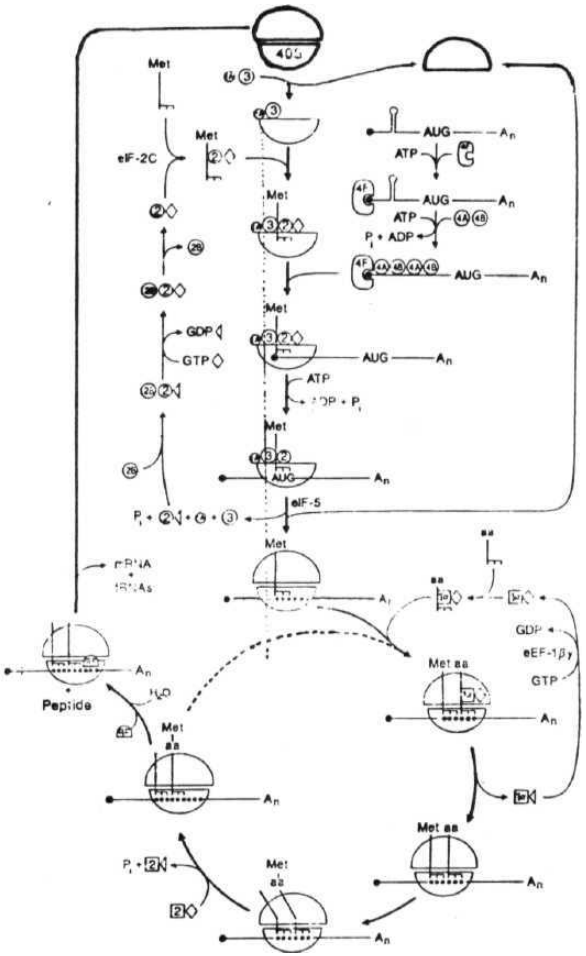


Fig. 2 Schematic representation of mammalian protein synthesis.

factors (eEF's) and termination factor(s) or the releasing factor(s) (eRFs) (Ochoa, 1983; Hershey, 1991).

Regulation of translation can occur at:

a) various stages of protein synthesis through the regulation of individual factors involved, or through the interplay of different factors (Hershey, 1989; Rhoads, 1993)

b) sequestration of mRNA's with proteins into a complex in which mRNA becomes inaccessible to the ribosomes. This kind of regulation is usually seen when the transcribed gene product is not of immediate use but has to be stored for later use eg. during development (Standart and Jackson, 1994).

c) selective regulation of certain mRNAs or subsets of mRNAs can occur because of structural features in the 5' and 3' sequences of mRNAs and/or mRNA binding proteins (Walden, 1993; Hershey, 1991; Jackson, 1991; Merrick, 1992; Redpath and Proud, 1994).

A schematic representation of the process of translation is illustrated in Fig 2. Each of the three phases of translation and regulation of protein synthesis in mammals is dealt with briefly in separate sections (1.1-1.5) of this chapter This is followed by a brief description of 'Translational control in plants' (section 1.6) and the objectives undertaken in the present work (1.7).

1.1 Initiation

The process of initiation starts with the dissociation of 80S monosome and culminates in the formation of 80S initiation complex with the initiator-tRNAi (Met-tRNAi) positioned at the correct AUG codon of mRNA on ribosome. The initiation step is the most complex of the three steps of protein synthesis, and can be further divided into the following substeps for easy understanding:

1.1.1 Dissociation of 80S ribosomes:

The monosomes (80S) and the subunits of ribosomes (60S and 40S) are present in equilibrium at physiological Mg^{2+} concentration with the equilibrium favouring the formation of 80S monosomes (Hershey 1991, Merrick, 1992; reviewed in detail in

Moldave, 1985). Therefore, substantial amount of free subunits are to be generated for the formation of 43 S complexes. Two initiation factors, eIF-3 and eIF-6, help in changing the equilibrium towards dissociation by binding the free subunits and preventing them from reassociation. eIF-3 is found associated with the smaller subunit (40S) of ribosome and this association is assisted by another factor eIF-4C (now called eIF-1A) (Goumans *et a!.*, 1980; Peterson *et al.*, 1979; Sonenberg, 1988; Benne and Hershey, 1978). The association of eIF-3 with 40S subunit is essential for the ternary complex to bind the ribosomal subunit. eIF-6 binds exclusively to 60S subunits (Raychaudhuri *et ah,* 1984; Russell and Spermulli, 1979) These initiation factors have been purified and characterized from reticulocytes (Benne and Hershey 1978; Schrier *et ai,* 1977; Raychaudhuri *et ai,* 1984) and wheat germ (Seal *et al.*, 1982; Russell and Spermulli, 1979). These factors which bind to the ribosomal subunits and prevent their association, are called anti-association factors.

1.1.2 Formation of ternary complex:

Ternary complex, as the name suggests, is formed by the interaction of three components, initiator tRNA (Met-tRNAi), eukaryotic initiation factor-2 (eIF-2) and the nucleotide GTP. eIF-2 is a heterotrimeric protein with a-, P-, and γ- subunits. This factor has binding motifs for GTP and Met-tRNA$_i$ (details will be dealt in section 1.5.2). The interaction of eIF-2 and Met-tRNAi is highly specific since eIF-2 does not recognize any other aminoacylated tRNA and Met-tRNAi is also specific for initiation since it does not participate in the elongation cycle (Moldave, 1985). This specificity is also in part influenced by the ribosomes (Schroer and Moldave, 1973). Binding of Met-tRNAi to eIF-2 occurs only when eIF-2 is bound to GTP i.e. formation of a binary complex between eIF-2 and GTP is essential for the ternary complex formation. The complex formation is also nucleotide specific since a binary complex formed between eIF-2 and GDP cannot bind Met-tRNAi (Walton and Gill, 1975). This specificity of nucleotide imparts the regulatory effect on the ternary complex formation. The ternary complex, eIF-2. GTP. Met-tRNAi then joins to 40S ribosomal subunit to form the 43S preinitiation complex.

1.1.3 Formation of 48S preinitiation complex; binding of 43S complex to mRNA:

Binding of 43 S preinitiation complex to mRNA requires the association of mRNA with certain initiation factors which help to localize mRNA on 40S ribosomes Unlike the small ribosomal subunit (30S) in prokaryotes, which have alignment capabilities for binding mRNA, the AUG codon, and the Shine-Dalgarno sequence (Merrick *et al,* 1990), eukaryotic mRNAs do not seem to have any specific sequence which can be recognized by the ribosomes. The initiation factors that mediate cap binding function are termed as Cap Binding Proteins (Edery *et al,* 1987). The formation of 48S preinitiation complex occurs in two stages: i) the factor bound to the cap of mRNA (m^7GpppN) interacts with the 43S preinitiation complex resulting in the binding of the later to the 5' end of the mRNA ii) the 40S ribosome subunit scans along the mRNA in a 5'→3' direction to locate the correct AUG codon, which serves as the start signal for peptide synthesis (reviewed in Kozak, 1992; Merrick, 1992; Hershey, 1991; Pain, 1986; Moldave, 1985).

1.1.3.1 Factors involved in the binding of mRNA to 40S subunit of ribosome:

Joining of the 43 S preinitiation complex to mRNA requires the participation of three eukaryotic initiation factors (eIF-4A, eIF-4B and eEF-4F) and ATP hydrolysis (Sonenberg, 1988). This is a multistep event which positions ribosome at the initiation codon (AUG). All eukaryotic cellular mRNAs (except organellar) contain a cap structure [$m^7G(5'ppp)(5'N)$; where N is any nucleotide] at their 5' terminus (Shatkin, 1985). The cap structure is a regulatory determinant of translational efficiency and functions to facilitate the attachment of the 43 S preinitiation complex to mRNA.

eIF-4F which mediates cap function (Tahara *et al,* 1981; Edery *et al.,* 1983; Grifo *et al,* 1983) is composed of three subunits: (i) a 24 kDa cap binding polypeptide called eIF-4E (Sonenberg *et al,* 1978), (ii) a 50 kDa polypeptide, eIF-4A which exhibits RNA-dependent ATPase and bi-directional RNA unwinding activities (Ray *et al,* 1985; Rozen *et al,* 1990) and (iii) a 220 kDa polypeptide, p220, whose integrity is required for eIF-4F activity in cap-dependent translation, as its cleavage following polio virus infection results in the shut down of host protein synthesis (Sonenberg, 1987).

In addition to the eIF-4F complex, eEF-4B protein (80 kDa) is found to stimulate the RNA-dependent ATPase and helicase activity of eIF-4A (Lawson *et al.* 1988, Rozen *et al.*, 1990; Abramson *et al.*, 1988).

1.1.3.2 The process of mRNA binding to 43S preinitiation complex:

The process occurs in stages with, first the eIF-4F recognizing the cap structure at the 5'end of mRNA (reviewed in Banerjee, 1980 Moldave, 1985; Pain, 1986, Hershey, 1991; Merrick, 1992). The eIF-4E component of eIF-4F binds to the cap via its cap recognition site. It is still unclear whether eIF-4E first binds to the cap *in vivo* as a monomer, as a part of the trimeric complex or as eIF-4E-p220 dimer (Lamphear and Panniers, 1990). This binding does not require the presence of eIF-4A or eIF-4B and is ATP independent (Grifo *et al,* 1984; Ray *et al,* 1985). The association between the cap structure and eIF-4F complex is stronger than that between cap and eIF-4E alone (Lee *el al.,* 1985). The translational efficiencies of different mRNA's correlate with the availability of m7G cap for interaction with eIF-4E subunit of eIF-4F (Godefroy-Colburn *et al,* 1985; Lawson *et al,* 1986).

The association of eIF-4F with cap is followed by the binding of eIF-4B, if it is not previously bound to eIF-4F (Grifo *et al,* 1983) and the unwinding of mRNA in the vicinity of the cap occurs (Lawson *et al.,* 1989, Ray *et al,* 1985) to facilitate the binding of 43 S ribosomal subunit complex. The unwinding of the distal part of mRNA requires eIF-4A, eEF-4B and the nucleotide ATP. The requirement of ATP in the 48S complex formation is mainly for the unwinding of mRNA (Jackson, 1991) though even the 'scanning' is also said to require ATP (Kozak, 1980). This suggestion is strengthened by the observation that mRNA's with less secondary structure are not dependent on ATP (Kozak 1980; Morgan and Shatkin, 1980; Sonenberg *et al,* 1981). There are many theories put forward to explain how the correct AUG is identified. The simplest of all is that the identification is done by the anti codon of the initiator tRNA and the first AUG encountered by the complex in the 3' region of the cap serves as the start signal (Kozak, 1989; Cigan, *et al,* 1988, 89, Donahue, *et al,* 1988). In certain cases it was observed that

initiation occurred at AUG codon other than the first. Studies by Kozak showed the presence of consensus sequences around AUG, A/GXXAUGG, which act as strong initiation sites (Kozak 1981, 84, 89). If two AUG codons are located in the preferred context of consensus sequences then initiation can occur at either of the sites resulting in the synthesis of two closely related proteins from a single monocistronic mRNA The presence of this consensus sequences is not ubiquitous as studies with many other eukaryotic systems has shown, but these studies also emphasize the preference for a purine at position -3 to AUG (Cavener and Ray, 1991).

The dependence of initiation on the secondary structure(s) in mRNA has also been investigated. It was suggested that AUG codon encountered by the complex during slow migration is utilized since the AUG crossed during fast scanning can be overlooked. This seems possible since any secondary structures in mRNA can hinder the migration of the scanning 40S subunit (Kozak, 1989a; Pelletier and Sonenberg, 1985). Studies of Pelletier and Sonenberg, (1985) have shown that insertion of hair pin loop in 5'UTR decreases the translational efficiency of mRNA. They have also shown that insertion of secondary structures in different regions of mRNA leads to decrease in initiation by different mechanisms, for example, an insertion very close to the 5' terminal cap appears to interfere with the interaction of eIF- 4B with cap structure while insertions further from the 5' end effects the migration of 43S subunit during scanning The role of secondary structures is also supported by the finding that eukaryotic mRNA lacks secondary structures in 5' UTR but contains extensive secondary structures in the coding region (Merrick, 1992). In addition to eIF-4E and eIF-4B, eIF-2 factor associated with the 43S complex may also assist to locate the initiation codon (Dasso *et ed.,* 1990).

1.1.4 Formation of the 80S initiation complex:

The 60S ribosomal subunit joins the 48S initiation complex to form the 80S initiation complex. This forms the last step of initiation. The formation of 80S initiation complex requires the release of 'anti-association' factors like eIF-3, eIF-4C and eIF-6. The GTPase activity associated with eIF-5 permits the hydrolysis of GTP in the ternary complex and releases the bound initiation factors (Maitra *et al.*, 1982). eIF-2 present in the

ternary complex is released here as eIF-2.GDP. Many models (Ochoa, 1983; Watson *et al*, 1987; Hershey, 1991; Merrick, 1992) indicate that eIF-2 GDP binary complex is released before the 80S initiation complex formation However, recent evidence (Thomas *et al*, 1985; Ramaiah *et al.*, 1992) suggests that eIF-2 GDP is translocated to the 60S subunits of the 80S initiation complexes and is released from there probably depending on the availability of a rate limiting multimeric protein factor called eIF-2B (please see below). A new model has also been presented to explain the presence of eIF-2 on 60S subunits of 80S initiation complex and on the recycling of eIF-2 (Ramaiah *et al*, 1992; Altman and Trachsel, 1993).

1.1.5 Recycling of eIF-2.GDP:

The eIF-2.GDP binary complex that is released at the end of initiation, can enter another initiation cycle only when the GDP in binary complex is replaced by GTP. This is critical since GDP inhibits the joining of Met-tRNA$_i$ to eIF-2. The replacement of GDP by GTP is catalyzed by guanine nucleotide exchange activity present in the eIF-2B protein factor. This factor enjoyed several names in literature. Previously it was known as Co-eIF-2C (Majumdar *et al.*, 1977; Das *et al*, 1979; Das *et al*, 1982), ESP (DeHaro *et al*, 1978) SF (Ranu and London, 1979), SP (Siekierka *et al*, 1981), GEF (Siekierka *et al*, 1982, 1983; Panniers and Henshaw, 1983, Goss *et al*, 1984), eIF-2B (Koniecizny and Safer, 1983; Safer 1983), eRF (Salimans *et al*, 1984), RF (Matts *et al*, 1983) and anti-heme regulated eIF-2α kinase (Amesz *et al*, 1979). This factor with five subunits (α,β, *y*, 8, and e) is often found to migrate with eIF-2 (Matts *et al*, 1983). In addition to exchanging guanine nucleotides on eIF-2 as mentioned above, eIF-2B protein helps to release eIF-2 from the 60S subunits of 80S initiation complexes (Thomas *et al*, 1985); thereby suggesting that it may have several activities critically important in the recycling of eIF-2 GDP (Ramaiah *et al*, 1992).

1.2 Elongation and Regulation:

During the elongation step in protein synthesis, the respective amino acids brought by the aminoacylated tRNAs are added to the template depending on the

sequence of nucleotides. Peptide bond formation then occurs between adjacent aminoacids (amino acid at the *A'* site and growing peptide at the 'P' site of the ribosome) and the ribosomes move by three bases towards the 3' end of mRNA. The whole process is essentially aided by two protein factors EF-1 and EF-2 (elongation factors). The binding of cognate aminoacyl-tRNA molecules to the 'A' site is mediated by elongation factor 1 (EF-1) EF-1 is a heterotrimeric protein containing three subunits of a, β and y. EF-1 a is equivalent to prokaryotic EF-Tu and helps in binding of the aminoacyl-tRNAs to ribosomes. EF-1β and γ-subunits are equivalent to EF-Ts in prokaryotes and assist in the recycling of EF-1 a by exchanging GTP for GDP in EF-1 a GDP. With the help of peptidyl transferase enzyme, presumably located on the large subunit of ribosomes, a peptide bond is formed between adjacent amino acids. Afterwards, the elongation factor 2 (EF-2) hydrolyzes GTP and catalyzes the translocation of aminoacyl-tRNA from 'A' site to the 'P' site on the ribosome with concomitant movement of the message. EF-2 corresponds to prokaryotic EF-G (Watson *et al,* 1987). EF-1 is methylated and phosphorylated (Janssen *et al,* 1988). The degree of methylation alters activity of the factor (Fonzi *et al,* 1986) while the role of phosphorylation is unknown. EF-2 is phosphorylated *in vitro* and *in vivo* by a Ca^{+2} / Calmodulin dependent protein kinase (Narin *et al,* 1987; Ryazanov *et al.,* 1987, 88). Enhanced phosphorylation of EF-2 inhibits protein synthesis (Carlberg *et al,* 1990; Redpath and Proud, 1989). Phosphorylation of EF-2 impairs its translocation activity rather than its inability to bind GTP and ribosomes (Ryazanov and Davydova, 1989).

Apart from phosphorylation, EF-2 is covalently modified by ADP-ribosylation. This ADP-ribosylation occurs on a modified histidine residue (diphthamide) which renders EF-2 inactive (Lee and Iglewski, 1984). Yeast EF-2 in which His-699 (diphthamide) is replaced, retains its activity since it becomes resistant to ADP-ribosylation but the yeast becomes temperature sensitive. This indicates that the presence of diphthamide some how confers heat resistance to EF-2 (Kimata and Kohno, 1994).

1.3 Termination :

Newly made polypeptide is released during this step when the ribosome reaches the stop codon. The releasing factor (RF), catalyzes this termination, in the presence of GTP (Caskey, 1977). Unlike in prokaryotes, a single releasing factor can recognize all the three stop codons in eukaryotes.

1.4 Nucleotide requirement in protein synthesis pathway:

Two nucleotides, ATP and GTP, are required for efficient translation. Hydrolysis of ATP provides necessary energy for the various reactions while hydrolysis of GTP induces conformational changes in the protein factors and ribosomes which facilitate attachment and detachment of specific factors to ribosomes (Spirin, 1986). While ATP is required for aminoacylation of tRNA's and the formation of 48S preinitiation complexes, GTP is required in initiation, elongation and termination as well.

Nucleotides also exert a regulatory effect on protein synthesis. Evidence has been provided by correlation's between small changes in the adenine nucleotide balance and the changes in the rate of protein synthesis in various systems (Lyons *et al.*, 1980; Rupniak and Quincey, 1975). The inhibition in protein synthesis under decreased energy charge was shown primarily to be due to inhibition in initiation in rat thymus cells (Mendelsohn *et al*, 1977). In reticulocyte lysates, evidence has been provided to show that changes in the energy charge can influence both initiation and elongation (Rupniak and Quincey, 1975). A possible mechanism for the nucleotide regulation of protein synthesis was given by Walton and Gill (1975; 76) and Proud (1986) (dealt in greater detail in the next sections). In Ehrlich cell lysates, it has been demonstrated that the initiation of protein synthesis is regulated by the ratios of nucleoside diphosphates to triphosphates (Hucul *et al*, 1985).

1.5 Regulation of Initiation:

Since the process of protein synthesis involves complex sequence of reactions, as mentioned above, it is natural for the cells to exert control at the first step of reaction, that is, at initiation. Translational regulation can be either for individual proteins or especially

for global protein synthesis through reversible covalent modifications of initiation factors and other components.

Phosphorylation of the protein factors is considered to be a principle means of translational regulation in eukaryotes (reviewed in Hershey, 1989; Proud, 1992; Rhoads, 1993). Two important rate-limiting factors whose phosphorylation is known to affect the regulation of protein synthesis in mammalian systems have been very well characterized These are eIF-2 and eIF-4E. Interestingly, enhanced phosphorylation of eIF-4E enhances translational activity of several mRNAs. In contrast, enhanced phosphorylation of the small subunit of eukaryotic initiation factor 2 (eIF-2 alpha) down regulates protein synthesis (Hershey, 1989). Whether a common cellular signal mediates these two events of phosphorylation is not yet known. But it is quite likely that an equilibrium in the phosphorylation of these two factors may also regulate gene expression and translation.

1.5.1 Regulation of protein synthesis at the level of eIF-4:

eIF-4E plays a key role in the regulation of translation (Hershey, 1991). It is present in limiting quantities in the cell (Hiremath *et al,* 1985; Duncun *et al,* 1987), consistent with a regulatory role in translation. There exists also a strong correlation between the phosphorylation state of eIF-4E and the rates of protein synthesis and cell growth. Increased eIF-4E phosphorylation occurs in response to growth factors, mitogens and cytokines (Morley and Traugh, 1989; Frederickson *et al,* 1991, 92; Kasper *et al,* 1990, Donaldson *et al,* 1991). eIF-4E is hypophosphorylated during mitosis (Bonneau and Sonenberg, 1987), following heat shock (Duncun *et al,* 1987) or infection with several viruses (Huang and Schneider, 1991; Feigenblum and Schneider, 1993) with a concomitant reduction in translation rates. Interestingly these conditions which reduce eIF-4E phosphorylation can enhance eIF-2 phosphorylation. However the connection, if any, between the phosphorylation states of these two proteins is not yet understood. Also the mechanism by which eIF-4E phosphorylation enhances translation is not well understood but, eIF-4E phosphorylation enhances its binding to the cap structure (Minich *et al,* 1994). Overexpression of eIF-4E results in transformation (Lazaris-Karatzas *et al,* 1990; Lazaris-Karatzas and Sonenberg, 1992) in rodent cells and deregulates HeLa cell

growth (De Bennedetti and Rhoads, 1990). Consistent with the transforming activity, eIF-4E is mitogenic, as its microinjection into quiescent NIH3T3 cells activates DNA synthesis (Smith *et al,* 1990). The microinjection of eIF-4E mRNA into early Xenopus laevis leads to mesodenn induction (Klein and Melton, 1994). One possible explanation for the transforming activity of eIF-4E is that its overexpression results in active eIF-4F complex, and hence increased unwinding activity and mitigation of the translational repression of the growth promoting genes that are important for the control of cell growth. Indeed enhanced or increased expression of cyclin D1 (Rosenwald *et al,* 1993), ornithine decarboxylase (Shantz and Pegg, 1994) and c-myc (De Benedetti *et al,* 1994) has been demonstrated in cells overexpressing eIF-4E.

Recent studies indicate that eIF-4E activity is also modulated by two specific binding proteins (BP) termed 4E-BP1 and 4E-BP2. These proteins have a high sequence homology to heat and acid stable protein, PHAS1 (Hu *et al,* 1994). 4E-BP1 is also a heat and acid stable protein, which is phosphorylated by mitogen activated protein (MAP) kinase on serine (ser) 64 in response to insulin and growth factors that signal through MAP kinase pathway (Lin *et al,* 1994). The association of 4E-BP1 with eIF-4E decreases exclusively the translation of capped but not uncapped mRNA's *in vitro* and in cultured cells (Pause *et al,* 1994). However, this interaction is diminished dramatically upon phosphorylation of 4E-BP1 in response to insulin, concomitant with the relief of translational repression of capped mRNA's. These findings while provide a basis for understanding the enhancement of eIF-4E activity and specific stimulation of cap-dependent translation following insulin treatment (Manzella *et al,* 1991, Gallie and Traugh, 1994), these observations also indicate a key role for 4E-BPs in the regulation of protein synthesis and cellular growth and differentiation.

The phosphorylation site in eIF-4E was initially thought to be Ser-53 (Rychlik *et al.,* 1987). The Ser-53 is important for physiological activity of the factor as evidenced by the inability of the mutant factor, in which Ser-53 is replaced by alanine, to participate in many of its biological functions (Lazaris-Karatazas *et al,* 1990; De Beneditti and Rhoads, 1990; Joshi-Barve *et al,* 1990, Koromilas *et al,* 1992; Rosenwald *et al,* 1993). It has

been shown that the major site of phosphorylation in eIF-4E is not Ser-53 but Ser-209 (Bhavesh-Joshi *et al*, 1995; Flynn and Proud, 1995) but this does not disprove the importance of Ser-53 in other activities of eIF-4E since the Ser-53 mutant fails to carry out the functions of wild type eIF-4E (Bhavesh-Joshi *et al*, 1995; Flynn and Proud 1995).

Translation of capped messages is also regulated by the activity of p220 (or eIF-4G) During viral infection the host cell p220 is proteolytically cleaved by the viral encoded proteases which leads to the inhibition of translation of capped messages (Etchison *et al*, 1982; Llyod *et al*, 1987). Under these conditions the translation of the uncapped messages is enhanced. The viral proteases cleave p220 into two fragments, a N-terminal fragment which has the binding site for eIF-4E and a C-terminal fragment containing the binding site for eIF-3 and eIF-4A. Since only the C-terminal fragment is necessary for internal initiation, as seen in uncapped messages, translation of these mRNA's continue but as the N-terminal fragment contains only cap binding domain and therefore cannot bind ribosomes, it fails to support the translation of capped messages (Lamphear *et al.*, 1995).

1.5.2 Regulation of translation initiation through el F-2:

1. *S.* ***2.1 eIF-2*** *structure, function, phosphorylation and regulation:*

Mammalian eEF-2 is a trimeric protein with three nonidentical subunits, a (~38 kDa), β (~50 kDa) and *y* (~52 kDa). Molecular weight of the p-subunit of eIF-2 has been variously reported as 36-55 kDa based on its migration in different gel systems (Colthurst and Proud, 1986; Lloyd *et al*, 1980, Meyer *et al*, 1981; Panniers and Henshaw, 1983). This difference in migration of the eIF-2p subunit in different gel systems used may be due to the presence of large blocks of lysine residues in the subunit which can alter its electrophoretic mobility (Pathak *et al*, 1988a). In certain cases, a two subunit preparation of eIF-2 has been reported (Ochoa, 1983) in which the P-subunit was missing. The disappearance of p-subunit in these preparations may be due to the proteolytic cleavage and subsequent loss of this sensitive subunit during purification or due to incomplete resolution of the P- and γ-subunits on the gel (Moldave, 1985).

eIF-2 is involved in bringing Met-tRNA$_i$ to 40S ribosomes The joining of eIF-2 with initiator tRNA requires GTP. The cDNA cloning and sequencing of subunits has helped in partially elucidating their structure and function in yeast and mammals (Ernst *et al,* 1987, Pathak *et al.,* 1988a; Gaspar *et al,* 1994). Both eIF-2|3 and eIF-2Y subunits appear to be involved in binding guanine nucleotides Various findings (Kurzchalia *et al,* 1984; Anthony *et al,* 1987 & 1990, Bommer *et al,* 1988, Bommer and Kurzchalia, 1989, Dholakia *et al,* 1989) indicate that guanine nucleotide binding site in eIF-2 is 'shared' between these two subunits. However, even the two-subunit eIF-2 preparations lacking the P-subunit, have been also shown to bind guanine nucleotides and mediate the GTP dependent translocation of Met.tRNAi to the 40S ribosomal subunit (Chaudhuri *et al,* 1981; Colthurst and Proud, 1986). This may be possible since preparations of eIF-2 which appear to be devoid of P-subunit, as judged by SDS-PAGE, may still contain fragments of P-subunit bound to a- and y-subunits by non-covalent forces (Kimball *et al,* 1987). The studies with cDNA encoding human eIF-2P and γ-subunits (Pathak *et al,* 1989; Gaspar *et al,* 1994) suggest that DXXG and NKXD, consensus elements for GTP binding, are present in both p- and γ-subunits, thereby reinforcing the possibility that both the subunits might be involved in GTP binding.

Also affinity labeling of eIF-2 with GTP derivatives occurs in both P- and y-subunits (Anthony *et al,* 1990; Bommer *et al,* 1988). But mutational studies, in which the asparagine (Asn) residue in the NKXD consensus sequences of γ-subunit of eIF-2 is altered, it has been shown that protein synthesis is strongly inhibited. In contrast, alteration in corresponding Asn residue in the P-subunit causes little change in protein synthesis compared to the wild type subunit. This result supports the view that GTP binding requires NKID element in eIF-2y but does not involve NKKD element in eIF-2P (Naranda *et al,* 1995). A possible explanation for the above conflicting observations could be that eIF-2p lies very close to the GTP binding site in the G-domain of eIF-2y (Naranda *et al.,* 1995).

eIF-2 also has the ability to bind mRNA and initiator-tRNA. eIF-2 recognizes a stem loop structure in the 5'end of mRNA, deletion of which decreases binding of eIF-2

to mRNA and also causes 2 fold reduction in protein synthesis (Liarakos *et al.*, 1994) suggesting that interaction of eIF-2 with mRNA may be functionally important. In *Saccharomyces cerevisiae,* two mutations which restore translation of mRNAs in which the start AUG codon is altered to UUG (termed SU12 and SU13) map to the a- and P-subunits of eIF-2 respectively (Cigan *et al.,* 1989, Donahue *et al.,* 1988). This suggests that eIF-2 plays an important role in correct selection of initiation site during scanning Another investigation by Dasso *et al.,* (1990) suggests that, in mRNAs containing two possible start codons of differing contexts, eIF-2 influences the choice of start codon. This again points to a role for eIF-2 in the selection process. Kaempfer's group presented evidence that competition between different mRNA species is relieved by excess eIF-2, and that there is a positive correlation between the ability of mRNA to compete in translation and its ability to bind eIF-2 (Di Segni *et al.,* 1979; Kaempfer *et al.,* 1981; Rosen *et al.,* 1981 & 82; Kaempfer and Konijn, 1983; Kaempfer, 1984). Also, the findings of Gupta and co-workers suggest that the presence of mRNA or trinucleotide AUG stimulates the eIF-2 mediated transfer of Met-tRNAj to 40S ribosomal subunit (Roy *et al.,* 1981, 84, 88; Chakravarty *et al.,* 1985). These observations substantiate the importance of eIF-2 interaction with mRNA.

Kaempfer's group has also reported that different functional sites on eIF-2 are involved in interactions with initiator tRNA and GTP on one hand, and mRNA on the other. Such a conclusion was reached based on differing abilities of different anti-eIF-2 antibodies to inhibit these binding functions of eIF-2 (Harary and Kaempfer, 1990). Also the eIF-2P subunit has structural features which can interact with mRNA in the form of lysine blocks and zinc finger structure (Pathak *et al.,* 1988a). The p-subunit also has an ATP binding site which may influence the ability of eIF-2 to bind mRNAs (Gonsky *et al.,* 1990). Further, a preparation of eIF-2 lacking the P-subunit is unable to modify the start site selection, again, suggesting a role for eIF-2p in interacting with mRNA and participating in the start codon selection.

Similar to mammalian eIF-2, plant eIF-2 can also interact with mRNA (Browning *et al.,* 1995) but the functional importance of this binding is still unclear.

Barrieux and Rosenfeld (1977) have shown that low concentrations of GDP can cause inhibition in ternary complex formation. The affinity of mammalian eIF-2 towards GDP is higher than for GTP (Proud, 1992). eIF-2 is released as eIF-2 GDP during the process of initiation (Peterson *et al*, 1979, Raychaudhuri *et al*, 1985) or eIF-2 can form binary complex with free GDP, which is stabilized in the presence of Mg^{+2} (Watson and Gill, 1975; 76) and this GDP has to be exchanged for GTP, for eIF-2 to enter the initiation cycle (Merrick, 1992). It has been found that nearly 50% of the purified eIF-2 is bound to GDP (Siekierka *et al*, 1983). Purified preparation of eIF-2 can join $Met\text{-}tRNA_i$ in the absence of Mg^{+2}. But the presence of Mg^{+2} inhibits the joining reaction (Bagchi *et al*, 1985).

Findings from other laboratories, which are consistent with the above observations, indicate that conversion of GDP to GTP by nucleoside diphosphate kinase in the presence of energy regenerating systems like phosphocreatine/creatine kinase and by enzymes which hydrolyze GDP to GMP and pi have been shown to stimulate eIF-2 activity, that is, its ability to form $eIF\text{-}2.GTP.Met\text{-}tRNA_i$ complex (Walton and Gill, 1976, Benne *et al*, 1979, Clemens *et al.*, 1980).

Later several protein factors like Co-eIF-2A, Co-eIF-2B and Co-eEF-2C (reviewed in Gupta *et al*, 1987), anti-heme regulated eIF-2α kinase (Amesz *et al*, 1979), guanine nucleotide exchange factor (Siekierka *et al*, 1982, 83; Gross *et al*, 1984; Panniers and Henshaw, 1983), eIF-2B (Konieczny and Safer, 1983) and reversing factor (Matts *et al*, 1983) were purified based upon their ability to stimulate the eIF-2 activity *in vitro*. Subsequently most of these preparations are found to contain a high molecular weight protein which can exchange GDP in eIF-2.GDP for GTP and is physiologically important, regulating eIF-2 activity. The factor is now designated as eIF-2B (please see section 1.5.2.2 dealing with the regulation of eIF-2 by eIF-2B).

eIF-2 protein, as mentioned above, binds to GDP and is also a phosphoprotein. Hence its activity is regulated by eIF-2B guanine nucleotide exchange factor, eIF-2 kinases and phosphatases.

Phosphorylation of **eIF-2:**

The a- and β-subunits of eIF-2 are phosphorylated both *in vitro* and *in vivo* by cyclic-AMP independent kinases. Phosphorylation of mammalian β-subunit occurs by multipotential kinase casein kinase II (CK-II), but the functional significance of this phosphorylation, if any, remains unknown (Proud, 1992).

In contrast, the phosphorylation of the a-subunit (38 kDa) of reticulocyte eIF-2 occurs under several biotic and abiotic conditions such as absence of heme (Levin *et al,* 1976; Kramer *et al,* 1976; Farrell *et al.*, 1977), presence of dsRNA (Farrell *et al.,* 1977), amino acid starvation (Wek, 1994), heat-shock (Duncan and Hershey, 1984, Clemens *et al.,* 1987, Murtha-Riel *et al.,* 1993), serum deprivation (Duncan and Hershey, 1985) and calcium deprivation (Preston and Berlin, 1992; Prostko *et al.,* 1992; Srivastava *et al.,* 1995) and, treatment with N-ethylmaleimide (Chen *et al.,* 1989), oxidized glutathione (Ernst *et al.,* 1979; Kan *et al.,* 1988), heavy metal ions (Matts *et al.*, 1991) and O-iodoisobenzoate (Gross and Rabinowitz, 1972).

Phosphorylation of the a-subunit of eIF-2 in translational control is well established With the development of cell-free protein synthesis systems (Zucker and Schulman, 1968; Adamson *et al.*, 1968), it became possible to study the regulation of translation in greater detail. In the absence of added hemin, protein synthesis in heme-deficient reticulocyte lysates continues for the first 5-10 minutes followed by an abrupt decline in the rate of synthesis (shut-off). Addition of hemin permits protein synthesis to continue for 60-90 minutes (Adamson *et al.,* 1968; Hunt *et al.,* 1972) and delayed addition of hemin can restore protein synthesis (Adamson *et al.*, 1969). Besides heme-deficiency, protein synthesis in actively translating cell-free systems is shown to be also inhibited by the addition of dsRNA (Ehrenfeld and Hunt, 1971) and by oxidized glutathione, GSSG (Kosower *et al.*, 1973). Also the gel-filtered lysates devoid of small molecular weight compounds show similar inhibition (Ernst *et al.*, 1978; Lenz *et al.*, 1978, Jackson *et al.*, 1983). In all these situations, protein synthesis proceeds at control rates for a few minutes before an abrupt decline in translation to a low rate of the control. This is preceded by the disappearance of Met.tRNAi/native 40S subunit complexes (Darnbrough *et al.*, 1972; Legon *et al.*, 1973). The inhibition can be overcome by the

addition of a relatively large amount of eIF-2 (Kaempfer, 1974; Clemens *et al,* 1975) and also by the addition of 5 mM 3',5'-cAMP, 2 aminopurine, caffeine and other related compounds (Legon *et al,* 1974; Ernst *et al,* 1976).

Absence of hemin or the addition of dsRNA activates inhibitors of translation (Maxwell *et al,* 1971; Hunter *el al,* 1975). The inhibitor which forms in the absence of hemin is called heme controlled repressor (HCR) or heme regulated inhibitor (HRI) and the inhibitor that is formed by the addition of low concentrations of dsRNA (1-100 ng/ml) or polyIC (100-500 ng/ml) is called dsI or DAI or PKR (dsRNA activated/induced inhibitor). Paradoxically, higher concentrations of dsRNA (>10 μg/ml) fail to inhibit protein synthesis (Hunter *et al,* 1975). The inhibitions are reversed by the addition of hemin or higher concentration of dsRNA respectively. Afterwards, it has been shown that HRI contains a protein kinase activity that can phosphorylate eIF-2 (Levin *et al,* 1976; Kramer *et al,* 1976). Later studies have shown that both HRI and PKR contain a protein kinase activity that can phosphorylate the small subunit of eIF-2 and inhibit protein synthesis (Levin *et al,* 1976; Farrell *et al,* 1977). In addition to HRI and PKR, recent studies have shown that amino acid starvation in yeast can lead to the activation of GCN2 kinase which phosphorylates yeast eIF-2a (Hinnebusch, 1994). The above three kinases (HRI, PKR and GCN2) have been very well characterized (Samuel, 1993).

The site of phosphorylation of eIF-2a *in vitro* (Colthurst *et al,* 1987) by HRI / PKR or in translating lysates (Price and Proud, 1990), is a single serine residue, the Ser-51. No second site of phosphorylation is observed even when lysates are supplemented with potent protein phosphatase inhibitors like microcystin (Price *et al,* 1991). Suzuki and Mukuoyama (1988) have also shown that purified eIF-2 from pig liver is also phosphorylated at this site (Ser-51) by HRI. Earlier evidence by Wettenhall *et al* (1986) suggested that Ser-48 in eIF-2a was the site which was phosphorylated by HRI, although their subsequent work showed that HRI phosphorylates only the equivalent Ser-51 residue in a synthetic peptide (Kudlicki *et al,* 1987).

With the help of amino acid sequencing (Colthurst *et al,* 1987), site specific mutagenesis (Pathak *et al,* 1988b), mutational studies (Vazquez de Aldana *et al,* 1993)

and through overexpression of mutant and wild type eIF-2a, it has been demonstrated that the phosphorylation of Ser-51 residue in wild type eIF-2a impairs protein synthesis (Kaufman *et al.*, 1989; Choi *et al.*, 1992; Murtha-Reil *et al.*, 1993) and eIF-2B activity *in vivo* (Ramaiah *et al.*, 1994).

Mutants of eIF-2a and PKR kinase have helped in demonstrating that calcium depletion from the endoplasmic reticulum activates PKR and the inhibition of protein synthesis is confirmed to be mediated by eIF-2a phosphorylation (Srivastava *et al.*, 1995). Since some mutants of eIF-2a can bypass the protein synthesis inhibition caused by endogenous wild type eIF-2a phosphorylation, they are also found to be helpful in overexpressing the eIF-2α kinases whose expression is otherwise inhibitory to protein synthesis (Chefalo *et al.*, 1994).

Studies by Chen *et al.*, (1991a) involving HRI cDNA coding sequence have shown that there exists extensive homology between HRJ and GCN2 protein kinase of yeast, and to human PKR. In addition, HRI has an unusual high degree of homology with three protein kinases, NimA, Wee1 and CDC2, that are involved in the regulation of cell cycle (Chen *et al.*, 1991b). HRI cDNA contains a unique insertion sequence of approximately 140 amino acids located between 5th and 6th domain. Both HRI and GCN2 have a much longer kinase insertion sequence than PKR, although it is possible that part of the insertion sequence is involved in the binding of heme and in the regulation of autokinase and eIF-2a kinase activities. The insertion sequence may be involved in the interaction with other proteins or with regulators (Chen, 1993). Amino acid sequence of GCN2 is found closely related to histidine tRNA synthetase of yeast, human and E.coli and is required for the translational activation of GCN4 (Hinnebusch, 1988).

Mammalian eIF-2α kinases can also phosphorylate insect and yeast cell eIF-2α and regulate the insect cell protein synthesis and GCN4 translation in yeast (Dever *et al.*, 1993).

1.5.2.2 Regulation of eIF-2 by eIF-2B:

The cessation of protein synthesis that occurs upon eIF-2a phosphorylation has been well studied in mammalian systems and it is not due to a direct effect of phosphorylation. This is because just 20-30% phosphorylation of eIF-2a is sufficient to cause maximum or complete inhibition of protein synthesis suggesting that there is yet another rate-limiting factor which is affected under these conditions (Leroux and London, 1982). Eventually the factor involved, eIF-2B, has been identified and purified eIF-2B catalyzes the exchange of GDP in eIF-2 GDP for GTP. eIF-2B is a pentameric protein with five subunits viz., a- (34 kDa), β- (40 kDa), *y*- (55 kDa), 8- (65 kDa) and e- (82 kDa) (Matts *et al.*, 1983).

Phosphorylation of eIF-2a reduces the guanine nucleotide exchange activity of eIF-2B *in vitro* (Clemens *et al.*, 1982). The affinity of eIF-2B for eIF-2(aP) GDP is higher than for eIF-2 GDP (Rowlands *et al*, 1988a). So eIF-2B is trapped in a 15S complex [eIF-2(αP).GDP.eIF-2B] in which eIF-2B becomes non-functional (Thomas *et al.*, 1984). Since eIF-2B is less abundant than eIF-2 (l/10th of eIF-2), a small increase in eIF-2α phosphorylation is proposed to sequester all of the available eIF-2B and prevent the recycling of eIF-2 (Thomas *et al.*, 1984; Ramaiah *et al.*, 1994).

An assay system was initially developed by Matts and London (1984) to study the correlation between eIF-2B activity and protein synthesis in reticulocyte lysates or extracts. This system measures the release of labeled GDP or exchange of labeled GDP in the preformed eIF-2[^{3}H]GDP, binary complex Conditions such as heme-deficiency, addition of dsRNA or oxidized glutathione which inhibit protein synthesis, are also found to inhibit eIF-2B activity while simultaneously enhancing eIF-2a phosphorylation in reticulocyte lysates. This assay system is also used to correlate the inhibition in protein synthesis with reduction in eIF-2B activity in cells under different physiological stresses (Kimball and Jefferson, 1990; Prostko *et al.*, 1992; Rowlands *et al.*, 1988b). More recently, this assay system has been used to measure the rapid activation of eIF-2B in insulin and growth hormone treated Swiss 3T3 fibroblasts (Welsh and Proud, 1992),

inactivation of eIF-2B in insect cells resulted due to the expression of mammalian recombinant eIF-2α kinase (Chefalo *et a/.*, 1994) and also in evaluating the overexpression of wild type and mutants of eIF-2a subunits in rescuing the inhibition in eIF-2B activity of Chinese hamster ovary (CHO) cells that is mediated by eIF-2ct phosphorylation (Ramaiah *et al.*, 1994). The latter study reveals that overexpression of mutants of eIF-2a subunits, in which Ser-48 and Ser-51 are replaced by alanine (Ala-48 or Ala-51 mutants), rescues eIF-2B activity in inhibited heat-shocked CHO cells. The phosphorylation of Ser-51 in wild type eIF-2α impairs the eIF-2B activity. This study proposes that the hydroxyl group of Ser-48 acts to maintain a higher affinity between phosphorylated eIF-2a and eIF-2B and thereby inactivating eIF-2B These findings suggest that phosphorylation of eIF-2a inhibits protein synthesis by reducing eIF-2B activity and also emphasizes the importance of Ser-48 and Ser-51 in the interaction with eIF-2B and in the regulation of eIF-2B activity.

Various models have been proposed to explain the eIF-2B catalyzed dissociation of bound GDP in the binary complex (Pain, 1986). The eIF-2(aP).GDP is regarded as a competitive inhibitor of eIF-2B competing with eIF-2.GDP and the magnitude of difference is so large that under physiological conditions this type of competitive inhibition could efficiently mimic sequestration (Rowlands *et al.*, 1988a). Apart from phosphorylation of eIF-2a, phosphorylation of the 82 kDa (e) subunit of eIF-2B by CK-II (Dholakia and Wahba, 1988) and changes in redox levels can also regulate the guanine nucleotide exchange activity of eIF-2B (Dholakia *et al.*, 1986; Akkaraju *et al.*, 1991). The phosphorylation of e-subunit of eIF-2B by CK-II is associated with an increase in the guanine nucleotide exchange activity of the factor (Dholakia and Wahba, 1988). This finding suggests that E-subunit of eIF-2B is apparently associated with the guanine nucleotide exchange activity. The functions of other subunits however are not clear.

Recent data suggest that eIF-2B may be involved in the release of eIF-2(αP).GDP from the 60S subunits of 80S initiation complexes (Thomas *et al*, 1985; Ramaiah *et al.*, 1992). It is important to note that CK-II appears to be under acute regulation of hormones (insulin) and other growth factors (EGF) (Ackermann and Osheroff, 1989,

Carroll and Marshak, 1989). Activation of CK-II, which in turn leads to the activation of eIF-2B, may provide a mechanism by which insulin stimulates recycling of eIF-2 and peptide chain initiation. Spermidine, a polyamine, has been reported to activate partially purified, but not highly purified eIF-2B (Wahba and Dholakia, 1991; Gross *et al*, 1991) suggesting that a factor which confers sensitivity to polyamines is removed. Since CK-II is activated by polyamines, it is conceivable that it is the activation of contaminating CK-II and consequent phosphorylation of eIF-2B that accounts for the stimulation of eIF-2B activity by spermidine. Not only polyamines, but other ligands like $NADP^+$, NADPH, ATP and heparin can also modulate eIF-2B activity (Dholakia *et al*, 1986; Kimball and Jefferson, 1995; Singh *et al*, 1995; Oldfield and Proud, 1992, Akkaraju *et al.*, 1991). An enhanced $NADPH/NADP^+$ ratio also enhances eIF-2B activity (Dholakia *et al*, 1986; Akkaraju *et al*, 1991). As $NADP^+$ inhibits the activity of eIF-2B in *in vitro* reactions, a recent study examines whether or not the activity of eIF-2B is modulated by ATP. Prior treatment of eIF-2B protein with ATP inhibits 50% of the activity approximately with 0.8 mM ATP. This inhibition is not due to phosphorylation of eIF-2B factor. The inhibition caused by ATP can be prevented by co-incubating with factors like NADPH or F-1,6-BisPi. Therefore it is possible that the activity of eIF-2B may be allosterically regulated *in vivo* not only by changes in the pyrimidine nucleotides but also by changes in relative amounts of NADPH and ATP (Kimball and Jefferson, 1995). Also, a recent study by Ramaiah *et al* (1994b) suggests that PQQ, a novel cofactor of many bacterial dehydrogenases, stimulates eIF-2B activity of CHO cell extracts which do not contain an active eIF-2a kinase. In contrast, the compound is shown to inhibit protein synthesis, enhance eIF-2α phosphorylation and inhibit eIF-2B activity of reticulocyte lysates. However, in the absence of the CHO cell extracts, the compound inhibits eIF-2α kinase activity and phosphorylation. These studies emphasize that the compound is reduced by extracts to $PQQH_2$ which in turn can stimulate the activities of eIF-2a and eIF-2B. However the increase in eIF-2B activity can be observed when eIF-2α kinase activity is minimal or lacking as in CHO cell extracts. These findings suggest that the reducing power of a lysate stimulates eIF-2B activity and protein synthesis if eJP-2a kinase activity is not interfering. Low concentrations of sugar phosphates like G6P which can generate

NADPH and also high concentrations of sugar phosphates are shown to enhance protein synthesis in gel-filtered reticulocyte lysates (London *et al,* 1987; Jackson *et al,* 1983). A recent study suggests that sugar phosphates probably regulate eIF-2B activity allosterically (Singh and Wahba, 1995). N-ethylmaleimide, a -SH reactive agent which alkylates protein -SH groups, also inhibits eIF-2B activity (Alcazar *et al,* 1995)

Cloning of one of the subunits of eIF-2B, that is, the a subunit of eIF-2B has been recently accomplished from rat cDNA library. It has sequence homology with GCN3 protein, an eIF-2B equivalent in yeast (Flowers *et al.,,* 1995). The P-subunit of eIF-2B has also been cloned (Craddock *et al,* 1995). Cloning and characterization of the various subunits of eIF-2B will facilitate to understand the mechanisms and consequences of eIF-2B/eIF-2 interactions in normal and in perturbed physiological conditions.

1.5.2.3 Regulation of eIF-2a phosphorylation by other proteins:

In addition to the regulation of eIF-2α kinase activity, eIF-2α phosphorylation is also influenced by other proteins. Gupta and co-workers have identified a glycosylated (GlcNAc) protein with a molecular weight of 67 kDa which comigrates with many of the eIF-2 preparations and is shown to affect eIF-2a phosphorylation. p67 contains 12-O-linked GlcNAc residues and evidence suggests that these glycosyl residues protect eIF-2a subunit from eIF-2a kinase catalyzed phosphorylation (Datta *et al,* 1988, 89). Further they have suggested that inhibition of protein synthesis that occurs during heme-deficiency and in serum-starved cells is due to deglycosylation and subsequent degradation of p67 (Ray *et al,* 1992). This may have lead to enhanced eIF-2a phosphorylation and concomitant inhibition in protein synthesis. Mitogen treated serum starved cells show high quantity of p67 with accompanying increase in protein synthesis. This suggests that p67 activity may directly correlate with the protein synthesis activity of the cell (Gupta *et al,* 1993). However the fate of p67 is not known when protein synthesis gets restored by the addition of hemin in inhibited lysates. p67 does not affect HRI auto-phosphorylation but interferes with HRI catalyzed eIF-2 phosphorylation (Datta *et al.,,* 1988). Wheat germ agglutinin (WGA), a lectin, inhibits p67 activity and promotes eIF-2a phosphorylation *in vitro* presumably by binding to glycosyl residues of p67 protein in the contaminant eIF-2

preparations (Datta *et al.*,, 1989). It is not known however if WGA can deglycosylate lysate p67 protein and can enhance lysate eIF-2a phosphorylation.

Heat shock proteins (HSP90 and HSP70) also seem to regulate eIF-2a phosphorylation by regulating HRI activity. HRI is found to be associated with HSP90 (Rose *et al.,* 1987). Binding of phosphorylated HSP90 to HRI increases the kinase activity (Szyszka *et al.,* 1989). The activity of PKR is modulated by polyamines such as heparin, dextran sulfate, chondroitin sulfate and poly L-glutamine (Hovanessian and Galabru, 1987). The only common feature between these compounds to PKR is their polyanionic nature, thus indicating that activation of PKR is dependent on the polyanionic nature of the activator. These results emphasize the possibility that various activators might exist in different types of cells to influence the PKR activity. Also the 5' untranslated region (leader region or Tar sequence) of an HIV mRNA can activate the PKR due to its stem loop structure (Sengupta and Silverman, 1989; Roy *et al.,* 1990). Also as a defense mechanism, different viruses have developed specific strategies to regulate the functioning of PKR activity, for example, adenovirus encoded VA_1 RNA complexes with PKR and inactivates the kinase (Katze *et al.,,* 1987); poliovirus infection induces the degradation of kinase (Black *et al.,,* 1989). While infection by another picornavirus, encephalomyocarditis virus, possibly causes its sequestration (Dubois and Hovanessian, 1990). HIV virus may mediate the down regulation of the kinase via action of the TAT regulatory protein (Roy *et al.,* 1990), whereas influenza virus blocks kinase activity by activation of a cellular inhibitor of PKR (Katze *et al.,* 1988; Lee *et al.,* 1990). Finally, reovirus and vaccinia virus appear to down regulate the kinase by encoding gene products that bind to and sequester an activator of PKR (Imani and Jacobs, 1988; Akkaraju *et al.,* 1989).

1.6 Translational control in plants: Regulation of Initiation factor- 2 activity.

As has been mentioned above, a lot of information has been accumulating recently to indicate that translational controls are facts and not a fantasy any more. However, most of this information comes from the animal systems and very little is known regarding regulation of protein synthesis in plants.

Several conditions such as the leader sequence in mRNA, light, hormones, embryogenesis and stresses such as heat shock, hypoxia, wounding, water and nutrient deficit alter rates of protein synthesis and translation of different plant mRNA's (reviewed in Gallie, 1993). In most of the cases, no correlation has been observed between changes in mRNA levels and transcription, and, between changes in protein synthesis and mRNA levels. Hence it is quite likely that post-trancriptional controls are playing an important role in the regulation of protein synthesis in many of those conditions mentioned above. Only in a few cases has the regulation been characterized.

Leader sequences at the 5'end of plant mRNA are somehow found to control the translational efficiency of mRNA's. Viral leader sequences which are naturally capped and uncapped (alpha alpha mosaic virus RNA 4, AMVRNA 4 vs tobacco etch virus genome respectively), with or without secondary structure (tobacco mosaic virus leader vs AMVRNA 4) and in which the natural cap is removed (AMV RNA 4) are found to enhance the translational efficiencies of chimeric plant mRNA's *in vitro* (Gallie *et ah,* 1987; Jobling and Gehrke, 1987). Although one expects lower levels of eIF-4F to be sufficient for the translation of these mRNA's with uncapped viral leader sequences and with little or no secondary structure, the findings indicate that some of the messages infact require higher levels of eIF-4F (Fletcher *et al.,* 1990). Such findings suggest that the need for a cap and the levels of eIF-4F required for efficient translation need not necessarily correlate. The mRNA's without cap may bind eIF-4F by alternative means. Since some of the leader sequences are found to enhance the translation in cis position and inhibit in trans position, it is likely that the sequences are recognized by leader specific RNA binding proteins (Gallie, 1993). The leader sequence of heat shock genes is required in order to

escape thermo-repression of translation in plant species (Pitto *et al*, 1992; Schoffl *et al.* 1989). The leader from genes encoding HSP17 was implicated to be necessary in soybean (Schoffl *et al.*, 1989). Direct evidence for the role was obtained using maize HSP70 leader in chimeric mRNA constructs that were delivered directly to maize protoplasts prior to the application of thermal stress. With the HSP70 leader present, translation of reporter mRNA's continued during heat shock (Pitto *et al.*, 1992)

The general decrease in cell metabolism in aging tissue is reflected in the translational machinery. Polysomes in aging pea decrease in quantity and size, and the levels of mRNA and ribosome drop (Schuster and Davies, 1993). Since the aged ribosomes were only 2% as active during translation *in vitro* as ribosomes isolated from non-aged tissue, the finding suggests that ribosomes in aged tissue are subject to some structural changes leading to a reduction in activity.

Wounding of plant storage organs leads frequently, if not invariably, to enhancement of polysome formation and protein synthesis *(KM,* 1978; Wielgat and Kahl, 1979; Ishizuka *et al.*, 1981). However wounding of aged, excised pea epicotyls (non-storage tissue) that have recovered from their initial wounding and are able to take-up precursors, have shown 75% inhibition of protein synthesis within the first 5min., without a change in the amount or proportion of polysomes (Davies *et al.*, 1986; Davies, 1987). Anaerobic conditions prevented the increase in polysomes and protein synthesis that occurs on wounding of a storage tissue like potato (Crosby and Vayda, 1991).

A 32 kDa protein associates with polysomes within the 1st hour of wounding in potato (Crosby and Vayda, 1991). The protein is loosely associated with ribosomes and exhibits a kinase activity. As the protein remains associated with polysomes in wounded tissue subjected to hypoxic conditions, it remains unclear if the protein plays a role in mRNA selection (Crosby and Vayda, 1991). Several conditions known, as mentioned above, can alter protein synthesis, but the regulatory mechanism involved in those cases have not been identified.

Auxins stimulate both rRNA and ribosomal protein mRNA synthesis (Gantt and Key, 1985). Moreover, auxins can stimulate phosphorylation of maize ribosomal proteins resulting in increased mRNA binding *in vitro* (Perez *et al.*, 1990). A 95 kDa protein

kinase has been identified from barley embryo's that exhibits an inverse correlation with protein synthesis and may be responsible for inhibiting translation of stored mRNA's through phosphorylation of a translational component (Reddy *et al.*, 1987).

To understand the effect of translational inhibitor(s), if any, in wheat germ cell-free translational systems, Rychlik *et al,* (1980) isolated from wheat germ a protein kinase (Mr. 20 kDa) that phosphorylates two polypeptides present in the preparation of unwashed wheat germ ribosomes. Salt washed ribosomes are no longer a substrate for the kinase, indicating that polypeptides phosphorylated are not ribosomal proteins. This kinase is found to inhibit selectively the translation of BMV RNA's 1 and 2, but not BMV RNA 4. The mechanism of this inhibition is not clear; however, translation of poly U directed polyphenylalanine synthesis is not inhibited, indicating that elongation is not affected. A second wheat germ kinase, partially purified by Ranu (1980) appears to phosphorylate the 38 kDa subunit from both wheat germ and rabbit reticulocytes. This kinase also inhibits the translation of reticulocyte lysate. The physical properties of this kinase and the mechanism of inhibition by this kinase have not been worked out so far. A third wheat germ kinase having a mol. wt. of 32 kDa has been purified to homogeneity by Yan and Tao (1982a). This kinase appears to be physically similar to the wheat germ kinase isolated by Davies and Polya (1983). The kinase isolated by Yan and Tao (1982) preferentially phosphorylates a 48 kDa polypeptide found in wheat germ. This kinase is also shown to catalyze the phosphorylation of the 41-42 kDa subunit of eukaryotic initiation factor-2 and the 107 kDa subunit of eIF-3. Also, three proteins (38 kDa, 14.8 kDa and 12.6 kDa) of the 60S ribosomal subunit are phosphorylated by this kinase but some how 40S ribosomal subunit proteins are not the substrates for this kinase (Yan and Tao, 1982b). No effects of phosphorylation on the activities of eIF-2, eIF-3 or 60S ribosomal subunits could be demonstrated *in vitro* (Browning *et al,* 1985).

Many earlier workers have purified initiation and elongation factors involved in translation from wheat germ (Lax *et al,* 1986), however, there has been little progress regarding understanding of the regulation of translation in plants involving the factors.

Attempts have been made to purify an eIF-2B like protein from wheat germ but have not met with any success (Osterhout *et al,* 1983; Lax *et al.,* 1982). Earlier reports

indicate that one of the subunit of wheat germ eIF-2 (p41-42) is phosphorylated by heme-regulated eIF-2α kinase from rabbit reticulocyte lysate (Benne *et al,* 1980, Ranu, 1980) and also by CK-II (Mehta *et al,* 1986). Phosphorylation of this doublet subunit by reticulocyte eIF-2α kinase like HRI (Benne *et al,* 1980; Ranu, 1980) or the dsRNA induced inhibitor (PKR or dsI) (Shaikin *et al,* 1992) does not affect translation. A recent study infact describes the expression of cloned HRI cDNA in wheat germ lysates (Chen *et al,* 1990).

Indeed there is considerable confusion in the literature regarding the phosphorylation of plant eIF-2 in large part, due to discrepancies in subunit identification. However, a careful analysis reveals that most of the reports suggest that one of the eIF-2 subunits (p41-42 doublet subunit) can be phosphorylated by some cyclic AMP independent kinases, as mentioned above. However, the physiological significance of this phosphorylation has not been understood (Benne *et al.,* 1980; Ranu, 1980; Seal *et al,* 1983; Shaikin *et al.,* 1992). Some recent studies atleast from one laboratory indicated that tobacco mosaic virus infection to uninfected host-cells enhances the phosphorylation of a host encoded protein (p68) which appear to share many properties with one of the mammalian eIF-2α kinases (PKR) (Crum *et al,* 1988). Recently, at the conclusion of the work, a paper appeared by the above group (Langland *et al,* 1996) indicating that addition of relatively higher concentrations of dsRNA (20-100 μg/ml) or, phosphorylated plant or mammalian eIF-2α can inhibit wheat germ translation. These observations contradict the earlier observations which suggest that the presence of dsRNA in plant systems in a wide range of concentrations does not result in suppression of protein synthesis, neither *in vitro* (Reijenders *et al,* 1975; Grill *et al,* 1976; Pratt *et al,* 1978) nor, apparently, *in vivo* (Dezoeten *et al,* 1989). The authors (Langland *et al,* 1996) however suggest that this discrepancy may be due to varying levels of a PKR inhibitor like the glycosylated protein p67 present in mammalian systems (Gupta *et al,* 1993). However the p67 protein does not inhibit autophosphorylation of eIF-2α kinase activity but may 'protect' eIF-2α phosphorylation catalyzed by eIF-2α kinase *in vitro* (Datta *et al,* 1988). Also higher concentrations of dsRNA that are required to stimulate eIF-2α kinase activity

of PKR in plant systems is found to inhibit the PKR activity in reticulocyte lysates (Clemens, 1994). While earlier observations of Osterhout *el al,* (1983) and Lax *et al.,* (1982) suggest that plants may not carry an eIF-2B like protein, the recent work by Langland *el al.,* (1996) suggests the existence of such a protein in *Arabidopsis thalliana* based on the information available from Expressed sequence tags (ESTs). However, these above authors have not shown any effect of eIF-2α phosphorylation on eIF-2B like activity or any mechanism by which eIF-2a phosphorylation in plants can inhibit protein synthesis.

1.7 Objectives:

The present work is taken up to identify conditions which can affect eIF-2 phosphorylation, protein synthesis and guanine nucleotide exchange activity of an eIF-2B like protein in translating wheat germ lysates. In order to carry out the work:

- Wheat germ protein synthesizing lysates have been modified with those agents which are known to enhance the phosphorylation of eIF-2a in mammalian systems. In this study, agents such as dithiothreitol (DTT), pyrroloquinoline quinone (PQQ) oxidized glutathione (GSSG) and N-ethylmaleimide (NEM) which are known to reduce, oxidize or alkylate protein -SH groups, have been used to study the lysate protein synthesis and phosphorylation. We have also used diamide and diaphorase to study overall protein synthesis. Also the affects of protein phosphatase inhibitors like okadaic acid on wheat germ protein synthesis has been studied. Besides the above mentioned inhibitor, we have also used heterologous serine-threonine kinases like HR1 (heme-regulated eIF-2a kinase), dsI (double stranded RNA induced inhibitor) and casein kinase II (CK-II) on wheat germ protein synthesis and eIF-2 phosphorylation.
- The effect of eIF-2 phosphorylation on the exchange of guanine nucleotides on wheat germ eIF-2 has also been assessed.
- Since the data suggest that wheat germ eIF-2 may not require an eIF-2B like protein and is consistent with a recent report (Shaikin *et al.,* 1992) which indicates that the

affinity of wheat germ eIF-2 to GDP is only 10 times higher compared to GTP, the effects of nucleoside di- and tnphosphates on wheat germ protein synthesis has also been investigated here. Since the nucleoside diphosphate to tnphosphates can be regulated by nucleoside diphosphate kinase (NDK) like activity, the present studies have also focused on the identification of such an activity in translating lysates and with purified wheat germ **eI F-2**

CHAPTER 2

MATERIALS AND METHODS

2.1 METHODS

2.1.1 Preparation of wheat germ lysate:

Wheat germ lysate was prepared as described (Roberts and Patterson, 1973; Ramaiah and Davies, 1985). All the necessary glassware and solutions were autoclaved (except Hepes and DTT which were prepared in autoclaved double distilled water). About 40g of wheat germ was floated on carbon tetrachloride and cyclohexane mixture in the ratio of 2.5:1. The floated wheat germ was vacuum dried (1hr in a hood) before processing further. The floated and dried wheat germ (3 g) was removed with the help of a spatula, powdered in liquid nitrogen and made into a paste with extraction buffer (40 mM Hepes-KOH, pH 7.6, 100 mM KOAc, 1 mM $Mg(OAc)_2$, 2mM $CaCl_2$ and 1 mM DTT) on ice. Extraction and the subsr juent steps were done as quickly as possible at 4°C. The paste was spun at 15,000 rpm for 15 min. in a high speed refrigerated centrifuge. The top 3/4th supernatant was collected and clarified again at 15,000 rpm for 15 min. Supernatant thus obtained was loaded on a 50 x 2.5 cm Sephadex G-25 column which was preequilibrated with the column buffer containing 40 mM Hepes-KOH pH 7.6, 100 mM KOAc, 5 mM $Mg(OAc)_2$ and 1 mM DTT. Elution was also carried out using column buffer and 3 ml fractions were collected. Highly turbid fractions were pooled and spun at 15,000 rpm for 20 min. The top 3/4th supernatant was collected and stored as 0.5 ml aliquots in liquid nitrogen.

2.1.2 Standardization of wheat germ protein synthesis :

The requirement of ATP, GTP, K^+, Mg^{2+} and the message, BMVRNA, for carrying out wheat germ protein synthesis for one of the lysate batches is shown (Fig 3a-e).

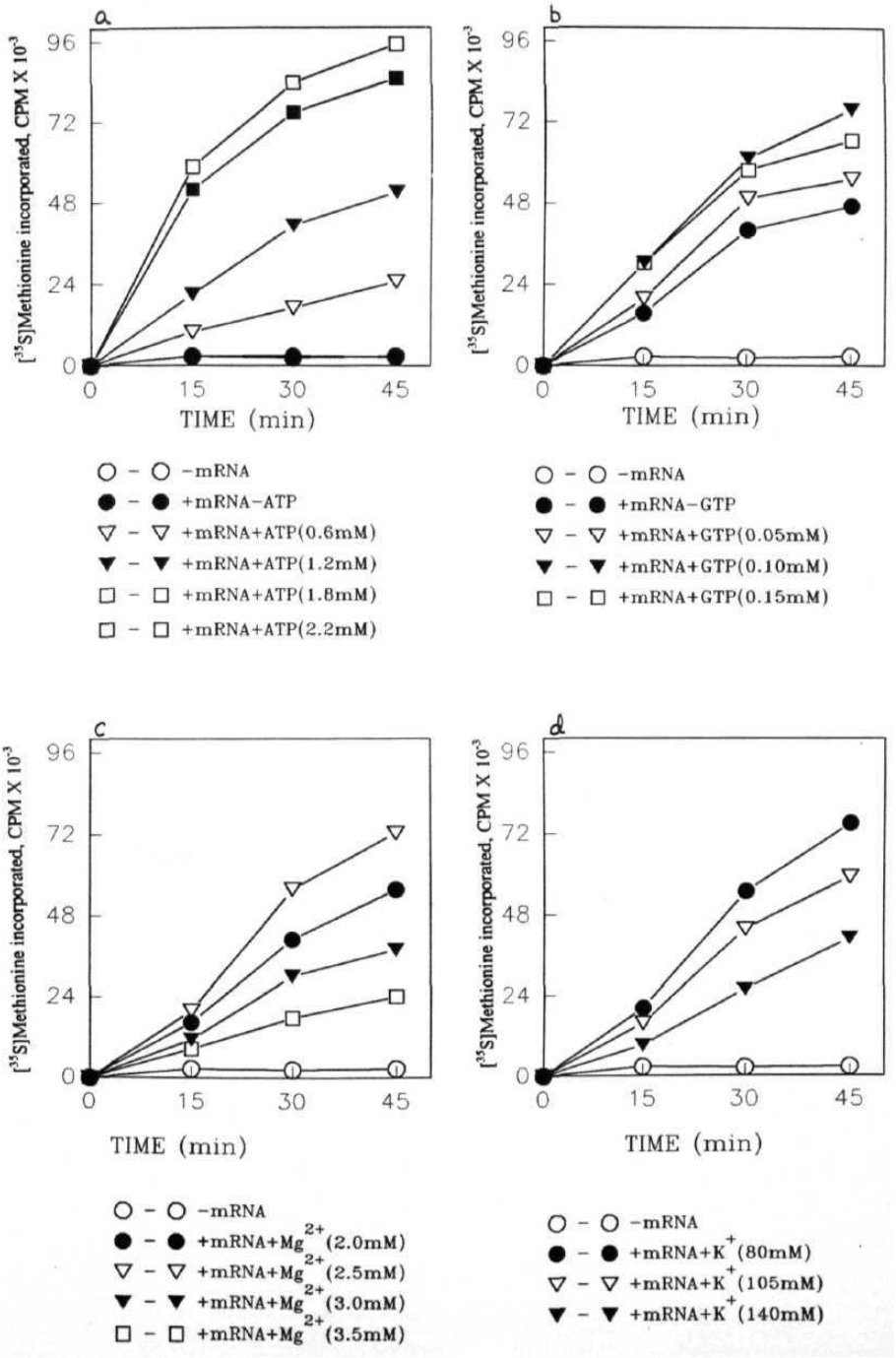
Fig.3a. shows protein synthesis in the presence of various concentrations of ATP (the concentrations of the rest of the components were kept constant). Optimal protein synthesis was observed in the presence of 1.8mM ATP (-•-). Increasing or decreasing the ATP concentration inhibited protein synthesis.

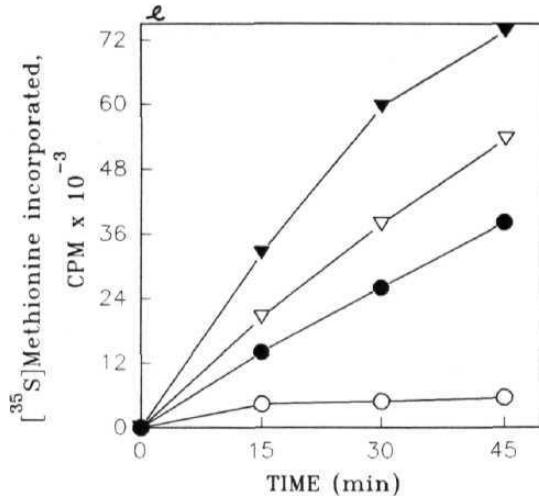
Fig. 3 **(a-e)** Standardization of wheat germ lysate protein synthesis.

Standard lysate protein synthesis assays **(25 µl)** were carried out in the presence pf BMV RNA (15 **µg/ml)** at **25°C** for 45 minutes as described in 'Materials and Methods'. The reaction mixture contained all the components required for protein synthesis except the component being standardized. The requirement for ATP (Fig. 3a); GTP (Fig. 3b); Mg^{2+} (Fig. 3c); K^{+}**(Fig**. 3d) and BMV RNA (Fig. 3e) to carry out optimal protein synthesis was assesses by the addition of different concentrations of these components to the reaction mixtures as shown in the respective figures. A control reaction without the addition of BMV RNA **(O-O, -mRNA)** was also carried out to assess the endogenous lysate protein synthesis.

Protein synthesis was assessed by the incorporation of **[^{35}S]methionine** into acid precipitable protein in a 5 µl aliquot of the reaction mixtures, taken at different time intervals **(15**, 30 and 45), as described in 'Materials and Methods'.

Fig. 3a-e


a
b
c
d
[35S]Methionine incorporated, CPM X 10-3
96
72
48
24
0
0
15
30
45
TIME (min)
○ - ○ -mRNA
● - ● +mRNA-ATP
▽ - ▽ +mRNA+ATP(0.6mM)
▼ - ▼ +mRNA+ATP(1.2mM)
□ - □ +mRNA+ATP(1.8mM)
□ - □ +mRNA+ATP(2.2mM)
○ - ○ -mRNA
● - ● +mRNA-GTP
▽ - ▽ +mRNA+GTP(0.05mM)
▼ - ▼ +mRNA+GTP(0.10mM)
□ - □ +mRNA+GTP(0.15mM)
○ - ○ -mRNA
● - ● +mRNA+Mg2+(2.0mM)
▽ - ▽ +mRNA+Mg2+(2.5mM)
▼ - ▼ +mRNA+Mg2+(3.0mM)
□ - □ +mRNA+Mg2+(3.5mM)
○ - ○ -mRNA
● - ● +mRNA+K+(80mM)
▽ - ▽ +mRNA+K+(105mM)
▼ - ▼ +mRNA+K+(140mM)



○ - ○ −BMVRNA
• - • +BMVRNA (lOug/ml)
V - V +BMVRNA (20ug/ml)
• - • +BMVRNA (30ug/ml)

Wheat germ protein synthesis in the presence of various concentrations of GTP is shown in Fig. 3b. Protein synthesis was best when 100 μM GTP (-V-) was used. Addition of higher concentration (150 μM, -•-) of GTP did not enhance the translation. Therefore all the protein synthesis reactions were carried out with 100 μM GTP.

The concentration curve of Mg^{2+} is shown in Fig. 3c. Optimal protein synthesis was seen in the presence of 2.5 mM Mg^{2+} (final concentration including the Mg^{2+} contributed by the lysate, -V-), further increase or decrease lowered the protein synthesis.

Fig. 3d shows wheat germ protein synthesis in the presence of different K^+ concentrations. Maximal protein synthesis was seen in the presence of 80 mM K^+ (-•-).

Wheat germ protein synthesis increased with the amount of the message (BMV RNA) used. Fig. 3e shows wheat germ protein synthesis in the presence of 10, 20, 30μg/ml BMV RNA. For all our experiments we have used 15 μg/ml of BMV RNA.

Based on our above standardizations, the protein synthesis reactions were routinely supplemented with 1.8 mM ATP, 100 uM GTP, 80 mM K^+ and 2.5 mM Mg^{2+} (final). Rarely the optimal concentration of Mg^{2+} required for some batches of lysates was found to be 3.0 mM. Hence for our comparision of protein synthesis activities under different conditions, lysates obtained from the same batch have been used.

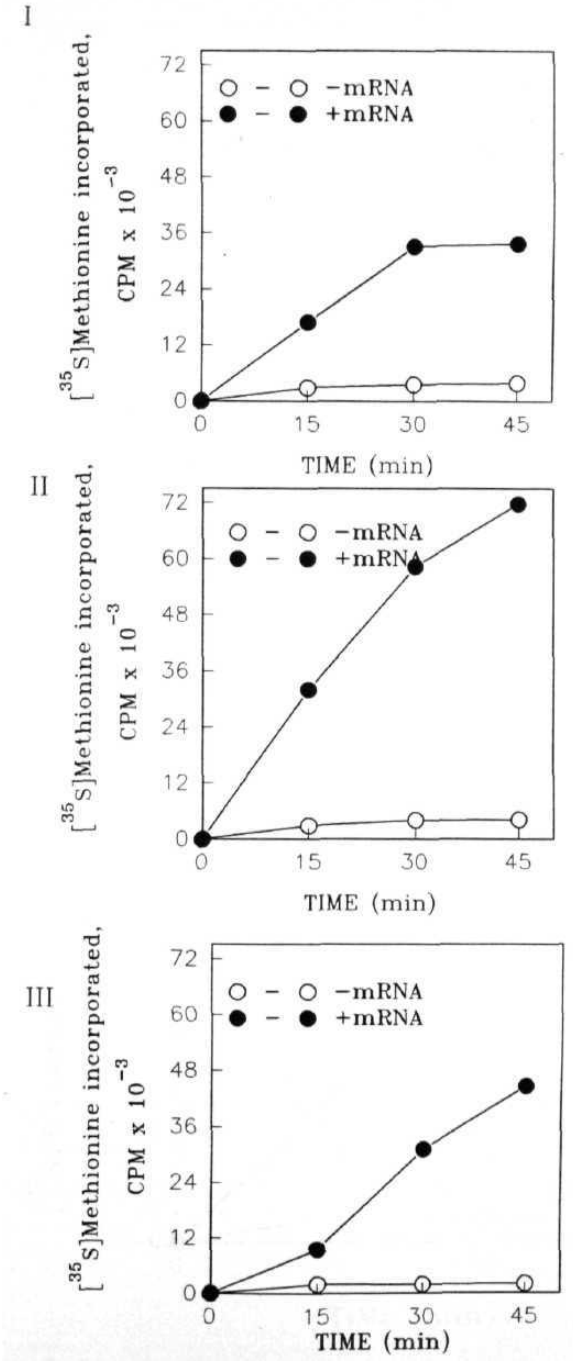
2.1.3 Wheat germ lysate protein synthesis:

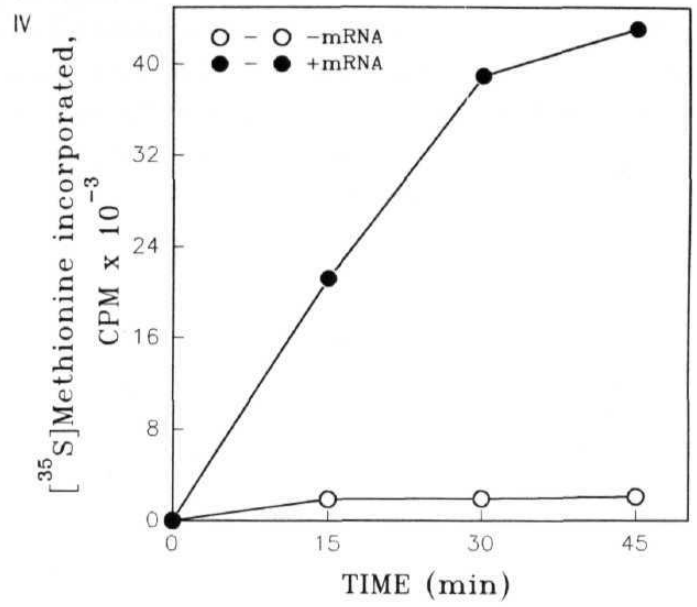
Wheat germ lysate protein synthesis was performed as described (Janaki et al 1995). Since the endogenous message (mRNA) in wheat germ lysate is almost absent, an exogenous message, Brome Mosaic Virus (BMV) mRNA (15 μg/ml) was used in all the translation experiments. Typically, the reaction mixture in a 25 ul volume contained 20 mM Hepes-KOH, pH 7.6, 1.8 mM ATP, 100 uM GTP, 4 mM CP, 64 μg CPK, 80 mM K^+, 2.5 mM Mg^{2+}, 0.5 mM Spermidine, 40 U/ml of RNase inhibitor (RNAsin), 20 uM of all the amino acids except methionine, 1 uCi of [^{35}S] methionine (1000 Ci / mmole) and 10ul lysate (40% final). The reactions were incubated at 25°C to carry out protein

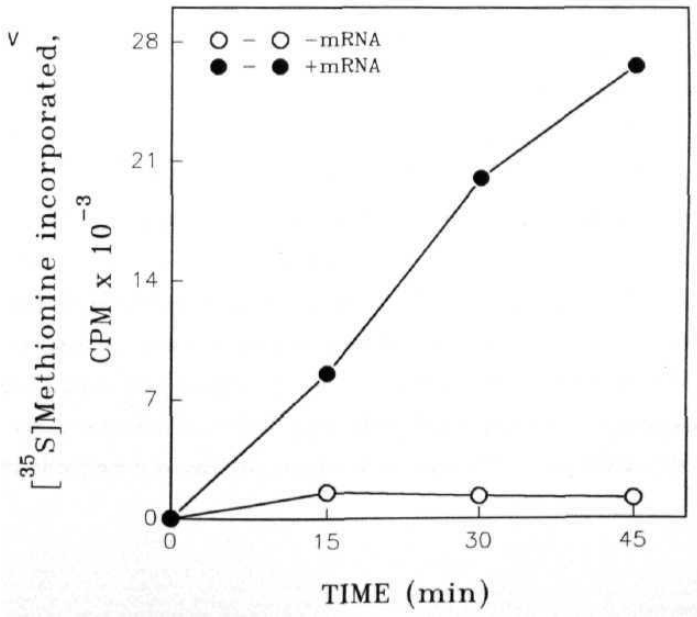
Fig. 4 Protein synthesis in different batches of wheat germ lysates (I-V).

Standard lysate protein synthesis assays (25 μl) were carried out in the presence of BMV RNA (15 μg/ml) at 25°C for 45 minutes as described in 'Materials and Methods' to assess the ability of the lysates to carry out protein synthesis. A control reaction without the addition of BMV RNA (O-O, -mRNA) was also carried out to assess the endogenous lysate protein synthesis.

Protein synthesis was assessed by the incorporation of [^{35}S]methionine into acid precipitable protein in a 5 μl aliquot of the reaction mixtures, taken at different time intervals (15, 30 and 45), as described in 'Materials and Methods'.


I
II
III
[35S]Methionine incorporated, CPM x 10-3
72
60
48
36
24
12
0
−mRNA
+mRNA
0
15
30
45
TIME (min)


IV
O – O –mRNA
● – ● +mRNA
[35S]Methionine incorporated,
CPM x 10-3
0
8
16
24
32
40
0
15
30
45
TIME (min)


V
O – O –mRNA
● – ● +mRNA
[35S]Methionine incorporated,
CPM x 10-3
0
7
14
21
28
0
15
30
45
TIME (min)

synthesis. The performance of lysates in 5 µl aliquots was assessed with time by their ability to incorporate labeled methionine into acid precipitable protein. Samples of 5 µl were removed at different time intervals and spotted on a Whatman No. 1 filter paper disc. Proteins in the samples were precipitated by immersing the filters in 10% cold TCA for 1 hour. Afterwards, the filters were washed with 5% hot TCA (3-5) min and 5% TCA at room temperature to remove any non-specific radioactivity. Later the filters were washed with ethanol and acetone and finally air dried. Radioactivity of the filters was counted in a liquid scintillation counter.

Performance of the various lysates prepared from different batches of wheat germ are shown in Fig. 4. Wheat germ protein synthesis was carried as mentioned above. Since very little endogenous message is present in wheat germ lysates, the incorporation of labeled amino acid into protein was not very significant without added mRNA (-mRNA, -O-). Addition of small amount (15 µg/ml) of BMV RNA stimulated the incorporation of the labeled amino acid into protein quite significantly (+mRNA, -•-).

2.1.4 Measuring reticulocyte lysate protein synthesis:

Reticulocyte lysates were prepared from anemic rabbit blood as described (Hunt et al., 1972). Unlike wheat germ lysates, reticulocyte lysates were not gel filtered. A standard incubation mixture for reticulocyte protein synthesis contains the following in a total reaction volume of 25 µl: 60% reticulocyte lysate, 4 mM creatine phosphate (CP), 250 µg creatine phosphate kinase (CPK), 80 mM KCl, 1 mM $Mg(OAc)_2$, 200 µM GTP, 33 µM amino acid mix without leucine, 33 µM [^{14}C] leucine (340 uCi / mmole) (Ernst et al., 1978). Where indicated, the reaction mixtures were also supplemented with hemin (20 uM). The components of the incubation mixture were mixed together at 0°C and the protein synthesis reaction was carried out at 30°C. The performance of lysates in 5 µl aliquots was assessed with time by their ability to incorporate labeled leucine into acid precipitable protein. Samples of 5 µl were removed at different time intervals and spotted on a Whatman No.1 filter paper discs. The proteins were precipitated by placing the filter discs in ice-cold 10% trichloroacetic acid (TCA) for 1 hour. The filters

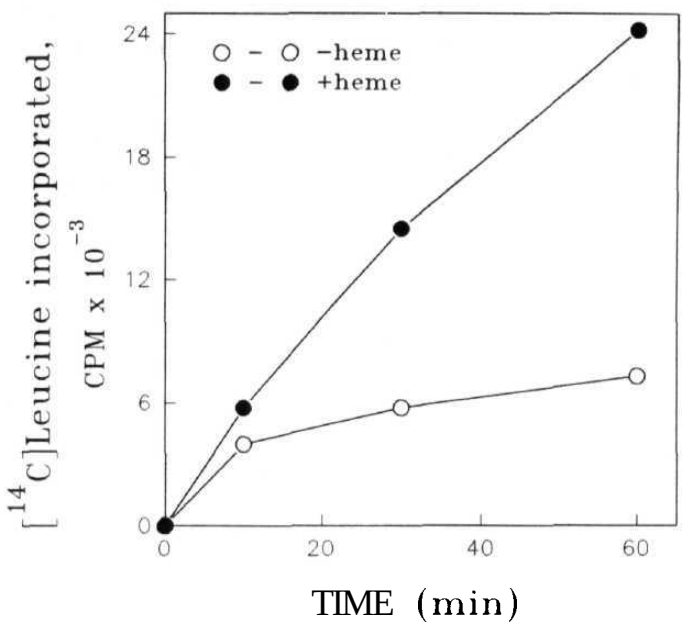


Fig. 5 Rabbit reticulocyte lysate protein synthesis.

Standard reticulocyte lysate protein synthesis assays (25 µl) were carried out at 30°C for 60 minutes as described in 'Materials and Methods' in the presence of 20 µM hemin. A control reaction, without any additions (O-O, +heme) was also carried out to assess the normal protein synthesis.

Protein synthesis was assessed by the incorporation of [^{14}C]leucine into acid precipitable protein in a 5 µl aliquot of the reaction mixtures, taken at different time intervals (10, 30 and 60), as described in 'Materials and Methods'.

were then washed with 5% boiling TCA (3-5 min.) and 5% TCA at room temperature The filters were then washed with ethanol and acetone. The filters were then air dried and transferred to H_2O_2 solution (1:1 diluted with water) for 10 min. to bleach the red color present on the filters. This is to avoid any quenching effects while reading the filters in a scintillation counter. The filters were again washed in ethanol and acetone before drying. The air dried filters were read in a liquid scintillation counter to determine the protein synthesizing capacity of the extracts.

Protein synthesis of a typical heme-deficient reticulocyte lysate in the presence and absence of added hemin is shown in Fig. 5. Translation of the endogenous message in reticulocyte lysate was dependent on the availability of hemin (+ heme, -•-). In the absence of heme (-heme, -O-) protein synthesis occured for a short period of time (7-10 min.) and then it was shut off. Addition of hemin (20 μM) maintained protein synthesis for over an hour.

2.1.5 Preparation of wheat germ binary complex, eIF-2.[H]GDP, and its dissociation in vitro:

The binary complex was prepared as described by Ramaiah et al., (1994). Purified wheat germ eIF-2 (2 μg) was incubated in 20 μl reaction mixtures containing Tris-HCl (20 mM, pH 7.8, KCl (100 mM), creatine phosphofructo kinase (CPK, 100 μg/ml) and [^{3}H]GDP (2 μM, ~1900 cpm/pmol) at 25 °C for 10 min. to form an eIF-2.[^{3}H]GDP binary complex and then incubated on ice for 10 min. The complex was stabilized by the addition of 2.5 mM Mg(OAc)2 followed by another 10 min. incubation on ice. The amount of binary complex formed was assessed by arresting the reaction mixture in 3ml of cold wash buffer (containing 20 mM Tris-HCl pH 7.8, 80 mM KCl and 2.5 mM $Mg(OAc)_2$) The reaction mixture was then filtered through HAWP 0025 nitrocellulose (0.45 uM) millipore filters and the filters were washed thrice with 3 ml of cold wash buffer. Filters were then dried and the amount of labeled GDP bound to eIF-2 on the filter paper was measured in a liquid scintillation counter.

Exchange of unlabeled GDP for labeled GDP was studied by the addition of 40 μM unlabeled GDP to the labeled binary complex. Reactions were carried out at 25°C for specified time intervals as given in the figure legends. The reaction mixtures were stopped by the addition of 3 ml of cold wash buffer and the contents were filtered through nitrocellulose filters as mentioned above. Undissociated eIF-2.[^{3}H]GDP, binary complex, was measured by the retention of the complex on the millipore filters. The filters were dried and the amount of labeled GDP bound to eIF-2 on the filter paper was measured in a liquid scintillation counter. Modifications (if any) are mentioned in the figure legends. Pmols of eIF-2.[^{3}H]GDP dissociated were determined by the difference of the total eIF-2.[^{3}H]GDP added and the amount remaining at the end of the reaction as described (Ramaiah et al., 1994).

2.1.6 Protein phosphorylation in translating reticulocyte lysates:

Protein synthesis in reticulocyte lysates was carried out (in 25 μl) as described above (section 2.3), except that unlabeled leucine was used instead of labeled leucine. Lysates were supplemented briefly with wheat germ eIF-2 (wherever mentioned) and [γ-^{32}P]ATP (3000 Ci/mmole, 10 μCi) during 10-15 min. of protein synthesis to facilitate the labeling of phosphoproteins Ten microliters of the reaction mixture was taken out at the end of the reaction and was concentrated by pH 5.0 precipitation as described (Ernst et al., 1978) by the addition 0.8 ml of NaF and EDTA (50mM and 5mM final respectively) followed by the addition of 10 ul of 0.5 M glacial acetic acid. The reaction mixtures were then left on ice for 1 hour for the proteins to precipitate and then spun at 10,000 rpm for 15 minutes. The supernatant obtained after centrifugation was discarded. The pellet was suspended in 20 ul of 1X SDS-sample buffer (Tris-HCl pH 6.8, 10% SDS, 10% glycerol, β-mercaptoethanol and bromophenol blue) and heated in boiling water bath for 3 minutes. Proteins were separated by sodium dodecyl sulfate-10% polyacrylamide gels as described (Ramaiah et al., 1992) and the phosphoproteins analyzed by autoradiography. Details and modifications (if any) are mentioned in the figure legends.

2.1.7 Protein phosphorylation in translating wheat germ lysates:

Protein synthesis in wheat germ iysates was carried out (in 25 u.1) as described above (section 2.2.1), except that unlabeled methionine was used instead of labeled methionine. Protein synthesizing lysates were treated with various agents (as mentioned in the figure legends) added at the start of the protein synthesis incubation, for 10 min at 25°C. The lysates were then pulsed with 10 µCi of [γ-^{32}P]ATP (3000 Ci / mmol) for 5 minutes. Reactions were terminated by the addition of 5 µl of 4X-SDS sample buffer heated in boiling water bath for 3 minutes and the denatured proteins separated on SDS-PAGE according to modified Laemmli method (1970). The phosphorylated proteins were analyzed by autoradiography. Modifications (if any) are mentioned in figure legends.

2.1.8 Autoradiography

The labeled proteins were separated on SDS-PAGE. The gel was then dried in a Bio-rad gel drier equipment and exposed to an X-ray film, Kodak X-Omat or Indu (manufactured locally), at -70 °C. The film, after exposure for the required time, was developed by a set of photographic solutions obtained commercially and as per the manufacturer's instructions.

2.1.9 Thin layer chromatography to assess the conversion of GDP to GTP:

The conversion of GDP to [γ-^{32}P]GTP by incubation with [γ-^{32}P]ATP in the presence of nucleoside diphosphate kinase (NDK) was studied as described by Siekierka et al., (1983) with few modifications. This assay was used to assess (i) the amount of GDP present in lysates (ii) GDP bound to wheat germ eIF-2 (iii) to search for the presence of NDK like activity in wheat germ lysates (iv) NDK activity associated with purified wheat germ eIF-2.

NDK catalyzes the reversible reaction GDP + [γ-^{32}P]ATP——[γ-^{32}P]GTP + ADP in the presence of Mg^{2+}. The conversion is assessed by autoradiography.

The standard reaction was carried out in a 10 µl reaction mixture containing 20 mM Tris-HCl, pH 7.6, 10 mM $Mg(OAc)_2$, 100 mM KC1, 2.5 mM β-mercaptoethanol, 100

pmols of [γ-^{32}P]ATP, 30 pmols of GDP and 3 units of NDK The reaction mixtures were incubated at 25°C for 10 minutes and then 2ul aliquot was spotted on polyethyleneimine-cellulose MN-300-coated plastic sheets. The reactions were spotted 1 cm apart leaving 1.5cm on either side of the sheet. The spots were dried in a stream of hot air and developed by ascending chromatography in 1.0 M potassium phosphate buffer (pH 3.4). The chromatograms were dried after the development and exposed to Indu X-ray film for 6 hours at -70°C. The autoradiograms were analyzed for the conversion of NTPs.

2.2 CHEMICALS:

Mono-, di- and triphosphates of adenosine and guanosine, CP, CPK, DTT, β-Nicotinamide compounds and glucose-6-phosphate were purchased from Boehringer and Mannheim. Poly IC was purchased from Calbiochem, USA [8-^{3}H] GDP (9 Ci/mmol), [γ-^{32}P] ATP (3500 Ci/mmol) [^{14}C] Leucine (340 mCi / mmol) and [^{35}S] Methionine (1100 Ci/mmol) were obtained from Dupont, NEN and BRIT, Bombay, India. Nitrocellulose membranes, HAWP 0025 nitrocellulose membranes (0.45 μM, 1.75 cm) were obtained from Millipore, USA. BMV RNA was obtained from Promega. X-ray films were purchased from Indu, India. Acrylamide, Bis-acrylamide, Ammonium persulfate, TEMED, SDS were obtained from Bio-rad Kinases like Casein Kinase II; lck kinase and protein phosphatases I; 2A were purchased from Upstate Biotech. Inc., USA. N-ethyl maleimide, GSSG, diamide, diaphorase and other chemicals like Hepes, potassium acetate and magnesium acetate etc. required for routine work were purchased from Sigma. Wheat germ was obtained from General Mills Inc., USA, Sigma, and also locally from Krishna Mills, Banglore, India.

Wheat germ eIF-2 was prepared by Mr. V. M. Krishna, University of Hyderabad, India. Reticulocyte eIF-2 and HRI were prepared by Dr. Ramaiah, University of Hyderabad, India.

CHAPTER 3

PROTEIN SYNTHESIS AND PHOSPHORYLATION IN WHEAT GERM LYSATE

Results and Discussion

Initially, several agents which are known to stimulate the eIF-2α phosphorylation and inhibit protein synthesis in reticulocyte lysates have been selected depending upon their availability with us and assessed their ability to effect wheat germ translation. These include redox agents, phosphatase inhibitors, purified kinases and phosphatases The protein synthesis results are described in section 3.1. Based on the data obtained from protein synthesis, some of these agents have been chosen for their ability to phosphorylate wheat germ lysate proteins and purified eIF-2 in translating lysates in order to determine the overall phosphorylation status and to assess the activation of a kinase(s) in lysate which can phosphorylate endogenous or added eIF-2. Phosphorylation results are described in section 3.2. Later, those agents which can inhibit protein synthesis and stimulate eIF-2 phosphorylation have been selected to determine the effect of eIF-2 phosphorylation on guanine nucleotide exchange (GNE) activity of wheat germ eIF-2 (Chapter 4).

The results presented here suggest that decreased protein synthesis and enhanced eIF-2 phosphorylation can occur without altering the GNE activity of wheat germ eIF-2 (sections 4 3-4.32), hence, protein synthesis in the presence and absence of ATP generating system has been carried out to evaluate the role of increasing concentrations of ADP / ATP or GDP / GTP on protein synthesis (Sections 5.1-5.4). Since GDP can substitute for GTP in protein synthesis reactions and higher concentrations of GDP (section 5.4) inhibit protein synthesis, the finding suggest the importance of an enzyme like nucleoside diphosphate kinase in the regeneration of GDP to GTP or ADP to ATP conversion. Hence, further experiments are carried out to identify such an enzyme activity. Interestingly, the purified enzyme is found comigrating with wheat germ eIF-2. Results of these experiments have been presented in sections 5.5-5.8.

Wheat germ lysate is a good model translation system among plants, and is routinely used to translate different mRNAs to the corresponding protein. The system is also being used to understand the regulation of protein synthesis in plants. Various protein factors involved in initiation and elongation of protein synthesis have been purified from

wheat germ lysate (Lax *et al.*, 1986). The system, however, is not yet explored well for studying the regulation of protein synthesis.

In animal systems it has been well demonstrated that phosphorylation of eIF-2α inhibits protein synthesis (London *et al.*, 1987; Jackson, 1991; Hershey, 1991). This is due to the sequestration of a rate limiting protein called eIF-2B in the form of a 15S complex [eIF-2(aP).eIF-2B]. Free eIF-2B exchanges GTP for GDP bound to eIF-2. The guanine nucleotide exchange reaction of eIF-2 is very critical for the peptide chain initiation. Unless the GDP exchanges for GTP, eIF-2 cannot form the ternary complex (eIF-2.GTP.Met-tRNA) and join the initiation cycle. Three eIF-2α kinases, HRI (Heme-regulated inhibitor, from rabbit reticulocytes), PKR (protein kinase regulated by RNA, from human and mouse) and GCN2 (general control non-derepressible kinase, from yeast) have been cloned and characterized (Meurs *et al.*, 1990; Chen *et al.*, 1991; Baier *et al.*, 1993; Wek *et al.*, 1989).

In addition to heme-deficiency, viral infection and amino acid starvation, eIF-2a phosphorylation also occurs in the presence of DTT, GSSG and NEM (Hunt, 1979; Kan *et al.*, 1988, Chen *et al.*, 1989), heavy metals (Hurst *et al.*, 1987), heat shock (Duncan & Hershey, 1984; Clemens *et al.*, 1987), serum deprivation (Duncun and Hershey, 1985) and other stresses. The responsible kinase(s) in these cases have not yet been identified. A lot of information is accumulating in animal systems indicating the importance of phosphorylation of eIF-2α in the regulation of protein synthesis. However there has been very little progress regarding the understanding of translational regulation in plants involving the factors. It is not known if eIF-2a phosphorylation mechanism regulates protein synthesis in plants. This chapter deals with the effects of various oxidizing-reducing agents, purified heterologous kinases and phosphatases, and certain inhibitors of kinases and phosphatases on wheat germ lysate protein synthesis. The studies are aimed at finding a condition of protein synthesis inhibition with concomitant phosphorylation of eIF-2 to understand its role in the regulation of plant protein synthesis.

3.1 Protein Synthesis in Wheat Germ Lysate:

3.1.1 Effect of -SH reactive agents on wheat germ protein synthesis:

In our earlier studies (Janaki, M.Phil dissertation) it has been observed that addition of 1mM dithiothreitol as recommended by many protocols (Erichson and Gunter, 1983) to translating wheat germ lysates does not improve the performance of wheat germ translation. Hence, all protein synthesis reactions have been carried out here in the presence of 0.4 mM DTT which comes along with the lysate preparations. Addition of GSSG or NEM which are known to inhibit reticulocyte lysate protein synthesis are also found here to inhibit wheat germ translation (Fig. 6). In contrast, pyrroloquinoline quinone (PQQ), (0.01-100 μM), a cofactor of many bacterial dehydrogenases (Salisbury *et al.*, 1979) which is shown to inhibit reticulocyte lysate protein synthesis (Ramaiah *et al.*, 1994) fails to cause any significant change in wheat germ translation (Fig. 7). A DMSO control was also carried out since PQQ suspension was prepared in it. The concentration of DMSO used was 0.05%, which corresponded to the concentration of DMSO present in the highest concentration of PQQ tested. At this concentration DMSO did not inhibit wheat germ protein synthesis (-V-).

Diamide, another potent oxidizing agent, inhibited both reticulocyte lysate (Fig. 8, -■-) and wheat germ lysate protein synthesis (Fig. 10, -■-, 0.5mM; -D-, 1.0mM). Addition of diamide to reticulocyte cells causes cessation of protein synthesis *in vivo*. This is because diamide converts the internal GSH pool to GSSG which in turn inhibits protein synthesis (Kosower *et al.*, 1969). We have tested if reducing conditions like DTT or addition of G6P which can generate NADPH in the reticulocyte lysate system (Kan *et al.*, 1988), can reverse the protein synthesis inhibition caused by diamide. Addition of equimolar concentration of DTT at 0 min (Fig. 8, -T-) to the diamide-treated reticulocyte lysate reversed the inhibition, whereas addition of DTT at 10 min. reversed the diamide induced inhibition partially (-D-). DTT alone has no significant effect on reticulocyte lysate protein synthesis (Fig. 8., -•-, 0 min.; -V-, 10 min). Addition of G6P (Fig. 9a., 50 μM; -V- and 9b., 500 μM, -V-) to the protein synthesizing reticulocyte lysate at 0 min. stimulated protein synthesis marginally but the addition of the same at 10 min. had no

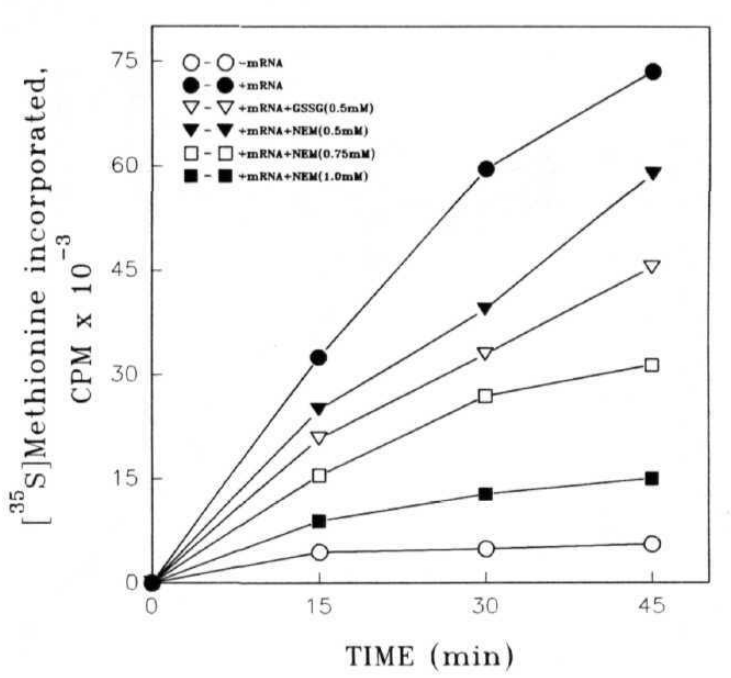


Fig. 6 Effect of NEM and GSSG **on wheat germ lysate protein** synthesis:

Standard lysate protein synthesis assays (25nl) were carried out in the presence of BMV **RNA** (15 **µg/ml)** at 25°C for 45 minutes as described in 'Materials and Methods'. Different concentrations of NEM (0.5 - 1.0 mM) or GSSG (0.5 **mM)** were added at 0 min. of protein synthesis as shown in the figure. A control **reaction,** without any additions (•-•, **+mRNA),** was carried out to assess the optimal protein synthesis. A reaction without the addition of BMV RNA **(O-O, -mRNA)** was also carried out to assess the endogenous lysate protein synthesis.

Protein synthesis was assessed by the incorporation of [S]methionine **mto acid** precipitable protein in a 5 **µl** aliquot of the reaction mixtures, taken at different time intervals (15, 30 **and** 45), as described in 'Materials and Methods'.

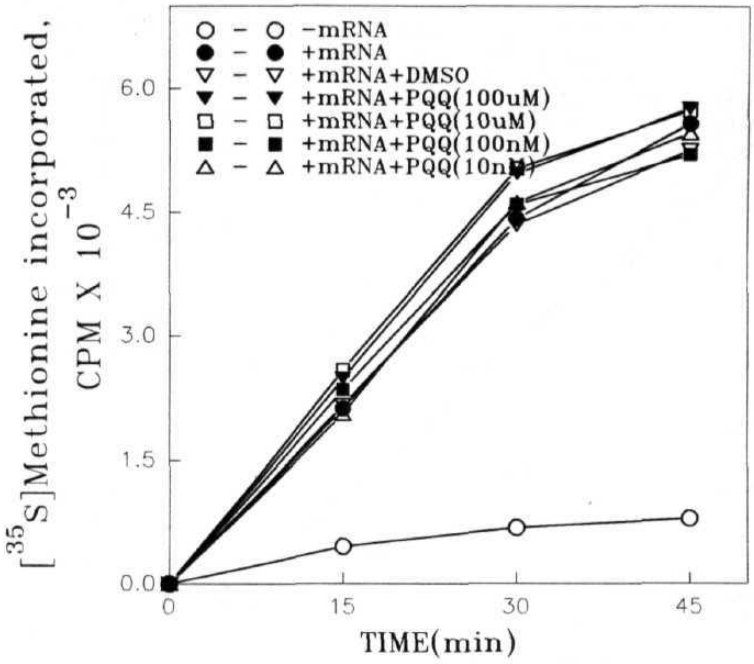


Fig. 7 Effect of PQQ on wheat germ lysate protein synthesis.

Standard lysate protein synthesis assays (25µl) were carried out in the presence of BMV RNA (15 µg/ml) at 25°C for 45 minutes as described in 'Materials and Methods'. Different concentrations of PQQ (10 nM - 100 µM) were added at 0 min. of protein synthesis as shown in the figure. The effect of 0.05% DMSO (V-V) on Wheat germ lysate protein synthesis was also studied since PQQ was prepared in DMSO. The concentration of DMSO tested corresponded to its concentration present in the higest concentration of PQQ tested (100 µM). A control reaction, without any additions (O-O, +mRNA), was carried out to assess the optimal protein synthesis. A reaction without the addition of BMV RNA (O-O, -mRNA) was also carried out to assess the endogenous lysate protein synthesis.

Protein synthesis was assessed by the incorporation of [' S]methionine into acid precipitable protein in a 5 µl ah'quot of the reaction mixtures, taken at different time intervals (15, 30 and 45), as described in 'Materials and Methods'.

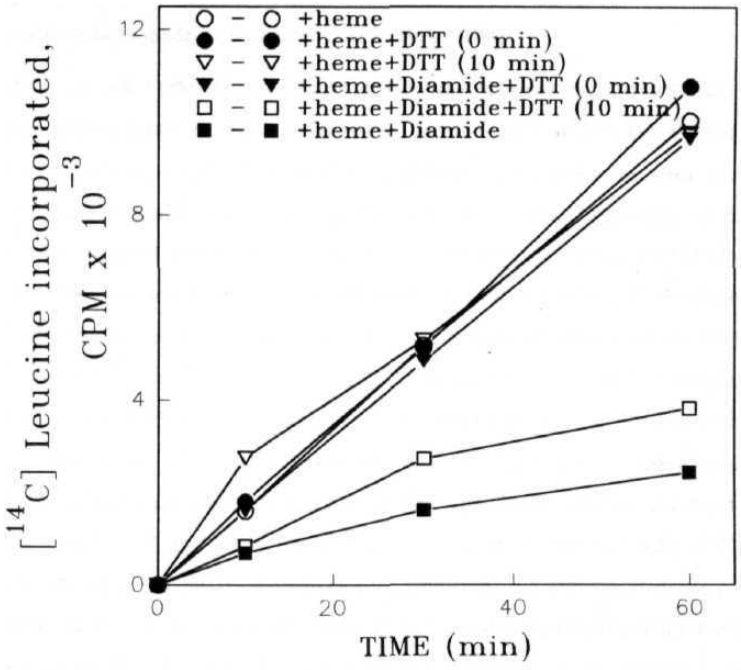


Fig. 8 Effect of diamide and I) II on protein synthesis in hemin-supplemented reticulocyte lysates.

Standard reticulocyte lysate protein synthesis assays (25 µl) were carried out at 30°C for 60 minutes as described in 'Materials and Methods' in the presence of 20 µM hemin. Whereever present, diamide (250 µM) was added at 0 min. DTT (250 µM) was supplemented either at 0 or 10 min. of protein synthesis as shown in the figure. A control reaction, without any additions (O-O, +heme) was also carried out to assess the normal protein synthesis.

Protein synthesis was assessed by the incorporation of [^{14}C]leucine into acid precipitable protein in a 5 µl aliquot of the reaction mixtures, taken at different time intervals (10, 30 and 60), as described in 'Materials and Methods'.

effect (Fig 9a., -V-; 9b., -∇-) Addition of G6P (500 μM, Fig 9b., -D-) at 0 min protected the lysates from diamide induced inhibition in translation . Delayed addition of G6P (10 min.) also causes restoration of protein synthesis but the reversal was only partial (Fig. 9b., -■-). Addition of low conc. of G6P (50 uM) at 0 (Fig. 9a., -□-) or 10 min. (Fig. 9a., -■-) could not reverse the inhibition caused by diamide. These results are consistent with the observations made by Kan *et al.*, (1988) using GSSG.

Addition of 0.5 mM DTT to 1.0 mM diamide-treated wheat germ lysates could not reverse the protein synthesis inhibition caused by diamide (Fig. 10, -A-) but addition of equimolar concentrations of DTT reversed the inhibition (-A-). Since wheat germ lysates are gel-filtered and therefore are devoid of low molecular weight compounds, we have added NADP along with G6P to see if generation of physiological levels of NADPH can reverse the protein synthesis inhibition in the presence of diamide (Fig. 11). Addition of G6P (50 uM; -•-) alone did not affect wheat germ translation but presence of NADP (50 uM; -V-) or G6P + NADP (50 uM each, -V-) reduced protein synthesis marginally compared to when NADP was present alone. The combination of G6P and NADP also failed to reverse the inhibition caused by diamide (-■-). As PQQ is a better reductant than NADPH, we studied the effect of PQQ along with G6P and NADP. PQQ alone (-A-) or in combination with G6P and NADP did not affect the protein synthesis (-A-) and the combination failed to reverse the diamide induced inhibition. We have not tried using 0.5 mM of G6P, as in reticulocyte lysates, since G6P inhibits wheat germ protein synthesis at this concentration (Fig 12., -■-) Similarly nicotinamide compounds also inhibited wheat germ protein synthesis (Fig. 12).

Diaphorase, a lipoyl dehyrogenase enzyme (Massey, 1958), at physiological pH produces NADPH from NADP is used here as an alternate physiological system for the production of NADPH instead of G6P. Addition of diaphorase inhibited protein synthesis in a concentration dependent manner. 50% inhibition is seen with 10 U of Diaphorase (Fig 13, -V-).

Fig. 9 Effect of G6P on diamide induced inhibition of hemin-supplemented reticulocyte lysate protein synthesis.

Standard reticulocyte lysate protein synthesis assays (25 µl) were carried out at 30°C for 60 minutes as described in 'Materials and Methods' in the presence of 20 uM hemin. Whereever present, diamide (250µM) was added at 0 min. G6P was supplemented either at 0 or 10 min. of protein synthesis as shown in the figure. The assay was carried out with 50µM (Fig. 9a) and 500 µM (Fig. 9b) of G6P. A control reaction, without any additions (O-O, +heme) was also carried out to assess the normal protein synthesis.

Protein synthesis was assessed by the incorporation of [^{14}C]leucine into acid precipitable protein in a 5 µl aliquot of the reaction mixtures, taken at different time intervals (10, 30 and 60), as described in 'Materials and Methods'.

Fig. 9a.

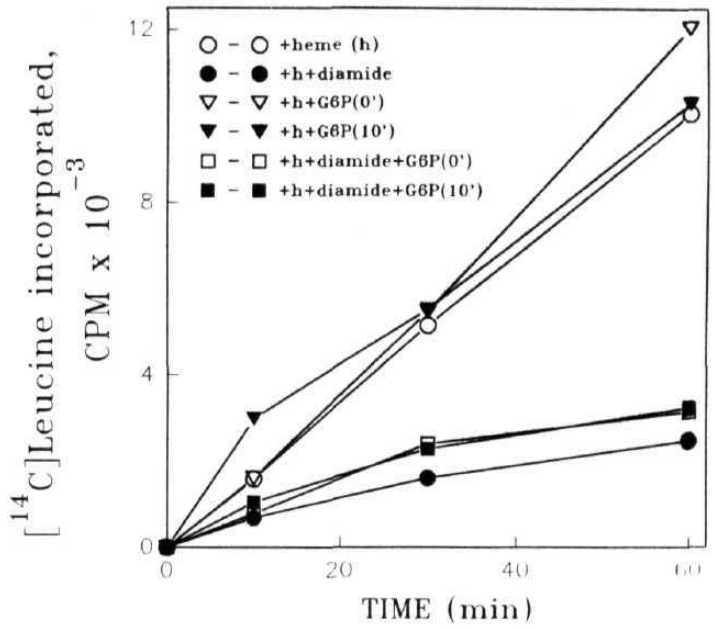

12
8
4
0
0
20
40
60
TIME (min)
[14C]Leucine incorporated, CPM x 10-3
+heme (h)
+h+diamide
+h+G6P(0')
+h+G6P(10')
+h+diamide+G6P(0')
+h+diamide+G6P(10')

Fig. 9b.

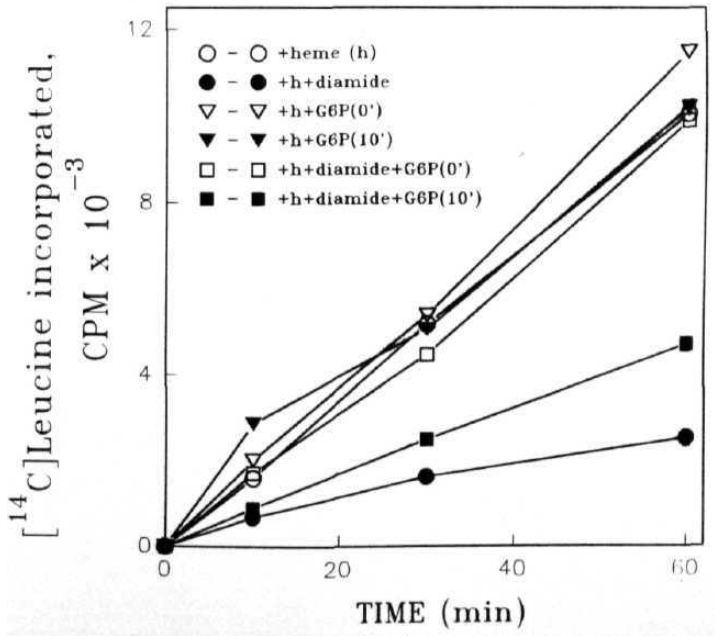

12
8
4
0
0
20
40
60
TIME (min)
[14C]Leucine incorporated, CPM x 10-3
+heme (h)
+h+diamide
+h+G6P(0')
+h+G6P(10')
+h+diamide+G6P(0')
+h+diamide+G6P(10')

Fig. 10 Effect of **diamide** on Wheat germ lysate protein synthesis.

Standard lysate protein synthesis assays (25|il) were **carried** out in the presence of BMV RNA (15 **μg/ml) at** 25°C for 45 minutes as described in 'Materials and Methods'. Different concentrations of diamide (0.5 and 1.0 mM) were added at 0 **min**. of protein synthesis as shown in the figure. The ability of DTT to reverse the diamide effect was also monitored. DTT (0.5 or 1.0 **mM)** was added at the start of incubation to lysates which are treated with or without diamide (1.0 **mM)**. A control reaction, without any additions (**O-O**, +mRNA), was carried out to assess the optimal protein synthesis. A reaction without the addition of BMV RNA **(●-●, -mRNA)** was also carried out to assess the endogenous lysate protein synthesis.

Protein synthesis was assessed by the incorporation of **[^{35}S]methionine** into acid precipitable protein in a 5 **μl** aliquot of the reaction mixtures, taken at different time intervals (15, 30 and 45), as described in 'Materials and Methods'.

Fig. 10

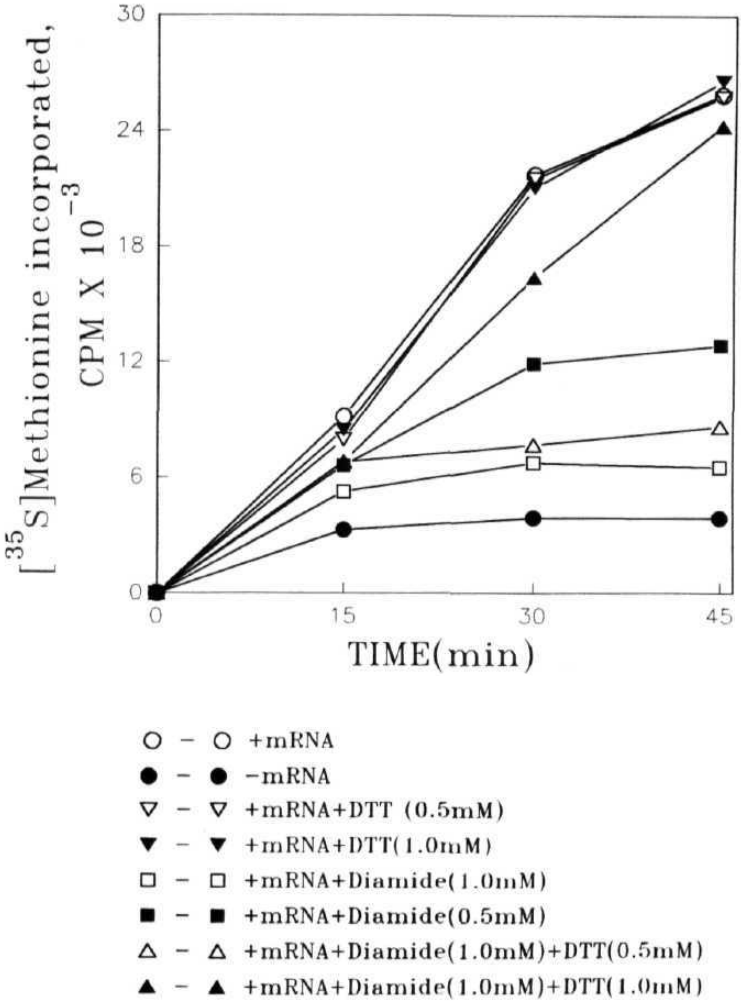

[35S]Methionine incorporated, CPM X 10-3
30
24
18
12
6
0
0
15
30
45
TIME(min)
○ – ○ +mRNA
● – ● –mRNA
▽ – ▽ +mRNA+DTT (0.5mM)
▼ – ▼ +mRNA+DTT(1.0mM)
□ – □ +mRNA+Diamide(1.0mM)
■ – ■ +mRNA+Diamide(0.5mM)
△ – △ +mRNA+Diamide(1.0mM)+DTT(0.5mM)
▲ – ▲ +mRNA+Diamide(1.0mM)+DTT(1.0mM)

Fig. 11 Effect **of G6P, NADP and PQQ on diamide induced inhibition in Wheat germ lysate protein synthesis.**

Standard lysate protein synthesis assays (25 **μl)** were **carried** out in the presence of BMV RNA **(15 μg/ml)** at **25°C** for 45 minutes as described in 'Materials and Methods'. G6P, NADP or PQQ or the combinations of these were added to diamide (1.0 **mM)**-treated and untreated lysates as shown in the figure. A control reaction, without any additions **(O-O, +mRNA),** was carried out to assess the optimal protein synthesis.

Protein synthesis was assessed by the incorporation of **[^{35}S]methionine** into acid precipitable protein in a **5 μl** aliquot of the reaction mixtures, taken at different time intervals **(15,** 30 and 45), as described in 'Materials and Methods'.

Fig. 11

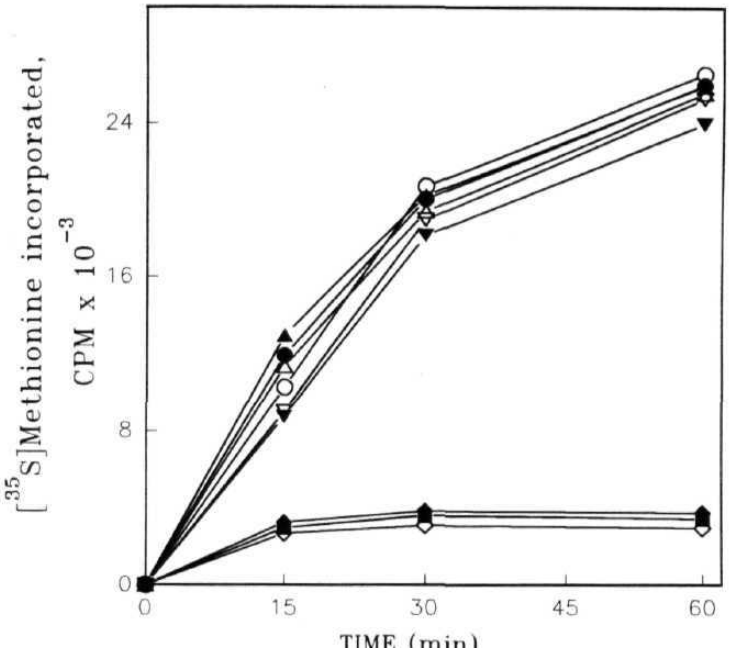
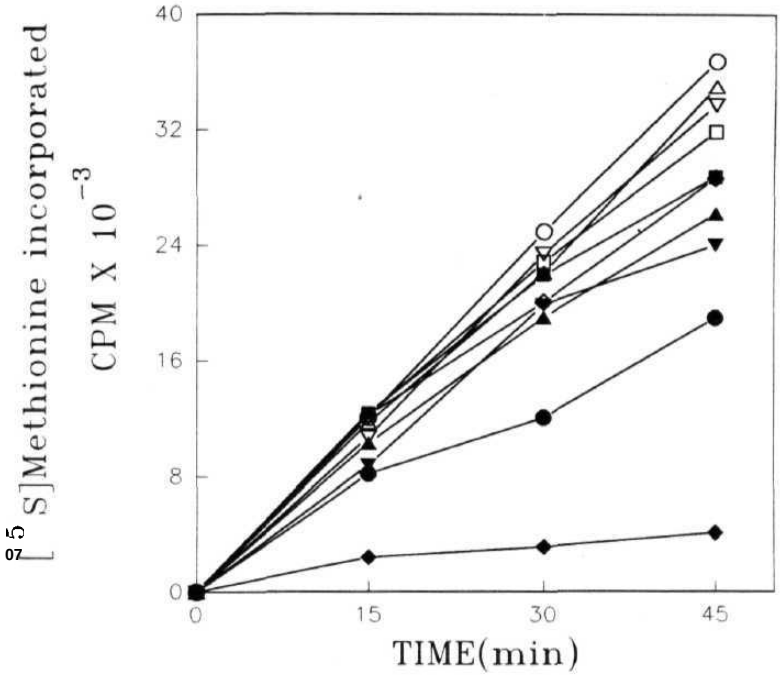

[35S]Methionine incorporated, CPM x 10-3
24
16
8
0
0
15
30
45
60
TIME (min)
○ – ○ +mRNA
● – ● +G6P
▽ – ▽ +NADP
▼ – ▼ +G6P+NADP
□ – □ +Diamide (DA)
■ – ■ +DA+G6P+NADP
△ – △ +PQQ
▲ – ▲ +PQQ+G6P+NADP
◆ – ◆ +DA+PQQ+G6P+NADP
◇ – ◇ +Diamide+PQQ

Fig. 12 Effect of G6P, NADP, NADPH and NADH on Wheat germ lysate protein synthesis.

Standard lysate protein synthesis assays (25µl) were carried out in the presence of BMV RNA (15 µg/ml) at 25°C for 45 minutes as described in 'Materials and Methods'. Two concentrations (50 µM and 500 µM) each of G6P, NADP, NADPH or NADH were added to the reaction mixtures as shown in the figure. A control reaction, without any additions (O-O, +mRNA), was carried out to assess the optimal protein synthesis. A reaction without the addition of BMV RNA (•-•, -mRNA) was also carried out to assess the endogenous lysate protein synthesis.

Protein synthesis was assessed by the incorporation of [^{35}S]methionine into acid precipitable protein in a 5 µl aliquot of the reaction mixtures, taken at different time intervals (15, 30 and 45), as described in 'Materials and Methods'.

Fig. 12



○ – ○ +mRNA
● – ● +mRNA+NADPH(0.5mM)
▽ – ▽ +mRNA+NADPH(0.05mM)
▼ – ▼ +mRNA+NADP(0.5mM)
□ – □ +mRNA+NADP(0.05mM)
■ – ■ +mRNA+Glu6P(0.5mM)
△ – △ +mRNA+Glu6P(0.05mM)
▲ – ▲ +mRNA+NADH(0.5mM)
◇ – ◇ +mRNA+NADH(0.05mM)
◆ – ◆ −mRNA

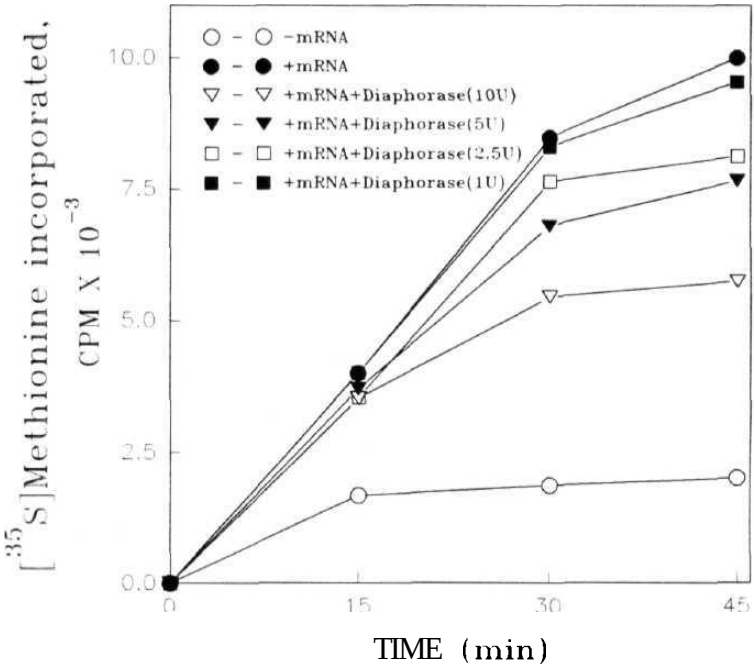


Fig. 13 Effect of diaphorase on wheat germ lysate protein synthesis.

Standard lysate protein synthesis assays (25 μl) were carried out in the presence of BMV RNA (15 μg/ml) at 25°C for 45 minutes as described in 'Materials and Methods". Different concentrations of diaphorase (1.0 - 10.0 U) was added at 0 min. of protein synthesis as shown in the figure. A control reaction, without any additions (•-•, +mRNA), was carried out to assess the optimal protein synthesis. A reaction without the addition of BMV RNA (O-O, -mRNA) was also carried out to assess the endogenous lysate protein synthesis.

Protein synthesis was assessed by the incorporation of [S]methionine into acid precipitable protein in a 5 μl aliquot of the reaction mixtures, taken at different time intervals (15, 30 and 45), as described in 'Materials and Methods'.

3.1.2 Effect of purified, heterologous kinases on wheat germ protein synthesis:

Previously an inhibitor was partially purified from wheat germ lysate which was shown to phosphorylate the small subunit of wheat germ eIF-2 and rabbit reticulocyte eIF-2 (Ranu, 1980). The same author had also used purified eIF-2α kinase from heme-deficient rabbit reticulocyte to phosphorylate the wheat germ eIF-2 in wheat germ lysates. Addition of HRI to wheat germ lysates did not diminish wheat germ protein synthesis (Ranu, 1980; Benne *et al.*, 1980). Consistent with these findings, addition of pure, activated HRI (prephosphorylated HRI) preparation obtained from heme-deficient rabbit reticulocyte lysates did not significantly alter wheat germ protein synthesis (Fig. 14).

In plant systems, it has been shown that tobacco mosaic virus infection or the addition of dsRNA to uninfected host cell extracts leads to the enhanced phosphorylation of a host encoded protein, P68, suggesting the involvement of a dsRNA dependent protein kinase like activity in the regulation of protein synthesis (Crum *et al.*, 1989). However, in our present study, addition of pure dsRNA (Fig. 14, 20 ng/ml; 40 ng/ml), obtained from reovirus, or poly IC (Fig. 15., 0.01-25 μg/ml) to wheat germ lysate did not inhibit protein synthesis. The inability of dsRNA or poly IC to inhibit protein synthesis in wheat germ extracts may be due to the absence of a right protein kinase, like P68, which can be activated by the dsRNA. These agents were however found to inhibit reticulocyte lysate protein synthesis (Babu and Ramaiah, 1996). Interestingly, other heterologous kinases like casein kinase II (Fig 16., lng, -•-) and lck (Fig. 17., -V-) inhibited wheat germ lysate protein synthesis. However HRI, which does not inhibit the wheat germ lysate translation phosphorylates the p41-42 doublet (see section on phosphorylation) in wheat germ eIF-2 like purified CK-II. In addition to the phosphorylation of p41-42 doublet, CK-II also phosphorylates the small subunit, p36, of wheat germ eIF-2 along with some other proteins (Krishna, Ph.D thesis). Hence, further experiments have been carried out (Please see Chapter 4, section 4.3.1) to determine if the enhanced eIF-2 phosphorylation by CK-II can inhibit the guanine nucleotide exchange activity and is the cause for protein synthesis inhibition.

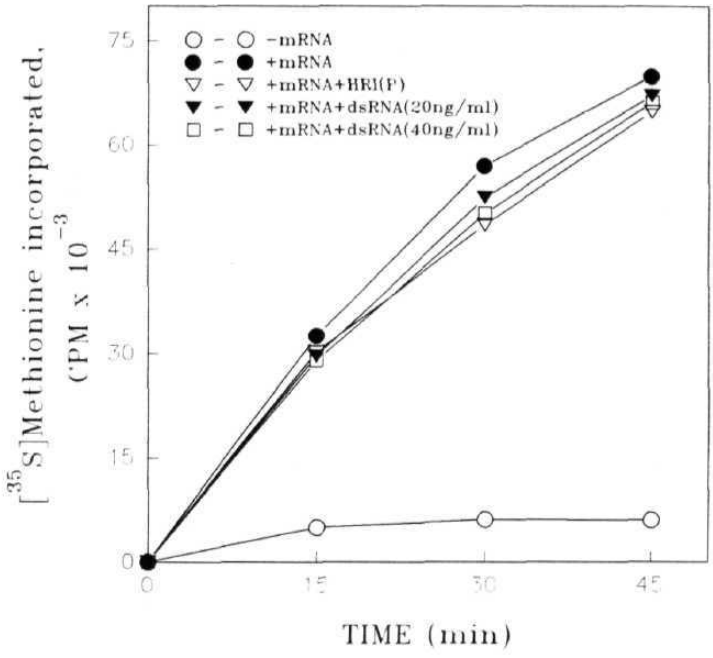


Fig. 14 Effect of activated **HRI** |**HR1(P)]** and dsRNA on wheat germ lysate protein synthesis.

Standard lysate protein synthesis assays **(25**μl) were carried out in the presence of BMV RNA (15 μg/ml) at 25"C for 45 minutes as described in 'Materials and Methods'. Prephosphorylated activated HRI **[HRI(P)]** or different concentrations of dsRNA (20 and 40 ng/ml) were added to the reaction mixtures, at 0 min. of protein synthesis, as shown in the figure. A control reaction, without any additions (•-•. -t-mRNA). was carried out to assess the optimal protein synthesis. A reaction without the addition of BMV RNA **(O-O, -mRNA)** was also carried out to assess the endogenous lysate protein synthesis.

Protein synthesis was assessed by the incorporation of [' S]methionine into acid precipitable protein in a 5 μl aliquot of the reaction mixtures, taken at different time intervals **(15,** 30 and **45),** as described in 'Materials and Methods'.

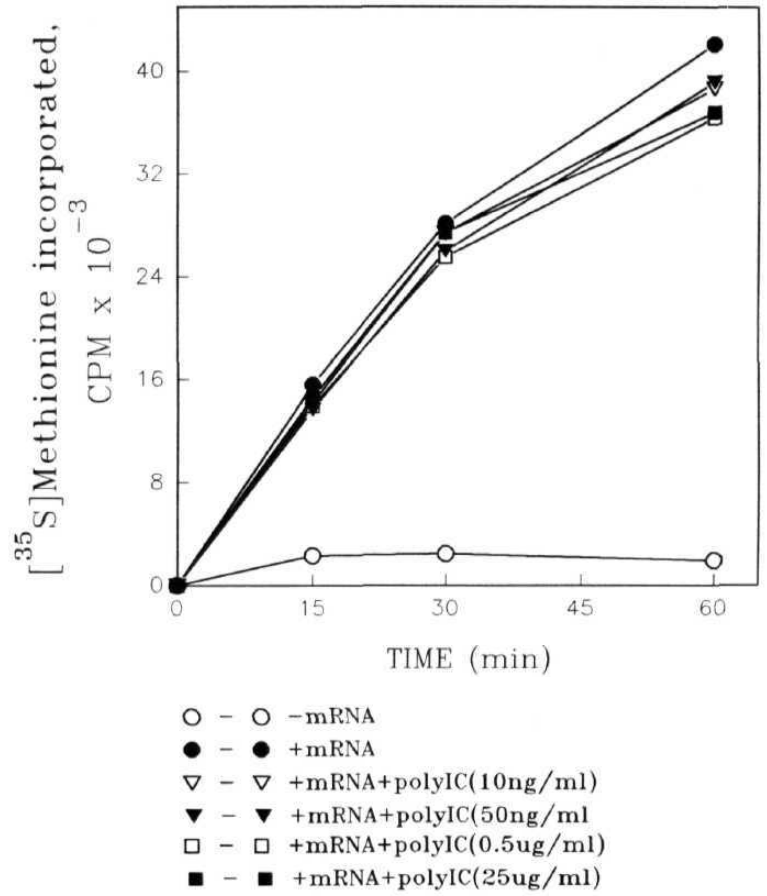


Fig. 15 Effect of poly **IC** on wheat germ lysate protein synthesis.

Standard lysate protem synthesis assays **(25µl)** were carried out in the presence of **BMV** RNA (15 **µg/ml)** at 25°C for 45 minutes as described in 'Materials and Methods'. Different concentrations of poly IC (10 **ng/ml** - 25 **µg/ml)** were added to the reaction mixtures, at 0 min. of protein synthesis, as shown in the figure. A control reaction, without any additions (•-•, +mRNA), was carried out to assess the optimal protein synthesis. A reaction without the addition of BMV RNA **(O-O, -mRNA)** was also carried out to assess the endogenous lysate protein synthesis.

Protein synthesis was assessed by the incorporation of **[^{35}S]methionine** into acid precipitable protein in a 5 **µl** aliquot of the reaction mixtures, taken at different time intervals (15, 30 and 45), as described in 'Materials and Methods'.

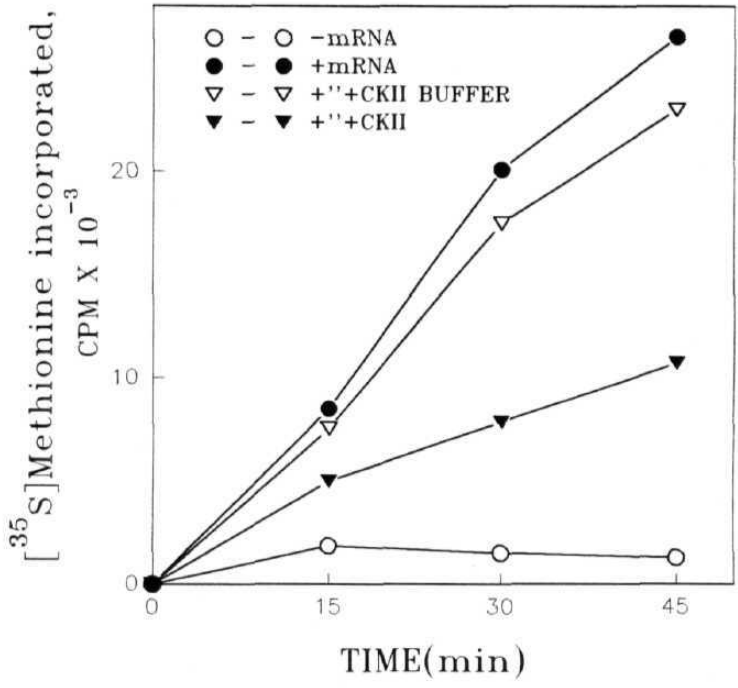


Fig. 16 Effect of casein kinase II (CK-II) on wheat germ lysate protein synthesis.

Standard lysate protein synthesis assays (25 µl) were carried out in the presence of BMV RNA (15 µg/ml) at 25°C for 45 minutes as described in 'Materials and Methods'. The enzyme CK-II (40 ng/ml) or the CK-II buffer (the buffer in which the enzyme was stored) were added to the reaction mixtures, at 0 min. of protein synthesis, as shown in the figure. A control reaction, without any additions (•-•, +mRNA), was carried out to assess the optimal protein synthesis. A reaction without the addition of BMV RNA (O-O, -mRNA) was also carried out to assess the endogenous lysate protein synthesis.

Protein synthesis was assessed by the incorporation of [^{35}S]methionine into acid precipitable protein in a 5 µl aliquot of the reaction mixtures, taken at different time intervals (15, 30 and 45), as described in 'Materials and Methods'.

3.1.3 Effect of phosphatases and kinase inhibitors on wheat germ protein synthesis:

Addition of genistein, a tyrosine kinase inhibitor, did not have any effect on wheat germ translation (Fig. 18, 50 μM, -V-). Protein synthesis was also carried out in the presence of DMSO (0.05% final, -•-) as genistein was prepared in it. Okadaic acid, a serine-theronine phosphatase inhibitor, in the range of 1nM-1μM did not inhibit wheat germ lysate protein synthesis (Fig. 20). The effect of purified protein phosphatases on wheat germ lysate translation was also studied (Fig. 19). The buffers in which the phosphatases were stored were found to be inhibitory to wheat germ lysate protein synthesis (PP1 buffer, -•-; PP2A buffer -T-). Addition of PP1 brought down protein synthesis (-V-) further, while PP2A stimulated protein synthesis marginally (-D-) over their respective controls. Since addition of type 1 phosphatase inhibits protein synthesis and type 2A stimulates, albeit marginally, it suggests that a standard amount of phosphorylation of these proteins is required for protein synthesis. Overall, the effects caused by the addition of purified type 1 and type 2 phosphatases do not appear to be very significant to study the protein synthesis regulation.

Addition of okadaic acid, an inhibitor of type 1 and type 2 protein phosphatases did not cause any marked inhibition on wheat germ translation (Fig. 20). This is consistent with the observation that no endogenous EF-2 kinase activity has been observed *in vitro* or *in vivo* (Smailov *el al.*, 1993). In contrast, low concentration of okadaic acid (25-50 nM) can inhibit protein synthesis significantly in reticulocyte lysates due to the enhanced phosphorylation of EF-2 (Redpath and Proud, 1989; Babu and Ramaiah, 1996).

Analysis of the overall results indicate that :

- Oxidizing and alkylating agents can cause potent inhibition of protein synthesis, some of which can be reversed by the addition of reducing agents, suggesting that some how the redox levels play a role in the regulation of protein synthesis.
- Genistein, an inhibitor of tyrosine phosphorylation does not effect wheat germ translation. In contrast, lck kinase, a partially purified tyrosine kinase is found to inhibit wheat germ protein synthesis. These findings suggest that probably the lysates

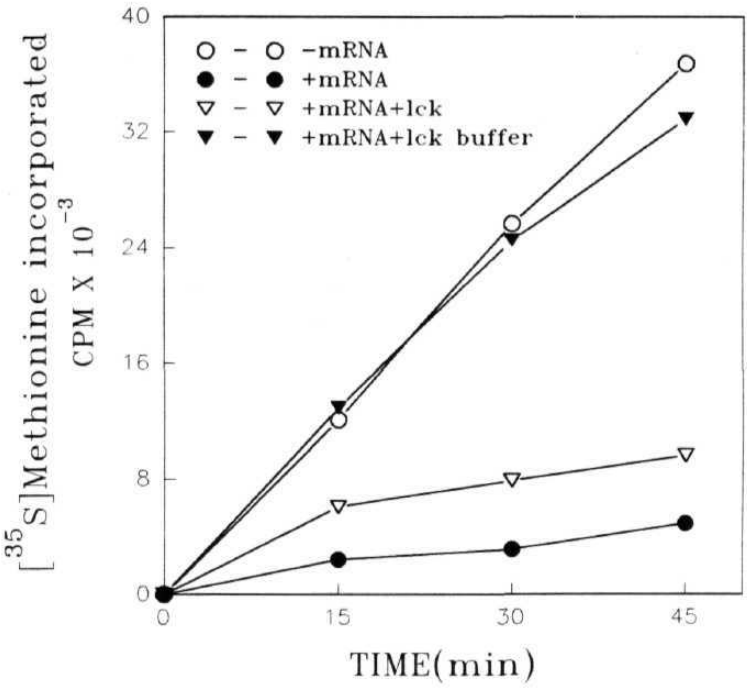


Fig. 17 Effect of lck on wheat germ lysate **protein synthesis.**

Standard lysate protein synthesis assays (25 µl) were carried out in the presence of BMV RNA (15 µg/ml) at 25°C for 45 minutes as described in 'Materials and Methods'. One microliter of lck or a microliter of lck buffer (buffer in which the lck enzyme was stored) were added to the reaction mixtures, at 0 min. of protein synthesis, as shown in the figure. A control reaction, without any additions (O-O, —mRNA), was carried out to assess the optimal protein synthesis. A reaction without the addition of BMV RNA (•-•, +mRNA) was also carried out to assess the endogenous lysate protein synthesis.

Protein synthesis was assessed by the incorporation of [^{35}S]methionine into acid precipitable protein in a 5 µl aliquot of the reaction mixtures, taken at different time intervals (15, 30 and 45), as described in 'Materials and Methods'.

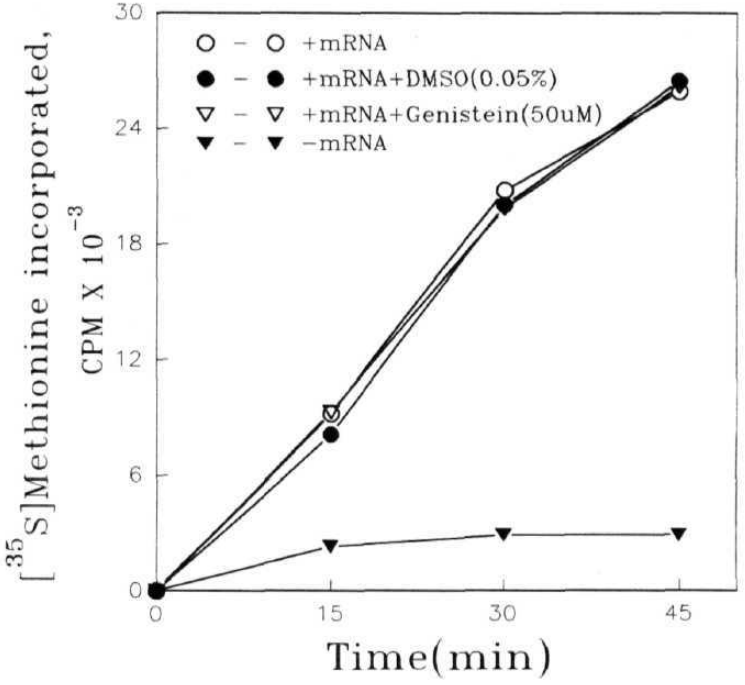


Fig. 18 Effect of genistein on wheat germ lysate protein synthesis.

Standard lysate protein synthesis assays (25 µl) were carried out in the presence of BMV RNA (15 µg/ml) at 25°C for 45 minutes as described in 'Materials and Methods'. Genistein (50 µM) or DMSO (0.05%) (concentration of DMSO present in 50 µM genistein solution) were added to the reaction mixtures, at 0 min. of protein synthesis, as shown in the figure. A control reaction, without any additions (O-O, +mRNA), was carried out to assess the optimal protein synthesis. A reaction without the addition of BMV RNA (▼-▼, -mRNA) was also carried out to assess the endogenous lysate protein synthesis.

Protein synthesis was assessed by the incorporation of [^{35}S]methionine into acid precipitable protein in a 5 µl aliquot of the reaction mixtures, taken at different time intervals (15, 30 and 45), as described in 'Materials and Methods'.

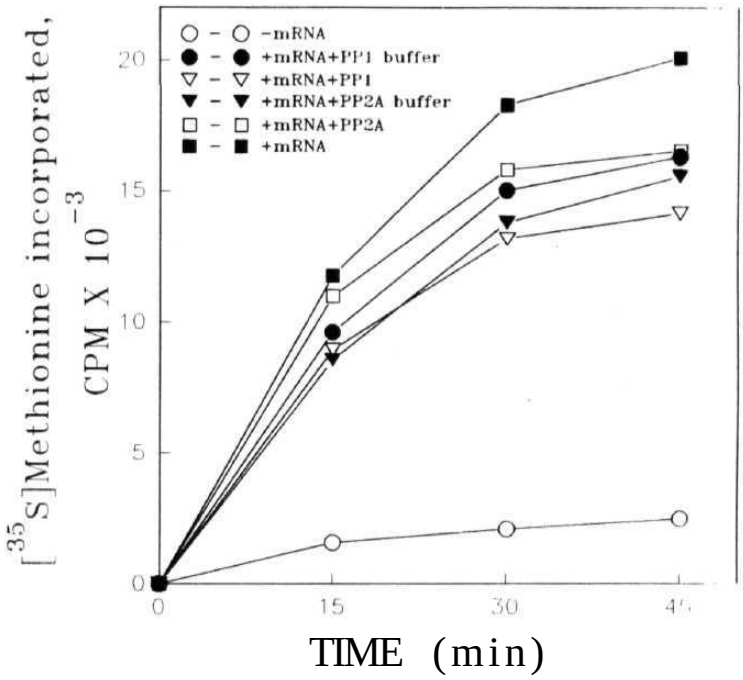


Fig. 19 Effect of purified protein phosphatases on wheat germ lysate protein synthesis.

Standard lysate protein synthesis assays (25 µl) were carried out in the presence of BMV RNA (15 µg/ml) at 25°C for 45 minutes as described in 'Materials and Methods'. One microliter of purified protein phosphatase type 1 or type 2A was added to the reaction mixtures, at 0 min. of protein synthesis, as shown in the figure. A control reaction, without any additions (■-■, +mRNA), was carried out to assess the optimal protein synthesis. Control reactions were also carried out without the addition of purified phosphatases, but these reactions contained the buffers in which the respective phosphatases (type 1 and type 2A) were stored. Endogenous lysate protein synthesis was assessed by eliminating BMV RNA in one of the reactions (○-○, -mRNA).

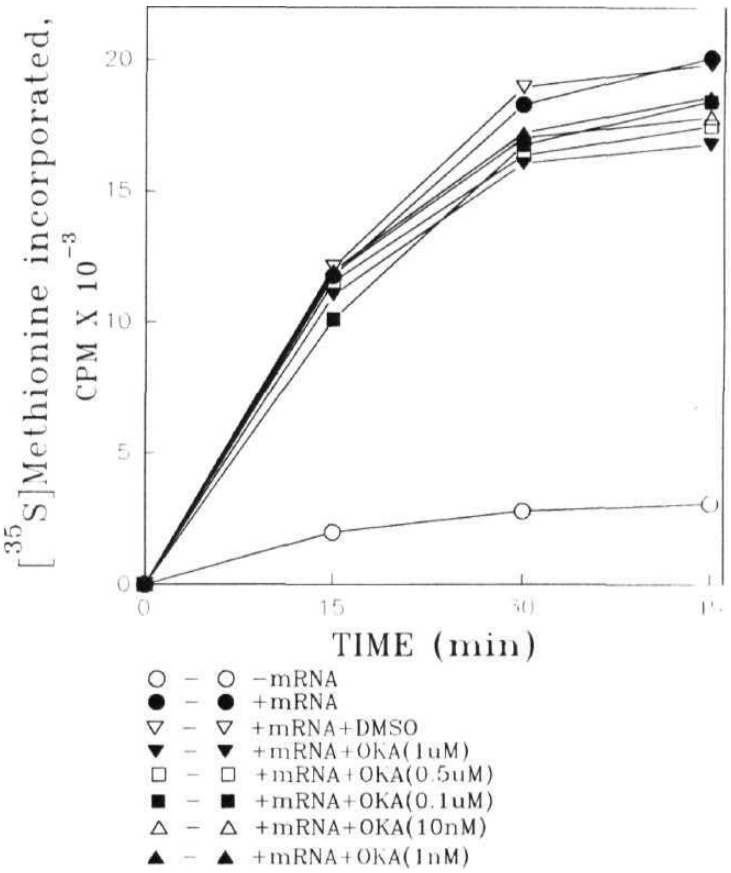
Protein synthesis was assessed by the incorporation of [^{35}S]methionine into acid precipitable protein in a 5 µl aliquot of the reaction mixtures, taken at different tinae intervals (15, 30 and 45), as described in 'Materials and Methods'.

Fig. 20 Effect of okadaic acid (OKA) on wheat germ lysate protein synthesis.

Standard lysate protein synthesis assays (25 μl) were carried out in the presence of BMV RNA (15 μg/ml) at 25°C for 45 minutes as described in 'Materials and Methods'. Different concentrations of OKA (1 nM - 1 μM) were added to the reaction mixtures, at 0 min. of protein synthesis, as shown in the figure. The effect of 0.05% DMSO (V-V) on Wheat germ lysate protein synthesis was also studied since OKA was prepared in DMSO. The concentration of DMSO tested corresponded to its concentration present in the higest concentration of OKA tested (1 μM). A control reaction, without any additions (•-•, +mRNA), was carried out to assess the optimal protein synthesis. A reaction without the addition of BMV RNA (O-O, -mRNA) was also carried out to assess the endogenous lysate protein synthesis.

Protein synthesis was assessed by the incorporation of [^{35}S]methionine into acid precipitable protein in a 5 μl aliquot of the reaction mixtures, taken at different time intervals (15, 30 and 45), as described in 'Materials and Methods'.

Fig. 20


[35S]Methionine incorporated, CPM X 10-3
20
15
10
5
0
0
15
30
TIME (min)
−mRNA
+mRNA
+mRNA+DMSO
+mRNA+OKA(1uM)
+mRNA+OKA(0.5uM)
+mRNA+OKA(0.1uM)
+mRNA+OKA(10nM)
+mRNA+OKA(1nM)

do not carry a tyrosine linase like activity. However, tyrosine phosphorylation may influence translational rates *in vitro.*

- Addition of okadaic acid a potent inhibitor of type I and type 2 serine-threionine phosphatases has however failed to elicit any significant reaction on protein synthesis, whereas addition of protein phosphatase I is found to inhibit and phosphatase 2A increases protein synthesis albeit marginally. These results are interesting when compared to the effects of okadaic acid on protein synthesis in reticulocyte lysates. In reticulocytes a type I phosphatase is implicated in the dephosphorylation of eIF-2a and type 2A phosphatase is implicated in the regulation of EF-2 phosphorylation Low concentrations of okadaic acid (25-50 nM) are found to inhibit reticulocyte protein synthesis through enhancement of EF-2 phosphorylation. Mammalian and plant type 1 and type 2A protein phosphatases have indistinguishable properties in terms of their sensitivity towards inhibition with okadoic acid and mammalian inhibitors 1 and 2 (MacKintosh and Cohen, 1989). The role of type 1 and type 2 protein phosphatases are implicated in translational regulation of mammalian protein synthesis (Cohen *et al.*, 1990). Based on these findings, the results here suggest that probably type 1 and type 2 protein phosphatase activity may not play any role in the regulation of wheat germ translation. This may also be due to the absence of active kinases in wheat germ lysate which can phosphorylate crucial translational factors like eIF-2 and EF-2 and inhibit protein synthesis.

Since one of the main objectives here is to identify the conditions that can promote wheat germ eIF-2 phosphorylation, we have used our rationale and judgement to select some of these compounds here for our further work to study the endogenous eIF-2 phosphorylation. The rationale is based on the preliminary results obtained on protein synthesis and the available resources.

3.2 Phosphorylation ***studies with translating wheat germ lysates:***

3.2.1 Effects of different -SH group modifying agents on wheat germ lysate phosphorylation:

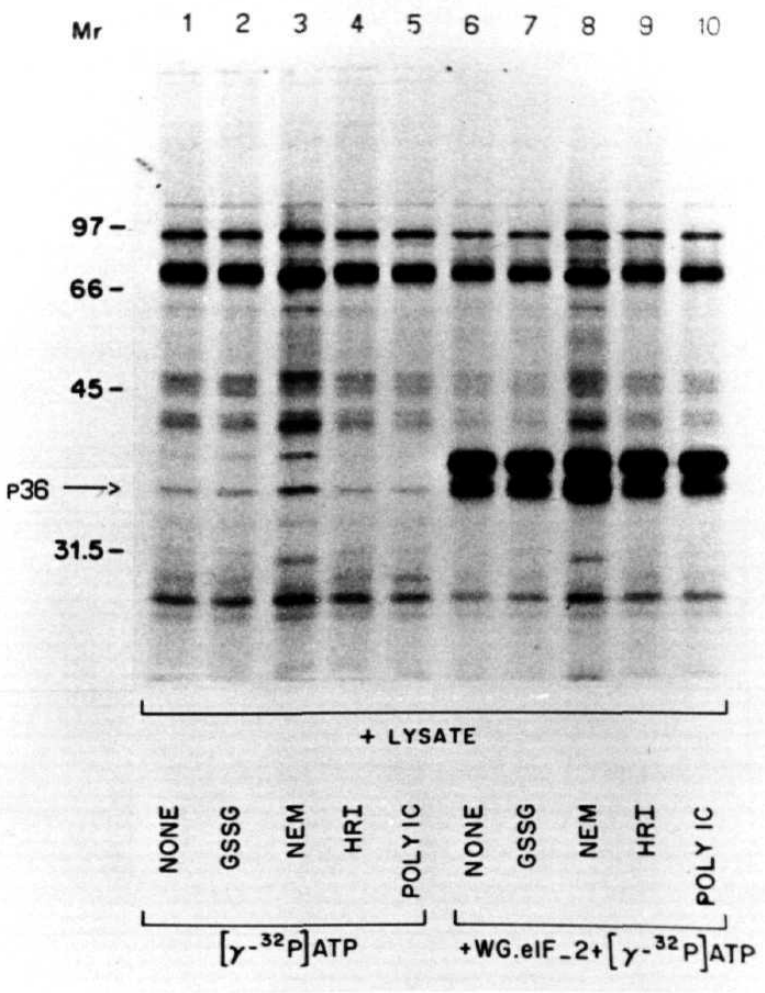
In order to determine the conditions that can enhance the phosphorylation of wheat germ proteins (possibly including eIF-2a subunit) and inhibit protein synthesis, we have used agents which act on protein -SH groups such as oxidized glutathione (GSSG), dithiothrietol (DTT), N-ethylmaleimide (NEM) and diamide. The selection of these agents is also based on the fact that they are known to enhance the reticulocyte eIF-2a phosphorylation (except for diamide, whose effects on reticulocyte eIF-2a phosphorylation are not investigated)

3.2.2 -SH reactive agents such as NEM and DTT enhance the phosphorylation of wheat germ lysate proteins including p36 of eIF-2 :

In our experiments here, it has been observed that GSSG and NEM (1 mM) (Fig. 6) are found to inhibit protein synthesis in wheat germ lysate. The extent to which these -SH reactive agents, which are known to activate the reticulocyte eIF-2α kinase(s) (Ernst *et al*, 1978; Hunt, 1979; Kan *et al*, 1988, Chen *et al*, 1989) can phosphorylate wheat germ eIF-2 in translating wheat germ lysates was investigated. Our studies showed that GSSG, which was shown to enhance reticulocyte eIF-2a phosphorylation (Kan *et al*, 1988), did not alter the phosphorylation of wheat germ lysate proteins (Fig. 21, lane 2 vs control lane 1), or that of exogenously added eIF-2 in wheat germ lysate (Fig 21, lane 7 vs lane 5). Under the same conditions, NEM treatment (1.0 mM) significantly enhanced the phosphorylation of several lysate proteins (Fig 21, lane 3 vs lane 1) including the small subunit, p36, of added wheat germ eIF-2 (Fig. 21, lane 8 vs lane 6). This probably suggests that the protein synthesis inhibition observed here in the presence of GSSG is not due to the activation of any protein kinase(s) but could be simply due to the potent oxidizing conditions created by GSSG. This may have an effect on the functioning of eIF-2B like proteins involved in translation, because mammalian eIF-2B protein has been found associated with NADPH (Dholakia *et al.*, 1986). It has been reported that the

Fig. 21 **[^{32}P]Phosphoprotein** profiles of wheat germ lysate and purified wheat germ **eIF-2.**

Wheat germ lysate protein synthesis was carried out in the presence of GSSG (0.5 **mM**, lanes 2 and 7), NEM **(1 mM**, lanes 3 and 8), **HRI (~50** ng, lanes 4 and 9), or poly **IC** (100 ng/mL lanes 5 and 10) at 25°C for 10 **min**. Ten **microliters** of protein synthesis reactions was then supplemented with 5 **µl** of **Tris-HCl** buffer (20 mM, pH 7.8) containing **[γ-^{32}P]ATP** (10 **µCi)** (lanes 1-5). Lanes 6-10 are reaction mixtures similar to I-5 but the reactions were carried out in the presence of **exogenously** added purified ell -2 **(~200** ng). Lanes 1 and 6 are control lysate reactions without and with added eIF-2. The final reaction mixtures containing 2.5 **mM Mg^{2+}** were incubated at 25°C for 5 min. Aliquots of 7.5 **µl** of the reaction mixtures were withdrawn and separated on SDS-PAGE as described in 'Materials and Methods'.


Mr
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6
7
8
9
10
97-
66-
45-
P36 ⟶
31.5-
+ LYSATE
NONE
GSSG
NEM
HRI
POLY IC
NONE
GSSG
NEM
HRI
POLY IC
[γ-32P]ATP
+WG.eIF_2+[γ-32P]ATP

oxidation of this bound NADPH to NADP would result in diminished activity of eIF-2B (Dholakia *et al.*, 1986 ; Kan *et al.*, 1988).

NEM enhanced phosphorylation of the lysate proteins has been tested here further to see if this is reversible by the addition of DTT. DTT is known to neutralize the action of NEM (Hunt, 1979). Neutralization of NEM with DTT should reverse the NEM-induced phosphorylation Hence the phosphorylation experiments were carried out with and without DTT. The NEM supplemented lysates were always labelled with [γ-^{32}P]ATP between 10-15 minutes of protein synthesis and it was observed that the phosphorylation of proteins under these conditions was always enhanced This suggests that NEM may be truly activating a protein kinase(s) rather than inhibiting any protein phosphatases This is substantiated by the fact that delayed addition of [γ-^{32}P]ATP to NEM-treated lysates at different time intervals, that is, between 10-15 and 15-20 minutes of protein synthesis can also stimulate the phosphorylation of several lysate proteins including the p36 subunit of wheat germ eIF-2 (Fig. 22)

To neutralize the effect of NEM, the translating NEM-treated wheat germ lysate was supplemented with an equimolar concentration of dithiothreitol (DTT) and the pattern of protein phosphorylation was studied. Contrary to our expectations, DTT enhanced the phosphorylation of several proteins including that of exogenously added wheat germ eIF-2(p36) (Fig. 23, lane 2 vs lane 1). DTT and NEM (lane 4) together led to increased phosphorylation of wheat germ eIF-2 and other lysate proteins than when either was present alone (lanes 2 & 3). These findings suggest that protection of protein -SH groups by either reduction or alkylation can enhance the phosphorylation of several lysate proteins including p36 of eIF-2. The fact that wheat germ lysate protein phosphorylation is enhanced by the sulfhydryl reagents like NEM and DTT also suggests that protein sulfhydryl groups are important for activation of kinases. The above hypothesis based on our observations reported here is consistent with a report which indicates that disulfide bond formation by heme causes the diminution in the activity of HR1 protein, an eIF-2α kinase (Chen *et al.*, 1989). Alternatively, these agents may also protect the protein -SH groups of the substrates which may be required for efficient phosphorylation.

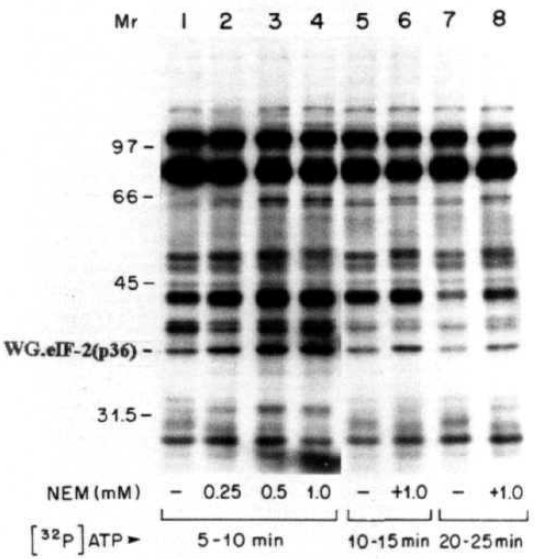

Mr
1
2
3
4
5
6
7
8
97 -
66 -
45 -
WG.eIF-2(p36) -
31.5 -
NEM (mM)
-
0.25
0.5
1.0
-
+1.0
-
+1.0
[32P] ATP ►
5-10 min
10-15 min
20-25 min

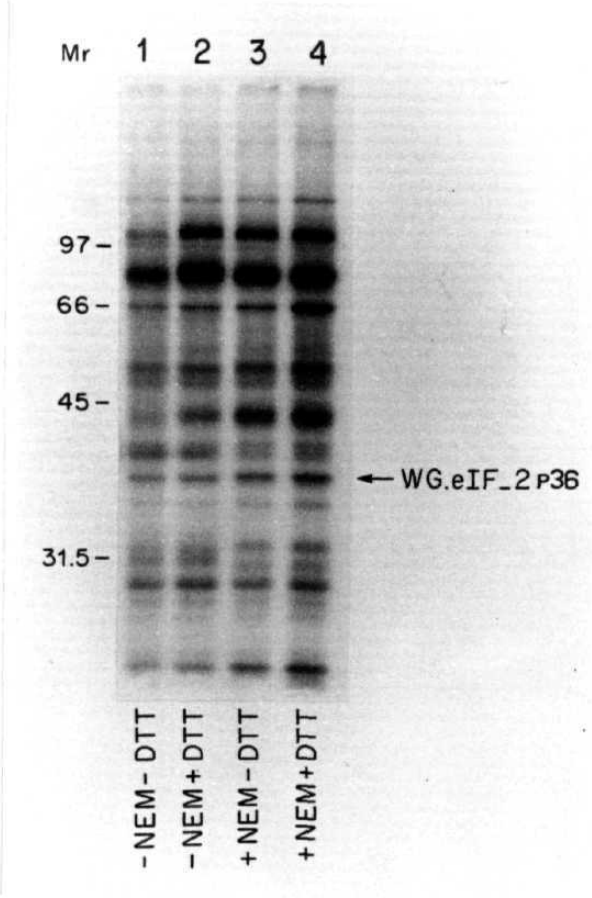
Fig. 22 **[^{32}P]Phosphoprotein** profile of wheat germ lysate during delayed addition of **[γ-^{32}P]ATP** to **NEM-treated lysates.**

Wheat germ lysate protein synthesis was carried out with and without **NEM (1mM)** at **25°C** for **10 min**. Ten microliter aliquots of protein synthesis reactions were then supplemented with 5 **µl** of **Tris-HCl** buffer (20 **mM**, pH 7.8) containing **[γ-^{32}P]ATP (10 µCi)** at different time intervals as indicated in the figure. The final reaction mixtures containing 2.5 **mM Mg^{2+}** were incubated at **25°C** for 5 min. Aliquots of 7.5 **µl** of the reaction mixtures were withdrawn and separated on SDS-PAGE as described in 'Materials and Methods'.

Fig. 23 Phosphorylation of purified eIF-2 in NEM- and DTT-treated wheat germ **lysates.**

Wheat germ lysate protein synthesis was carried out with or without the addition of NEM (1 mM) as described in 'Materials and Methods'. Wherever indicated, at 15 min. of protein synthesis, the reaction mixtures were supplemented with DTT (1 mM). Six microliters of protein synthesizing lysate was then incubated with another 6 μl of buffer (Tris-HCl, pH 7.8) containing 2.5 mM Mg^{2+}, 10 μCi [γ-^{32}P]ATP, and 1 μl of eIF-2 (~150 ng) for 5 min. at 25°C. The reaction mixtures were terminated with the addition of SDS smaple buffer and the proteins were separated by SDS-PAGE. The figure is an autoradiogram.

Lane 1,-NEM,-DTT; lane 2, -NEM,+DTT; lane 3, +NEM,-DTT; lane 4, +NEM,+DTT.


Mr
1
2
3
4
97-
66-
45-
31.5-
WG.eIF_2 p36
-NEM-DTT
-NEM+DTT
+NEM-DTT
+NEM+DTT

The idea that phosphorylation of proteins in wheat germ lysate may require protein sulfhydryl groups has been here further substantiated by using wheat germ lysates which differ in their translational activities and also their response to NEM Fig. 24 shows the phosphorylation pattern of 3 different lysates to NEM-treatment. Reactions in lanes 1-4 contain lysate I. This lysate is translationally weak compared to lysate II and lysate III (Fig 4 I, II, and III respectively) Lysate II is optimally active lysate (lanes 5-8) and lysate III is moderately active lysate (lanes 9-12). The 'weak' lysate I was more strongly phosphorylated in the presence of NEM compared to an optimally or moderately translating lysates (Fig. 24, compare lanes 1 vs 2 with 5 vs 7 or 9 vs 11) When these lysates were subjected to heat-shock (40°C) it was observed that heat-shock caused an overall reduction in the incorporation of labelled phosphate and reduced the overall phosphorylation of lysate proteins Enhanced phosphorylation was observed in NEM-treated lysates (lanes 3 vs 4, 6 vs 8 and 10 vs 12). An analysis of these findings reveals that:

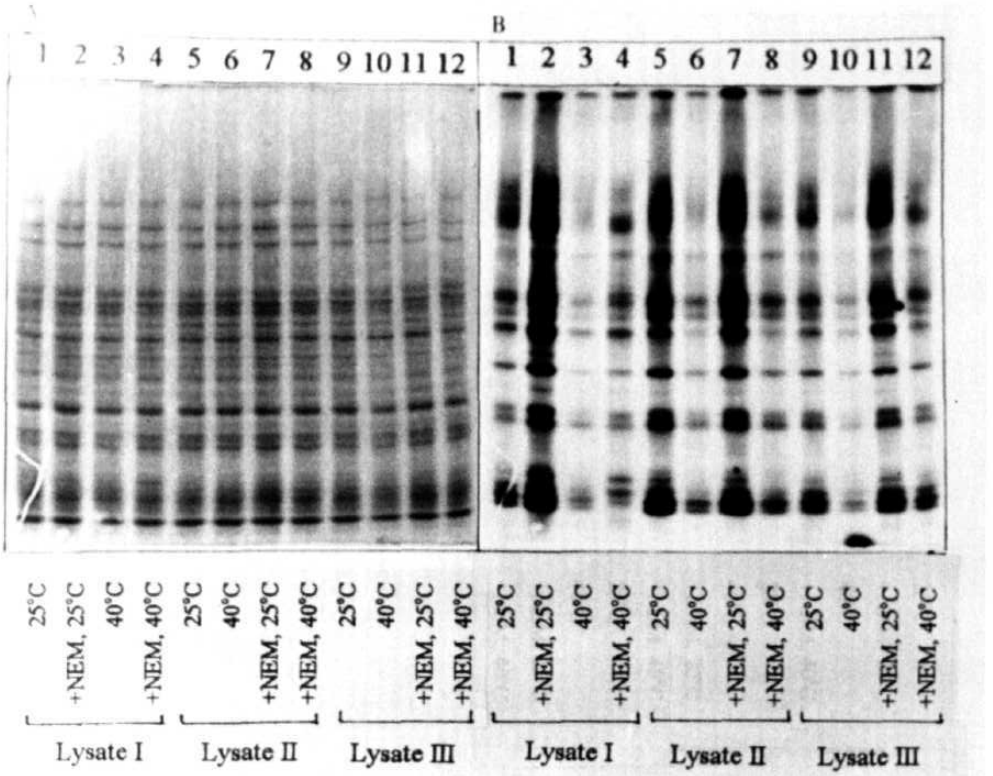
- Heat-shock decreases and NEM enhances the general lysate phosphorylation
- The effect of NEM appears to be dependent on the translational ability of the lysates used A 'weak' lysate (with low translational ability) responds more readily to the NEM-treatment.
- The phosphorylation pattern of these 3 lysates without NEM-treatment is also different. The phosphorylation of proteins of a 'weak' lysate is less compared to those of optimally or moderately translating lysates

These findings illustrate the importance of protein -SH groups presumably playing a role in the protein phosphorylation Heat-shock may be causing some amount of denaturation of proteins and can enhance the formation of disulfide bonds.

'Weak' lysates are poor in translation and this is probably due to the modification of protein sulfhydryl groups. Addition of NEM, which can alkylate -SH groups and therefore maintain them, elicits better response. Actively translating lysates showed higher

Fig. 24 [^{32}P]Phosphoprotein profiles of three batches of wheat germ lysates with and **without N KM during heat shock (40'C).**

Three batches of wheat germ lysates, with different **translational** abilities (lysate 1, weak; lysate II optimal; and lysate **III,** moderate) were used for the experiment. Protein synthesis was **carried** out in standard 25 μl reaction mixtures at 25"C and **40°C** (heat shock) for 10 **min,** with or without NEM (1 **mM)** as shown in the figure. Ten microliter aliquots of protein synthesis reactions were then supplemented with 5 μl of **Tris-HCl** buffer (20 mM, pH 7.8) containing **[γ-^{32}P]ATP** (10 **μCi)**. The final reaction mixtures containing 2.5 mM **Mg^{2+}** were incubated at **25°C** for 5 min. Aliquots of 7.5 μl of the reaction mixtures were withdrawn and separated on SDS-PAGE as described in 'Materials and Methods'.


B
1 2 3 4 5 6 7 8 9 10 11 12
1 2 3 4 5 6 7 8 9 10 11 12
25°C
+NEM, 25°C
40°C
+NEM, 40°C
Lysate I
Lysate II
Lysate III
Lysate I
Lysate II
Lysate III

phosphorylation even without NEM treatment, indicating that -SH groups may be naturally preserved in such lysates.

NEM can only be used as a probe to illustrate the point that protein phosphorylation requires -SH groups but it cannot be used to correlate the protein synthesis activity and protein phosphorylation, whereas different preparations of lysates with different translational abilities can be used to suggest that protein phosphorylation requires protein -SH groups.

In contrast, diamide, another -SH reactive agent which enhanced the eIF-2a phosphorylation in reticulocyte system (Fig. 25a.) reduced the overall phosphorylation in wheat germ lysates (Fig. 25b.) but however enhanced phosphorylation of a single polypeptide of molecular weight 45 kDa in wheat germ lysates (Fig 25, lane 2). The phosphorylation of this polypeptide was not seen when DTT was added to the diamide treated-wheat germ lysate indicating that the diamide effects could be reversed (lane 4). It would be interesting to identify this polypeptide, to study if the diamide induced inhibition of wheat germ lysate is due to the phosphorylation of this peptide or is due to the general oxidizing environment created by diamide. Addition of pyrroloquinoline quinone (PQQ), which inhibits reticulocyte lysate protein synthesis by enhancing eIF-2a phosphorylation (Ramaiah *et al.*, 1994), had no effect on wheat germ lysate protein phosphorylation (lane 6). These results indicate that wheat germ and reticulocyte systems differ in their ability to phosphorylate their lysate eIF-2 during different treatments The findings of diamide on the phosphorylation pattern of wheat germ lysate are consistent with the idea that phosphorylation of a majority of proteins, at least in wheat germ lysates, require the maintenance of protein -SH groups. The increased phosphorylation of the 45 kDa protein in diamide-treated lysates is interesting and requires to be further characterized.

3.2.3 Phosphorylation of purified wheat germ eIF-2 in reticulocyte lysate:

Whether native reticulocyte eIF-2α kinases can phosphorylate wheat germ eIF-2 was tested by adding a small amount of the latter to heme-deficient, hemin-treated, or heme and NEM-treated reticulocyte lysates (Fig. 26). Heme-deficiency, or NEM-treatment activated endogenous reticulocyte eIF-2α kinase(s) which is known to

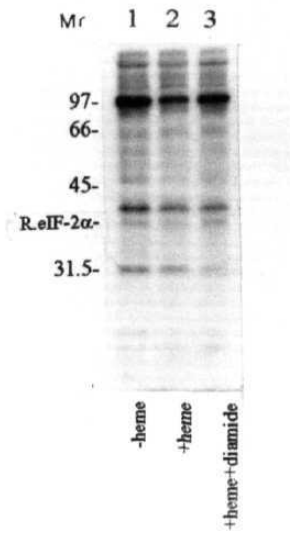


Fig. 25a. [^{32}P]Phosphoprotein profile of diamide-treated rabbit reticulocytc lysate.

Protein synthesis was carried **out** at 30"C in 10 μl reticulocyte lysates as described in 'Materials and Methods' **without** any added hemin (lanes 1), with hemin (20 μM, lanes 2). or **with** heinin and **diamide** (20 μM / 250 μM, lanes 3) **for** 10 min. A 5 μl aliquot of protein **synthesizing** lysate was then incubated with a 5 μl Tris buffer (20 mM Tris-HCl, pH 7.8) containing 10 μCi [γ-^{32}P]ATP and Mg^{2+} (1 mM) for 5 **min,** at 30"C. The reactions were then **terminated** and **the** samples separated on SOS-PAGE as described in 'Materials and Methods'. The figure is an **autoradiogram.**

Fig. 25b. **[^{32}P]Phosphoprotein profiles of wheat germ lysate.**

Wheat germ lysate protein synthesis was carried out in the presence of diamide (1.0 **mM**, lanes 2), DTT (1 **mM**, lanes 3), diamide and DTT (1.0 mM each, lanes), PQQ (500 **μM**, **lane** 5), or G6P (500 **μM**, lanes 6) at 25°C for 10 **min**. Ten **microliters** of protein synthesis reactions was then supplemented with 5 **μl** of **Tris-HCl** buffer (20 **mM**, pH 7.8) containing **[γ-^{32}P]ATP (10 μCi)** (lanes 1-5). Lane 1 is a control lysate reactions without any additions. The final reaction mixtures containing 2.5 mM **Mg^{2+}** were incubated at 25''C for 5 min. **Aliquots** of 7.5 **ul** of the reaction mixtures were withdrawn and separated on SDS-PAGE as described in 'Materials and Methods'.

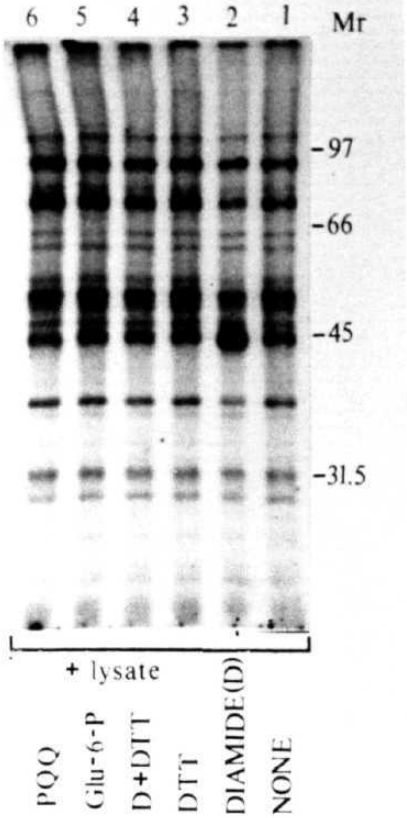
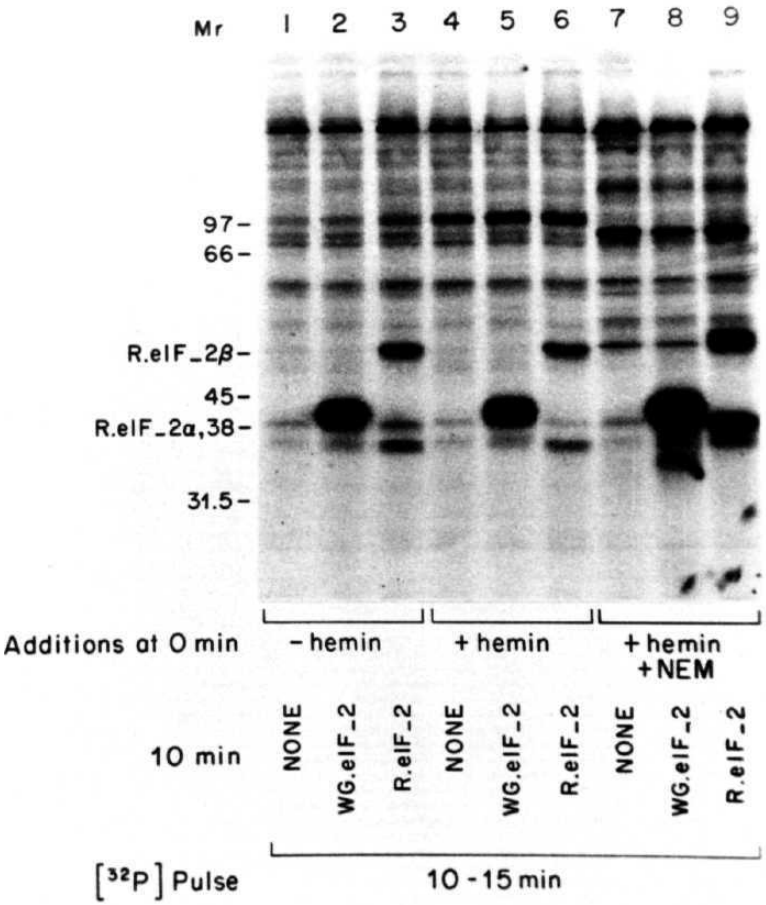

6
5
4
3
2
1
Mr
-97
-66
-45
-31.5
+ lysate
PQQ
Glu-6-P
D+DTT
DTT
DIAMIDE(D)
NONE

Fig. 26 Phosphorylation of wheat germ eIF-2 in rabbit reticulocyte lysates.

Protein synthesis was carried out at 30°C in 10 μl reticulocyte lysates as described in 'Materials and Methods' without any added hemin (lanes 1, 2, and 3), with hemin (20 μM, lanes 4, 5, and 6), or with hemin and NEM (20 μM/5mM, lanes 7, 8, and 9) for 10 min. A 5-μl aliquot of protein synthesizing lysate was then incubated with a 5 μl reaction mixture containing purified wheat germ eIF-2 (200 ng) (lanes 2, 5, and 8) or reticulocyte eIF-2 (150 ng) (lanes 3, 6, and 9), 10 μCi [γ-^{32}P]ATP and Mg^{2+} (1 mM) for 5 min. at 30°C. Protein synthesis reaction mixtures were also incubated without any added eIF-2 (lanes 1, 4, and 7). The reactions were then terminated and the samples separated on SDS-PAGE as described in 'Materials and Methods'. The figure is an autoradiogram.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9
97-
66-
R.eIF_2β-
45-
R.eIF_2α,38-
31.5-
Additions at 0 min
- hemin
+ hemin
+ hemin
+NEM
10 min
NONE
WG.eIF_2
R.eIF_2
NONE
WG.eIF_2
R.eIF_2
NONE
WG.eIF_2
R.eIF_2
[32P] Pulse
10 - 15 min

phosphorylate endogenous (Fig. 26, Lines 1 & 7), or exogenously added (Fig 26, lanes 3 & 9) reticulocyte eIF-2 (Hunt, 1979). Addition of hemin inhibited the eIF-2a kinase activity of HRI. Hence, relatively very little endogenous or exogenously added reticulocyte eIF-2α was phosphorylated in hemin-treated lysates (Fig. 26, lanes 4 & 6) when compared to heme-deficient, or heme and NEM-treated lysates (Fig. 26, lanes 1, 3, 7 & 9). In contrast, the phosphorylation of p41-42 doublet of wheat germ eIF-2 was found to be similar both in heme-deficient and hemin-supplemented lysates (Fig. 26, lanes 2 & 5) but was enhanced in NEM-treated reticulocyte lysates (Fig 26, lane 8) These findings suggest that in addition to heme-regulated eIF-2α kinase (HRJ), other kinases in reticulocyte lysates can phosphorylate the doublet subunit, p41-42, of wheat germ eIF-2 We could not however assess the phosphorylation status of the small subunit, p36, of wheat germ eIF-2 in reticulocyte lysates.

We have also analyzed the phosphorylation pattern of translating wheat germ lysates treated with highly purified reticulocyte HRI and other agents which are known to stimulate the phosphorylation of reticulocyte eIF-2α (Fig 21). Purified HRI did not alter the phosphorylation pattern of translating wheat germ lysate (Fig 21, lane 4 vs lane 1). Further, when a small amount of purified wheat germ eIF-2 and HRI were added to translating wheat germ lysates, variable phosphorylation of the subunits p36 and p41-42 was noted (Fig 21, lane 9). However, the pattern of phosphorylation was not different from the control reaction (Fig. 21, lane 7) suggesting that some amount (basal level) of phosphorylation of wheat germ eIF-2 subunits occurs under normal conditions. Low levels of poly IC treatment (which facilitates eIF-2a phosphorylation through the activation of dsI in reticulocyte lysates) did not alter the phosphorylation pattern in wheat germ lysates (Fig. 21, lanes 5 & 10 vs control lanes 1 & 6) suggesting that wheat germ may not carry any endogenous kinase which can be activated by poly IC or dsRNA. Alternatively, the wheat germ lysates may contain some inhibitors of eIF-2a phosphorylation which 'protect' eIF-2 from being phosphorylated. This may be like p67 protein (Gupta *et ed.*, 1993), heat shock proteins (Chen and London, 1995), small nuclear RNA or their products (Clemens, *et ed.*, 1994).

The findings here indicate that mammalian eIF-2α kinases can phosphorylate the p41-42 doublet subunit of wheat germ eIF-2, where as NEM or NEM and DTT-treated lysates phosphorylate the p41-42 doublet and p36 subunits of wheat germ eIF-2. Addition of purified heme-regulated eIF-2a kinase, or low concentrations of dsRNA or poly IC does not inhibit wheat germ translation but addition of NEM is found to inhibit protein synthesis in wheat germ lysate. In addition to these above results it has been demonstrated here that CK-II inhibits protein synthesis (Fig. 16) and has been shown in our laboratory that it can phosphorylate both the p36 and the p41-42 subunits of wheat germ eEF-2 (Krishna, 1996, Ph. D Thesis). Also a recent study suggests that protein synthesis in wheat germ lysates is inhibited upon phosphorylation of p41-42 doublet subunit of wheat germ eIF-2. The authors have shown that relatively high concentrations (10-100 μg/ml) of dsRNA or poly IC are required for the activation of a plant eIF-2α kinase However, our lysates treated with lower-higher concentrations (10 ng- 25 μg/ml) of poly IC were unable to stimulate the phosphorylation of any protein (Krishna, 1996, Ph D. Thesis) or inhibit wheat germ translation (Fig. 15). This observation is itself consistent with the observations of Langland *et al.*, 1996, who mention in their paper that many lysates do not respond to dsRNA-treatment because of varying concentrations of p67 protein, while these authors think that p67 inactivates eIF-2a kinases (as per their unpublished observations) It has been demonstrated by others that p67 protects eIF-2α phosphorylation by interfering in the second step of kinase catalyzed phosphorylation of the substrate (Gupta *et al*, 1993). Since eIF-2a phosphorylation inhibits GNE activity of eIF-2B and thereby inhibits protein synthesis, it becomes important to determine if this activity is inhibited upon phosphorylation of eIF-2α. Langland *et al*, (1996) have not investigated the mechanism by which their eIF-2α phosphorylation inhibits protein synthesis. Here, in this work, we tried to determine if the eIF-2B like activity is inhibited in NEM and CK-II-treated lysates which can inhibit protein synthesis and promote the eEF-2 phosphorylation (p36 and p41-42 subunits).

CHAPTER 4

STUDIES ON GUANINE NUCLEOTIDE EXCHANGE ON WHEAT GERM eIF-2

At the end of protein chain initiation, eIF-2 is released as eIF-2 GDP in mammalian systems and this complex cannot join the initiator tRNA (Met-tRNA$_i$) and enter into the initiation pathway unless the GDP is exchanged for GTP. This is because eIF-2 has greater affinity for binding to GDP than GTP (Merrick, 1992). Further, phosphorylation of eIF-2α impairs the eIF-2B activity *in vitro* and in translating lysates (Clemens *et al.*, 1982, Matts and London, 1984). The affinity of eIF-2B for eIF-2(aP).GDP is higher than that for eIF-2.GDP. And also, eIF-2B constitutes a small portion of eIF-2 (Rowlands *et al.*, 1988) Consequently, phosphorylation of a limited portion of eIF-2a (10-20%) sequesters all the available eIF-2B into a 15S complex [eIF-2(αP).eIF-2B], in which eIF-2B becomes non-functional. This can lead to the inhibition in protein synthesis (Thomas *et al*, 1984, 85).

The eIF-2B like activity is apparently conserved in other systems like yeast (Dever *et al.*, 1992) and insects (Chefalo *et al.*, 1994). However it is not known whether a similar protein is required for the exchange of guanine nucleotides from wheat germ eIF-2 Hence labeled binary complex, eIF-2.[3H]GDP, was prepared here with wheat germ eIF-2 *in vitro* and studied the exchange of the labeled GDP complexes under a variety of conditions that have been observed to affect wheat germ eIF-2 phosphorylation and lysate protein synthesis.

*4.1 Binary **complex**, eIF-2.GDP, formation requires* Mg^{2+}*:*

Wheat germ eIF-2 can bind to added labeled GDP to form a binary complex, eIF-2.[^{3}H]GDP. The binary complex was formed as described (Ramaiah *et al.*, 1994). The formation of the binary complex is highly dependent on Mg^{2+} (Fig. 27).

The efficiency of binary complex formation was found better with carboxy-methyl-Sephadex (CMS, highly purified) purified eIF-2 than with phosphocellulose (P11, one step less purified than CMS-eIF-2) eIF-2 (Fig. 28).

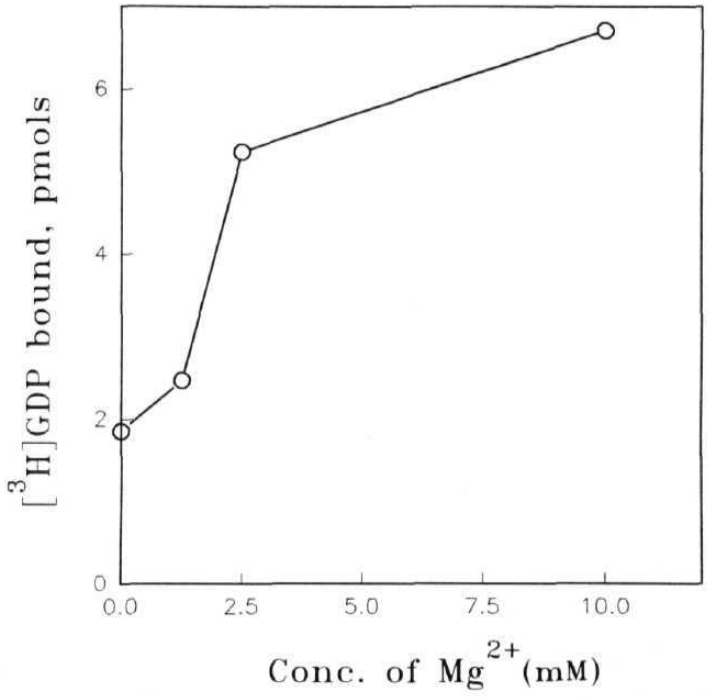


Fig. 27 Mg^{2+} is required for efficient binary complex formation.

Wheat germ binary complex was formed using purified wheat germ eIF-2 (2 μg) as described in 'Materials and Methods'. The requirement of Mg^{2+} for stabilization of the wheat germ binary complex was studied using different concentrations of Mg^{2+} (as indicated in the figure) during the formation of binary complex. The figure indicates the pmols of labeled wheat germ binary complex formed.

4.2 Dissociation of the preformed binary complex:

In order to assess whether the dissociation of preformed binary complex or exchange of unlabeled GDP for labeled GDP present in the reaction mixture, requires any additional protein factors, the guanine nucleotide exchange in the presence of excess unlabeled GDP (40 μM) and physiological concentration of Mg^{2+} (2 5 mM) was measured as described (Ramaiah *et al,* 1994). Interestingly, the exchange reaction occurred without the addition of any protein factor (Fig. 29).

This finding suggests that

- the wheat germ eIF-2 preparation may be contaminated with small amounts of eIF-2B like activity,
- one of the eIF-2 subunits may be serving the function of eIF-2B or, the exchange reaction may not require an eIF-2B like protein and occurs probably due to mass action.

4.3 Guanine nucleotide exchange on wheat germ eIF-2:

The above observation that the exchange of unlabeled GDP for labeled GDP in the preformed wheat germ binary complex, eIF-2.[^{3}H]GDP, can proceed without the addition of any protein factor is consistent with the previous report on the guanine nucleotide exchange on wheat germ eIF-2 (Osterhout *et al,* 1983; Shaikin *et al.,* 1992). Despite rigorous purification, earlier workers however could not purify eIF-2B like activity from wheat germ (Osterhout *et al.,* 1983; Lax *et al.,* 1982). In many mammalian systems, it has been shown that eIF-2 can be contaminated with eIF-2B like preparations. Infact many times it was observed that the eIF-2B co-migrates with eEF-2 preparations during purification (Matts *et al,* 1983; Reichel *et al,* 1985; Panniers and Henshaw, 1983).

To determine if the exchange of guanine nucleotides is helped by an eIF-2B like protein associated with wheat germ eEF-2, it becomes important to show that the eIF-2B activity is specifically inhibited upon eIF-2 phosphorylation. Since purified CK-II (Krishna *et al,* 1994), or NEM-treated lysates were shown here to phosphorylate the p36 and p41-42 subunits of wheat germ eIF-2 (Fig. 21), we have determined here the effect of purified

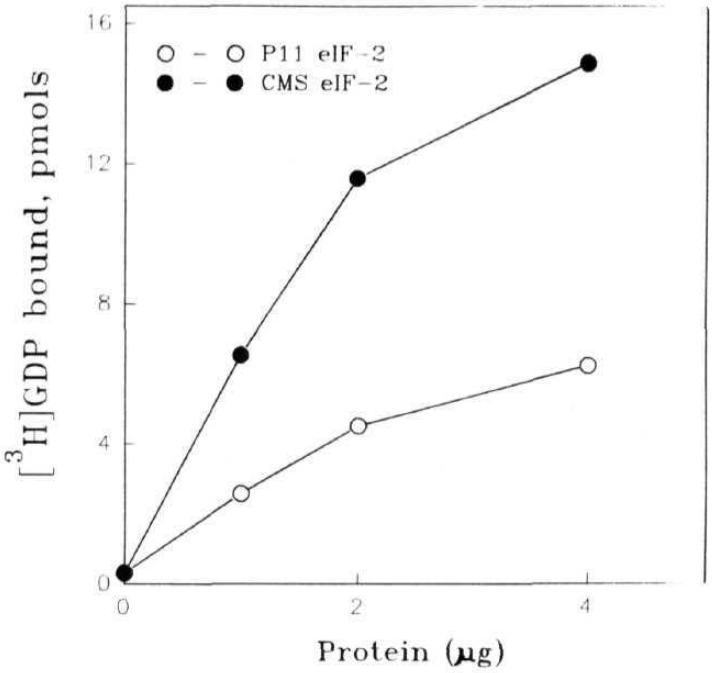


Fig. 28 Formation of wheat germ binary complex using partially purified **(P11)** and highly purified (CMS) **eIF-2.**

Wheat germ binary complex was formed in the presence of 2.5 **mM Mg^{2+}** as described in 'Materials and Methods' using different concentrations of **P11-eIF-2** or **CMS-eIF-2** as indicated in the figure. The figure indicates the amount of wheat germ binary complex formed (in pmols).

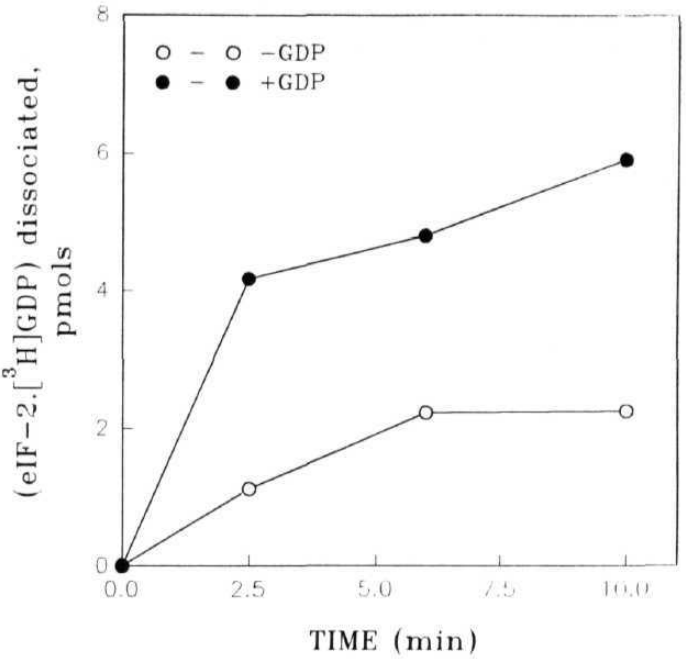


Fig. 29 Exchange of unlabeled GDP for labeled GDP in wheat germ binary complex. Labeled binary complex, eIF-2[^{3}H]GDP, was prepared as described in 'Materials and Methods'. Dissociation of the preformed binary complex (70 µl, 25 pmols) was studied at 25°C with and without the addition of 40 µM unlabeled GDP. At different time intervals (2.5, 6 and 10 min.), 20 µl aliquots of the reaction mixtures were withdrawn and the reactions were stopped with the addition of 3 ml of cold wash buffer. The reaction mixtures were then filtered through millipore filters (HAWP 0.45 µm), air dried and the radioactivity bound to the filters was measured in a liquid scintillation counter. One pmol of eIF-2[^{3}H]GDP gives ~1900 cpm. Pmols of eIF-2[^{3}H]GDP dissociated were determined as described in the 'Materials and Methods'. The figure indicates the dissociation of labeled GDP from eEF-2.

CK-II or NEM-treated lysate on the guanine nucleotide exchange activity associated with wheat germ eIF-2 *in vitro*

4.3.1 Effect of CK-II phosphorylation on GNE activity associated with wheat germ eIF-2

The experiment was carried out in two different ways: 1) in the presence and absence of the phosphorylating enzyme, CK-II (Fig 30) and 2) in the presence and absence of the phosphate donor, ATP (Fig. 31).

In the first set of experiments, ATP was present in both the reactions. However, CK-II was added to only one of the reactions to determine the effect of phosphorylation of eIF-2 by CK-II on GNE activity. In another set of reactions, CK-II was present in both the reactions, however, ATP was included only in one of them to facilitate phosphorylation. After carrying out the phosphorylations of eIF-2 in the respective experiments, binary complexes were formed and GNE activity was studied as described in 'Materials and Methods'

Kinetics of the GDP exchange reaction were found similar for phosphorylated and unphosphorylated eIF-2 (Fig 30 and Fig. 31). Presence of ATP however reduced the GDP exchange marginally (Fig 31).

4.3.2 Effect of NEM on the GNE activity of wheat germ eIF-2:

Translating wheat germ lysates were incubated with and without 1 mM NEM and 100 uM ATP, for 15 minutes to activate the endogenous kinase(s). Wheat germ binary complex was formed as described in 'Materials and Methods' and added to the translating lysates to study the GNE activity.

GNE activity was decreased in NEM-treated lysates compared to the non-NEM treated lysates (Fig. 32). This decrease in the GNE activity could be due to either NEM induced phosphorylation of eIF-2 or due to a direct modification of eIF-2 by NEM. Studies by Suzuki *el al.*,(1990) show that the formation of ternary complex, eIF-2GTP.Met-tRNAi, is inhibited by NEM. This inhibition is shown to be mediated, atleast partially, due to the binding of NEM to the γ-subunit of eIF-2. Four NEM binding sites,

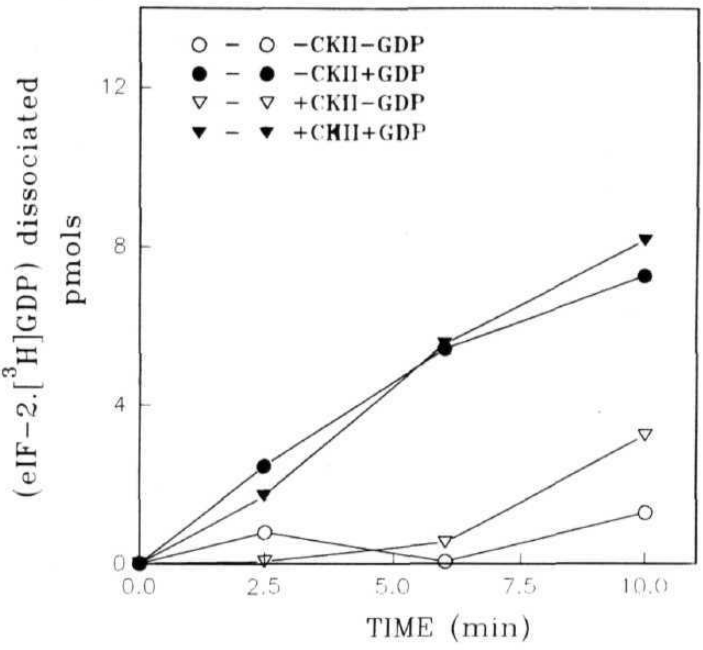


Fig. 30 Effect of phosphorylation of wheat germ eIF-2 by **CK-II** on the dissociation of **eIF-2[^{3}H]GDP.** *(Control reactions contain **ATP** and lack **CK-II**).*

The experiment was carried out in 3 steps. In step 1, phosphorylation of wheat germ eIF-2 (1 **μg)** was carried out at **30°C** by **CK-II** (10 ng) for 5 min. in a 10 ml reaction mixture in the presence of Tris **buffer** (20 **mM Tris-HCl**, pH 7.6; 80 **mM** KC1; and 2.5 **mM Mg^{2+})** and **100 μM** ATP. **The** control **un phosphor vla ted reaction mixtures lacked the CK-Il protein.** In step 2, the phosphorylated and the **unphosphorylated** reaction mixtures (70 **μl)** were incubated with **[^{3}H]GDP** to form **eIF-2[^{3}H]GDP** binary complex as described in 'Materials and **Methods**'. **In** step 3, the dissociation of the preformed binary complex (70 **μl**, 31.5 **pmols)** was studied at **25°C** with and without the addition of 40 **μM** unlabeled GDP to the reaction mixtures. At different time intervals (2.5, 6 and 10 min.), 20 **μl** aliquots of the reaction mixtures were withdrawn and the dissociation of the preformed binary complex monitored as described in 'Materials and Methods'. One **pmol** of **eIF-2[^{3}H]GDP** gives **~1900 cpm**. Pmols of **eIF-2[^{3}H]GDP dissociated were** determined as described in the 'Materials and Methods'. The figure indicates the dissociation of labeled GDP fron eIF-2.

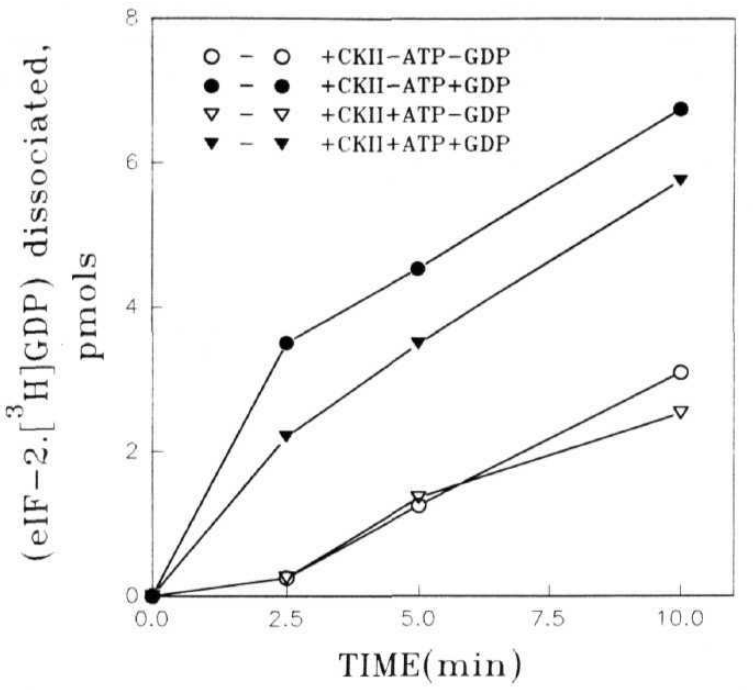


Fig. 31 Effect of phosphorylation of wheat germ **eIF-2** by < **K-II** on the dissociation of **eIF-2[^{3}H]GDP:** *(Control reactions contain **CK-II** and lack **ATP).***

In step 1, phosphorylation of wheat germ **eIF-2** (1 **μg)** was carried out at **30°C** by **CK-II** (10 ng) for 5 min. in a 10 **μl** reaction mixture in the presence of Tris buffer (20 mM **Tris-HCl**, pH 7.6; 80 **mM** KC1; and 2.5 mM **Mg^{2+})** with and without the addition of 100 **μM** ATP. The control unpbosphorylated reaction mixtures contained **CK-II** protejn without ATP. In step 2, the phosphorylated and the unpbosphorylated reaction mixtures (70 **μl)** were incubated with **[^{3}H]GDP** to form **eIF-2[^{3}H]GDP** binary complex as described in 'Materials and Methods'. In step 3, the dissociation of the preformed binary complex (70 **μl**, 31.5 pmols) was studied at **25°C** with and without the addition of 40 **μM** unlabeled GDP to the reaction mixtures. At different time intervals (2.5, 6 and 10 **min.**), 20 **μl** aliquots of the reaction mixtures were withdrawn and the dissociation of the preformed binary complex was **monitored** as described in 'Materials and Methods'. One **pmol** of **eIF-2[^{3}H]GDP** gives ~**1900 cpm**. Pmols of **eIF-2[^{3}H]GDP** dissociated were determined as described in the 'Materials and Methods'. The figure indicates the dissociation of labeled GDP fron eIF-2.

out of which two highly reactive ones, were identified in the Y-subunit. Binding of NEM to the y-subunit decreases the binding of guanine nucleotides to the subunit, thereby preventing the formation of ternary complex. NEM also binds to the a-subunit of eIF-2, but this binding did not affect the binding of the guanine nucleotides to eIF-2 (Suzuki *el al.*, 1990).

To eliminate the possibility of direct modification of eIF-2 by NEM, we carried out the above experiment with a 0-70% ammonium sulfate cut fraction of NEM-treated and untreated lysates instead of total lysates. In contrast to the above observations, the 0-70% ammonium sulfate cut fraction of NEM-treated lysates did not decrease the GNE activity of wheat germ eIF-2 (Fig. 33).

As can be seen from the data, neither conditions (CK-II or NEM induced phosphorylations of wheat germ eIF-2) inhibit significantly the guanine nucleotide exchange on wheat germ eIF-2. These findings suggest that the protein synthesis inhibition caused by CK-II or NEM-treatment are atleast not mediated by a reduction in the GNE activity of wheat germ eIF-2.

The NEM-treated lysates or purified CK-II may not be phosphorylating wheat germ eIF-2 at a proper site that can effectively inhibit the eIF-2B activity. A recent study infact emphasizes the importance of Ser-51 phosphorylation and the importance of adjacent unphosphorylated Ser-48 residue in the wild type human eIF-2(aP) to effectively sequester the eIF-2B guanine nucleotide exchange activity (Ramaiah *et al.*, 1994). Further, studies in our laboratory (Krishna, 1996, Ph.D. Thesis) indicate that guanine nucleotide exchange on wheat germ eIF-2 can also proceed in inhibited heme-deficient, or heme and poly IC-treated reticulocyte lysates. Under these conditions reticulocyte eIF-2B activity is impaired due to the phosphorylation of Ser-51 residue in reticulocyte eIF-2α. These lysates cannot support the guanine nucleotide exchange on reticulocyte eIF-2 but however can support the GDP exchange on wheat germ eIF-2.

Since significant dissociation of wheat germ eIF-2.[3H]GDP occurs in heme-deficient and poly IC treated lysates, one can eliminate the possibility of a small contaminant of eIF-2B or of a p67 like protein in the eIF-2 preparations. A careful analysis of purified wheat germ eIF-2 preparation did not indicate any major contaminants

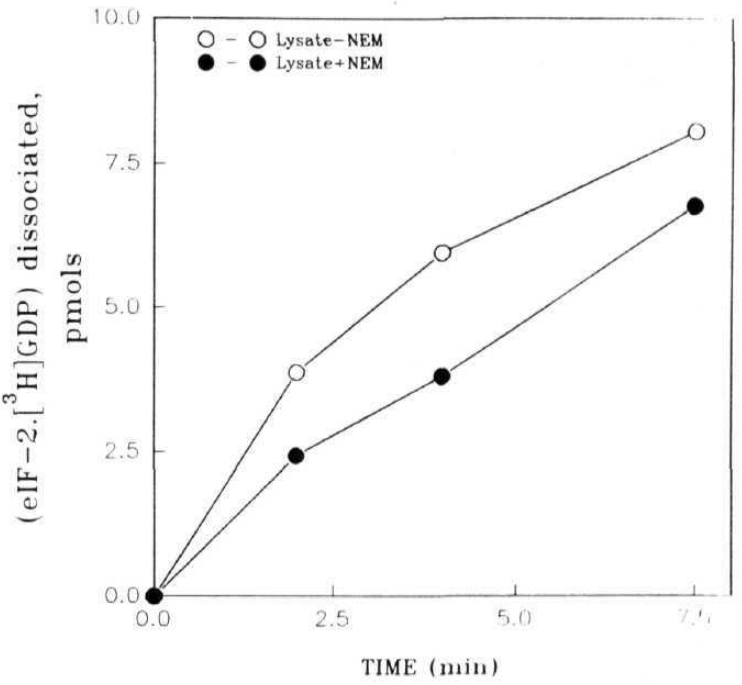


Fig. 32 Effect of NEM on wheat germ eIF-2[^{3}H]GDP dissociation.

The experiment was carried out in three steps. The wheat germ lysates were treated with 100 μM ATP and incubated at 25°C for 10 min. with or without the addition of NEM (1.5 mM) to facilitate the activation of endogenous eIF-2 kinase(s) and eIF-2 phosphorylation. In step 2, the eIF-2[^{3}H]GDP, binary complex was prepared as described in 'Materials and Methods'. In step 3, The NEM-treated and untreated lysates (7 μl) were then incubated for 5 min. at 25°C with the preformed binary complex (25 pmols) in the presence of 100 μM ATP in a final volume of 77 μl to facihtate the phosphorylation of preformed wheat germ binary complex. Afterwards the dissociation of the preformed binary complex was monitored in 22 μl reaction mixtures at different time intervals with and without the addition of 40 μM unlabeled GDP as described in 'Materials and Methods'. One pmol of bound eIF-2[^{3}H]GDP gives -2200 cpm. The figure indicates the dissociation of labeled GDP from eIF-2.

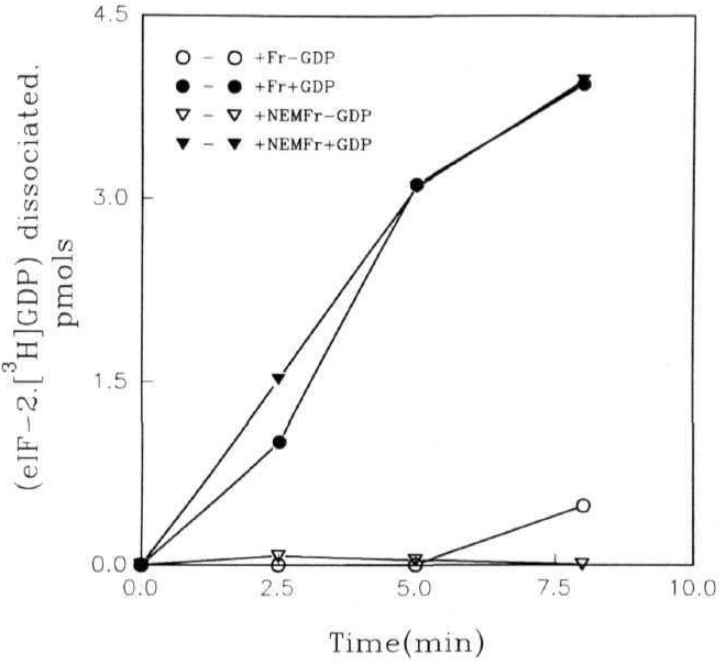


Fig. 33 Effect of NEM-treated *lysate fraction* on wheat germ **eIF-2[^{3}H]GDP** dissociation.

The experiment was carried out in three steps. The wheat germ **lysates** were treated with **100 μM**ATP and incubated at **25°C** for 10 **min**. with or without the addition of NEM (1.5 **mM)** to **facilitate** the activation of endogenous **eIF-2 kinase(s)** and **eIF-2** phosphorylation. The lysate proteins were then immediately precipitated by the addition of 2.5 volumes of saturated ammonium sulfate (70% final). In step 2, the **eIF-2[^{3}H]GDP,** binary complex was prepared as described in 'Materials and Methods'. **In** step 3, The NEM-treated and untreated lysate **fractions** (7 **μl)** were then incubated for 5 min. at 25°C with the preformed binary complex (25 pmols) in the presence of 100 **μM** ATP in a final volume of 77 **μl** to facilitate the phosphorylation of preformed wheat germ binary complex. Afterwards the dissociation of the preformed binary complex was monitored in 22 **μl** reaction mixtures at different time intervals with and without the addition of 40 **μM** unlabeled GDP as described in 'Materials and Methods'. One **pmol** of bound **eIF-2[^{3}H]GDP** gives **~2200** cpm. The figure indicates the dissociation of labeled GDP from eIF-2.

(Krishna *et al.*, 1994). It is likely that the exchange reaction is mediated by mass reaction (depends upon the concentration of GDP or GTP). These observations are consistent with the recent studies (Shaikin *et al*, 1992) which suggested that the affinity of wheat germ eIF-2 for GDP is only 10 times higher than that for GTP. In contrast, mammalian eIF-2 has much higher affinity for GDP than for GTP (Walton and Gill, 1975). These findings suggest that eIF-2B analogs may not be required for the exchange of GTP for GDP in wheat germ eIF-2 and the latter appears to be different from mammalian eIF-2 preparations. The findings are thus consistent with the idea that phosphorylation of eIF-2 may not regulate protein synthesis in higher plants (Shaikin *et al.*, 1992). Alternatively, one of the eIF-2 subunits may be serving the function of eIF-2B activity. If this is true, a high level of eIF-2 phosphorylation is required to sequester completely the eIF-2B activity.

The findings presented here suggest that wheat germ eIF-2 behaves different from mammalian eIF-2 since it exchanges guanine nucleotides independent of an eIF-2B like protein. Hence it raises a question regarding the functional significance of eIF-2B like factor, if any, in wheat germ lysates and also the mechanism of inhibition in protein synthesis mediated by eIF-2α phosphorylation in plants.

A recent report however, indicates that phosphorylation of p4!-42 subunit in wheat germ lysates can occur by relatively higher concentrations of dsRNA. Presumably these conditions stimulate an eIF-2α kinase like PKR and phosphorylation of wheat germ eIF-2 subunits. These authors have also demonstrated that phosphorylation of the above subunit of wheat germ eIF-2 impairs protein synthesis. However, a) they have not studied the mechanism of protein synthesis under those conditions. It is not known if these conditions can also decrease the lysate eIF-2B like activity, b) the authors also mention that significant variations exist between wheat germ lysate preparations in terms of dsRNA levels required for inhibition, with several showing no response to dsRNA (like the results that we have mentioned). The required response or the lack of response of wheat germ lysates towards dsRNA is presumed to be due to varying levels of a PKR inhibitor present in the lysates. In this regard, the authors cite the role for a glycosylated p67-like protein which can inhibit eIF-2α kinase phosphorylation. As far as we are aware of, the p67 protein cannot inhibit the eIF-2α kinase activity (that is its autophosphorylation) but it can

interfere in eIF-2α phosphorylation of *in vitro* reactions (personal observations of Babu and Ramaiah; Chen and London).

Also the role of purified p67 in lysates for its affects on protein synthesis, eIF-2α phosphorylation and on eIF-2B activity during protein synthesis has not been worked out so far. While we do not rule out the possibility of such a protein involved in the regulation of eIF-2α phosphorylation and protein synthesis, however we think that this may not be a reason for various workers in failing to identify an eIF-2α phosphorylation mechanism in plants that can inhibit protein synthesis. Our findings presented here suggest two most important points:

- The guanine nucleotide exchange on wheat germ eIF-2 can occur independent of an eIF-2B like protein.
- The phosphorylation of wheat germ eIF-2 occurs under several conditions but does not lead to an inhibition in GNE activity associated with wheat germ eEF-2.

The findings mentioned above suggest that probably wheat germ eIF-2 is able to exchange guanine nucleotides both by mass exchange and a reaction catalyzed by eIF-2B like protein. The latter is substantiated by findings of Krishna *et at.,* (1994) which indicate that a functional reticulocyte eIF-2B activity can enhance the GDP exchange on wheat germ eIF-2. This may reflect two states of the protein and it may be similar to guanine nucleotide exchange of eIF-2 in Drosophila embryo or Artemia (Mateu and Sierra, 1987, Mateu *et al.,* 1989).

Also consistent with these interpretations, ribosomal salt wash did not yield significant amount of eIF-2. We could purify eIF-2 only from the post ribosomal supernatant. Recently, it was suggested that functional eIF-2B activity is required to release eIF-2 from the 60S subunits of 80S initiation complexes in reticulocyte lysates Otherwise eIF-2 stays bound to the 60S subunits of polysomes (Ramaiah *el at.,* 1992; Thomas *et at.,* 1985). Since GDP in wheat germ eIF-2 can be readily exchanged either by mass exchange or because of an intrinsic eIF-2B like activity, it is quite likely that most of the eIF-2 is released from the ribosomes without any difficulty and is found in the post ribosomal supernatant.

There is considerable confusion regarding the designation of the various subunits of wheat germ eIF-2. Going by the criteria of the size of the different subunits in eIF-2, the 38 kDa of wheat germ eIF-2 is considered to be the smallest and is equivalent to the smallest subunit in reticulocyte eIF-2 (eIF–2α). However, the reticulocyte eIF–2α kinases phosphorylate the 41-42 kDa of wheat germ eIF-2. This suggests that part of reticulocyte eIF-2a sequences are conserved in the 41-42 kDa of wheat germ eIF-2 Consistent with this notion, the recent observations based on molecular cloning and cDNA sequencing of wheat germ eIF-2 indicate (unpublished observations cited by Langland *et al.*, 1996) that the p41-42 doublet subunit is equivalent to reticulocyte eIF-2α. However the report suggests that 17% sequences in plant eIF-2α around the phosphorylation domain (45-56 region) may be different from human eIF-2a sequence. A modification in an amino acid adjacent to phosphorylation site has been shown to overcome the protein synthesis inhibition and inhibition in eIF-2B activity mediated by human eIF-2α phosphorylation (Murtha-Reil *et al*, 1993; Ramaiah *et al.*, 1994). Also a study with yeast cells identified several mutants within 40 amino acids of the phosphorylation site that can overcome the inhibitory effect of eIF-2a phosphorylation at residue Ser-51 mediated by GCN2 kinase (Hinnebusch, 1994). Also a mutation in eIF-2B protein can counter the phosphorylation of eEF-2a (Vazquez de Aldana *et al.*, 1993). So it is possible that modification in some of the amino acid sequences in wheat germ eIF-2 may be the reason for its inability to inactivate eIF-2B like activity associated with it, as mentioned above, or of reticulocyte lysates (Krishna, 1996, Ph. D. Thesis).

CHAPTER 5

EFFECT OF NUCLEOSIDE DI- AND TRIPHOSPHATES ON WHEAT GERM LYSATE PROTEIN SYNTHESIS

Protein synthesis reactions require continuous supply of ATP and GTP. Since gel-filtered lysates do not carry adequate amounts of these small molecular weight compounds, protein synthesis reactions are usually supplemented with free ATP and GTP. However free ATP and GTP are usually hydrolyzed during the course of protein synthesis reactions and also by lysate enzymes. The results reported in the earlier chapters here do not indicate that any of the agents used here can inhibit wheat germ protein synthesis due to increased eIF-2 phosphorylation and decreased GNE activity associated with eIF-2. It is likely that in the absence of eIF-2 phosphorylation mechanism, the GDP / GTP ratios may also be regulating eIF-2 recycling and overall protein synthesis. Hence, studies have been carried out here to determine the effects of increasing concentrations of the hydrolyzed products to their respective triphosphates on protein synthesis and also the conditions that are important in generating ATP and GTP. We have carried out here protein synthesis reactions with the addition of mono, di and/ triphosphates of adenosine and guanosine in the presence and absence of creatine phosphate (CP) and creatine phosphokinase (CPK).

5.1 CP / CPK system is required to maintain wheat germ protein synthesis:

Protein synthesis reactions were found optimal in the presence of complete system i.e., in the presence of ATP, GTP, CP, and CPK (Fig. 34). Omission of CP and CPK lead to about 40% drop in protein synthesis (-V-). Inclusion of CP alone without CPK caused a slightly higher level of inhibition (~50%) (-T-). Also, CP and CPK failed to stimulate protein synthesis in gel-filtered lysates if ATP was not provided to the system (-O-). These observations indicate that ATP is essential to carry out protein synthesis. Free ATP that is supplied to the protein synthesizing system is hydrolyzed and is not generated in the absence of CP/CPK. Therefore, CP / CPK are required to maintain a continuous supply of ATP that is essential for efficient translation.

5.2 ADP can substitute for ATP in the presence of energy generating system:

Wheat germ lysate protein synthesis occurred in the presence of ADP if CP and CPK were present suggesting that ADP can be substituted for ATP in the protein

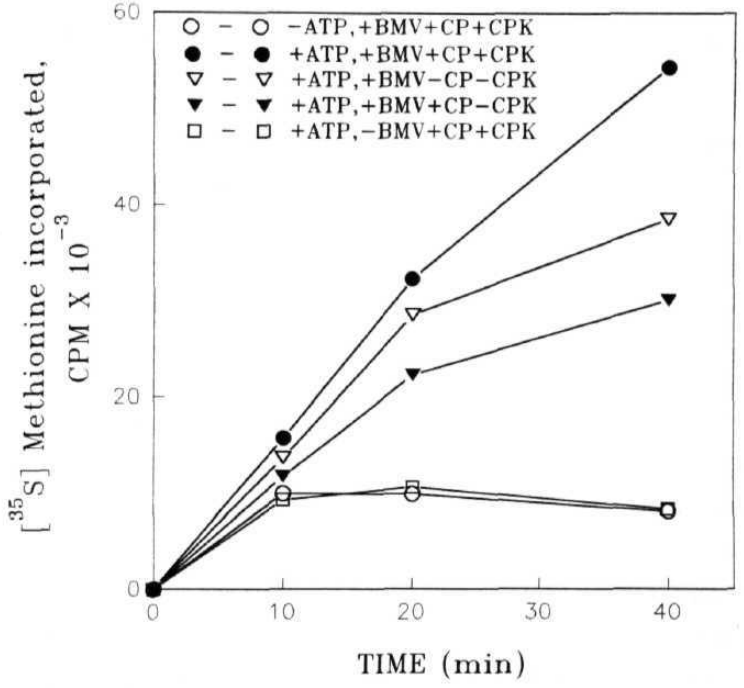


Fig. 34 Effect of **ATP** and CP / CPK on wheat germ lysate protein synthesis.

Standard lysate protein synthesis assays **(25µl)** were carried out in the presence of BMV RNA (15 **µg/ml)** at **25°C** for 45 minutes as described in 'Materials and Methods'. The experiment was carried out without ATP **(O-O)**; without CPK **(▼-▼)**; or without CP and CPK (V-V) in the reaction mixtures. A control reaction with the addition of ATP, CP, and CPK (•-•) was carried out to assess the optimal protein synthesis. A reaction without the addition of BMV RNA (D-D) was also carried out to assess the endogenous lysate protein synthesis.

Protein synthesis was assessed by the incorporation of **[^{35}S]methionine** into acid precipitable protein in a 5 **µl** aliquot of the reaction mixtures, taken at different time intervals (15, 30 and 45), as described in 'Materials and Methods'.

synthesizing lysates (Fig. 35a., -V-). Similar substitution of ADP for ATP was not seen if CP and CPK were omitted from the system (Fig. 36a., -T-). Addition of AMP, in the absence of ATP, did not stimulate protein synthesis, even in the presence of an energy generating system (Fig. 35a. -▼-; Fig. 36a. -•-). These results substantiate the regeneration of ATP from ADP in the presence of CP and CPK and show that ATP but not ADP maintains protein synthesis. The results also show that AMP is not efficiently converted to ATP in the lysates and cannot be substituted for ATP. Alternatively, AMP may be inhibitory to wheat germ protein synthesis.

5.3 GDP can substitute for GTP in the presence or absence of energy generating system:

GDP was found promoting protein synthesis in the place of GTP (Fig. 35b., -V-). This could be because of the conversion of GDP to GTP. Relatively good amount of protein synthesis was also observed even in the absence of both GTP and GDP (-□-) This may be because the lysate may contain some amount of endogenous GDP (probably bound to proteins) which may be used for the process.

In the absence of energy generating system (CP and CPK), the general protein synthesis was reduced to approximately half, and was further reduced when GTP was eliminated from the system (Fig. 36b., -■-) Addition of GDP instead of GTP, under those conditions, also maintained protein synthesis partially (that is in the absence of CP and CPK) (-▼-). This suggests that CP and CPK are not directly involved in the generation of GTP from GDP. The activity of nucleoside diphosphate kinase may be involved since it can use the available ATP to generate GTP from GDP.

Similarly, addition of GMP under both the conditions (presence and absence of an energy generating system) supported protein synthesis to some extent in the absence of GTP (Fig. 35b., -•-; Fig. 36b., -D-). This shows that unlike AMP, which cannot substitute for ATP in wheat germ protein synthesis, GMP may be getting converted to GTP in wheat germ lysates and supporting the translation.

Fig. 35 Effect of nucleoside mono- and diphosphates on wheat germ lysate protein synthesis.

a) Ability of adnosine mono- and diphosphates to substitute for adenosine triphosphate in Wheat germ lysate protein synthesis:

Standard lysate protein synthesis assays (25 µl) were carried out in the presence of BMV RNA (15 µg/ml) at 25°C for 45 minutes as described in 'Materials and Methods' except that ATP was eliminated from the reaction mixtures. The experiment was carried with the addition of 1.8 mM of ADP (V-V) or AMP (▼-▼) to the reaction mixtures. A control reaction with the addition of ATP (•-•, +mRNA), was carried out to assess the optimal protein synthesis. A reaction without the addition of BMV RNA (O-O, -mRNA) was also carried out to assess the endogenous lysate protein synthesis.

Protein synthesis was assessed by the incorporation of [^{35}S]methionine into acid precipitable protein in a 5 µl aliquot of the reaction mixtures, taken at different time intervals (15, 30 and 45), as described in 'Materials and Methods'.

b) Ability of guanosine mono- and diphosphates to substitute for guanosine triphosphate in Wheat germ lysate protein synthesis:

Standard lysate protein synthesis assays (25 µl) were carried out in the presence of BMV RNA (15 µg/ml) at 25°C for 45 minutes as described in 'Materials and Methods' except that GTP was eliminated from the reaction mixtures. The experiment was carried without any additions (□-□, to assess the effect of elimination of GTP) or, with the addition of 100µM of GDP (V-V) or GMP (•-•) to the reaction mixtures. A control reaction with the addition of GTP(100 µM) (•-•, +mRNA), was carried out to assess the optimal protein synthesis. A reaction without the addition of BMV RNA (O-O, -mRNA) was also carried out to assess the endogenous lysate protein synthesis.

The extent of protein synthesis that occurred was measured as mentioned above.

Fig. 35a.

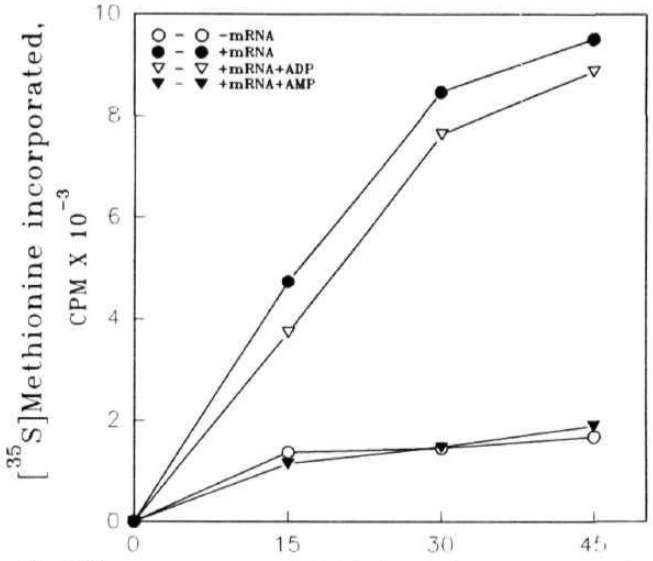


Fig. 35b.

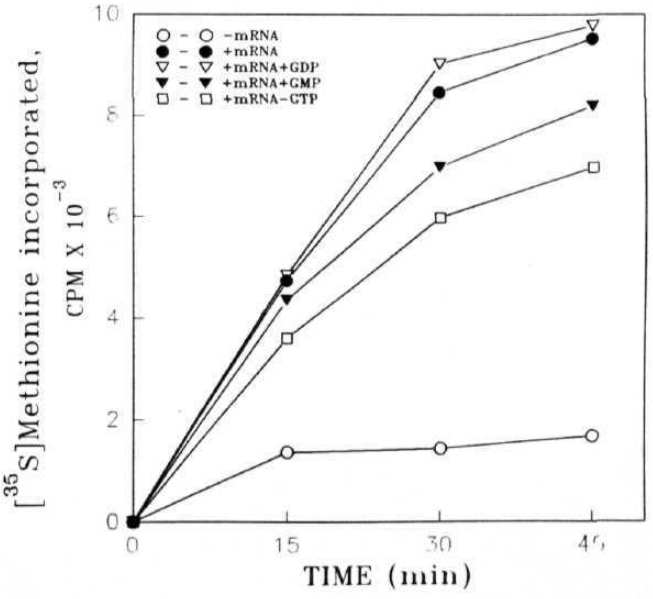


Fig. 36 Effect of nucleoside mono- and diphosphates on wheat germ lysate protein synthesis *in the absence of an energy regenerating system.*

Standard lysate protein synthesis assays (25 µl) were carried out in the presence of BMV RNA (15 µg/ml) at 25°C for 45 minutes as described in 'Materials and Methods' except that the energy regenerating system (CP and CPK) was eliminated from the reaction mixtures. Wherever indicated, the CP and CPK were added exogenously. ATP (Fig. 36a) and GTP (Fig. 36b) were also eliminated from the reactions in the respective experiments.

a) Ability of adnosine mono- and diphosphates to substitute for adenosine triphosphate in Wheat germ lysate protein synthesis in the absence of an energy regenerating system:

The experiment was carried with the addition of 1.8 mM of ADP (▼-▼) or AMP (D-D) to the reaction mixtures. Control reactions were also carried out with the addition of ATP alone (•-•, to assess the effect of elimination of CP and CPK) and with ATP, CP and CPK (V-V, +mRNA), to assess the optimal protein synthesis. A reaction without the addition of BMV RNA (O-O, -mRNA) was carried out to assess the endogenous lysate protein synthesis.

Protein synthesis was assessed by the incorporation of [^{35}S]methionine into acid precipitable protein in a 5 µl aliquot of the reaction mixtures, taken at different time intervals (15, 30 and 45), as described in 'Materials and Methods'.

b) Ability of guanosine mono- and diphosphates to substitute for guanosine triphosphate in Wheat germ lysate protein synthesis in the absence of an energy regenerating system:

The experiment was carried without any additions (•-•, to assess the effect of elimination of GTP, CP and CPK); with the addition of 100 µM of GTP (•-•, to assess the effect of the elimination of CP and CPK); with 100 µM GDP(T-T); or with 100 µM GMP (C-D) to the reaction mixtures. A control reaction with the addition of GTP, CP, and CPK (V-V, +mRNA), was carried out to assess the optimal protein synthesis. A reaction without the addition of BMV RNA (O-O, -mRNA) was also carried out to assess the endogenous lysate protein synthesis.

The extent of protein synthesis that occurred was measured as mentioned above.

Fig. 36a.

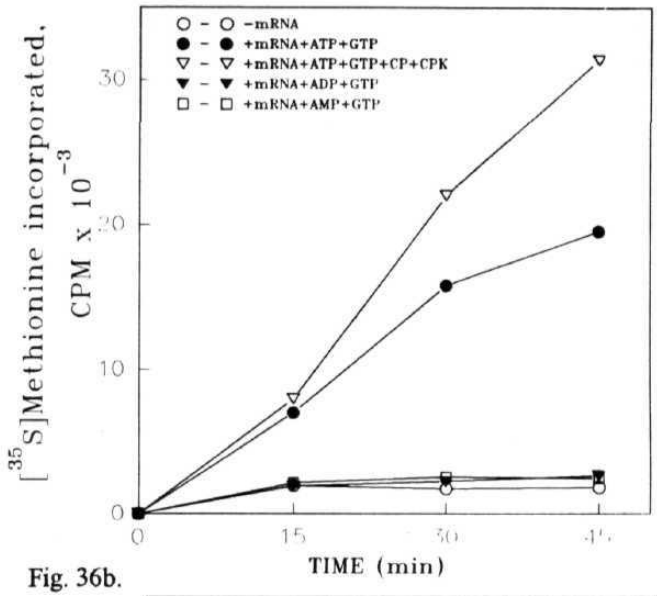
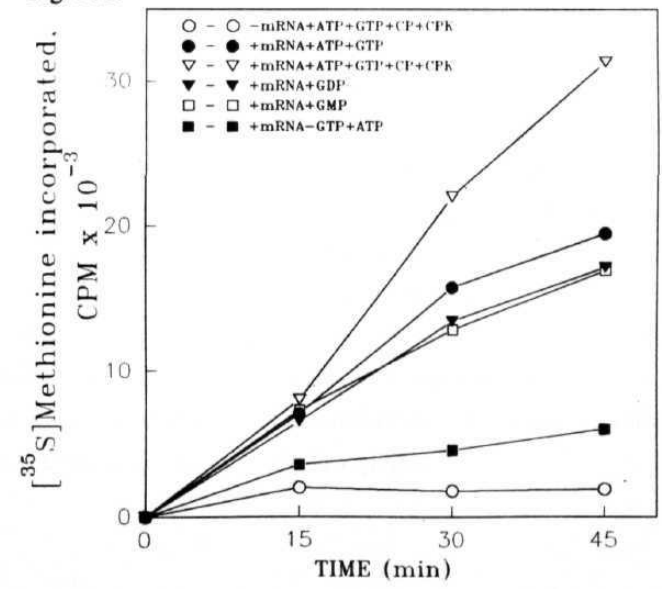


Fig. 36b.



5.4 Increasing concentrations of GDP are inhibitory to wheat germ protein synthesis:

As can be seen from the above results, GDP can substitute for GTP in the presence or absence of an energy generating system (Figs. 35b. & 36b). Equimolar concentration of GDP stimulated wheat germ protein synthesis in the absence of GTP (Fig. 37b., -•-), however, addition of increasing concentrations of GDP inhibited protein synthesis (Fig.37b., -D-). Addition of equimolar or higher concentrations of GDP, in the presence of GTP, inhibited protein synthesis in a concentration dependent manner (Fig. 37a., -V-0 5 mM; -T-, 0.1 mM). These findings suggest that higher ratios of GDP / GTP in the lysate can lead to a decrease in protein synthesis.

In the absence of any other regulatory mechanisms that involves the covalent modifications of protein factors (previous chapters), it is likely that the rates of protein synthesis may be regulated by GDP /GTP ratios. Since wheat germ eIF-2.GDP recycling to eIF-2 GTP can occur without the requirement of a guanine nucleotide exchange factor like eIF-2B, it is likely that the GDP/GTP ratios regulate formation of eIF-2 GTP that is critical for the formation of ternary complex

The above findings suggest that nucleoside diphosphate kinase (NDK) or adenylate kinase like enzymes operate and facilitate the conversion of added GDP and ADP to their corresponding triphosphates in wheat germ lysates.

To study the presence of NDK like activity in wheat germ lysates, we have standardized the separation of labeled nucleotides on TLC. The presence of NDK like activity is assessed here by the conversion of [γ–^{32}P] ATP to labeled GTP in the presence of exogenously added GDP. The products are separated by TLC and exposed to X-ray film as described by Siekierka *et al.*, (1983) and mentioned in 'Materials and Methods'.

Purified NDK (obtained from Sigma) when added to the reaction mixture, converted GDP to [γ-^{32}P]GTP in the presence of [γ-^{32}P]ATP (Fig. 38, lane 3). Reactions containing only labeled GTP or labeled ATP were spotted in lane 4 and lane 5 respectively, to see the migration of labeled GTP and ATP. Lane 2 has additional GDP in the reaction mixture as described in the figure legend.

Fig. 37 Higher concentrations of GDP inhibit wheat germ lysate protein synthesis.

Standard lysate protein synthesis assays **(25µl)** were **carried** out in the presence of BMV RNA (15 **µg/ml)** at **25°C** for 45 minutes as described in 'Materials and Methods' except that the energy regenerating system (CP and **CPK)** was **eliminated** from the reaction mixtures. Wherever indicated, the CP and CPK were added.
The reactions were carried out in the presence (Fig. 37a) and absence (Fig. 37b) **of** GTP **(100 µM)** to assess the effect of GDP on Wheat germ lysate protein synthesis.

a) Effect of increasing concentrations of GDP on Wheat germ lysate protein synthesis in the presence of GTP.

The experiment was carried with the addition of **100µM** (V-V) or 500 **µM** GDP (T-T) to the reaction mixtures. Control reactions were also carried out without any additions (•-•, to assess the effect of elimination of CP and CPK) and with the additon of CP and CPK (D-D, to assess the optimal protein synthesis). A reaction without the addition of BMV RNA **(O-O, -mRNA)** was carried out to assess the endogenous lysate protein synthesis.

Protein synthesis was assessed by the incorporation of **[^{35}S]methionine** into acid precipitable protein in a 5 **µl** aliquot of the reaction **mixtures,** taken at different time intervals (15, 30 and 45), as described in 'Materials and Methods'.

b) Effect of increasing concentrations of GDP on Wheat germ lysate protein synthesis in the absence of GTP.

The experiment was carried with the addition of **100µM** (•-•) or 500 **µM** GDP (D-D) to the reaction mixtures. Control reactions were also carried out without any additions (•-•, to assess the effect of elimination of GTP along with CP and CPK), with the addition of 100 **µM** GTP (•-•, to assess the effect **of** elimination of CP and CPK) and with the addition of 100 **µM** GTP, CP and CPK (V-V, to assess the optimal protein synthesis). A reaction without the addition of BMV RNA **(O-O, -mRNA)** was carried out to assess the endogenous lysate protein synthesis.

The extent of protein synthesis that occurred was measured as mentioned above.

Fig. 37a.

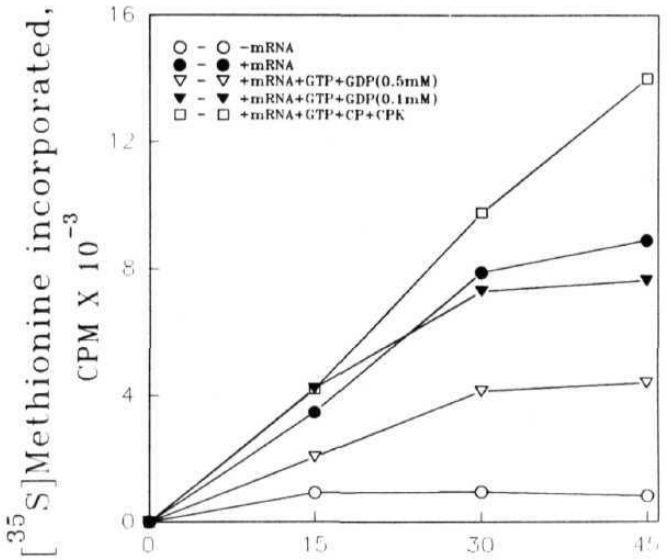
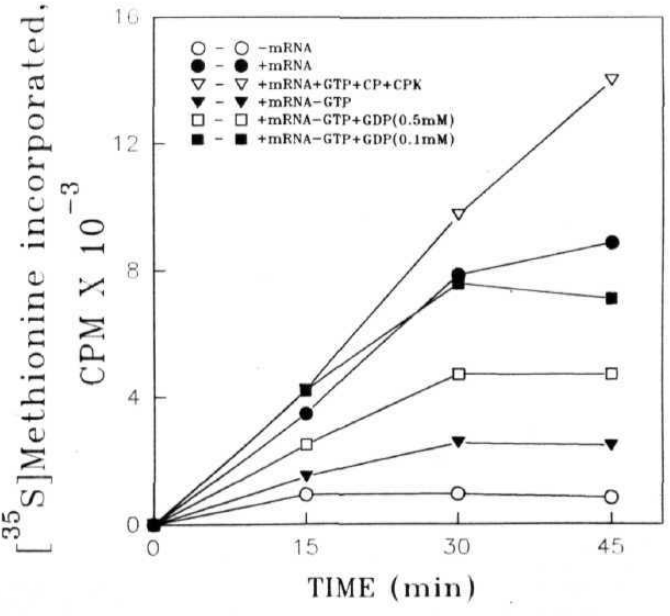


Fig. 37b.



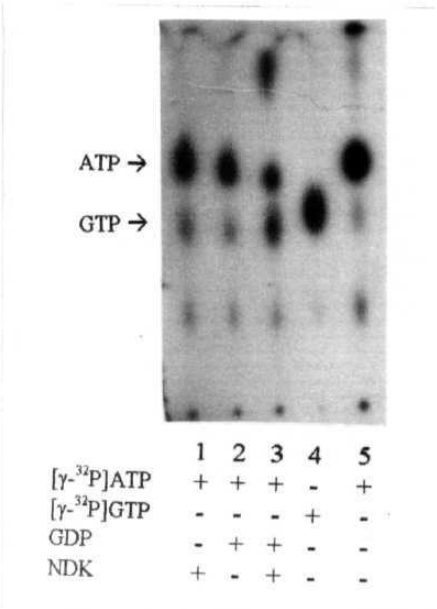


Fig. 38 Conversion **of GDP** to **labeled GTP** by **NDK** in the presence of labeled **ATP.**

The experiment were carried out in a 10 µl reaction mixture containing 22 mM Tris-HCl **(pH 7.6).** 10 **mM** $Mg(OAc)_2$, 100 mM KCl, 2.5 mM β-mercaptoethanol and 100 pmols **[γ- P]ATP.** Other additions were made as indicated in the figure. A blank reaction was carried out using only labeled ATP in the reaction mixture. The reaction mixtures were incubated at 25°C for 10 **rain,** to facilitate the conversion. Two microliter aliquots of the reaction mixtures were then withdrawn and spotted on the TLC sheets. The sheet was subjected to ascending chromatography to separate NTPs as described in 'Materials and Methods'. The chromatograms were air dried and autoradiogiaplied using Indu films. **The** figure is an **autoradiogram.**

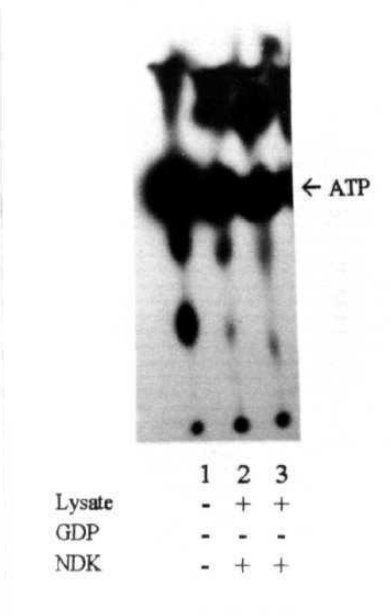


Fig. 39 Wheat germ lysates do **not contain appreciable amount of endogenous GDP.** The experiment were carried out in a 10 µl reaction mixture containing 22 mM Tris-HCl (pH 7.6), 10 mM $Mg(OAc)_2$, 100 mM KCl, 2.5 mM β-mercaptoethanol and 100 pmols [γ-^{32}P]ATP. Other additions were made as indicated in the figure. A blank reaction was carried out using only labeled ATP in the reaction mixture. The reaction mixtures were incubated at 25"C for 10 min. to facilitate the conversion. Two microliter aliquots of the reaction mixtures were then withdrawn and spotted on the TLC sheets. The sheet was subjected to ascending chromatography to separate NTPs as described **in** 'Materials and Methods' The chromatograms were air dried and autoradiograplied using Indu films. The figure is an autoradiogram.

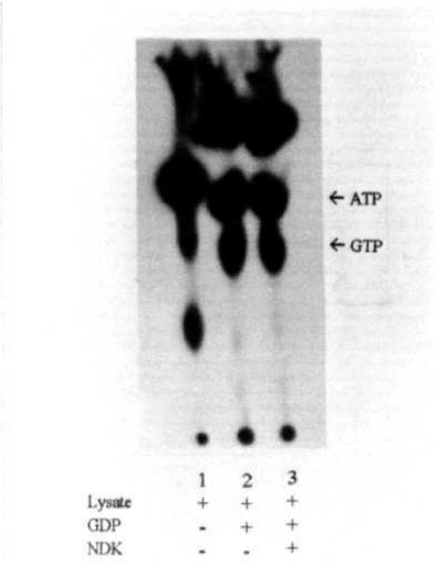


Fig. **40 Wheat germ lysates have** endogenous **NDK** like **activity.**

The **experiment** was carried **out in** a 10 ul reaction mixture containing 22 mM Tris-HCl **(pH** 7.6). **10** mM $Mg(OAc)_2$, 100 mM KC1. 2.5 mM β-mercaptoethanol and loo pmols **[y-"P]ATP.** Oilier additions were made as indicated in **the** figure. A blank reaction was carried out using only labeled ATP in the reaction mixture. The reaction **mixtures** were incubated at 25°C for 10 **mill** to facilitate **the conversion.** Two microliter **aliquots of** the **reaction** mixtures were then withdrawn and spotted on the **TLC** sheets. **The** sheet was subjected to ascending chromatography to separate NTPs as described in 'Materials and Methods'. The chromatograms were air dried and autoradiographed using Indu films. The figure is an **autoradiogram.**

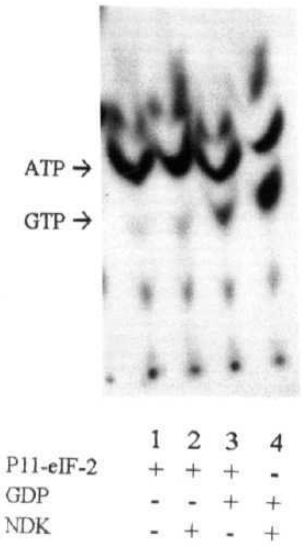


Fig. 41 Partially purified wheat germ **eIF-2** does not contain bound **GDP** but the **eIF-2** fraction has endogenous **NDK** like activity.

The **experiment** was carried out in a 10 µl reaction mixture containing 22 mM Tris-HCl (pH 7.6), 10 mM $Mg(OAc)_2$, 100 mM KCl, 2.5 mM β-mercaptoethanol and 100 pmols [γ-^{32}P]ATP. The experiments were carried out in the presence of partially purified wheat germ eIF-2 (2 µg). Oilier additions were made as indicated in the figure. A blank reaction was carried **out** using only labeled ATP in the reaction mixture. The reaction mixtures were incubated at 25°C for 10 min. to facilitate the conversion. Two microliter aliquots of the reaction mixtures were then withdrawn and spotted on the TLC sheets. The sheet was subjected to ascending **chromatography** to separate NTPs as described in 'Materials and Methods'. The chromatograms were air dried and autoradiographed using Indu **films.** The figure is an autoradiogram.

5.5 *Actively translating lysates have low levels of GDP, but can convert added GDP to GTP In the presence of ATP:*

As already mentioned in this chapter GDP can substitute for GTP in wheat germ lysato protein synthesis and it may be due to the presence of an NDK like activity To test this possibility the separation of labeled nucleotides has been standardized (as mentioned above). Further, a) the amount of endogenous GDP in the lysates and b) ability of lysates to convert GDP to GTP has been determined here. In the first experiment no external GDP was provided in the reaction mixture (Fig. 39). Lane 1 is a blank with only labeled ATP in the reaction while the other lanes contain lysate as well in the reactions. No conversion of GDP to labeled GTP was observed in lysates when NDK was supplemented to the reaction mixture (Fig. 39, lane 3) suggesting that the endogenous levels of GDP are probably inadequate to demonstrate the conversion of GDP to GTP. In the next experiment, GDP (30 pmols) was supplemented to the reaction to search for NDK like activity in lysates (Fig. 40). The reaction was carried out in the presence of labeled ATP and lysate with the addition of GDP at the start of incubation. A clear conversion of GDP to GTP was observed in lane 2 confirming the presence of NDK like activity in wheat germ lysates. No further enhancement was seen when purified NDK was also used with GDP (lane 3). Lane 1 contains labeled ATP as blank.

The results of the above two experiments suggest that the lysate GDP levels are too low to be detected by this assay system, and that the lysates contain NDK like activity.

5.6 *Partially purified eIF-2 does not contain bound GDP but can convert added GDP to GTP:*

Since purified mammalian eIF-2 is found associated with GDP (Siekierka *et al.*, 1983), we tried to determine the GDP bound to partially purified wheat germ eIF-2 (Fig. 41). It is interesting that no conversion of GDP to GTP occurred when eIF-2 was incubated with NDK (lane 3), but some amount of conversion occurred when the fraction was incubated with GDP (lane 4). These findings suggest a) that the partially purified eIF-2 is not associated with GDP which can be detected here by the assay system and b) NDK like activity exists in partially purified preparations of wheat germ eIF-2.

Since the eIF-2 preparation used above was only partially purified, similar studies were also carried out with highly purified (85%) wheat germ eIF-2

5.7 *Highly purified fraction of eIF-2 contains NDK like activity:*

The experiment was carried out as mentioned for partially purified wheat germ eIF-2 (Fig 42). Lane 1 contained only [γ–^{32}P]ATP and the reaction mixture to assess the migration of ATP. Since labeled GTP could not be formed in the absence of NDK like activity, lane 3 reaction was carried out with NDK (3 units). Lane 4 reaction contained eIF-2 and unlabeled GDP (30 pmols), while lane 5 reaction was similar to Lane 4 but it was supplemented with the enzyme NDK

Since labeled GTP was not formed in lane 3 even in the presence of NDK, it is concluded here that eIF-2 preparations do not contain any significant bound GDP. In contrast, the eIF-2 preparation converted added unlabeled GDP to labeled GTP in the absence of NDK (lane 4). Addition of purified commercially available NDK enhanced further the formation of GTP from GDP (lane 5). Based on these findings it is concluded that this eIF-2 fraction contains NDK like activity. It has to be determined however if the NDK like activity belongs to a co-purifying contaminant protein or is a part of the eIF-2 itself.

5.8 NDK like activity can convert GDP bound to eIF-2, in a binary complex, to GTP:

We wanted to further test the possibility of this NDK like activity to convert the GDP bound to eIF-2 in binary complex to GTP. Therefore, wheat germ binary complex was prepared as described in 'Materials and Methods' with 2 ug of wheat germ eIF-2 and 2 uM unlabeled GDP. The reaction mixture was then filtered through a nitrocellulose to remove the unbound GDP. The nitrocellulose membrane containing the binary complex (eIF-2.GDP) was then incubated with [γ-^{32}P]ATP (100 pmols) in the presence of 20 mM Tris-HCl buffer, pH 7.6; 80 mM KC1; and 10 mM Mg^{2+}, to see the conversion of GDP in the binary complex to [γ-^{32}P]GTP The samples were incubated at 25°C for 5 minutes. The reaction was then terminated and the excess labeled ATP was removed by washing the

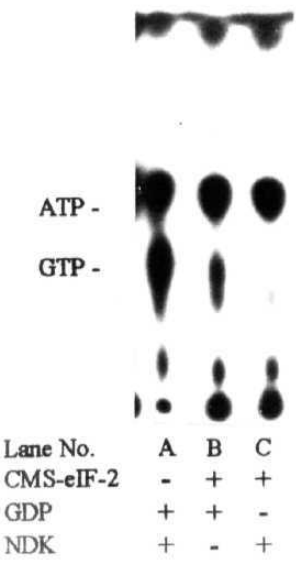


Fig. 42 Highly purified wheat germ **eIF-2 (CMS-eIF-2)** also does not contain bound GDP but the **eIF-2** fraction **has** endogenous NDK like activity.

The experiment was carried out in a 10 µl reaction mixture containing 22 mM Tris-HCl (pH 7.6), 10 mM $Mg(OAc)_2$, 100 mM KCl, 2.5 mM β-mercaptoethanol and 100 pmols [γ- "P]ATP. **The** experiments were carried out in the presence of highly purified CMS-eIF-2 (2 µg). Other additions were made as indicated in the figure. A blank reaction was carried out using only labeled ATP in the reaction mixture. The reaction mixtures were incubated at 25°C for 10 **rain,** to facilitate the conversion. Two microliter **aliquots** of the reaction mixtures were then withdrawn and spotted on the TLC sheets. The sheet was subjected to ascending chromatography to separate NTPs as described in 'Materials and Methods'. **The** chromatograms were air dried and autoradiographied using lndu films The figure is an autoradiogram.

Table 1. Conversion **of unlabeled GDP in wheat germ eIF-2.GDP binary complex to labeled GTP in the presence of [γ-^{32}P]ATP:**

Experimental conditions	[γ-^{32}P] bound (CPM)	[γ-^{32}P] bound (pmol)
WGBC +[γ-^{32}P]ATP	1,96,122	11.14
WGBC +[γ-^{32}P]ATP+WGeIF-2	2,34,122	13.302
WGBC+[γ-^{32}P]ATP+NDK	3,75,240	21.32
Wheat germ eIF-2 +[γ-^{32}P]ATP	1,558	-

WGBC - Wheat germ binary complex (eIF-2.GDP)

Wheat germ binary complex was prepared as described in 'Materials and Methods' except that unlabeled GDP was used instead of labeled GDP to prepare the binary complex. The formed binary complex was filtered through Millipore filter to remove excess GDP and then incubated with [γ-^{32}P]ATP for 5 min. at 25°C with or without any further addition, as indicated in the table. The reaction was then terminated by the addition of excess cold wash buffer and the contents were filtered. The filters were then washed thrice with 10 ml cold wash buffer, air dried and read in a liquid scintillation counter. one pmol of bound [γ-^{32}P] gives ~17,600 CPM.

nitrocellulose membrane with cold wash buffer (20 mM Tris-HCl, pH 7.6; 80 mM KCl and 2.5 mM Mg^{2+}). The nitrocellulose filters were dried and read in a liquid scintillation counter to study the conversion of unlabeled GDP to labeled GTP.

Since the NDK like activity may not be an integral part of eIF-2 and may be separated from eEF-2 due to filtration, additional experiments have been carried out by adding 2μg of eIF-2 (so as to provide NDK like activity) or 3 units of purified (commercial) NDK to the incubation mixture.

Conversion of GDP to GTP in the wheat germ binary complex occured through the endogenous NDK like activity of the fraction (table 1). The conversion was further aided by the addition of either wheat germ eIF-2 fraction or purified NDK. A control reaction (without GDP) was also carried out to determine the non-specific binding of labeled ATP to wheat germ eIF-2 protein fraction. Counts ($[\gamma\text{-}^{32}P]ATP$ bound) from the control filter were found negligible (table 1).

These findings are consistent with the earlier observations reported here that wheat germ eIF-2 is associated with NDK like activity that can convert the bound and free GDP to GTP.

CHAPTER 6

SUMMARY

The major observations of the present thesis are as follows:

- Oxidizing and alkylating agents can cause potent inhibition of protein synthesis.
- Inhibition caused by oxidizing agents like diamide can be reversed in wheat germ lysates by equimolar addition of a reducing agent like DTT.
- Addition of purified mammalian eIF-2α kinase like heme-regulated inhibitor (HRI) or, dsRNA or poly IC to translating wheat germ lysate does not inhibit wheat germ protein synthesis.
- Purified CK-II, a serine-threonine kinase, from sea star inhibits wheat germ protein synthesis.
- Genistein, an inhibitor of tyrosine kinase, does not effect wheat germ translation.
- Partially purified tyrosine kinase, p^{56} lck, inhibits wheat germ protein synthesis.
- Okadaic acid, a potent inhibitor of type 1 and type 2 serine-threonine phosphatases do not cause any significant effect on wheat germ translation.
- NEM and DTT enhance wheat germ lysate protein phosphorylation, including the p36 subunit of wheat germ eIF-2.
- NEM and DTT act synergistically and enhance the phosphorylation of lysate proteins.
- Diamide causes a general decrease in protein phosphorylation. However it enhances the phosphorylation of a single polypeptide of approx. 46 kDa.
- Heat shock causes a general reduction in lysate protein phosphorylation. In contrast, the general phosphorylation is enhanced in the presence of NEM. NEM also enhances the general protein phosphorylation during heat shock.
- Native or purified reticulocyte eIF-2a kinases phosphorylation p41-42 doublet subunit of wheat germ eIF-2.
- Wheat germ eIF-2 can exchange GDP *in vitro* in the absence of an eIF-2B like protein.
- The guanine nucleotide exchange activity associated with wheat germ eIF-2 is not inhibited *in vitro* due to eIF-2 phosphorylation.
- Protein synthesis cannot proceed in the absence of ATP.
- Protein synthesis drops by approx. 40% in the absence of an energy regenerating system.
- ADP and GDP can substitute for their respective triphosphates in translation in the presence of an energy regenerating system.
- In the absence of an energy regenerating system, ADP cannot but GDP can substitute for their respective triphosphates.
- GDP can substitute for GTP even in the absence of CP / CPK since it can be converted to GTP in the presence of ATP and nucleoside diphosphate kinase (NDK).
- Though GDP can support protein synthesis in the absence of GTP, increasing the concentration of GDP inhibits wheat germ protein synthesis.

- Addition of equimolar or higher concentrations of GDP to protein synthesizing lysates containing GTP inhibits translation suggesting that the GDP/GTP ratios play a role in the regulation of translation.
- NDK like activity is present in lysates and is also found associated with purified wheat germ **eIF-2**
- NDK like activity associated with wheat germ eIF-2 can convert GDP bound to eIF-2, in a binary complex, to GTP.

Keeping in view of the above results, it is likely that the regulation of wheat germ translation mediated by eIF-2 phosphorylation appears to be different from mammalian systems. Phosphorylation of wheat germ eIF-2 may not be involved in the regulation of protein synthesis. Changes in redox conditions or GDP / GTP and ADP / ATP ratios may play a role in the regulation of protein synthesis in wheat germ lysates.

CHAPTER 7

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ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS
Vol. 324, No I, December 1, pp. 1-8, 1995

Phosphorylation of Wheat Germ Initiation Factor 2 (eIF-2) by /V-Ethylmaleimide-Treated Wheat Germ Lysates and by Purified Casein Kinase II Does Not Affect the Guanine Nucleotide Exchange on eIF-2

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Received May 23, 1995, and in revised form August 28, 1995

Phosphorylation of the small subunit of eukaryotic initiation factor-2 (eIF-2α) impairs protein synthesis in mammalian systems. It is not known, however, if a similar regulatory mechanism exists in plants. Previous reports indicate that one of the wheat germ eIF-2 subunits, the p40-41 doublet, is phosphorylated by heterologous eIF-2α kinases. Here we report that phosphorylation of the small subunit in wheat germ eIF-2, p36, occurs in translating wheat germ lysates which are pretreated with *N*-ethylmaleimide (NEM) and dithiothreitol. Also, a purified sea star casein kinase II (CKII) phosphorylates the p41-42 doublet and p36 subunits of wheat germ eIF-2. While heme-regulated eIF-2a kinase from reticulocyte lysates does not inhibit wheat germ protein synthesis, CKII and NEM are found to be inhibitory. To determine whether phosphorylation of the small subunit (p36) is the cause for protein synthesis inhibition, we have further studied the exchange of labeled GDP for unlabeled GDP in the preformed eIF-2 · [^{3}H]GDP complex *in vitro* in the presence of CKII and ATP. The GDP exchange in ³*V*-2 - GDP complex can occur without the addition of any protein factor and the exchange reaction is marginally inhibited by CKII. A 0-70% ammonium sulfate cut fraction, prepared from NEM-treated wheat germ lysate, also does not inhibit the guanine nucleotide exchange reaction. These findings suggest that the protein synthesis inhibition in these cases is not mediated by eIF-2 phosphorylation. C 1995 Academic Press, Inc.

Phosphorylation of the protein factors involved in translation is a principal means of regulating protein synthesis in eukaryotic cells (reviewed in 1). Eukaryotic initiation factor 2 (eIF-2),[2] a heterotrimer, is a key protein factor involved in the initiation step of protein synthesis. At the end of protein synthesis initiation, the factor is released as eIF-2 • GDP. The exchange of GTP for GDP in eIF-2 • GDP is a prerequisite for eIF-2 to join the initiator tRNA and enter into another round of initiation. The guanine nucleotide exchange in eIF-2 is catalyzed by a rate-limiting multimeric protein factor called eIF-2B (reviewed in 2-5). Phosphorylation of a small portion of the small subunit (α-subunit) in eIF-2 (eIF-2α) that occurs due to the activation of eIF-2α kinases under several conditions (reviewed in 6 and 7) can impair the eIF-2B activity (8, 9) and protein synthesis *in vitro* (reviewed in 10 and 11). These events affect the recycling of eIF-2 (12-15). With the help of amino acid sequencing (16), site-specific mutagenesis (17), and mutational studies (18) and through the overexpression of mutant and wild type eIF-2α, it has been demonstrated that phosphorylation of Ser51 residue in wild-type eIF-2α impairs protein synthesis (19-21) and eIF-2B activity *in vivo* (22). Recent studies also suggest that mammalian eIF-2α kinases can phosphorylate insect and yeast cell eIF-2α and regulate the insect cell protein synthesis and GCN4 translation in yeast (23, 24). However, it is not known if eIF-2α phosphorylation occurs or if a similar regulatory mechanism exists in plants.

It has been reported that tobacco mosaic virus infec-

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[2] Abbreviations used: eIF-2, eukaryotic initiation factor-2; eIF-2(αP), eIF-2 phosphorylated in the a-subunit; eIF-2 • GDP, binary complex of eIF-2 and GDP; eIF-2B, guanine nucleotide exchange factor (also called GEF or reversing factor); dsRNA, double-stranded RNA; dsI, dsRNA-dependent eIF-2a kinase; HRI, heme-regulated eIF-2a kinase; SDS, sodium dodecyl sulfate, PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; CKII, casein kinase II; TCA, trichloroacetic acid; GSSG, oxidized form of glutathione; $AmmSO_4$, ammonium sulfate; BMV, brome mosaic virus.

0003-9861/95 $12.00

tion, or addition of dsRNA to uninfected host cell extracts, enhances the phosphorylation of a host-encoded protein (p68) which shares many properties with one of the eIF-2a kinases (double-stranded RNA-regulated eIF-2a kinase) of mammalian cells (25). Whether p68 has an eIF-2α kinase-like activity is not yet known. Earlier reports indicate that one of the subunits, p41-42, a doublet subunit in wheat germ eIF-2, is phosphorylated by heme-regulated eIF-2α kinase (HRI) of reticulocyte lysates (26) and also by casein kinase II (27). Phosphorylation of this doublet subunit (p41–42) was shown to inhibit wheat germ translation *in vitro* (26). In contrast, recent studies (28) have shown that phosphorylation of the above subunit by double-stranded RNA-induced inhibitor (PKR or dsI), yet another eIF-*2a* kinase, does not affect wheat germ translation. Based on the mobility of wheat germ eIF-2 subunits in sodium dodecyl sulfate and polyacrylamide gels and stain intensity, the p41-42 subunits are referred to as *0* and *y* subunits, respectively (28), or designated as the β-doublet (29). The small subunit, p36, is designated as the a-subunit by both these groups (28 and 29). However, some groups (26 and 27) referred to the β-doublet as a-subunit, probably because this doublet is phosphorylated by heterologous eIF-2α kinases. We refer to these subunits by molecular weights. None of the earlier reports have shown that the small subunit, p36, in wheat germ eIF-2 can be phosphorylated.

In our studies here we find that the small subunit in wheat germ eIF-2 can be phosphorylated in translating lysates which are treated with *N*-ethylmaleimide (NEM) and/or dithiothreitol (DTT). Also, a purified casein kinase II (CKII) phosphorylates the p41-42 doublet and p36 subunits of eIF-2. Partially purified HRI, which is shown to phosphorylate the doublet subunit of wheat germ eIF-2, cannot inhibit wheat germ translation, whereas CKII is found here to cause inhibition in protein synthesis. However, this inhibition in protein synthesis by CKII does not appear to be mediated by eIF-2 phosphorylation since eIF-2·GDP exchange activity associated with purified eIF-2 preparation is not inhibited *in vitro* by CKII.

MATERIALS AND METHODS

Materials. Purified rabbit reticulocyte eIF-2α kinases (HRI and dsI) and reoviral dsRNA were kind gifts received from Drs. Jane-Jane Chen and Daniel Levin (MIT, Cambridge, MA). A small amount of purified wheat germ eIF-2 used in these experiments was a kind gift from Dr. J. M. Ravel (University of Texas at Austin, TX). We have also purified wheat germ eIF-2 as described by Dr. Ravel's group (29). The preliminary characteristics of this preparation have been reported (30). Reticulocyte eIF-2 was prepared as described (31). BMV RNA was obtained from Promega and casein kinase II was purchased from Upstate Biotech, Inc., New York. Wheat germ was obtained locally. [^{35}S]Methionine (1100 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were obtained from BRIT, Bombay, and Jonaki Centre in CCMB, Hyderabad, respectively. [^{3}H]GDP (9 Ci/mmol) was purchased from Dupont NEN. DTT was obtained from Boehringer Mannheim. All other chemicals were purchased from Sigma (St. Louis, MO).

Protein synthesis in lysates. Gel-filtered wheat germ lysate (32, 33) and heme-deficient rabbit reticulocyte lysates (34) were prepared as described previously. Wheat germ and reticulrcyte protein synthesis was carried out at 25 and 30°C, respectively. DTT (1 mM) was added during the preparation of wheat germ lysates but no additional DTT was added to lysates during protein synthesis, as has been recommended by other protocols (45). Reticulocyte lysates were not supplemented with DTT at any stage. Wheat germ and reticulocyte protein synthesis was measured by the incorporation of [^{35}S]-methionine and [^{14}C]leucine, respectively, into TCA-precipitable protein in 5-μl aliquots with time. Other modifications of the standard protocols, if any, are mentioned in the figure legends.

Phosphorylation assays. Lysates were pulsed with [γ-^{32}P]ATP (3000 Ci/mmol) for a brief period (5 min) with or without exogenously added eIF-2. Phosphorylation assays of wheat germ eIF-2 and reticulocyte eIF-2 were carried out at 25 and 30°C with the addition of 2.5 and 1.0 mM $Mg(OAc)_2$, respectively, in 20 mM Tris–HCl buffer (pH 7.5) containing 80 mM KC1. Samples were separated by 10% polyacrylamide and 0.1% sodium dodecyl sulfate gels (35). Gels were analyzed by autoradiography.

Preparation of binary complex, eIF-2·[^{3}H]GDP, and its dissoeiation. Purified wheat germ eIF-2 (2 μg) was incubated in 20-μl reaction mixtures containing Tris-HCl (20 mM, pH 7.8), KC1 (100 mM), creatine phosphofructokinase (100 μg/ml), and [^{3}H]GDP (2 μM, ~1900 cpm/pmol) at 25°C for 10 min to form an eIF-2 • [^{3}H]GDP binary complex as described (9, 22). The complex was stabilized by the addition of 2.5 mM $Mg(OAc)_2$. Exchange of unlabeled GDP (GDP) for labeled GDP was studied with the addition of 40 μM unlabeled GDP. Reactions were carried out at 25°C for specified time intervals as described in the figure legends and stopped with the addition of 3 ml cold wash buffer (20 mM Tris-HCl, pH 7.8, 2.5 mM $Mg(OAc)_2$, and 100 mM KC1). Reaction contents were filtered through Millipore filters (HAWP, 0.45 μm). The amount of radioactivity bound to filters was counted in a Beckman liquid scintillation counter. Modifications (if any) are mentioned in the figure legends. Picomoles of eIF-2 • [^{3}H]-GDP dissociated were determined by the difference of the total eIF-2 • [^{3}H]GDP added and the amount remaining at the end of the reaction as described (22).

RESULTS AND DISCUSSION

Purified or Native Reticulocyte eIF-2a Kinases Phosphorylate the p41-42 Doublet of Wheat Germ eIF-2

Purified wheat germ eIF-2 was resolved into three subunits in our polyacrylamide gels (10% acrylamide, 0.26% bis, and 0.1% SDS). The molecular weights of these polypeptides are 36,000, 41,000-42,000 (doublet), and 52,000 (data not shown). The preparation matches with the preparations made by Lax *et al.* (29) and also with the recent preparations described by Shaikin *et al.* (28). Purified reticulocyte kinases, HRI (Fig. 1A) or dsI (Fig. 1B) which could phosphorylate the small subunit (38 kDa) of reticulocyte eIF-2 (eIF-2a) (Fig. 1A, lane 1, and Fig. 1B, lane 1) phosphorylated the p41-42 doublet subunit of wheat germ eIF-2 *in vitro* (Fig. 1A, lane 2, and Fig. 1B, lane 2). The small subunit, p36, of wheat germ eIF-2, however, was not phosphorylated under those conditions.

In a separate experiment (Fig. 1C), the phosphoryla-

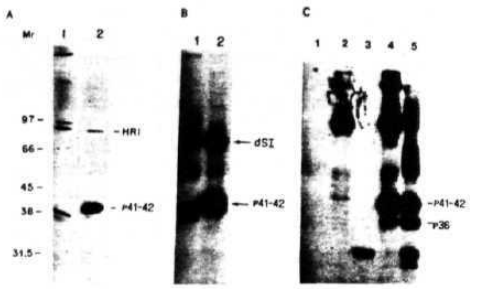

FIG. 1. **Phosphorylation** of reticulocyte and wheat germ **eIF-2** proteins by **purified HRI, dsI,** and **CKII kinases** *in vitro*. Phosphoryiation reactions were carried out in standard **20-**μl reaction mixtures containing 20 **mM** Tris-HCl (pH 7.7), 2.5 **mM** $Mg(OAc)_2$, 30 μ**M** [γ-^{32}P]**ATP** (26 Ci/mmol), and with purified wheat germ or reticulocyte eIF-2 (~**150** ng). Reaction mixtures were incubated at **30°C** for 10 **min** and the samples were separated by SDS-PAGE as described under Materials and Methods. The figure is an **autoradiogram**. (A) Phosphoryiation of eIF-2 by a relatively pure **HRI** preparation (44). Lane 1, **+reticulocyte** eIF-2; lane 2, +wheat germ eIF-2. (B) Phosphoryiation by **dsI** kinase. Lane 1, **+reticulocyte** eIF-2; lane 2, +wheat germ eIF-2. (C) Phosphoryiation of wheat germ eIF-2 without any added kinase (lane 1), a relatively impure **HRI** kinase alone (lane 2), highly **purified CKII** alone (lane 3), wheat germ eIF-2 by a partially purified HRI (lane 4), and wheat germ eIF-2 by CKII (lane 5).

tion of purified wheat germ eIF-2 was tested without the addition of any kinase (lane 1) or in the presence of an HRI preparation that was relatively less purified and also by a highly purified multipotential kinase like CKII. Little or no phosphoryiation of purified wheat germ eIF-2 occurred in the absence of any kinase (lane 1). Partially purified HRI was autophosphorylated (lane 2) **and** it phosphorylated both the p36 and the p41-42 doublet subunits in wheat germ eIF-2 (lane 4). The highly purified CKII was also autophosphorylated (lane 3) and it phosphorylated the p41-42 doublet subunit and the small subunit, p36, of eIF-2 (lane 5). These findings suggest that the p41-42 doublet subunit of wheat germ eIF-2 is phosphorylated by HRI and CKII kinases. In contrast, the phosphoryiation of the small subunit of wheat germ eIF-2 occurs by CKII or by a contaminating kinase present in the relatively impure HRI preparations.

Whether native reticulocyte **eIF-2**α kinases can phosphorylate wheat germ eIF-2 was also tested by adding a small amount of the latter to heme-deficient, hemin-treated, or **heme-** and NEM-treated reticulocyte lysates (Fig. 2). Heme deficiency or **NEM** treatment activated endogenous reticulocyte **eIF-2**α kinase(s) which is known to phosphorylate endogenous (Fig. 2, lanes 1 and 7) or exogenously added (Fig. 2, lanes 3 and 9) reticulocyte eIF-2 (36). Addition of **hemin** inhibited the **eIF-2**α kinase activity of HRI. Hence, relatively very little endogenous or exogenously added reticulocyte **eIF-2**α was phosphorylated in hemin-treated **ly** sates (Fig. **2**, lanes 4 and 6) when compared to heme-deficient, or heme- and NEM-treated lysates (Fig. 2, lanes 1, 3, 7, and 9). In contrast, the phosphoryiation of p41-42 doublet of wheat germ eIF-2 was found to be similar in both heme-deficient and **hemin-supplemented** lysates (Fig. 2, lanes 2 and 5) but was enhanced in NEM-treated reticulocyte lysates (Fig. 2, lane 8). These findings suggest that in addition to HRI, other kinases in reticulocyte lysates can phosphorylate the doublet subunit, p41-42, of wheat germ eIF-2. We could not, however, assess the phosphoryiation status of the small subunit, p36, of wheat germ eIF-2 in reticulocyte lysates.

We have also analyzed the phosphoryiation pattern of translating wheat germ lysates treated with highly purified reticulocyte HRI and other agents which are known to stimulate the phosphoryiation of reticulocyte **eIF-2**α (Fig. 3). Purified HRI did not alter the phosphoryiation pattern of the translating wheat germ lysate

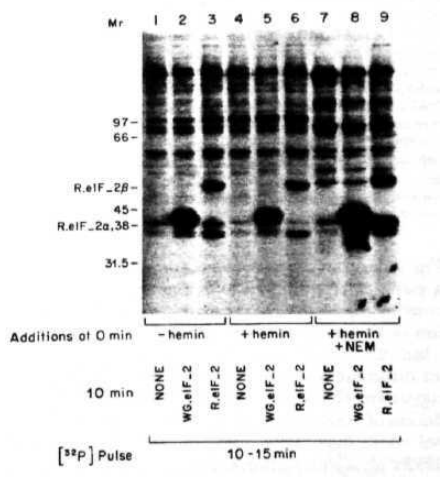

FIG. 2. Phosphoryiation of wheat germ eIF-2 in rabbit reticulocyte lysates. Protein synthesis was carried out at **30°C** in **10-**μl reticulocyte lysates as described under Materials and Methods without any added hemin (tracks 1, 2, and **3**), with hemin (20 μ**M**, tracks 4, 5, and 6), or with hemin and NEM (20 μ**M/5 mM**, tracks 7, 8, and 9) for 10 min. A **5-**μl aliquot of protein synthesizing lysate was then incubated with a **5** μl reaction mixture containing purified wheat germ eIF-2 (200 ng) (tracks 2, 5, and 8) or reticulocyte eIF-2 (150 **ng**) (tracks 3, 6, and 9), 10 μ**Ci** [γ-^{32}P]**ATP** and Mg^{2+} (1 mM) for 5 min at **30°C**. Protein synthesis reaction mixtures were also incubated without any added reticulocyte eIF-2 (tracks 1, 4, and 7). Samples were separated on SDS-PAGE. The figure is an autoradiogram.

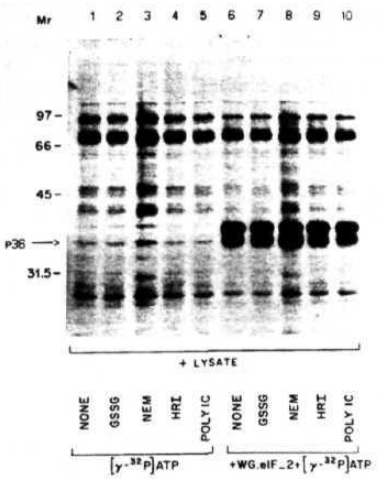

FIG. 3. [^{32}P]Phosphoprotein profiles of wheat germ lysate and purified wheat germ eIF-2. Wheat germ protein synthesis was carried out in the presence of GSSG (0.5 mM, tracks 2 and 7), NEM (1.0 mM, tracks 3 and 8), HRI (-50 ng, tracks 4 and 9), or poly(IC) (100 ng/ml, tracks 5 and 10) at 25°C for 15 min. Ten microliters of the protein synthesis reactions was then supplemented with 5 μl of Tris–HCl buffer (20 mM, pH 7.8) containing [γ-^{32}P]ATP (10 μCi) (tracks 1–5). Tracks 6-10 are reaction mixtures similar to 1-5 but the reactions were carried out in the presence of exogenously added purified eIF-2 (—200 ng). Tracks 1 and 6 are control lysate reactions without and with added eIF-2. The final reaction mixtures containing 2.5 mM Mg^{2+} were incubated at 25°C for 5 min. Aliquots of 7.5 μl of the reaction mixtures were withdrawn and separated on SDS–PAGE as described under Materials and Methods.

(Fig. 3, lane 4 vs lane 1). Further, when a small amount of purified wheat germ eIF-2 and HRI were added to translating wheat germ lysates, variable phosphorylation of the subunits p36 and p41-42 was noted (Fig. 3, lane 9). However, the pattern of phosphorylation was not different from the control reaction (Fig. 3, lane 7), suggesting that some amount (basal level) of phosphorylation of wheat germ eIF-2 subunits occurs under normal conditions. Poly(IC) treatment (which facilitates eIF-2α phosphorylation through the activation of dsI in reticulocyte lysates) did not alter the phosphorylation pattern in wheat germ lysates (Fig. 3, lanes 5 and 10 vs control lanes 1 and 6), suggesting that wheat germ may not carry any endogenous kinase which can be activated by poly(IC) or dsRNA.

SH-Reactive Agents such as NEM and DTT Enhance the Phosphorylation of Wheat Germ Lysate Proteins Including p36 of eIF-2

The extent to which some SH-reactive agents like oxidized glutathione (GSSG) and NEM, which are known to activate the reticulocyte eIF-2α kinase(s) (36-38), can phosphorylate wheat germ eIF-2 in translating wheat germ lysates was investigated. Our studies showed that GSSG did not alter the phosphorylation of wheat germ lysate proteins (Fig. 3, lane 2 vs control lane 1), or that of exogenously added eIF-2 in wheat germ lysate (Fig. 3, lane 7 vs lane 5). Under the same conditions, NEM treatment (1.0 mM) significantly enhanced the phosphorylation of several lysate proteins (Fig. 3, lane 3 vs lane 1) including the small subunit, p36, of added wheat germ eIF-2 (Fig. 3, lane 8 vs lane 6).

In another experiment (Fig. 4), in order to neutralize the effect of NEM, the translating NEM-treated wheat germ lysate was supplemented with an equimolar concentration of DTT and the pattern of protein phosphorylation was studied. DTT enhanced the phosphoryla-

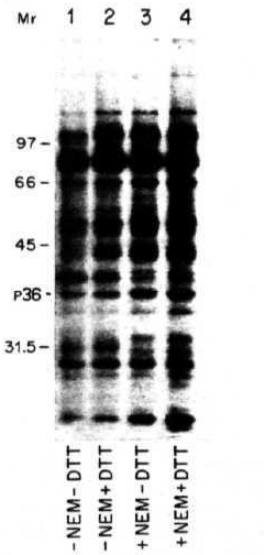

FIG. 4. Phosphorylation of purified eIF-2 in NEM- and DTT-treated wheat germ lysates. Wheat germ lysate protein synthesis was carried out with or without the addition of NEM (1 mM) as described under Materials and Methods. Wherever indicated, at 15 min of protein synthesis, the reaction mixtures were supplemented with DTT (1 mM). Six microliters of protein synthesizing lysate was then incubated with another 6 μl of buffer (Tris-HCl, pH 7.8) containing 2.5 mM Mg^{2+}, 10 μCi [γ-^{32}P]ATP, and 1 μl of eIF-2 (~150 ng) for 5 min at 25°C. The reaction mixtures were terminated with the addition of SDS sample buffer and the proteins were separated by SDS-PAGE. The figure is an autoradiogram. Track 1, -NEM, –DTT; track 2, -NEM, +DTT; track 3, +NEM, -DTT; track 4, +NEM, +DTT.

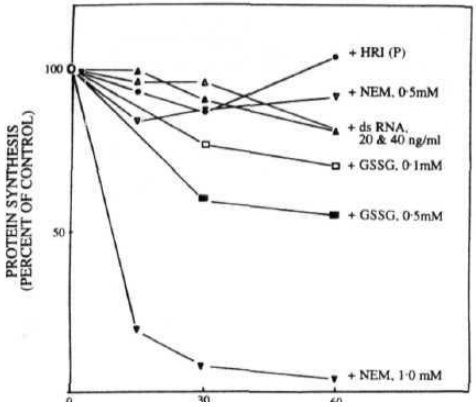

FIG. 5. Protein synthesis in wheat germ lysate in the presence of various agents. Protein synthesis was carried out in the presence of various agents as described under Materials and Methods. Protein synthesis was determined by the incorporation of [^{35}S]methionine into protein in 5-μl aliquots taken at different time intervals. The lines represent the results of three separate experiments. The values indicate the percentage of protein synthesis of the control in the presence of various agents. In experiment 1, the lysate protein synthesis was studied using globin mRNA (50 fig/ml) to assess the effect of phosphorylated HRI (~50 ng, • - •). The control reaction contains the phosphorylation reaction mixture lacking HRI. The [^{35}S]-methionine incorporation in this reaction was 7000, 22,000, and 38,500 cpm for 15, 30, and 60 min, respectively. In another experiment, the effect of GSSG was investigated (D-D, 0.1 mM, and • • •, 0.5 mM) on the protein synthesis of BMV RNA (50 μg/ml). The values of the control reaction were 32,000, 57,000, and 68,000 cpm for 15, 30, and 60 min, respectively. The effect of NEM (V - V, 0.5 mM, and T - T, 1.0 mM) and dsRNA (A - A, 20 ng/ml, and ▲ - A, 40 ng/ml) was investigated in a different experiment on protein synthesis of BMV RNA (75 μg/ml). The values of the control reaction were 25,837, 75,128, and 134,864 cpm for 15, 30, and 60 min, respectively.

tion of several proteins including that of exogenously added wheat germ eIF-2(p36) (Fig. 4, lane 2 vs lane 1). DTT and NEM (lane 4) together led to increased phosphorylation of wheat germ eIF-2 and other lysate proteins compared to when either was present alone (lanes 2 and 3). These findings suggest that protection of protein SH groups either by alkylation or reduction can enhance the phosphorylation of several lysate proteins including p36 of eIF-2.

Protein Synthesis in Wheat Germ Lysate

Protein synthesis was not significantly altered by the addition of activated HRI (prephosphorylated HRI) or dsRNA (Fig. 5). These agents were, however, found to inhibit reticulocyte lysate protein synthesis (data not

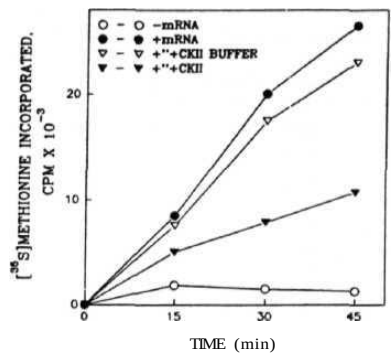

FIG. 6. Protein synthesis in wheat germ lysate in the presence of CKII. The translation of BMV RNA (15 μg/ml) in wheat germ lysate was studied in the presence and absence of CKII (50 ng/ml) as described (30 and 31). Control reactions which are carried out without the addition of CKII, however, contain the buffer (20 mM Tris–HCl, pH 7.5, 0.1 mM EDTA, 1 mM DTT) in which the enzyme was stored (V - V). Endogenous lysate protein synthesis was measured by omitting BMV RNA in the reaction mixtures (○ - ○). At different time intervals, protein synthesis was determined in 5-μl aliquots of the reaction mixtures by the incorporation of [^{35}S]methionine into the TCA-precipitable protein.

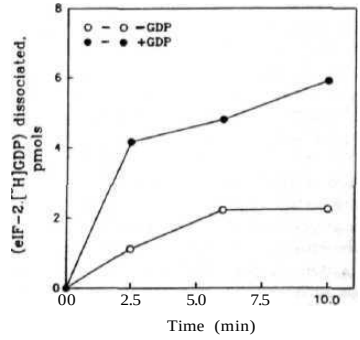

FIG. 7. Exchange of unlabeled GDP for labeled GDP in wheat germ binary complex. Labeled binary complex, eIF-2 • [^{3}H]GDP, was prepared as described under Materials and Methods. Dissociation of the preformed binary complex (70 μl, 8.5 pmol) was studied at 25°C with and without the addition of 40 μM unlabeled GDP. At different time intervals (2.5, 6, and 10 min), 20-μl aliquots of the reaction mixtures were withdrawn and stopped with the addition of cold wash buffer. The reaction mixtures were then filtered through Millipore filters (HAWP, 0.45 μm) and air dried and the radioactivity bound to the filters was measured in a liquid scintillation counter. One picomole of bound eIF-2 • [^{3}H]GDP gives ~1900 cpm. Picomoles of eIF-2 • [^{3}H]-GDP dissociated were determined as described under Materials and Methods. The figure indicates, however, the dissociation of labeled GDP from eIF-2.

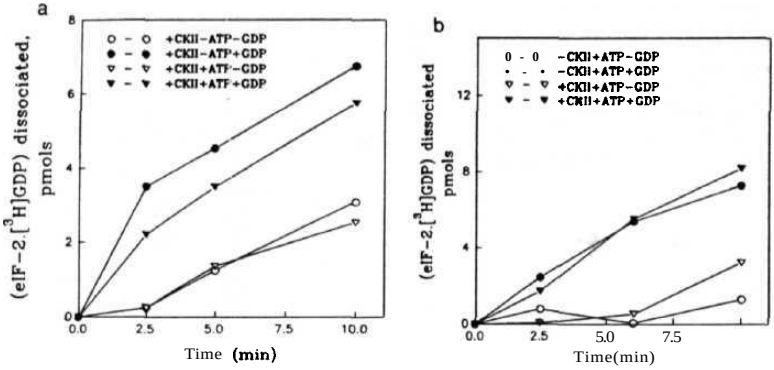

FIG. 8. Effect of phosphorylation of wheat germ eIF-2 by CHI on the dissociation of eIF-2·[^{3}H]GDP (a) In step 1, phosphorylation of wheat germ eIF-2 (1 μg) was carried out at 30°C by CKII (10 ng) for 5 min in a 10-μl reaction mixture in the presence of Tris buffer (20 mM Tris–HCl, pH 7.6, 80 mM KC1, and 2.5 mM Mg^{2+}) with and without the addition of 100 μM ATP. The control unphosphorylated reaction mixtures contained CKII protein without ATP. In step 2, the phosphorylated and unphosphorylated reaction mixtures (70 μl) were incubated with [HJGDP to form eIF-2 • [^{3}H]GDP binary complex as described under Materials and Methods In step 3, the dissociation of the preformed binary complex (70 μl, 31.5 pmol) was monitored with or without the addition of 40 μM unlabeled GDP to phosphorylated and unphosphorylated reaction mixtures. Picomoles of eIF-2 • [^{3}H]GDP dissociated were determined as described in the legend to Fig. 8 (22). (b) Dissociation of phosphorylated wheat germ eIF-2 • ["HIGDP was carried out in a way similar to that mentioned above. However, the control (unphosphorylated reaction) lacked the enzyme CKII but was supplemented with 100 μM ATP.

shown). Addition of GSSG (0.5 mM), or NEM (above 0.5 mM) inhibited wheat germ lysate protein synthesis (Fig. 5). Further, higher concentrations of DTT (>0.5 mM) did not improve the performance of lysate protein synthesis and was found to be slightly inhibitory (data not shown). Hence all protein synthesis reactions were carried out here with 0.4 mM DTT, which comes along with the lysate preparation. Interestingly, casein kinase II (1 ng) inhibited wheat germ lysate protein synthesis (Fig. 6). This finding of CKII on wheat germ translation may explain some of the earlier findings which suggested that reticulocyte eIF-2α kinases inhibit wheat germ protein synthesis (26) and were found to be subsequently untrue. As has been shown by us here, relatively impure HRI preparations may contain CKII-like activity (Fig. 1C, lane 4, vs Fig. 1B, lane 2) and this CKII activity may be the cause for translational inhibition rather than HRI. However, HRI, which does not inhibit the wheat germ lysate translation (Fig. 5), can phosphorylate the p41-42 doublet in wheat germ eIF-2, like purified CKII. In addition to the phosphorylation of p41-42 doublet, CKII also phosphorylates the small subunit, p36, of wheat germ eIF-2 along with some other proteins (Fig. 1C, lane 5) and inhibits protein synthesis as well (Fig. 6). Hence we carried out further experiments (mentioned below) on the exchange of guanine nucleotides on wheat germ eIF-2 to determine if the phosphorylation of the small subunit by CKII is the cause for protein synthesis inhibition.

Guanine Nucleotide Exchange on Wheat Germ eIF-2

The exchange of unlabeled GDP for labeled GDP in the preformed wheat germ eIF-2 • [^{3}H]GDP complex can proceed without the addition of any protein factor (Fig. 7). This observation is consistent with previous reports on the guanine nucleotide exchange of wheat germ eIF-2 (39, 28). Despite rigorous purification, earlier workers could not purify eIF-2B-like activity from wheat germ (39, 40). In many mammalian systems, it has been shown that eIF-2 can be contaminated with eIF-2B-like preparations. In fact, many times it was observed that the eIF-2B comigrates with eIF-2 preparations during purification (9, 41, 42).

To determine if the exchange of guanine nucleotides is helped by an eIF-2B protein associated with wheat germ eIF-2, it becomes important to show that the eIF-2B activity is specifically inhibited upon phosphorylation of the small subunit (p36) of eIF-2. Since purified CKII- and NEM-treated lysates were shown here to phosphorylate the p36 subunit of wheat germ eIF-2, we have also determined the guanine nucleotide exchange ability of such a phosphorylated eIF-2 *in vitro* (Figs. 8a, 8b, and 9). As can be seen from the data, the rate of guanine nucleotide exchange is not significantly inhibited upon phosphorylation of wheat germ eIF-2 by CKII (compare Figs. 8a, CKII+ATP vs CKII–ATP and 8b, ATP+CKII vs ATP–CKII) and also by NEM-treated lysate fraction (0-70% $AmmSO_4$ cut) *in vitro* (Fig. 9). These findings suggest that the protein synthe-

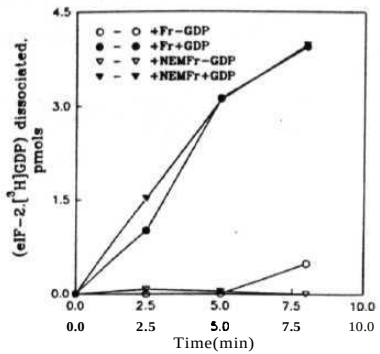

FIG. 9. Effect of NEM-treated fraction on wheat germ eIF-2 • [^{3}H]-GDP dissociation. Wheat germ lysates were treated with 100 μM ATP and incubated at 25°C for 10 min with or without the addition of NEM (1.5 mM) to facilitate the activation of endogenous eIF-2 kinase(s) and eIF-2 phosphorylation. The lysate proteins were then immediately precipitated by the addition of 2.5 volumes of saturated ammonium sulfate (70%). The NEM-treated and untreated lysate fractions (7 μl) were then incubated for 5 min at 25°C with preformed binary complex (25 pmol) in the presence of 100 μM ATP in a final volume of 77 μl to facilitate the phosphorylation of the preformed wheat germ binary complex. Afterward, the dissociation of preformed binary complex was monitored in 22-μl reaction mixtures at different time intervals with and without the addition of 40 μM unlabeled GDP as described under Materials and Methods. Fr, untreated fraction; NEM Fr, NEM-treated fraction. One picomole of bound eIF-2 • [^{3}H]GDP gives 2200 cpm. The plot indicates the dissociation of labeled GDP from eIF-2.

sis inhibition caused by CKII- or NEM-treatment are not mediated by eIF-2 phosphorylation (p36 or p41-42).

The NEM-treated lysates or purified CKII may not be phosphorylating wheat germ eIF-2 at a proper site that can effectively inhibit the eIF-2B activity. A recent study in fact emphasizes the importance of Ser51 phosphorylation and the importance of adjacent unphosphorylated amino acid residues like Ser48 in the wild-type human eIF-2(αP) to effectively sequester the eIF-2B guanine nucleotide exchange activity (22). Further, our own studies (V. M. Krishna *et al.,* unpublished observations) indicate that guanine nucleotide exchange on wheat germ eIF-2 can also proceed in inhibited heme-deficient or heme and poly(IC)-treated reticulocyte lysates in which reticulocyte eIF-2B activity is impaired due to the phosphorylation of Ser51 residue in reticulocyte eIF-2α. These lysates cannot support the guanine nucleotide exchange on reticulocyte eIF-2, but, however, can support the GDP exchange on wheat germ eIF-2. These observations are consistent with a recent study (28) which suggests that the affinity of wheat germ eIF-2 for GDP is only 10 times higher than that for GTP. In contrast, mammalian eIF-2 has much higher affinity for GDP than for GTP (43). These findings also suggest that, unlike in mammalian systems, no eIF-2B analogs are required for the exchange of guanine nucleotides on wheat germ eIF-2 and is consistent with the idea that phosphorylation of eIF-2 may not regulate protein synthesis in higher plants (28).

ACKNOWLEDGMENTS

This investigation was supported by a grant from the Department of Science and Technology, New Delhi, to K.V.A.R. We thank Dr. Joanne Ravel (University of Texas at Austin) for a generous gift of purified wheat germ eIF-2, which was used to carry out some of the initial experiments described here. We also thank Drs. Jane-Jane Chen and D. H. Levin (MIT) for providing us with their purified rabbit reticulocyte eIF-2α kinase preparations and reoviral dsRNA, respectively. Preliminary observations were abstracted in a meeting on "Translational Control" held in Cold Spring Harbor, New York (September 9-13, 1992) and at the XVIth IUBMB congress held at New Delhi, India (September 16-23, 1994). N.J. and V.M.K. were recipients of a Senior Research Fellowship from the CSIR and UGC, New Delhi, respectively.

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