Regulation of Protein Synthesis in Rabbit Reticulocyte Lysates Mediated by Initiation Factor 2 alpha (eIF-2α) Phosphorylation

Thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

By

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Certificate

This is to certify that the thesis entitled, **Regulation of Protein synthesis in rabbit reticulocyte lysates mediated by initiation factor 2 alpha (eIF-2a) phosphorylation** is based on the results of the work done by **Mr S.V.Naresh Babu** for the degree of **Doctor of Philosophy** under my supervision. This work has not been submitted for the award of degree or diploma of any other University or Institution.

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DECLARATION

I here by declare that the work presented in the present thesis entitled, **Regulation of Protein synthesis in rabbit reticulocyte lysates mediated by initiation factor 2 alpha (eIF-2α) phosphorylation,** is entirely original and was carried out by me under the guidance of **K.V.A.Ramaiah, Ph.D,** Department of Biochemistry, University of Hyderabad, Hyderabad, INDIA. I also declare that this has not been submitted before for the award of degree or diploma of any other University or Institution.

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Naresh..

ABBREVIATIONS

ADP	Adenosine 5' diphosphate
Ala	Alanine
cAMP	Adenosine 3' 5' monophosphate
AP	Alkaline phosphatase
APS	Ammonium per Sulfate
Asp	Aspartic acid
ATP	Adenosine 5' triphosphate
BOP	5-bromo-4-chloro-3-indolyl phosphate
Bis-acrylamide -	N, N'-methylene-bis-acrylamide
BSA	Bovine serum albumin
Ci	Curie
CH	Cycloheximide
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
DIA	Datura innoxia agglutinin
DNA	Deoxy ribonucleic acid
cDNA	Complementary deoxy ribonucleic acid
DAI/dsI/PKR -	double stranded RNA activated inhibitor
DEAE	Diethyiaminoethyl
DTT	dithiothreitol
EDTA	Ethylene diamine tetra acetic acid
EGF	Epidermal growth factor

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 eEF-2
EtBr	-	Ethidium bromide
FAD	-	Flavin adenine dinucleotide
Fru -1,6-BisP	-	Fructose 1, 6-diphosphate
GCN	-	General control non derepressable
GlcNAc	-	N-acetyl glucosamine
GDP	-	Guanosine 5' diphosphate
GEF/eIF-2B/RF	-	Guanine nucleotide exchange factor of eIF-2 or reversing factor
GSSG	-	Oxidized glutathione
GTP	-	Guanosine 5' triphosphate
HCR/HRI	-	Heme regulated inhibitor
HEPES	-	N-[2-hydroxyethyl] piperzine-N'-[2-ethane-sulfonic acid]
HIV	-	Human immuno deficiency virus
HSP	-	Heat shock protein
I-2	-	Inhibitor 2
H_2O_2	-	Hydrogen peroxide
I.U.	-	International Units
NAD^+	-	Nicotinamide adenine dinucleotide, reduced
NADPH	-	Nicotinamide adenine dinucleotide phosphate, reduced
NBT	-	Nitro blue tetrazonium
NEM	-	N-ethylmaleimide
min	-	minutes
PAGE	-	Polyacrylamide gel electrophoresis
PC	-	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
RNase	-	Ribonuclease
S	-	Svedberg
SDS	-	Sodium dodecyl sulphate
Ser	-	Serine
TCA	-	Trichloro acetic acid
TEMED	-	N',N N,N', N'-tetramethyl ethyl-ethylene diamine
Tris	-	Tris (hydroxymethyl) amino methane
WGA	-	Wheat germ agglutinin
μ	-	micro

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1.0. INTRODUCTION

The genetic information stored in the sequences of deoxyribonucleotide (DNA) molecules flows via three fundamental processes, namely, replication (copying the DNA template), transcription (synthesis of ribonucleic acids, RNA from DNA) and translation (decoding of the information present in RNA molecules to the amino acids in proteins by ribosomes). Each of these processes is very complex and is governed by specific cellular machinery that includes a variety of enzymes, proteins and RNA molecules. Protein synthesis in biological systems is an integral part of the overall pathway of gene expression. Gene expression can be controlled through gene rearrangements, transcription, translation and also through post-translational modifications. Since the present work is concerned with protein synthesis and its regulation particularly mediated by changes in the phosphorylation of the small or alpha-subunit (38 kDa) of initiation factor 2 (eIF-2a) in eukaryotic cells, the current information available on protein synthesis regulation due to changes in eIF-2 activity are mentioned here briefly.

Translational control, encompassing several kinds of regulation at the level of protein synthesis, is defined as a change in the efficiency of mRNA translation, that is, the number of amino acids polymerized per unit time per messenger RNA (mRNA) molecule (Hershey, 1991). This control may affect a quantitative change in the overall amount of protein synthesized, or qualitative change in the species of proteins produced. Translational control is a fact and not a fantasy any more (Hunt, 1980). This is evident from the mounting information.

For convenience, protein synthetic pathway is divided into three phases; initiation, elongation and termination and is mediated by several specific protein factors which are known as initiation, elongation and termination factors (Ochoa, 1983; Watson *et al*, 1987).

1.1. Global view of eukaryotic protein synthesis:

1.1.1. **Initiation:** It starts with the dissociation of the 80S ribosomal subunits (40S and 60S) and is divided into six phases.

i). At physiological Mg^{2+} concentration, the 40S and the 60S ribosomal subunits are in equilibrium with each other and remain separated due to the association of factors like eIF-3 and eIF-4C. These factors are also called as anti-association factors. Their association with the subunits help them to remain dissociated.

ii). Initiator tRNA (Met tRNAj) joins the 40S ribosomal subunit to form the 43 S preinitiation complex. This step requires initiation factor 2 (eEF-2) and GTP. Initiator tRNA joins the eIF-2.GTP to form a ternary complex, eIF-2.GTP.Met.$tRNA_i$, which subsequently joins the 40S ribosomal subunit to form the 43 S preinitiation complex.

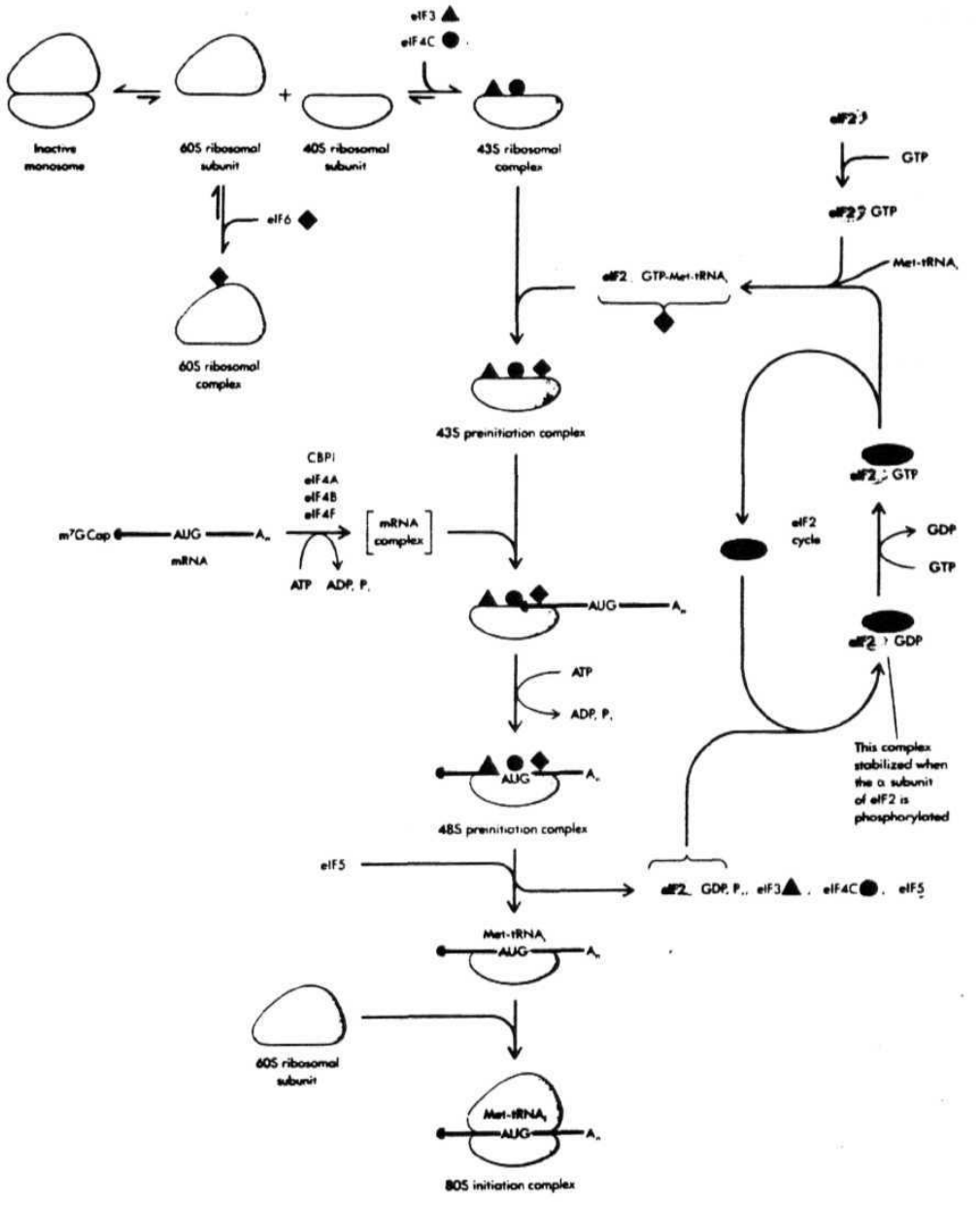
iii). The mRNA then joins the 43S preinitiation complex to form the 48S preinitiation complex. This joining reaction of 43 S to mRNA requires additional factors such as eIF-4E, eIF-4F and eIF-4A.

iv). The 48S preinitiation complex then scans the mRNA to determine the start' nucleotide sequence or start codon on the mRNA where the initiator tRNA can be positioned

v). The joining of 60S ribosomal subunit then occurs with the 48S initiation complex to form the 80S initiation complex. This step requires the release of anti-association' factors like eIF-3 and eEF-4C and also the activity of eIF-5 protein. The GTPase activity associated with eIF-5 permits the hydrolysis of GTP in the ternary complex and releases eIF-2 as eIF-2.GDP binary complex. Many models (Hershey, 1991; Merrick, 1992; Watson *et al,* 1987; Ochoa, 1983) indicate that eIF-2.GDP binary complex is released before the 80S initiation complex formation (Fig. 1) 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. Since these findings are incompatible with the conventional models, a new model has been presented to explain the presence of eIF-2 on the 60S subunits of 80S initiation complex and on the recycling of eEF-2 (Ramaiah *et al,* 1992; Altman and Trachsel, 1993).

vi). For eIF-2 to enter into another round of initiation, the GDP in eIF-2.GDP binary complex has to be exchanged for GTP. At physiological conditions, eIF-2 has a higher affinity for GDP and also the joining of Met.tRNAj to eIF-2 is inhibited in the presence of

Fig.1 Initiation of protein synthesis in eukaryotes (scheme from Watson, *et al*, 1987)



GDP. Hence the exchange of GDP for GTP on eIF-2 is very critical and this is catalyzed by eIF-2B protein factor.

1.1.2.£longation: In this step of protein synthesis, the incoming aminoacylated-tRNAs join the 60S subunits of 80S initiation complexes depending on the sequence information of mRNA in the 'A' site of ribosome. The `P' site of ribosome contains the Met.tRNA$_i$ or growing peptidyl tRNA complex. 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 -γ. EF-1 a is equivalent to prokaryotic EF-Tu and helps in the binding of 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 α.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 the aminoacyl-tRNA from the `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).

1.1.3.Termination: Protein synthesis continues till the ribosome reaches a termination codon. The completed nascent polypeptide chain is then released from ribosomes at this point with the help of a releasing factor (RF). The RF in eukaryotes recognizes all three stop codons and the termination requires GTP hydrolysis.

1.2. Importance of Initiation: In such a complex sequence of reactions, it is natural for the cells to exert a control at the first step of reaction, that is, at the initiation. Two important steps in polypeptide chain initiation have been identified which can control protein synthesis initiation either selectively or globally. One of them is the availability of Met.tRNAi to the 40S ribosomal subunit which is mediated by eIF-2 as mentioned above. Another step in the initiation which can regulate protein synthesis is the joining of 43 S initiation complex (40S ribosome.eIF-2.GTP.Met.tRNAj) to mRNA. This step requires 5' cap binding proteins like eIF-4α (also known as eIF-4E), eIF-4B and eIF-4y.

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 (Hershey, 1991; Jackson, 1991; Merrick, 1992; Redpath and Proud, 1994).

When viewed from the perspective of translational control, certain features of the initiation process are highlighted. Regulation of eIF-2 and formation of Met.tRNA-40S complex may affect protein synthesis globally, since this step is common to the translation of all mRNAs. However partial inactivation of eIF-2 can result in a more severe inhibition of translation of `weak' mRNAs compared to strong' mRNAs (Lodish, 1976). Indeed, this is true and several examples are known today which suggest that most of the regulation of protein synthesis in eukaryotes occurs at the formation of 43 S or 48S preinitiation complex.

These examples include heat-shock (Duncan and Hershey, 1984); deprivation of serum, hemin, amino acids and nutrients (Duncan and Hershey, 1985; Surolia and Padmanaban, 1991; Clemens, 1990; Hinnebusch, 1990; Scorsone *et al.*, 1987); fertilization (Bonneau and Sonenberg, 1987); growth and development (Donaldson *et al,* 1991); pathological conditions such as viral infection (O'Malley *et al,* 1989) and diabetes (Kimball and Jefferson, 1988); changes in redox levels (Kan *et al,* 1987); sequestration of Ca^{2+} (Prostko *et al,* 1992); heavy metal stress (Hurst *et al,* 1987) and treatment with cytokines, growth hormones and phorbal esters (Boal *et al,* 1993; Bu and Hagedorn, 1991; Donaldson *et al,* 1991; Frederickson *et al,* 1992; Kaspar *et al,* 1990 & 91, Welsh and Proud, 1992).

Temporal regulation either for individual proteins or especially for global protein synthesis is likely through reversible covalent modifications of initiation factors and other components Two important rate limiting factors whose phosphorylation is known to affect the regulation of protein synthesis 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

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.

1.3. eIF-2 structure and function:

eIF-2 is a heterotrimer with three subunits of a (~38 kDa), p (~50 kDa) and y (~52 kDa). The -β subunit of eIF-2 is found to vary in its molecular weight depending on the gel system used. It may migrate with apparent Mr. values of 36-55 kDa (Colthrust and Proud, 1986; Lloyd *et al,* 1980; Meyer *et al.*, 1981; Panniers and Henshaw, 1983). The anomalous behavior may be a consequence of large blocks of lysine residues in eIF-2P subunit that can alter its electrophoretic mobility (Pathak *et al,* 1988a). Three homologous subunits have been identified in *Saccharomyces cerevisiae* which are required for cell viability (Donahue, 1988).

The structure has been partially elucidated by cloning and sequencing of cDNAs encoding a, -P and -γ subunits of eIF-2 of *Saccharomyces cerevisiae* and certain mammalian cells (Ernst *et al,* 1982; Pathak *et al,* 1988a; Gaspar *et al,* 1994). Both eIF-2p and eIF-2y subunits appear to be involved in binding guanine nucleotides. Various findings (Anthony *et al,* 1987 & 1990; Bommer *et al,* 1988a; Bommer and Kurzchalia, 1989; Dholakia *et al,* 1989; Kurzchalia *et al,* 1984) indicate that guanine nucleotide binding site in eIF-2 is "shared" between these two subunits. However, this raises a question regarding some early observations in which the preparations of eIF-2 apparently lacking eIF-2p have been shown to still bind guanine nucleotide and mediate the GTP dependent translocation of Met.tRNAi to the 40S ribosomal subunit (Chaudhuri *et al,* 1981; Colthrust and Proud, 1986). To accommodate this old finding, a recent study indicates that preparations of eIF-2 which appear to be devoid of -β subunit as judged by SDS-PAGE may still contain fragments of -β subunit bound to the -a and -y subunits and presumably to one another by non-covalent forces (Proud, 1992; Kimball *et al,* 1987).

The nucleotide sequence information of eIF-2β suggests that it has an ATP binding site and this may influence the ability of eIF-2 to bind mRNAs (Gonsky *et al,* 1990). eIF-2 is also shown to interact with mRNAs and with initiator tRNA Since eEF-2 binds very well to conventional cation exchangers such as phosphocellulose, it is somewhat amazing to understand the binding of eIF-2 to RNA-Cellulose. It is not clear whether the binding reflects the cation exchange properties of the matrix or conversely, the binding of eIF-2 to phosphocellulose is a consequence of having binding sites for the phosphate group of RNA. But the sequence information of eIF-2β suggests that it has structural features, that is, the three blocks of lysine residues and a zinc finger motiff which may interact with RNA (Pathak *et al,* 1988a). Support for the idea that, the interaction of eIF-2 with mRNA may be functionally important, comes from two different investigations. 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 -β 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. Further, a preparation of eEF-2 lacking the -β subunit is unable to modify the start site selection again suggesting a role for eIF-2p in interacting with mRNA and participating in start codon selection. In support of a functional role for eIF-2/mRNA interaction, 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 (Kaempfer, 1984; Kaempfer and Konijn, 1983; Kaempfer *et al,* 1981; Di Segni *et al,* 1979; Rosen *et al,* 1981 & 82). Kaempfer's group has also reported that different functional sites on eIF-2 are involved in the interactions with the initiator tRNA and GTP on one hand and mRNA on the other. Such a conclusion was reached based on differing abilities of different anti-eEF-2 antibodies to inhibit these binding functions of eIF-2 (Harary and Kaempfer, 1990). Also, the findings of Gupta and

co-workers suggest that the presence of mRNA or trinucleotide AUG stimulate the eIF-2 mediated transfer of Met.tRNAi to 40S ribosomal subunit indicates the importance of eIF-2 interaction with mRNA (Ray *et al,* 1981, 84 & 88; Chakravarty, 1985).

The studies with cDNA encoding human eIF-2β and -γ subunits (Pathak *et al,* 1988 and Gaspar *et al,* 1994) suggest that DXXG and NKXD, consensus elements for GTP binding, are present in both -β and -γ subunits, thereby reinforcing the possibility that both the subunits might be involved in GTP binding. When the Asn residue is altered in the NKXD consensus sequences of -y subunit of eIF-2, protein synthesis is strongly inhibited. In contrast, alterations in the corresponding Asn in the -β subunit cause little change in protein synthesis compared to the wild type subunit. This result . further supports the view that GTP binding requires the NKID element in eIF-2y but does not involve in NKKD element in eIF-2β (Naranda *et al,* 1995). This one however cannot explain the result obtained from the affinity labelling of eIF-2 with GTP derivatives which is shown to occur in both -β and -y subunits (Anthony *et al,* 1990; Bommer *et al,* 1989). A possible explanation is that eIF-2P lies very close to the GTP binding site in the G-domain of eIF-2y (Naranda *et al,* 1995).

As mentioned above, at the end of initiation, eIF-2 is released as eIF-2.GDP binary complex and this binary complex cannot bind Met.tRNAi (Walton and Gill, 1975). It is shown that eIF-2 has a 400 fold higher affinity for GDP than for GTP (Rowlands *et al,* 1988a) and the exchange of GDP in eIF-2.GDP binary complex for GTP requires eIF-2B protein factor. Since eIF-2 is a phosphoprotein and has the ability to interact with eIF-2B for exchanging the guanine nucleotides, its regulation is dependent on the kinases and phosphatases that can phosphorylate and dephosphorylate eIF-2 and is also mediated by changes in eIF-2B activity.

1.4.Phosphorylation of eIF-2: Both the -a and -β subunits of eIF-2 can be phosphorylated *vitro* as well as *in vivo* (intact cells). Phosphorylation of -β subunit can

occur by casein kinase II but the functional significance, if any, of this phosphorylation remains unknown (Proud, 1992).

Phosphorylation of the small subunit, 38 kDa or the -a subunit of eIF-2 in translational control is well established. The regulatory role of phosphorylation of eIF-2a was studied first in translating reticulocytes. In the 1950s it was discovered that inorganic iron stimulates protein synthesis in immature erythroid cells (Kruh and Borsook, 1956; Kassenaar *et al,* 1957; Morell *et al,* 1958). Later in 1965, the importance of hemin on globin synthesis in intact reticulocytes was demonstrated (Bruns and London, 1965). Further studies indicated that desferroximine, an iron chelating agent does not block the stimulatory effect of heme (Grazyel *et al,* 1966). Iron deficiency causes disaggregation of polyribosomes in intact reticulocyte and this is reversed by the addition of iron or hemin (Waxman and Rabinowitz, 1966). Then came the development of cell-free reticulocyte lysates for determining protein synthetic activity *in vitro* (Zucker and Schulman, 1968; Adamson *et al,* 1968). In the absence of added hemin, protein synthesis 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). In addition, hemin added after shut-off of protein synthesis is capable of restoring protein synthesis and polyribosomes (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 there is 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). The striking common chara-

cteristics of the inhibition caused by such diverse agents and conditions suggest that each inhibitory condition acts in an independent series of events leading to a common termination step, which is either, the inactivation of eIF-2 itself or of some factor(s) necessary for the repeated functioning of eIF-2. When the inhibition of protein synthesis that occurs in the presence of dsRNA or in the absence of hemin was analyzed, it was found to be due to the activation of inhibitors of initiation (Maxwell *et al,* 1971; Hunter *et 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 concentration of dsRNA (>10 μg/ml) fails to inhibit protein synthesis (Hunter, 75). Both 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 and can phosphorylate the small subunit of eIF-2 and inhibit protein synthesis (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, 1988 & 90). The above three kinases (HRI, PKR and GCN2) have been very well characterized (Samuel, 1993).

Several conditions such as heat-shock (Duncan and Hershey, 1984; Clemens *et al,* 1987; Murtha-Riel *et al,* 1993), 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), o-iodosobenzoate (Gross and Rabinowitz, 1972), serum deprivation (Duncan and Hershey, 1985) and calcium deprivation (Preston and Berlin, 1992; Prostko *et al,* 1992) are found to enhance eIF-2α phosphorylation. However, the respective eIF-2α kinase(s) have not been well characterized yet (Samuel, 1993).

The location of phosphorylation site in eIF-2a has been carried out by Colthrust *et al* (1987) and has been shown that, when purified eIF-2 is labelled *in vitro* by HRI or PKR, a single serine residue, Ser-51 is labelled. This is true for rabbit or rat eIF-2. 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) suggests that Ser-48 in eIF-2α was the site which was phosphorylated by HRI, although their subsequent work showed that HRI phosphorylates only the equivalent Serine 51 residue in a synthetic peptide (Kudlicki *et al,* 1987b).

In translating reticulocyte lysates, inhibited by heme-deficiency or dsRNA treatment, only the Ser-51 residue in eIF-2a is phosphorylated (Price and Proud, 1990). No second site of phosphorylation is observed even when lysates are supplemented with potent protein phosphatase inhibitors like microcystin (Price *et al,* 1991a). The recent studies with mutants of eIF-2a generated by site-directed mutagenesis have shed more light on the phosphorylation of Ser-51 residue and its role in translational regulation (Pathak *et al,* 1988b; Kaufman *et al,* 1989; Davies *et al,* 1989). One of the mutants of eIF-2a, in which the Ser-51 residue is replaced by alanine (Ala-51), is not phosphorylated. In the case of another mutant in which the Ser 48 residue is replaced by alanine (Ala-48), phosphorylation of eIF-2α still occurs on the 51 serine residue. Also, a mutant eIF-2a, in which Ser-51 is altered to Asp, is found to inhibit translation presumably because Asp mimics phosphoserine at this position. In contrast, the expression of other mutants as mentioned above (Ala-48 or 51) is found to bypass the protein synthesis inhibition mediated by eIF-2a phosphorylation (Choi *et al,* 1993; Murtha-Riel *et al,* 1993). These studies have shown that the mutant eIF-2α exchanges out the eIF-2a in the native trimeric endogenous eIF-2 (Choi *et al,* 1993). Since one of these mutants (Ala-51) cannot be phosphorylated, it is predicted that the expression of this mutant eIF-2a can bypass protein synthesis inhibition by protecting eIF-2B activity. But it is unclear as to how the phosphorylated mutant eIF-2a (Ala-48) can overcome the inhibition in protein synthesis. Subsequent studies by Ramaiah *et al* (1994) have suggested that perhaps alteration of

Ser-48 affects the interaction of phosphorylated mutant eIF-2α with eIF-2B or the relative affinities of this mutant eIF-2 for GDP and GTP may be altered in such a way as to modify its requirement for eIF-2B for recycling. This is because the expression of either mutant reduces the inhibition in eIF-2B guanine nucleotide exchange activity that is mediated by eIF-2a phosphorylation. Further, the availability of mutant eIF-2a clones are helping to characterize the inhibition of protein synthesis mediated by eIF-2α phosphorylation. For example, calcium depletion is known to inhibit protein synthesis and increase eIF-2a phosphorylation (Preston and Berlin, 1992; Prostko *et al,* 1992). With the help of mutants of eIF-2a and also mutants of PKR kinase, it has been recently demonstrated 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 the mutants of eEF-2a can bypass protein synthesis inhibition caused by endogenous wild type eIF-2α 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).

Sequence surrounding the phosphorylation site in eIF-2a is found to be MILLSEL S51RRRIR The sequence adjacent to Serine 51 on the C-terminal side is rich in basic residues. Both HRI and dsI can phosphorylate peptides containing clusters of arginines at the C-terminal to the target' serine residue, provided that these residues are present at positions +3/ and +4 relative to the serine. However, peptides containing only N-terminal basic residues are poor substrates for these kinases (Proud *et al,* 1991b).

eIF-2 protein is isolated from ribosomes by high salt (0.5 M KC1), suggesting that it is not an integral protein of ribosome and is associated with ribosomes (Andrews *et al,* 1985). Consistent with these findings, eEF-2 is also found distributed on the 60S subunits of 80S initiation complexes and on polysomes in hemin-supplemented actively translating lysates (Gross *et al,* 1985; Thomas *et al,* 1985; Ramaiah *et al,* 1992). Immobilization of polysomes in inhibited heme-deficient lysates by the addition of cycloheximide enhances

the phosphorylation of eIF-2a. This finding suggests that eIF-2a is readily phosphorylated on the 60S subunits of 80S initiation complexes in heme-deficient lysates or in physiological conditions (Ramaiah *et al,* 1992). But, no reports are available to indicate that HRI is associated with ribosomes. In contrast other eIF-2a kinases such as PKR and GCN2 are associated with ribosomes (Chen, 1993; Ramirez *et al,* 1991). The preferential association of GCN2 with 60S ribosomes and the presence of PKR on ribosomes also suggest that eIF-2a phosphorylation probably occurs on ribosomes in translating lysates. The findings of Ramaiah *et al.* (1992) showing increased phosphorylation of eIF-2a when polysomes are maintained by the addition of cycloheximide may also serve to explain the observation of Clemens and co-workers (Clemens *et al,* 1987; Pollard *et al,* 1989) that a diminished rate of chain elongation that results from diminished aminoacid-tRNA synthetase activity is associated with increased phosphorylation of eIF-2a with no changes in eIF-2α kinase or phosphatase activity.

The recent studies (Chen *et al,* 1991a) of HRI cDNA coding sequence has shown extensive homology to 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, 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 HRJ 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 the autokinase and eIF-2α 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). Autophosphorylation of these kinases may be important for their ability to phosphorylate the -α subunit of eIF-2.

1.5. eIF-2B regulates eIF-2 activity:

The mechanism by which phosphorylation of eIF-2α inhibits its activity was not clear until the early 80's. It is found that less than 20-30% of eIF-2 phosphorylation is enough to inhibit protein synthesis completely or maximally (Leroux and London, 1982). In addition, it is observed that addition of purified eIF-2 can rescue protein synthesis activity in lysates incubated without hemin. It is puzzling to note that rescue is less effective the purer the preparation of eIF-2 (Jackson, 1991). These facts suggest that there must be yet another protein factor that regulates eIF-2 activity and is probably rate limiting (Leroux and London, 1982). The solution for this problem came from the identification of eIF-2B which catalyzes the exchange of GDP in eIF-2.GDP for GTP. This factor is purified from the post-ribosomal supernatant and also from the ribosomal eIF-2 preparations (Dholakia *et al,* 1986; Siekierka *et al,* 1981; Matts *et al,* 1983). It contains five subunits. These are -a (34 kDa), -β (40 kDa), -y (55 kDa), -5 (65 kDa) and -e (82 kDa). This factor has been variously called, GEF (Proud, 1992), anti-HRI (Amesz *et al,,* 1979), SP (Siekierka *et al.,,* 1982) or RF (Siekierka *et al,,* 1981; Matts *et al,,* 1983; Gross *et al,,* 1982). The factor here is referred as eIF-2B and it restores protein synthesis catalytically in inhibited heme-deficient lysates (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,* 1988b). So eIF-2B is trapped in a 15S complex [eIF-2(aP).eIF-2B] in which eIF-2B becomes non-functional (Thomas *et al,* 1984). Since eIF-2B is less abundant than eIF-2 (1/10th of eIF-2), a small increase in eIF-2a 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 labelled GDP or exchange of labelled 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-2α 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,* 1988). 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 which are expressing mammalian recombinant eIF-2α kinase (Chefalo *et al,* 1994) and also in evaluating the overexpression of wild type and mutant eIF-2α subunits in rescuing the inhibition in eIF-2B activity of CHO cells that is mediated by eIF-2α phosphorylation (Ramaiah *et al,* 1994). The latter study reveals that over expression of mutant eIF-2α 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-2a impairs the eIF-2B activity. This study proposes that Ser-48 acts to maintain a higher affinity between phosphorylated eIF-2α and eIF-2B and thereby inactivating eIF-2B. This finding suggests that phosphorylation of eIF-2α inhibits protein synthesis directly 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 labelled binary complex (Pain, 1986). Recently, two models have been proposed to explain the eEF-2B activity but the conclusions are in conflict. One group suggests an enzyme displacement mechanism and another group proposes a sequential mechanism (Panniers *et al,* 1988; Dholakia and Wahba, 1989). Notwithstanding the divergence of opinion, there is a general agreement that phosphorylation of eIF-2 impairs eIF-2B catalyzed exchange reaction as has been suggested above. The eIF-2(αP).GDP is regarded as a competitive inhibitor of eIF-2B interaction with eIF-2.GDP and the magnitude of differences is so large that under physiological conditions this type of competitive inhibition could efficiently mimic sequestration (Rowlands *et al,* 1988).

Not only phosphorylation of eIF-2a regulates eIF-2B activity, the recent studies suggest that conditions such as phosphorylation of one of the subunits (82 kDa) of eIF-2B and changes in redox levels can also regulate the guanine nucleotide exchange activity of eIF-2B. The phosphorylation of 82 kDa subunit of eIF-2B by CK-II (Dholakia and Wahba, 1988) is associated with an increase in the guanine nucleotide exchange activity of the factor. This finding suggests that -e subunit of eIF-2B is apparently associated with guanine nucleotide exchange activity. The functions of other subunits however are not clear. Some recent data suggest that eIF-2B may be involved in the release of eIF-2(aP).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). Hence 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. A polyamine, spermidine, 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 eEF-2B activity (Dholakia *et al,* 1986; Kimball and Jefferson, 1995, Singh *et al,* 1995; Oldfield and Proud, 1992; Akkaraju *et al,* 1991). While there is no good evidence for changes in intracellular concentrations of polyamines, the ratio of NADPH and NADP+ can be altered in cells under certain conditions. An enhanced $NADPH/NADP^+$ ratio also enhances eIF-2B activity (Dholakia *et al,* 1986; Akkaraju *et al,* 1991). As $NADP^+$ inhibits the activity of eEF-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-l,6-BisPi. Therefore it is possible that the

activity of eIF-2B may be allosterically regulated *in vivo* not only by changes in the pyridine nucleotides but also by changes in relative amounts of NADPH and ATP (Kimball and Jefferson, 1988). Also, a recent study by Ramaiah *et al* (1994b) suggests that PQQ, a novel cofactor of many bacterial dehydrogenases, stimulates eIF-2B activity of Chinese hamster ovary cell extracts which do not contain an active eIF-2α kinase. This is because some of the eEF-2a kinases like HRI are shown to be activated under reducing conditions like DTT or reduced PQQ ($PQQH_2$) and inactivated by hemin due to intramolecular disulfide bond formation (Ramaiah *et al,* 1994b; Chen *et al,* 1989). Lower concentrations of PQQ (10-100 nM) stimulate protein synthesis and eIF-2B activity marginally, where as higher concentrations (1-20 μM) activate heme-regulated eIF-2α kinase of reticulocyte lysates and inhibit protein synthesis. These findings suggest that the reducing power of a lysate stimulates eIF-2B activity and protein synthesis if eIF-2α kinase activity is not interfering. Consistent with the earlier findings which indicate enhancement in protein synthesis in gel-filtered lysates by the addition of sugar phosphates (Jackson *et al,* 1983), a recent study suggests that sugar phosphates probably regulate eIF-2B activity allosterically (Singh and Wahba, 1995).

Cloning of one of the subunits of eIF-2B, that is, the α 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). 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.

1.6. Role of heat shock and other proteins in the regulation of eIF-2a phosphorylation: Hardesty and his colleagues have shown that HSP90 is present in their preparation of highly purified HRI (Rose *et al,* 1987). They have observed that the eIF-2α kinase activity of HRI is increased with the addition of phosphorylated HSP90 but not by dephosphorylated HSP90 (Szyszka *et al,* 1989). The stimulation in HRI activity by

HSP90 is about 2 fold with atleast 20 fold excess of HSP90 in molar ratio (Szyszka *et al*, 1989b; Rose *et al,* 1989). Addition of purified HSP90 to hemin-supplemented reticulocyte lysates results in the inhibition of protein synthesis (Rose *et al,* 1989). However, the inhibition that is observed is not biphasic as typically observed in heme-deficiency and is also partially reversed by the addition of purified eIF-2 . They have not examined the activation of HRI or the phosphorylation of endogenous eIF-2a under those conditions. Recently, Matts and Hurst (1989) have provided evidence for the association of HRI with HSP90 in reticulocyte lysates. The extent of the co-absorption of HRI with HSP90 by anti-HSP90 monoclonal antibody 8D3 depends on the concentration of hemin (Matts and Hurst, 1989). However, dissociation of HSP90 from HRI is not a requirement for the activation of HRI since HSP90 remains associated with HRI when it is activated in hemin-supplemented lysates by heat-shock, NEM or Hg^{2+} (Chen, 1993). Therefore, the role of interactions of HSP90 and HRI on the regulation of HRI activity and activation is currently unclear. Also 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,* 1991). Also as a defense mechanism, different viruses have developed specific strategies to regulate the functioning of PKR activity; for example i) adenovirus encoded VA_I RNA complexes with PKR and inactivates the kinase (Katze *et al,,* 1987) ii) poliovirus infection induces the degradation of kinase (Black *et al,,* 1989). While infection by another picornavirus, encephalomyo-carditis 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).

In addition to the regulation of eIF-2α kinase activity, eIF-2a phosphorylation is also influenced by other proteins. Gupta and co-workers have recently 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-2α phosphorylation. p67 contains 12-0 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 eEF-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, 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-2α 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.

1.7. Dephosphorylation of eIF-2(αP):

eIF-2 phosphorylation must be dependent on the kinase and phosphatase activities. In shut-off heme-deficient lysates addition of hemin restores protein synthesis with the concomitant dephosphorylation of eIF-2(aP) under those conditions. According to the studies of Matts *et al* (1986), dephosphorylation of eIF-2(aP) must occur to restore eIF-2B activity and protein synthesis in inhibited heme-deficient lysates which are treated with

the delayed addition of hemin Also a standard amount of eIF-2α phosphorylation can be seen in translating lysates at different time points of protein synthesis in inhibited heme-deficient lysates. The net phosphorylation of eIF-2a does not change in inhibited lysates at different time points of translation suggesting that the phosphate on eIF-2a is turned over and is dependent on the eIF-2α kinase/ phosphatase activities

Work carried out before the establishment of the different classes of protein phosphatases, involves the isolation of protein phosphatases with activity against eIF-2 labelled in its a subunit. Crouch and Safer (1980) have purified a type II protein phosphatase from reticulocyte lysates which dephosphorylates eIF-2(αP) *in vitro.* Type 1 phosphatase can also dephosphorylate eIF-2(aP) although it hasa lower activity than type II phosphatase (PP-2A) does, when compared to common control substrates (Stewart *et al,* 1981; Ingebritsen and Cohen, 1983). A type 1 phosphatase has also been isolated from reticulocyte lysates that dephosphorylates eEF-2(aP) *in vitro* (Grankowski *et al,* 1980a; Tipper *et al,* 1986). But it is not known whether the purified phosphatase can dephosphorylate eIF-2(aP) in translating lysates under physiological conditions of protein synthesis. A previous study suggests that addition of inhibitor 2 (a thermostable protein which inhibits only PP-1) to reticulocyte lysates causes increased phosphorylation of eIF-2a and decreased polypeptide chain initiation (Ernst *et al,* 1982), apparently by directly inhibiting eIF-2α phosphatase activity. This is interesting and surprising because the lysates contain substantial amounts of PP2A which dephosphorylate eIF-2(aP) more efficiently than PP1 *in vitro* but is unaffected by inhibitor 2. Recently, Redpath and Proud (1990 & 91) have studied the roles of PP1 and PP2A in the dephosphorylation of eIF-2(αP) in reticulocyte lysates using the protein phosphatase inhibitors such as okadaic acid and microcystin. Okadaic acid inhibits PP2A much more strongly than PP1 at lower concentrations (Bialojan and Takai, 1988, Cohen, 1989). Microcystin has approximately equal potency against PP1 and PP2A (Mackintosh *et al,* 1990). Since low concentrations of okadaic acid can inhibit protein synthesis without affecting the eIF-2a phosphorylation,

it has been suggested that major eIF-2a phosphatase in reticulocyte lysates is PP1 (Redpath and Proud, 1989 & 91). The association of eIF-2(aP) with eIF-2B inhibits markedly its dephosphorylation when added to reticulocyte lysates (Crouch and Safer, 1984). This finding raises doubts regarding the substrate for the eIF-2(aP) phosphatase. It is known that in inhibited heme-deficient lysates, the formation of 15S complex [eIF-2(aP).eIF-2B] results in the inhibition of protein synthesis. Addition of hemin facilitates dephosphorylation of eIF-2(αP) and restoration in eIF-2B activity. So it is thought that under those conditions a physiological phosphatase must be able to dephosphorylate the eIF-2(aP) in the 15S complex. There are no reports to date indicating that addition of a protein phosphatase can restore protein synthesis in inhibited lysates mediated by eIF-2a phosphorylation. This may be due to the effect of the phosphatase on the dephosphorylation of other phosphorylated protein factors whose phosphorylation may be important in the regulation of protein synthesis.

1.8.OBJECTIVES:

The present work is initiated with a long term objective to further understand the mechanism of regulation of eukaryotic initiation factor 2 activity in reticulocyte lysates. The present studies are undertaken to determine

a) the type of phosphatase involved in the physiological dephosphorylation of eIF-2(aP) and in the restoration of eIF-2B activity in shut-off heme-deficient lysates treated with the delayed addition of hemin,

b) the distribution of eIF-2 and HRI in translating lysates where polysomes are maintained due to an active initiation or a block in elongation and

c) the effects of Datura lectin, which resembles WGA in its specificity to bind N-acetyl glucosamine residues, on protein synthesis and on eIF-2 phosphorylation.

2.0 MATERIALS AND METHODS

2.1. Materials used: [8-^{3}H]GDP (9 Ci/mmol), [γ-^{32}P]ATP (3000 Ci/mmol), [^{14}C] Lecuine (340 mCi/mmol), [^{35}S]Methionine (1100 Ci/mmol), [^{32}P]Orthophosphate (100 mCi/ml) were obtained from Dupont, NEN, USA. and from BRIT, Bombay, India ATP, GTP, GDP, CPK, FDP, NAD^{+}, DTT and CP were obtained from Boehringer and Mannheim. BMV RNA was obtained from Promega. DE-52, Sephadex G-25, Sephacryl-300, Phosphocellulose and CM sephadex column materials were purchased from Whatman, England. Filter paper discs (1.75 cm) were obtained from Schleicher and Schuell, USA. X-ray films were brought from Indu, India. Acetyl-phenyl hydrazine and chitin affinity matrix were purchased from Sigma. Other chemicals required for the study were purchased either from Sigma (St.Louis, MO) or from local market.

New Zealand white male rabbits were procured from Indian Immunologicals Limited, Hyderabad.

eIF-2a and HRI monoclonal antibodies, okadaic acid and inhibitor 2 protein were given by Drs.Jane Jane Chen and I.M.London, MIT as a kind gift. WGA and abrin were received as a kind gift from Dr. N.Siva Kumar and from Dr.K.Seshagirrao, University of Hyderabad.

Methods:

2.2. Preparation of cell-free translation system from rabbit reticulocytes:

Reticulocyte lysate was prepared from New Zealand white male rabbits as described (Hunt *et al.*, 1972). Each of several rabbits were injected subcutaneously with 2.5 ml of 1% acetyl phenylhydrazine daily for 4 days. After 5 days, the rabbits were bled. The blood was collected by cardiac puncture into a 500 ml beaker coated with heparin. Around 400 ml of blood was collected from 10 rabbits. To prevent the clotting of blood, 300 LU. of heparin was added to 40-50 ml of blood. The red blood cells were isolated by centrifuging at 2000 rpm for 10 minutes in a Remi high speed centrifuge. The supernatant was carefully removed with a pasteur pipette and the cells were resuspended in buffered saline containing 0.14 M NaCl, 5 mM KC1, 5 mM $Mg(OAc)_2$, 5 mM glucose, 5 mM HEPES (pH 7.2) and spun at 2000 rpm for 10 min. This step was repeated thrice and at

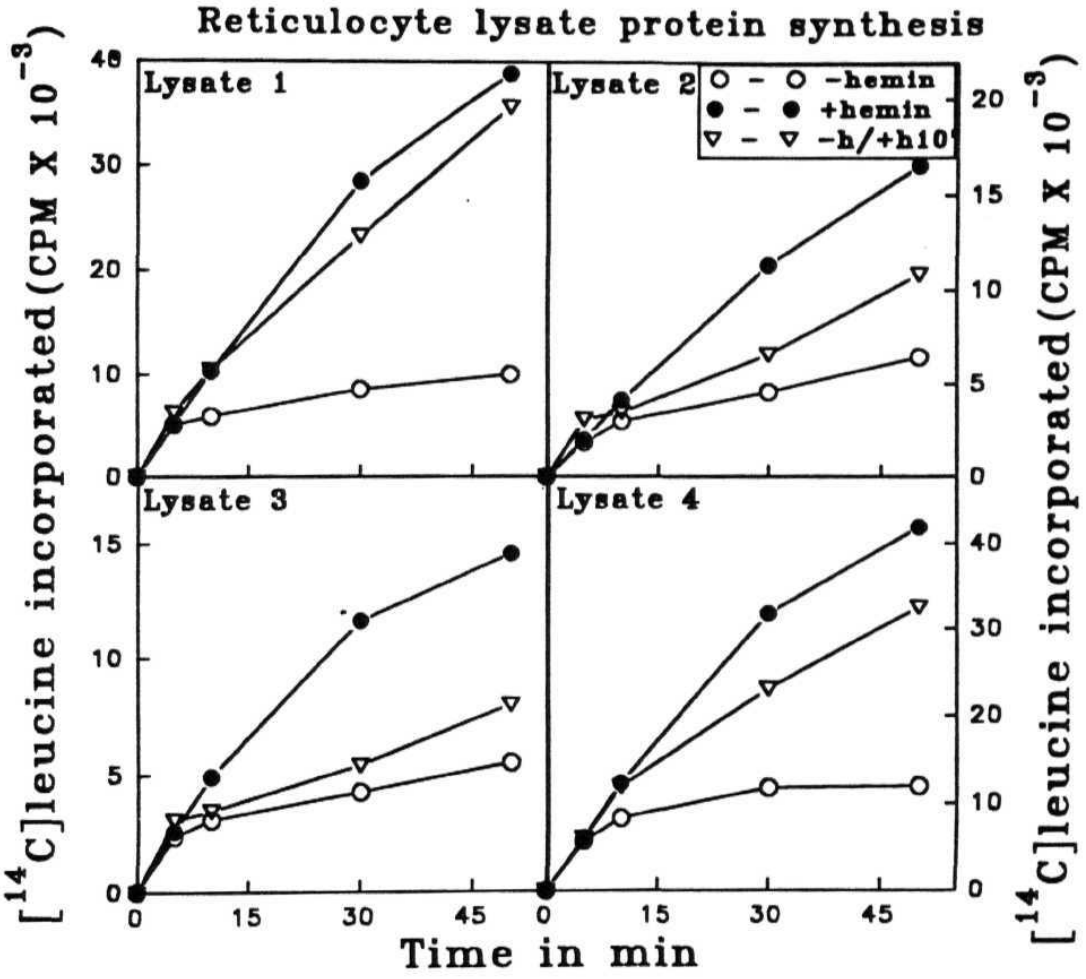
each step, the white buffy coat present on the top of the cells was carefully removed without touching the pellet. After the final step, the supernatant was carefully aspirated and the cells were lysed in equal volume of ice cold double distilled water. The lysed cells were spun at 10,000 rpm for 20 min. The 10 K supernatant was carefully removed and part of the lysate was stored in 1 ml aliquots in liquid N_2 for determining protein synthesis efficiency. The remaining lysate was further diluted with equal volume of distilled water and it was used for purification of eIF-2 and HRI. The whole process was carried out at 4°C. The translating lysates were stored in liquid nitrogen and the lysate used for protein purification was saved at -70°C.

2.2.1 Reticulocyte lysate **protein** synthesis : The reticulocyte lysate protein synthesis was performed in a 20 µl reaction volume. The reaction mixture contained the following ingredients. 60% lysate, 80 mM KC1, 1 mM Mg^{2+}, 33 µM amino acids mix minus leucine, 200 µM GTP, 4 µM CP, 250 µg CPK and 33 uM [^{14}C]leucine (Ernst *et al*, 1980).

The reaction was incubated without hemin (-h) or with the addition of hemin (+h) at 30°C for 60 min. At different time intervals, 5 µl of protein synthesizing lysates was spotted on Whatman No.1 filter discs. The filters were dried and then suspended in 10% cold TCA for 20 min to precipitate the proteins. These filters were then transferred to 5% boiling TCA for 5 min. Afterwards, the filters were washed in 5% TCA at room temperature for 5 min and then were washed with ethanol and acetone. The filters were then soaked in H_2O_2 solution for 10 min. to bleach the color. Once again the filters were washed with ethanol and acetone and were air dried. The dried filters were counted in a toluene based scintillation fluid in a Beckman Liquid Scintillation counter.

Since commercially available lysates are not heme-sensitive and can carry protein synthesis even in the absence of hemin, heme-sensitive lysates are routinely prepared. Protein synthesis in four different lysates which were obtained from four different rabbits is shown in Fig.2.

Fig.2


Reticulocyte lysate protein synthesis
Lysate 1
Lysate 2
Lysate 3
Lysate 4
○ - ○ -hemin
● - ● +hemin
▽ - ▽ -h/+h10'
[14C]leucine incorporated (CPM X 10-3)
Time in min
0
15
30
45
5
10
20
40

In all these lysates, protein synthesis was linear for only a few minutes and was then shut-off in the absence of added hemin. In the presence of 20 μM hemin, protein synthesis was linear for about 40 min. Also, the delayed addition of hemin at 7 min (-h/+h) to heme-deficient lysates was found to restore protein synthesis. These lysates were thus found to be heme-sensitive.

Although, the incorporation of labelled amino acid into protein varied from one lysate to another, the trend or direction of these results did not alter. The incorporation of amino acid into protein or overall protein synthesis is dependent on several conditions.

2.3. Preparation of wheat germ lysate:

Lysate was prepared as described by Roberts and Patterson (1973). Unroasted, dried wheat germ was obtained from General Mills Inc., California, USA. 20 gms of the material was floated on carbon tetrachloride and cyclohexane mixture (2.5:1). 3 gms of the floated wheat germ was taken and vacuum dried. Then, the material was powdered in liquid N_2 and soaked in an extraction buffer containing HEPES, pH 7.2 (40 mM), KOAc (100 mM), $Mg(OAc)_2$ (1 mM), $CaCl_2$ (2 mM) and DTT (5 mM) and made a paste with a glass rod for 10 min. The paste was spun at 15,000 rpm for 15 min in a high speed cooling centrifuge (Beckman). The 15 K supernatant was collected and loaded on G-25 sephadex column (50 X 2.5 cm) which was preequilibrated with the column buffer. The column buffer contains HEPES, pH. 7.6 (40 mM), KOAc (120 mM), $Mg(OAc)_2$ (5 mM) and DTT (4 mM). G-25 sephadex, distilled water for making buffers and all the glassware were autoclaved before use. 2 ml fractions were collected and the fractions which were turbid (peak fractions) were pooled and centrifuged at 15000 rpm for 15 min. The supernatant was carefully removed and stored in aliquots in liquid nitrogen or at -80°C.

2.3.1. Wheat germ lysate protein synthesis:

Wheat germ lysate prepared as above was tested for its ability to carry protein synthesis in the absence and presence of Brome mosaic virus RNA (50 μg/ml) at 25°C. Translation was carried out in a 25 μl reaction mixture containing the following

ingredients: 40% lysate, HEPES, pH 7.5 (20 mM), ATP (1.2 mM), GTP (80 uM), CP (8 mM), CPK (64 μg/ml), amino acid mix except methionine (20 μM). KOAc (80 mM), $Mg(OAc)_2$ (2 mM), DTT (1.5 mM) and 20 uM [^{35}S]methionine. Protein synthesis was determined by the incorporation of labelled methionine into protein in 5 |il aliquots taken at different time intervals and spotted on filter papers discs (Schleicher and Schuell). The filters were dried and transferred to 10% ice cold TCA for 20 min to precipitate proteins. Then, the filters were transferred to 5% boiling TCA for 5 min. Later, the filters were washed with 5% TCA at room temperature and was followed by ethanol and acetone. Filters were dried and counted in a toluene based scintillation fluid in a Beckman radioactive counter.

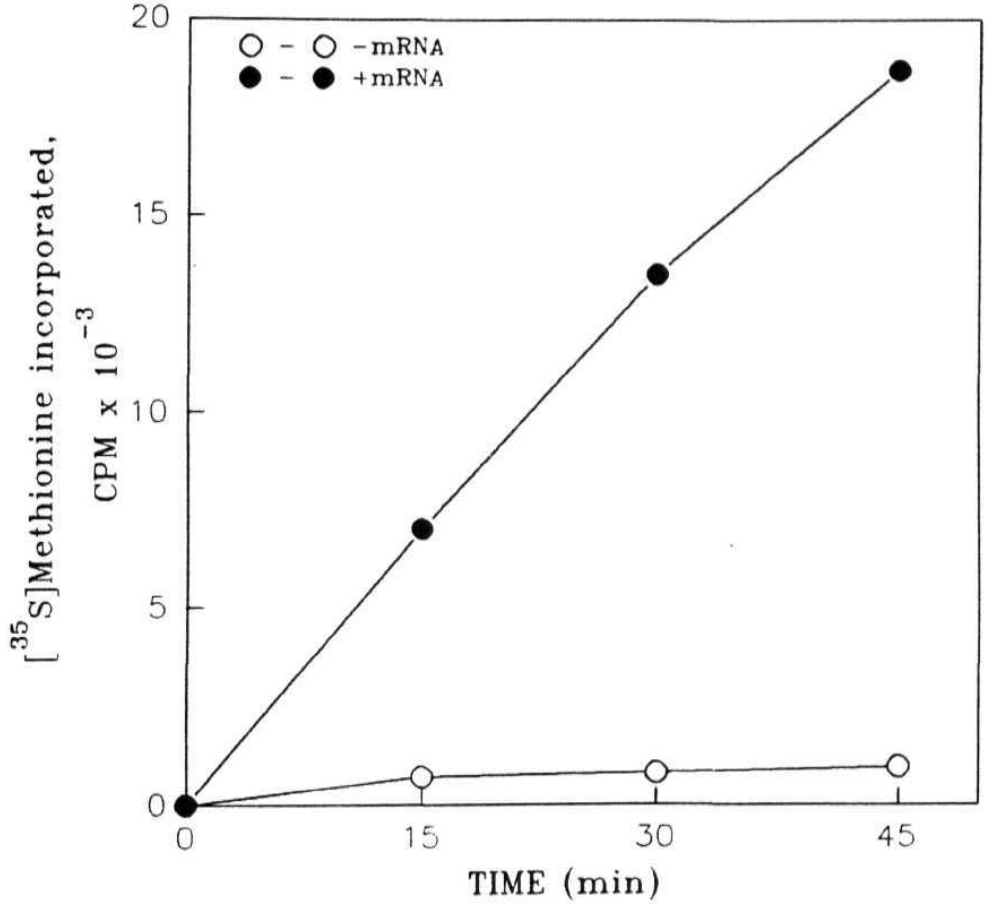
The results of a typical wheat germ lysate translation is shown in Fig. 3. Unlike reticulocyte lysates, wheat germ lysates do not carry any significant endogenous mRNA. In the absence of added BMV RNA (-BMV), protein synthesis is very minimal. In the presence of BMV RNA (+BMV), the incorporation of labelled amino acid into protein is 20 fold more than the control.

2.4. Purification of reticulocyte eIF-2:

eIF-2 was purified from the ribosomal salt wash of rabbit reticulocyte lysates as described (Andrews *et al,* 1985).

Reticulocyte lysate was layered on a 50% glycerol pad containing 10 mM Tris-HCl (pH 7.8), 5 mM NaCl, 25 mM KC1, 2 mM $Mg(OAc)_2$. 5 ml of 50% glycerol pad was used for 30 ml of the lysate. It was centrifuged at 45,000 rpm for 3.30 hrs in a Ti 70 rotor in Beckman ultracentrifuge. The post-ribosomal supernatant was carefully removed and kept frozen at -80°C. The ribosomal pellet was resuspended in 12 ml of ribosome suspension buffer (20 mM Tris-HCl, pH 7.8/2 mM $Mg(OAc)_2$/80 mM KCl/ 5% Glycerol/ 0.1 mM EDTA). The ribosomal suspension was then treated with 1.5 ml of high salt solution (4 M stock) to bring the final salt concentration to 0.5 M. The salt washed ribosomes were centrifuged at 50,000 rpm for 3 hrs in a Ti 80 rotor in a Beckman

Fig.3. Wheat germ lysate protein synthesis



ultracentrifuge. The supernatant was taken and the proteins were concentrated with 0-80% ammonium sulphate. The protein pellet was suspended in 1 ml of TDEG buffer (20 mM Tris-HCl, pH 7.8/1 mM DTT/0.1 M KCl/0.1 M EDTA/10% glycerol) and dialyzed against the same buffer. The salt wash of ribosomes obtained as mentioned above was loaded on DE-52 column which was preequilibrated with TDEG . The column was washed with TDEG buffer and eIF-2 was eluted with 0.2 M KC1. The 0.2 M KC1 eluate was concentrated by 0-80% ammonium Sulfate fraction and dialyzed prior to loading on phosphocellulose (PC) column. The PC column was equilibrated with TDEG buffer (0.1M KC1) and the pH of the column was checked (~7.8) before loading the 0.2 M DEAE eluate. Proteins from the PC column were eluted with 0.2, 0.4 and 0.7 M KC1. eIF-2 was eluted in 0.7 M fraction. 0.7 M KC1 eluate was concentrated, dialyzed and used for the study. The phosphocellulose purified eIF-2 was further loaded on CM sephadex which was equilibrated with TDEG containing 0.1 M KC1. eIF-2 was eluted with 0.4 M KC1. Highly purified eIF-2 was concentrated and dialyzed as mentioned above.

The various eIF-2 fractions were phosphorylated by HRI kinase and were separated on 10% SDS-PAGE to determine the purity of eIF-2 fraction and the phosphorylation of eIF-2a subunit (Fig. 4a & b). 2 µl of fractions obtained at various stages of purification of eIF-2 were phosphorylated for 5 min at 30°C with 5 µCi of [y-^{32}P]ATP and with purified HRI (~30 ng) in 20 µl reaction mixtures containing 20 mM Tris-HCl (pH 7.8), 80 mM KC1, 40 uM unlabelled ATP and 2 mM Mg^{2+} as described (Ramaiah *et al,* 1992). The reaction mixtures were supplemented with 2X SDS sample buffer and briefly heated for 2 min in boiling water. The samples were separated on 10% SDS-PAGE and stained with coomassie blue. Stained gel is shown in Fig. 4a. The dried gel was exposed to an X-ray film to determine the phosphorylation of eIF-2α (Fig. 4b).

The results indicate i) The CM-S 0.4 M KC1 purified eIF-2 fraction (lane 9 of Fig. 4; coomassie stained gel) has 4 bands of which three of them are stoichiometric and appear to be eIF-2 subunits (a- 38 kDa, β-51 kDa and y-52 kDa). This preparation is relatively rich in eIF-2 compared to other fractions.

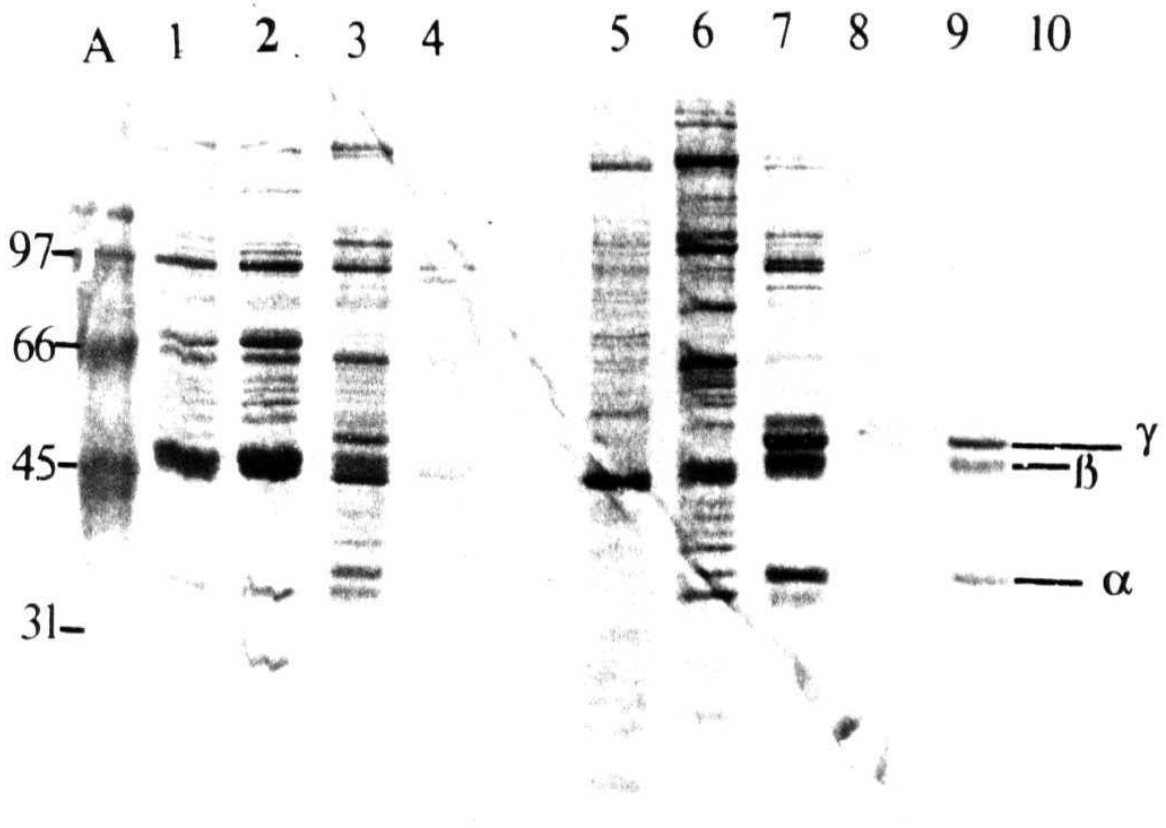


Fig. 4a. Purificiation of reticulocyte eIF-2:

A coomassie stained gel of the fractions obtained at various stages of eIF-2 purification. Lane A, Molecular weight markers;. Lane 1, ribosomal salt wash; lanes 2, 3 and 4 represent 0.1, 0.2 and 0.3 M KCl fractions of DEAE-52 column; lanes 5, 6 and 7 represent 0.2, 0.4 and 0.7 M KCl eluate fractions of phosphocellulose column and lanes 8 and 9 represent the 0.15 and 0.4 M KCl fractions obtained from CM Sephadex 52 respectively. Lane 10 contains purified HRI (~50 ng; please see the corresponding autoradiogram, Fig. 4b).

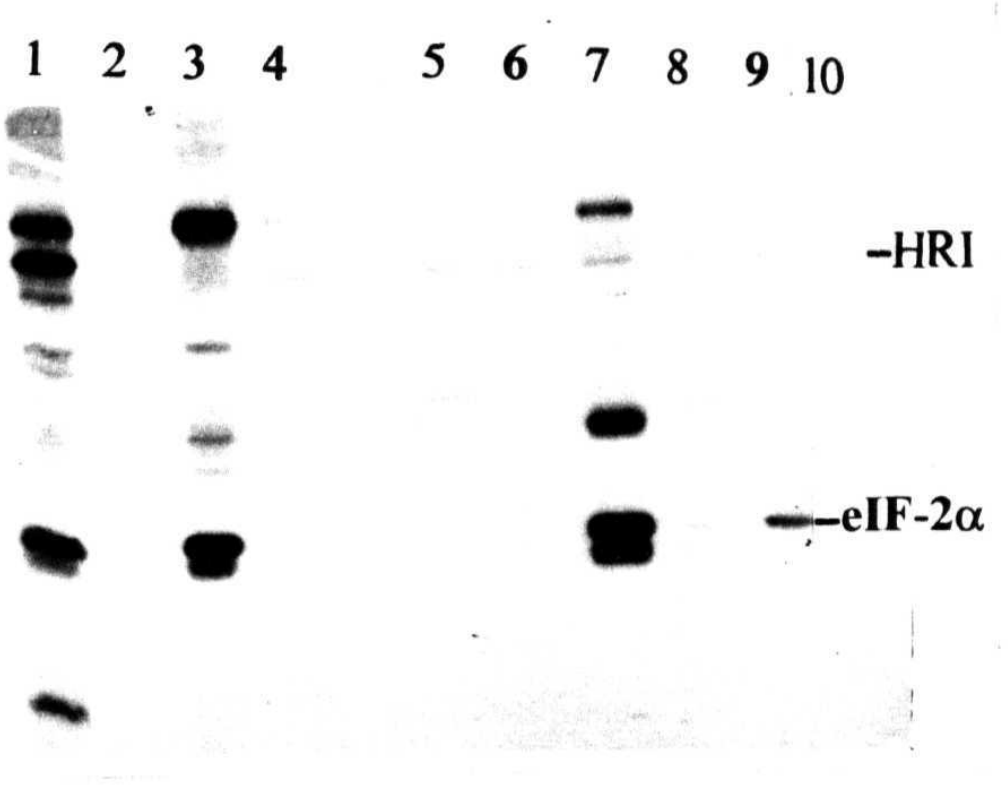


Fig. 4b. Phosphorylation of eIF-2 fractions by HRI kinase:

Autoradiogram of the above fractions phosphorylated by purified HRI kinase *in vitro.* The fractions were incubated with 5 uCi of [γ-^{32}P]ATP and 2 mM Mg^{2+} at 30°C for 5 min in a standard reaction mixture of 20 ul as described in Methods before separating them on 10% SDS-PAGE as mentioned above.

ii) Further, the phosphorylation of these fractions by HRI kinase indicates that the small subunit (38 kDa) of CMS 0.4 M fraction (in lane 9 of coomassie stained gel) is most efficiently phosphorylated (please see the corresponding lane 9 in the autoradiogram of Fig. 4b). The other fractions that contain this phosphorylated 38 kDa subunit are the ribosomal salt wash (lane 1, starting material), DE 0.2 M KCl fraction (lane 3) and PC 0.7 M KCl fraction (lane 7). Purified HRI (lane 10), although cannot be seen in the stained gel due to low quantities, is phosphorylated and does not contain any other stained or phosphorylated bands. The phosphorylation of the small subunit of eIF-2 in CMS 0.4 M KCl fraction is however strikingly enhanced in the presence of HRI (lane 9) suggesting that HRI specifically phosphorylates eIF-2a. The other phosphorylated bands in crude eIF-2 fractions may be due to the contamination of other kinase activities.

2.5. Formation and dissociation of binary complex, **eIF-2.[^{3}H]GDP**:

Assays were carried out to determine the ability of various fractions obtained during eIF-2 purification for their ability to bind [^{3}H]GDP in the presence of Mg^{2+} . Since partially purified eIF-2 preparations are known to contain trace amounts of eIF-2B like activity, the dissociation or exchange of labelled GDP in the binary complex, eIF-2.[^{3}H]GDP is also checked by the addition of unlabelled GDP (GDP°). Typically duplicate sets of 20 µl reaction mixtures containing approximately 1.5 µg of protein fraction was incubated with 2 µM [^{3}H]GDP (specific activity 7975 cpm/pmol) in 20 mM Tris-HCl (pH 7.8), 80 mM KCl, 0.1 mg CPK and 1 mM DTT buffer at 30°C for 10 min. The reaction mixtures were then kept on ice for 10 min and were later supplemented with Mg^{2+} (1 mM) to stabilize the binary complex, eIF-2.[^{3}H]GDP. Ten minutes after the addition of Mg^{2+}, the samples were taken out from ice and incubated at 30°C for 10 min with or without the addition of 40 µM GDP^0 to determine the formation and dissociation of the eIF-2.[^{3}H]GDP complex. The reactions were terminated by the addition of 3 ml cold wash buffer (20 mM Tris-HCl, pH 7.8, 100 mM KCl) containing 1 mM Mg^{2+}. They were then filtered through the Millipore filters (HAWP, 0.45 µM). The filters were dried and counted in a toluene based scintillation fluid in a Beckman liquid scintillation counter. The amount of [^{3}H]GDP bound to eIF-2 or to the protein fractions in the presence and absence

of unlabelled GDP is shown in Table 1. The amount of [^{3}H]GDP bound was always found lesser in the presence of GDP^0. This may be because of traces of eIF-2B contamination with eIF-2 preparations. Further the results indicate that 0.7 M PC and 0.4 M CMS fractions are enriched with eIF-2.

Table 1: Formation and dissociation of eIF-2[^{3}H]GDP, binary complex ability with fractions obtained at various stages of eIF-2 purification

Ser. No.	Fraction#	CPM (-GDP°)	Picomoles*	CPM (+GDP^0)	Picomoles*
1.	-	300	—	275	—
2.	Ribosomal salt wash	1250	0.15	958	0.12
3.	DEAE 0 1M KCl	900	0.11	848	0.10
4.	DEAE 0.2M KC1	1800	0.22	1154	0.14
5.	DEAE 0.3M KC1	870	0.09	806	0.10
6.	PC 0.2M KC1	800	0.10	750	0.09
7.	PC 0.4M KC1	1150	0.14	810	0.10
8.	PC 0.7 M KC1	18,450	2.32	15050	1.88
9.	CM 0.4M KC1	23,375	2.94	19851	2.48

Each fraction contains 1.5 μg/ml protein

* 1 picomole ~ 7975 cpm

2.5.1. Assay for eIF-2B activity in reticulocyte lysates:

The lysate eIF-2B activity was measured from the dissociation of labelled preformed added binary complex, eIF-2[^{3}H]GDP as described (Matts and London, 1984).

Reticulocyte lysates were incubated under protein synthesis conditions at 30°C. The time of incubation and modifications (if any) are mentioned in the legends to the figures. Lysate protein synthesis reaction was carried out without the addition of any labelled amino acid. Then, the binary complex (eIF-2.[^{3}H]GDP) prepared as above was added to the protein synthesis reactions immediately and the incubation was continued at 30°C. Reactions were stopped by the addition of 3 ml cold wash buffer and passed through the Millipore filter as mentioned above. Filters were oven dried and the radio-activity bound to the filter was determined in a 5 ml scintillation fluid in a Beckman scintillation counter. Undissociated eIF-2.[^{3}H]GDP was measured by the retention of the complex on Millipore filters. Picomoles of eIF-2.[^{3}H]GDP dissociated were determined by calculating the difference between the total eIF-2.[^{3}H]GDP added and that remaining in an assay mixture after incubation for the time stated in each figure.

2.6. Purification of HRI:

HRI was purified from the post ribosomal supernatant of rabbit reticulocyte lysate as described (Trachsel *et al,* 1978).

The proteins were precipitated at pH 5.4 and centrifuged at 12,000 rpm for 15 min. The supernatant was removed and the pellet was resuspended in TDEG buffer containing 0.1 M KC1. To this, few drops of 1 M KOH was added to bring up the pH of the solution to 7.5 to 7.8 and subsequently the suspension was homogenized. Then the proteins were precipitated with 0-40% ammonium sulphate and centrifuged at 10000 rpm for 30 min. The pellet was suspended in TDEG having 0.05 M KCl and dialyzed against the same. The ammonium sulphate precipitated protein fraction was loaded on DE-52

column which was equilibrated with TDEG buffer containing 0.05 M KCl. The column was then washed with the same salt concentration Then the HRJ protein was eluted with 0.3 M KCl. Peak fractions were pooled, precipitated with 0-70% ammonium sulphate and centrifuged at 10000 rpm for 30 min. The pellet was suspended in buffer A containing 20 mM potassium phosphate (pH 6.8), 50 mM KCl, 1 mM DTT, 0.1 mM EDTA and 10% glycerol and dialyzed against the same. DE-52 purified HRI was loaded on phosphocellulose column which was equilibrated with buffer A. The column was washed with buffer A and the proteins were eluted with a linear gradient of 50-300 mM KCl (50 ml). 1 ml fractions were collected. Since HRI inhibits the protein synthesis in reticulocyte lysate, 2 µl of every third fraction of the eluate has been tested for its ability to inhibit protein synthesis in hemin supplemented reticulocyte lysates. Results are shown in Table 2.

The fractions causing highest inhibition (28-34) were pooled and the proteins were precipitated with 0-80% ammonium sulphate. The pellet was suspended in TDEG buffer and dialyzed against the same. The dialyzed sample was stored at -80°C in aliquots.

Table 2. Protein synthesis in hemin-supplemented lysate with the addition of PC HRI fractions.

Sl.No.	Fraction No.	CPM
1	- Fraction	12075
2	3	11250
3	10	7668
4	13	5783
5	16	5578
6	19	6062
7	22	5926
8	25	5996
9	31	2468
10	34	4345
11	37	5560
12	40	4580
13	46	8580

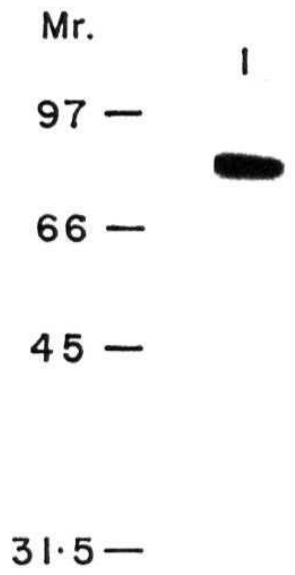


Fig. 5. Autophosphorylation of HRI:

Autophosphorylation of HRI (~50 ng) was perfomed in the presence of [γ-P]ATP (5 μCi) at 30°C for 5 min as described in this section. The reactions were terminated with 2X SDS sample buffer and heated briefly for 2 min. The samples were separated in 10% SDS-PAGE. The figure is an autoradiogram.

***2.7. In vitro* phosphorylation:** *In vitro* phosphorylation assays were carried out by incubating eIF-2 with HRI and [γ-^{32}P]ATP (5 uCi) in a cocktail (20 ul) containing 20 mM Tris-HCl (pH 7.6), 2 mM Mg^{2+}, 80 mM KC1 and 30 uM ATP for 5 min at 30°C. The reactions were terminated by the addition of 20 ul of 2X SDS sample buffer and were heated for 2-3 min in boiling water. Proteins were separated on 10% SDS-PAGE and analyzed by autoradiography. Modifications are mentioned in the legends to the figures.

Purified HRI is autophosphorylated (Fig. 5, lane 1) and is able to phosphorylate the small subunit of eIF-2 (38 kDa) in various eIF-2 fractions (DE 0.2 M, PC 0.7 M and CMS 0.4 KC1 fractions, Fig. 4b).

2.7.1. In situ **phosphorylation:** Protein synthesizing lysates containing any unlabelled amino acid were pulsed with [γ-^{32}P]ATP or inorganic [^{32}Pi] at different time points of protein synthesis for 5 min. The reactions (10 ul) were terminated by the addition of 800 ul of 50 mM NaF and 5 mM EDTA. Then the proteins were pH 5.0 precipitated with the addition of 0.5 M acetic acid. The samples were kept on ice for 45 min. and centrifuged at 12000 rpm for 20 min in a Remi high speed centrifuge. The supernatant was carefully aspirated and the pellet was suspended in 10 ul of 2X protein dissociation buffer containing SDS. The samples were briefly heated for 2-3 min. The gels were analyzed by SDS-PAGE and by autoradiography. Modifications are mentioned in the legends to the figures.

2.8. Purification *of Datura innoxia* agglutinin (DIA):

The agglutinin from the seeds of *Datura innoxia* was prepared as described (Petrescu *et al.*, 1993). Seeds of *D.innoxia* were ground and defatted by using CH_2Cl_2. The dry powder was extracted for 1 hr at room temperature with 10 vol. of 0.5 M HC1

containing 0.01 M EDTA and 5 mM thiourea. After centrifugation at 17600 x g for 30 min, the supernatant was brought to pH 5.0. with 1 M NaOH. Then the crude extract was incubated at 70°C for 30 min to eliminate some of the impurities that can bind chitin matrix and the solution was recentrifuged. Afterwards, the centrifuged extract was loaded on the chitin (affinity) matrix and the lectin was eluted with 0.5 M acetic acid. The peak lectin fractions were pooled and dialyzed exhaustively against distilled water for 48 hr and lyophilized. The lyophilized powder was dissolved in 25 mM Tris-HCl buffer (pH 7.5).

The affinity purified lectin was subjected to 7.5% SDS-PAGE under reducing and non-reducing conditions as described by Laemmli (1971). In the presence of β-mercaptoethanol, DIA has four subunits of molecular weight 101, 55, 39 and 24 kDa (Fig. 6, lanes 2 and 3). In the presence of β-mercaptoethanol, it migrates as two high molecular weight bands (189 and 122 kDa; Fig. 6, lanes 4 and 5).

2.8.1. Lectin Activity (agglutination):

Lectin activity was assayed by hemagglutination using rabbit erythrocytes in Greiner microtitre plate by serial dilution, using 100 ul of protein solution and 100 ul of 4% trypsinized erythrocytes in saline. Rabbit blood was added to an equal volume of Alsever's solution. The Alsever's solution was prepared by dissolving 2.05 gm of glucose, 0.8 gm of sodium citrate and 0.42 gm of sodium chloride in 80 ml distilled water. The pH was adjusted to 6.1 with 1% citric acid and the volume was made up to 100 ml with distilled water. The erythrocytes were isolated by centrifuging the suspension at 1000 rpm for 5 min at room temperature. The cells were washed 3-4 times with cold saline (5 ml saline for each ml of packed erythrocytes). The washed erythrocytes were incubated with trypsin (0.0025% w/v trypsin per 1% erythrocytes) for one hour at 37°C. After incubation, cells were washed 5-6 times with cold saline for removing the traces of trypsin and diluted to 4% with saline for use in the experiments.

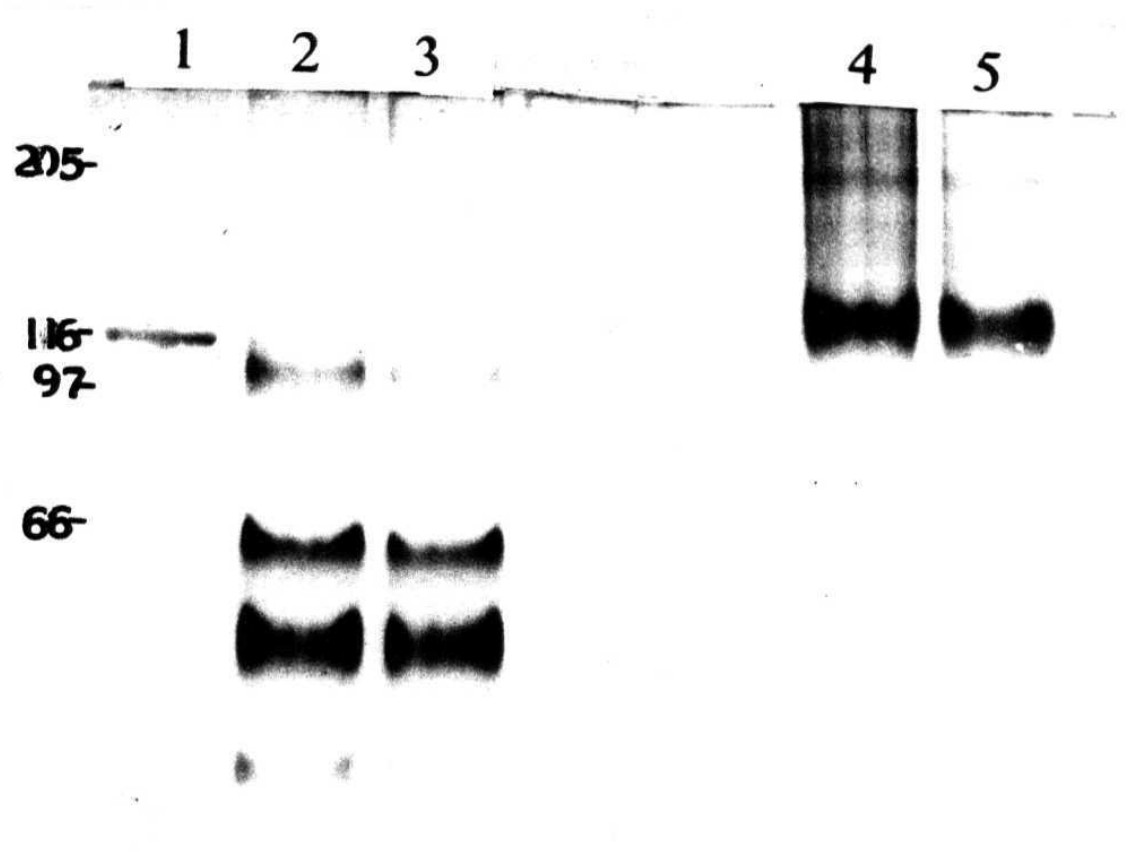


Fig.6. SDS-PAGE of affinity purified DIA in the presence and absence of β-mercaptoethanol: Purified DIA protein (5 μg) was resuspended in SDS-PAGE sample buffer in the presence of P-mercaptoethanol (lanes 2 and 3) and also in the absence of P-mercaptoethanol (lanes 4 and 5). The protein was separated on 7.5% SDS-PAGE and the gel was stained with silver nitrate as described in Materials and Methods.

One unit of lectin activity is expressed as the minimum amount of protein required to agglutinate 2% suspension of trypsin treated rabbit erythrocytes kept for 1 hour in saline.

The agglutination activity of crude and affinity purified DIA protein is carried out as mentioned above and the results are shown in Table 3.

Table 3. Purification and hemagglutination activity of *D.innoxia* agglutinin

Purification step	Protein (mg)	Specific* activity	Total activity (titre x mg)	Yield %	Purification factor
Crude extract	418	3413	1426634	100	1
Affi.purified	47	27306	1283382	11.2	8

* Specific activity is expressed as titre, the reciprocal of maximal dilution of protein that gives visible agglutination with 2% trypsinized rabbit erythrocytes.

Separation of ribosomes on 10-50% sucrose gradients:

Ribosomes of reticulocyte lysates were separated on 10-50% sucrose gradients and analyzed by ISCO density gradient fractionator as described (Ramaiah and Davies, 1985).

10 and 50% sucrose solutions were made in TKM buffer containing 20 mM Tris-HCl (pH. 7.8), 100 mM KC1, 1 mM Mg^{2+}. 2.5 ml of 50% sucrose was poured into a 5 ml SW 50.1 rotor tube. Then, 2.5 ml of 10% sucrose was carefully layered on 50% sucrose solution. The gradients were capped and kept horizontally for 3 hrs at room temperature and then lifted carefully. The gradients thus formed by diffusion were kept in cold room for 40 min. prior to centrifugation.

Protein synthesizing (typically 80-100 µl) reticulocyte lysates were incubated at 30°C for few minutes and the assays were terminated by adding equal volume of cold TKM buffer. This was carefully layered on the top of 10-50% sucrose gradient. Samples were spun at 45000 rpm for 45 min in a SW 50.1 rotor in a Beckman ultracentrifuge. The gradients were analyzed at A_{258m} nm using ISCO density gradient fractionator. Modifications are mentioned in the legends to the figures.

2.10. Sodium dodecyl Sulfate - polyacrylamide gel electrophoresis (SDS-PAGE): Proteins were separated on a modified Laemmli method (1970). The separation gel mix, 30 ml, was prepared with the following ingredients: 1.5 M Tris-HCl, pH 8.8 (7.5 ml), 30:0.8 of Acrylamide:Bis (10 ml), 10% SDS (0.3 ml), 10% ammonium persulphate (100 µl) and H_20 (12.1 ml). The stacking gel mix contained 4.5% acrylamide, 0.1% ammonium persulphate and 10% SDS in 125 mM Tris-HCl (pH 6.8). Protein samples were prepared in protein dissociation buffer containing Tris-HCl (pH 6.8), glycerol, SDS, β-mercaptoethanol and bromophenol blue. Samples were briefly heated for 2-3 minutes and then loaded in the gel wells. Electrophoresis was carried out at 120 volts with Tris-SDS-Glycine buffer until the bromophenol blue dye front had run off from the bottom of the gel. The gel was fixed and stained with coomassie or silver nitrate.

2.11. Autoradiography: The labelled proteins were analyzed by separating on SDS-PAGE and then by autoradiography. For carrying the autoradiography, the gels were vacuum dried and exposed to X-ray film with or without intensifier. The film was developed by a set of photographic solutions obtained commercially and as per the manufacturer's instructions.

2.12. Western Blotting: Proteins were separated on 10% SDS-PAGE. The separated proteins were electrophoretically transferred to nitrocellulose membrane using Tris-SDS-Glycine as an electrode buffer. Transfer was carried out for 3 hrs at 75 V. After the transfer, the nitrocellulose membrane was carefully removed and soaked in TBST buffer

containing 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, 0.05% Tween 20. The membrane was washed for a few minutes with TBST and the buffer was replaced with blocking solution (TBST buffer containing 1% BSA). After 1 hr, the blocking solution was decanted and the membrane was incubated with TBST containing the appropriate dilution of primary antibody for 1 hr. The membrane was washed thrice with TBST for 5-10 min each to remove the unbound antibody. Later the membrane was incubated with TBST containing the appropriate anti IgG-AP conjugate for 30 min. The membrane was washed in TBST three times for 5-10 min each as above. Then the membrane was developed with a color development solution which was prepared as follows: for every 10 ml of AP solution, 66 u1 of NBT and 33 ul of BCIP substrates were added. AP solution contains 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 5 mM $MgCl_2$. When the color was developed to the desired intensity, the reaction was arrested by rinsing the membrane in distilled water for several times.. The membrane was air dried and stored at 4°C. The membrane was protected from light during the color development.

2.13. Protein estimation: Protein was estimated by standard Bio-rad method and as per the instructions of the manufacturer.

2.14. RNA Isolation: RNA was isolated from reticulocyte lysates by phenolization and ethanol precipitation as has been reported earlier (Sambrook *et al*, 1989).

70 µl of reticulocyte lysate was mixed with 80 ul of buffer A which contains 20 mM Tris-HCl (pH 7.8), 50 mM KC1, 10 mM DTT and 2 mM Mg^{2+}. 500 ul of buffer B, which contains 0.5% SDS and 50 mM Tris-HCl (pH 7.8) was added and mixed. To this, equal volume of buffer saturated phenol was added and thoroughly mixed for 30 min. The samples were spun at 10 K for 20 min. The aqueous layer was carefully removed and to this 2 volumes of distilled ethanol and 0.1 volume of 20% sodium acetate was added and the samples were kept overnight at -20°C. RNA was obtained by centrifuging the samples at 10 K for 10 min. The pellet was lyophilized briefly and it was dissolved in an appropriate volume of autoclaved distilled water. The RNA was stored at -70°C.

2.15. **Acrylamide-urea gel**: For the separation of lysate RNA, acrylamide-urea gels were used as described (Sallustio and Stanley, 1990).

The separation gel mix contains Tris-borate buffer of pH 8.3 (89 mM Tris, 89 mM Borate, 2.5 mM EDTA), 4.5% acrylamide, 7 M urea, 0.1 ml of 10% ammonium sulphate and 7.5 ul TEMED and it does not contain any stacking gel. RNA samples were suspended in Tris borate buffer that contains 7 M urea and 100 mg/ml sucrose. Samples were briefly heated for about one minute at 90°C and were loaded on the gel. Electrophoresis was done at 20 milli amp. using Tris-borate buffer as an electrode buffer until the bromophenol blue dye front had run off from the bottom of the gel. Then, the gel was stained with EtBr.

30 CHAPTER I

TYPE I PHOSPHA TASE INHIBITORS REDUCE THE GUANINE NUCLEOTIDE EXCHANGE A CTIVITY OF eIF-2B IN INHIBITED L YSA TES RESCUED B Y HEM IN

Phosphorylation of eIF-2a leads to the inhibition in the guanine nucleotide exchange activity of eIF-2B *in vitro* (Clemens *et al,* 1982). In heme-deficient lysates, the phosphorylation of eIF-2α gives rise to the formation of a 15S phosphorylated complex [eIF-2B.eIF-2(aP)], in which eIF-2B is tightly sequestered and unable to catalyze the guanine nucleotide exchange (Thomas *et al,* 84 & 85; Gross *et al,* 1985). Since the concentration of eIF-2B relative to that of eIF-2 in the lysate is low, phosphorylation of a portion (20-40%) of eIF-2a is sufficient to bind all of the lysate eIF-2B in this nonfunctional 15S complex (Thomas *et al,* 1985). It was shown previously (Thomas *et al,* 1984) that alkaline phosphatase treatment of the eIF-2B.eIF-2(αP) complex from heme-deficient lysates results in the recovery of eIF-2B activity. The rescue of protein synthesis in heme-deficient lysates by the delayed addition of hemin (20 μM) or MgGTP (2 mM) is also closely correlated with the dephosphorylation of lysate eIF-2(aP) and the restoration of eIF-2B activity (Matts *et al,* 1986, Kan *et al,* 1988). Both hemin and MgGTP exert their effects by inhibiting HRI activity, thus permitting dephosphorylation of lysate eIF-2(αP) by endogenous protein phosphatase (Matts *et al,* 1986). These findings indicate that the dephosphorylation of lysate eIF-2(αP) is a critical event in the rescue of protein synthesis by hemin and that both eEF-2B activity and the rate of protein synthesis are regulated by the equilibrium between eIF-2α kinase and phosphatase activities.

The physiological mechanism of dephosphorylation of eIF-2(aP) and the restoration of eIF-2B activity has not been clear. Other studies (Mumby and Traugh, 1979 &1980; Grankowski *et al,* 1980; Crouch and Safer, 1984; Stewart *et al,* 1980; Wollny et al; 1984; Fullilove *et al,* 1984; Redpath and Proud, 1990) with isolated protein phosphatases which dephosphorylate purified eIF-2(aP) *in vitro* have not demonstrated that these phosphatases can also dephosphorylate endogenous eIF-2B.eIF-2(αP) complex or restore eIF-2B activity in heme-deficient lysates. In this study, some characteristics of the dephosphorylation of eIF-2(αP) by endogenous protein phosphatase(s) in the lysate have been examined. We have measured eIF-2B activity directly in protein synthesizing lysates and have found a correlation of changes in this activity with changes in phosphorylation and dephosphorylation of eIF-2a. The specific effect of phosphorylation

of eIF-2α on eIF-2B activity is also indicated by our finding that the recovery of eIF-2B activity in inhibited lysates on addition of hemin is unaffected by the addition of inhibitors of protein synthesis (pactamycin, puromycin, or cycloheximide) whose action is not dependent on phosphorylation of eIF-2α. The endogenous protein phosphatase activity which restores eEF-2B activity in hemin-rescued lysates displays type 1 protein phosphatase characteristics.

3.1. Results:

3.2. Restoration of eIF-2B activity in heme-deficient lysates is dependent on the concentration of added hemin and HRI activity:

Protein synthesis in reticulocyte lysates is dependent upon the concentration of hemin, which binds to and inactivates HRI by promoting intersubunit disulfide bond formation (Chen *et al,* 1989; Fagard and London, 1981; Yang *et al,* 1992). In heme-deficient lysates, protein synthesis is inhibited due to the activation of HRI, the phosphorylation of eEF-2a, and the sequestration of eIF-2B in a nonfunctional 15S phosphorylated complex [eIF-2B.eIF-2(αP)]. To understand the physiological phosphatase activity which is responsible for the dephosphorylation of eIF-2(aP) and restoration of eEF-2B activity, we have studied here the restoration of guanine nucleotide exchange activity of eIF-2B in inhibited heme-deficient lysates which are supplemented with the delayed addition of hemin or phosphatase inhibitors or both. The eIF-2B activity is assayed by measuring the extent of dissociation of added labelled binary complex eIF-2.[^{3}H]GDP. As shown in Table 4, eIF-2B activity in heme-deficient lysates is very low (0%), whereas eIF-2B activity is maximal in the presence of optimal concentration of hemin (20 μM). This is consistent with the earlier reports (Matts and London, 1984, Matts *et al,* 1986) and correlates with the ability to carry out protein synthesis. While eIF-2B activity can fluctuate significantly in different lysate preparations depending on their ability to carry out protein synthesis and respond to added hemin, the general direction of these results does not change; that is, the protein synthesis and eIF-2B activity are always higher in hemin-supplemented reticulocyte lysates than in heme-deficient lysates.

Table 4

Effect of hemin concentration on eIF-2B activity in reticulocyte lysates

Protein synthesis	eIF-2B activity	
	eIF-2.[^{3}H]GDP dissociated, pmol	% Activity
I. -Hemin	0.52	0
+Hemin (5 μM)	0.69	13
+Hemin (10 μM)	1.43	70
+Hemin (20 μM)	1.82	100
II. -Hemin	0.61	0
+Hemin (5 μM)	0.64	5
+Hemin (10 μM)	1.12	100
+Hemin (20 μM)	1.12	100
-Hemin/+Hemin 7' (10 μM)	0.87	45
-Hemin/+Hemin 7' (20 μM)	1.03	72

Protein synthesizing lysates (30 μl) containing 5, 10, or 20 uM hemin were incubated at 30°C for 12 min. In one experiment (II), heme-deficient lysates were supplemented with 10 or 20 μM hemin at 7 min, and incubation was continued for 5 min. At 12 min of protein synthesis, 2.6 or 2.48 pmol (in 20 ul) of eIF-2.[^{3}H]GDP was added to lysates in Expt.I and II respectively to determine the eIF-2B activity. The activity was assayed for 15 min. at 30°C as described in Materials and Methods. The results of two independent experiments from two differnt lysate preparations are shown. Values are expressed as pmoles of dissociated binary complex.

Addition of optimal concentration of hemin to inhibited heme-deficient lysates restores eIF-2B activity more efficiently than suboptimal concentrations of hemin (Table 4). The restoration of eIF-2B activity in lysates which are treated with the delayed addition of hemin occurs gradually and is time-dependent (Fig. 7), Maximum recovery occurs within 15-20 min. The recovery of eIF-2B activity is, however, inhibited significantly if the lysates are incubated for a longer duration of time without hemin and is correlated to the restoration of protein synthesis (Table 5). These findings suggest that the recovery of eIF-2B activity is dependent on the concentration of added hemin and the time at which hemin is supplemented to lysates. Since heme inhibits the eIF-2a kinase activity of HRI, the recovery of eIF-2B activity is dependent on the kinase activation.

3.3. Protein synthesis inhibitors that have no effect on eIF-2a phosphorylation do not affect eIF-2B activity:

The specificity of eIF-2a phosphorylation in regulating eIF-2B activity in lysates is demonstrated by the results obtained with other translational inhibitors of protein synthesis, namely, pactamycin, puromycin, and cycloheximide. The inhibition elicited by these agents is not mediated by the phosphorylation of eIF-2α and has no effect on the recovery of eIF-2B activity promoted by the addition of hemin to inhibited heme-deficient lysates (Table 6).

3.4. Okadaic acid inhibits the restoration of eIF-2B activity and dephosphorylation of eIF-2(αP) mediated by the delayed addition of hemin to inhibited lysates:

Okadaic acid, a polyether fatty acid found in certain marine fauna (sea sponges, dinoflagellates), is a potent inhibitor of protein phosphatases (Bialojan and Takai, 1988; Cohen *et al,* 1990). Type 2A protein phosphatase is selectively inhibited by low levels of okadaic acid (1-20 nM), whereas inhibition of type 1 protein phosphatase requires higher concentrations of okadaic acid (>50 nM) (Cohen *et al,* 1990). This property of okadaic acid has been used to characterize the protein phosphatase involved in the dephosphorylation of eIF-2B.eIF-2(aP) and the recovery of eIF-2B activity in lysates. As

Fig. 7. Kinetics of eIF-2.[^{3}H]GDP dissociation in reticulocyte lysates during the delayed addition of hemin:

In step I, protein synthesis was carried out in lysates (70 μl) with and without the addition of 20 uM hemin (-hemin or +hemin, 0 min) at 30°C for 10 min as described under Materials and Methods At 7 min of protein synthesis, 20 uM heme was added to one of the heme-deficient inhibited lysates (-heme, 0 min +heme). Soon after the addition of hemin, eEF-2B activity of the lysates was determined from the dissociation of preformed labelled eIF-2.[^{3}H]GDP binary complex (12.60 pmol in 70 ul to a lysate volume of 70 μl). At each time interval, as indicated, a 40 ul aliquot was withdrawn from each of the reactions to determine the amount of labeled GDP bound to the Millipore membrane as described in Materials and Methods. The values plotted represent picomoles of eIF-2.[^{3}H]GDP dissociated with time.

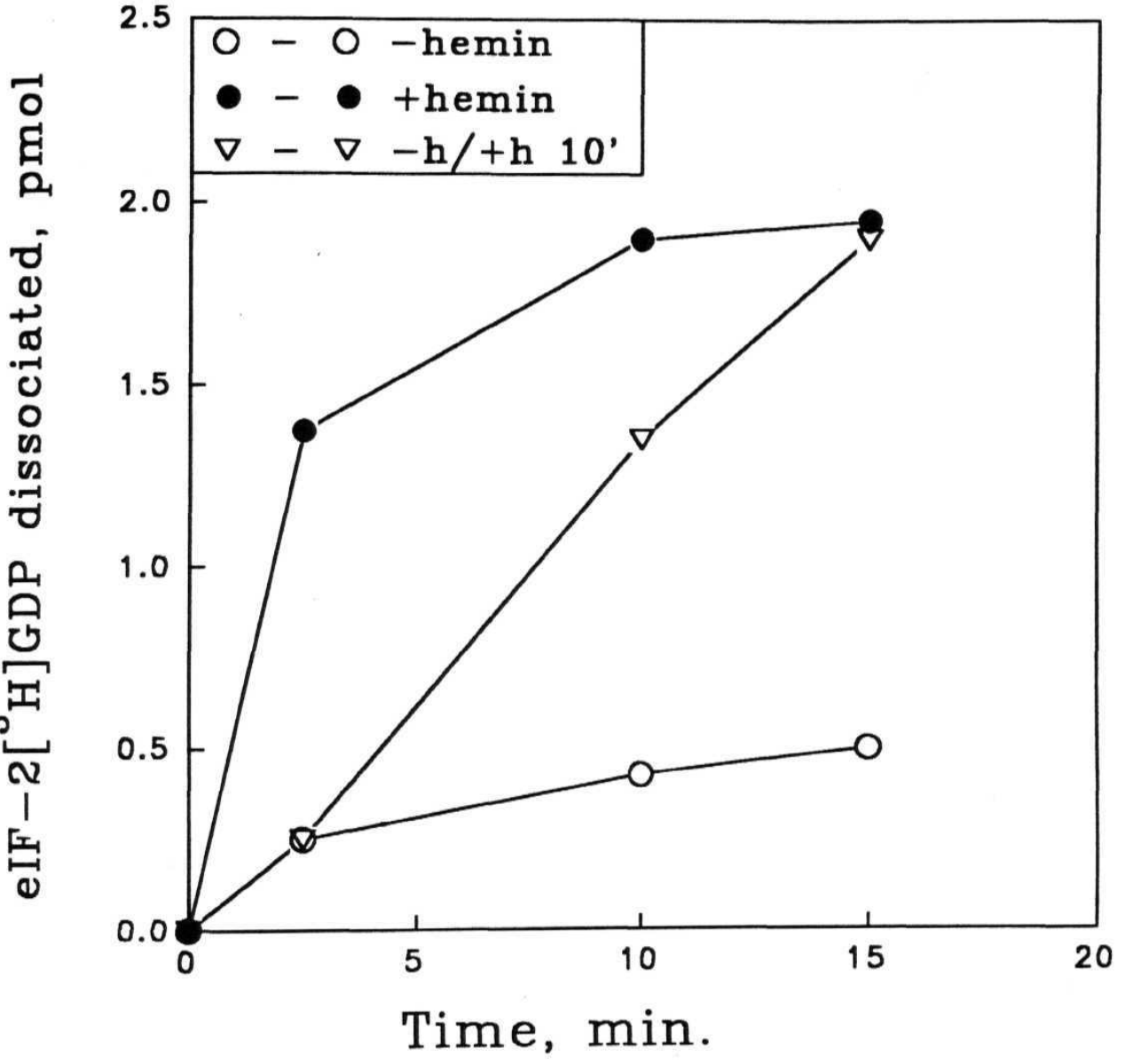

−hemin
+hemin
−h/+h 10'
eIF−2[³H]GDP dissociated, pmol
2.5
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
Time, min.

Table 5

Recovery of eIF-2B activity and protein synthesis in heme-deficient lysates teated with hemin at different time intervals

Protein synthesis conditions	eIF-2B Activity	Protein synthesis at 30 min.
	eIF-2.[^{3}H]GDP Dissociated, pmol	[^{14}C]Leucine inc. cpm
-Hemin	1.03	8642
+Hemin 0'	1.82	16652
-Hemin/+Hemin 5'	1.70	15754
-Hemin/+Hemin 12'	1.25	12776
-Hemin/+Hemin 20'	1.08	9050

Heme-deficient lysates (30 µl x 2) were incubated for different time periods (0, 5, 12 and 20 min) before the adddition of 20 uM hemin to determine the effect of prolonged incubation without heme on eIF-2B activity (eIF-2.[^{3}H]GDP dissociated) and on protein synthesis ([^{14}C]Leucine incorporated, cpm). Protein synthesis was measured in 5 ul aliquots at 30 min. as described in Materials and Methods. eIF-2B activity was assayed in lysates (20 µl) for 15 min from the dissociation of labelled binary complex, eIF-2.[^{3}H]GDP (1.99 pmol in 20 µl aliquots). The labelled binary complex was added to lysates at 10 min (-h; +h, 0 min; -h/+h, 5 min) or at 12 min of protein synthesis (-h/+h, 12 min; -h/+h, 20 min).

Table 6

Effects of cycloheximide, pactamycin and puromycin on eIF-2B activity in reticulocyte lysates

Protein synthesis	eIF-2B activity	
	eIF-2.[^{3}H]GDP dissociated, pmol	% Activity
-Hemin	0.65	0
-Hemin [+Hemin 10']	1.45	100
-Hemin [+Hemin + cycloheximide10']	1.46	100
-Hemin [+Hemin + pactamycin10']	1.46	100
-Hemin [+Hemin + puromycin10']	1.43	96

Lysate protein synthesis was carried out at 30°C for 10 min as described under Materials and Methods. Incubations (30 μl) were supplemented at 10 min with hemin (20 μM), cycloheximide (10 μg/ml), pactamycin (2 μM) or puromycin (10 μg/ml) as indicated. At 15 min, lysate eIF-2B activity was assayed in 20 ul samples with the addition of 3.0 pmol of labelled eIF-2.[^{3}H]GDP. eIF-2B activity was assayed for 15 min at 30°C as described under Materials and Methods.

shown in Table 7, the addition of increasing levels of okadaic acid to hemin-supplemented lysates (+h, 0 min) does not affect the functional eIF-2B activity that is available in these lysates, although the protein synthesis is progressively inhibited (Fig. 8); eIF-2B activity is not affected because the inhibition of protein synthesis by okadaic acid is not primarily due to eIF-2a phosphorylation (Redpath and Proud, 1989). This is discussed below.

In our experience, it has been always observed that some amount of eIF-2B activity is available in inhibited heme-deficient lysates to dissociate the preformed binary complex (Tables 4-8). The eIF-2B activity that is available in heme-deficient lysates is further inhibited by high concentrations of okadaic acid (Table 7). The recovery of eIF-2B activity that is observed by the delayed addition of hemin (at 10 min) to lysates is also inhibited by the addition of high concentrations of okadaic acid. These results indicate that a type 1 phosphatase is largely responsible for the recovery of eIF-2B activity. This conclusion is further supported by the data in Fig.9A, which displays [^{32}P] phosphoprotein profiles generated in heme-deficient lysates by delayed ^{32}P pulse (7-12 min). The addition of high levels of okadaic acid (125-250 nM) causes an increase in eIF-2(αP) (tracks 5 and 7) compared to assays with no okadaic acid (track 1) or low levels (25 nM) of okadaic acid (track 3). At the same time, as expected, hemin-supplemented control lysates display very little eIF-2(αP) (track 2) and okadaic acid does not affect this result (tracks 4, 6 and 8). This finding is also consistent with the maintenance of functional eIF-2B activity in hemin and okadaic acid-supplemented lysate (Table 7). In a separate experiment (Fig. 9B), we examined the effect of high concentrations of okadaic acid on the [^{32}P]phospho-protein profile derived from 0-12 min of [^{32}P] pulse in heme-deficient lysates rescued by the delayed addition (at 7 min) of hemin. In the absence of okadaic acid, a low level of eIF-2α phosphorylation is observed in hemin-supplemented (+heme, 0 min, track 2) lysates and also in lysates treated with the delayed addition of hemin (-heme, +heme at 7 min, track 3) when compared to inhibited heme-deficient lysates (track 1). These findings, which are in agreement with a previous report (Matts *et al,* 1986), suggest that a block in the eIF-2α kinase activity of HRI by hemin will allow one to monitor the dephospho-rylation of eIF-2(aP) caused by an endogenous phosphatase in the lysate (track 3 vs 1).

Table 7

Effect of okadaic acid on restoration of eIF-2B activity in reticulocyte lysates by the delayed addition of hemin

Protein synthesis Conditions	eIF-2B activity		
	eIF-2.[^{3}H]GDP dissociated, pmol		
	(+)hemin	(-)hemin	-h + h (10 min)
----	2.33	0.51	2.28
+ 10 nM OA	2.01	0.91	2.25
+ 50 nM OA	2.32	0.52	2.22
+100 nM OA	2.29	0.29	2.08
+250 nM OA	2.25	0.00	1.85
+500 nM OA	2.19	0.00	1.43

Protein synthesizing lysates (30 μl) were incubated under three conditions: (i) Plus 20 μM hemin (+hemin), (ii) minus hemin (-hemin); and (iii) minus hemin and plus 20 uM hemin added at 10 min (-h/+h, 10 min) Increasing concentrations of okadaic acid (OA) were added at 0 min to separate assays as indicated. After 17 min at 30°C, lysate eIF-2B activity (in 30 μl) was assayed by the addition of 4.5 pmol of eIF-2.[^{3}H]GDP (in 20 μl) as described in Materials and Methods. Values represent net pmols of labelled eIF-2.[^{3}H]GDP dissociated by endogenous eIF-2B under standard conditions.

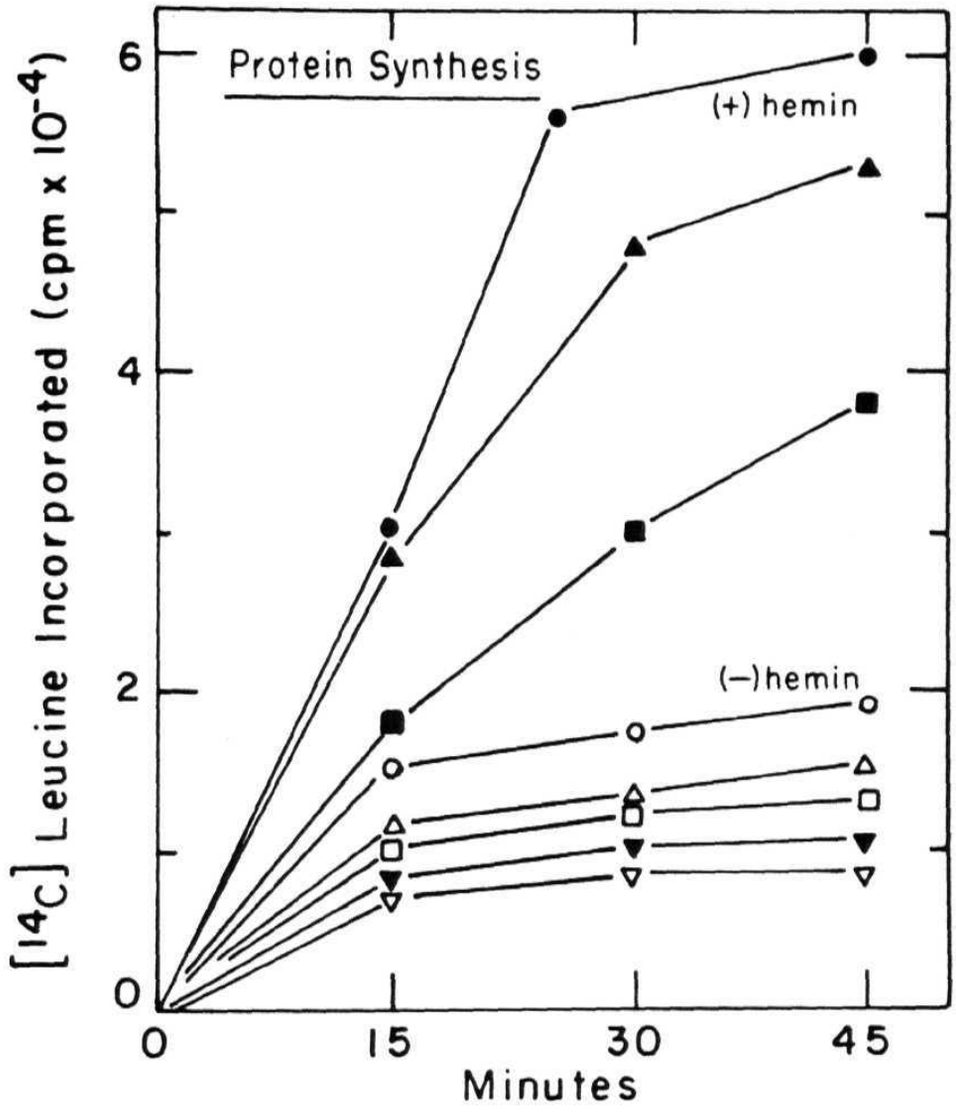
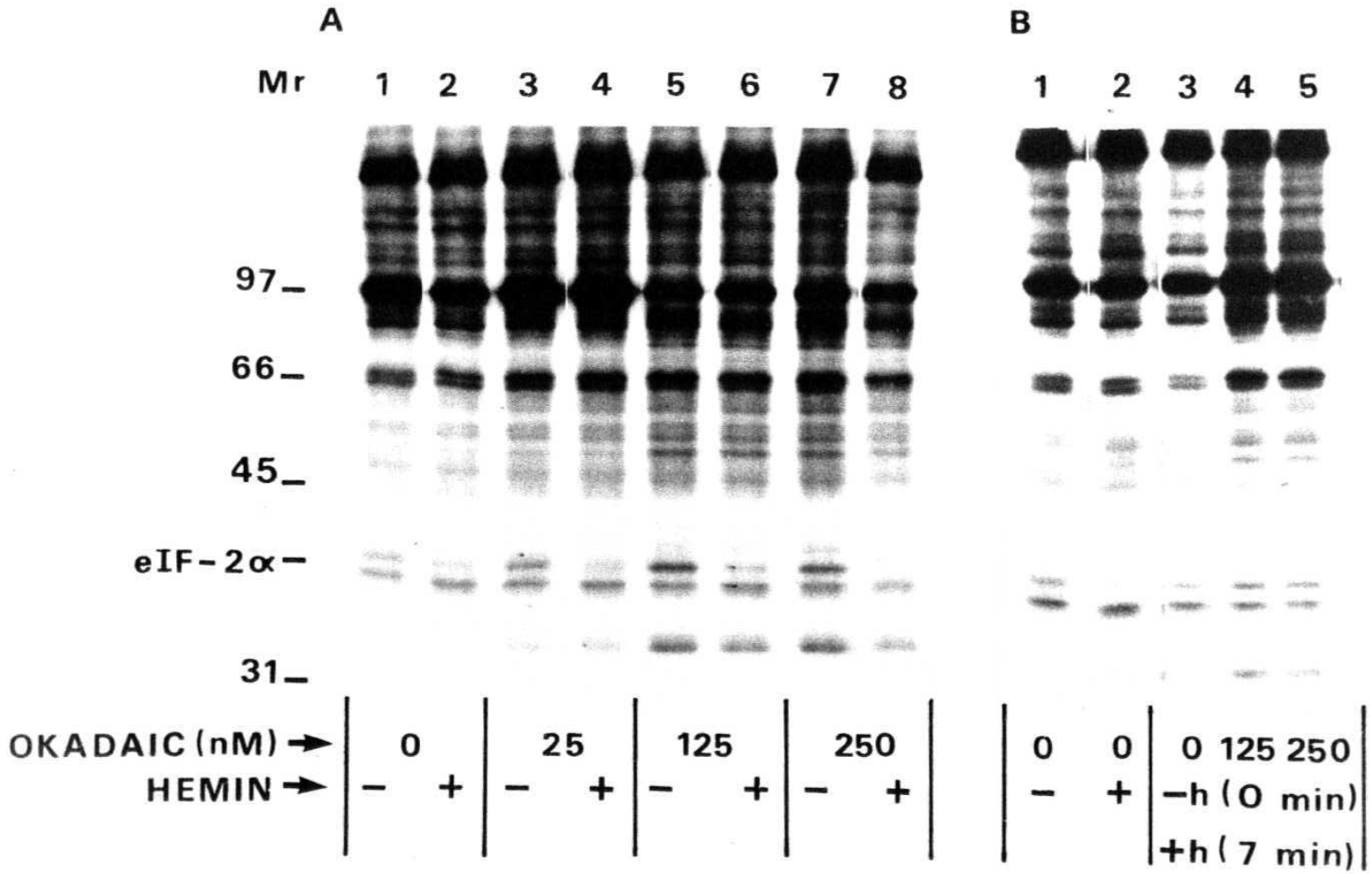


Fig. 8. Effect of okadaic acid on reticulocyte lysate protein synthesis:

Protein synthesis was carried out under two conditions: i) -hemin ii) +hemin. The heme-deficient lysates were incubated with 25 nM (A), 75 nM (D), and 125 nM (V) of okadaic acid. The hemin-supplemented lysates were also treated with 25 nM (▲), 75 nM (•), andl25 nM (T) of okadaic acid. The incorporation of labelled amino acid into protein in 5 μl aliquots was measured with time as described in Materials and Methods.

Fig.9. Effect of okadaic acid on [^{32}P]phosphoprotein profiles of protein synthesizing lysates. Protein synthesis reactions (30 μl) were incubated at 30°C for 17 min with or without 20 μM hemin as indicated. At the beginning of protein synthesis reactions, assays were supplemented where indicated with 0, 25, 100 and 250 nM okadaic acid. Assays were pulse-labeled with ^{32}P at 12-17 min (A, tracks 1-8) or at 0-12 min (B, tracks 1-5). Assays, in B, 3-5, were incubated without hemin for 7 min (-h) and then supplemented with 20 μM hemin and incubated for an additional 5 min. Samples of each assay were pH 5.0 precipitated and then separated in sodium dodecyl sulfate-10% polyacrylamide gels as described in Materials and Methods. The figure is an autoradiogram.


A
B
Mr
1 2 3 4 5 6 7 8
1 2 3 4 5
97
66
45
eIF-2α
31
OKADAIC (nM)
HEMIN
0 25 125 250
− + − + − + − +
0 0
− +
0 125 250
−h (0 min)
+h (7 min)

When high concentrations of okadaic acid are present (tracks 4 and 5), dephosphorylation of eIF-2(aP) is, however, prevented in response to rescue by hemin.

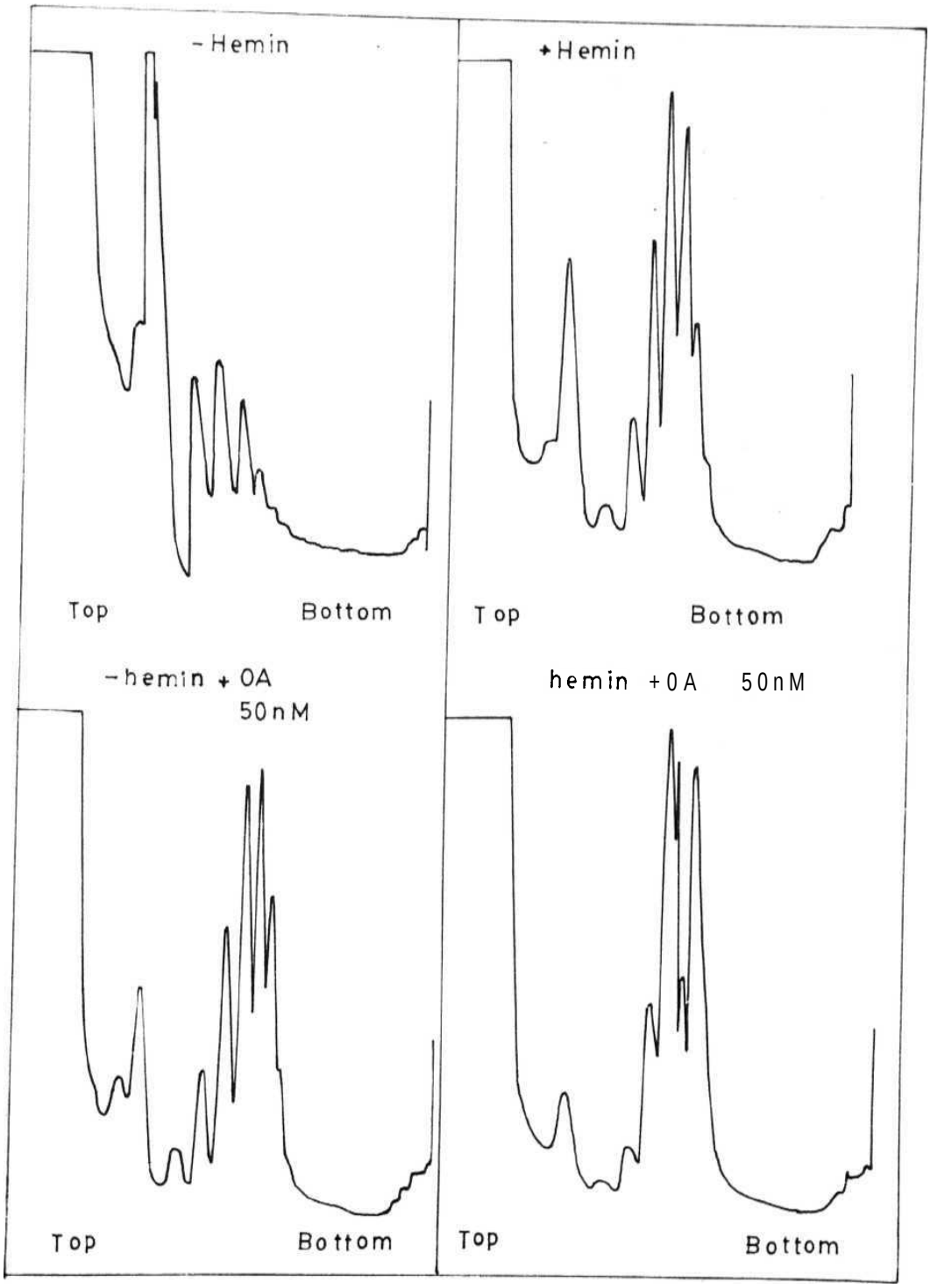
Hence, when HRI is active, high levels of okadaic acid enhance eIF-2α phosphorylation by inhibiting type 1 protein phosphatase. At 20 μM hemin, at which HRI is not active, high levels of okadaic acid cause an inhibition of protein synthesis, but this inhibition is not due to phosphorylation of eIF-2a (Fig. 9A, tracks 2, 4, and 8) and accordingly eIF-2B activity is not affected. In these experiments, we noticed an effect of okadaic acid on 97-kDa polypeptide which is probably elongation factor 2 (Fig. 9) and is phosphorylated in both heme-deficient and hemin-supplemented lysates. At low concentrations of okadaic acid (25 nM), phosphorylation of EF-2 is enhanced, probably due to a partial inhibition of a protein phosphatase (Fig. 9 A, tracks 3 and 4). At high levels of okadaic acid (125-250 nM) the phosphorylation is reduced in the profiles generated by the delayed ^{32}P pulse (Fig.9A, tracks 5-8) but this is probably due to the combination of unlabelled phosphorylation of EF-2 prior to the addition of the ^{32}P pulse and the prevention of phosphate turnover after the pulse. These results on EF-2 phosphorylation (97 kDa), protein synthesis inhibition in hemin-supplemented lysates treated with low concentration of okadaic acid and polyribosome formation (Figs.8 and 10) are in accordance with the results reported by Redpath and Proud (1989). Recently, Ramaiah et al. (1992) reported that enhanced eIF-2α phosphorylation occurs in cycloheximide-treated heme-deficiency lysates in which HRI is active and polyribosomes are maintained, a finding that indicated polysome-bound eIF-2a is a target of eIF-2a kinase under quasiphysiological conditions. The diminution in eIF-2B activity in okadaic acid-treated heme-deficient lysates (Table 7) may be due therefore to a combination of active HRI, decreased eIF-2α phosphatase activity, and increased polyribosomes.

3.5. Protein phosphatase inhibitor 2 inhibits hemin-mediated restoration of eIF-2B activity in lysates:

Protein phosphatase inhibitor 2 (I-2), a heat stable protein, is a selective inhibitor of type 1 protein phosphatases (Haystead *et al,* 1989, Cohen *et al,* 1990, Redpath and

Fig.10. Polysome profiles of protein synthesizing reticulocyte lysates in the presence of okadaic acid:

Protein synthesis reaction mixtures (80 μl) containing heme-deficient lysates (-h) were supplemented, where indicated, with hemin (20 u.M) or okadaic acid (50 nM) or hemin and okadaic acid. The reactions were incubated for 15' at 30°C. Reactions were terminated with the addition of equal volume of buffer containing 20 mM Tris-HCl (pH 7.8), 1 mM $Mg(OAc)_2$ and 80 mM KC1. Ribosomes were separated on a 10-50% sucrose gradients in SW 50.1 rotor as described in Materials and Methods. The gradients were fractionated and analyzed at 258 nm by ISCO density gradient fractionator. The top and bottom of the gradients are shown in the figure.


-Hemin
Top
Bottom
+Hemin
Top
Bottom
-hemin + OA
50nM
Top
Bottom
hemin +OA 50nM
Top
Bottom

Proud, 1989; Cohen, 1989). Previous studies have shown that addition of inhibitor-2 protein enhances eIF-2a phosphorylation and inhibits protein synthesis in hemin-supplemented lysates (Ernst *et al,* 1982). In those experiments, the hemin-treated lysates were incubated with I-2 from the beginning of protein synthesis reactions. A small amount of I-2 obtained as a free gift from the above laboratory has been used here to determine the effect of I-2 directly on the eIF-2B activity of lysates and also on the restoration of eIF-2B activity in inhibited lysates supplemented with the delayed addition of hemin. To determine if I-2 affects the functional eIF-2B activity in lysates directly, the eIF-2 guanine nucleotide exchange ability of the hemin-treated lysates has been carried out here immediately after the addition of I-2. Our results (Table 8) suggest that addition of I-2 at 5 min of protein synthesis to translating hemin-supplemented lysates just before measuring eIF-2B activity, does not affect the functional eEF-2B activity which is available in these lysates and catalyzes readily the dissociation of preformed eIF-2.[^{3}H]GDP binary complex. However, the restoration of eIF-2B activity that occurs in inhibited heme-deficient lysates upon delayed addition of hemin is inhibited in the presence of I-2 (Table 8). These results are consistent with the idea that a protein phosphatase, preferably type 1, plays a dominant role in the physiological dephosphorylation of eIF-2(aP) (Ernst *et al,* 1982; Proud, 1992) and in the restoration of eIF-2B activity in heme-deficient lysates.

3.6. Discussion:

The critical events in the inhibition of protein synthesis in heme-deficiency are the activation of HRI, the phosphorylation of eIF-2a, and the sequestration of eIF-2B by phosphorylated eIF-2α into a complex, in which eIF-2B becomes nonfunctional (Thomas *et al,* 1984 & 1985; Gross *et al,* 1985). Previously, several protein phosphatases have been reported to act on eIF-2(aP) *in vitro* (Mumby and Traugh, 1979 & 1980; Grankowski *et al,* 1980; Crouch and Safer, 1984; Stewart *et al,* 1980; Wollny *et al,* 1984; Fullilove *et al,* 1984). A recent report (Redpath and Proud, 1990) indicates that the protein phosphatases 1 and 2A dephosphorylate the eIF-2(aP) at similar relative rates *in vitro.* There was no indication, however, to date that such preparations could restore eIF-2B activity or reverse the inhibition of protein synthesis in heme-deficient lysates.

Table 8

Effect of inhibitor-2 on the recovery of eIF-2B activity in heme-deficient lysates

Protein synthesis Conditions	Delayed additions	eIF-2B activity	
		eIF-2.[^{3}H]GDP dissociated, pmol	
		(-)I-2	(+)I-2
I. +Hemin	----	1.22	1.25
-Hemin	----	0.47	0.51
-Hemin	+hemin	0.99	0.67
II. +Hemin	----	1.94	*
-Hemin	----	0.60	0.67
-Hemin	+hemin	1.65	1.22

Protein synthesizing lysates (30 μl) were incubated at 30°C for 12 min with or without hemin (20 μl) as described under Materials and Methods. At 5 min, I-2 was added to one set of reaction mixtures at a final concentration of 0.45 μM. At 7 min, heme-deficient lysates were supplemented with hemin (20 μM) and the eIF-2B activity was immediately assayed by the addition of 3.58 (Expt. I) or 3.7 (Expt. II) pmol of eIF-2.[^{3}H]GDP (in 20 μl). The dissociation assay was carried out for 15 min at 30°C as descibed in Materials and Methods. The results of two independent experiments from two different lysate preparations are shown.

* could not be assayed due to lack of I-2.

Results reported by Thomas et al. (1984) indicate that dephosphorylation of eIF-2(αP) in eIF-2(aP).eIF-2B complex *in vitro* by alkaline phosphatase can lead to the restoration of eIF-2B activity. The restoration of eIF-2B activity in fully inhibited lysates can be achieved by the addition of hemin which inhibits HRI activity and permits an endogenous protein phosphatase to dephosphorylate the eIF-2(aP) (Matts *et al,* 1986). We provide here further evidence that this endogenous phosphatase, which is required to dephosphorylate eIF-2(aP) and restore eIF-2B activity, is sensitive to inhibitor-2 and higher concentrations of okadaic acid.

The extent of eIF-2α phosphorylation defines the extent of inhibition in eIF-2B activity. In the equilibrium between phosphorylation of eIF-2a and dephosphorylation of eIF-2(aP), a marked shift to dephosphorylation not only requires the phosphatase activity but also the inhibition of eIF-2α kinase activity. This point is further substantiated here by showing that the recovery of eIF-2B activity by the delayed addition of hemin is dependent a) on the concentration of added hemin (Table 4), b) the time at which eIF-2B activity is studied following the addition of hemin (Fig. 7), and c) the time when hemin is supplemented to heme-deficient lysates (Table 6). It has to be emphasized here that addition of hemin promotes the inactivation of HRI, so that endogenous phosphatase can dephosphorylate eIF-2(aP), and facilitates the restoration of eIF-2B activity. The release of GDP under those conditions is not due to a nonspecific dissociation of added hemin on the eIF-2.[^{3}H]GDP binary complex. This is because the dissociation of eIF-2.[^{3}H]GDP in not uniform in heme-deficient lysates treated with the delayed addition of hemin. Lysates which are incubated for longer period without hemin cannot restore eIF-2B activity as efficiently as those lysates which are incubated for shorter intervals before the addition of hemin (Table 5). Also, the activation of double-stranded RNA-dependent eIF-2a kinase that occurs in response to the addition of dsRNA in hemin-supplemented lysates inhibits the eIF-2B activity due to increased eIF-2a phosphorylation (Matts and London, 1984).

The measurement of eIF-2B activity in whole cell extracts was initially developed by Matts and London (1980) to study the correlation between eIF-2B activity and protein synthesis in reticulocyte lysates which were exposed to several conditions that enhance endogenous eEF-2a phosphorylation. This assay system was subsequently used by others to correlate the inhibition of protein synthesis with reduction in eIF-2B activity in cells under different physiological stress (Rowlands *et al,* 1988; Kimball and Jefferson, 1990; Prostko *et al,* 1992). More recently this assay system was used to measure the rapid activation of eIF-2B in insulin and growth factor treated Swiss 3T3 fibroblasts (Welsh and Proud, 1992) and the inactivation of eIF-2B in insect cells which are expressing mammalian recombinant eIF-2α kinase (Chefalo *et al,* 1994), and it was also used in evaluating the overexpression of wild-type and mutant eIF-2a subunits in rescuing the inhibition of eIF-2B activity in Chinese hamster ovary cells that is mediated by eIF-2a phosphorylation (Ramaiah *et al,* 1994).

Here, the restoration of eIF-2B activity is used as a parameter to characterize the physiological phosphatase that dephosphorylates eIF-2(aP) in inhibited heme-deficient lysates which are supplemented with the delayed addition of hemin and phosphatase inhibitors like okadaic acid and inhibitor 2. To demonstrate that eIF-2B activity is specifically diminished due to eIF-2α phosphorylation in heme-deficient lysates and is not related to the total protein synthesis activity, it has been shown here that inhibitors of protein synthesis, namely pactamycin, puromycin, and cycloheximide, which do not affect eIF-2a phosphorylation, do not affect eIF-2B activity (Table 6). The recovery of eIF-2B activity promoted by the delayed addition of hemin is maintained although protein synthesis is inhibited in these lysates.

Inhibitor-2 and okadaic acid do not affect the functional eIF-2B activity. Okadaic acid inhibits type 2A and type 1 phosphatases in a concentration-dependent manner. Somewhat higher concentrations of okadaic acid are required to inhibit type 1 phosphatase than type 2A phosphatases (Cohen *et al,* 1990). Okadaic acid at 25-50 nM, which causes accumulation of polyribosomes and inhibition of protein synthesis (Figs. 8 & 10), does not

affect eIF-2 phosphorylation (Fig 9) or eIF-2B activity (Table 7) but, however, is shown to enhance EF-2 phosphorylation (Redpath and Proud, 1989). Consistent with these findings, we find here that relatively higher concentrations of okadaic acid are required to inhibit the restoration of eIF-2B activity and dephosphorylation of eIF-2(aP) in inhibited lysates treated with the delayed addition of hemin (Table 7 and Fig.9). Also I-2, a specific inhibitor of protein phosphatase 1, inhibits the restoration of eIF-2B activity in inhibited lysates (Table 8). These findings suggest that a type 1 phosphatase plays a dominant role in the dephosphorylation of eIF-2(aP) and restoration of eIF-2B activity in translating reticulocyte lysates. In addition, these observations are also consistent with the findings of Wek et al. (1992) who have demonstrated that a type 1 phosphatase is involved in the modulation of the extent of eIF-2a phosphorylation in yeast. In contrast, the findings of some recent *in vitro* studies indicate that both protein phosphatases, 1 and 2A, can dephosphorylate eIF-2(αP) significantly (Redpath and Proud, 1990). However, these authors have pointed out that this need not be the case in translating lysates since phosphorylated eIF-2a can interact with eIF-2B, Met-tRNAi, ribosomes, and several other components of translational machinery which can alter the relative activities of the phosphatases against eIF-2(αP) as has been previously suggested (Crouch and Safer, 1984).

A further analysis of results indicates that eIF-2B activity is not completely inhibited in heme-deficient lysates (Table 4 to 8). Addition of higher concentrations of okadaic acid further enhances the phosphorylation of eIF-2α (Fig. 9) and sequesters all the available eIF-2B activity (Table 6) in heme-deficient lysates. This is possible because of the following events. While measuring eIF-2B activity, large quantity of unphosphorylated binary complex is used which may be in dynamic equilibrium with [eIF-2(αP).eIF-2B] complex as proposed by Rowlands et al. (1988a);this might lead to the release of phosphorylated eIF-2a and functional eIF-2B activity depending on the eIF-2α kinase and phosphatase activities under those conditions. In inhibited heme-deficient lysates eEF-2(aP) is accumulated on 60S subunits of 80S initiation complexes (Gross *et*

al, 1985; Thomas *et al,* 1984, Ramaiah *et al,* 1992) In the presence of kinase inhibitor like hemin, the eIF-2(aP) is presumably readily dephosphorylated by a phosphatase that is bound to ribosomes and is resistant to lower concentrations of okadaic acid Phosphorylated eIF-2a accumulates, however, in okadaic acid treated heme-deficient lysates because the heme-regulated eIF-2α kinase activity is not inhibited and eIF-2α phosphatase activity is diminished In addition, okadaic acid maintains polysomes due to a block in elongation as has been previously suggested (Redpath and Proud, 1989) and has been shown also here (Fig. 10). This can lead to enhanced eIF-2α phosphorylation since the eIF-2 bound to 60S subunits of 80S initiation complexes has been reported to be readily phosphorylated in heme-deficient lysates in which polysomes are maintained due to a block in elongation cycle (Ramaiah *et al,* 1992) Also a type 1 protein phosphatase activity is reported to be present on ribosomes (Foulkes *et al,* 1983). Together, these findings substantiate the currently available notion that phosphorylation-dephosphorylation of eIF-2a occurs on ribosomes in physiological conditions The dephosphorylation is evidently mediated by a type 1 phosphatase in physiological conditions. These findings are novel, consistent with previous studies that show only about 30% of total eIF-2α is phosphorylated in heme-deficient lysates, and indicate that the degree of phosphorylation of eIF-2α can be influenced by more than the activation of HRI, namely the inhibition in phosphatase activity and by the distribution/ localization of eEF-2, its kinase and phosphatase among free and ribosomal-bound compartments

40 CHAPTER II

DISTRIBUTION OF EUKARYOTIC INITIATION FACTOR 2 AND HEME-REGULATED eIF-2a KINASE IN RIBOSOME AND NON-RIBOSOMAL FRACTIONS OF TRANSLATING RABBIT RETICULOCYTE LYSATES

Recent studies by Ramaiah et al. (1992) suggest that eIF-2 is carried to the polysomes and this polysomal eIF-2α is readily phosphorylated by HRI. Moreover total eIF-2(aP) levels are higher in cycloheximide treated heme-deficient lysates than in heme-deficient lysates. Since elongation is blocked and polysomes are maintained, these authors are led to the conclusion that polysomal eIF-2α is a target of HRI under physiological conditions. Since the presence of eIF-2 on the 60S subunits of polysomes is incompatible with the conventional models in which eIF-2 is shown to be recycled during the joining of the 48S preinitiation complex and the 60S subunits to form the 80S initiation complex, these authors have presented a modified model with emphasis on the translocation of eIF-2 from the 40S ribosomal subunit of 48S preinitiation complex to the 60S subunit of 80S initiation complex. A similar model is also shown by Altman and Trachsel (1993). The correlation between polysomal integrity and enhanced eIF-2a phosphorylation is further emphasized by demonstrating that a similar increase in eIF-2a phosphorylation does not occur with other inhibitors of protein synthesis namely pactamycin and puromycin. Although cycloheximide does not appear to affect the eIF-2a kinase/phosphatase activities, these authors however have not shown the presence of HRI on ribosomes or the mechanism by which eIF-2a phosphorylation is enhanced in inhibited lysates in which polysomes are maintained. Moreover HRI was purified from post-ribosomal supernatant than from ribosomes by earlier workers (Trachsel et al., 1978; Chen et al., 1989). Hence here we tried to determine if HRI is associated with ribosomes in translating lysates and to further understand the mechanism of enhanced eIF-2a phosphorylation in cycloheximide-treated heme-deficient lysates.

4.1. Small but significant amount of HRI is associated with ribosomal fractions in translating lysates:

Ribosomal fractions were separated from non-ribosomal fractions using sephacryl-300 column (Fig. 11a). The S-300 column chromatography is found advantageous because the total ribosomes of the lysate are eluted out in 2-3 fractions soon after the void volume (Fig. 11 a, Fractions 15-18). The ribosomal fractions can be neatly separated out

without shearing force. In contrast, such a neat separation of non-ribosomal fraction is difficult to be achieved by centrifuging the samples on 10-50% gradients. Moreover, the shearing force that develops during centrifugation may release ribosome bound proteins. However, one difficulty with the S-300 column is that it cannot resolve the large size class ribosomes from monosomes and subunits. Other gel-filtration columns (like Sepharose 6B), are found to resolve the large size class ribosomes from the smaller ones, but the samples are found quite diluted. Hence S-300 fractionation was carried out here, in order to determine the distribution of HRI and eIF-2 all along the column fractions with the help of respective monoclonal antibodies.

Analysis of the western blot (Fig. 11b) indicates that most of the HRI is associated with non-ribosomal fraction (fractions 19-24 corresponding to lanes 5-10). However, a small but significant level of HRI is found associated with ribosomes (fractions 15 to 18 corresponding to lanes 1-4). In contrast, eIF-2 is found equally abundant both on ribosome and in non-ribosome fractions The latter finding is consistent with the earlier observation (Ramaiah *et al,* 1992; Thomas *et al,* 1984).

4.2. Polyribosomes carry higher HRI levels than dissociated ribosomes:

Further studies have been carried out here to determine the level of ribosome bound HRI in translating lysates in which polysomes are maintained due to active initiation or a block in elongation and is compared to the ribosome bound HRI level in inhibited lysates in which polysomes are disaggregated. This study is taken up to find a relation between ribosome bound HRI and increased eIF-2a phosphorylation. Since a previous study (Ramaiah *et al,* 1992) has shown that eIF-2α phosphorylation is enhanced in heme-deficient lysates which are treated with cycloheximide, here we want to investigate whether it is related to increased level of ribosome bound HRI or to any other reasons (like activation of the kinase due to associated ribosomal proteins).

Protein synthesis was carried out typically in 250 μl reaction mixtures under three different conditions, viz. a) In hemin-supplemented lysates in which polysomes are

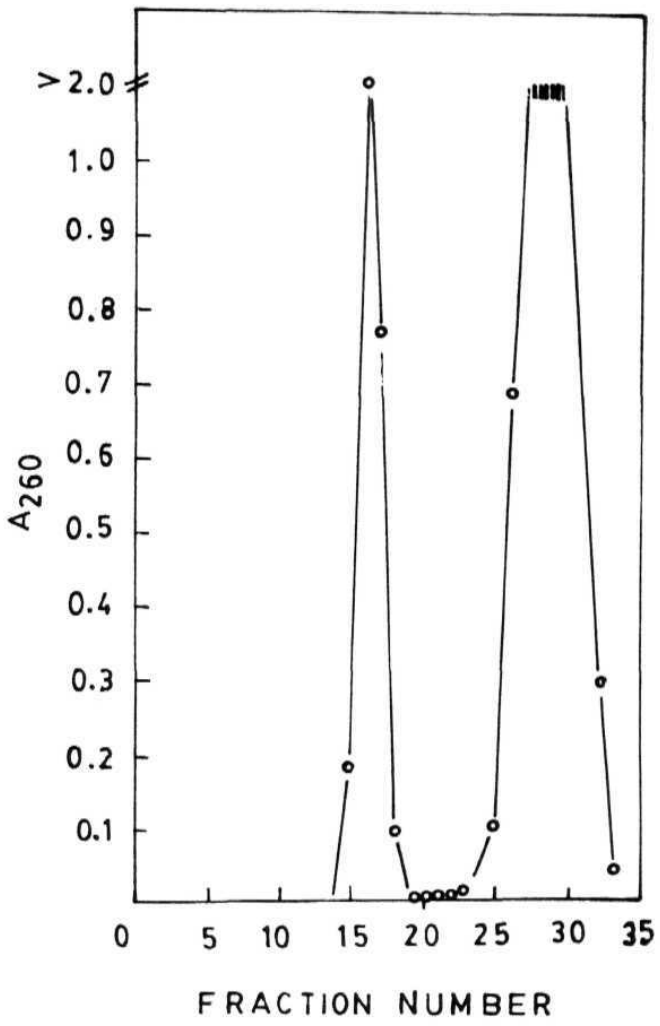
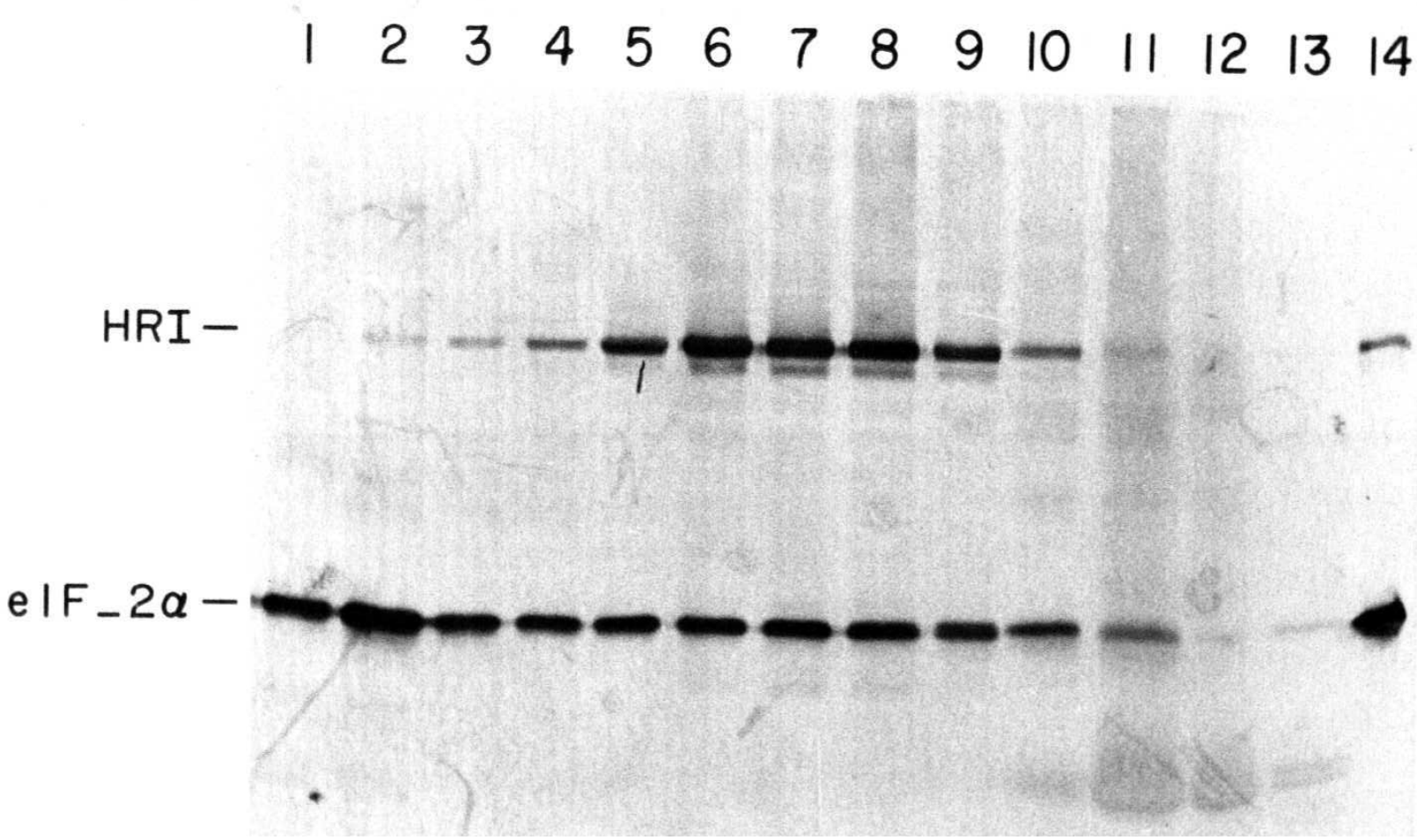


Fig. 11a. Separation of ribosomes and non-ribosomal fractions of heme-supplemented protein synthesizing lysates:

Protein synthesis was carried out typically in 250 µl reaction mixtures containing 60% lysate and 20 µM hemin at 30°C for 15 min. Afterwards, the reaction mixtures were supplemented with equal volume of TKM buffer containing 20 mM Tris-HCl (pH 7.8), 2 mM Mg^{2+}, 80 mM KC1, 1 mM DTT and 10% glycerol. The lysate was loaded on S-300 column which was equilibrated with TKM buffer. 0.8 ml fractions (5.5 min) were collected and the O.D of each fraction was checked at 260 and 280nm and buffer was used as a blank. The figure is an elution profile of S-300 column.

Fig. 11b. Distribution of eIF-2 and HRI on ribosome and non-ribosomal fractions of the translating lysates:

The fractions (0.5 ml) obtained from the above S-300 column were pH 5.0 precipitated and the samples were resuspended in SDS-PAGE sample buffer Samples were briefly kept for two minutes in boiling water bath and were separated on 10% SDS-PAGE. Gels were kept in transfer buffer and then transferred to nitrocellulose membrane. The nitrocellulose membrane with transferred proteins was immunoblotted with eIF-2 and HRI monoclonal antibodies as described in Materials and Methods. Lanes 1-4 represent ribosomal fractions and lanes 5-13 represent non-ribosomal fractions. Lane 14 is a marker lane containing purified HRI (~25 ng) and eIF-2 (~100 ng). The figure is an immunoblot..


1 2 3 4 5 6 7 8 9 10 11 12 13 14
HRI
eIF_2α

maintained due to active initiation, b) heme-deficient lysates treated with cycloheximide in which polysomes are maintained due to a block in elongation, and c) heme-deficient lysates in which polysomes are disaggregated due to an impairment in the initiation step of protein synthesis.

Since these assays are carried out with cell-free translational systems *in vitro,* the above conditions do not affect the total quantity of ribosomes. However, the ribosomal configuration can be different. Polysomes are maintained or dissociated depending on the conditions used. Soon after the protein synthesis (15 min at 30°C) the reaction mixtures were supplemented with 250 µl column buffer (20 mM Tris-HCl, pH 7.8; 1 mM $Mg(OAc)_2$ and 80 mM KC1). Afterwards, the reaction mixtures were fractionated by passing through the same S-300 column one after another under identical conditions. The fractions were pH 5.0 precipitated and separated on 10% SDS-PAGE. The proteins were transferred to nitrocellulose membrane and the levels of HRI and eIF-2 were detected in all the fractions with the help of respective monoclonal antibodies by western blot analysis.

The amount of HRI associated with ribosomes in hemin-treated lysates and in cycloheximide treated heme-deficient lysates (Fig. 12, lanes 3, 4 and 5 vs lanes 7, 8 and 9) is comparable and is relatively higher compared to the level of ribosome bound HRI of the heme-deficient lysates (Fig. 12, lanes 11, 12 and 13). In contrast, the eIF-2 levels are not significantly different among these fractions but they are found to be considerably higher than HRI levels. These findings correlate well with the enhanced eIF-2a phosphorylation observed in heme-deficient lysates treated with cycloheximide than in heme-deficient lysates (Fig. 13). Although the ribosome bound HRI is significantly higher in hemin-supplemented lysates than in heme-deficient lysates, the HRI of hemin-supplemented lysates is inactive (Chen *et al,* 1989) and cannot phosphorylate eIF-2a efficiently. Hence eIF-2 alpha phosphorylation does not occur significantly in hemin-supplemented lysates.

Fig. 12. Western blot analysis of HRI and eIF-2a distribution in the ribosome fractions of hem in-supplemented, heme-deficient and cycloheximide treated, and heme-deficient lysates: Protein synthesizing reticulocyte lysates (250 µl) were incubated for 20 min. at 30°C under three different conditions. a) in the absence of hemin, b) in the presence of hemin and c) in the absence of hemin and presence of CH (20 µg/ml). Reactions were terminated by the addition of equal volume of TKM buffer and stored at -80°C. Each reaction was passed through the S-300 column which was equilibrated with TKM buffer as described in the previous figure legend. 0.8 ml ribosome fractions (corresponding to the first peak of Fig. 11a) were collected and of which 0.5 ml fractions were pH 5.0 precipitated. The pH 5.0 samples were resuspended in protein dissociation buffer and were briefly heated for 2 min before separating them on 10% SDS-PAGE. The proteins in the gel were transferred to nitrocellulose membrane. The eIF-2 and HRI proteins in the fractions were immunoblotted with respective monoclonal antibodies. Lane 1 contains purified eIF-2 and HRI which serve as markers. Lanes 3, 4 and 5 represent ribosomal fractions of hemin-supplemented reticulocyte lysates; lanes 7, 8 and 9 were represent the ribosomal fractions of cycloheximide treated heme-deficient lysates ; lanes 11, 12 and 13 represent the ribosomal fractions of heme-deficient lysates.

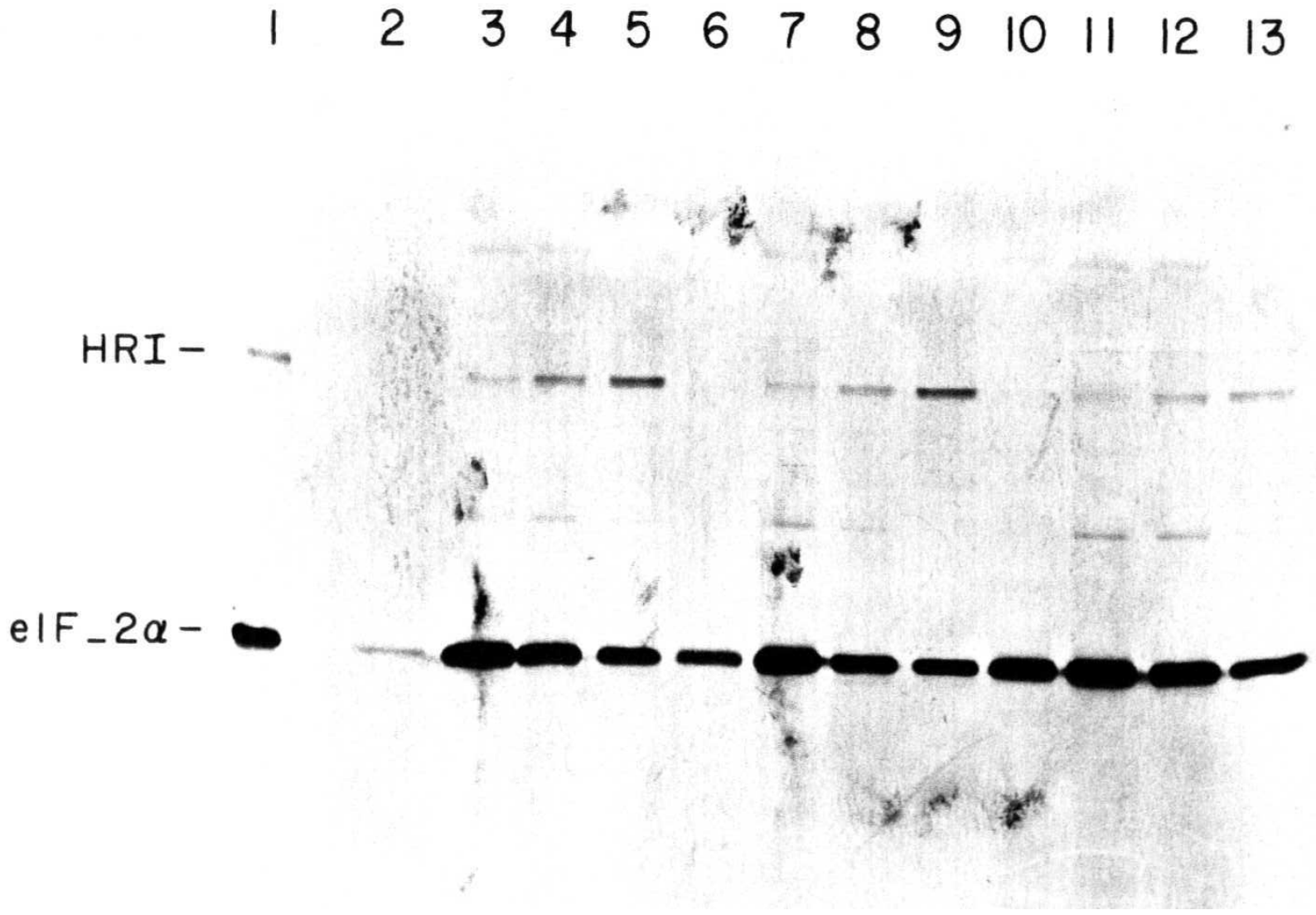
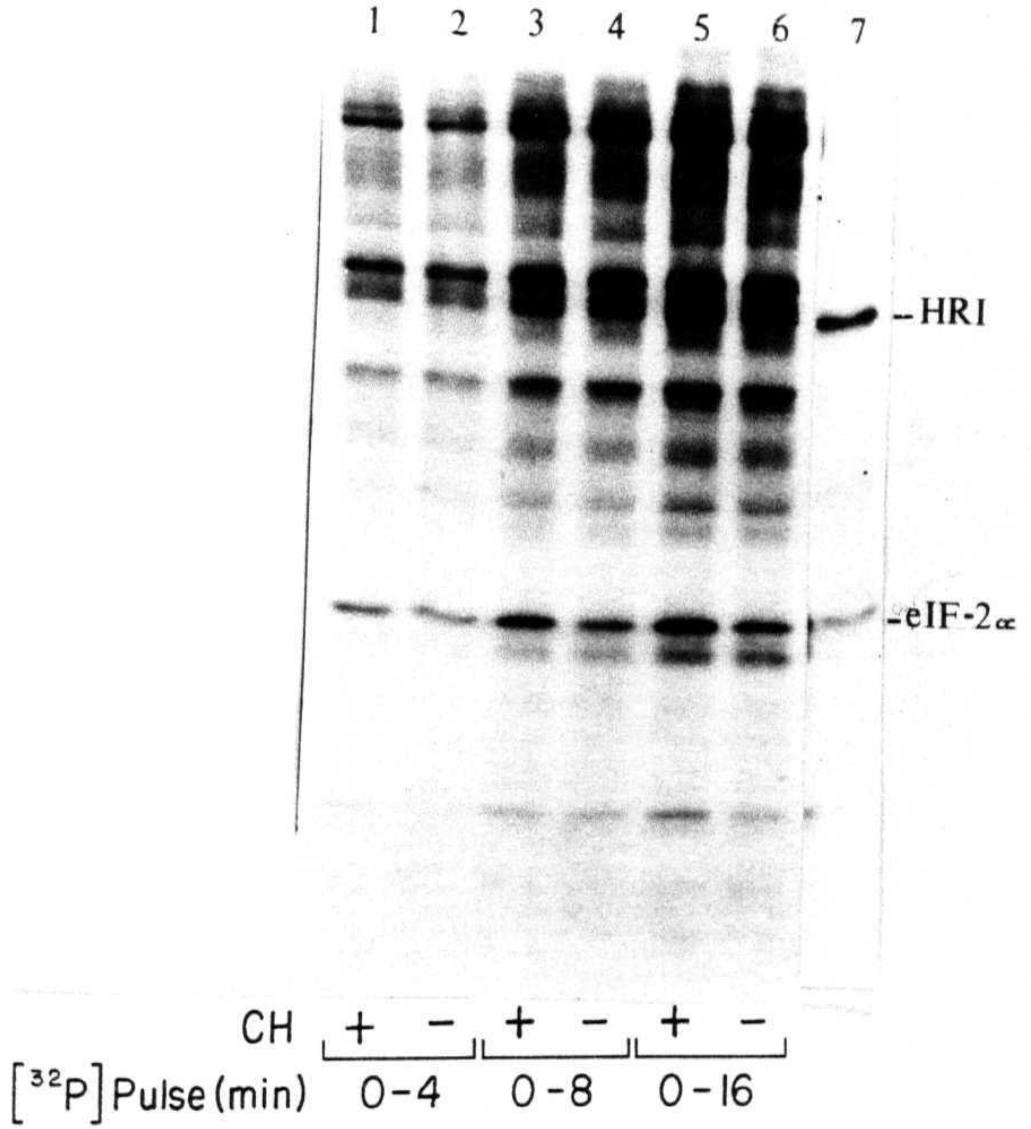

1 2 3 4 5 6 7 8 9 10 11 12 13
HRI –
eIF_2α –

Fig. 13. ^{32}P labelled phosphoprotein profiles of heme-deficient reticulocyte lysates in the presence and absence of cycloheximide: Protein synthesis reaction mixtures (25 μl) were supplemented where indicated with cycloheximide (20 μg/ml) and incubated in the absence of hemin. All incubations contained 20 μCi of [^{32}P]orthophosphoric acid. The reactions were pulsed at different time points as indicated in the figure. The reactions were concentrated by pH 5.0 precipitation and separated by electrophoresis in 10% polyacrylamide/0.1% SDS as described in Materials and Methods. The figure is an autoradiogram.


1 2 3 4 5 6 7
HRI
eIF-2α
CH + − + − + −
[32P] Pulse (min) 0-4 0-8 0-16

4.3. HRI autophosphorylation correlates with eIF-2α phosphorylation:

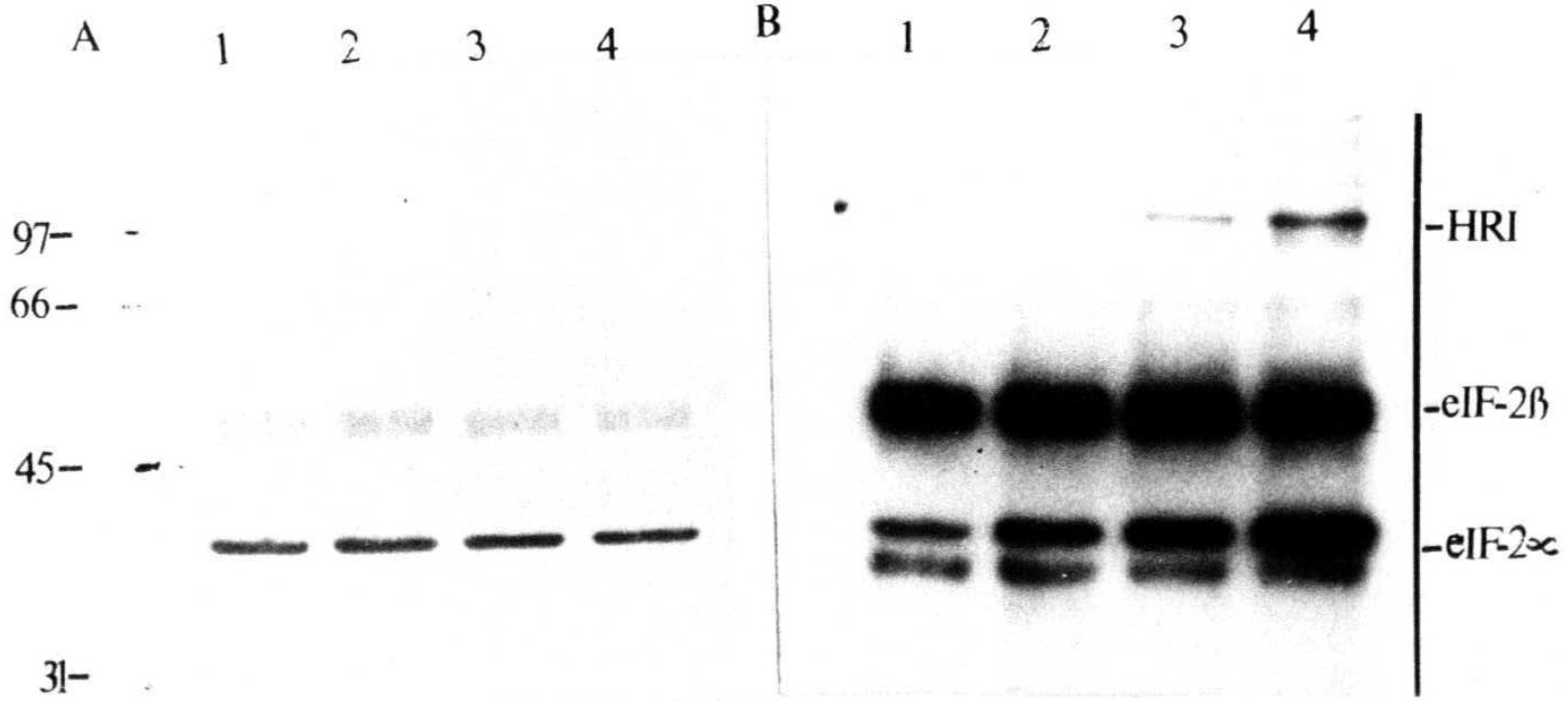
Ribosome bound HRI appears to be 10-20% of the non-ribosomal HRI. Maintenance of polysomes increases the level of ribosome bound HRI by a small proportion. Hence we want to determine if the small increase in HRI can correlate to the increased eIF-2a phosphorylation. The phosphorylation of eIF-2α *in vitro* increased with a small increase in the HRI concentration (Fig. 14, lanes 1, 2, 3 & 4). Hence these findings suggest that the enhanced eIF-2a phosphorylation observed in cycloheximide-treated heme-deficient lysates is due to increased HRI concentration rather than due to a change in HRI activity.

4.4. Significance of ribosome bound HRI:

Consistent with the previous findings (Ramaiah *et al,* 1992), the present findings indicate that a small proportion of HRI joins or associates with ribosomes during protein synthesis. This is the first report indicating the association of HRI with ribosomes. However, it does not provide any further clues as to how it joins the ribosomes. Alternatively it may be the newly made HRI protein in lysates that is still associated with ribosomes. This suggests that the lysate contains active HRI message. Although we thought of carrying an experiment to inhibit the translation of endogenous HRI mRNA with the addition of HRI cDNA, but, we could not do so due to some practical limitations.

The above findings indicating a) the presence of eIF-2 on polysomes and b) association of HRI with ribosomes, may also serve to explain the regulation of translation by the availability of essential amino acids (Clemens *et al,* 1987). One might expect that this regulation would be exerted at the level of peptide chain elongation, where amino acids are required as precursors for amino acyl-tRNAs. However, the primary effect is found to be on initiation. A diminished rate of chain elongation that results from diminished tRNA synthetase activity is found associated with increased eIF-2a phosho-rylation but, with no change in eIF-2a kinase/phosphatase activities. Furthermore, in cells containing a temperature sensitive amino acyl-tRNA synthetase (which can be regarded as

Fig. 14. Effect of increasing concentrations of HRI on eIF-2a phosphorylation: eIF-2 (~400 ng)was phosphorylated by incubating increasing concentrations of HRI and [γ-^{32}P]ATP for 5 min. at 30°C as described in Materials and Methods. The samples were separated on 10% SDS-PAGE and transfered to nitrocellulose membrane. eIF-2 was western blotted by monoclonal antibodies (Fig. 14a). Figure 14b represents an autoradiogram of the western blot. Each lane contains 0.4 μg of eIF-2 and lanes 1, 2, 3, 4 contains 10, 20, 40, 80 ng of HRI respectively.


A
1
2
3
4
B
1
2
3
4
97–
66–
45–
31–
-HRI
-eIF-2ß
-eIF-2∝

a model for absence of essential amino acids), translation is again inhibited at the level of peptide chain initiation. At non permissive temperature, cells containing the temperature sensitive leucyl-tRNA synthetase (tsH1 cells) also exhibit increased phosphorylation of eIF-2a and inhibition in eIF-2B activity (Austin *et al,* 1986; Clemens *et al,* 1987 & 1989, Polard *et al,* 1989). Based on these findings Clemens (1990) suggested that a link with amino acylation of tRNA may involve a cascade of protein phosphorylation events initiated by amino acyl-tRNA synthetase itself and ultimately leading, directly or indirectly, to increased eIF-2α phosphorylation. This proposal or hypothesis thus implies a role for amino acyl-tRNA synthetases in activating the protein kinase. This idea appears to be true, in particular to explain the link between eIF-2 phosphorylation and amino acid availability in yeast (Wek, 1994).

When yeast cells are starving for one or several amino acids, increased transcription of several unlinked genes encoding enzymes involved in different biosynthetic pathways occurs. Because the induced enzymes are solely involved in the synthesis of limiting amino acids, this regulatory system is referred to as general amino acid control. Yeast strains containing a defective amino acyl-tRNA synthetase exhibit high level of GCN4 expression in the presence of corresponding amino acid. The translation of GCN4 is also regulated by GCN2. Recent studies indicate that GCN2 is an eIF-2a kinase of yeast and displays significant homology to HR1 and PKR of eIF-2α kinases Interestingly, the carboxy terminal portion of GCN2 contains a region related to the sequence of histidine tRNA synthetase (Wek, 1994; Ramirez *et al,* 1992). This domain is thought to monitor amino acid availability via the levels of uncharged tRNA. Amino acid deprivation leads to enhanced eIF-2a phosphorylation in yeast. This may be due to binding of uncharged tRNA to this synthetase like domain in GCN2 which might produce a confirmation change in protein, resulting in the activation of adjacent protein kinase moiety, thus enhancing the GCN2 phosphorylation of the eIF-2 substrate.

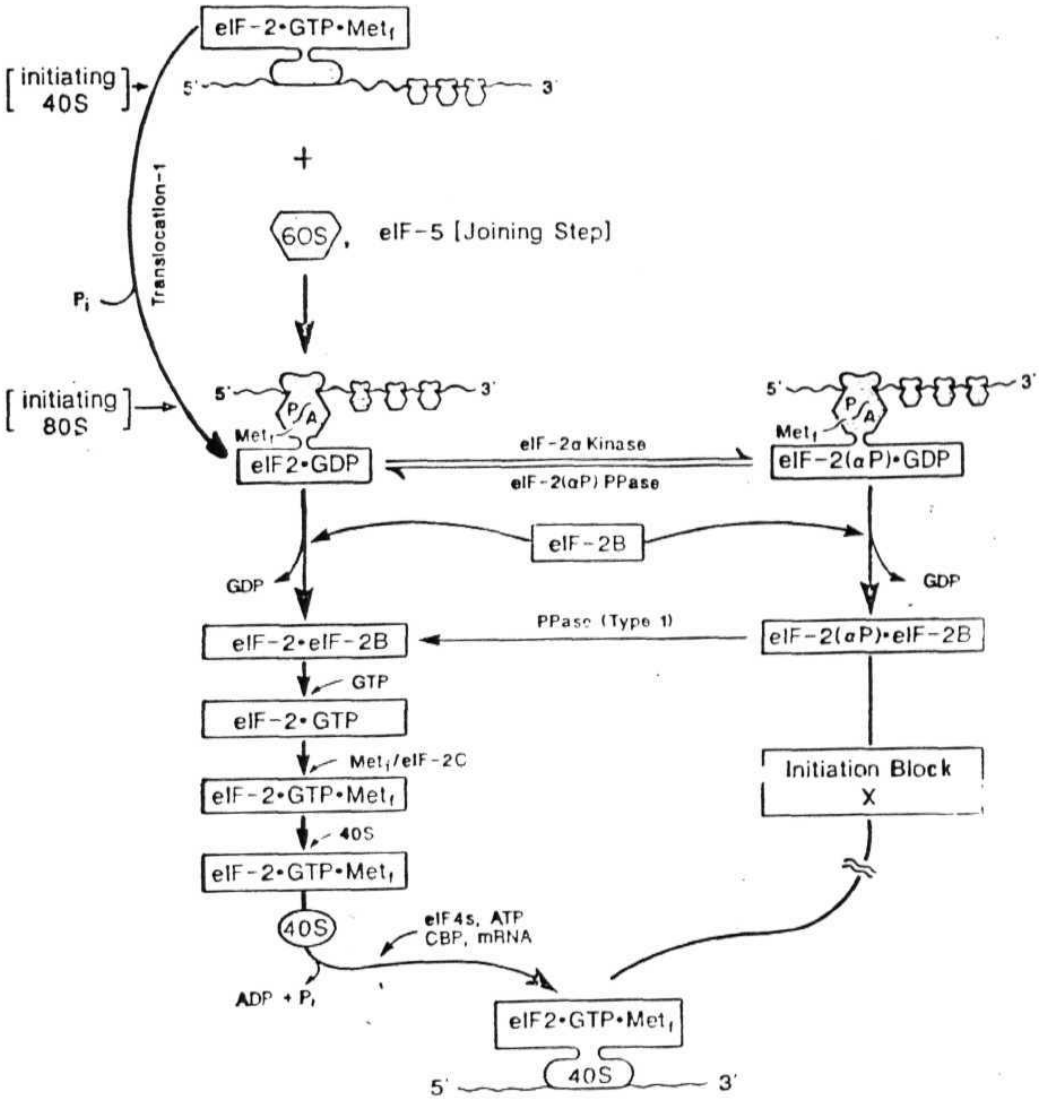
In mammalian systems, phosphorylation of eIF-2 down regulates initiation. In contrast, phosphorylation of yeast eIF-2 by GCN2 during amino acid starvation does not decrease protein synthesis globally, but enhances the translation of GCN4 mRNA.

Recently, the GCN2 protein kinase in yeast is shown to be also associated with ribosomal subunits and polysomes. This interaction requires sequences in the carboxy terminal segment of GCN2 (Ramirez *et al,* 1992). The amino terminal portion contains a sequence related to the subdomains of VIB and XI of protein kinases. The truncated segment appears to be required for GCN2 function, since an in-frame deletion of this region abolishes GCN2 stimulation of general control (reviewed by Wek, 1994).

In contrast, HRI and PKR, the well studied mammalian eIF-2α kinases, do not contain a sequence related to histidine synthetase or any other amino acyl-tRNA synthetase. It is also not clear if they contain any region in the protein kinase domain that can interact with ribosomes. However, PKR is generally found associated with ribosomes but HRI is predominantly found in the non-ribosomal fractions as shown here (Fig. 11b). Our studies further suggest enhanced eIF-2α phosphorylation that occurs when polysomes are maintained in heme-deficient lysates by the addition of cycloheximide is due to the presence of more HRI associated with ribosomes. No direct or indirect evidence is yet available to indicate that amino acyl synthetases or uncharged tRNAs or sequence in the kinase in HRI can play a role in enhancing the phosphorylation of eIF-2 in lysates, which are inhibited by an elongation block.

Some of the results presented here such as a) association of eIF-2 with ribosomes, b) association of HRI (although a small fraction) with ribosomes and c) enhanced eIF-2 phosphorylation during a block in elongation cycle of protein synthesis are consistent with previous reports (Ramaiah *et al,* 1992; Clemens *et al,* 1990; Wek *et al,* 1992). These findings also suggest that eIF-2 is released from 80S initiation complexes or from the polysomes and the phosphorylation of eIF-2α occurs on ribosomes in physiological conditions. Based on the findings of previous and present studies, the following model for

Fig.15. Proposed Model for the Recycling and Phosphorylation of eIF-2



the recycling and phosphorylation of eIF-2 has been presented. (Fig. 15). The model suggeststhat eIF-2 is translocated from the 40S subunit to 60S subunit of 80S initiation complex as has been suggested by Ramaiah et al. (1992) and is released from there depending on the availability of eIF-2B. This is because eIF-2B has been shown to release eIF-2.GDP from the 60S subunits of initiating monosomes (Thomas *et al,* 1985). Furthermore by immuno blot analysis utilizing anti-eIF-2B antibodies, eIF-2B is readily detected not only on 60S subunits and 80S monosomes but also on 40S subunits as well (Matts *et al,* 1988). These findings raise the possibility that eIF-2B activity is not only required in the GDP/GTP exchange of eIF-2 but also in the recycling of eIF-2. Besides these two points this model also suggests that phosphorylation and dephosphorylation events probably occur on ribosomes in physiological conditions.

5.0 CHAPTER III

CHARACTERIZATION OF N-ACETYLGLUCOSAMINE OLIGOMER SPECIFIC LECTIN ISOLATED FROM DATURA INNOXIA AS A PROTEIN SYNTHESIS INHIBITOR

Several conditions are known to regulate eIF-2α phosphorylation (Jackson, 1991). Recently, it has been demonstrated that eIF-2a phosphorylation is also regulated by a glycosylated 67 kDa protein (p67) (Datta *et al,* 1989; Gupta, 1993). p67 has 12-0 linked N-acetylglucosamine residues. Dr. Gupta's group have reported that p67 protects eIF-2a from eIF-2α kinase catalyzed phosphorylation. Inhibition in p67 activity is observed when the p67 containing eIF-2 preparation is incubated with wheat germ agglutinin (WGA), a lectin which has sugar specificity towards N-acetylglucosamine residues (Datta *et al,* 1989). This result suggests that sugar residues on p67 are important for its activity. All these experiments have been carried out *in vitro* with purified factors. However, the effect of WGA on the degradation of endogenous p67 in lysates is not shown.

WGA is a lectin Lectins are proteins of non-immune origin that agglutinate cells and bind specifically and reversibly to sugar molecules or carbohydrate moieties of glycoconjugates (Goldstein and Poretz, 1986). It has been known that lectins in plants may contribute to host defense against fungal, bacteria, viral and insect pathogens (Janzen *et al,* 1976; Etzler *et al,* 1986). Lectins also serve as valuable tools in biological and medical research for the separation and characterization of glycoconjugates and glycoproteins, histochemistry of cells and tissues and in the study of cell differentiation (Liener *et al,* 1983; Gabius and Gabius, 1993). Recently, interest in lectins has gone up with the discovery that a potent toxic protein chain, present in some of the lectins, inactivate ribosomes from phylogenetically distant species including animal and fungi. These are called ribosome inactivating proteins (RIPs). RIPs are of two types. Type 1 RIPs exist as a single polypeptide chain, while type II RIPs consist of an A-chain with RIP properties linked to a B-chain having lectin properties (Stirpe *et al,* 1990). The lectin in type II RIPs is generally galactose specific. The most well characterized type II RIPs are ricin, abrin, viscumin, modeccin and volkensin which are highly toxic and contain galactose specific lectins. These toxic proteins are gaining importance for their potential

uses as a toxic moiety in immunotoxins for clinical use (Pastan *et al,* 1992). All RIPs share a common property of inactivating ribosomes, hence inhibiting protein synthesis. This is due to their highly specific RNA N-glycosidase activity that cleaves the glycosidic bond of $Adenine_{4324}$ in 28S rRNA (Endo *et al,* 1987). The removal of one adenine base renders the 60S subunit of eukaryotic ribosomes unable to bind the elongation factor 2 (EF-2). Treatment of depurinated rRNA with acid aniline results in the release of a specific fragment called as aniline' or endo's' band which is a diagnostic feature of RIP's action.

Recent studies suggest i) that RIPs need not be toxic, like Ebulin 1 which has been isolated from *Sambucus ebulus L.* Leaves (Girbes *et al,* 1993), ii) the lectin chain need not be galactose specific as in the case of RIP that is purified from the bulbs of *Eranthis hyemalis*. This protein has a lectin with specificity toward N-acetylgalactosamine (Kumar *et al,* 1993) and finally iii) the RNA N-glycosidase activity of type II RIPs can cleave ribosomal RNA at multiple sites rather than at a specific site. This is true with the RIPs obtained from a plant called *Saponaria officinalis* which are called saporins. Saporins depurinate ribosomal RNA at multiple sites (Barbieri *et al,* 1992).

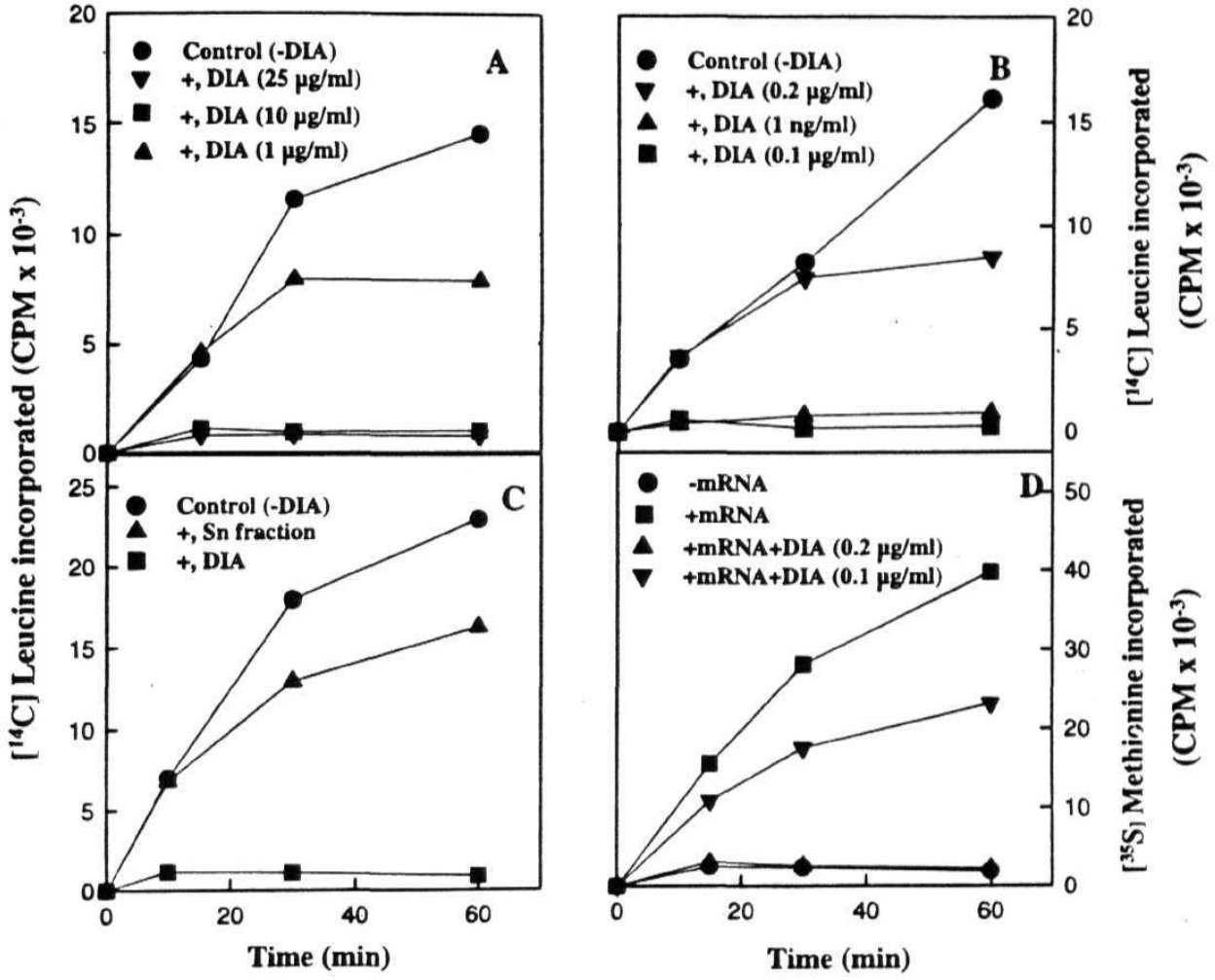
We are drawn into this area because we have purified a lectin from the seeds of *Datura innoxia* (DIA) which has a high affinity for N-acetyl glucosamine oligomers and resembles WGA in its sugar specificity. Since the lectin inhibits protein synthesis *in vitro* at very low concentrations, further investigations are carried out to determine if it can stimulate eIF-2α phosphorylation and to characterize the mechanism of inhibition in protein synthesis in the presence of the lectin. Like WGA, DIA also stimulated eIF-2α phosphorylation. However, interestingly DIA inhibits protein synthesis but WGA has no affect on protein synthesis. The inhibition of protein synthesis caused by DIA appears to be due to RNase like activity than RIP like activity. Again a similar activity is not associated with WGA. These findings are presented and discussed here.

5.1. Results and Discussion:

5.1.1. Protein synthesis inhibition: In our preliminary studies, crude agglutinin from Datura seeds was found to inhibit protein synthesis of hemin-supplemented reticulocyte lysates (Fig. 16A). Addition of another lectin purified from *Euphorbia nerifolia* did not inhibit protein synthesis in these lysates (8). Hence we have further purified DIA by chitin chromatography as described (Petrescu *et al,* 1993) and tested again for its effect on cell-free protein synthesis. In the absence of added lectin, the hemin-supplemented lysates were able to support protein synthesis linearly for a period of 60 min. Addition of purified lectin inhibited protein synthesis in a concentration dependent manner (Fig. 16B). As low as, 1 ng/ml affinity purified DIA was sufficient to cause 50% inhibition in protein synthesis. In contrast, a 100 fold higher concentration (100 ng/ml) of purified DIA was required to inhibit 50% of protein synthesis in wheat germ lysates (Fig.l6D). These kind of marked differences in the translational sensitivity between animal and plant systems may be related to the conformational and -or, structural differences that can be recognized by some lectins but not by others. To determine if the inhibition of protein synthesis in lysates treated with lectin is due to any RNase like contaminant in the solutions, we have reextracted the purified DIA by addition of chitin matrix. The supernatant, obtained after a brief centrifugation, has also been tested for its ability to inhibit protein synthesis. The supernatant devoid of DIA could not inhibit protein synthesis (Fig. 16C) suggesting that it is not due to a contaminant RNase like activity. This is consistent with the inhibition of protein synthesis by DIA in a concentration dependent manner and also that the severity of inhibition by lectin increases with its purity.

5.1.2. Purity of the lectin: The lectin purity was assayed on 7.5% SDS-PAGE in the presence and absence of reducing conditions like p-mercaptoethanol and the gel was silver stained (Fig 6). Two high molecular weight proteins (189 kDa and 122 kDa) appeared in the absence of P-mercaptoethanol. Since the addition of p-mercaptoethanol

Fig. 16. Effect of DIA on reticulocyte and wheat germ lysate protein synthesis:
Translating lysates from rabbit reticulocytes (Fig A, B and C) were treated with crude (Fig. 16A) or affinity purified DIA (Fig. 16B). In one case (Fig. 16C), protein synthesis was also determined by the addition of supernatant fraction, free of DIA (Sn.fraction) to determine the RNase activity of the solution. The supernatant fraction was obtained by centrifuging briefly (10 min at 10K) the purified lectin with chitin affinity matrix (Fig. 16 C). Since wheat germ lysates do not carry significant levels of any endogenous message, the translation of wheat germ was studied by the addition of Brome mosaic virus RNA (BMV RNA). The effect of different concentrations of purified lectin on wheat germ translation is shown in Fig. 16D. The incorporation of labelled amino acid into protein in 5 µl aliquots was measured in with time as described in Materials and Methods. The protein synthesis of the supernatant fraction (A) is compared to the original purified DIA extract (■).


A
Control (-DIA)
+, DIA (25 µg/ml)
+, DIA (10 µg/ml)
+, DIA (1 µg/ml)
B
Control (-DIA)
+, DIA (0.2 µg/ml)
+, DIA (1 ng/ml)
+, DIA (0.1 µg/ml)
C
Control (-DIA)
+, Sn fraction
+, DIA
D
-mRNA
+mRNA
+mRNA+DIA (0.2 µg/ml)
+mRNA+DIA (0.1 µg/ml)
[14C] Leucine incorporated (CPM x 10-3)
[35S] Methionine incorporated (CPM x 10-3)
Time (min)
Time (min)

generated four bands (101 kDa, 55 kDa, 39 kDa and 24 kDa) and the electrophoretic pattern of purified DIA appeared to be similar to the DIA reported by previous studies (Petrescu *et al,* 1993). It is likely that the upper band (189 kDa) observed in the absence of β-mercaptoethanol may be an aggregate of DIA and the protein subunits are held by disulfide bridges. The preparation thus appears to be quite homogenous and does not contain any other impurities. The preparation also showed agglutination activity (please see methods). However, the molecular nature of the DIA in the absence of β-mercapto-ethanol requires to be characterized further.

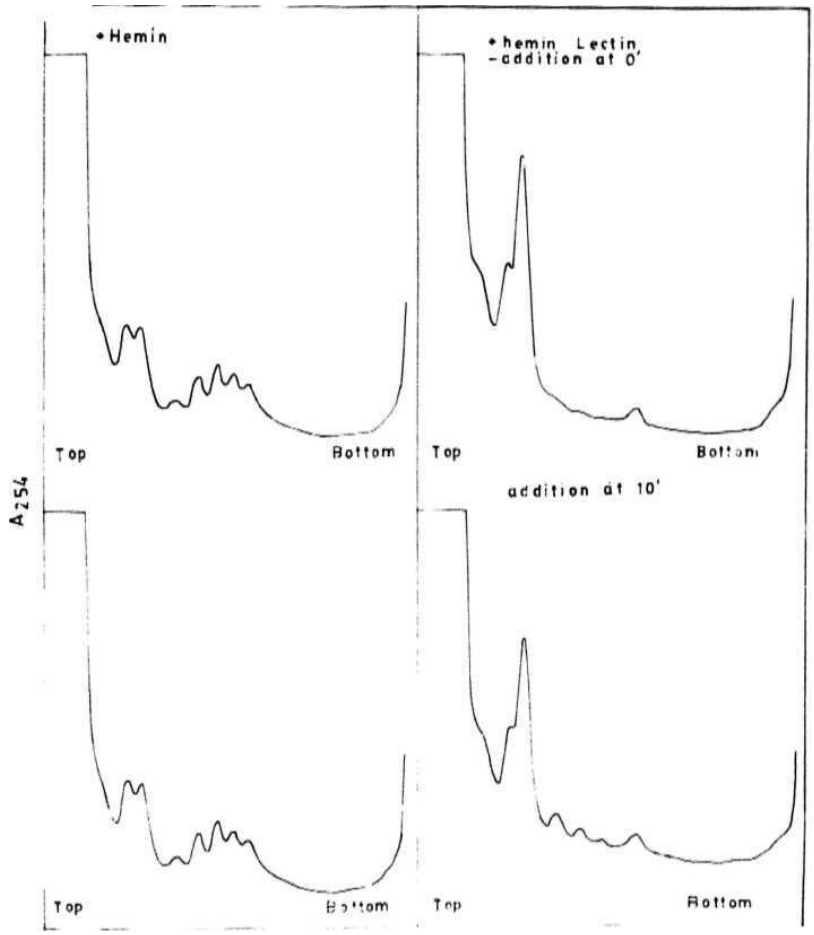
5.1.3. Effect of DIA on polysomal profile of reticulocyte lysates:

Addition of DIA 0' or at 10' to hemin-supplemented reticulocyte lysates, causes disaggregation of the polysomes suggesting that it affects primarily at initiation (Fig. 17). This is because when initiation is rapid and polysomes are formed at the beginning of protein synthesis, inclusion of DIA is found to inhibit the formation of polysomes. Also, DIA addition at 10 min of protein synthesis, to lysates that carry active polysomes, causes the disaggregation of polysomes suggesting that it is impairing initiation as well as reinitiation. However, the analysis of polysome profiles does not reveal that DIA affects some of the steps in elongation of protein synthesis.

5.1.4. Effect of DIA on Ivsate RNA: We have tested the ability of DIA to cleave the ribosomal RNA to determine if it contains any type II RIP like activity. The action of Datura lectin is compared with a known type II RIP, like abrin here. We have also made a comparison with WGA which is known to have an affinity for NAcGlc oligomers like DIA (Fig. 18). The results indicated that the action of these three lectins are different for their ability to modify lysate RNA. To identify the Endo's' fragment which is typical of RIP's action, the RNAs extracted from lysates (treated with and without the above lectins) were treated without and with aniline (Fig. 18, lane 1-5 and 6-10) as described (Girbes *et al,* 1993). In the presence of low concentrations of DIA (0.2 μg), the pattern of RNA products separated on the gel was similar to RNA obtained from control lysates both in the absence and presence of aniline treatment (lane 2 vs 1 and 7 vs 6 respectively). When

Fig. 17. Poly so me profiles of reticulocyte lysates in the presence of DIA:

Protein synthesis reaction mixtures (80 μl) containing heme-deficient lysates (-h) were supplemented, where indicated, with hemin (20 μM) or heme and DIA (0.2 μg). DIA protein was added at the beginning of protein synthesis (0 min) or at 10 min of protein synthesis and the reactions were incubated for a total period of 15 min at 30°C. Reactions were terminated by the addition of equal volume of buffer containing 20 mM Tris-HCl (pH 7.8), 1 mM $Mg(OAc)_2$ and 80 mM KC1. The ribosomes were separated on 10-50% sucrose gradients as described in Materials and Methods. The gradients were fractionated and analyzed by I SCO density gradient fractionator. The top and bottom of the gradients are shown in the figure.


+Hemin
+hemin Lectin
-addition at 0'
Top
Bottom
Top
Bottom
A254
addition at 10'
Top
Bottom
Top
Bottom

lysates were treated with higher concentrations (1 μg/ml) of DIA, several fragments of RNA appeared in the absence or presence of aniline treatment (lane 3 and 8). In contrast, the RNA products obtained from lysates treated with abrin showed a similar pattern to the control RNA in the absence of aniline treatment (lane 4 vs lane 1). However, a distinct additional Endo's' fragment was observed when this RNA was treated with aniline (lane 9). This is a typical property of RIPs. Wheat germ agglutinin, however does not behave like DIA or abrin. It does not possess any activity that can modify the lysate RNA because the RNA obtained from the lysates treated with WGA (5 μg/ml) appears to be similar both in the absence and presence of aniline (lanes 5 and 10) and these profiles resemble more like control reactions (lanes 1 and 6).

Some RIPs like saporins (obtained from seeds, leaves and roots of *Saponaria officinalis*) are shown to depurinate 28S rRNA at multiple sites and produce several bands on aniline treatment (Barbieri *et al*, 1992). Since the electrophoretic pattern of RNA obtained from DIA treated lysates is similar in the presence and absence of aniline, these findings suggest that DIA does not contain RIP like activity but is associated with RNase like activity. However this is not a contaminant activity of the solutions but appears to be an intrinsic property of purified DIA

5.1.5. Phosphorylation of eIF-2α *in vitro:*

As DIA resembles WGA in its affinity for sugar residues, the DIA has also been tested for its ability to stimulate the phosphorylation of two batches of purified reticulocyte eIF-2 *in vitro.* As shown in figure 19, the heme-regulated eIF-2α kinase was autophosphorylated typically (lane 1). The autophosphorylted kinase phosphorylated the 38 kDa subunit of reticulocyte eIF-2 preparations (lanes 2 and 4). Since these preparations are partially purified, other contaminant proteins like casein kinase II associated with the preparations can phosphorylate the (3 subunit (50 kDa) of eIF-2 and probably other proteins as well. Addition of DIA enhanced the phosphorylation of 38 kDa subunit of eIF-2 significantly but the phosphorylation of eIF-2a kinase was unaffected (lane 3 and 5). DIA alone did not affect HRI autophosphorylation (lanes 1 to 5). These findings suggest

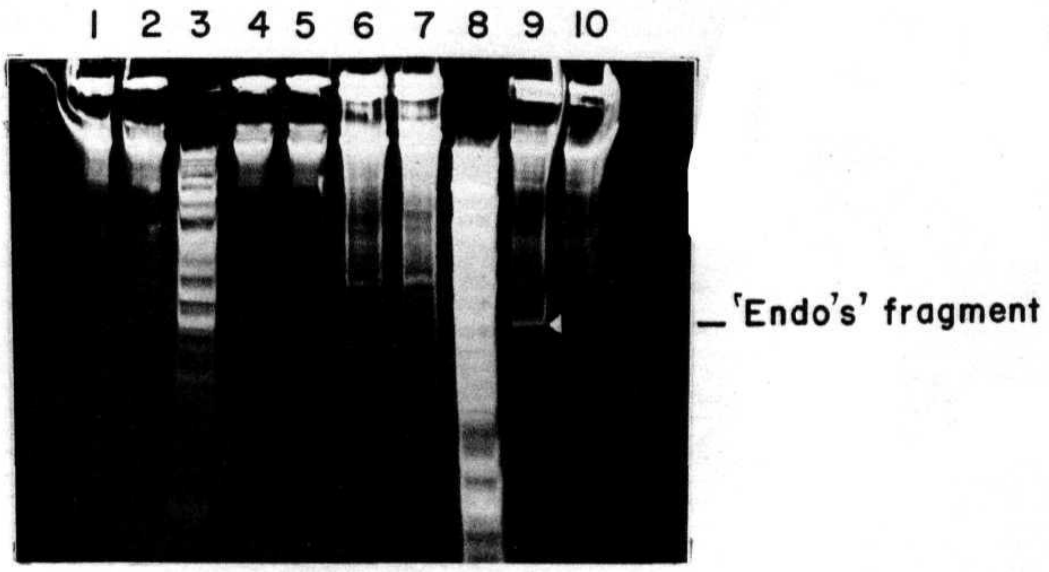


Fig. 18. Effect of DIA, WGA and Abrin on lysate RNA:

RNA was extracted from the lysates treated with and without the above agents as described in Materials and Methods. RNA extracted as above, is then treated with acid aniline (1M) and incubated in dark for about 10 minutes to determine if the aniline treatment releases the typical 'Endo's' fragment from 28S rRNA. Aniline was removed after the treatment by diethyl ether, and RNA was precipitated by ethanol. RNA samples of lanes 1-5 were treated without aniline and of lanes 6-10 were treated with aniline. Lanes 1 and 6 contain RNA from control lysate; Lanes 2 and 7, plus DIA (0.2 μg); Lanes 3 and 8, plus DIA (1 jig), Lanes 4 and 9, plus abrin (0.2 μg); Lanes 5 and 10, plus WGA (2 μg).

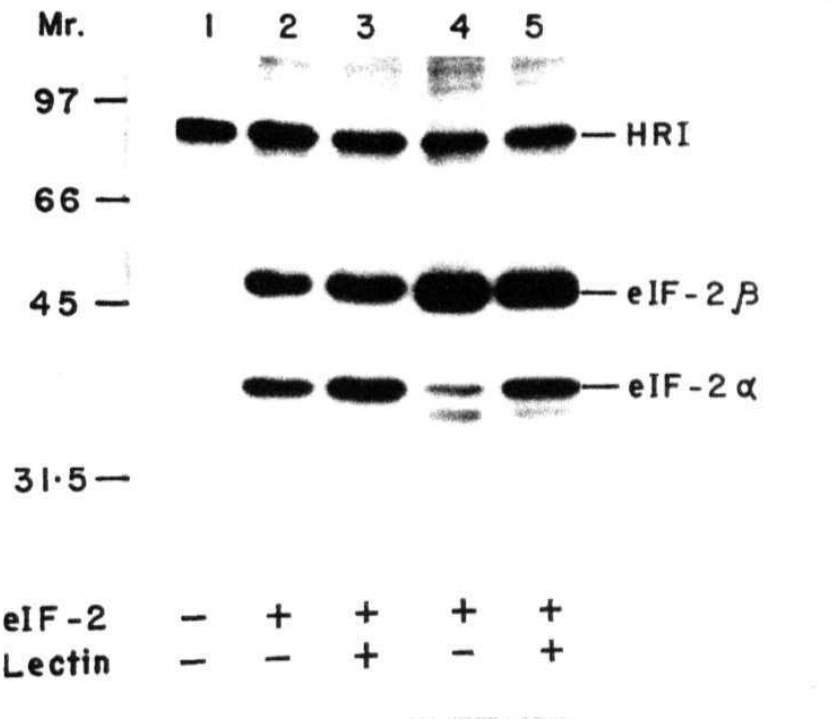


Fig. 19 Effect of **DIA** on **phosphorylation** of eIF-2a:

The eIF-2 preparation was incubated with 0.5 μg of DIA and 50 ng of HRI and the phosphorylation was performed in the presence of [γ-^{32}P]ATP as described under Materials and Methods. The effect of DIA on two different preparations of eIF-2 were carried out. Lane 1, HRI; lane 2, eIF-2 (prep I) + HRI; lane 3, eIF-2 (prep. I) + HRI + DIA; lane 4, eIF-2 (prep.II) + HRI; lane 5, eIF-2 (prep.II) + HRI + DIA.

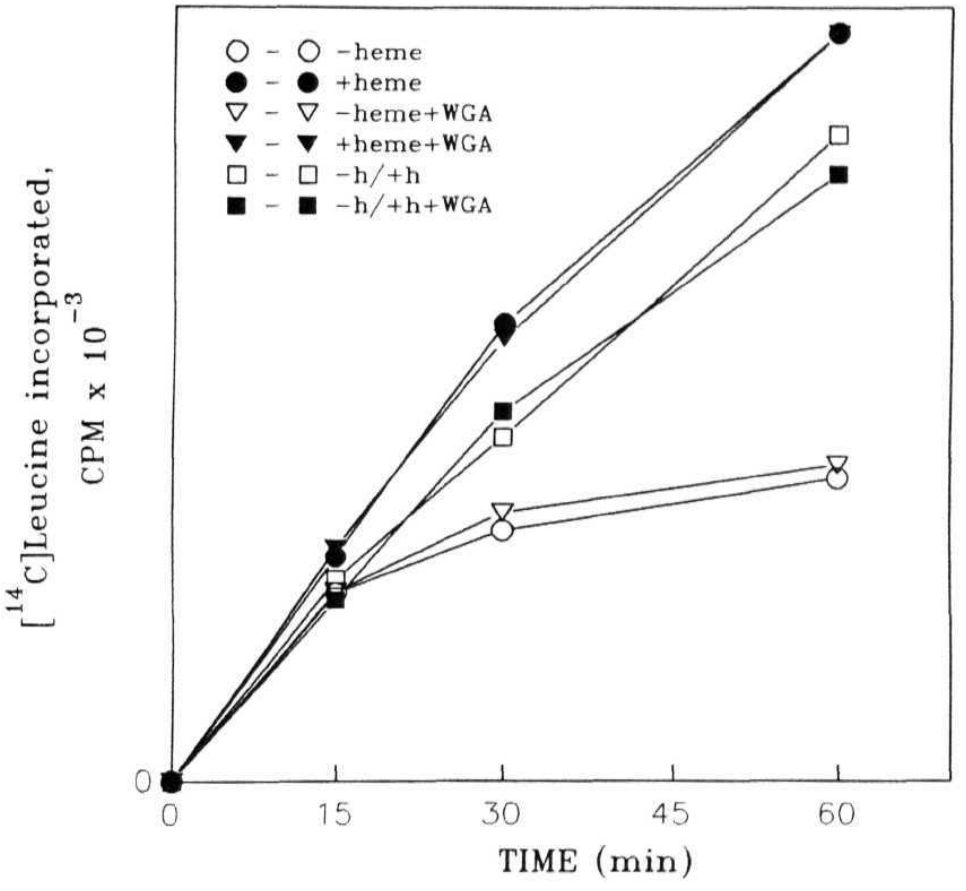


Fig. 20. Effect of WGA on reticulocyte lysate protein synthesis:

Protein synthesis reaction mixtures (25 μl) were incubated under three conditions: i)-hemin ii) +hemin iii)-hemin/+hemin at 7 min with and without WGA (1 μg). The incorporation of labeled amino acid into protein in 5 μl aliquots was measured with time as described in Materials and Methods.

that probably the p67 like contaminant protein associated with partially purified eIF-2 is modified upon the addition of DIA and loses its ability to protect eIF-2 from being phosphorylated as has been suggested (Datta *et al.*, 1989)

The inhibition in protein synthesis by DIA appears to be due to its RNase like activity than its ability to increase eIF-2α phosphorylation *in vitro.* This is consistent with the results obtained here by WGA. The latter, WGA did not inhibit protein synthesis in hemin-supplemented (+h, 0') or in inhibited heme-deficient reticulocyte lysates treated with the delayed addition of hemin (-h/+h, 7') (Fig. 20) and also WGA preparations lacked any RNase or RIP like activity (Fig. 18, lanes 5 and 10). However, WGA like DIA is known to enhance eIF-2a phosphorylation *in vitro* (Datta *et al,* 1989). These findings also suggest that these agglutinins (WGA and DIA) can stimulate eIF-2a phosphorylation *in vitro,* however their action in lysates appear to differ. This raises also a question on the role of p67 like protein in the regulation of protein synthesis and eIF-2a phosphorylation in translating lysates.

The nuclease activity of DIA requires to be further characterized to determine its specificity and advantage (if any) over other nucleases that are currently being used in molecular biology research.

6.0 SUMMARY

Active cell-free translational systems are prepared from reticulocyte lysates which respond to added hemin. In the absence of hemin, protein synthesis is linear for a few minutes and then shuts-off. In the presence of hemin (20 μM), protein synthesis is linear upto 60 min. Delayed addition of hemin to shut-off lysates is found to restore protein synthesis.

The eukaryotic initiation factor-2 is purified from the ribosomal salt wash of reticulocyte lysate. The eIF-2 preparation is checked for its ability to form eIF-2.[^{3}H]GDP binary complex in the presence of physiological concentration of Mg^{24}. The eIF-2B activity of translating lysate is estimated from the exchange of labeled GDP in the preformed binary complex, eIF-2.[^{3}H]GDP. The heme-regulated eIF-2a kinase is purified from the post-ribosomal supernatant of reticulocyte lysates based on its ability to inhibit protein synthesis in hemin-supplemented lysates. The purified HRI is autophosphorylated and also phosphorylates the alpha subunit of eIF-2.

In heme-deficient reticulocyte lysates, the a-subunit of eukaryotic initiation factor-2 (eIF-2a) is phosphorylated due to the activation of the heme-regulated eIF-2a kinase. Previous studies have shown that 30% phosphorylation of eIF-2a impairs the guanine nucleotide exchange activity of eIF-2B and thereby inhibits or shuts-off protein synthesis. Since eIF-2B is a rate-limiting protein and is present 20-30% of total eIF-2, a small increase in eIF-2a phosphorylation is able to inhibit protein synthesis completely. Delayed addition of hemin to shut-off lysates inhibits the eIF-2α kinase activity of HRI and restores protein synthesis; under those conditions, the endogenous phosphatase of the lysate dephosphorylates phosphorylated eIF-2a and restores eIF-2B activity. In this study we present evidence that the restoration of eIF-2B activity is dependent on the concentration of added hemin and is related to HRI activity in lysates. The recovery of eIF-2B activity is not affected by protein synthesis inhibitors such as cycloheximide, pactamycin, and puromycin, which do not affect the eIF-2a phosphorylation. Also, the functional eIF-2B activity that is available in hemin-supplemented lysates is not affected by phosphatase

inhibitors such as okadaic acid and heat-stable inhibitor-2. However, the recovery of eIF-2B activity that is observed by the delayed addition of hemin to inhibited heme-deficient lysates is reduced by inhibitor-2 and high concentrations of okadaic acid. These findings suggest that a type 1 phosphatase is involved in the recovery of eIF-2B activity and protein synthesis upon delayed addition of hemin to heme-deficient lysates.

Earlier, Ramaiah et al. (1992) have shown that eIF-2α phosphorylation occurs more readily on the 60S subunits of 80S initiation complexes. These findings suggest that HRI and eIF-2 should be present on the ribosomes. The presence of eIF-2 is shown previously on ribosomes but no reports are available so far to indicate that HRI is also present on ribosomes. HRI has been purified mostly from non-ribosomal fraction. So it is not clear as to how the phosphorylation of eIF-2a is enhanced in heme-deficient lysates in which polysomes are maintained. Hence we have studied the distribution of eIF-2 and HRI in ribosome and non-ribosomal fractions of protein synthesizing rabbit reticulocyte lysates with the help of eIF-2a and HRI monoclonal antibodies Although eIF-2a is present equally well both in the ribosome and non-ribosomal fractions, the findings indicate that most of the HRI is located in the non-ribosomal fraction. A small but significant amount of HRI is also found associated with ribosomes. The level of ribosome bound HRI is enhanced in heme-supplemented and in heme-deficient cycloheximide treated reticulocyte lysates in which polysomes are maintained. This observation is consistent with the observations made by Ramaiah et al. (1992) which suggested that enhanced eIF-2a phosphorylation occurs in cycloheximide-treated heme-deficient lysates in which polysomes are maintained. Since HRI autophosphorylation is correlated with eIF-2 phosphorylation in our studies, we suggest that the enhanced eIF-2a phosphorylation occurs in cycloheximide treated heme-deficient lysates (where polysomes are maintained) and it is due to an increase in the ribosome bound kinase fraction. These findings also indicate that a fraction of HRI is somehow recruited on to the ribosomes during protein synthesis. These findings are novel, consistent with previous studies that show only about 30% of total eIF-2α is phosphorylated in heme-deficient lysates, and indicate that the

degree of phosphorylation of eIF-2α can be influenced by more than the activation of HRI, namely the inhibition in phosphatase activity and by the distribution /localization of eIF-2, its kinase and phosphatase among free and ribosomal bound compartments.

A glycoprotein with high affinity for N-acetyl glucosamine (GlcNAc) oligomers obtained from the seeds of *Datura innoxia L.* has hemagglutinating activity and inhibits cell-free translation in wheat germ and reticulocyte lysates.

It has been observed here that Datura lectin can stimulate eIF-2a phosphorylation catalyzed by heme-regulated eIF-2a kinase *in vitro.* Its effect on eIF-2α phosphorylation *in vitro* is found to be similar to wheat germ agglutinin, a lectin with affinity towards N-Acetyl glucosamine oligomers (Datta et al., 1989). These findings suggest that probably a p67 like contaminant protein in eIF-2 preparations interferes with eIF-2α phosphorylation.. The effects of these lectins in translating lysates however are different. While wheat germ agglutinin does not inhibit protein synthesis, Datura lectin is found to inhibit protein synthesis. Further, Datura lectin has been tested to determine if it contains any ribosome inactivating protein (RIP) like activity which can cut ribosomal RNA (Stirpe et al., 1990). But the findings indicate that it contains RNase like activity than RIP like activity. These findings suggest that the protein synthesis inhibition caused by Datura lectin is not mediated by eIF-2α phosphorylation but it may be due to the RNase like activity associated with the lectin. Since purified WGA does not inhibit protein synthesis recovery in inhibited heme-deficient lysates treated with the delayed addition of hemin, the role of p67 in the regulation of eIF-2α phosphorylation in physiological conditions requires to be studied more carefully.

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Type 1 Phosphatase Inhibitors Reduce the Restoration of Guanine Nucleotide Exchange Activity of Eukaryotic Initiation Factor 2B in Inhibited Reticulocyte Lysates Rescued by Hemin

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In heme-deficient reticulocyte lysates, the α-subunit of eukaryotic initiation factor-2 (eIF-2α) is phosphorylated due to the activation of the heme-regulated eIF-2α kinase (HRI). Phosphorylation of eIF-2α impairs the guanine nucleotide exchange activity of eIF-2B and thereby inhibits or shuts off protein synthesis. Delayed addition of hemin to shut-off lysates inhibits the eIF-2α kinase activity of HRI and restores protein synthesis; under those conditions, the endogenous phosphatase of the lysate dephosphorylates phosphorylated eIF-2α and restores eEF-2B activity. In this report we present evidence that the restoration of eIF-2B is dependent on the concentration of added hemin and is related to HRI activity in lysates. The recovery of eEP-2B activity is not affected by protein synthesis inhibitors such as cycloheximide, pactamycin, and puromycin, which do not affect the eIF-2α phosphorylation. Also, the functional eIF-2B activity that is available in hemin-supplemented lysates is not affected by phosphatase inhibitors such as okadaic acid and heat-stable inhibitor-2. However, the recovery of eIF-2B activity that is observed by the delayed addition of hemin to inhibited heme-deficient lysates is reduced by inhibitor-2 and high concentrations of okadaic acid. These findings suggest that a type 1 phosphatase is involved in the recovery of eIF-2B activity and protein synthesis upon delayed addition of hemin to heme-deficient lysates. c 1996 Academic Press, Inc.

The initiation of protein synthesis in heme-deficient reticulocyte lysates is inhibited as a result of the activation of heme-regulated eIF-2α[2] kinase (HRI) and the phosphorylation of the α subunit of eukaryotic initiation factor-2 (reviewed in 1-7). In normal lysates, the binary complex eIF-2 • GDP is formed by GTP hydrolysis during the joining of the 43S and 60S ribosomal subunits to form the 80S initiation complex in the final step of each initiation cycle. The recycling of eIF-2 • GDP to form the ternary complex (eIF-2 • GTP • BMet tRNAf) requires the guanine nucleotide exchange factor eIF-2B (previously called reversing factor) which catalyzes the replacement of GDP by GTP (8-13). Phosphorylation of eIF-2α leads to the inhibition in the guanine nucleotide exchange activity of eIF-2B *in vitro* (49). In heme-deficient lysates, the phosphorylation of eIF-2α gives rise to the formation of a 15S phosphorylated complex [eIF-2B · eIF-2(αP)], in which eIF-2B is tightly sequestered and unable to catalyze the guanine nucleotide exchange (14-17). Since the concentration of eIF-2B relative to that of eIF-2 in the lysate is low, phosphorylation of a portion (20-40%) of eIF-2α is sufficient to bind all of the lysate eIF-2B in this nonfunctional 15S complex (14, 15). It was shown previously (16) that alkaline phosphatase treatment of the eIF-2B • eIF-2(αP) complex from heme-deficient lysates results in the recovery of eIF-2B activity. The rescue of protein synthesis in heme-deficient lysates by the delayed addition of hemin (20 μM) or MgGTP (2 mM) is also closely correlated with the dephosphorylation of lysate eIF-2(αP) and the restoration of eIF-2B activity (17, 18). Both hemin and MgGTP exert their effects by inhibiting HRI activity, thus permitting dephosphory-

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[2] Abbreviations used: eIF-2, eukaryotic initiation factor-2; eIF-2α, α subunit (38 kDa) of eIF-2; eIF-2(αP), phosphorylated eIF-2α; eIF-2 · [^{3}H]GDP, binary complex labeled in GDP; HRI, heme-regulated eIF-2α kinase; I-2, heat-stable inhibitor-2; eIF-2B, guanine nucleotide exchange factor, DTT, dithiothreitol; EF-2, elongation factor-2.

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lation of lysate eIF-2(αP) by endogenous protein phosphatase (17). These findings indicate that the dephosphorylation of lysate eIF-2(αP) is a critical event in the rescue of protein synthesis by hemin and that both eIF-2B activity and the rate of protein synthesis are regulated by the equilibrium between eIF-2α kinase and phosphatase activities.

The physiological mechanism of dephosphorylation of eIF-2(αP) and the restoration of eIF-2B activity has not been clear. Other studies (19-26) with isolated protein phosphatases which dephosphorylate purified eIF-2(αP) *in vitro* have not demonstrated that these phosphatases can also dephosphorylate endogenous eIF-2B · eIF-2(αP) complex or restore eIF-2B activity in heme-deficient lysates. In this report, some characteristics of the dephosphorylation of eIF-2(αP) by endogenous protein phosphatase(s) in the lysate have been examined. We have measured eIF-2B activity directly in protein-synthesizing lysates and have found a correlation of changes in this activity with changes in phosphorylation and dephosphorylation of eIF-2α. The specific effect of phosphorylation of eIF-2α on eIF-2B activity is also indicated by our finding that the recovery of eIF-2B activity in inhibited lysates on addition of hemin is unaffected by the addition of inhibitors of protein synthesis (pactamycin, puromycin, or cycloheximide) whose action is not dependent on phosphorylation of eIF-2α. The endogenous protein phosphatase activity which restores eIF-2B activity in the hemin-rescued lysates displays type 1 protein phosphatase characteristics.

MATERIALS AND METHODS

Materials. [8-^{3}H]GDP (9 Ci/mmol), [γ-^{32}P]ATP (3000 Ci/mmol), [^{14}C]leucine, and [^{32}P]orthophosphate (100 mCi/ml) were obtained from Dupont-NEN and from BRIT, Bombay, India. ATP, GDP, GTP, CPK, FDP, NAD^+, and dithiothreitol were purchased from Sigma.

Preparation of eIF-2. The protein factor eIF-2 was purified from the ribosomal salt wash of reticulocyte lysates as described (27). The salt wash preparations were concentrated by ammonium Sulfate (0-80%) and dialyzed in a buffer containing Tris (20 mM, pH 7.8), KCl (100 mM), EDTA (0.2 mM), DTT (1 mM), and glycerol (10%) and loaded onto DEAE cellulose eIF-2 was eluted with 0.2 M KCl from DEAE cellulose. The ammonium sulfate-concentrated DEAE eIF-2 was further purified on a phosphocellulose column and the 0.5-0.7 M KCl eluate was concentrated and used here in these experiments.

Preparation of eIF-2 · [^{3}H]GDP and its dissociation in lysates. The binary complex eIF-2 · [^{3}H]GDP was prepared as described (9, 29). To estimate the eIF-2B activity in lysates, the preformed binary complex was added to 30 μl of translating lysates and the dissociation of labeled GDP was studied as described (29, 30). Modifications and experimental conditions are mentioned in the legends to the tables.

Protein-synthesizing rabbit reticulocyte lysates were prepared as described (28) and the protein synthesis was carried out at 30°C with or without the addition of any labeled amino acid as described (30). eIF-2B activity was measured in lysates which were incubated without the addition of any labeled amino acid.

Phosphorylation of reticulocyte lysate proteins. Phosphorylation of lysate eIF-2a was carried out at various time intervals with [^{32}P]orthophosphoric acid (~1 mCi/ml) in the presence of an energy-regenerating system consisting of 1 mM fructose-1,6-bisphosphate and 100 μM NAD^+. This glycolytic system drives protein synthesis by the generation of ATP and replaces the need for creatine phosphate and phosphocreatine kinase. Reactions were carried out at 30°C and the reaction mixtures were concentrated by pH 5.0 precipitation (31, 32). The samples were analyzed by 10% SDS-PAGE and autoradiography.

RESULTS

Restoration of eIF-2B Activity in Heme-Deficient Lysates Is Dependent on the Concentration of Added Hemin and HRI Activity

Protein synthesis in reticulocyte lysates is dependent upon the concentration of hemin, which binds to and inactivates HRI by promoting intersubunit disulfide bond formation (33-35). In heme-deficient lysates, protein synthesis is inhibited due to the activation of HRI, the phosphorylation of eIF-2α, and the sequestration of eIF-2B in a nonfunctional 15S phosphorylated complex [eIF-2B · eIF-2(αP)]. To understand the physiological phosphatase activity which is responsible for the dephosphorylation of eIF-2(αP) and restoration of eIF-2B activity, we have studied here the restoration of guanine nucleotide exchange activity of eIF-2B in inhibited heme-deficient lysates which are supplemented with the delayed addition of hemin or phosphatase inhibitors or both. The eIF-2B activity is assayed by measuring the extent of dissociation of added labeled binary complex eIF-2 · [^{3}H]GDP. As shown in Table I, eIF-2B activity in heme-deficient lysates is very low (0%), whereas eIF-2B activity is maximal in the presence of optimal concentration of hemin (20 μM). This is consistent with the earlier reports (17, 30) and correlates with the ability to carry out protein synthesis in those lysates (data not shown). While eIF-2B activity can fluctuate significantly in different preparations depending on their ability to carry out protein synthesis and respond to added hemin, the general direction of these results does not change; that is, the protein synthesis and eIF-2B activity are always higher in hemin-supplemented reticulocyte lysates than in heme-deficient lysates.

Addition of optimal concentration of hemin to inhibited heme-deficient lysates restores eIF-2B activity more efficiently than suboptimal concentrations of hemin (Table I). The restoration of eIF-2B activity in lysates which are treated with the delayed addition of hemin occurs gradually and is time-dependent (Fig. 1). Maximum recovery occurs within 15-20 min. The recovery of eIF-2B activity is, however, inhibited significantly if the lysates are incubated for a longer duration of time without hemin and is correlated to the restoration of protein synthesis (Table II). These findings suggest that the recovery of eIF-2B activity is dependent on the concentration of added hemin and the time at

TABU: I

Effect of Hemin Concentration on eIF-2B Activity in Reticulocyte Lysates

Protein synthesis conditions	eIF-2B activity	
	eIR-2·[^{3}H]GDP dissociated (pmol)	Activity (%)
Expt. I		
−Hemin	0.52	0
+Hemin (5 μM)	0.69	13
+Hemin (10 μM)	1.43	70
+Hemin (20 μM)	1.82	100
Expt. II		
−Hemin	0.61	0
+Hemin (5 μM)	0.64	5
+Hemin (10 μM)	1.12	100
+Hemin (20 μM)	1.12	100
−Hemin/+Hemin 7 min (10 μM)	0.87	45
−Hemin/+Hemin 7 min (20 μM)	1.03	72

Note. Protein-synthesizing h/sates (30 μl) containing 5, 10, or 20 μM hemin were incubated at 30°C for 12 min. In one experiment (II), heme-deficient lysates were supplemented with 10 or 20 μM hemin at 7 min and incubation was continued for 5 min. At 12 min of protein synthesis, 2.6 or 2.48 pmol (in 20 μl) of eIF-2·[^{3}H]GDP was added to lysates in Expt. I and II, respectively, to determine the eIF-2B activity. The activity was assayed for 15 min at 30°C as described under Materials and Methods. The results of two independent experiments from two different lysate preparations are shown. Values are expressed as picomoles of dissociated binary complex.

which hemin is supplemented to lysates. Since heme inhibits the eIF-2α kinase activity of HRI, the recovery of eIF-2B activity is dependent on the kinase activation.

Protein Synthesis Inhibitors That Have No Effect on eIF-2α Phosphorylation Do Not Affect eIF-2B Activity

The specificity of eIF-2α phosphorylation in regulating eIF-2B activity in lysates is demonstrated by the results obtained with other translational inhibitors of protein synthesis, namely, pactamycin, puromycin, and cycloheximide. The inhibition elicited by these agents is not mediated by the phosphorylation of eIF-2α and has no effect on the recovery of eIF-2B activity promoted by the addition of hemin to inhibited heme-deficient lysates (Table III).

Okadaic Acid Inhibits the Restoration of eIF-2B Activity and Dephosphorylation of eIF-2(αP) Mediated by the Delayed Addition of Hemin to Inhibited Lysates

Okadaic acid, a polyether fatty acid found in certain marine fauna (sea sponges, dinoflagellates), is a potent inhibitor of protein phosphatases (36, 38). Type 2A protein phosphatase is selectively inhibited by low levels of okadaic acid (1-20 nM), whereas inhibition of type 1 protein phosphatase requires higher concentrations of okadaic acid (>50 nM) (38). This property of okadaic acid has been used to characterize the protein phosphatase involved in the dephosphorylation of eIF-2B*eIF-2(αP) and the recovery of eIF-2B activity in lysates. As shown in Table IV, the addition of increasing levels of okadaic acid to hemin-supplemented lysates (+ h, 0 min) does not affect the functional eIF-2B activity that is available in these lysates, although the protein synthesis is progressively inhibited (data not shown); eIF-2B activity is not affected because the inhibition of protein synthesis by okadaic acid is not primarily due to eIF-2α phosphorylation (39). This is discussed below.

In our experience, it has been always observed that some amount of eIF-2B activity is available in inhibited heme-deficient lysates to dissociate the performed binary complex (Tables I–V) (please see Discussion). The eIF-2B activity that is available in heme-deficient lysates is further inhibited by high concentrations of okadaic acid (Table IV). The recovery of eIF-2B activity that is observed by the delayed addition of hemin (at

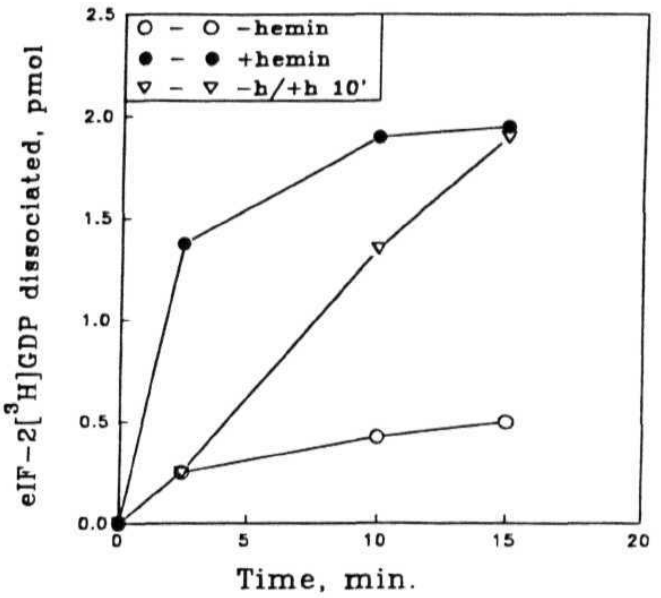

FIG. 1. Kinetics of eIF-2·[^{3}H]GDP dissociation in reticulocyte lysates during the delayed addition of hemin. In step 1, protein synthesis was carried in lysates (70 μl) with and without the addition of 20 μM hemin (-hemin or +hemin, 0 min) at 30°C for 10 min as described under Materials and Methods. At 7 min of protein synthesis, 20 μM heme was added to one of the heme-deficient inhibited lysates (-heme, 0 min +heme). Soon after the addition of hemin, eIF-2B activity of the lysates was determined from the dissociation of preformed labeled eIF-2·[^{3}H]GDP binary complex (12.60 pmol in 70 μl to a lysate volume of 70 μl). At each time interval, as indicated, a 40-μl aliquot wa6 with drawn from each of the reactions to determine the amount of labeled GDP bound to the Millipore membrane as described (29). The values plotted represent picomoles of eIF-2·[^{3}H]GDP dissociated with time.

TABLE II

Recovery of eIF-2B Activity and Protein Synthesis in Heme-Deficient Lysates Treated with Hemin at Different Time Intervals

Protein synthesis conditions	eIF-2B activity (eIF-2·[^{3}H]GDP dissociated, pmol)	Protein synthesis at 30 min ([^{14}C]leucine incorporated, cpm)
−Hemin	1.03	8642
+Hemin 0 min	1.82	16652
−Hemin/+Hemin 5 min	1.70	15754
−Hemin/+Hemin 12 min	1.25	12776
−Hemin/+Hemin 20 min	1.08	9050

Note. Heme-deficient lysates (30 μl × 2) were incubated for different time periods (0, 5, 12, and 20 min) before the addition of 20 μM hemin to determine the effect of prolonged incubation without heme on eIF-2B activity (eIF-2·[^{3}H]GDP dissociated) and on protein synthesis ([^{14}C]leucine incorporated, cpm). Protein synthesis was measured in 5-μl aliquots at 30 min as described (30). eIF-2B activity was assayed in lysates (20 μl) for 15 min from the dissociation of labeled binary complex, eIF-2·[^{3}H]GDP (1.99 pmol in 20-μl aliquots). The labeled binary complex was added to lysates at 10 min (−h; +h, 0 min; −h/+h, 5 min) or at 12 and 20 min of protein synthesis (−h/+h, 12 min; −h/+h, 20 min).

10 min) to lysates is also inhibited by the addition of high concentrations of okadaic acid. These results indicate that a type 1 phosphatase is largely responsible for the recovery of eIF-2B activity. This conclusion is further supported by the data in Fig. 2A, which displays [^{32}P]phosphoprotein profiles generated in heme-deficient lysates by delayed ^{32}P pulse (7–12 min). The addition of high levels of okadaic acid (125–250 nM) causes an increase in eIF-2(αP) (tracks 5 and 7) compared to assays with no okadaic acid (track 1) or low levels (25 nM) of okadaic acid (track 3). At the same

TABLE III

Effects of Cycloheximide, Pactamycin, and Puromycin on eIF-2B Activity in Reticulocyte Lysates

Protein synthesis conditions	eIF-2B activity	
	eIF-2·[^{3}H]GDP dissociated, (pmol)	Activity (%)
−Hemin	0.65	0
−Hemin [+Hemin 10 min]	1.45	99
−Hemin [+Hemin + cycloheximide 10 min]	1.46	100
Hemin [+Hemin + pactamycin 10 min]	1.46	100
−Hemin [+Hemin + puromycin 10 min]	1.43	96

Note. Lysate protein synthesis was carried out at 30°C for 10 min as described under Materials and Methods. Incubations (30 μl) were supplemented at 10 min with hemin (20 μM), cycloheximide (10 μg/ml), pactamycin (2 μM), or puromycin (10 μg/ml) as indicated. At 15 min, lysate eIF-2B activity was assayed in 20-μl samples with the addition of 3.0 pmol of labeled eIF-2·[^{3}H]GDP. eIF-2B activity was assayed for 15 min at 30°C as described under Materials and Methods.

TABLE IV

Effect of Okadaic Acid on Restoration of eIF-2B Activity in Reticulocyte Lysates by the Delayed Addition of Hemin

Protein synthesis conditions	eIF-2B activity (eIF-2·[^{3}H]GDP dissociated, pmol)		
	(+)hemin	(−)hemin	−h + h (10 min)
—	2.33	0.51	2.28
+10 nM OA	2.01	0.91	2.25
+50 nM OA	2.32	0.52	2.22
+100 nM OA	2.29	0.29	2.08
+250 nM OA	2.25	0.00	1.85
+500 nM OA	2.19	0.00	1.43

Note. Protein-synthesizing lysates (30 μl) were incubated under three conditions: (i) plus 20 μM hemin (+hemin), (ii) minus hemin (−hemin), and (iii) minus hemin plus 20 μM hemin added at 10 min (−h/+h 10 min). Increasing concentrations of okadaic acid (OA) were added at 0 min to separate assays as indicated. After 17 min at 30°C, lysate eIF-2B activity (in 30 μl) was assayed by the addition of 4.5 pmol of eIF-2·[^{3}H]GDP (in 20 μl) as described under Materials and Methods. Values represent net picomoles of labeled eIF-2·[^{3}H]GDP dissociated by endogenous eIF-2B under standard conditions.

TABLE V

Effect of Inhibitor-2 on the Recovery of eIF-2B Activity in Heme-Deficient Lysates

Protein synthesis conditions	Delayed additions	eIF-2B activity (eIF-2·[^{3}H]GDP dissociated, pmol)	
		(−)I-2	(+)I-2
Expt. I			
+Hemin	—	1.22	1.25
−Hemin	—	0.47	0.51
−Hemin	+hemin	0.99	0.67
Expt. II			
+Hemin	—	1.94	—
−Hemin	—	0.60	0.67
−Hemin	+hemin	1.65	1.22

Note. Protein-synthesizing lysates (30 μl) were incubated at 30°C for 12 min with or without hemin (20 μM) as described under Materials and Methods. At 5 min, I-2 was added to one set of reaction mixtures at a final concentration of 0.45 μM. At 7 min, heme-deficient lysates were supplemented with hemin (20 μM) and the eIF-2B activity was immediately assayed by the addition of 3.58 (Expt. I) or 3.7 (Expt. II) pmol of eIF-2·[^{3}H]GDP (in 20 μl). The dissociation assay was carried out for 15 min at 30°C as described under Materials and Methods. The results of two independent experiments from two different lysate preparations are shown.

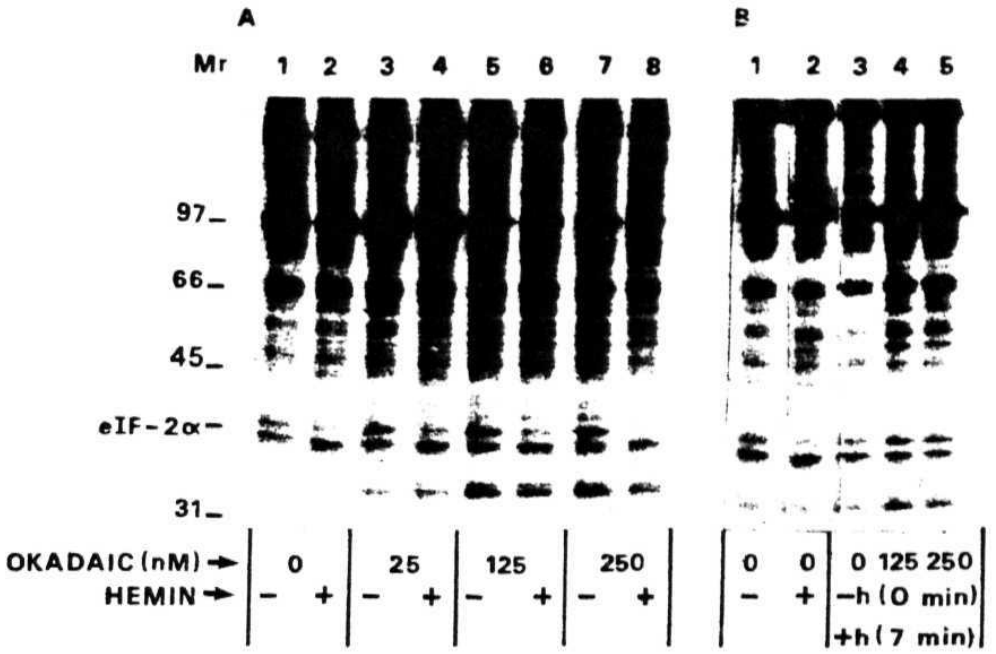

FIG. 2. Effect of okadaic acid on [^{32}P]phosphoprotein profiles of protein-synthesizing lysates. Protein synthesis reactions (30 μl) were incubated at 30°C for 17 min with or without 20 μM hemin as indicated At the beginning of protein synthesis reactions, assays were supplemented where indicated with 0, 25, 100, and 250 nM okadaic acid Assays were pulse-labeled with ^{32}P at 12-17 min (A, tracks 1-8) or 0-12 min (B, tracks 1–5) Assays, in B, 3-5, were incubated without hemin for 7 min (–h) and then supplemented with 20 μM hemin and incubated for an additional 5 min. Samples of each assay were pH 5.0-precipitated and then separated in sodium dodecyl sulfate–10% polyacrylamide gels as described (31, 32). The figure is an autoradiogram.

time, as expected, hemin-supplemented control lysates display very little eIF-2(αP) (track 2) and okadaic acid does not affect this result (tracks 4, 6, and 8). This finding is also consistent with the maintenance of functional eIF-2B activity in hemin and okadaic acid-supplemented lysate (Table IV). In a separate experiment (Fig. 2B), we examined the effect of high concentrations of okadaic acid on the [^{32}P]phosphoprotein profile derived from 0-12 min of ^{32}P pulse in heme-deficient lysates rescued by the delayed addition (at 7 min) of hemin. In the absence of okadaic acid, a low level of eIF-2α phosphorylation is observed in hemin-supplemented (+ heme, 0 min, track 2) lysates and also in lysates treated with the delayed addition of hemin (–heme, +heme at 7 min, track 3) when compared to inhibited heme-deficient lysates (track 1). These findings, which are in agreement with a previous report (17), suggest that a block in the eIF-2α kinase activity of HRI by hemin will allow one to monitor the dephosphorylation of eIF-2(αP) caused by an endogenous phosphatase in the lysate (track 3 vs 1). When high concentrations of okadaic acid are present (tracks 4 and 5), dephosphorylation of eIF-2(αP) is, however, prevented in response to rescue by hemin.

Hence, when HRI is active, high levels of okadaic acid enhance eIF-2α phosphorylation by inhibiting type 1 protein phosphatase. At 20 μM hemin, at which HRI is not active, high levels of okadaic acid cause an inhibition of protein synthesis, but this inhibition is not due to phosphorylation of eIF-2α (Fig. 2A, tracks 2, 4, and 8) and accordingly eIF-2B activity is not affected. In these experiments, we noticed an effect of okadaic acid on 97-kDa polypeptide which is probably elongation factor 2 (EF-2) (Fig. 2) and is phosphorylated in both heme-deficient and hemin-supplemented lysates. At low concentrations of okadaic acid (25 nM), phosphorylation of EF-2 is enhanced, probably due to a partial inhibition of a protein phosphatase (Fig. 2A, tracks 3 and 4). At high levels of okadaic acid (125-250 nM) the phosphorylation is reduced in the profiles generated by the delayed ^{32}P pulse (Fig. 2A, tracks 5-8) but this is probably due to the combination of unlabeled phosphorylation of EF-2 prior to the addition of the ^{32}P pulse and the prevention of phosphate turnover after the pulse. These results on EF-2 phosphorylation (97 kDa), protein synthesis inhibition in hemin-supplemented lysates treated with low concentrations of okadaic acid, and polyribosome formation (data not shown) are in accordance with the results reported by Redpath and Proud (39). Recently, we reported (31) that enhanced eIF-2α phosphorylation occurs in cycloheximide-treated heme-deficient lysates in which HRI is active and polyribosomes are maintained, a finding that indicated poly some-bound eIF-2α is a target of eIF-2α kinase under quasiphysiological conditions. The diminution in eIF-2B activity in okadaic acid-treated heme-

deficient lysates (Table IV) may be due therefore to a combination of increased HR1 activity, decreased eIF-2α phosphatase activity, and increased polyribosomes.

Protein Phosphatase Inhibitor-2 Inhibits Hemin-Mediated Restoration of eIF-2B Activity in Lysates

We have used phosphatase inhibitor-2 (I-2), a selective inhibitor of type 1 protein phosphatase (37, 40), to characterize further the endogenous phosphatase responsible for the recovery of eIF-2B activity in heme-deficient lysates on delayed addition of heme.

Previous studies have shown that addition of inhibitor-2 protein enhances eIF-2α phosphorylation and inhibits protein synthesis in hemin-supplemented lysates (32). In those experiments, the hemin-treated lysates were incubated with I-2 from the beginning of protein synthesis reactions. We have also observed that prolonged incubation of hemin-treated lysates (+h 0 min) with I-2 can lead to enhanced eIF-2α phosphorylation (data not shown). Hence to determine if I-2 affects the functional eIF-2B activity in lysates directly, the eIF-2 guanine nucleotide exchange ability of the hemin-treated lysates has to be carried out soon after the addition of I-2. Our results (Table V) suggest that addition of I-2 at 5 min of protein synthesis to translating hemin-supplemented lysates just before measuring eIF-2B activity does not affect the functional eIF-2B activity which is available in these lysates and catalyzes readily the dissociation of preformed eIF-2 • [^{3}H]-GDP binary complex. However, the restoration of eIF-2B activity that occurs in inhibited heme-deficient lysates upon delayed addition of hemin is inhibited in the presence of I-2 (Table V). These results are consistent with the idea that a protein phosphatase, preferably type 1, plays a dominant role in the physiological dephosphorylation of eIF-2(αP) (32, 26, 48) and in the restoration of eIF-2B activity in heme-deficient lysates.

DISCUSSION

The critical events in the inhibition of protein synthesis in heme-deficiency are the activation of HRI, the phosphorylation of eIF-2α, and the sequestration of eIF-2B by phosphorylated eIF-2α into a complex, in which eIF-2B becomes nonfunctional (14-16). Previously, several protein phosphatases have been reported to act on eIF-2(αP) *in vitro* (19-25). A recent report (26) indicates that the protein phosphatases 1 and 2A dephosphorylate the eIF-2(αP) at similar relative rates *in vitro.* There was no indication, however, to date that such preparations could restore eIF-2B activity or reverse the inhibition of protein synthesis in heme-deficient lysates. Results reported by Thomas *et al* (16) indicate that dephosphorylation of eIF-2(αP) in eIF-2(αP)•eIF-2B complex *in vitro* by alkaline phosphatase can lead to the restoration of eIF-2B activity. The restoration of eIF-2B activity in fully inhibited lysates can be achieved by the addition of hemin which inhibits HRI activity and permits an endogenous protein phosphatase to dephosphorylate the eIF-2(αP) (17). We provide here further evidence that this endogenous phosphatase, which is required to dephosphorylate eIF-2(αP) and restore eIF-2B activity, is sensitive to inhibitor-2 and higher concentrations of okadaic acid.

The extent of eIF-2α phosphorylation defines the extent of inhibition in eIF-2B activity. In the equilibrium between phosphorylation of eIF-2α and dephosphorylation of eIF-2(αP), a marked shift to dephosphorylation not only requires the phosphatase activity but also the inhibition of eIF-2α kinase activity. This point is further substantiated here by showing that the recovery of eIF-2B activity by the delayed addition of hemin (at 7 min) is dependent (a) on the concentration of added hemin (Table I), (b) the time at which eIF-2B activity is studied following the addition of hemin (Fig. 1), and (c) the time when hemin is supplemented to heme-deficient lysates (Table II). It has to be emphasized here that addition of hemin promotes the inactivation of HRI, so that endogenous phosphatase can dephosphorylate eIF-2(αP), and facilitates the restoration of eIF-2B activity. The release of GDP under those conditions is not due to a nonspecific dissociation of added hemin on the eIF-2 • [^{3}H]GDP binary complex. This is because the dissociation of eIF-2 • [^{3}H]GDP is not uniform in heme-deficient lysates treated with the delayed addition of hemin. Lysates which are incubated for longer periods without hemin cannot restore eIF-2B activity as efficiently as those lysates which are incubated for shorter intervals before the addition of hemin (Table II). Also, the activation of double-stranded RNA-dependent eIF-2α kinase that occurs in response to the addition of dsRNA in hemin-supplemented lysates inhibits the eIF-2B activity due to increased eIF-2α phosphorylation (30).

The measurement of eIF-2B activity in whole-cell extracts was initially developed by Matts and London (30) to study the correlation between eIF-2B activity and protein synthesis in reticulocyte lysates which were exposed to several conditions that enhance endogenous eIF-2α phosphorylation. This assay system was subsequently used by others to correlate the inhibition of protein synthesis with reduction in eIF-2B activity in cells under different physiological stress (42-44). More recently this assay system was used to measure the rapid activation of eIF-2B in insulin and growth factor-treated Swiss 3T3 fibroblasts (45) and the inactivation of eIF-2B in insect cells which are expressing mammalian recombinant eIF-2α kinase (47), and it was also used in evaluating the overexpression of wild-type and mutant eIF-2α subunits in rescuing the inhibition of

eIF-2B activity in Chinese hamster ovary cells that is mediated by eIF-2α phosphorylation (29).

Here, the restoration of eIF-2B activity is used as a parameter to characterize the physiological phosphatase that dephosphorylates eIF-2(αP) in inhibited heme-deficient lysates which are supplemented with the delayed addition of hemin and phosphatase inhibitors like okadaic acid and inhibitor-2. To demonstrate that eIF-2B activity is specifically diminished due to eIF-2α phosphorylation in heme-deficient lysates and is not related to the total protein synthesis activity, it has been shown here that inhibitors of protein synthesis, namely pactamycin, puromycin, and cycloheximide, which do not affect eIF-2α phosphorylation, do not affect eIF-2B activity (Table III). The recovery of eIF-2B activity promoted by the delayed addition of hemin is maintained although protein synthesis is inhibited in these lysates.

Inhibitor-2 and okadaic acid do not affect the functional eIF-2B activity. Okadaic acid inhibits type 2A and type 1 phosphatases in a concentration-dependent manner. Somewhat higher concentrations of okadaic acid are required to inhibit type 1 phosphatases than type 2A phosphatases (38). Okadaic acid at 25-50 nM, which causes accumulation of polyribosomes and inhibition of protein synthesis (data not shown), does not affect eIF-2 phosphorylation (Fig. 2) or eIF-2B activity (Table IV) but, however, is shown to enhance EF-2 phosphorylation (39). These concentrations of okadaic acid are expected to inhibit protein phosphatase 2A more efficiently than protein phosphatase 1 (39). Consistent with these findings, we find here that relatively higher concentrations of okadaic acid are required to inhibit the restoration of eIF-2B activity and dephosphorylation of eIF-2(αP) in inhibited lysates treated with the delayed addition of hemin (Table IV and Fig. 2). Also I-2, a specific inhibitor of protein phosphatase 1, inhibits the restoration of eIF-2B activity in inhibited lysates (Table V). These findings suggest that a type 1 phosphatase plays a dominant role in the dephosphorylation of eIF-2(αP) and restoration of eIF-2B activity in translating reticulocyte lysates. In addition, these observations are also consistent with the findings of Wek *et al.* (51) who have demonstrated that a type 1 phosphatase is involved in the modulation of the extent of eIF-2α phosphorylation in yeast. In contrast, the findings of some recent *in vitro* studies indicate that both protein phosphatases, 1 and 2A, can dephosphorylate eIF-2(αP) significantly (26). However, these authors have pointed out that this need not be the case in translating lysates since phosphorylated eIF-2α can interact with eIF-2B, Met-tRNAi, ribosomes, and several other components of translational machinery which can alter the relative activities of the phosphatases against eIF-2(αP) as has been previously suggested (22).

A further analysis of results indicates that eIF-2B activity is not completely inhibited in heme-deficient lysates (Tables I and IV). Addition of higher concentrations of okadaic acid further enhances the phosphorylation of eIF-2α (Fig. 2) and sequesters all the available eIF-2B activity (Table IV) in heme-deficient lysates. This is possible because of the following events. While measuring eIF-2B activity, large quantity of unphosphorylated binary complex is used which may be in dynamic equilibrium with [eIF-2(αP) • eIF-2B] complex as proposed by Rowlands *et al.* (46); this might lead to the release of phosphorylated eIF-2α and functional eIF-2B activity depending on the eIF-2α kinase and phosphatase activities under those conditions. In inhibited heme-deficient lysates eIF-2(αP) is accumulated on 60S subunits of 80S initiation complexes (15, 16, 31). In the presence of kinase inhibitor like hemin, the eIF-2(αP) is presumably readily dephosphorylated by a phosphatase that is bound to ribosomes and is resistant to lower concentrations of okadaic acid. Phosphorylated eIF-2α accumulates, however, in okadaic acid-treated heme-deficient lysates because the heme-regulated eIF-2α kinase activity is not inhibited and eIF-2α phosphatase activity is diminished. In addition, okadaic acid maintains polysomes due to a block in elongation (39). This can lead to enhanced eIF-2α phosphorylation since the eIF-2 bound to 60S subunit of 80S initiation complexes has been reported to be readily phosphorylated in heme-deficient lysates in which polysomes are maintained due to a block in elongation cycle (31). Also a type 1 phosphatase activity is reported to be present on ribosomes (50). Together, these findings substantiate the currently available notion that phosphorylation–dephosphorylation of eIF-2α occurs on ribosomes in physiological conditions. The dephosphorylation is evidently mediated by a type 1 phosphatase in physiological conditions.

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