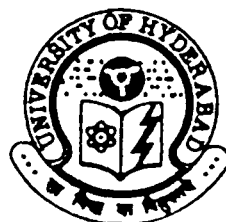


STRUCTURE—FUNCTION RELATIONSHIP
IN RIBOSOMAL PROTEIN S1 FROM *E.coli*
AND EVOLUTION OF ITS DOMAIN STRUCTURE

A THESIS
SUBMITTED TO THE UNIVERSITY OF HYDERABAD
FOR THE DEGREE OF
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IN BIOCHEMISTRY

BY

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To the memory of my Late Father,
and to
my Mother.

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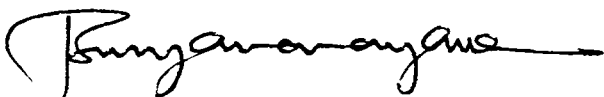
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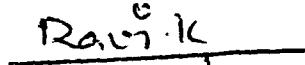
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DECLARATION

Dated 27 November, 1993

I here by declare that the work presented in this thesis has been carried out by me under the supervision of Prof. T. Suryanarayana and that this has not been submitted for a degree or diploma of any other University.

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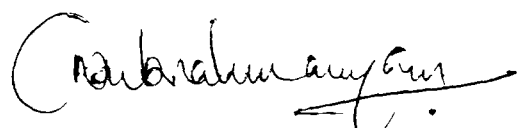
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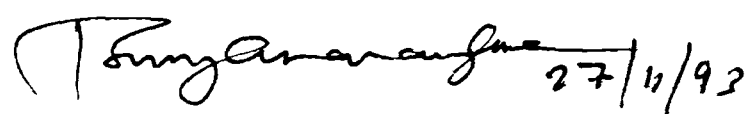
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CERTIFICATE

This is to certify that Mr. KAMBAMPATI RAVI, has carried out the research work embodied in the present thesis under my supervision and guidance for the full period prescribed under the Ph.D ordinance of this University. I recommend his thesis entitled **STRUCTURE-FUNCTION RELATIONSHIP IN RIBOSOMAL PROTEIN S1 FROM *E.coli* AND EVOLUTION OF ITS DOMAIN STRUCTURE** for submission for the degree of Doctor of Philosophy of this University.



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K. Ravi

ABBREVIATIONS

Abbreviations of units of measurement and of physical and chemical quantities, trival names for nucleic acids and amino acids listed in the instructions to authors, The Journal of Biological Chemistry, that may be used without definition are not mentioned here.

CNBr	cyanogen bromide
CPM	counts per minute
DEAE-	diethylaminoethyl-
DEPC	diethyl pyrocarbonate
DTT	dithiothreitol
NEM-S1	N-ethylmaleimide treated S1
NTCB	2-nitro-5-thiocyanobenzoic acid
PFU	plaque forming units
PLP	pyridoxal 5-phosphate
POPOP	1,4-bis[5-phenyl-2-oxazolyl]benzene
PPO	2,5-Diphenyloxazole
RB	rose bengal
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
TNM	tetranitromethane
Tris	tris(hydroxymethyl)aminomethane
TPCK	N-tosyl-L-phenylalanine chloromethyl ketone

Chapter I

INTRODUCTION

This thesis deals with the study of structure–function relationships in ribosomal protein S1 from *Escherichia coli* and evolution of its domain structure.

A brief introduction will be presented in the following pages on the bacterial ribosome structure followed by detailed account of literature review on ribosomal protein S1.

Proteins are linear polymers of L–amino acids that are joined together by peptide bonds. Amino acids are assembled into proteins by the ribosomes. Ribosomes are present in all organisms and they decode mRNA with high speed and accuracy. The 20,000 or so ribosomes present in the intact bacterium, *E.coli* are responsible for the synthesis of all cellular proteins.

I.1 RIBOSOME STRUCTURE, TOPOLOGY OF THE RIBOSOME AND ACTIVE CENTERS

Ribosomes are large ribonucleoprotein particles on which actual process of translation occurs. In prokaryotic cells, they occur in the free form as monosomes or are associated with mRNA as polysomes. About two thirds of the weight of *E.coli* ribosome is RNA, where as the other third is protein. The 70S ribosomes of bacteria consist of two subunits. The small 30S subunit contains 21 distinct proteins (S1–S21) and one molecule of 16S RNA. The large 50S subunit contains 33 distinct proteins (L1–L33) and two RNA molecules namely 23S RNA and 5S RNA. Almost all proteins are present only one copy per subunit. The only exception to the unimolarity of ribosomal proteins is L7/L12. L7 differs from L12 only in the presence of an acetyl group on the N–terminus, there are four copies of L7/L12 dimer per ribosome (Subramanian, 1975). The primary structures of all r–proteins and r–RNAs

have been determined (see Giri *et al* 1984 for complete r-protein sequences). Ribosomes self assemble from the constituent RNA and protein molecules. The techniques used to investigate ribosome structure include identification of locations of individual ribosomal proteins by immune electron microscopy, cross linking of neighboring components to determine the relationships of adjacent proteins, ribosome reconstitution from the isolated ribosomal RNAs and proteins, analysis of interactions between ribosomal proteins and rRNA by neutron scattering of subunits containing specific deuterated proteins. The genes of all the ribosomal components of *E.coli* have been mapped on the bacterial linkage map. Specific ribosomal proteins are directly involved in the binding of mRNA and tRNA. Ribosomal RNAs are involved directly and/or indirectly in all the steps of protein synthesis. Together with accessory factors, the ribosome provides the full range of activities required for all the steps of protein synthesis.

The results obtained so far indicate that ribosomes have several active centers contributed by both rRNA and r-proteins. e.g. mRNA binding site, initiation and elongation factor binding sites, aminoacyl tRNA binding site (A site), peptidyl tRNA binding site (P site), peptidyl transferase site, 5S rRNA site and antibiotic binding sites. The work on the ribosome structure and protein synthesis has been extensively reviewed (see for example Nomura *et al* 1974; Chambliss *et al* 1980; Hardesty and Cramer, 1986; Hill *et al* 1990).

I.2 RIBOSOMAL PROTEIN S1

Initially, S1 was found as a component of *E.coli* 30S ribosomal subunit (Kurland *et al* 1969; Nomura *et al* 1969; Traut *et al* 1969; Kaltschmidt and Wittmann, 1970). In the early years of ribosome research there were serious

questions about the molar stoichiometry of S1 and its relation to ribosome (Nomura *et al* 1969; Voynow and Kurland, 1971). Subsequently it has been established that S1 is a component of 70S ribosome performing essential function in protein synthesis (Van Knippenberg *et al* 1974; Van Duin and Van Knippenberg, 1974; Suryanarayana and Subramanian, 1983). The S1 protein has been independently isolated as i (interference) factor (Groner *et al* 1972a) and subunit I or α of Q β replicase (Kamen *et al* 1972; Groner *et al* 1972b). The i factor was shown to inhibit translation of synthetic and natural mRNA (Groner *et al* 1972c; Miller *et al* 1974). Subunit I prepared from Q β replicase, i factor isolated from 1 M NH₄Cl ribosomal wash, and S1 prepared from 30S particles were shown to be identical (Wahba *et al* 1974; Inouye *et al* 1974).

I.3 PURIFICATION OF S1

Protein S1 as an interference factor was purified from high salt ribosomal wash (Groner *et al* 1972a,b; Miller *et al* 1974; Jay and Kaempfer, 1974 & 1975). Tal *et al* (1972) isolated S1 by exposing the 70S ribosomes to low ionic strength buffers. S1 was selectively removed from the 30S subunit at 1 mM concentration of Tris buffer. Protein S1 was also purified directly from the nucleic acid free crude cell extracts by poly (C)–cellulose chromatography (Carmichael, 1975). Suryanarayana and Subramanian (1979 & 1983) purified S1 in high yield by affinity chromatography of ribosomes on poly (U)–Sepharose.

I.4 PHYSICAL PROPERTIES OF S1

The physical constants of S1 have been determined in several laboratories (see Subramanian, 1983 for review). S1 is the largest (Mr 61,159) and

the longest (23 nm) of all the ribosomal proteins. Its large radius of gyration (7 nm) and intrinsic viscosity (9.8 ml g^{-1}) as well as low sedimentation coefficient (3.2S) and diffusion constant ($4.5 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$) indicated a very asymmetric elongated shape (Giri and Subramanian, 1977). Small angle X-ray scattering analysis revealed that protein S1 in solution behaves like a rod shaped molecule (Laughrea and Moore, 1977; Labischinski and Subramanian, 1979). The iso-electric point (pI) of the protein is 4.5. S1 shows anomalously low electrophoretic mobility (Subramanian, 1983). It is interesting that S1 (23 nm) is slightly longer than the biggest dimension of the ribosome (22 nm), which may be important in the function of the protein.

I.5 PRIMARY STRUCTURE OF S1

The structural gene for S1 (rps A) has been mapped at 20 min on the *E. coli* K-12 chromosome (Ono *et al* 1979). Kitakawa *et al* (1980) isolated transducing λ phage carrying rps A. Both, amino acid sequencing of the protein and nucleic acid sequencing of S1 gene rps A have been reported (Schnier *et al* 1982; Schnier and Isono, 1982). S1 from *E. coli* strain MRE 600 is a single chain of 557 amino acid residues.

HOMOLOGOUS REPEAT SEQUENCES IN S1

The primary structure of S1 revealed the presence of four contiguous, homologous repeats in the central and C-terminal parts of the molecule (Kimura *et al* 1982). Each repeat consists of about 86 amino acids. These were referred as R1, R2, R3 and R4 (Suryanarayana and Subramanian, 1984; Subramanian, 1983). The homologies were also observed in the nucleotide sequence of the structural gene (Schnier *et al* 1982; Schnier and Isono, 1982). The homologous

repeats are probably evolved from a single short gene after repeated duplication and fusion during evolution. Positions of the four repeat sequences (R1 to R4) and their beginning and ending positions are given in Fig. 1.1.

Regnier *et al* (1987) observed primary structural homology between a 69 amino acid stretch of polynucleotide phosphorylase and the four homologous repeats of protein S1. This discovery establishes that homologous domains are conserved among proteins of different functions which bind to non-specific sites on RNA and polynucleotides. These domains may have evolved from duplicate of a single ancestral gene coding for an RNA binding molecule. RNA helicase-like protein PRP22 (required for the release of mRNA from yeast spliceosomes) contain RNA binding motif of ribosomal protein S1 (Company *et al* 1991). Ribosomal protein S1 is known to contain a repeated sequence motif (S1-RM), also found in the bacterial and chloroplast translation initiation factor IF-1 sequences, and in sequences of eukaryotic initiation factor eIF-2 α chains. The significance in the similarity of the sequences is very high suggesting that the occurrence of the S1-RM in these diverse proteins represents homology. The similarity of S1 to IF-1 further suggests that S1 has evolved from an IF-1 like ancestor. The most obvious common function of the proteins containing the S1-RM seems to be RNA binding (Gribskov, 1992).

-SH GROUPS IN S1

S1 contains two cysteine residues (Cys-292 and Cys-349), of which only one is reactive in the native form. Both the cysteine residues are located on the homologous stretch R2 (Subramanian, 1980). Cys-349 is located near the carboxyl end of R2 (see Fig. 1.1). The reactive -SH group of S1 (Cys-349) is

Fig. 1.1: Schematic representation of *E.coli* ribosomal protein S1 and its polypeptide fragments.

F2a, F2b and F3 are CNBr fragments of S1. F2a contains the ribosome binding domain of S1. S1F4 is produced by the cleavage of S1 at SH-2 with NTCB. S1F1 is produced by the trypsin cleavage of S1 and contains the nucleic acid binding domain. M1S1 is the mutant protein. The four contiguous homologous repeats (R1-R4) are shown striped and are present in the central and C terminal parts of the molecule.

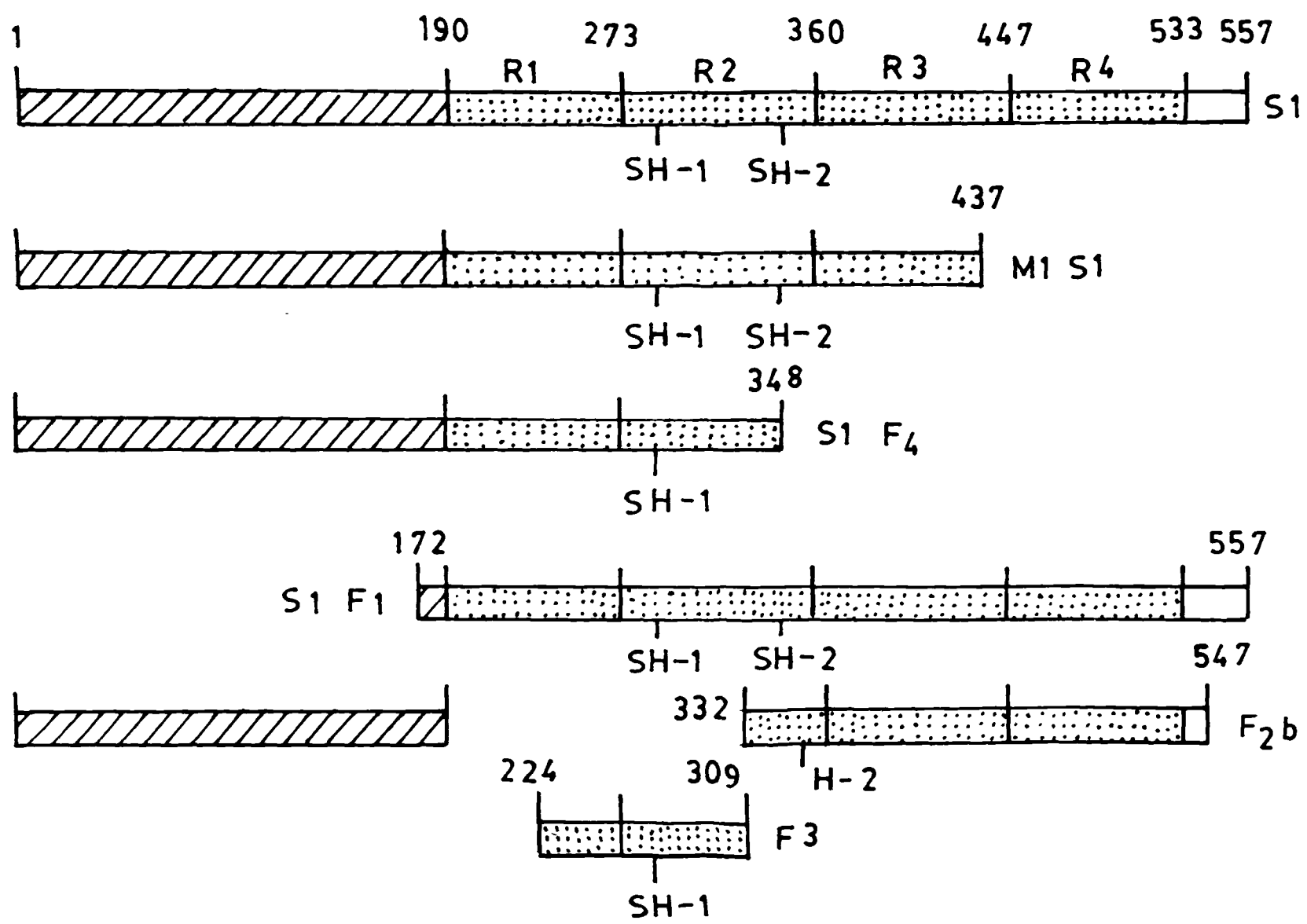


Fig.1.1

exposed in the free protein (Linde *et al* 1979; Kolb *et al* 1977; Kahan *et al* 1974; Subramanian, 1980) as well as in 30S subunits, but becomes inaccessible to reaction when treated with poly or oligo nucleotides (Acharya and Moore, 1973; Ginzberg *et al* 1973; Ginzberg and Zamir, 1976; Lipecky *et al* 1977).

I.6 STRUCTURAL DOMAINS AND FRAGMENTS OF S1

Small angle X-ray scattering studies of S1 (Labischinski and Subramanian, 1979; Osterberg *et al* 1978), NMR studies of S1 (Moore and Laughrea, 1979), fluorescent polarization measurements of S1 (Chu and Cantor, 1979) and limited proteolytic digestion of S1 (Suryanarayana and Subramanian, 1979) indicated that there are at least two distinct structural domains in S1.

Treatment of protein S1 with TPCK-trypsin under mild conditions resulted in the production of high molecular weight fragment denoted as S1F1 (Suryanarayana and Subramanian, 1979). It contains the sequence from amino acid residues 172 to 557 of S1 with Mr. 41,330. S1F1 contains all the homologous repeats R1 to R4.

Methionine specific cleavage of S1 by CNBr yields three large and three small fragments. Two of these, F2a and F3 retain the structural domains. The fragment F2a from the N-terminal region of S1 contains the sequence from amino acid residues 1 to 193 with Mr. 21,304 (Subramanian *et al* 1981; Giorginis and Subramanian, 1980). The fragment F3 contains the sequence from amino acid residues 224 to 309 with Mr. 9,725.

Cleavage of S1 at its reactive -SH group (Cys-349) with 2-nitro-5-thiocyanobenzoic acid resulted in the production of a fragment denoted as S1F4 (Suryanarayana and Subramanian, 1984). This fragment contains the

sequence from amino acid residues 1 to 348, containing the N-terminal domain and two of the four repeating sequences (R1 & R2).

A mutant form of S1 lacking approximately 20% of the chain length from the C-terminus was isolated (Subramanian and Mizushima, 1979). The mutant S1 denoted as M1S1 contains the sequence from amino acid residues 1 to 437. M1S1 does not contain the C-terminal 120 amino acids of S1 that corresponds to the homologous repeat R4 (Kimura *et al* 1982; Schnier and Isono, 1982). All the fragments of S1 are shown in Fig. 1.1.

I.7 FUNCTIONAL PROPERTIES OF S1 FRAGMENTS

E.coli mutant S1 (M1S1) was able to restore about 75% activity to S1-lacking ribosomes in the translation of natural and synthetic mRNA (Subramanian and Mizushima, 1979). Therefore, the C-terminal sequence absent from M1S1 is considered non-essential. M1S1 also restored full functional activity to S1 lacking Q β replicase (Guerrier Takada *et al* 1983).

S1F1 was totally inactive in restoring function to S1-lacking ribosomes (Suryanarayana and Subramanian, 1979) and to S1-lacking Q β replicase (Guerrier Takada *et al* 1983). S1F1 binds to poly (U)-Sepharose as strongly as S1 and contains the major nucleic acid binding domain of S1 (Suryanarayana and Subramanian, 1979).

The fragment F3 binds to poly (U) and the binding is abolished by low concentration of aurintricarboxylate (Subramanian *et al* 1981). This fragment is from the central region of the protein possessing the core of nucleic acid binding domain. F2a binds to S1-lacking 30S subunits and competes with S1 in its binding to the ribosome. This indicates that F2a contained more or

less intact ribosomal binding domain of S1.

The S1F4 fragment contains the ribosome binding domain and the repeat sequences R1 & R2, including RNA binding core of F3. S1F4 binds to 30S subunits lacking S1 and competes with S1 in the binding (Suryanarayana and Subramanian, 1984). S1F4 binds to poly (U) and confers the ability to S1-lacking subunits to bind MS2 phage RNA. These results show that S1F4 contains functionally active ribosome and RNA binding domains. S1F4 was able to stimulate translation of poly (U) and poly (A) with considerable efficiency. However S1F4 is inactive in translation of MS2-RNA. The results indicated that the repeat stretches of S1 are necessary for the correct initiation of natural mRNA i.e placing mRNA on the AUG initiation codon at the P site of the ribosome (Suryanarayana and Subramanian, 1984).

The fragments S1F1, F2a and F3 are all inactive in translation assays. Nucleic acid binding domain of S1 is located within the four repeat structures (R1 to R4). Two of the repeats R1 and R2 represent the core of the nucleic acid binding domain. The ribosome binding domain of S1 is located outside the beginning of repeated sequences. Two arginyl peptide bonds of S1 (at positions 190 and 192) in the sequence are highly sensitive to trypsin. The sequence around these arginine residues is highly hydrophobic and probably function as a hinge between globular ribosomal binding domain and highly organized trypsin resistant RNA binding domain. This inter-domain region probably permits free movement of the domains relative to each other (Odom *et al* 1984). Deletion and insertion studies in the structural gene for ribosomal protein S1 suggest that S1 has a general regulatory role during protein synthesis (Schnier *et al* 1986).

I.8 POLYNUCLEOTIDE BINDING PROPERTIES OF S1

Ribosomal protein S1 is known to bind various RNAs independent of ribosomes (Tal *et al* 1972; Carmichael, 1975; Lipecky *et al* 1977). The interaction of S1 with polynucleotides has been studied by various investigators using a wide range of physical and biochemical techniques such as,

- (a) millipore filter binding (Smolarski and Tal, 1970; Katunin *et al* 1980; Suryanarayana and Subramanian, 1979 & 1983).
- (b) equilibrium dialysis (Lipecky *et al* 1977).
- (c) quenching of intrinsic fluorescence of tryptophan residues of S1 by polynucleotides (Draper *et al* 1977; Draper and von Hippel, 1978a,b).
- (d) high resolution electron microscopy (Thomas *et al* 1978).
- (e) temperature-jump relaxation procedures (Yuan *et al* 1979; Mulsch *et al* 1981).
- (f) ability to lower the melting temperature of polynucleotides (Szer *et al* 1976).
- (g) alteration in the circular dichroic spectrum (Bear *et al* 1976).
- (h) change in the fluorescence properties of free or ethidium bromide bound polynucleotides (Kolb *et al* 1977; Bear *et al* 1976) and
- (i) use of matrix bound nucleic acids (Carmichael, 1975; Suryanarayana and Subramanian, 1979).

S1 can bind to either ssRNA or ssDNA, but it only displays strong, characteristic binding to RNA. The nucleic acid binding site of S1, therefore, distinguishes between the ribose and deoxy ribose residues of a polynucleotide

chain. The RNA-binding site has been estimated to extend over 10–12 nucleotides (Gassen, 1980; Draper and von Hippel, 1978; Thomas and Szer, 1982). The S1. oligonucleotide binding constant is $3 \times 10^6 \text{ M}^{-1}$, was found to be independent of the base sequence. The S1.RNA- binding is salt-insensitive. S1 elutes from poly (A) with 1.0 M NH_4Cl , but 8 M urea is required to elute S1 from poly (C) and poly (U) columns (Carmichael, 1975; Suryanarayana and Subramanian, 1979). The binding of S1 to polypyrimidines is stronger than to polypurines. Draper and von Hippel (1978b) observed the existence of strong co-operativity for poly (C) binding and its absence in the case of poly (A) binding.

S1 has been shown to bind the denatured DNA and to synthetic poly-deoxynucleotides (Thomas *et al* 1978 & 1979; Draper *et al* 1977; Draper and von Hippel, 1978). S1 binding to denatured DNA is salt-sensitive. Studies of Draper and von Hippel (1978) on the nucleic acid binding properties of the *E.coli* protein S1 indicated that it has two independent nucleic acid binding sites. They reported that Site I is non specific and binds to either single stranded DNA or RNA and Site II binds to single stranded RNA only. It was further reported that the binding affinities of site I is independent of base and sugar composition of nucleic acid ligand and Site II binds polypyrimidines with three fold higher affinity than polypurines. Hahn *et al* (1987) using monoclonal anti bodies identified two distinct sites for mRNA binding in the regions 349–437 and 438–547 located in the C-terminal domain of protein S1.

I.9 UNFOLDING OF POLYNUCLEOTIDES BY S1

Studies on the interaction of ribosomal protein S1 with polynucleotides

by optical methods (UV hyperchromicity and CD ellipticity) revealed that S1 causes disruption of secondary structure of polynucleotides. Alteration of poly pyrimidine conformation by protein S1 is similar to that resulting from thermal denaturation (Bear *et al* 1976; Szer *et al* 1976 & 1977). S1 does not unstack double- and triple- stranded helical polynucleotides, but perturbs the conformation of RNA-DNA hybrid poly (A)-poly (dT) resulting in the release of intercalated ethidium bromide. The translation inhibitor, aurintricarboxylic acid completely inhibits the helix unwinding action of S1 on polypyrimidines (Bear *et al* 1976). S1 prevents formation of poly (A)-poly (U) double helix. MS2-RNA, 16S RNA, and tRNA are all partially denatured in the presence of large amounts of S1 (Szer *et al* 1976).

It was found that N-ethylmaleimide modified S1 (NEM S1) still binds to RNA, 30S subunits, but does not disrupt the RNA secondary structure (Kolb *et al* 1977; Thomas *et al* 1978; Thomas and Szer, 1982). The ribosomes reconstituted with NEM S1 are reported to be inactive in binding fMet-tRNA and initiation complex formation with MS2-RNA (Kolb *et al* 1977; Chu and Cantor, 1979).

I.10 INTERACTION OF S1 WITH 30S RIBOSOMAL SUBUNIT

S1 is loosely associated with the 30S subunit. It is partially lost from ribosomes during the isolation procedures. Furthermore, S1 dissociates from 30S at 1 mM Tris concentration (Tal *et al* 1972). S1, free in solution, exchanges rapidly with the bound S1 (Laughrea and Moore, 1977). Dahlberg (1974) separated two forms of 30S subunits by agarose acrylamide gel electrophoresis, the slower migrating form contained S1 and the faster migrating form is devoid of it. Two moles of S1 bind to the inactive 30S subunit, while only

one copy binds per active 30S subunit (Laughrea and Moore, 1977). The thermodynamic constant for the association of S1 with the 30S subunit is $1.7 \times 10^8 \text{ M}^{-1}$ (Laughrea and Moore, 1978; Draper von Hippel, 1979a).

S1 binds to 30S subunit mostly through protein-protein interactions (Boni *et al* 1982). The N-terminal fragment of S1 containing residues 1-195 (F2a) binds strongly to 30S subunit and competes with S1 for binding (Subramanian *et al* 1981; Georginis and Subramanian, 1980). S1 plays no role in ribosome assembly (Mizushima and Nomura, 1970; Held *et al* 1973 & 1974). The ribosome and RNA binding fragments of S1 have no specific affinity to ribosomal RNA but the protein binds to free 16S and 23S rRNA (Krol *et al* 1977) and to tRNA (Li *et al* 1979) like any synthetic polynucleotide. The target of RNA binding site of S1 which is free in the protein bound to the 30S subunit is mRNA and not rRNA (Subramanian, 1983). S1 binds near the 3' terminus of the 16S rRNA in the 30S subunit.

Protein S1 has been cross linked to the 3' terminus of 16S rRNA in intact subunits (Kenner, 1973; Czernilovsky *et al* 1975). Protein S1 binding site on the ribosome is reported to be near the proteins S2, S10 and S18 (Boilaeu *et al* 1981). Colicin E3 removes the terminal 50 nucleotides of 16S rRNA and S1 from the ribosomes (Dahlberg, 1974). Protein S1 has been reported to bind to the cloacin fragment of 16S rRNA (Dahlberg and Dahlberg, 1975). S1 can bind to 30S subunits subjected to mild nucleolytic cleavage but not to 30S subunits subjected to mild proteolysis indicating that S1 binds to ribosome through protein-protein interactions (Boni *et al* 1982). Protein S1 was cross linked to mRNAs by irradiation of intact *E.coli* cells with ultraviolet light (Schouten, 1985). The binding sites of S1 in cloacin fragment was found to be rich in pyrimidines (Shine and Dalgarno, 1974). However, it has been

reported that S1 does not protect cloacin fragment of 16S rRNA from nuclease digestion (Wickstrom, 1986). Based on the fluorescence anisotropy studies Goss *et al* (1983) suggested Mg^{2+} -dependent conformational changes in intact ribosomes (including RNA) upon binding of S1.

I.11 FUNCTION OF S1 IN $Q\beta$ REPLICASE

$Q\beta$ replicase is the enzyme responsible for replication of $Q\beta$ viral RNA. $Q\beta$ replicase is a hetero tetramer, containing four polypeptides designated as subunit I, II, III and IV (Kamen, 1970) or α , β , γ and δ (Kondo *et al* 1970). Synthetase, the only phage coded polypeptide, is primarily responsible for polymerization activity of the enzyme. Subunit I is identical to ribosomal protein S1 (Inouye *et al* 1974; Wahba *et al* 1974). The binding constant of S1 with S1-lacking $Q\beta$ replicase is same as the binding constant of S1 with S1-lacking 30S subunits (Cole *et al* 1982). This indicates that the same surface of S1 is involved in both these interactions.

$Q\beta$ replicase binds strongly to two regions (S and M) on the plus strand of $Q\beta$ RNA. Protein S1 is absolutely required for replicase binding to both S and M sites. Protein S1 was able to bind to specific sites (S and M) of $Q\beta$ RNA in the absence of other subunits (Senear and Steitz, 1976; Goelz and Steitz, 1977). The presence of S1 enables replicase to bind specifically to the central region of $Q\beta$ RNA, which contains the ribosome binding sites. Kolakofsky and Weissmann (1971) demonstrated that replicase cannot dislodge ribosomes from the template and that replicase binding to $Q\beta$ RNA prevents protein synthesis initiation. S1 as a part of replicase represses protein synthesis specifically which is necessary for $Q\beta$ phage growth.

$Q\beta$ minus RNA strands are not bound or translated by ribosomes. Studies on reconstitution of S1 lacking $Q\beta$ replicase with discrete fragments of S1 revealed that NH_2 -terminal region of S1 is required for S1 interactions in replicase. The $COOH$ -terminal region of S1 is not essential for S1 function in replicase (Cole *et al* 1982). The RNA unwinding property of S1 is not implicated in the $Q\beta$ replicase function. (Guerrier-Takada *et al* 1983). $Q\beta$ replicase in the absence of added template was shown to synthesize RNA. Removal of ribosomal protein S1 from replicase results in the loss of RNA synthesis in the absence of added template (Hill and Blumenthal, 1983). In summary, the role of S1 in the transcription reaction catalysed by $Q\beta$ replicase is probably similar to the role of S1 in translation i.e. in this case also S1 serves to bind and hold viral RNA strands that are being copied. The ribosome-MS2 RNA interaction involves at least two different regions of the phage RNA—the internal region of the replicase gene (S1-binding site) and the ribosome binding site of the coat protein gene (Boni *et al* 1986).

I.12 FUNCTION OF S1 IN PROTEIN SYNTHESIS

Ribosomes efficiently translate synthetic homopolynucleotides like poly (U) and poly (A) that lack AUG initiation codon like any natural mRNA. Removal of S1 from the ribosome curtails its ability to translate mRNA (Yokata *et al* 1977; Van Duin and Van knippenberg, 1974; Sobura *et al* 1977; Suryanarayana and Subramanian, 1983). The S1 dependence of the system was first demonstrated by Van Duin and Van knippenberg (1974). This dependence is greatest at low amounts of synthetic messenger. Addition of purified S1 in stoichiometric amounts (1 mol/mol ribosome) to S1-free translating system results in stimulation of activity comparable to normal level. Addition of S1 in excess is inhibitory (Van Dieijen *et al* 1975; Miller and Wahba, 1974). This

inhibition depends on poly (U)/ribosomes ratio, there is strong inhibition when the ratio is low, but none when large excess of poly (U) is present in the system (Adochi *et al* 1980; Van Duin and Van knippenberg, 1974). When antibodies specific for S1 were added to an S1 containing system, there is total inhibition which can be reversed by the addition of S1 (Van Dieijen *et al* 1975, 1976 & 1977).

Interesting results were observed when the translation was carried with increasing amounts of mRNA using S1-containing and S1-lacking ribosomes. S1-containing ribosomes were able to translate to the maximum level at low concentration of either synthetic or natural mRNA. S1-lacking ribosomes poorly translated natural mRNA irrespective of its concentration. S1-lacking ribosomes overcame S1 requirement at high concentrations of synthetic mRNA (Suryanarayana and Subramanian, 1983).

The translation of phage MS2-RNA is strongly dependent on the presence of S1. The effect of S1 on individual steps in the translation of MS2 RNA has been examined. The presence of S1 on 30S subunits is strictly necessary for the ribosome to bind MS2- RNA (Szer and Leffler, 1974; Szer *et al* 1975). Ribosomes programmed with MS2-RNA are S1 dependent for the f-Met-tRNA binding and this binding is inhibited by S1 antibodies (Van Dieijen *et al* 1976).

Mild pretreatment of MS2-RNA with formaldehyde abolishes the requirement of S1 in translation. Formaldehyde treatment disrupts small amounts of secondary structures present in MS2- RNA (Van Dieijen *et al* 1976). This

led to the conclusion that, the main function of S1 is to disrupt mRNA secondary structure for initiation of protein synthesis. Addition of S1 to *Bacillus stearothermophilus* ribosomes stimulates translation by about 10 fold at 39 °C. Raising the temperature of the assay to 49 °C stimulates translation to the same extent as adding S1 at 39 °C, implying again that the stimulation observed at 39 °C with S1 was as a result of melting of secondary mRNA structure by S1 (Isono and Isono, 1975).

There were two early proposals for the specific function of S1. A model has been proposed that S1 denatures the Shine–Dalgarno complementary sequence at the 3' end of 16S RNA in order to promote base pairing with Shine–Dalgarno sequences on mRNA (Dahlberg and Dahlberg, 1975). This was later disapproved when detailed studies revealed no specificity in the interaction between S1 and the 3' end of 16S RNA. The second proposal was based on ability of S1 to unfold the secondary structure of mRNA (Van Dieijen *et al* 1976). This was also rendered untenable when the studies with NEM modified S1 revealed that, S1 with out RNA helix unwinding activity could support *in vitro* protein synthesis as well as Q β RNA transcription.

I.13 A MODEL FOR THE SPECIFIC FUNCTION OF S1 IN PROTEIN SYNTHESIS

Suryanarayana and Subramanian (1983) proposed a model for the action of S1. Their model was based on striking structural and functional features of S1. S1 is attached to the ribosome only by its N-terminal domain and the long flexible RNA binding domain searches a large area around ribosome for mRNA. The trypsin sensitive peptide sequence present in between the two domains functions as a hinge and facilitates the mRNA catching function of S1. Another specific function of S1 is, precise fitting of the mRNA containing

AUG codon at the P-site of the ribosome to initiate translation.

Boni *et al* (1991) identified S1 binding sites on several phage RNAs in preinitiation complexes. Targets for S1 on phage RNAs are localized upstream from the SD domain which contain oligo U sequences. It is suggested that the U-rich sequences represent S1 binding sites on bacterial mRNAs that are situated at different distances from the initiation codon. The U-rich region preceding the SD domain may help efficient translation initiation. They have proposed that S1 binding sites, represented by pyrimidine-rich sequences upstream from the SD region, serve as determinants involved in the recognition of mRNA by the ribosome.

I.14 S1-LIKE PROTEINS IN OTHER ORGANISMS

S1-like protein is not present in ribosomes from *Bacillus stearothermophilus* (Isono and Isono, 1976). *Bacillus subtilis* also does not have a protein showing structural homology with *E.coli* S1 (Higo *et al* 1982). Muralikrishna and Suryanarayana (1985) made comparison of several Gram-negative and Gram-positive eubacteria for the presence of S1 using several criteria. They observed that most Gram-negative bacteria (all enterobacteria) contained protein S1 while most Gram-positive tested with the exception of *Micrococcus luteus* did not contain protein S1. Yamada (1982) isolated a ribosomal protein from *Mycobacterium smegmatis* which binds to poly (U). However, this protein was found to be non homologous to *E.coli* protein S1 either immunologically or functionally. Schnier and Faist (1985) compared the structural gene *rp-s A* coding for protein S1 and its preceding regulatory region (having four promoters) from *E.coli* with other bacterial species using southern blot technique. Their results indicated high degree of homology in S1 structural genes

of *E.coli* and other Gram-negative but not Gram-positive bacteria. However, the regulatory sequence was not highly conserved. It was concluded that a gene coding for a protein similar to S1 is present in all Gram-negative species which they have analyzed. Hahn and Stiegler (1986) tested bacteria belonging to both Gram-negative and Gram-positive families for protein S1 using immunoblotting technique. They have concluded that S1-like protein is immunologically conserved in majority of Gram-negative bacteria. Mikulik *et al* (1986) isolated S1-like protein (SS1) from Gram-positive *Streptomyces aureofaciens* which showed weak immunological cross reactivity that was detectable only by immuno dot binding assay.

Muralikrishna and Suryanarayana (1987a,b) purified S1 from Gram-negative nitrogen fixing bacterium *Azotobacter vinelandii* (Av-S1) and Gram-positive *Micrococcus luteus* (Ml-S1) by employing poly (U)-Sepharose chromatography. Both Av-S1 and Ml-S1 have been characterized with respect to structural, immunochemical and functional properties. Av-S1 cross reacted poorly with antiserum raised against N-terminal portion (having ribosome binding domain) of *E.coli* S1 while Ml-S1 cross reacted poorly with antiserum raised against central and C-terminal portion (having nucleic acid binding domain) of *E.coli* S1. Av-S1 showed greater structural and immunological homology in the nucleic acid binding domain of the protein while Ml-S1 showed greater homology in the ribosome binding domain. Mild trypsin digestion of both Av-S1 and Ml-S1 resulted in the production of trypsin resistant core (S1F1). Schnier *et al* (1988) cloned and characterized rps A gene from *Rhizobium melilotii*. DNA sequence analysis revealed that RS1 has 568 amino acids which showed 47% homology to *E.coli* protein S1.

Hahn *et al* (1988) identified S1-like protein in spinach chloroplast ribosomes by means of immunoblotting technique. The central core of chloroplast r-protein S1 (CS1) shows 31.5% amino acid homology to the *E.coli* protein S1. The CS1 has a Mr. of 40 kDa. The core fragment contains three degenerated repeats which show homology to both the ribosome and the RNA-binding domain of S1. The CS1 binds to poly (A) in contrast to S1 which binds strongly to poly (U). This may be due to the presence of poly (A)-rich regions in chloroplast transcripts of higher plants (Franzetti *et al* 1992). The chlamydial gene encoding 70 kDa protein has been cloned in *E.coli*. The sequence analysis of the 5' portion of the gene identified its product as the chlamydial homologue of the *E.coli* protein S1 (Sardinia *et al* 1989).

I.15 S1 IN BACTERIA AND TRANSLATION SPECIFICITY

Stallcup *et al* (1974 & 1976) have reported that ribosomes from Gram-positive bacteria could not translate mRNA from Gram-negative bacteria while the ribosomes from Gram-negative bacteria translated mRNA from both types of bacteria. This difference in translation has been attributed to the weak Shine-Dalgarno interaction operating between Gram-positive ribosomes and Gram-negative messenger RNA (McLaughlin *et al* 1981a,b; Murray and Rabinowitz, 1982). The Gram-positive mRNA appear to possess strong Shine-Dalgarno regions. Higo *et al* (1982) suggested that the absence of S1 from bacterial ribosomes may account for species-specific translation.

Roberts and Rabinowitz (1989) studied translation activity of *E.coli* S1 on *B.subtilis* ribosomes. S1 is active on *B.subtilis* ribosomes in translation of poly (U) but that is unable to overcome the characteristic inability to translate *E.coli* mRNA. The removal of S1 from *E.coli* ribosomes causes them to

become translationally specific to preferentially translate messengers having strong SD regions. In summary there appears to be an evolutionary correlation between the presence of S1, the type of Shine and Dalgarno sequence on mRNA and the translational specificity exhibited by bacteria (Roberts and Rabinowitz, 1989). Detailed analysis on the occurrence of S1 in Gram-positive bacteria by Rabinowitz and co-workers had led them to suggest that, translational specificity of the ribosomes from Gram-positive bacteria is correlated with low genomic G+C content and lack of ribosomal protein S1. The Gram-positive organisms with high G+C content have S1-like proteins on their ribosomes and are translationally non-specific (Farwell and Rabinowitz, 1991). Balakin *et al* (1992) showed that the mRNA with an extended Shine-Dalgarno sequence is translated independent of S1.

OBJECTIVES AND SCOPE OF THE PRESENT INVESTIGATION

Ribosomal protein S1 of *E.coli* plays a major role in the initiation of translation. Protein S1 has been extensively studied with respect to structure, nucleic acid binding properties and its involvement in protein synthesis. Protein S1 possesses distinct structural domains which have been isolated and characterized (Suryanarayana and Subramanian, 1979 & 1984; Giorginis and Subramanian, 1980; Subramanian and Mizushima, 1979; Subramanian *et al* 1981). Search for the S1-like proteins in eubacteria revealed that majority of Gram-negative bacteria contained S1 (Hahn *et al* 1988; Muralikrishna and Suryanarayana, 1985 & 1987a,b; Schnier and Faist, 1985).

The objectives of the present investigation are the study of structure-function relationship in protein S1 from *E.coli* and analysis of archaeobacterial ribosomes for the presence of protein S1.

Several amino acid residues of *E.coli* S1 were modified with specific chemical reagents and the effect of such modifications on the protein-nucleic acid interaction and in stimulating poly (U) directed *in vitro* translation were studied. This study may be helpful in the identification of amino acid functional groups of protein S1 involved in the interaction of nucleic acids (chapter III).

It is reported that S1 possesses two independent polynucleotide binding sites. Site I binds either ssDNA or ssRNA and site II binds to ssRNA only (Draper *et al* 1977; Draper and von Hippel 1978a,b). The ssDNA binding property of the protein is not well studied. It is not known whether the DNA

binding site of the protein S1 is also part of the RNA binding site. *E.coli* S1 was cleaved both enzymatically and chemically to isolate several structural/functional domains. Binding of these fragments to M13 ssDNA were carried out to localize the ssDNA binding domain in the protein (chapter IV).

There was no major study on the occurrence of S1-like proteins in archaeobacteria. The detection and characterization of S1-like proteins in archaeobacteria will help in understanding the evolutionary relationship of S1 in different organisms. So two families of archaeobacteria (a thermoacidophile and an extreme halophile) were chosen to look for the protein S1 using the western blotting technique (chapter V). Further work is feasible to purify the S1 homologues from archaeobacteria and to analyze archaeobacterial S1 in terms of structural/functional homology to eubacterial S1.

Chapter II

MATERIALS AND METHODS

II.1 CHEMICALS

Poly (U), pyruvate kinase, ATP, GTP, coomassie blue R 250, TPCK-trypsin, soyabean trypsin inhibitor, DNase I, BSA, unlabelled amino acid alumina, diethyl pyrocarbonate, N-ethylmaleimide, 2,3-butanedione (diacetyl), rose bengal dye, *E.coli* tRNA, sucrose, alkaline phosphatase conjugate anti rabbit Ig G (goat) and ethidium bromide were purchased from Sigma chemical company, U.S.A.

DEAE-cellulose (DE-52) and glass fibre filters GF/A were from Whatman, England. Nitrocellulose paper was obtained from Schleicher & Schull, Germany. Anti rabbit Ig G (goat) coupled peroxidase and 4-chloro-1-naphthol were from Pierce, U.S.A. Casaminoacids, Freund's adjuvant (complete and incomplete) were purchased from Difco laboratories, U.S.A. Poly (U)Sephadex 4B, Sepharose-4B, Sephadex G-150 and Sephadex G-100 were procured from Pharmacia fine chemicals, Sweden. Molecular weight protein standards kit was prepared by mixing individual proteins, that are procured from Sigma chemical company, U.S.A. SDS, DTT, and 2-nitro 5-thiocyanobenzoic acid were obtained from Serva, Germany. Phosphoenol pyruvate was from Boehringer mannheim, Germany.

Uniformly labelled [^{14}C]-phenylalanine was procured from Board of Radiation and Isotope Technology (BRIT), Bombay, India. Acrylamide, N,N-bisacrylamide, and agarose were from Sisco research laboratories Pvt Ltd, India. TEMED and 2-mercaptoethanol were obtained from Loba Chemie, India. Tetranitromethane, PPO, POPOP, Tris buffer, and Folin-Ciocalteu reagent were from Spectrochem Pvt Ltd, India. Bactotryptone and Yeast

extract were purchased from Himedia laboratories Pvt Ltd, India. All other chemicals used in this study were of analytical grade, available locally.

II.2 BACTERIAL STRAINS

The strains used in this study were *Sulfolobus acidocaldarius* strain DSM 639 obtained from Deutsche Sammlung Von Mikroorganismen, Gottingen, Germany and *Escherichia coli* A19 from Dr. A.R. Subramanian, Max-Planck Institute for Molecular genetics, Berlin, Germany. Three strains of halobacteria viz., *Halobacterium halobium*, *H. salinarium*, and *H. cutirubrum* were obtained from Dr. W. Alteckar, Baba Atomic Research Center (BARC), Bombay, India.

II.3 BACTERIAL GROWTH

E. coli A19 cells were grown in Luria broth at 37 °C with aeration (Minks *et al* 1978). *S. acidocaldarius* was grown at 75 °C for 40–48 h with aeration in a medium containing 0.1% yeast extract, 0.1% bacto tryptone, 0.5% -casaminoacids, 0.1% glucose, 0.02% NaCl, 0.13% ammonium sulphate, 0.03% KH₂PO₄, 0.025% MgSO₄, 0.07% CaCl₂ and a required amount of 10 N H₂SO₄ to adjust the pH to 3.0 (Kikuchi and Asai, 1984). Halobacterial cells were grown at 37 °C in a medium containing 25% sodium chloride, 0.3% sodium citrate, 0.2% potassium chloride, 0.02% calcium chloride, 0.3% yeast extract, 0.5% bacto tryptone, 2% magnesium sulfate and required amounts of 2 N NaOH to adjust the pH to 7.5, with aeration for 36–44 h. All bacterial cultures were grown to mid-logarithmic phase, harvested by centrifugation. *Sulfolobous* cells were harvested after neutralizing the culture with 1 M Tris base. Bacterial cells excepting Halobacteria were washed once with TKM10 buffer

(10 mM Tris-HCl, pH 7.6, 50 mM KCl, 10 mM magnesium acetate and 7 mM 2-mercaptoethanol) and stored frozen at -80°C until further use.

II.4 PREPARATION OF CRUDE CELL LYSATES

S.acidocaldarius and halobacterial cells were grown to an OD of 0.6 to 0.8 at 660 nm. The cells were harvested and lysed directly in electrophoretic sample buffer containing SDS, boiled at 100°C for 5 min. Crude cell lysates were then clarified by a low speed centrifugation.

II.5 ISOLATION OF RIBOSOMES, SUPERNATANT FRACTION (S-100)

Ribosomes, supernatant fraction (S-100) from *E.coli* and *S.acidocaldarius* were isolated according to Minks *et al* (1978). All operations were carried out at $0-4^{\circ}\text{C}$. Cells were ground with twice the weight of alumina and extracted with 3 ml of TKM10 buffer containing $2\text{ }\mu\text{g}$ DNase I per ml. The suspension was centrifuged for 10 min at $10,000 \times g$ to sediment alumina and cell debris and the supernatant obtained was again centrifuged at $30,000 \times g$ for 30 min to obtain S-30 extract. The S-30 supernatant was centrifuged at $100,000 \times g$ for 4 h to pellet ribosomes. The upper 2/3 rd of supernatant (S-100) was collected separately and dialyzed against TKM10 buffer containing 10% glycerol for 4 h. S-100 was stored at -80°C in aliquots and used as a source of aminoacid activating enzymes and protein synthesis elongation factors. Ribosomal pellet was suspended in TKM10 buffer containing 1 M NH_4Cl and left at 4°C for 12 h. The ribosomes present in the high salt wash were pelleted by centrifugation at $100,000 \times g$ for 4 h. The 1 M NH_4Cl washed ribosomal pellet was suspended in TKM10 buffer containing 15% glycerol and stored

frozen at -80°C .

Ribosomes from Halobacteria were isolated according to Sanz *et al* (1988). Halobacterial cells were harvested when the O.D. at 600 nm was 0.6 to 0.8. Cells were once washed with buffer (30 mM Tris-HCl, pH 7.6, 2 M ammonium sulfate, 60 mM magnesium acetate and 5 mM 2-mercaptoethanol), ground with twice the weight of alumina and extracted with 1 ml of buffer (30 mM Tris-HCl, pH 7.6, 2 M ammonium sulfate, 60 mM magnesium acetate and 5 mM 2-mercaptoethanol) per gm cells containing $2\text{ }\mu\text{g}$ DNase I. The extract was centrifuged at $10,000 \times g$ for 10 min. The supernatant was again centrifuged at $30,000 \times g$ for 30 min to remove cell debris. The S-30 supernatant obtained was recentrifuged at $100,000 \times g$ for 4 h to pellet ribosomes. The ribosomal pellet was resuspended in buffer (30 mM Tris-HCl, pH 7.6, 2 M ammonium sulfate, 60 mM magnesium acetate and 5 mM 2-mercaptoethanol) and stored at -80°C until further use.

II.6 ISOLATION OF RIBOSOMAL SUBUNITS BY DENSITY GRADIENT CENTRIFUGATION

S. acidocaldarius 70S ribosomes were dialysed against high salt buffer (0.5 M ammonium chloride, 0.5 mM magnesium acetate and 10 mM Tris-HCl, pH 7.6). The dialysed ribosomal solution was clarified by low speed centrifugation and was carefully layered on top of 10–30% linear sucrose gradients and centrifuged for 2 h at 40,000 rpm in VTi-50 Beckman rotor. Fractions were collected from the bottom of the gradient tube. The absorbance of the fractions was measured at 260 nm after appropriate dilution. Fractions corresponding to 30S and 50S subunits are pooled. The subunits were immediately brought to 10 mM magnesium by the addition of 1 M magnesium acetate.

The subunits were then concentrated by DEAE-cellulose chromatography and were stored in aliquots at -80°C .

II.7 ISOLATION OF SUBUNITS BY HYDROPHOBIC CHROMATOGRAPHY

Ribosomal subunits were separated by the method developed by Kirillov *et al* (1978). 70S ribosomes were freed of magnesium ions by gel filtration on Sephadex G-50 column equilibrated with buffer (20 mM Tris-HCl, pH 7.6 and 0.6 M ammonium sulfate). Ribosomes from G-50 column were dialyzed against 20 mM Tris-HCl, pH 7.6 and 1.5 M ammonium sulfate and then loaded on Sepharose-4B and the column was eluted by a linear reverse salt gradient from 1.25 M to 0.02 M ammonium sulfate. Fractions corresponding to 30S and 50S subunits were pooled, concentrated by DEAE-cellulose chromatography.

II.8 PURIFICATION OF RIBOSOMAL PROTEIN S1

Protein S1 from *E.coli* has been purified taking advantage of its property of strong binding to polypyrimidines. The method is based on the procedure developed by Suryanarayana and Subramanian (1983). This involves affinity chromatography of ribosomes on poly (U)-Sepharose followed by ion-exchange chromatography on DEAE-cellulose. *E.coli* ribosomes were passed through a poly (U)-Sepharose column equilibrated with TKM10 buffer containing 1 M NH_4Cl . The column was washed with the same buffer to recover ribosomes devoid of S1. S1 bound to the column was eluted with 7 M urea. S1 which was contaminated with small amounts of ribosomal proteins and RNA was further purified by DEAE-cellulose chromatography. Protein S1 was dialysed against 10 mM Tris-HCl, pH 7.6, 6 M urea and 7 mM

2-mercaptoethanol. The dialysed S1 was passed through DEAE cellulose column, which was previously equilibrated with the dialysis buffer and then eluted with a linear gradient of 0–0.2 M ammonium chloride. Fractions containing protein S1, eluting at ammonium chloride concentration of about 0.1 M as determined by SDS-PAGE, were pooled and dialysed against 10mM Tris-HCl, pH 7.6, 7 mM 2-mercaptoethanol and stored at -80°C .

II.9 REMOVAL OF PROTEIN S1 FROM RIBOSOMES AND POST RIBOSOMAL SUPERNATANT

Protein S1 from ribosomes was removed by affinity chromatography on poly (U)-Sepharose (Suryanarayana and Subramanian, 1983). Ribosomes in TKM10 1 M NH_4Cl buffer were passed through poly (U)-Sepharose equilibrated with the same buffer. Washing the column with the equilibrating buffer resulted in the elution of ribosomes free of S1 protein. The ribosomes eluted from the column were completely free of S1. No other ribosomal protein was missing from these ribosomes. Ribosomes lacking S1 were dialysed against TKM10 buffer containing 10% glycerol and stored at -80°C .

A similar procedure was followed to remove small amounts of S1 present in the post ribosomal supernatant fraction (S-100).

II.10 ASSAY SYSTEM FOR IN VITRO PROTEIN SYNTHESIS

Poly (U)-dependent polyphenylalanine synthesis was carried as described by Suryanarayana and Subramanian (1984). Protein synthesis was carried out in 100 μl of 50 mM Tris-HCl, pH 7.6, 100 mM NH_4Cl , 16 mM magnesium acetate, 1 mM DTT, 1.6 mM ATP, 0.3 mM GTP, 8 mM PEP, 30 μM each of the 20 unlabelled amino acids, 0.3 μM [^{14}C]-phenylalanine (0.1 μCi), 5 μg of pyruvate kinase, 200 μg *E.coli* tRNA, 100 μg of ribosomes, 200 μg S-100

protein and 5 μ g poly(U).

All additions were done at 0–4 °C. The mixture was incubated at 37 °C for 20 min and then 1.5 ml of 6% trichloroacetic acid containing 0.1% casaminoacids was added, heated to 95 °C for 15 min, filtered through glass fibre filters GF/A. The filters were washed with ice cold 6% trichloroacetic acid and dried. Radioactivity was determined by counting the filters in a liquid scintillation counter.

When the effect of S1 on the protein synthesis was studied ribosomes and post ribosomal supernatant fraction both depleted of S1 were used. The mixtures, after addition of protein S1 were pre incubated at 37 °C for 5 min before the addition of poly (U).

II.11 ANTISERA

Antibodies to S1, S1F1 and F2a were raised in rabbits as described (Stoffler and Wittmann, 1971). 100 μ g of protein was emulsified with Freund's complete adjuvant and injected subcutaneously into rabbit at multiple sites. After 4 weeks a second booster injection of 50 μ g of protein in Freund's incomplete adjuvant was given subcutaneously. Rabbit was bled after 1 week of booster injection through pinna vein. Antiserum was collected after centrifugation of clotted blood. Ouchterlony immunodiffusion was performed as per standard procedure.

II.12 ISOLATION OF Ig G FROM ANTISERUM

Antibodies were purified by the following procedure. The antisera were heated at 56 °C for 30 min to inactivate the complement proteins. The antiserum was fractionated with ammonium sulphate. Antiserum was brought to 37.5% saturation by adding saturated ammonium sulphate made in 0.02 M Tris-HCl, pH 8.0, 1 mM EDTA. The precipitate was collected by centrifugation at 15,000 rpm for 10 min. The precipitate was dissolved in small volume of 0.02 M Tris-HCl, pH 8.0, 1 mM EDTA and the ammonium sulphate precipitation was repeated once again. The final precipitate was dissolved in phosphate buffer (0.07 M sodium phosphate, pH 6.3) and dialysed against the same buffer.

The crude IgG solution was further purified by ion-exchange chromatography (DE-52). The dialysed protein solution was applied on the DE-52 column and eluted with phosphate buffer. Fractions of 1 ml volume were collected and the absorbance at 280 nm was measured. The peak fractions were pooled and concentrated by adding equal volume of saturated ammonium sulphate (precipitated at 50% saturation). The Ig G precipitate was collected by centrifugation at 15,000 rpm for 10 min and dissolved in phosphate buffered saline and dialysed against the same buffer.

II.13 SLAB GEL ELECTROPHORESIS IN THE PRESENCE OF SDS

Protein samples were electrophoresed on 18% polyacrylamide slab gels containing SDS as described by Thomas and Kornberg (1975) with some modifications (Reddy and Suryanarayana, 1988). The ratio of acrylamide

to bisacrylamide was 40: 0.4. The 18% separating gel was in 0.75 M Tris-HCl, pH 8.8 and 0.1% SDS and the stacking gel was of 5% polyacrylamide in 0.08 M Tris-HCl pH 6.8 and 0.1% SDS. Protein samples were treated with 0.1% SDS and 1% 2-mercaptoethanol at 65 °C for 15 min. Electrophoresis was carried out at 180 volts until the tracking dye (bromophenol blue) reached the bottom of the gel. The electrode buffer comprised of 0.05 M Tris, 0.38 M glycine, and 0.1% SDS.

After electrophoresis, the gels were washed in 7.5% glacial acetic acid for 20 min, stained with 0.15% coomassie blue R 250 in 50% methanol and 7.5% acetic acid and destained with a solution containing 5% methanol and 7.5% acetic acid.

II.14 IMMUNOBLOTTING

It was done according to Towbin *et al* (1979) with some modifications. Proteins were electrophoresed on 18% polyacrylamide gels as described above. The proteins were electrophoretically transferred to nitrocellulose membrane (0.45 μ M pore size) using the electrode buffer (25 mM Tris, 192 mM glycine and 20% methanol). The electroblots were gently washed in tween buffer (10 mM Tris-HCl, pH 7.6, 150 mM NaCl and 0.1% tween-20) for 10 min. The additional protein binding sites were saturated by incubating the blot with the blot buffer (5% skimmed dry milk in tween buffer) for 1 h. The nitrocellulose sheet was incubated with diluted antiserum (1: 100) in blot buffer for 2 h and washed 6 times (6 x 5 min) with tween buffer. The blots were then incubated with peroxidase conjugated anti rabbit Ig-G (1: 2000 dilution) in blot buffer for 2 h. The nitrocellulose sheet was once again washed 6 times (6 x 5 min) with tween buffer. Finally, the blots were developed in the

enzyme substrate solution. The substrate solution was prepared freshly by adding 1 ml stock solution of 4-chloro-1-naphthol (3 mg/ml in methanol) and 10 μ l of 30% H_2O_2 to 10 ml Tris-buffered saline (10 mM Tris-HCl, pH 7.6, 150 mM NaCl) The reaction was terminated after 10 to 15 min by washing with distilled water and the membrane was air dried.

II.15 SILVER STAINING

Silver staining of the gels was performed according to Blum *et al* (1987) with some modifications. After completion of electrophoresis the gels were removed and fixed in 50% methanol, 12% glacial acetic acid for 1 h and then washed with 50% ethanol for 1 h. The gels were treated with sodium thiosulfate (0.2 g/L) for 1 min and washed 3 times with distilled water (3 x 20 s). The gels were then impregnated with silver nitrate (0.5 g/L) containing 0.75 ml of 37% formaldehyde for 30 min. The gels were washed 2 times with distilled water (2 x 20 s). The protein bands were developed with sodium carbonate (60 g/L) containing 0.5 ml of 37% formaldehyde and sodium thiosulfate (4 mg/L). After the protein bands were developed, the gels were washed with distilled water and placed in fixing solution for 1 h.

II.16 ESTIMATION OF PROTEIN

The concentration of protein in different samples was determined according to Lowry *et al* (1951) using bovine serum albumin as standard.

II.17 ISOLATION OF SINGLE STRANDED M13 DNA

Single stranded DNA from M13 Phage was isolated as described by Messing (1983) with some modifications. We have used MV 1190 as a host for M13

phage infection. A stock of M13 lysate having a titre of 10^{11} plaque forming units per ml (PFU/ml) were prepared initially by growing some infected cells in 2x YT medium. A single plaque (will have about 10^7 to 10^8 PFU/ml) is picked with a tooth pick and the infected cells are placed in 2 ml 2x YT medium and allowed to grow for 3 h. The infected culture was centrifuged at 10,000 rpm for 5 min. The clear supernatant having a titre of 10^{11} PFU/ml was used as stock M13 lysate.

A culture of non infected cells was grown in 2x YT medium by transferring a single colony from M9 glucose plate. The culture was allowed to grow until the absorbance at 600 nm reached 0.3. The culture was then infected with M13 phage with a multiplicity of infection (MOI) of 1 to 100. Incubation is continued at 37 °C for 4 to 5 h. The culture was centrifuged to obtain supernatant which contained M13 phage.

A overnight culture of MV1190 was grown in M9 liquid medium having glucose as carbon source and supplemented with thiamin. 2 ml of overnight grown MV1190 was diluted with equal amount of 2x YT medium and then infected with 30 μ l of M13 lysate stock having 10^{11} PFU/ml. The culture was grown for 4 h at 37 °C with vigorous aeration. The culture was centrifuged at 5,000 rpm for 5 min. The clear supernatant was recentrifuged at 15,000 rpm for 15 min to make sure that all the cells are removed from the supernatant.

The M13 phages present in the supernatant were precipitated with polyethylene glycol/sodium chloride. The supernatant was brought to 6% PEG and 0.5 M sodium chloride. The solution was mixed gently and kept at 4 °C for 1 h. The turbid solution was centrifuged at 10,000 rpm for 5 min and the supernatant was discarded with out disturbing the M13 phage pellet. The

pellet was then suspended in 10 mM Tris-HCl, pH 7.6 and 0.1 mM Na₂EDTA. The M13 ssDNA from the resuspended phage particles was isolated by extracting once with buffered phenol followed by buffered phenol-chloroform. The M13 ssDNA was concentrated by precipitating with ethanol. M13 ssDNA was finally dissolved in 10 mM Tris-HCl, pH 7.6, 0.1 mM Na₂EDTA, two drops of chloroform was added and stored at 4 °C.

II.18 GEL MOBILITY SHIFT ASSAY

It was carried out as described by Lohman *et al* (1986). The binding of S1 and of its fragments to circular M13 ssDNA was analysed by electrophoresis using 0.5% agarose gels. The binding of protein to DNA results in the retardation in the migration of bound DNA compared to free DNA. In all the experiments, DNA concentration was held constant (in the range of 0.1 to 0.5 µg) and the protein was taken in increasing amounts (in the range of 1 to 6 µg). Complexes were formed in 30 µl buffer (10 mM Tris-HCl, pH 7.6, 0.1 mM Na₂EDTA and 22 mM NaCl) by adding DNA to protein. Loading dye (2 µl of 50% v/v glycerol, 0.04% w/v bromophenol blue) was added to 30 µl sample and the sample was loaded directly on to the slots in the agarose gel submerged in the low-salt running buffer (20 mM Tris (pH 7.8), 0.4 mM sodium acetate, 0.2 mM Na₂EDTA). Electrophoresis was carried out at 25 °C with a constant voltage (7 V/cm) for 3 to 3.5 h. The gels are then stained for 10 min in distilled water containing 0.5 µg/ml ethidium bromide. The gel was photographed immediately with a camera using UV transilluminator.

II.19 BINDING OF S1 TO 70S RIBOSOMES

10 A₂₆₀ Units of poly (U)-Sepharose passed *E.coli* ribosomes (devoid of S1)

or *S.acidocaldarius* 70S were incubated with 45 μ g of protein S1 in 0.1 ml of TKM10 buffer (10 mM Tris-HCl, pH 7.6, 50 mM KCl, 10 mM magnesium acetate, 7 mM 2-mercaptoethanol). Binding of S1 to *H.salinarium* 70S was carried out at both high and low salt conditions. In the low salt condition all the buffers were supplemented with 0.2 M NH_4Cl while in high salt condition the buffers contained 1.5 M NH_4Cl . After the addition of S1, samples were incubated at 37 $^{\circ}\text{C}$ for 10 min. The reaction mixtures were then layered over 4 ml of 15% sucrose cushion in TKM10 buffer and centrifuged at 50,000 rpm for 5 h at 4 $^{\circ}\text{C}$ to pellet ribosomes. Ribosome pellet was suspended directly in SDS sample buffer and electrophoresed on 18% gel slabs. Proteins from the gel were transferred to nitrocellulose and blotted with S1 antiserum to detect bound S1.

II.20 BINDING OF S1 TO *E.coli* 30S RIBOSOMES DEVOID OF S1

4 A_{260} units of *E.coli* 30S ribosomes devoid of S1 were incubated with 18 μ g of protein S1 in 0.15 ml of Tris-HCl, pH 7.6, 50 mM KCl, 10 mM magnesium acetate and 7 mM 2-mercaptoethanol for 10 min at 37 $^{\circ}\text{C}$. The reaction mixture was passed through 1.0 ml DE-52 column previously equilibrated with 10 mM Tris-HCl, pH 7.6, 10 mM magnesium acetate, 0.2 M NH_4Cl , and 7 mM 2-mercaptoethanol. The column was washed with ten bed volumes of equilibrating buffer. The 30S ribosomal subunits bound to DE-52 were finally eluted with equilibrating buffer containing 0.6 M NH_4Cl . Fractions containing 30S ribosomes were pooled, and treated with 10% trichloroacetic acid to precipitate the ribosomes. The precipitates were collected by centrifugation, washed with chilled acetone, heated in 0.1% SDS, 1% 2-mercaptoethanol (65 $^{\circ}\text{C}$ for 10 min) and electrophoresed according to Reddy and Suryanarayana, 1988).

II.21 TRYPSIN CLEAVAGE OF S1: ISOLATION AND PURIFICATION OF S1F1 AND N-TERMINAL FRAGMENTS

The tryptic resistant core (S1F1) and N-terminal fragment of S1 was isolated as described earlier (Suryanarayana and Subramanian, 1979). Protein S1 in 0.01 M triethanolamine-HCl (pH 8.0), at 0 °C was treated with TPCK-trypsin (400: 1 ratio w/w). Soyabean trypsin inhibitor (five fold excess over S1) was added after 15 min to stop the reaction. The reaction mixture was made to 6 M urea/0.1 M NH₄Cl and chromatographed on a Sephadex G-150 column. Peak fractions containing S1F1, N-terminal fragments were determined after SDS-PAGE. S1F1 emerged at about 45% of the column volume followed by N-terminal fragment. Fractions containing the fragments were pooled, concentrated and dialysed against 10 mM Tris-HCl, pH 7.6, 100 mM NH₄Cl, 1 mM dithiothreitol, and 10% glycerol.

II.22 NTCB CLEAVAGE OF S1: ISOLATION AND PURIFICATION OF S1F4 AND C-TERMINAL FRAGMENTS

Protein S1 was cleaved with NTCB and S1F4, C-terminal fragments were isolated as described earlier (Suryanarayana and Subramanian, 1984). Protein S1 (1 mg/ml) in 0.1 M Tris-HCl, pH 7.6, and 1 mM 2-mercaptoethanol was reacted with 10 mM 2-nitro-5-thiocyanobenzoic acid (NTCB) at 37 °C for 30 min. The protein after cyanylation was dialysed at room temperature for 48 h against 1000 volumes of 0.1 M sodium tetraborate with four changes. The reaction mixture was then chromatographed on a Sephadex G-100 column. Equilibration and elution were carried out with 10 mM Tris-HCl, pH 7.6, 100 mM NH₄Cl, 1 mM dithiothreitol, and 1 mM EDTA. Peak fractions containing fragments S1F4, C-terminal fragments were determined by electrophoresis (Reddy and Suryanarayana, 1988), pooled, concentrated

and dialyzed against 10 mM Tris-HCl, pH 7.6, 100 mM NH₄Cl, 1 mM dithiothreitol and 10% glycerol.

II.24 BINDING OF S1, FRAGMENTS OF S1 TO POLY (U)-SEPHAROSE

Protein S1 or its fragments were passed through 0.2 ml of poly (U)-Sephadex 4B. The column was washed successively with 5 column volumes of 0.1 M, 0.25 M, 0.5 M, 1.0 M, 2.0 M NH₄Cl and finally with 1.0 M NH₄Cl/7 M urea. The protein in each eluate was precipitated with 6% trichloroacetic acid after adding myoglobin as carrier. The precipitate was electrophoresed on 18% acrylamide slab gels.

II.25 MODIFICATION OF HISTIDINE RESIDUES OF S1 WITH DIETHYL PYROCARBONATE

Commercially available diethyl pyrocarbonate (DEPC) was diluted with anhydrous methanol before use. Treatment was carried out as described (Baxter *et al* 1978). The concentration of diluted DEPC was determined quantitatively according to Melchior and Fahrney (1970). An aliquot of the diluted DEPC was added to 1 ml of 0.1 M imidazole (pH 7.0), the increase in the absorbance at 242 nm was measured. The concentration of diluted DEPC was determined from the known absorption coefficient of $3.0 \times 10^3 \text{ cm}^{-1} \text{ M}^{-1}$ for N-ethoxyformyl imidazole (Setlow and Mansour, 1970).

A typical DEPC reaction carried out in the present study is described below. Two cuvetts containing aliquots of protein S1 (0.6 mg/ml) in 10 mM potassium phosphate buffer, pH 6.0 and 100 mM KCl) are placed in the sample and reference compartments of a double-beam spectrophotometer at 14 °C. 1 M DEPC in methanol was added to protein in the sample cuvette to a final concentration of 2 mM. The reference cuvette received an equal

amount of methanol. Immediately after the addition of DEPC, the increase in the absorption of protein at 242 nm and 280 nm was monitored with time. The DEPC reaction was terminated by adding 0.1 M histidine to a final concentration of 10 mM. The reaction mixture was immediately dialyzed against 10 mM Tris- HCl, pH 7.6 and 7 mM 2-mercaptoethanol.

II.26 MODIFICATION OF HISTIDINE RESIDUES OF S1 WITH ROSE BENGAL

Histidine residues of protein S1 were also modified using rose bengal dye (RB) as the photosensitizer. The reaction was carried out as described (Jonak and Rychilik, 1980). A 230 W slide projector was used as a light source. The lens of projector was placed at a distance of 32 cm from sample and focused to give the most concentrated light possible. Protein S1 (0.28 mg/ml in 20 mM Tris- HCl, pH 7.6, 5 mM 2-mercaptoethanol) was taken in a glass tube, placed in a glass beaker containing water (maintained at 25 °C). 2 mM RB in water was added to protein S1 to a final concentration of 8 μ M and immediately exposed to the light source. The reaction was terminated after 35 min by dialyzing against 10 mM Tris-HCl, pH 7.6 and 7 mM 2-mercaptoethanol. The control protein was also processed in the above manner in the absence of RB dye.

II.27 MODIFICATION OF ARGININE RESIDUES OF S1 WITH 2,3-BUTANEDIONE

Arginine residues of protein S1 were modified with 2,3-butanedione (diacetyl). The treatment was carried out as described (Rohrbach and Bodely, 1977). 2,3-butanedione in water was added to protein S1 (0.7 mg/ml in 50 mM borate, pH 9.0 and 5 mM 2-mercaptoethanol) to a final concentration

of 4 mM. The reaction mixture was incubated at 30 °C for 30 min. Excess reagent was removed by dialyzing against 50 mM borate, pH 9.0 and 5 mM 2-mercaptoethanol. This dialysis also stabilizes the modification process. Modification of arginine residues by 2,3-butanedione was reversed by exhaustively dialyzing a part of the reaction mixture against 10 mM Tris-HCl, pH 7.6 and 7 mM 2-mercaptoethanol.

II.28 MODIFICATION OF TYROSINE RESIDUES OF S1 WITH TETRANITROMETHANE

Tetranitromethane (TNM) was diluted with absolute methanol before use. The modification was carried out as described (Riordan and Vallee, 1972). TNM was added to protein S1 (0.3 mg/ml in 50 mM Tris-HCl, pH 8.0, 100 mM KCl at 21 °C for 30 min, the reaction was terminated and the unreacted TNM was removed by dialyzing the reaction mixture against 10 mM Tris-HCl, pH 7.6 and 7 mM 2-mercaptoethanol.

II.29 MODIFICATION OF LYSINE RESIDUES OF S1 WITH PYRIDOXAL 5-PHOSPHATE

Modification reaction was carried out as described (Ohsawa and Gualerzi, 1981). Pyridoxal 5-phosphate (PLP) was added to protein S1 (0.5 mg/ml in 20 mM Triethanolamine-HCl, pH 7.8, 30 mM KCl) to a final concentration of 1 mM. The reaction mixture was incubated at 37 °C for 12 min and the reaction was stopped by the addition of sodium borohydrate (NaBH₄). The reaction mixture was left for 10 min at 0 °C to ensure complete reduction. before further processing. The final concentration of NaBH₄ is 10 times that of PLP concentration. The reaction mixture was immediately dialyzed against 10 mM Tris-HCl, pH 7.6 and 7 mM 2-mercaptoethanol.

II.30 MODIFICATION OF CYSTEINE RESIDUES OF S1 WITH N-ETHYLMALEIMIDE

Modification reaction was carried out as described (Suryanarayana and Subramanian, 1983). Protein S1 (0.5 mg/ml in 0.1 M Tris-HCl, pH 7.6, 5 mM Na₂EDTA) was incubated with 20 fold molar excess of N-ethylmaleimide (over total thiols) at room temperature for 30 min. After the reaction, the protein solution was dialyzed extensively against 10 mM Tris-HCl, pH 7.6 and 7 mM 2-mercaptoethanol. In order to modify both SH groups of S1, the reaction was performed in buffer containing 8 M urea.

Chapter III

CHEMICAL MODIFICATION STUDIES ON S1

Chemical modification of amino acid residues of proteins with specific reagents is widely used in probing complex active site chemistry and the relationship between structure and function in proteins. There are several reports on amino acid modifications of intact 70S ribosomes or ribosomal subunits or ribosomal protein(s) and identification of functional groups responsible for mRNA recognition. Using chemical modification as a strategy, the role played by an amino acid in macromolecular interaction can be elucidated.

We have selected histidine, arginine, lysine, tyrosine and cysteine residues in S1 for the modification studies because these amino acid residues are implicated in the protein–nucleic acid interactions in model systems and in the active sites of many enzymes/proteins acting on nucleic acids. Ribosomal protein S1 was chemically modified with specific reagents and the effect of such modification on the function of protein, namely protein–nucleic acid interaction and *in vitro* translation, was studied. A brief introduction to the chemical reagents used in our study will be presented below.

Diethyl pyrocarbonate ($\text{C}_2\text{H}_5\text{OCO}$)₂O (DEPC), also known as ethoxyformic anhydride or diethyloxydiformate or diethyldicarbonate reacts with histidyl residues in model systems (Ehrenberg *et al* 1976; Muhlrads *et al* 1967 & Ovadi *et al* 1967) and in proteins (Ovadi *et al* 1967; Ovadi and Keleti, 1969). DEPC rapidly acylates accessible imidazole groups at pH 7.0 to yield an N-carbethoxyhistidyl derivative (Fig. 3.1 A). This reaction may be conveniently followed spectrophotometrically by the increase in absorbance, which has a maximum between 230 and 250 nm (Ovadi *et al* 1967; Ovadi and Keleti, 1969).

The number of modified histidyl residues is calculated from the molar absorption coefficient for N-carbethoxy histidine at 240 nm (ϵ 3200 m⁻¹ cm⁻¹) (Ovadi *et al* 1967). Inactivation of the enzyme by DEPC may be correlated with the modification of histidyl residues (Ovadi *et al* 1967). Calculation of the extent of modification of liver alcohol dehydrogenase by a 200– fold excess of DEPC gave a number of modified histidyl residues greater than the known histidine content of the enzyme. These results led to a study of model reactions of histidyl derivatives with excess DEPC (Morris and Mckinsley–Mckee, 1972). Excess DEPC can react to form a disubstituted histidyl derivative, which has a higher absorbance at 242 nm than the N–carbethoxyhistidyl derivative resulting in overestimation of the number of modified histidyl residues.

Photo chemical oxidation is a mild and under some conditions, a selective method for modifying amino acids in proteins. In the case of histidine, oxidation in the presence of rose bengal results in unknown product because of ring cleavage. The mechanism of photo chemical oxidations proposed by Oster *et al* (1959) is presented in Fig. 3.2 (B) in an abbreviated form by equations 1–3, where D represents the dye, D* an excited electronic state of the dye and S the substrate.

Riordan (1973) developed 2,3–butanedione (diacetyl) as a highly specific arginine modifying reagent. Using this reagent, several enzymes acting on phosphate–containing substrates were shown to have arginyl residues at their active sites (alcohol dehydrogenase, alkaline phosphatase). Rohrbach and

Bodely (1977) studied the interaction of butanedione with elongation factor-G (EF-G). Their results indicate that EF-G contains only one essential arginine residue that is located in the guanine nucleotide binding site of the protein. Reaction of 2,3-butanedione with EF-G results in the inactivation of the enzyme leading to loss of guanine nucleotide binding. The mechanism of 2,3-butanedione reaction with arginine residues in proteins is outlined in Fig. 3.1 (C).

Riordan *et al* (1966) introduced tetranitromethane (TNM) to the protein field for the modification of tyrosyl residues. TNM is highly specific and selective in nitrating tyrosyl residues of protein around pH 8.0 to 3-nitrotyrosine. Nitration of tyrosine is an irreversible reaction (Riordan *et al* 1968). Intermolecular covalent crosslinking of protein in presence of TNM has been shown by Doyle *et al* (1968), Boesel and Carpenter (1970) and Williams and Lowe (1971). Polymerization reaction always occurs at the expense of tyrosine nitration (Vincent *et al* 1970). The mechanism of nitration of tyrosyl residues by TNM probably involves the participation of nitronium ions. Nitrotyrosine may also participate in the crosslinking reaction (Hugli *et al* 1971). Modification of cysteine, the only other amino acid reactive to TNM proceeds at pH 6.0. Oxidation of cysteine by TNM is partially reversible by dialyzing against 2-mercaptoethanol. Nitration displaces two protons and produces 1 mole of nitroformate/mole of tyrosine at pH 8.0, suggesting the formation of 3-nitrotyrosine (Fig. 3.1 D).

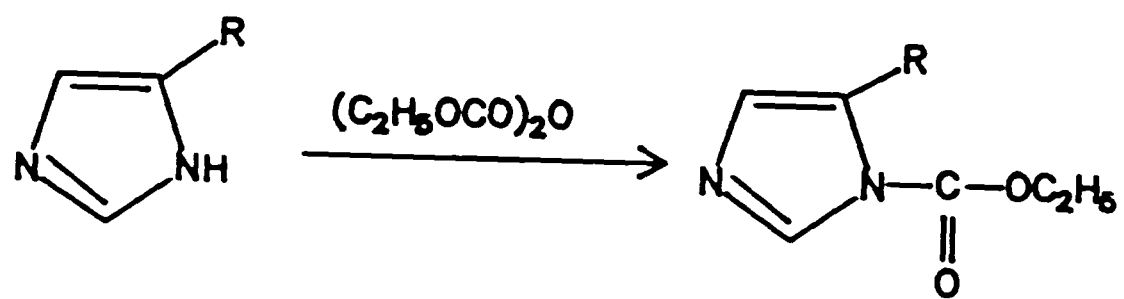
Site-specific chemical modification of proteins with pyridoxal-5-phosphate (PLP) has been widely used to inactivate phosphate-binding enzymes. In many instances, this reaction proved to be selective for those lysyl residues

displaying affinity for negatively charged phosphate groups. The reaction is potentially useful in the study of structure–function relationships in the protein synthetic machinery where many nucleic acid (and nucleotide)–protein interactions occur. The active sites of both *E.coli* ribosomal subunits as well as initiation and elongation factors were studied using PLP. ϵ -NH₂ group of lysine forms a schiff base with the aldehyde group of pyridoxal phosphate, the schiff base is then reduced by NaBH₄ to form ϵ -(5-phosphopyridoxyl)lysine (Fig. 3.1 E). This compound has an absorption maxima at 325 nm and fluorescence emission maxima around 390 nm.

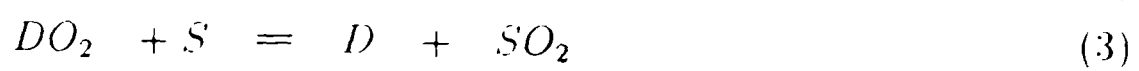
Chemical modification has long been an important method for studying the role of cysteines in enzyme function, because many reagents are known which react preferentially with sulfhydryl groups. The sulfhydryl groups of enzymes possess a high degree of reactivity and interact with a wide variety of thiol reagents. This property permits their chemical modification with highly selective and specific reagents that react stoichiometrically under relatively mild conditions. These reagents are particularly attractive for modification studies on ribosomes because cysteine is the scarcest residue in ribosomal proteins. Variety of thiol reagents have been found to impair the activity of ribosomes in protein synthesis (Traut and Haenni, 1967; Retsema and Conway, 1969 & 1971; Moore, 1973). N-ethylmaleimide (NEM) is one such reagent which reacts with sulfhydryl groups of proteins to form stable thiol esters (Fig. 3.1 F). The alkyl derivative formed is resistant to acid hydrolysis.

There are very few reports on the chemical modification of protein S1.

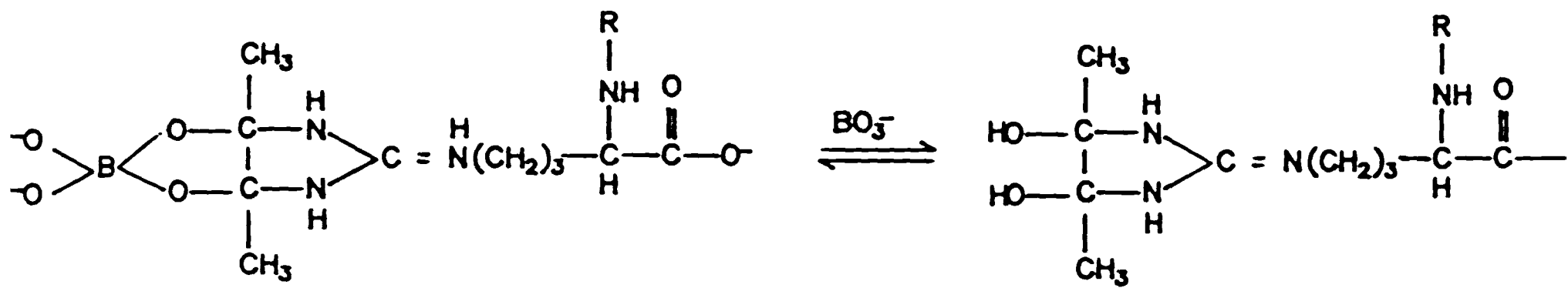
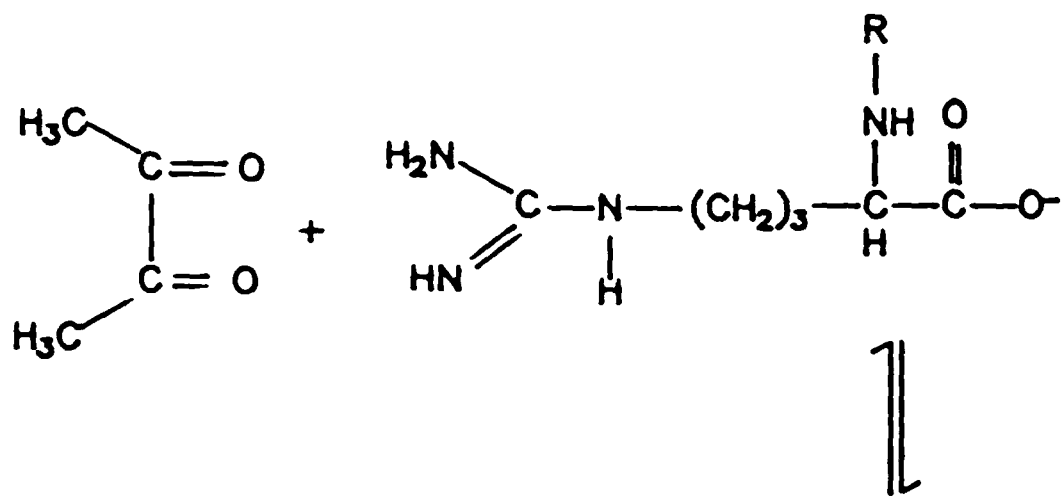
- ig. 3.1 (A): Reaction of diethyl pyrocarbonate (DEPC) with histidine residues in proteins. 6
 DEPC ($\text{C}_2\text{H}_5\text{OCO})_2\text{O}$) reacts with histidyl residues to yield N-carbethoxyhistidyl derivative.
- (B): Mechanism of photochemical oxidations (Oster *et al* 1959).
 D, D^* and S represent the dye, an excited electronic state of the dye and substrate respectively. $h\nu$ represents light radiation.
- (C): Mechanism of reaction of 2,3-butanedione with arginyl residues in proteins.
 A cyclic borate ester is formed following initial condensation of the guanidinium group with 2,3- butanedione.
- (D): Mechanism of reaction of tetranitromethane (TNM) with tyrosyl residues in proteins.
 TNM ($\text{C}(\text{NO}_2)_4$) reacts with tyrosine and forms 3-nitrotyrosine.
- (E): Mechanism of reaction of pyridoxal 5-phosphate (PLP) with lysine residues in proteins.
 PLP reacts with ϵ - NH_2 group of lysine and forms schiffs base. This reaction is reversible and upon NaBH_4 reduction yields ϵ -(5'- phosphopyridoxyl)lysines.
- (F): Mechanism of reaction of N-ethylmaleimide (NEM) with cysteine residues in proteins.
 NEM reacts with cysteine and yields S-(ethylsuccinimido)-cysteine.



A



B



C

Fig. 3.1

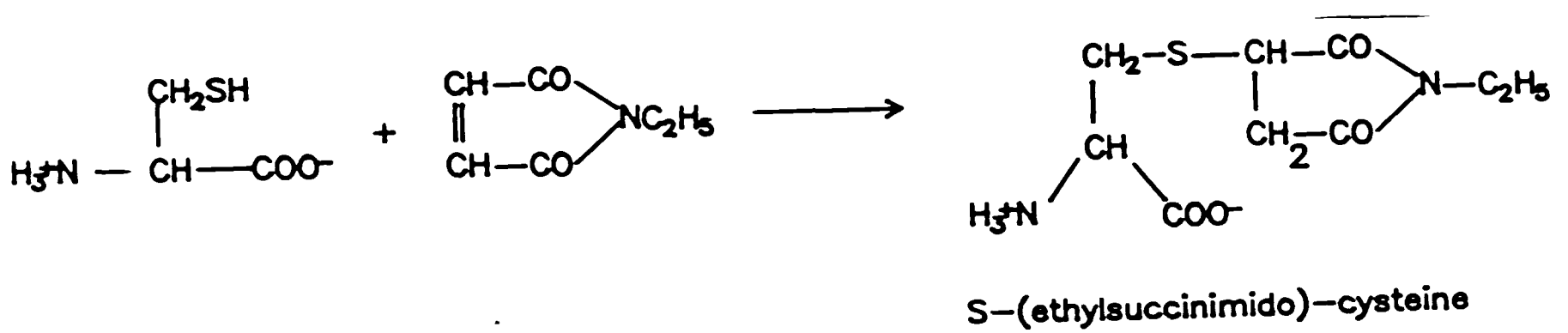
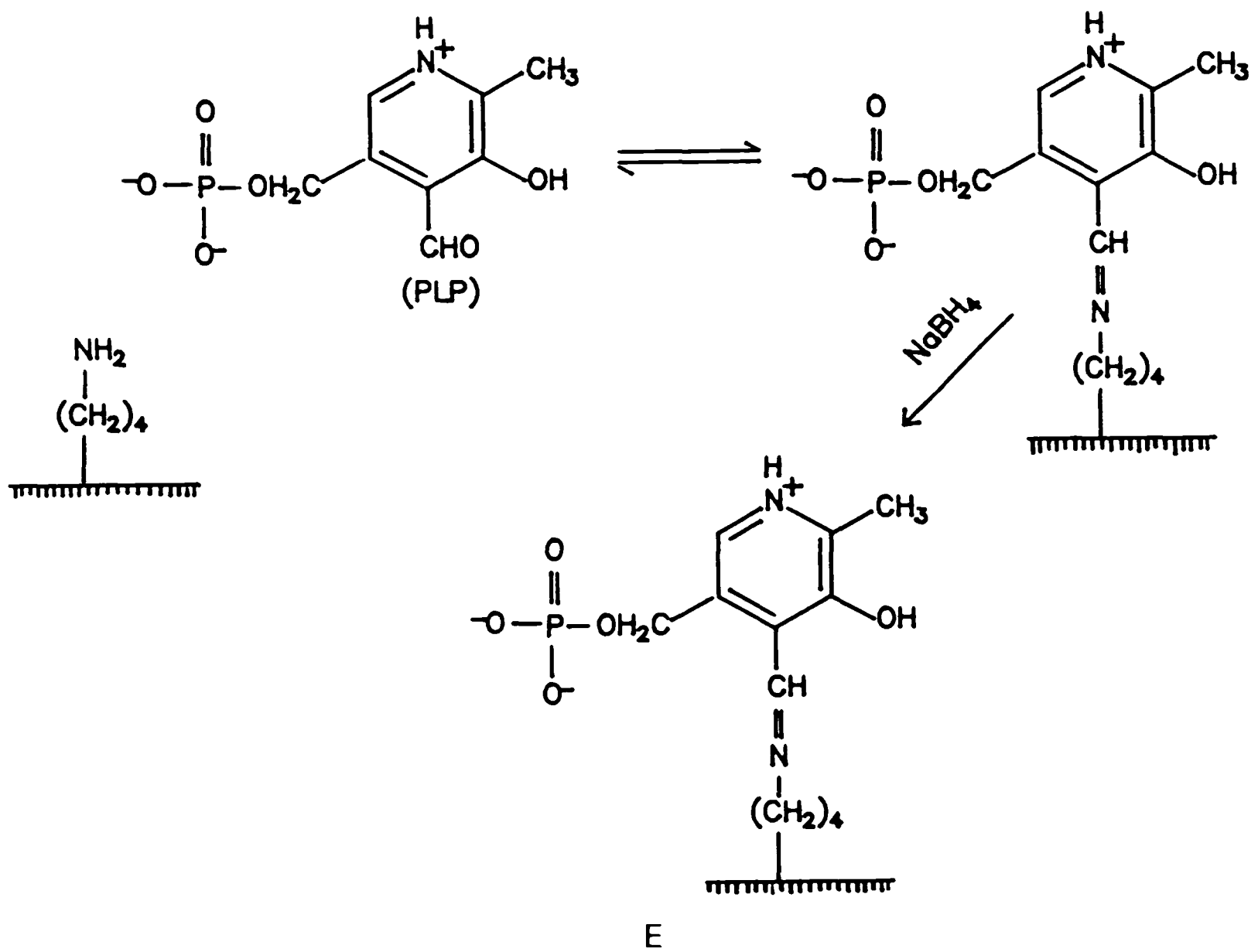
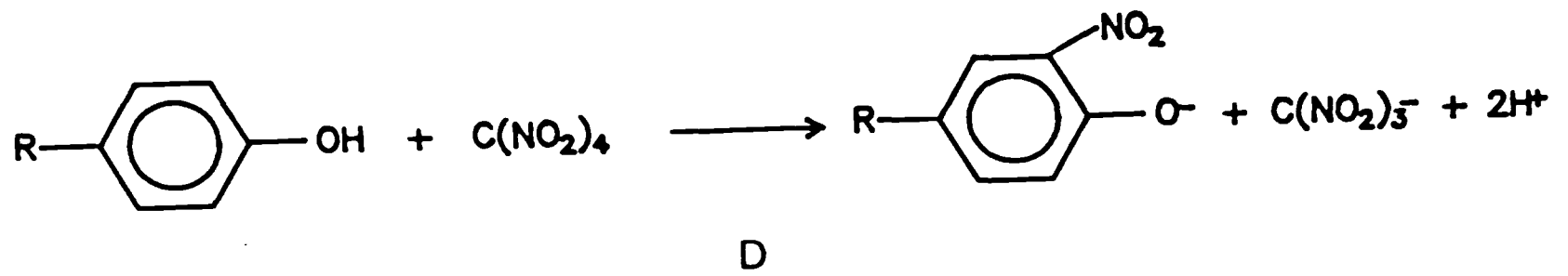


Fig. 3.1

Khanah *et al* (1978) modified lysine residues by reductive methylation. Protein S1 was treated with formaldehyde and then reduced with NaBH_4 . They have reported that, modification of lysine residues abolished the polyuridylylate binding of S1. NEM modification of S1 has been reported to impair the helix destabilizing property of ribosomal protein S1 (Kolb *et al* 1977; Thomas *et al* 1978; Thomas and Szer, 1982). However, NEM treated S1 with both SH groups alkylated was functionally active in *in vitro* protein synthesis (Suryanarayana and Subramanian, 1983).

MATERIALS AND METHODS

Ribosomal protein S1 was purified from *E.coli* by affinity chromatography of ribosomes on poly (U)–Sepharose 4B followed by DEAE cellulose chromatography (for details see chapter II). Histidine, arginine, tyrosine, lysine, and cysteine residues in S1 were modified by treating with specific chemical reagents. Complete details of modification reactions carried out have been described in chapter II. Summary of the reaction conditions for amino acid modifications are given in Table 3.1. Excess or unreacted reagent is generally removed by extensive dialysis against 10 mM Tris–HCl, pH 7.6 buffer containing 7 mM 2–mercaptoethanol. The effect of chemical modification of amino acid residues on the function of protein S1 was studied by following assays.

1. Binding of S1 to ssRNA.
2. Binding of S1 to ribosomes.
3. Binding of S1 to ssDNA and
4. Stimulation of poly (U) directed *in vitro* translation by S1.

Reagent	Modified amino acid residue	Buffer	Temp	Time (min)	Ref
Diethylpyro carbonate	His	10 mM Phosphate pH 6.0, 0.1 M KCl	14 °C	60	Baxter et al (1978)
Rose bengal dye	His	10 mM Tris-HCl pH 7.6 5 mM 2-Me	25 °C	35	Jonak and Rychilik (1980)
2,3-butane-dione	Arg	50 mM Borate pH 9.0, 5 mM 2-Me	30 °C	30	Rohrbach and Bodely (1977)
Tetranitro methane	Tyr	50 mM Tris-HCl pH 8.0 100 mM KCl	21 °C	30	Riordan and Vallee (1972)
Pyridoxal-5-phosphate	Lys	20 mM Triehanolam- mine-HCl pH 7.8 30 mM KCl	37 °C	12	Ohsawa and Gualerzi (1981)
N-ethylmale-imide	Cys	0.1 M Tris-HCl pH 7.6 5 mM Na ₂ EDTA	37 °C	30	Suryanarayana & Subramanian, 1983

Table 3.1. Summary of reaction conditions.

Methods used for binding of S1 to ssRNA, ssDNA and ribosomes, assay system used for poly (U)-dependent polyphenylalanine synthesis are described in chapter II. Specific details of the methods used are mentioned below.

BINDING OF S1 TO ssRNA

The ssRNA binding activity of protein S1 was tested by passing S1 through poly (U)-Sepharose column (Carimichael, 1975; Suryanarayana and Subramanian, 1979) and then eluting with increasing concentration of salt and finally with salt plus urea. Normal S1 is adsorbed to poly (U)-Sepharose strongly even in the presence of high concentration of salt (1 M NH_4Cl) and is released only when eluted with 6–8 M urea. From this experiment not only binding of S1 to poly (U) but also salt sensitivity of the binding can be analyzed.

BINDING OF PROTEIN S1 TO ssDNA

ssDNA binding activity of protein S1 was analyzed by agarose gel mobility shift assay using M13 ssDNA. Protein S1 was incubated with M13 ssDNA and the complexes formed were electrophoresed in agarose gels.

BINDING OF S1 TO 30S SUBUNITS

30S ribosomal subunits were isolated from 70S tight couples by density gradient centrifugation. 30S ribosomal subunits lacking S1 (30S(-1)) were prepared by passing 30S through poly (U)-Sepharose 4B column followed by elution of the S1 deprived subunits with 1 M NH_4Cl . Reconstitution of S1 deprived 30S ribosomal subunits with S1 was performed at two fold excess of S1 to 30S(-1). Ribosomal subunits were separated from excess S1 by DEAE

Fig. 3.2 (A): SDS-polyacrylamide slab gel electrophoretic analysis of fractions obtained after poly (U)-Sepharose chromatography of *E.coli* ribosomes.

Lane 1: *E.coli* ribosomes (1.0 A₂₆₀ unit).

Lane 2: Ribosomes eluted by 1 M NH₄Cl buffer (1.0 A₂₆₀ unit).

Lane 3: Purified protein S1 (2.0 μg).

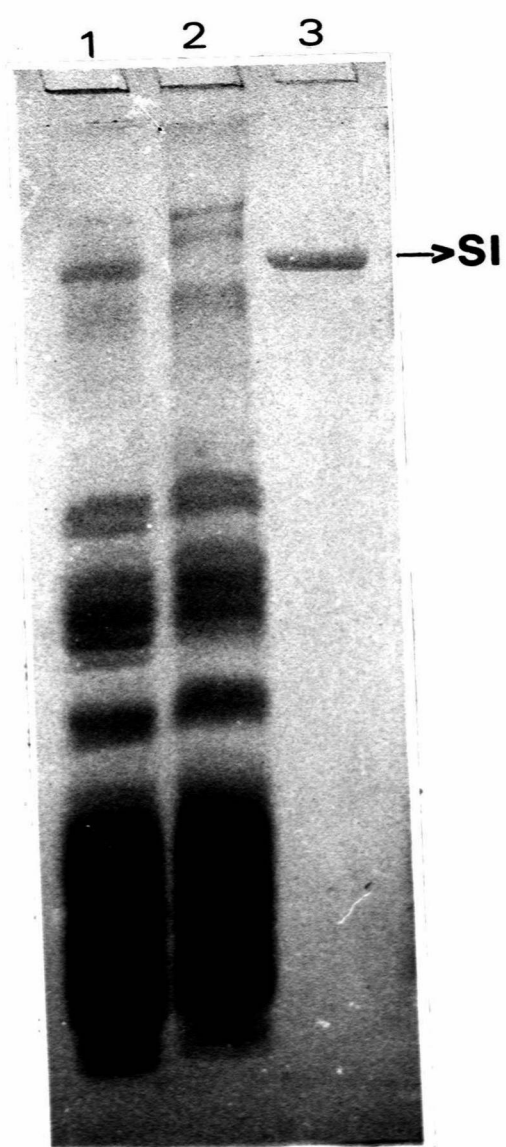


FIG 3-2(A)

Fig. 3.2 (B): DEAE-cellulose chromatography of S1 fraction obtained from poly (U)-Sepharose column.

Column chromatography was carried out as described in chapter II.

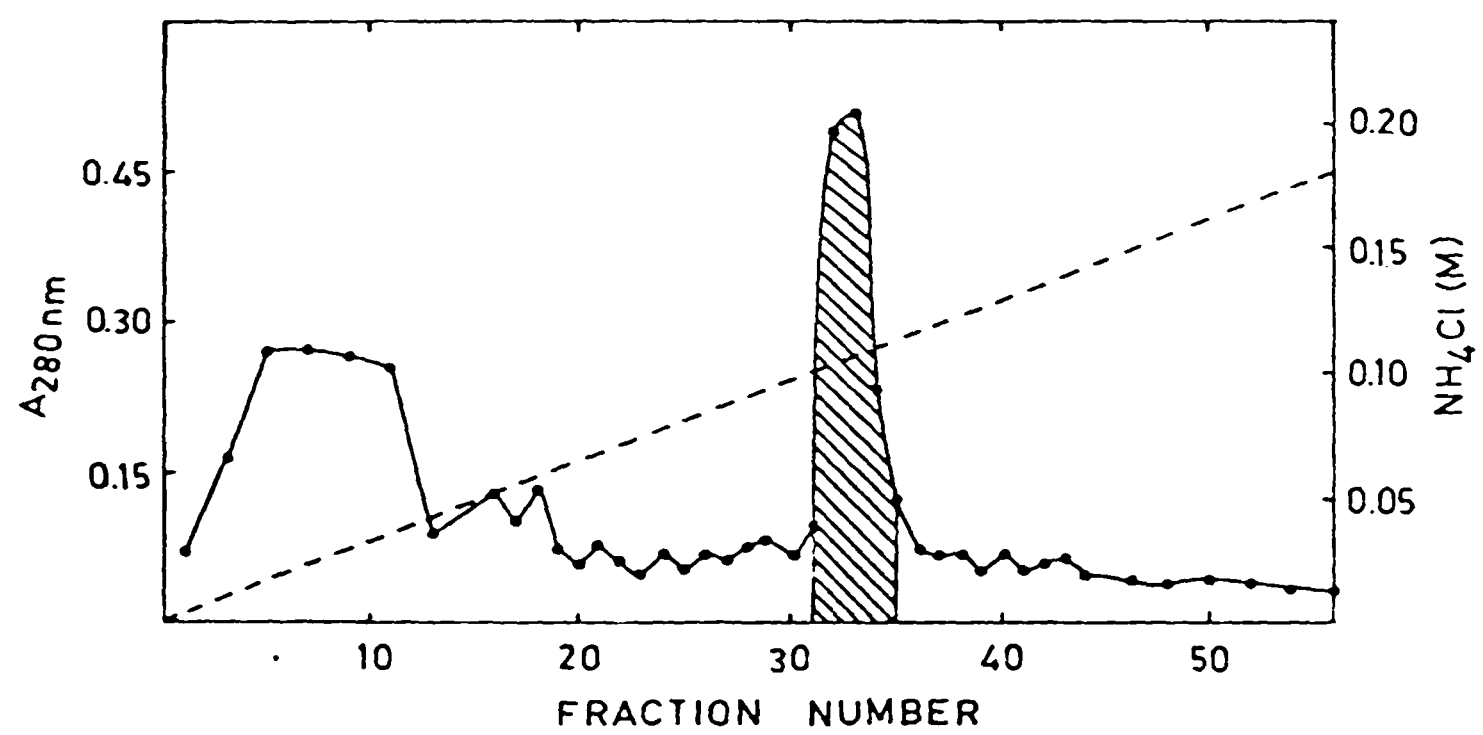


Fig. 3.2 (B)

SPECTRAL PROPERTIES OF CHEMICALLY MODIFIED S1

The time course of the DEPC reaction with S1 is presented in Fig. 3.3 (A). The addition of DEPC to protein resulted in a rapid increase in the absorbance of protein at 242 nm. However, there was no change in the absorption of protein at 280 nm during DEPC reaction. When there is no further increase in the absorbance at 242 nm (after 60 min), the DEPC reaction was terminated by adding 0.1 M histidine to a final concentration of 10 mM. The reaction mixture was immediately dialyzed against 10 mM Tris-HCl, pH 7.6 and 7 mM 2-mercaptoethanol.

The UV absorption and 4th derivative spectra of unmodified and DEPC modified S1 are presented in Fig. 3.3 (B) & (C) respectively. The increase in the absorbance at 242 nm during DEPC treatment indicates that available histidine(s) are modified to N-ethoxyformyl histidine(s). Both the UV absorption maxima (at 280 nm) and their 4th derivative spectra of unmodified and DEPC modified S1 were similar in the region between 275 to 300 nm. The spectrophotometric study of DEPC reaction clearly indicates that, the DEPC reagent is selective in modification of histidine(s). No tyrosine residues are modified under the above conditions. The difference in the absorbance at 242 nm between an equal concentration of DEPC modified S1 and unmodified S1 proteins was used to calculate the number of ethoxyformyl histidine(s). Out of 8 histidines, two were modified by the reagent.

The UV absorption and 4th derivative spectra of unmodified and RB modified S1 are shown Fig. 3.4 (A) & (B) respectively. The UV absorption spectra of the RB treated S1 showed a blue shift with λ maximum of 270 nm. However, the 4th derivative spectra of unmodified S1 and RB modified S1 did

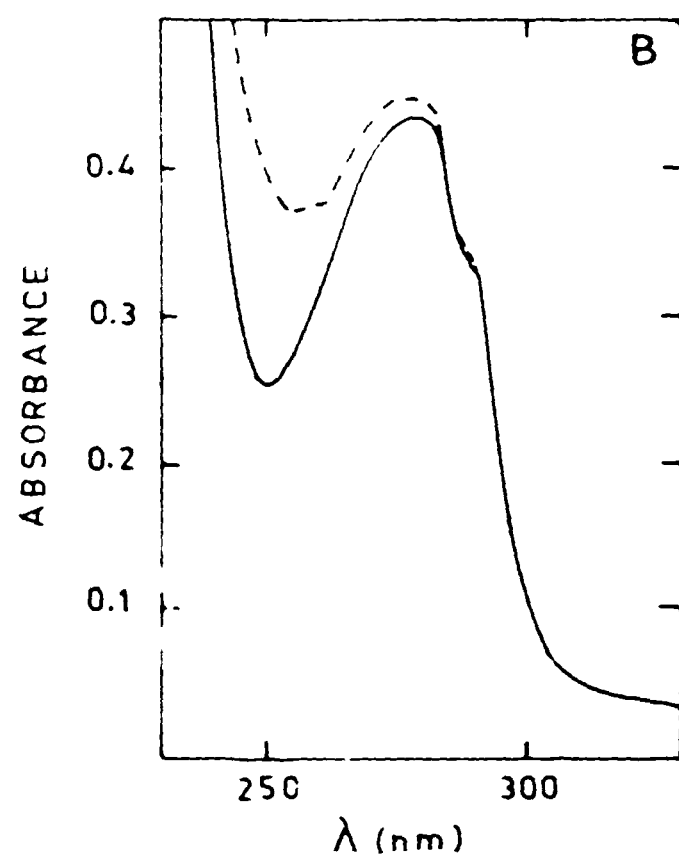
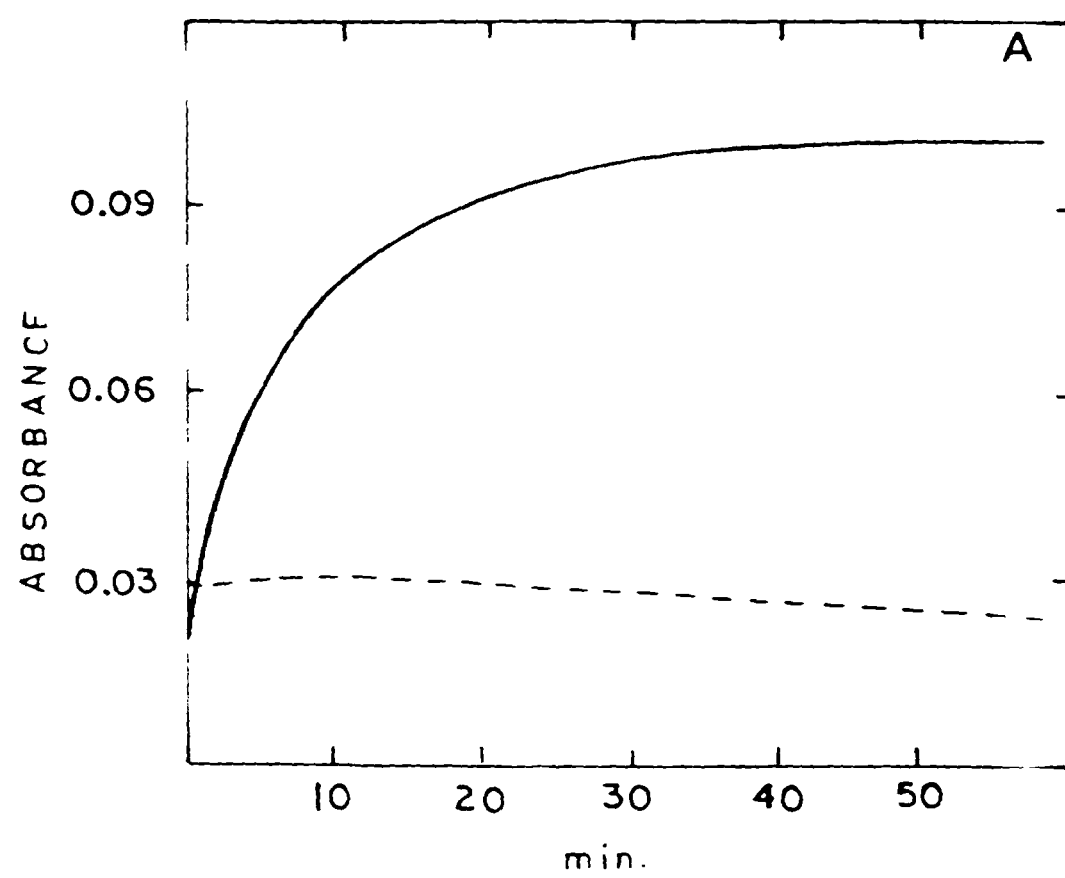


FIG. 3.3

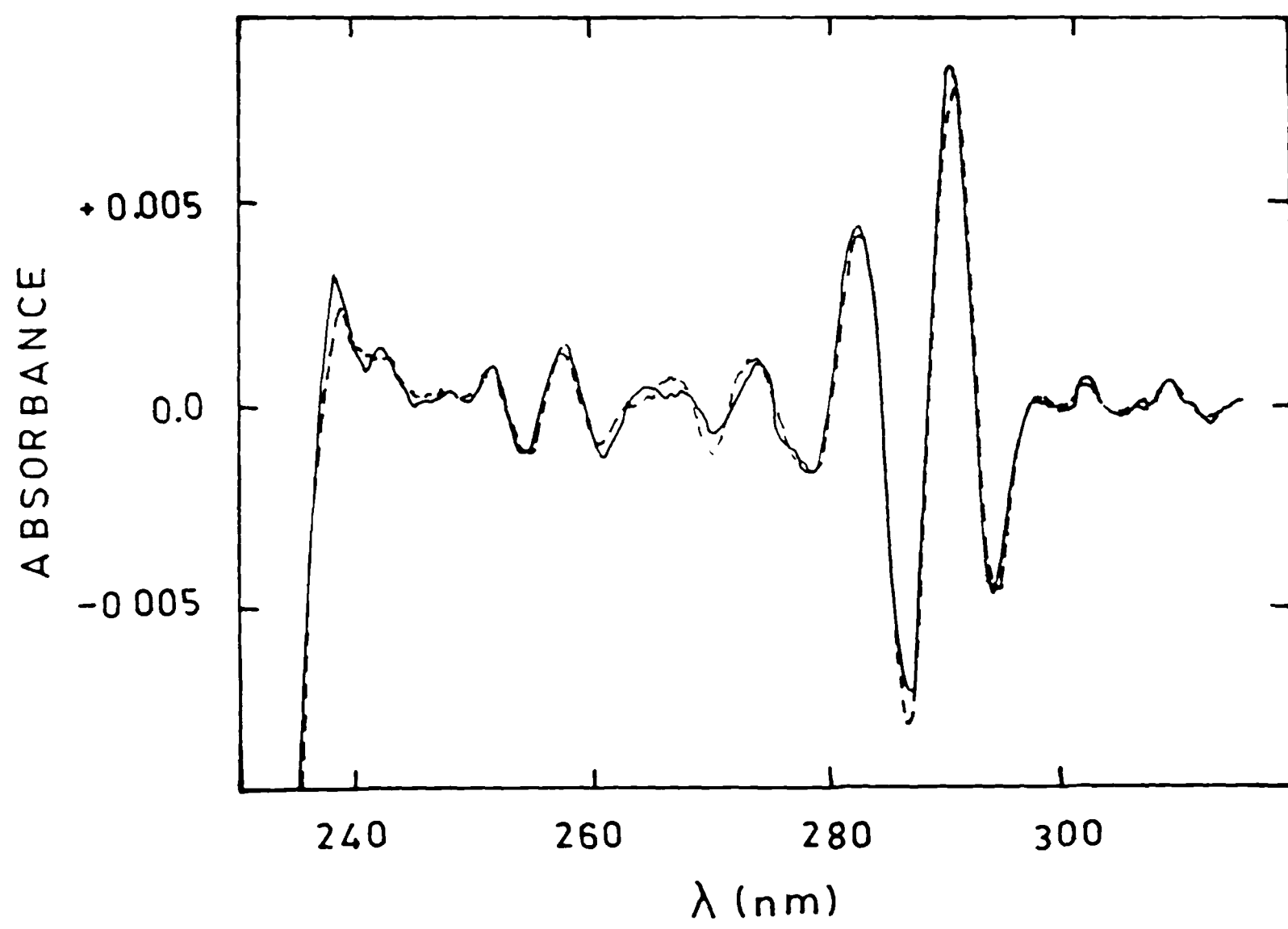


Fig. 3.3 (C)

g. 3.4 (A): UV absorption spectra of unmodified S1 (solid line) and S1 modified with rose bengal (RB) (broken line).

The concentration of unmodified and modified S1 were both 0.2 mg/ml in 10 mM Tris-HCl (pH 7.6) and 7 mM 2- mercaptoethanol.

(B): Fourth derivative spectra of unmodified S1 (solid line) and S1 modified with rose bengal (RB) (broken line).

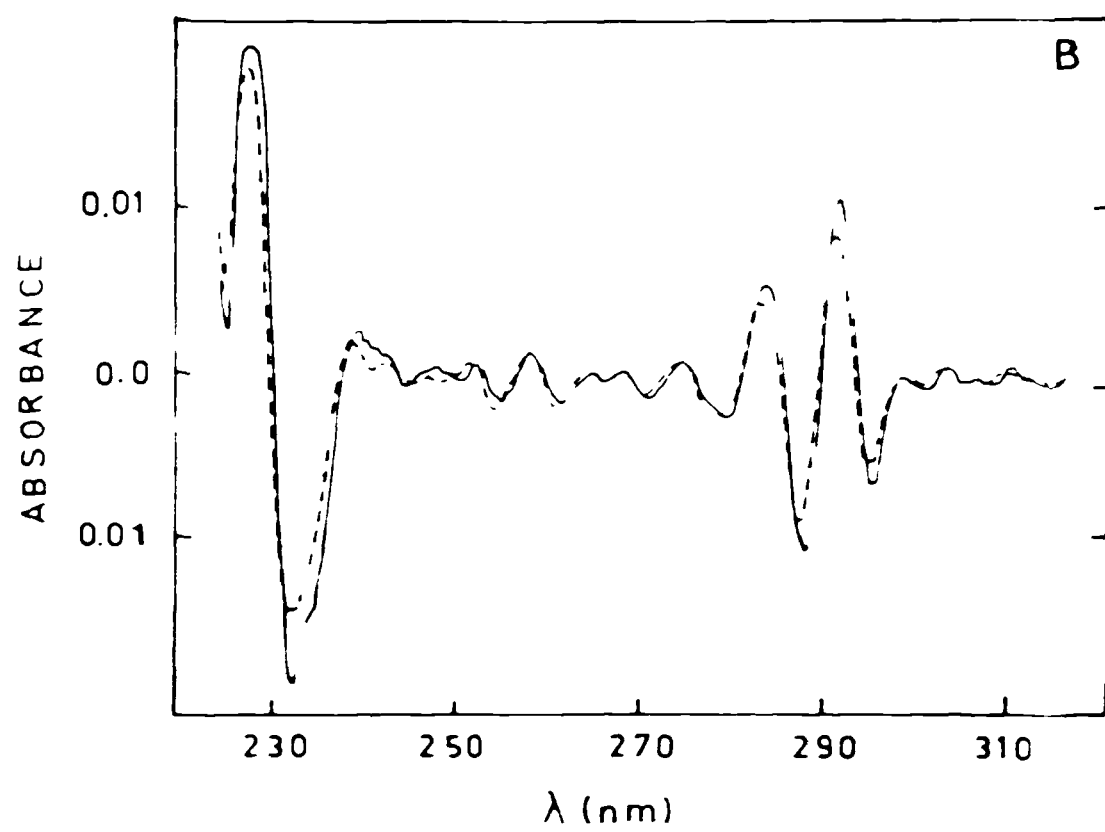
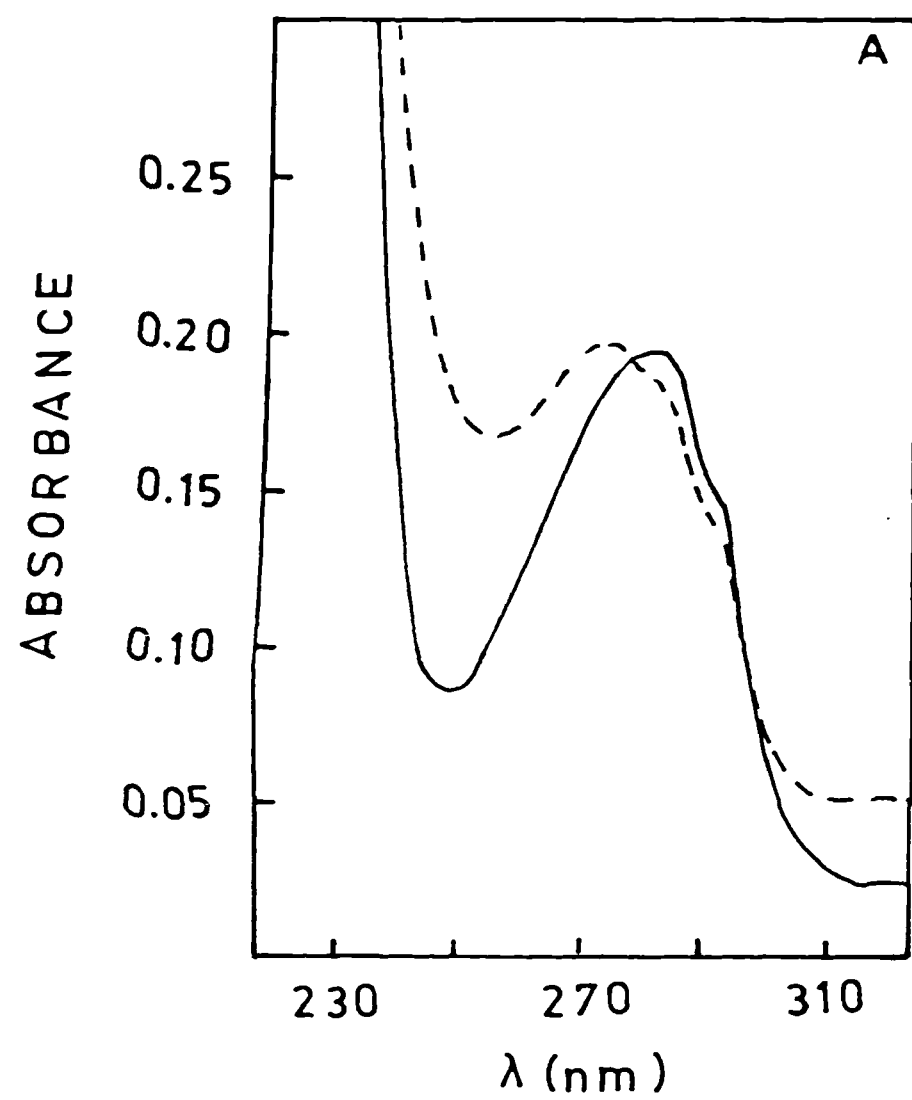


Fig.3.4

not reveal any changes. Polypeptide fragments of S1 namely S1F4 and S1F1 were also photooxidized with RB dye as described above.

The UV absorption (Fig. 3.5 A) and 4th derivative (Fig. 3.5 B) spectra of 2,3-butanedione modified S1 were recorded and compared with those of unmodified S1. The spectral properties of both unmodified and modified S1 samples were similar. However, differences were observed in the lower wavelength region (< 265 nm). The treated S1 showed greater absorption in region between 220 to 240 nm. Further derivative spectrum showed reduced intensity of the bands due to phenylalanine. These results indicate possible structural changes in the polypeptide backbone.

The UV absorption (Fig. 3.6 A) and 4th derivative (Fig. 3.6 B) spectra of both unmodified and TNM modified S1 were recorded in a spectrophotometer. TNM modifies tyrosine residues of the protein to 3-nitrotyrosine. This compound has an absorption maximum at 425 nm. A broad absorption peak of 3-nitrotyrosine at 400–450 nm was observed in the case of TNM modified S1 (Fig. 3.6 C). The 4th derivative spectrum of TNM modified S1 shows high absorption bands for tryptophan and lower intense bands in the regions corresponding to tyrosine.

The UV absorption and 4th derivative spectra of unmodified and PLP modified S1 are presented in Fig. 3.7 (A) & (B) respectively. The visible absorption spectra (Fig. 3.7 C) of PLP modified S1 clearly showed the presence of an absorption peak at 325 nm, indicating the presence of modified lysine residues. The characteristic S1 absorption spectrum has also changed with a large blue shift with λ maximum of 260 nm.

Fig. 3.5 (A): UV absorption spectra of unmodified S1 (solid line) and S1 modified with 2,3-butanedione (broken line).

The concentration of unmodified and modified S1 were both 0.7 mg/ml in 10 mM Tris-HCl (pH 7.6) and 7 mM 2-mercaptoethanol.

(B): Fourth derivative spectra of unmodified S1 (solid line) and S1 modified with 2,3-butanedione (broken line).

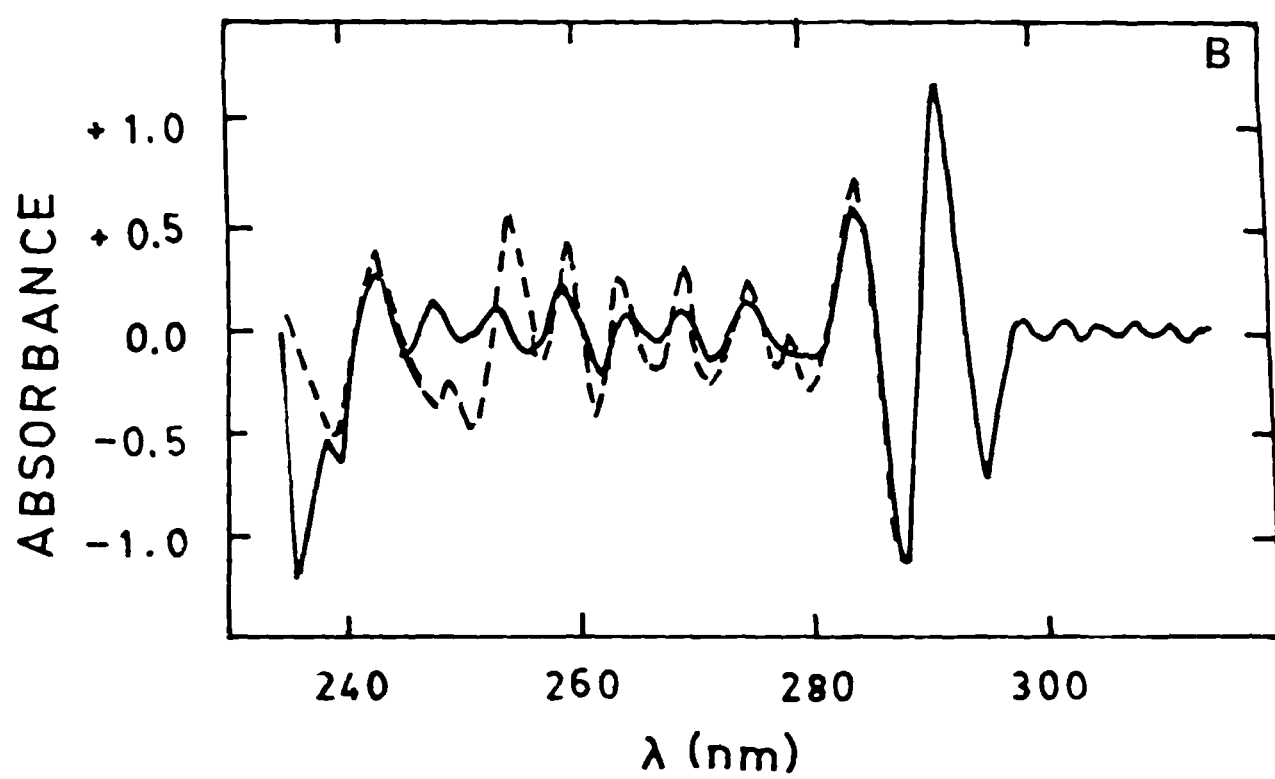
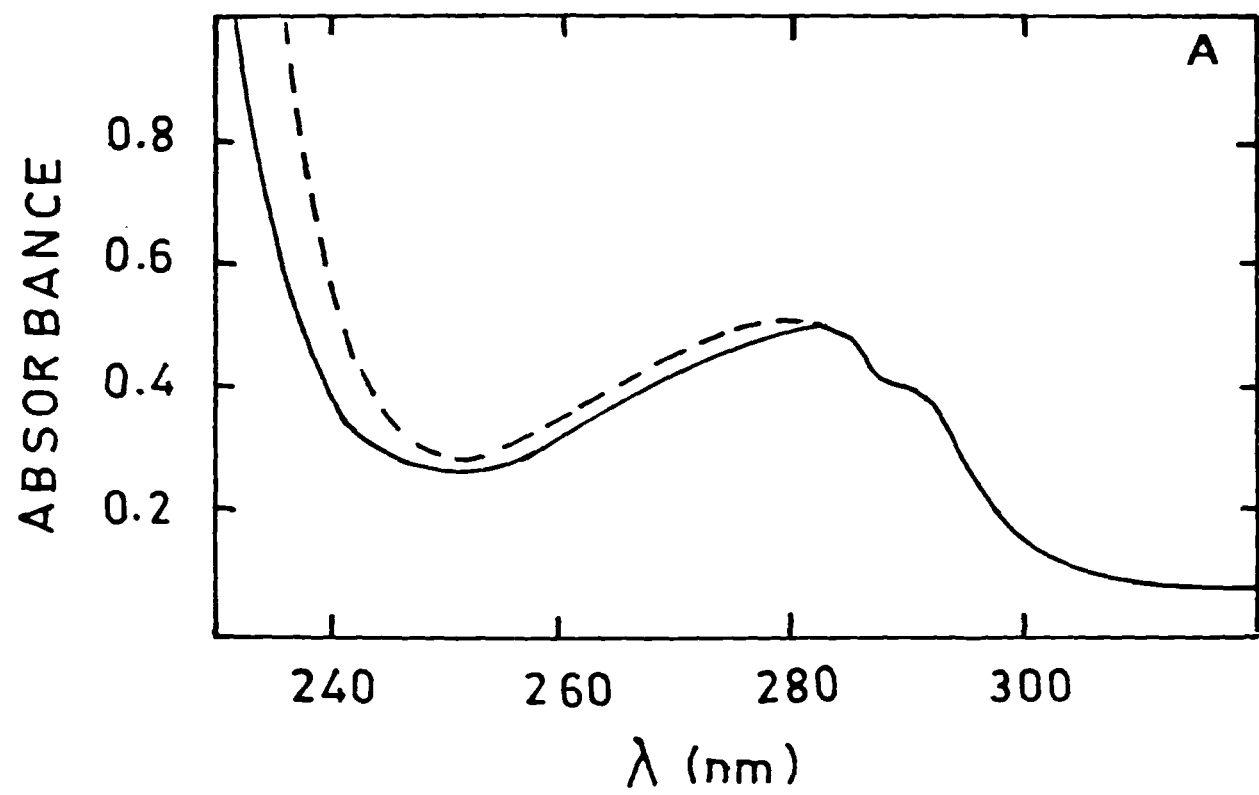


Fig. 3.5

Fig. 3.6 (A): UV absorption spectra of unmodified S1 (solid line) and S1 modified with tetranitromethane (TNM) (broken line).

The concentration of unmodified and modified S1 were both 0.3 mg/ml in 10 mM Tris-HCl (pH 7.6) and 7 mM 2-mercaptoethanol.

(B): Fourth derivative spectra of unmodified S1 (solid line) and S1 modified with TNM (broken line).

(C): Visible absorption spectra of unmodified S1 (solid line) and S1 modified with tetranitromethane (TNM) (broken line).

The concentration of unmodified and modified S1 were both 0.3 mg/ml in 10 mM Tris-HCl (pH 7.6) and 7 mM 2-mercaptoethanol.

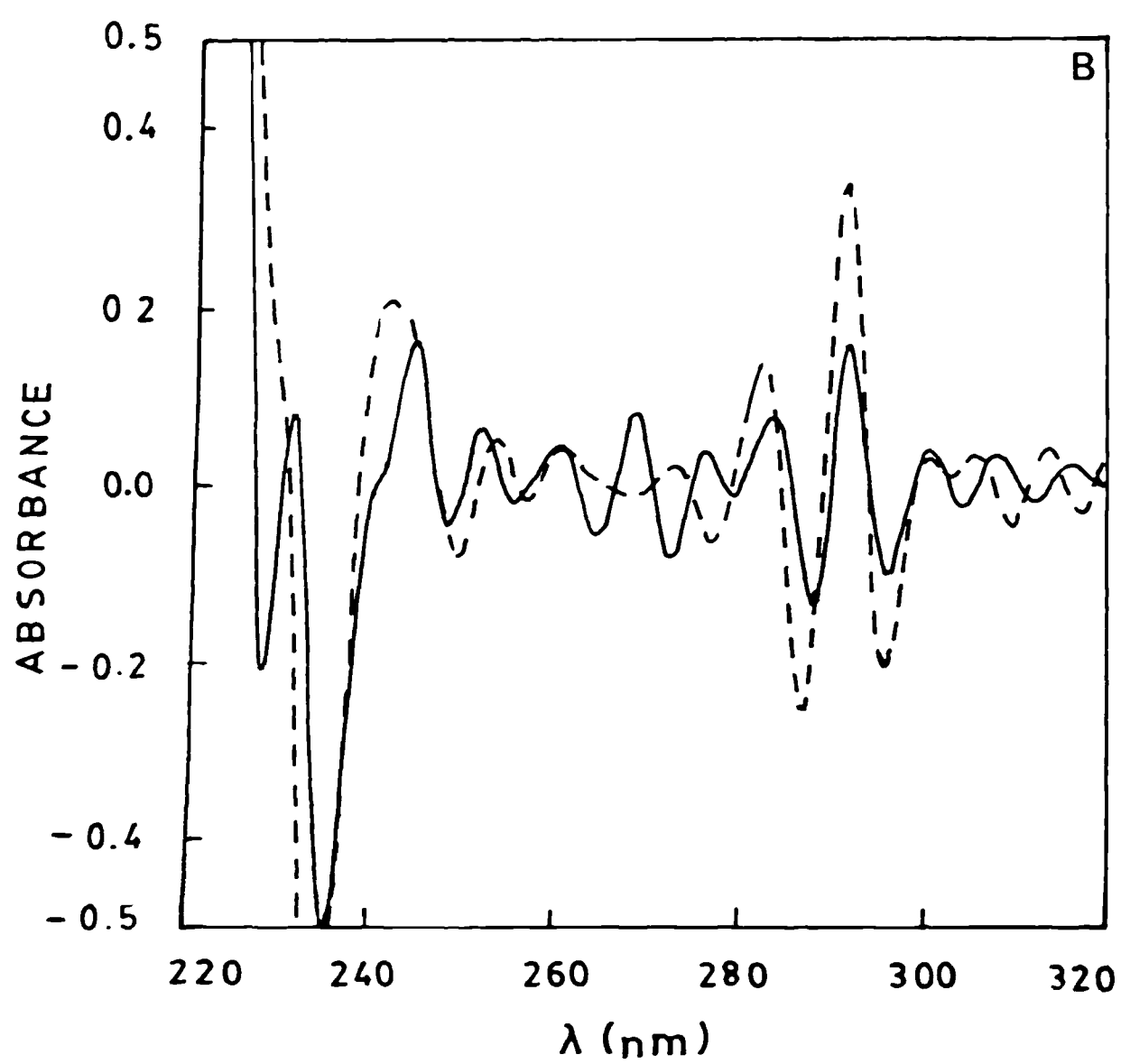
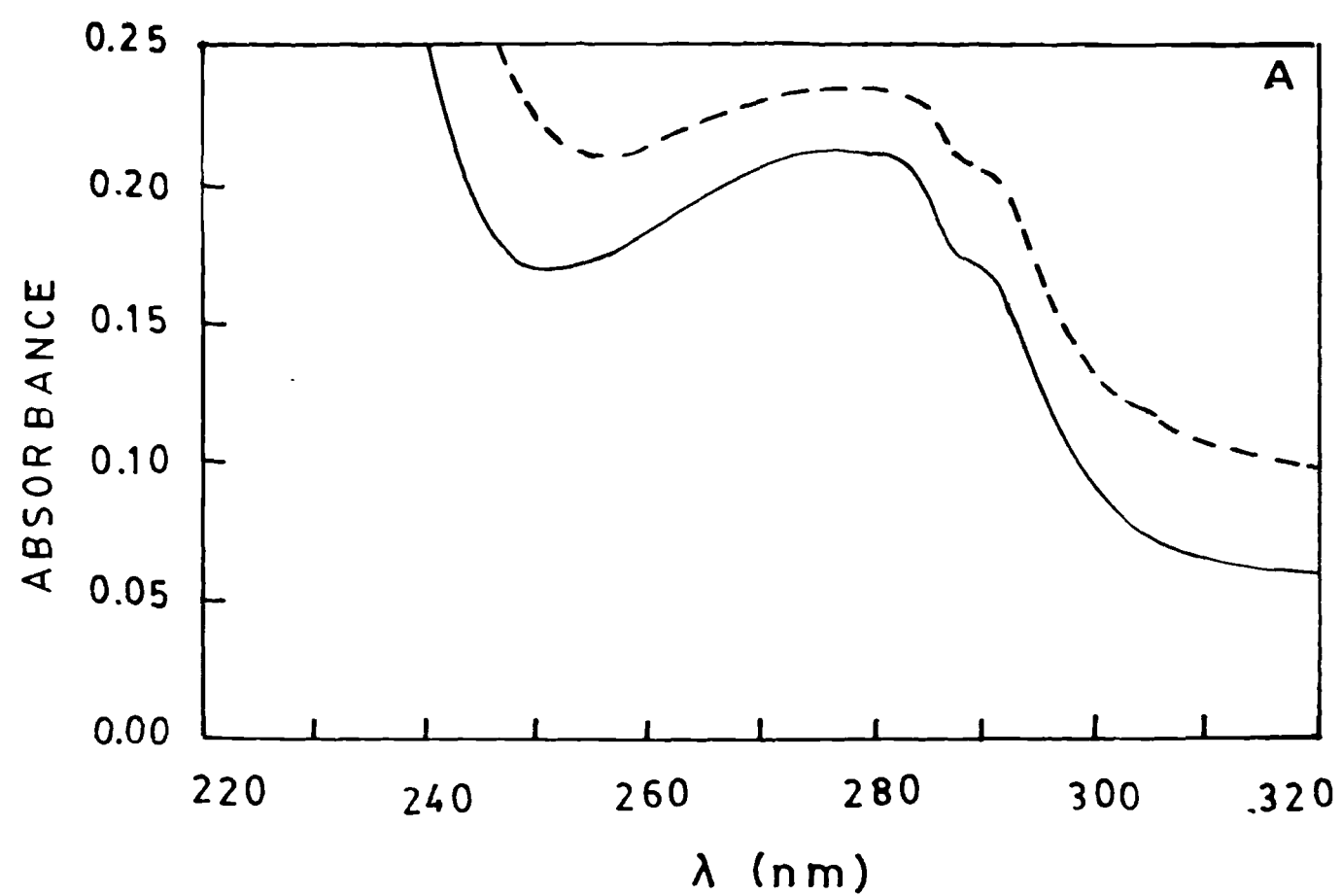


Fig. 3.6

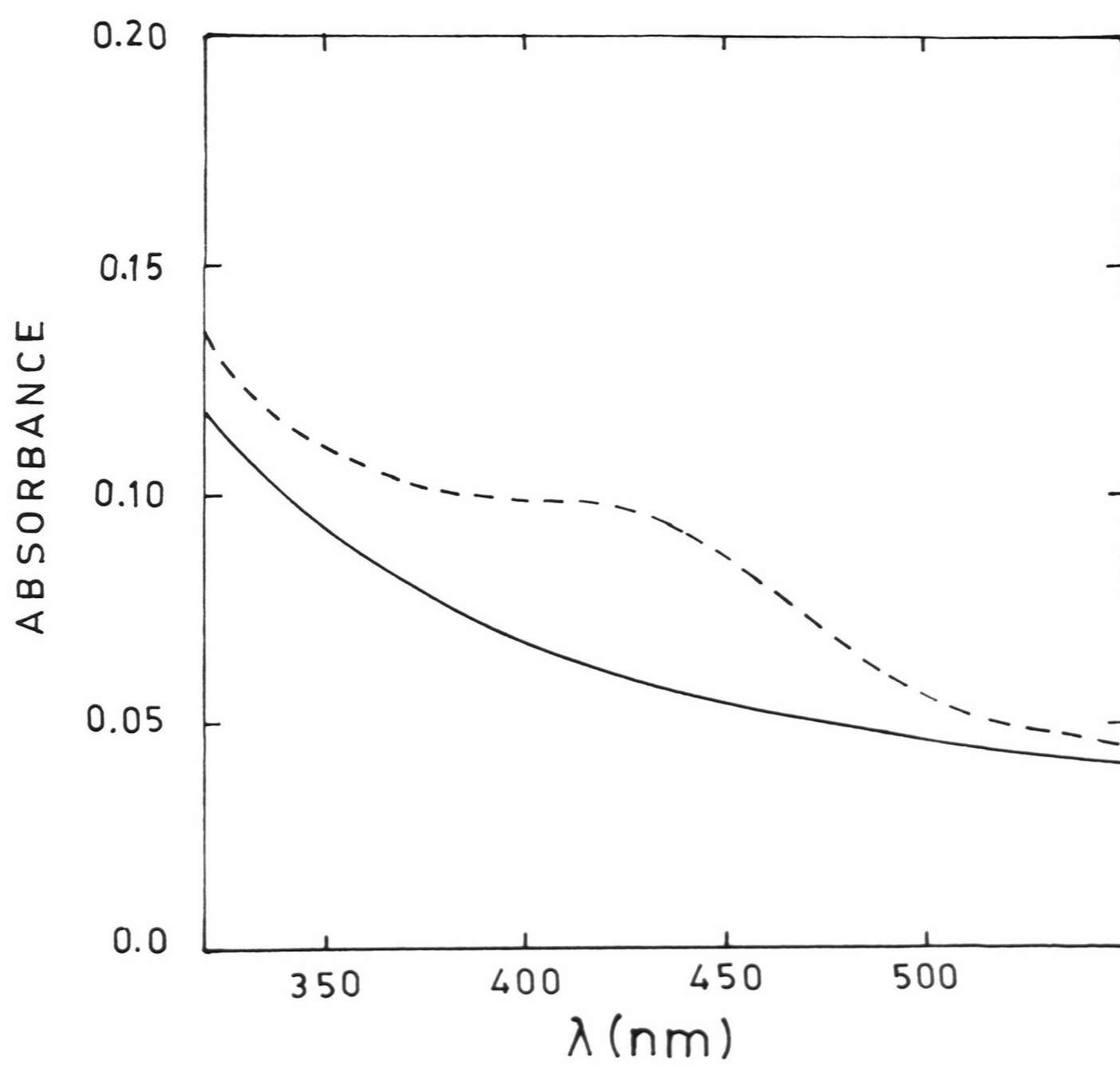


Fig. 3.6(C)

3.7 (A): UV absorption spectra of unmodified S1 (solid line) and S1 modified with pyridoxal 5-phosphate (PLP) (broken line).

The concentration of unmodified and modified S1 were both 0.5 mg/ml in 10 mM Tris-HCl (pH 7.6) and 7 mM 2-mercaptoethanol.

(B): Fourth derivative spectra of unmodified S1 (solid line) and S1 modified with PLP modified S1 (broken line).

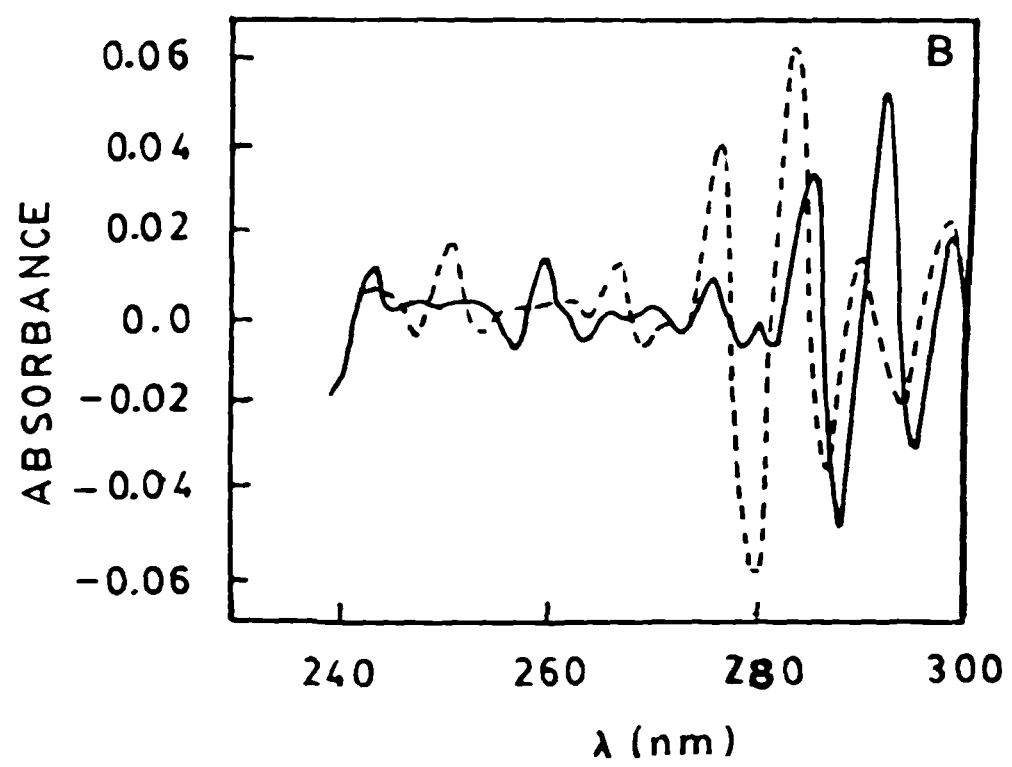
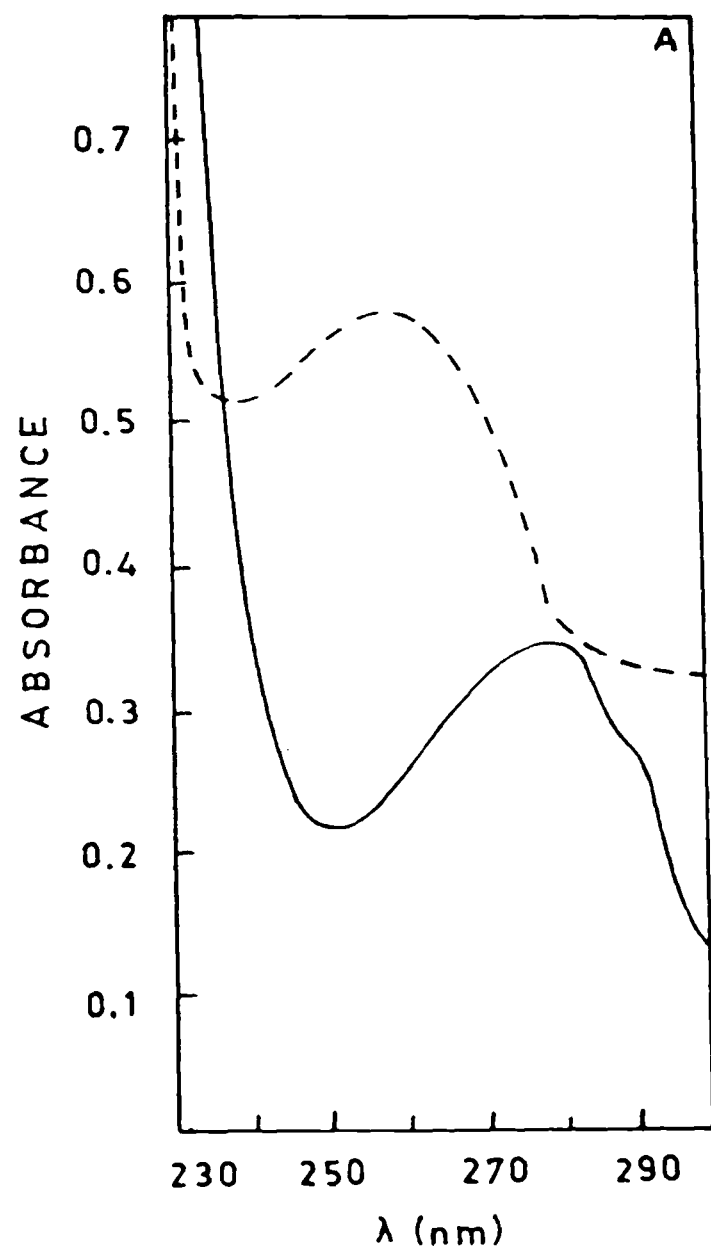


Fig. 3.7

3.7 (C): Visible absorption spectra of unmodified S1 (solid line) and S1 modified with pyridoxal 5-phosphate (PLP) (broken line).

The concentration of unmodified and modified S1 were both 0.5 mg/ml in 10 mM Tris-HCl (pH 7.6) and 7 mM 2-mercaptoethanol.

(D): Fluorescence emission spectra of unmodified S1 (solid line) and S1 modified with PLP (broken line).

Emission spectra were recorded by excitation at 325 nm. The concentration of unmodified and modified S1 were both 4.5 μ g/ml in 10 mM Tris-HCl (pH 7.6) and 7 mM 2-mercaptoethanol.

(E): Fluorescence emission spectra of protein S1 modified with pyridoxal 5-phosphate (PLP) in the presence (broken line) and absence (solid line) of poly (U) .

Emission spectra were recorded by excitation at 325 nm. The concentration of S1 modified with PLP in the presence and absence of poly (U) were both 5.0 μ g/ml in 10 mM Tris-HCl (pH 7.6) and 7 mM 2-mercaptoethanol. Protein S1 used was 5.0 μ g/ml in 10 mM Tris-HCl (pH 7.6) and 7 mM 2-mercaptoethanol.

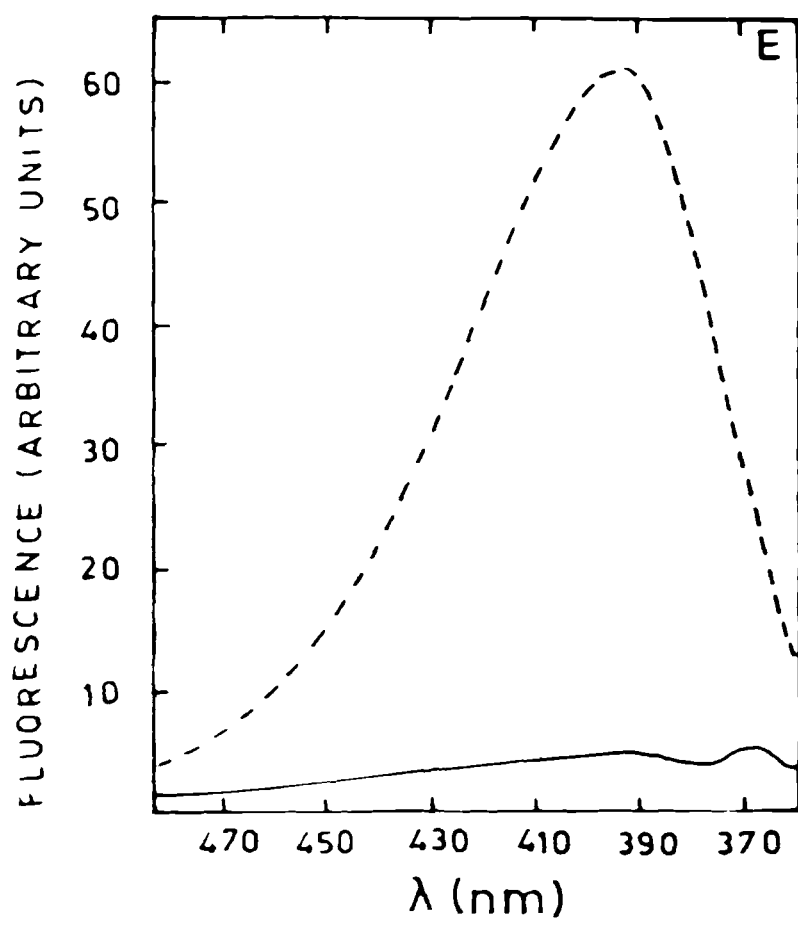
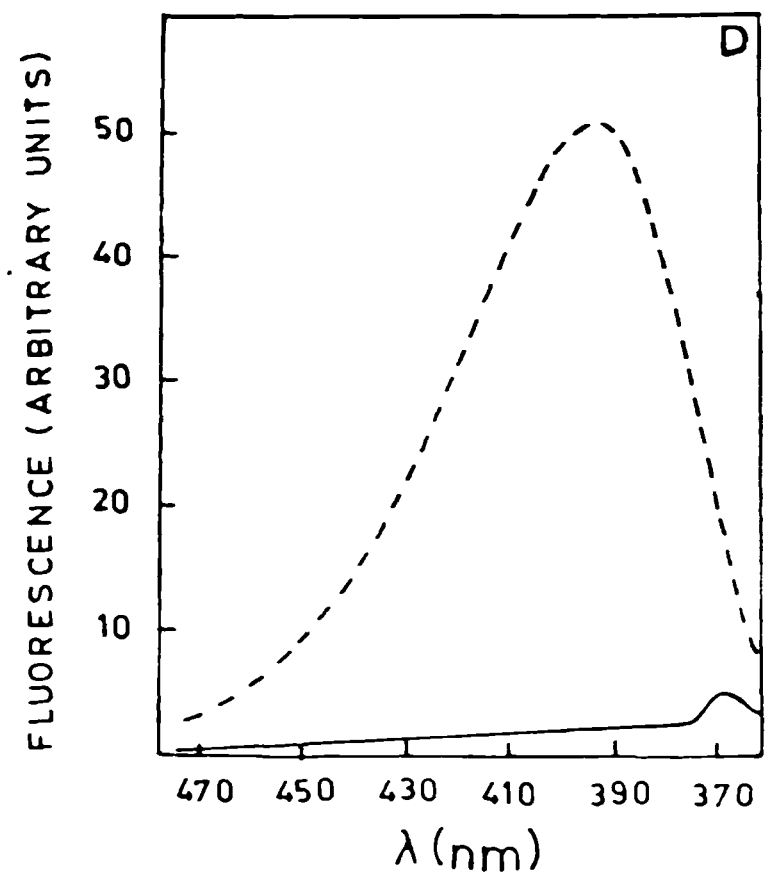
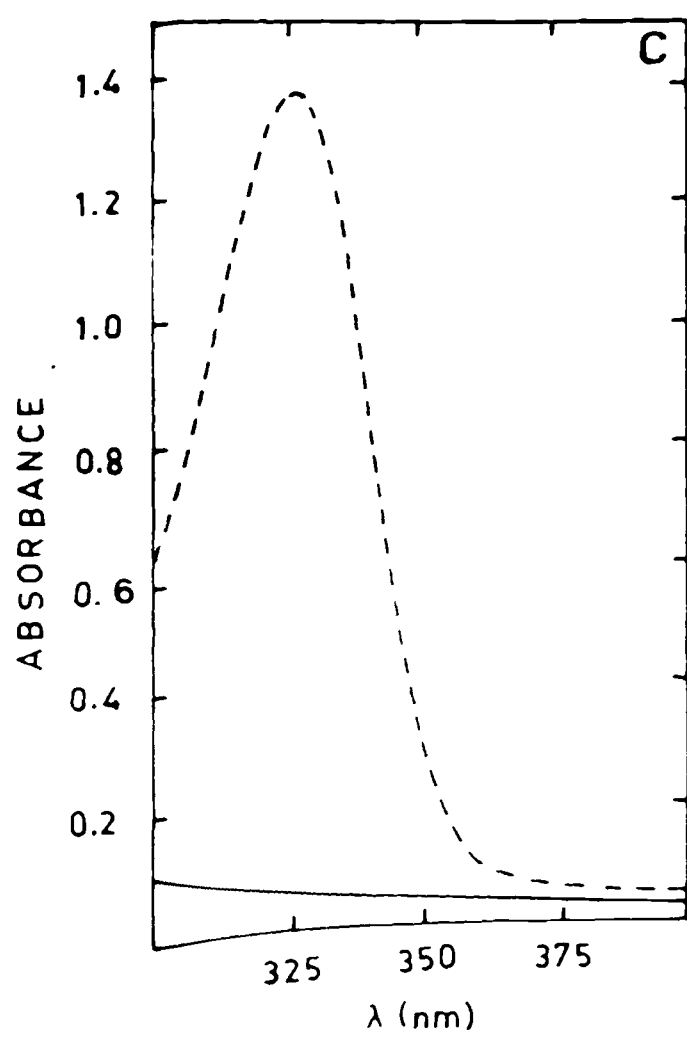


Fig. 3.7

Fluorescence emission spectra of PLP treated S1 was recorded to look for the presence of ϵ -(5-phosphopyridoxyl)lysine. The fluorescence emission spectra of both PLP modified S1 and unmodified S1 is given in Fig. 3.7 (D). When excitation was carried out at 325 nm, the PLP modified S1 gave an emission maximum around 395 nm unlike unmodified S1. The absorption and fluorescence emission spectra of PLP modified S1 indicate the formation of ϵ -(5-phosphopyridoxyl)lysine residues which showed absorption maximum at 390 nm. Calculation from the known molar absorption coefficient of ϵ -(5-phosphopyridoxyl) lysine (15, 800) at 325 m μ showed that 8 lysine residues in S1 were modified with PLP under these conditions. We have also carried out PLP modification of S1 in the presence of poly (U). PLP was added to protein S1 previously incubated with poly (U) and the modification was carried out as described above. The fluorescence emission spectra of both PLP modified S1 and PLP modification carried in the presence of poly (U) were recorded (Fig. 3.7 E). The characteristic fluorescence emission maxima of ϵ -(5-phosphopyridoxyl)lysine is not seen when the PLP modification was carried out in the presence of poly (U) indicating that, poly (U) protects S1 from PLP modification. Binding of poly (U) to protein S1 probably masks lysine residues (Fig. 3.7 E).

The suitability of reagents for chemical modification of proteins depends not only on their specificity towards a particular functional group but also on the reaction conditions. The reaction conditions used in the present study are mild and do not expose the proteins to extreme conditions of pH or temperature.

EFFECT OF CHEMICAL MODIFICATION OF S1 ON RNA BINDING

Binding of protein S1 to ssRNA was studied by affinity chromatography on poly (U)–Sephadex column. The strength of the binding of the protein to matrix bound nucleic acid was determined by the salt concentration required for elution of the protein. Protein S1 binds strongly to poly (U)–Sephadex even in the presence of high concentration of salt and is released only when eluted with urea (Carmichael, 1975; Suryanarayana and Subramanian, 1979). Using this procedure ssRNA binding activity of the protein S1 was analyzed. Binding of unmodified S1 to poly (U)–Sephadex was carried out as a control.

Rosebengal (RB) modified S1 was passed through a small column of poly (U)–Sephadex 4B and the column was washed successively with 0.1 M, 0.25 M, 0.5 M, 1.0 M, 2.0 M NH_4Cl and finally with 1.0 M NH_4Cl /7 M urea buffer. Results from this experiment are summarized in a histogram (Fig.3.8 A). RB modified S1 bound to poly(U)–Sephadex was eluted by 0.5 M NH_4Cl buffer unlike unmodified S1 which was eluted at 1 M NH_4Cl /7 M urea buffer. Treatment with RB did not result in the loss of ssRNA binding (poly (U)) of the protein. But the RB modification has affected the salt insensitivity of the nucleic acid binding. Treatment with DEPC also resulted in decreased affinity to poly (U) and the protein eluted with 0.5 M NH_4Cl as in the case of RB modification. Thus modification of histidyl residues of S1 results in the loss of strong affinity towards nucleic acid i.e poly (U). This indicates that histidyl residues of S1 probably play an important role in the strong binding the the protein to poly (U).

We have also modified histidine residues of a functional fragment of S1

(S1F1) which retains the nucleic acid binding domain of the protein. Protein S1 was cleaved with TPCK-trypsin and the functional fragment S1F1 was isolated as described in chapter II. S1F1 was also photo chemically modified by treating with RB. The binding of the RB modified S1F1 to poly (U) was tested as described above. RB modified S1F1 did not bind to poly (U)-Sephadex and was eluted with equilibration buffer, 0.1 M NH_4Cl (Fig. 3.8 B). Unmodified S1F1 binds to poly(U)-Sephadex as strongly as protein S1 and elutes from the column with 1 M NH_4Cl /7 M urea buffer. Thus R-B modification of S1F1 results in the complete loss in the ssRNA binding activity. Our results indicate that histidyl residues present in the fragment S1F1 are involved in the ssRNA binding of protein S1 and that a region in S1 that overlaps ribosome binding region and N-terminal part of the first homologous repeat (R1) may also contribute to binding of S1 to poly (U) or stabilize the interaction of homologous repeats with poly (U).

The ssRNA binding activity of both arginine(2,3-butanedione) and tyrosine (TNM) modified proteins was analyzed as described above. Both the arginine and tyrosine modified proteins bound to poly (U)- Sephadex and were eluted with 1 M NH_4Cl /7 M urea buffer (Fig. 3.8 C & D). Thus the modification of arginyl and tyrosinyl residues by 2,3-butanedione and TNM has no effect on the RNA binding ability of the protein. Our results indicate that neither arginine nor tyrosine residues play any role in the binding of protein to poly (U).

The capacity of lysine (PLP) modified S1 to bind poly (U) was also studied. Results shown in Fig. 3.8 (E) indicate that modification with PLP resulted in complete loss of poly (U) binding of the protein. No binding to poly (U)-Sephadex was observed even at 20 mM NH_4Cl buffer. Our results indicate

Fig. 3.8: Binding of chemically modified protein S1 to poly (U)–Sepharose 4B.

Binding of protein S1 to poly (U)–Sepharose was carried out as described in chapter II.

- (A): Binding of rose bengal (RB) modified S1.
- (B): Binding of RB modified S1F4.
- (C): Binding of 2,3–butanedione modified S1
- (D): Binding of tetranitromethane (TNM) modified S1
- (E): Binding of pyridoxal 5–phosphate (PLP) modified S1.
- (F): Binding of N-ethylmaleimide (NEM) modified S1 (both mono-NEM S1 and di-NEM S1).

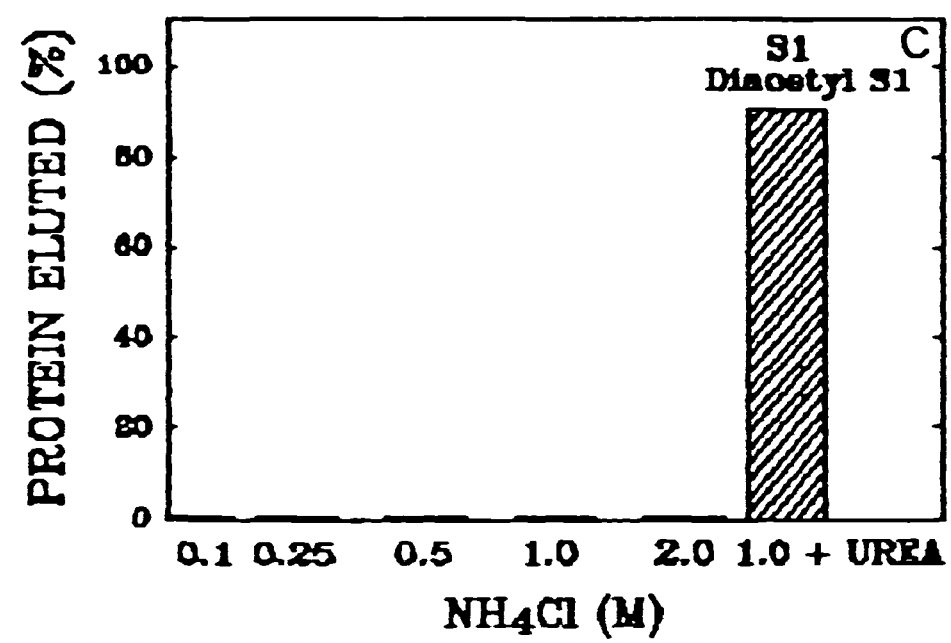
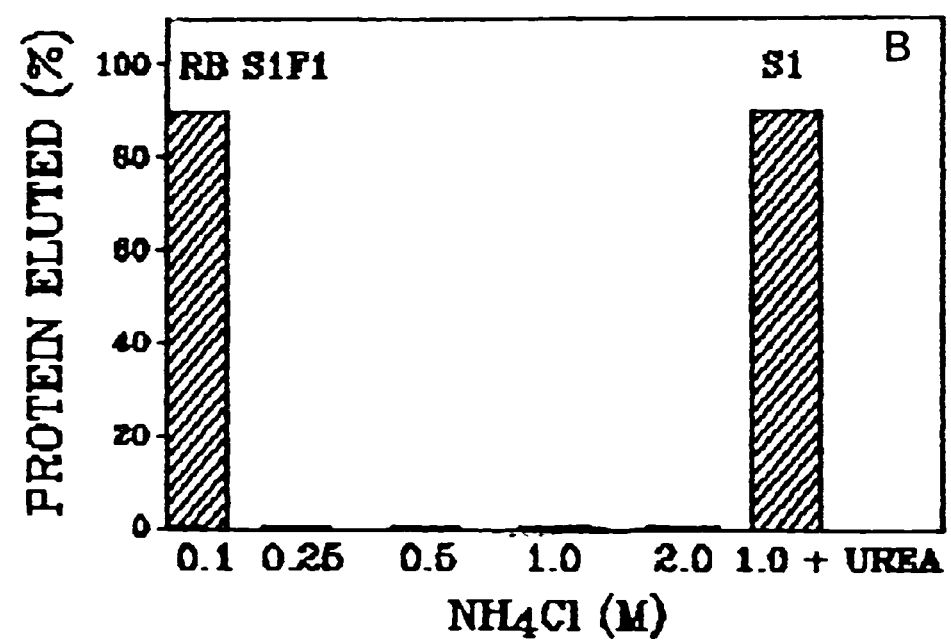
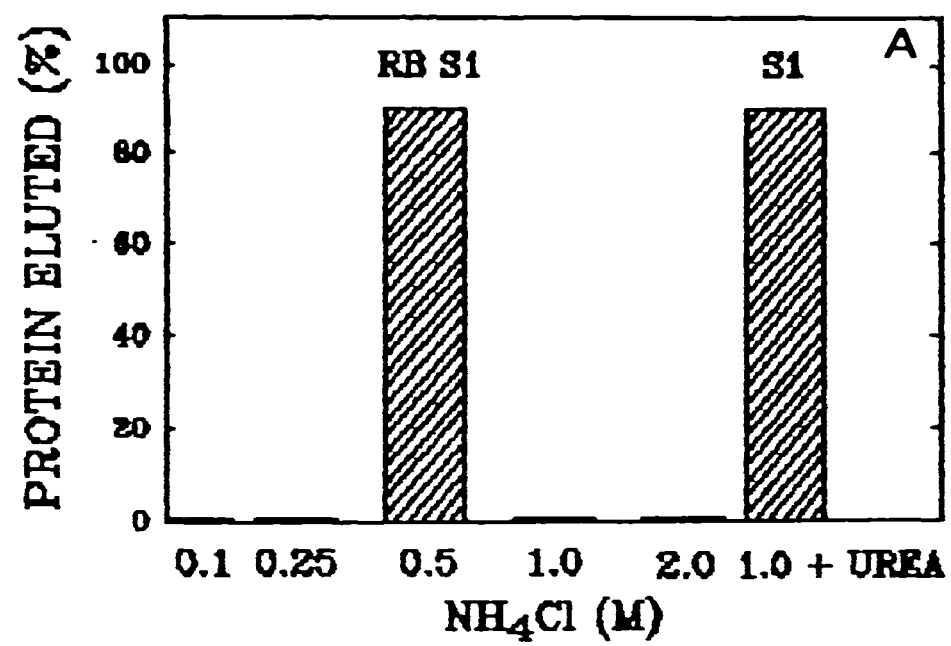


Fig. 3.8

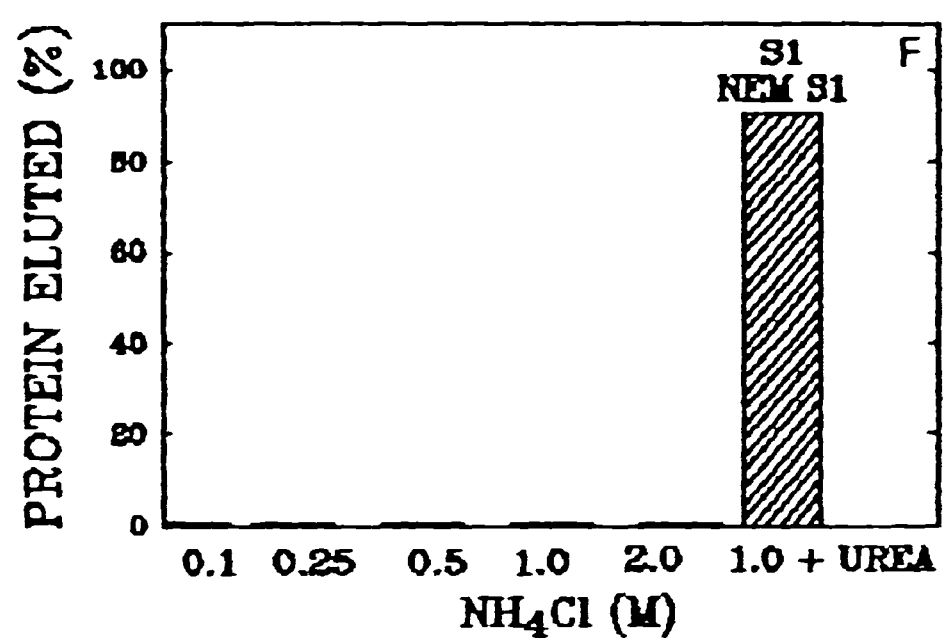
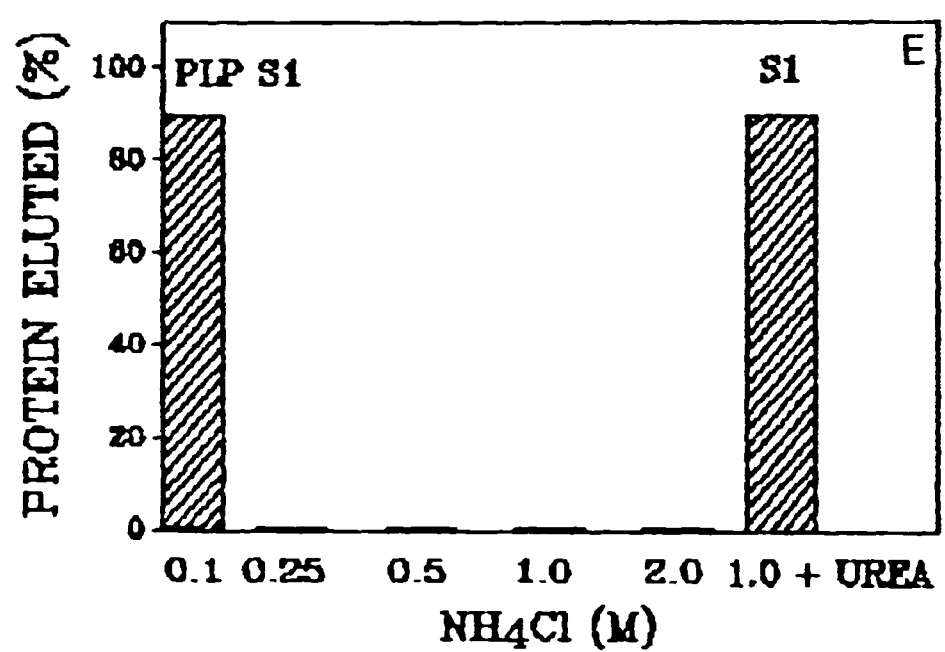
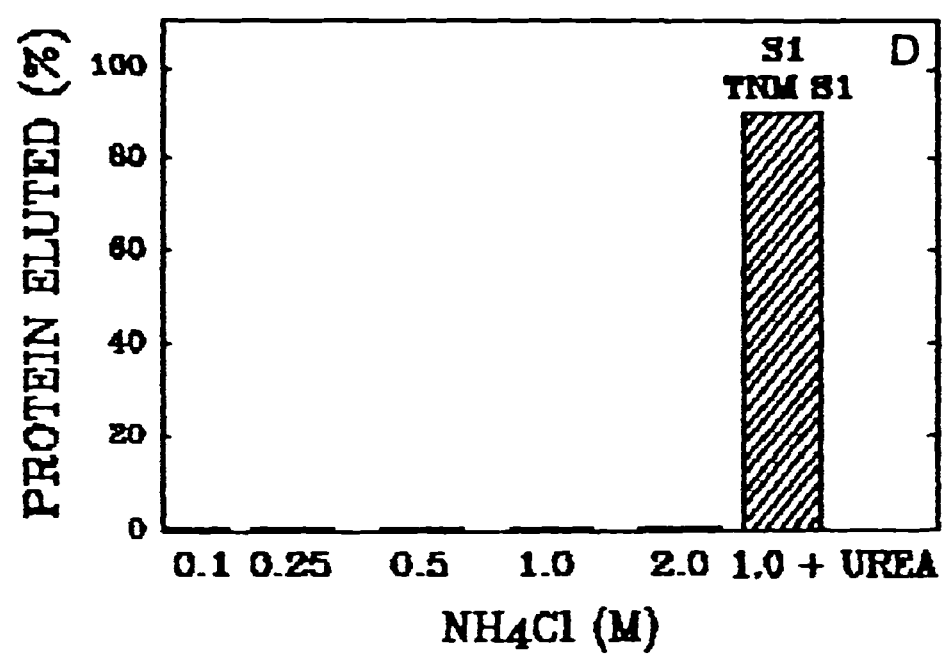


Fig. 3.8

that lysyl residues are involved in the binding of protein S1 to ssRNA (poly (U)). Khanah *et al* (1978) have reported that S1 was inactivated in binding poly (U) upon reductive methylation of lysyl-ammonium groups.

We have modified either single or both the cysteine(-SH groups) of S1 by treating with N-ethylmaleimide (NEM) in native and denatured conditions. The ssRNA binding activity of NEM modified S1 was also studied using poly (U)-Sepharose. The results obtained are presented in Fig. 3.8 (F). Both the mono-NEM derivative of S1 (Cys-349 modified) and di-NEM derivative of S1 (Cys-349 and Cys- 292 modified) eluted from the poly (U)-Sepharose column with 1 M NH₄Cl/7 M urea buffer. This indicates that the modification of either single or both the cysteine residues do not affect the RNA binding property of the protein. It has been reported that mono-NEM-S1 lost the helix unwinding property and the modified protein showed reduced ability to inhibit *in vitro* protein synthesis when present in molar excess to ribosome (Kolb *et al* 1977; Thomas *et al* 1978; Thomas and Szer, 1982). However Suryanarayana and Subramanian (1983) showed that NEM-S1 retained 80-100% activity in stimulating protein synthesis directed by synthetic RNAs and about 50% activity in the translation of natural mRNA.

EFFECT OF CHEMICAL MODIFICATION OF S1 ON RIBOSOME BINDING

The ability of the chemically modified protein S1 to bind ribosomes were tested. For this *E.coli* 30S subunits devoid of S1 were isolated as described in chapter II. Binding of unmodified S1 to 30S subunits devoid of S1 was carried out as a control. The experiment was performed first by reconstituting 30S subunits devoid of S1 with S1. Reconstituted 30S subunits were separated from excess S1 by DEAE cellulose chromatography. The results obtained are

presented in Fig. 3.9 (A-E).

Fig. 3.9 (A) shows the binding of histidine (RB) modified S1 to subunits devoid S1. RB modification of S1 results in the inactivation of the protein to bind 30S subunits. Our results indicate that histidyl residues play important role in the binding of S1 to ribosome and these residues are also involved in the strong binding of S1 to poly (U). We have also analyzed binding of RB modified S1F4 (containing R1 and R2) to 30S subunits. Binding of S1F4 to 30S subunits was carried out as described for S1. The gel instead of staining was western blotted and S1F4 was detected by using anti S1 & peroxidase coupled anti rabbit IgG. Results presented in Fig. 3.9 (B) show that unmodified S1F4 bound to 30S while RB modified S1F4 did not bind to 30S subunits.

Binding of arginine (2,3-butanedione), tyrosine (PLP), lysine (PLP) and cysteine (NEM) modified S1 to 30S subunits was studied. The results are presented in Fig. 3.9 (C-F). Modification of tyrosine residues only resulted in the substantial loss (>50%) in the ability of protein to bind ribosomes. All the other modifications did not affect the binding of S1 to ribosomes. The results indicate that arginine, lysine, cysteine residues do not contribute to the activity of the protein to bind ribosomes. Szer and coworkers already reported that NEM-S1 retained the ability to bind 30S subunits (Kolb *et al* 1977)

In summary, histidine and to some extent tyrosine residues play an important role in the binding of S1 to ribosomes. Normal binding of lysine and arginine modified S1 to 30S subunits clearly indicate that no strong electrostatic interactions are possibly involved in the binding of protein to ribosomes. Earlier studies of (Boni *et al* 1982) indicated that S1 binds to ribosome

Fig. 3.9: Binding of chemically modified protein S1 to ribosomes.

Binding of protein S1 to 30S subunits devoid of S1 was carried out as described in chapter II. Arrow indicates the position of S1.

(A): Binding of rose bengal (RB) modified S1 to 30S subunits.

Lane 1: S1 depleted 30S subunits.

Lane 2: Unmodified protein S1 incubated with S1 depleted 30S ribosomes.

Lane 3: RB modified protein S1 incubated with S1 depleted 30S ribosomes.

(B): Binding of RB modified S1F4.

Bound S1F4 was detected by immunoblotting. 0.1 A₂₆₀ units of 30S subunits were electrophoresed and blotted with anti *E.coli* S1.

Lane 1: S1 depleted 30S ribosomes.

Lane 2: Unmodified S1F4 incubated with S1 depleted 30S ribosomes.

Lane 3: Rose bengal modified S1F4 incubated with S1 depleted 30S ribosomes.

(C): Binding of 2,3-butanedione modified S1.

Lane 1: S1 depleted 30S subunits.

Lane 2: Unmodified protein S1 incubated with S1 depleted 30S ribosomes.

Lane 3: 2,3-butanedione modified protein S1 incubated with S1 depleted 30S ribosomes.

(D): Binding of tetranitromethane (TNM) modified S1.

Lane 1: S1 depleted 30S subunits.

Lane 2: Unmodified protein S1 incubated with S1 depleted 30S ribosomes.

Lane 3: TNM modified protein S1 incubated with S1 depleted 30S ribosomes.

(E): Binding of pyridoxal 5-phosphate (PLP) modified S1.

Lane 1: S1 depleted 30S subunits.

Lane 2: Unmodified protein S1 incubated with S1 depleted 30S ribosomes.

Lane 3: PLP modified protein S1 incubated with S1 depleted 30S ribosomes.

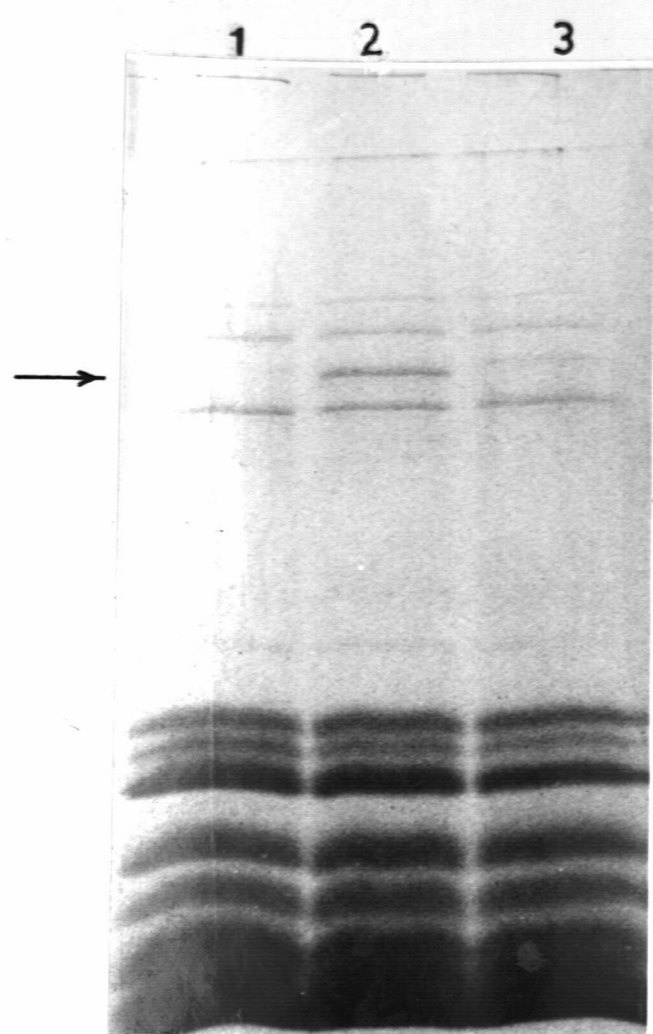
(F): Binding of N-ethylmaleimide (NEM) S1.

Lane 1: S1 depleted 30S ribosomes.

Lane 2: Unmodified S1 incubated with S1 depleted 30S ribosomes.

Lane 3: mono-N-ethylmaleimide (Cys-349) modified S1 incubated with S1 depleted 30S ribosomes.

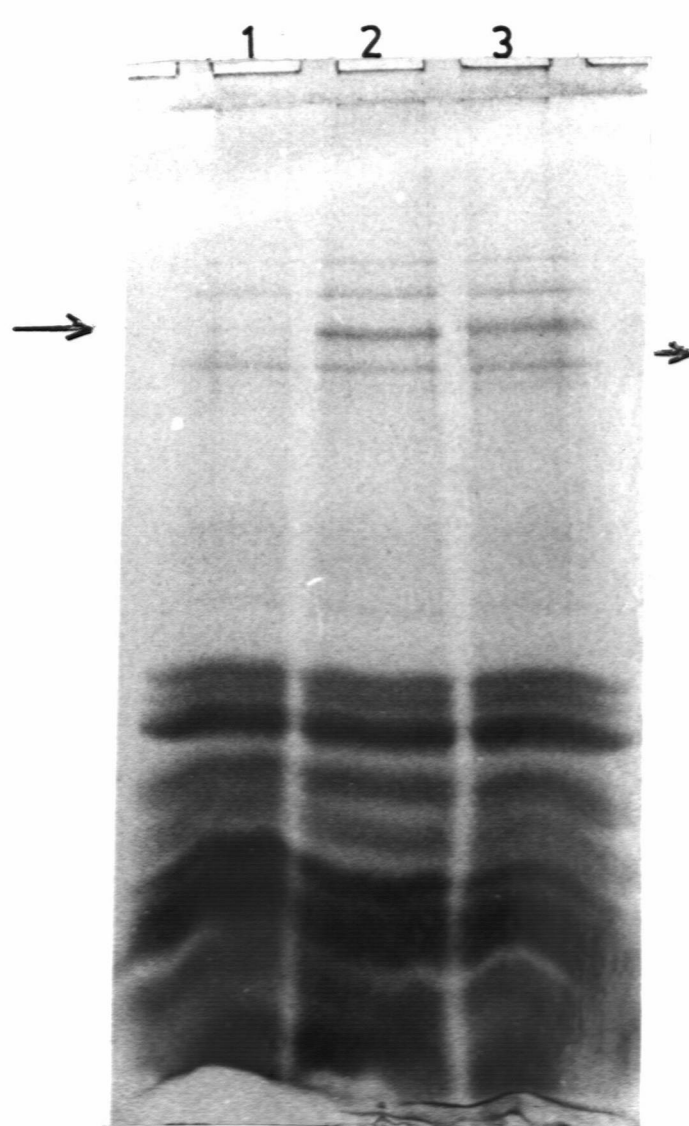
Lane 4: di-NEM (Cys-349 & Cys-292) modified S1 incubated with S1 depleted 30S ribosomes.



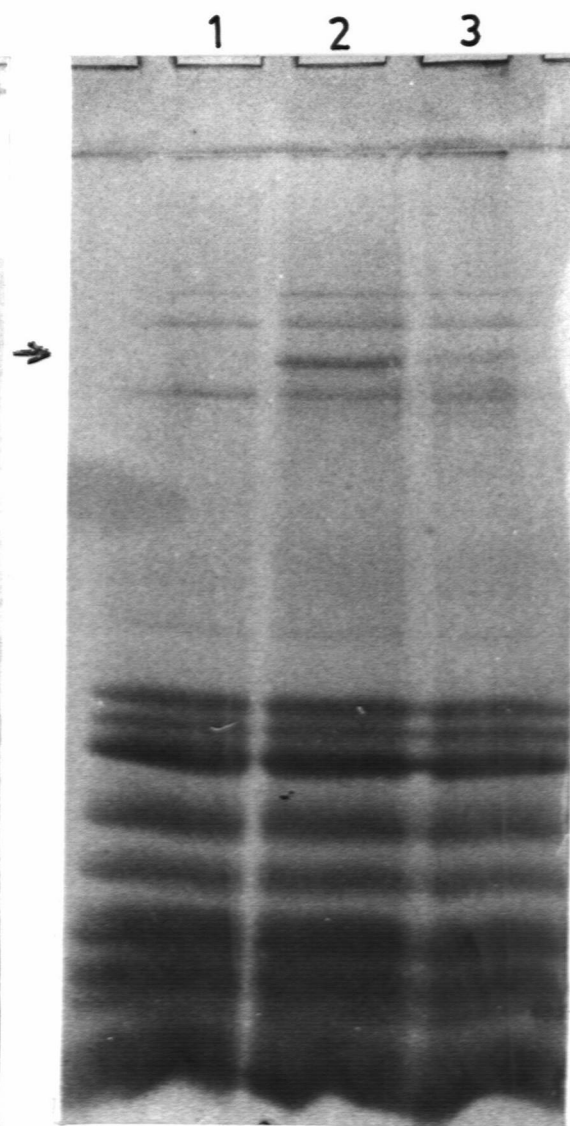
A



B



C



D

FIG. 3.9

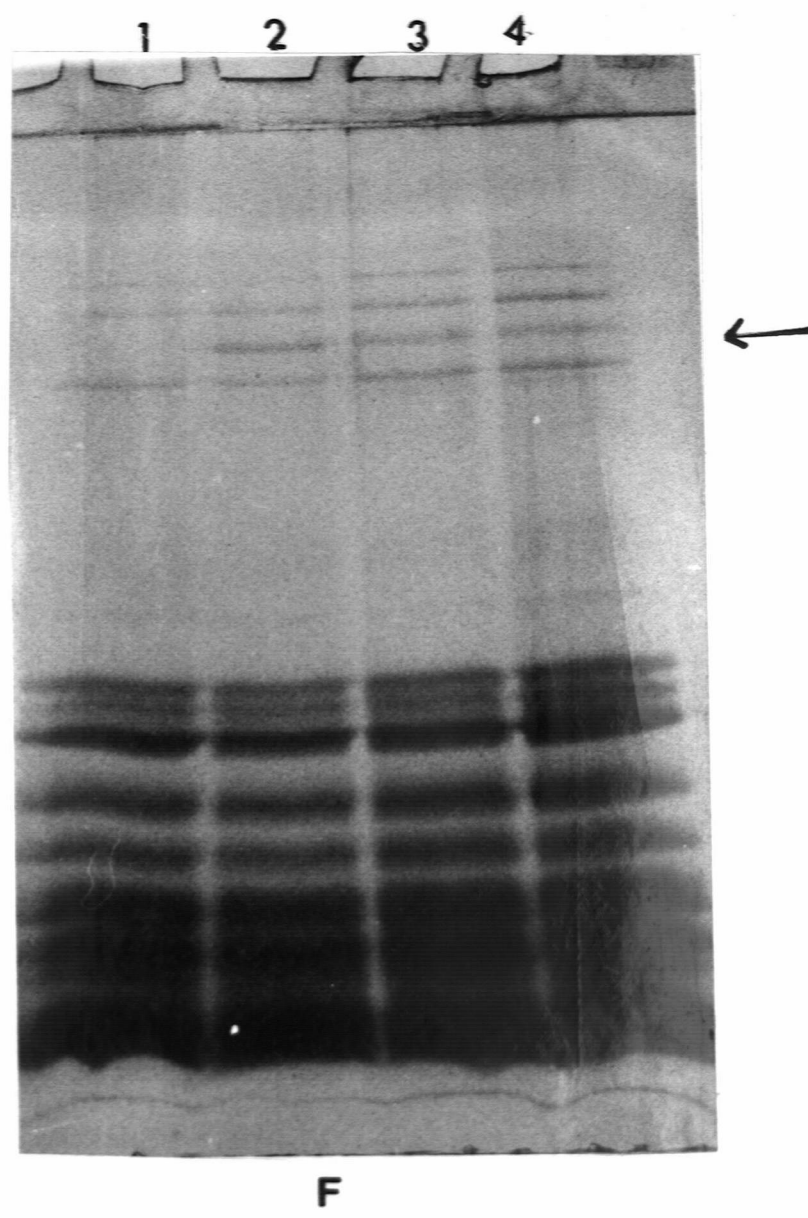
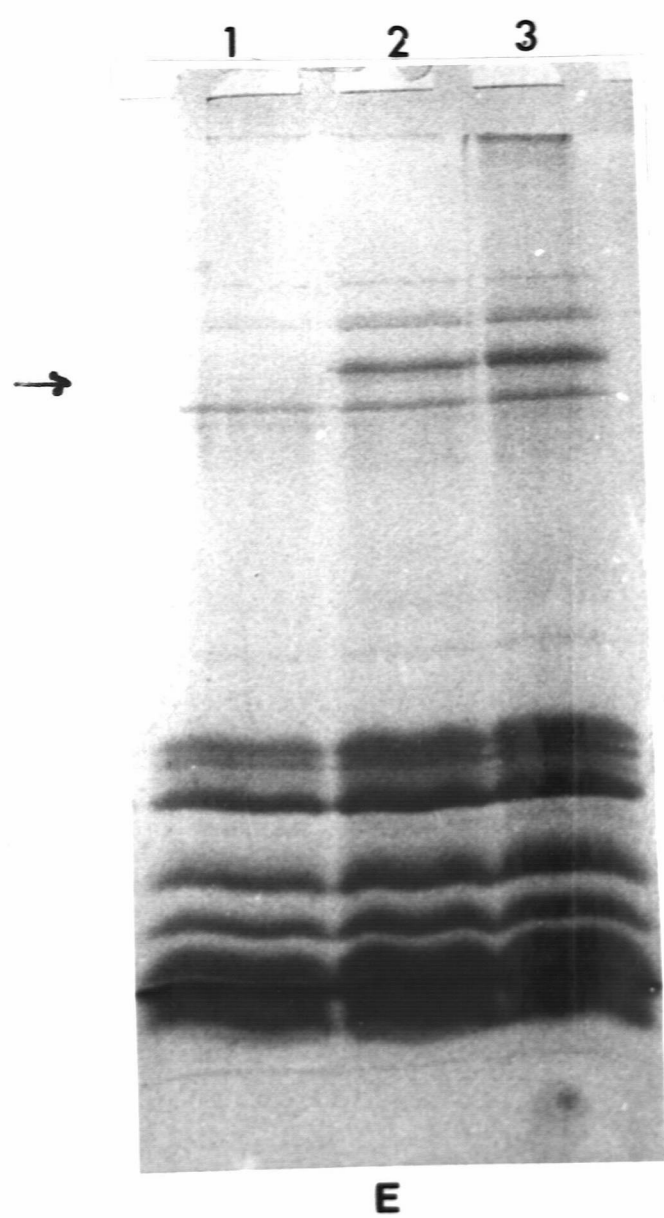


FIG . 3 . 9

through protein–protein interaction. These protein–protein interactions may involve hydrogen bonding and/or hydrophobic interaction.

EFFECT OF CHEMICAL MODIFICATION OF S1 ON ssDNA BINDING

The ability of the chemically modified protein S1 to bind ssDNA was tested by agarose gel mobility shift assay using M13 ssDNA. In all the binding experiments, binding of unmodified S1 to M13 ssDNA was carried out as a control. The results obtained are presented in Fig. 3.10 (A–F).

Binding of unmodified S1 to ssDNA resulted in progressively decreased mobility of ssDNA (S1 complexes) (Fig. 3.10 A). ssDNA binding of S1 is drastically affected by treatment with RB, PLP and to much lesser extent by TNM (Fig. 3.10 B, C, and D). Other modifications with 2,3– butanedione (Fig. 3.10 E) and NEM (Fig. 3.10 F) had no affect on the ssDNA binding of S1. These results indicate involvement of lysine and histidine residues in the binding of S1 to ssDNA. TNM modification of tyrosine resulted only in decreased retardation of the ssDNA.S1 complexes indicating that tyrosine residues are not essential for the binding but may be required for the stability of the complexes.

It is interesting to note that lysine modification also caused complete loss of activity to bind poly (U) and histidine modification resulted in the loss of salt insensitive binding of S1 to poly (U). These results also can be interpreted to suggest two components in the interaction of S1 with polynucleotides namely, electrostatic interaction through lysine & histidine and other noncovalent interactions (hydrogen bonding, hydrophobic (stacking) interaction) through histidine. It is known that S1 binds to poly (U) very strongly eluting from

Fig. 3.10: Binding of chemically modified protein S1 to M13 ssDNA.

Electrophoresis of complexes of S1–single stranded M13 mp7 ssDNA in 0.5% agarose. Complexes were formed by incubating M13 ssDNA with increasing amounts of protein S1 at 25 °C in 10 mM Tris–HC, pH 7.6, 0.1 mM Na₂EDTA, 22 mM NaCl. Electrophoresis was carried out as described in chapter II. Control experiment(s) with unmodified S1 were also carried out as above.

(A): Binding of unmodified S1

Lane 1 : M13 ssDNA (0.5 µg).

Lane 2-4 : 0.5 µg of M13 ssDNA incubated with 2.0, 4.0, and 6.0 µg of unmodified S1 respectively.

(B): Binding of rose bengal (RB) modified S1

Lane 1 : M13 ssDNA (0.5 µg).

Lane 2-4 : 0.5 µg of M13 ssDNA incubated with 2.0, 4.0, and 6.0 µg of RB modified S1 respectively.

(C): Binding of pyridoxal 5–phosphate (PLP) modified S1

Lane 1 : M13 ssDNA (0.5 µg).

Lane 2-4 : 0.5 µg of M13 ssDNA incubated with 2.0, 4.0, and 6.0 µg of PLP modified S1 respectively.

(D): Binding of tetranitromethane (TNM) modified S1

Lane 1 : M13 ssDNA (0.375 µg).

Lane 2-4 : 0.375 µg of M13 ssDNA incubated with 2.0, 4.0, and 6.0 µg of TNM modified S1 respectively.

(E): Binding of 2,3-butanedione modified S1

Lane 1 : M13 ssDNA (0.375 µg).

Lane 2-4 : 0.375 µg of M13 ssDNA incubated with 2.0, 4.0, and 6.0 µg of 2,3–butanedione modified S1 respectively.

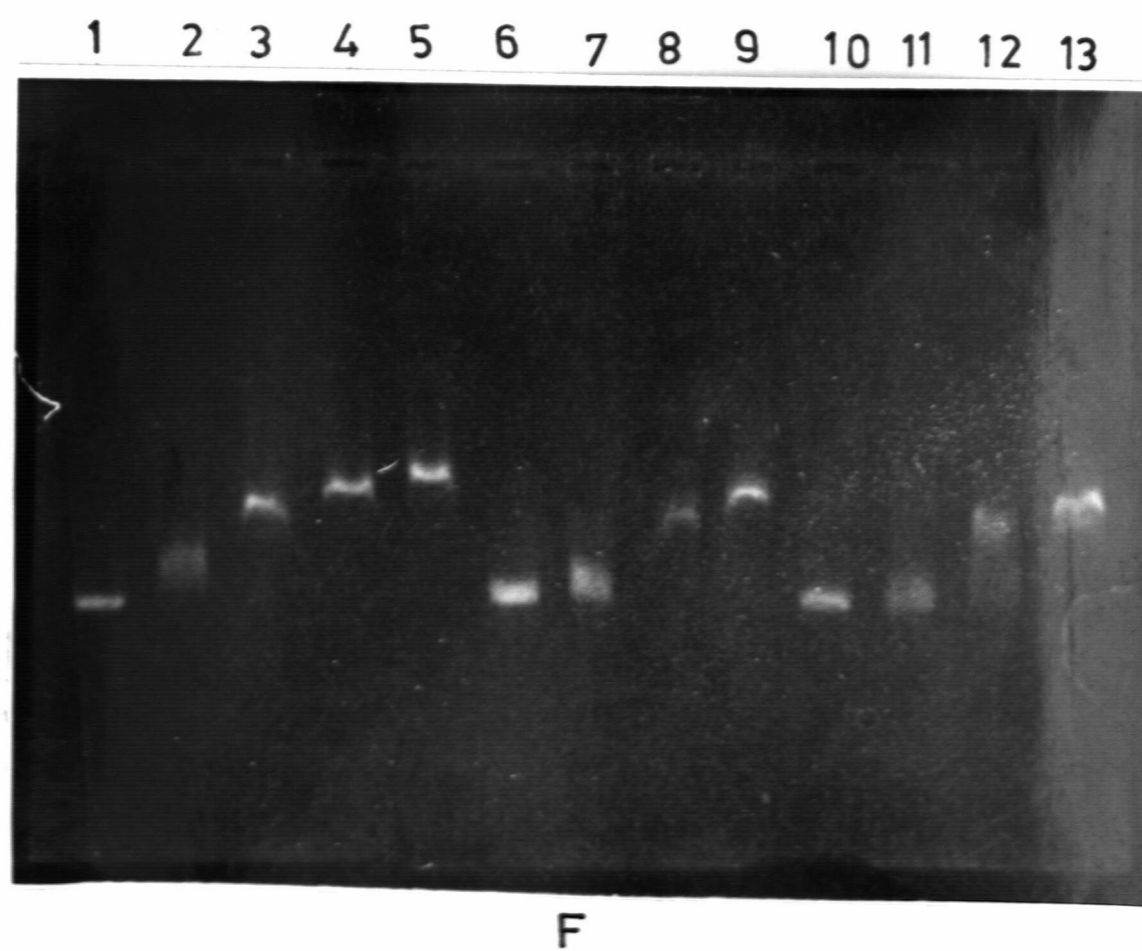
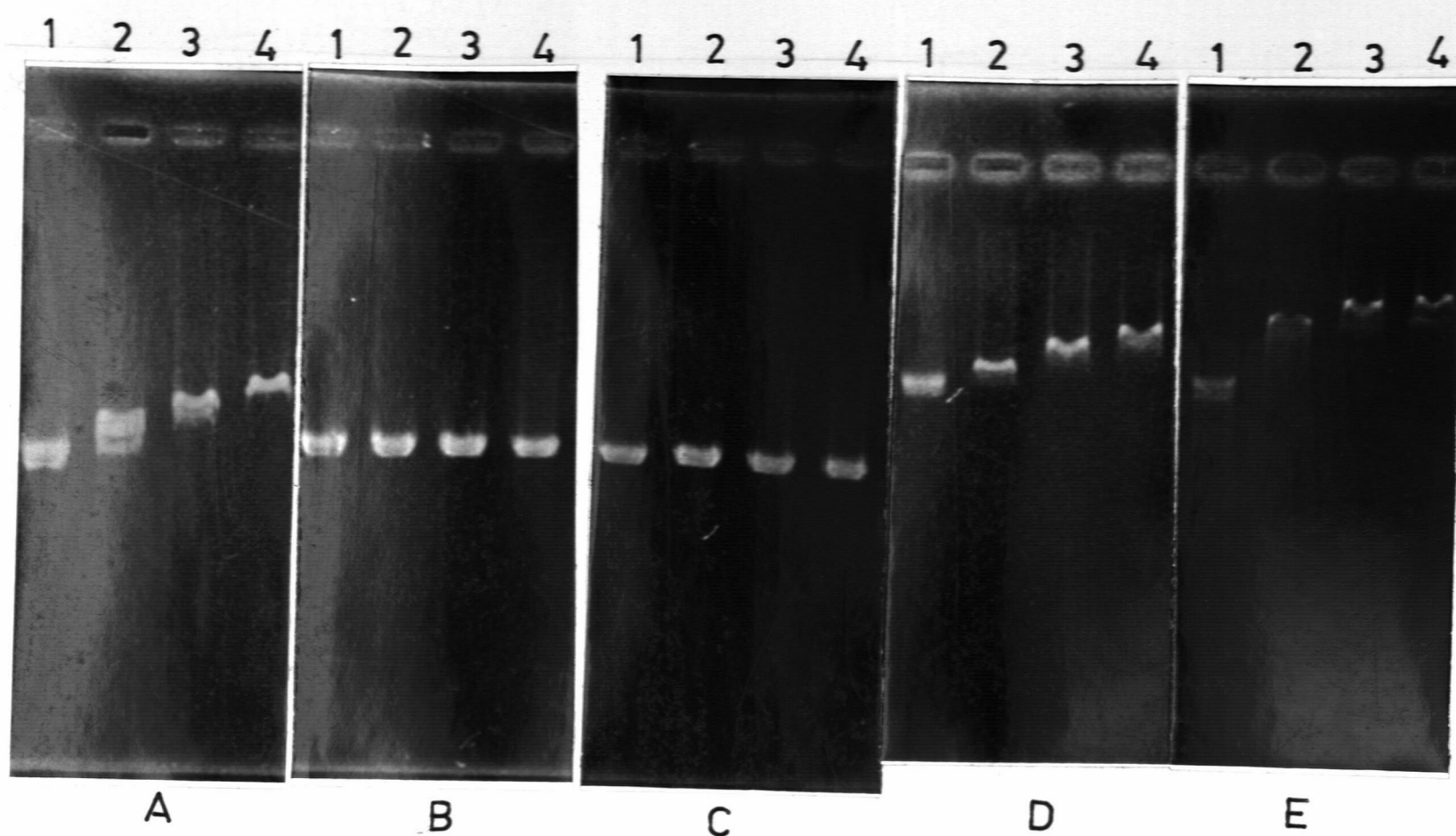
(F): Binding of N–ethylmaleimide (NEM) modified S1.

Lane 1 : M13 ssDNA (0.375 µg).

Lane 2-5 : 0.375 µg of M13 ssDNA incubated with 1.0, 2.0, 3.0, and 5.0 µg of unmodified S1 respectively.

Lane 6-9 : 0.375 µg of M13 ssDNA incubated with 1.0, 2.0, 3.0 and 5.0 µg of mono–NEM (Cys–349) modified S1 respectively.

Lane 10-13: 0.375 µg of M13 ssDNA incubated with 1.0, 2.0, 3.0, and 5.0 µg of di–NEM (Cys– 349 & Cys–292) modified S1 respectively.



F
Fig 3.10

polypyrimidine columns with urea buffer (Carimichael, 1975; Suryanarayana and Subramanian, 1979; Draper von Hippel, 1978a,b). However S1 shows salt sensitive binding to both poly (rA) and denatured DNA cellulose columns which elutes with salt buffer (0.5 to 1 M NH_4Cl) (Carmichael, 1975; Draper von Hippel, 1978a,b). Taking into account of all these results, it might be argued that the binding of S1 to both poly (U) and denatured DNA might be through a single site and that the binding strength varies with the type of polynucleotide.

Furthermore, Draper and von Hippel (1977 & 1978b) suggested that the site II, which was reported to be specific for polyribonucleotides binds to polynucleotides with no protein-polynucleotide phosphate ion pairs. This indicates that the interaction at this site is not electrostatic and hence no basic amino acids are involved in the binding (Draper and von Hippel, 1978b). However, our results with PLP modification and those of Khanah *et al* (1978) clearly indicate involvement of lysine residues in the binding of S1 to poly (U). Our results further show that electrostatic component is involved in the binding of S1 to both polyribo (poly (U)) and polydeoxyribonucleotides (M13 ss DNA).

Both mono-NEM (Cys-349) and di-NEM (Cys-349 & Cys-292) modified S1 retained the ability to bind ssDNA . Kolb *et al* (1977); Thomas *et al* (1978) & Thomas and Szer (1982) have earlier reported that mono-NEM S1 could bind polynucleotides (MS2 RNA, ϕX 174 ssDNA) and was unable to unwind them.

POLY (U) TRANSLATION BY RIBOSOMES: Mg^{2+} -OPTIMUM DETERMINATION

We have determined the optimal magnesium ion concentration requirement of *E.coli* ribosomes before studying the functional activity of S1. 70S ribosomes and S-100 fraction were used for carrying out this experiment. Figure 3.11 shows that translation of poly (U) by *E.coli* (RNase I⁻) ribosomes required an optimum magnesium ion concentration of 14 mM.

EFFECT OF CHEMICAL MODIFICATION OF S1 ON *IN VITRO* TRANSLATION

Specific removal of S1 from the protein synthesis system (both ribosomes and S-100) by poly (U)-Sephadex chromatography resulted in the drastic reduction in the protein synthetic activity. Addition of exogenous S1 to S1 depleted *E.coli* system results in the stimulation of protein synthetic activity. The ability of all chemically modified S1 samples to stimulate poly (U) translation by S1 depleted ribosomes was studied. The results obtained are presented in Fig. 3.12.

The functional activity of both DEPC modified and RB modified S1 in protein synthesis was analyzed (Fig. 3.12 A, B). When the modification was carried out at 14 °C for 60 min with 2.0 mM DEPC, the modified protein showed only about 25 to 30% activity of that of unmodified S1 (Fig. 3.12 A). RB modified S1 is virtually inactive in stimulating poly (U) dependent protein synthesis (Fig. 3.12 B). Modification of histidyl residues by DEPC is reversible and the modified residues (N-carbethoxy histidyl or N-ethoxy-formyl imidazoles) autoreactivate with time. DEPC modified S1 on storage for 10 days at 0 °C regained the activity upto 82% activity compared to unmodified S1 (Fig.3.12 C). This suggests that, DEPC modified exclusively

histidyl residues in the conditions we have used for the modification. Our results with both DEPC and RB modified S1 indicate that modification of histidine residues results in the loss of functional activity of the protein. This inactivity to stimulate poly (U) dependent polyphenylalanine synthesis could be as a result of loss of ribosome binding activity, since the modified protein is still able to bind poly (U) in salt sensitive mode .

Arginine (2,3-butanedione) modified S1 stimulated poly (U)-dependent protein synthesis like unmodified S1. There was no difference even after modification was reversed (Fig. 3.12 D). Arginine modification does not affect either ribosome binding or poly (U) binding by S1. Hence it is not surprising that this modification has not affected its ability to stimulate protein synthesis.

Tyrosine (TNM) modification of S1 did not result in substantial loss of activity of the protein in protein synthesis (Fig. 3.12 E). There was about 25% reduction which could be due to nonspecific affect of the modification due to the modification of some other residues.

Unexpected results were obtained with lysine (PLP) modified S1. The modified S1 is able to stimulate polyphenylalanine synthesis almost to the same extent as unmodified S1 (Fig. 3.12 F). Modification of lysyl residues with PLP has drastically affected poly (U) binding activity of the protein (Fig. 3.8 E) as measured by the affinity of the modified protein to bind poly (U)-Sepharose. However, Khanah *et al* (1978) reported that the lysine modification of S1 by reductive methylation inactivates the protein to bind poly (U) as well as in stimulating the phenyl-t-RNA binding to ribosomes. Our results are at variance with the above. Lysine modification has not affected

Fig. 3.11: Poly (U) translation by *E.coli* ribosomes as a function of magnesium concentration. The assay was performed as described in chapter II. Blank values in the absence of poly (U) have been subtracted.

Fig. 3.12: Poly (U) dependent polyphenylalanine synthesis was performed as described in chapter II using *E.coli* system depleted of S1. The reaction mixtures were preincubated with increasing amounts of S1 at 37 °C for 5 min. Translation reaction was initiated by adding 5 µg of poly (U). Blank values in the absence of poly (U) have been subtracted.

- (A): Stimulation of Poly (U)–dependent polyphenylalanine synthesis by unmodified S1 (solid line) and diethyl pyrocarbonate (DEPC) modified S1 (broken line).
- (B): Autoreactivation of diethyl pyrocarbonate (DEPC) modified S1. 3.0 µg of protein S1 was used in the assay.
 - 1: Unmodified S1.
 - 2: Diethyl pyrocarbonate (DEPC) modified S1.
 - 3: Autoreactivated DEPC modified S1.
- (C): Stimulation of Poly (U)–dependent polyphenylalanine synthesis by unmodified S1 (solid line) and rose bengal (RB) modified S1 (broken line).
- (D): Stimulation of Poly (U)–dependent polyphenylalanine synthesis by unmodified S1 (solid line) and tetranitromethane (TNM) modified S1 (broken line).
- (E): Stimulation of Poly (U)–dependent polyphenylalanine synthesis by unmodified S1 (solid line) and pyridoxal 5– phosphate (PLP) modified S1 (broken line).

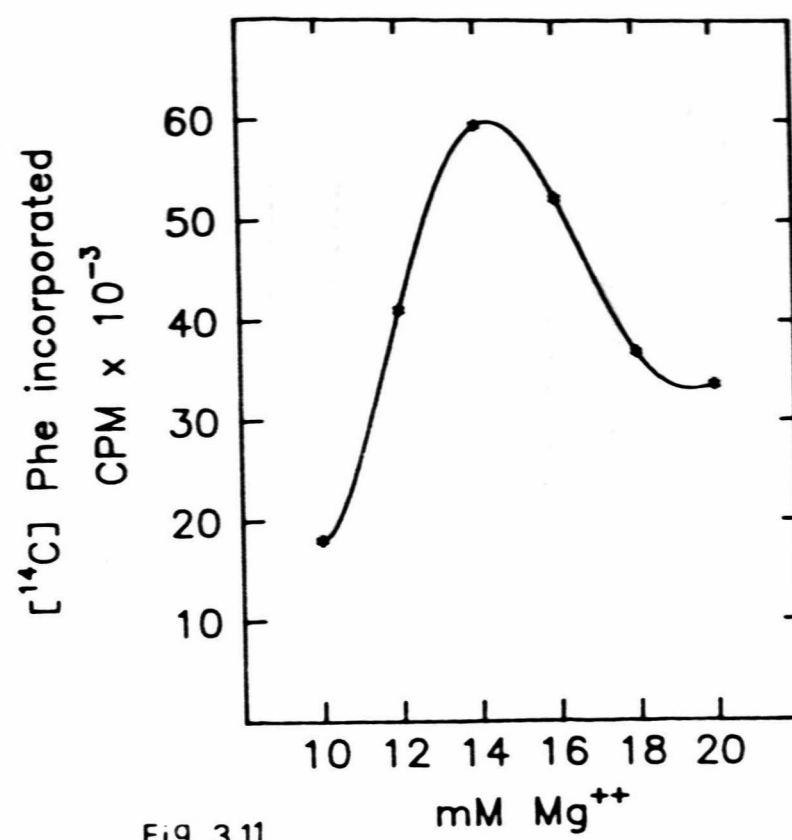


FIG 3.11

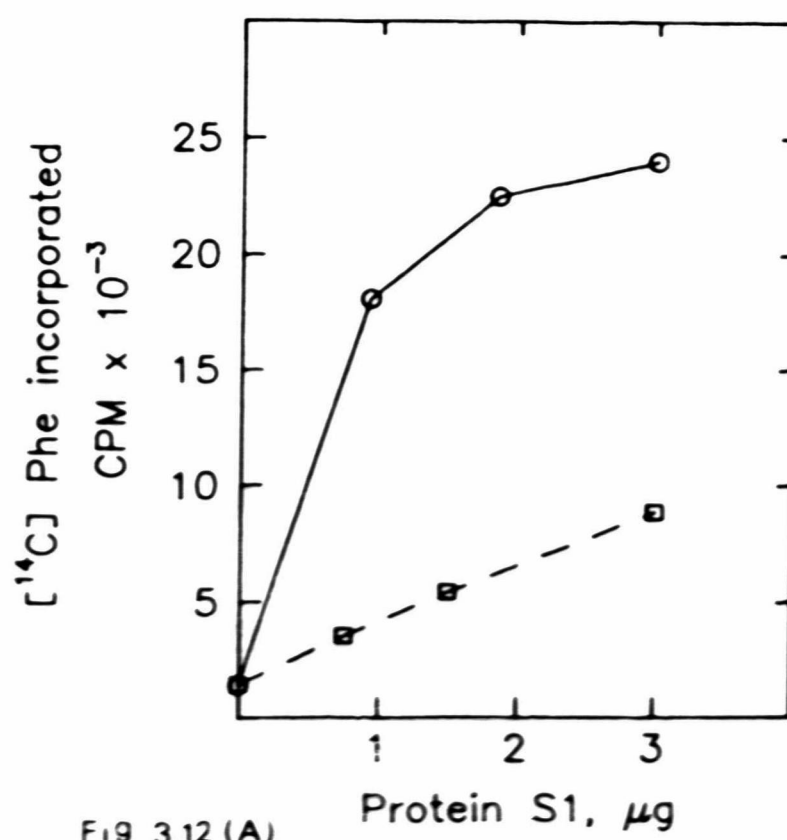
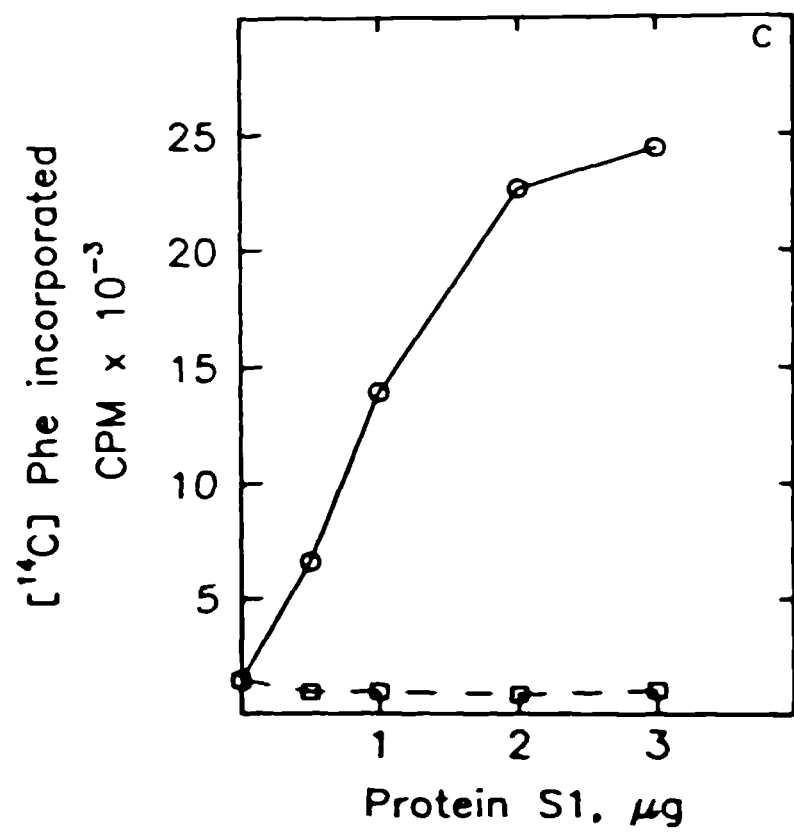
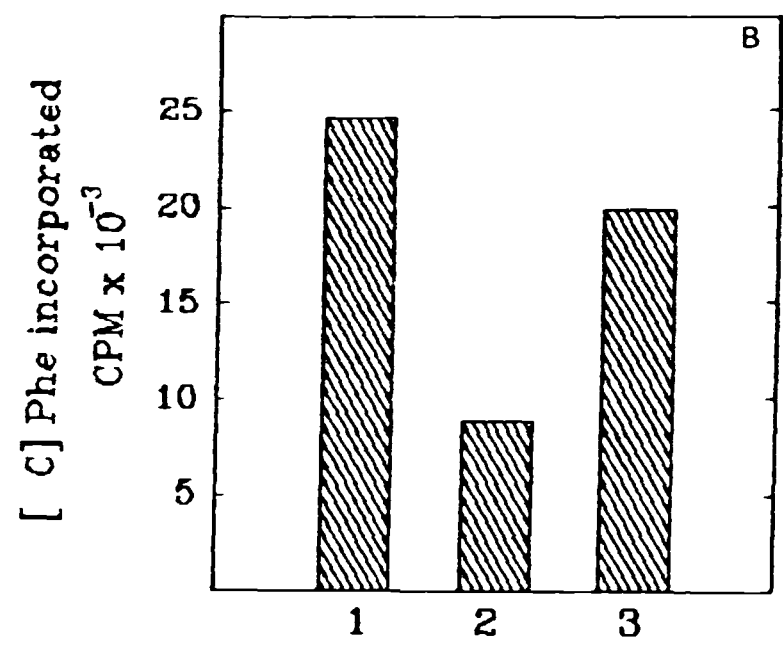


FIG 3.12 (A)



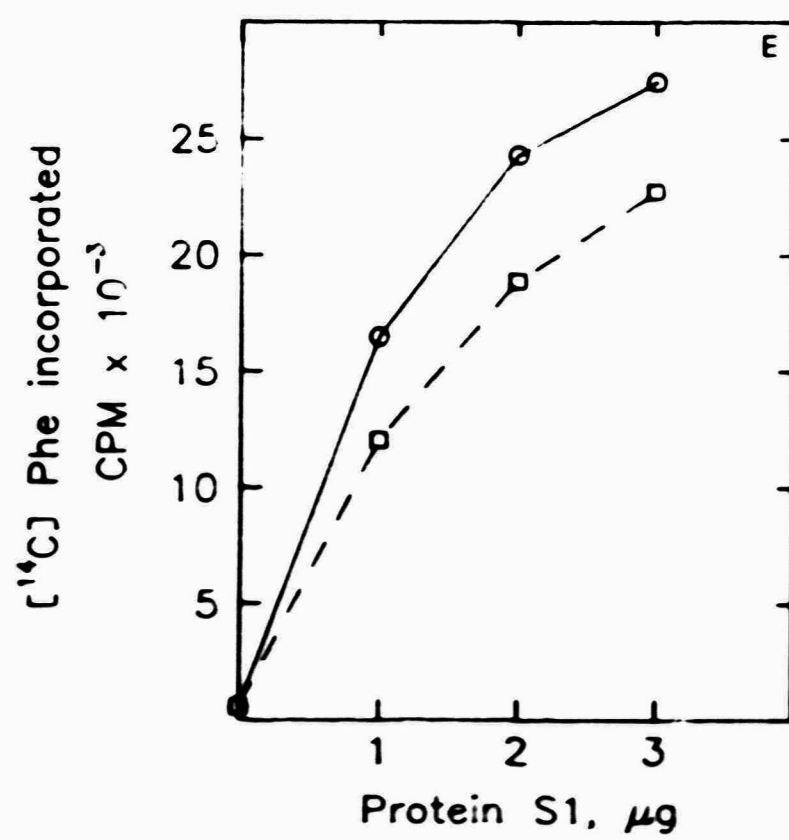
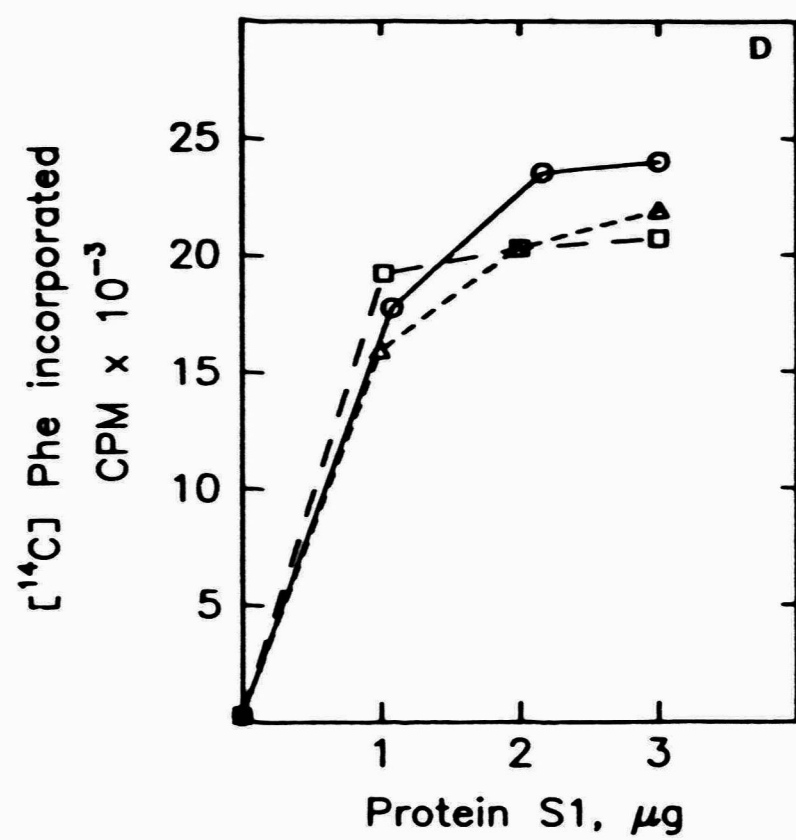


FIG. 3.12

Fig 3.13: Fluorescence titration of unmodified S1 (solid line) and S1 modified with pyridoxal 5-phosphate (broken line) with poly (U).

Titration experiments were carried out with 20 μg of protein S1 in 1 ml buffer (10 mM Tris-HCl pH 7.6, 10 mM magnesium acetate, 100 mM NH_4Cl and 7 mM 2-mercaptoethanol). Aliquots (2 μl) of poly (U) were added to the protein, the change in the fluorescence emission intensity at 350 nm (5 nm slit width) was recorded by excitation at 280 nm (5 nm slit width).

the ribosome binding of the protein. Hence under *in vitro* protein synthesis conditions the protein may be able to stimulate the polyphenylalanine synthesis. Modification of lysine residues with PLP and subsequent reduction with borohydride makes the modification irreversible. We have not tested whether there was any reversal of the modification by incubation under *in vitro* protein synthesis conditions. The protein may still show some weak interaction with poly (U) which could not be measured by poly (U) –Sepharose chromatography. It is known that intrinsic fluorescence of most of the nucleic acid binding proteins (including S1) is quenched by polynucleotides (Draper and von Hippel, 1977 & 1978a,b). We have tested whether PLP modified S1 can bind poly (U) by fluorescence titration. Results presented in Fig. 3.13 show that there is quenching of fluorescence of PLP modified S1 by poly (U). However, it is much less than that observed with normal S1. These results indicate that PLP modification did not totally abolish the poly (U) binding ability of the protein. There could be some weak hydrophobic interaction between PLP modified S1 and poly (U) which may be stabilized under *in vitro* protein synthesis conditions.

The results obtained with the chemical modification of S1 are summarized in Table 3.2. Modifications that effect the RNA binding also effected the ssDNA binding. Lysine modification caused loss of binding to both RNA and DNA. Histidine modification resulted in loss in the ability of protein to bind ssDNA. The RNA binding is also severely effected. The histidine modified S1 bound to poly (U)–Sepharose was eluted with 0.5 M NH_4Cl buffer i.e the salt insensitive binding mode is abolished. The salt sensitive binding seen the case of histidine modified S1 can not be the same as binding of poly (U) to site I reported by Draper and von Hippel (1978b) because the modified protein

Modification	Poly (U) binding	ssDNA binding	Ribosome binding	Stimulation of Polyphenylalanine synthesis
Histidine	loss of salt insensitive binding	—	—	—
Lysine	—	—	—	+
Arginine	+	+	+	+
Tyrosine	+	+	↓	+
Cysteine	+	+	+	+

Table 3.2. Effects of chemical modifications of protein S1.

failed to bind ssDNA. These results can be explained by assuming a single site on S1 which binds both types of nucleic acids (RNA and ssDNA). The binding mode depends on the type of nucleic acids. Binding requires the functional groups of both histidine and lysine. Tyrosine also may partly contribute to the stability of the binding of protein to ssDNA. Furthermore, Thomas and Szer (1982) have reviewed the experimental observations which are inconsistent with the two-site model such as inability to detect RNA–RNA crosslinks or RNA–DNA crosslinks involving protein S1 either by electron microscopy or by sedimentation analysis.

Protein S1 is the largest and the longest of all the ribosomal proteins. Using classical methods of protein fragmentation, several domains and truncated forms of S1 have been characterized. Structural and functional features of different polypeptide fragments of S1 have been described in chapter I.

The nucleic acid binding properties of different peptide fragments of S1 were studied mainly with respect to RNA binding (Suryanarayana and Subramanian, 1979; Giorginis and Subramanian, 1980; Kimura *et al* 1982). The binding of fragments to poly (U) was assayed using radiolabelled poly (U) and membrane filtration or passing through poly (U)-Sepharose. Draper and von Hippel (1978) reported the presence of two polynucleotide binding sites in S1: site I, capable of binding either ssDNA or RNA and site II, binding only RNA. Even though the nucleic acid binding properties of the two sites have been studied in great detail, their exact location on the polypeptide chain has not been determined.

In this chapter, we have studied the binding of S1 and its fragments viz., S1F1, S1F4, N-terminal fragment and C-terminal fragment to ssDNA in an attempt to localize the ssDNA binding site on protein S1. We have used, closed circular single stranded M13mp7 DNA in our studies. The DNA binding was analyzed by gel mobility shift assay. The assay originally developed by Garner and Revzin (1981), is the most widely used technique to detect binding of proteins to nucleic acids. This technique is based on the differential electrophoretic mobilities of nucleic acid-protein complexes relative to free nucleic acids.

MATERIALS AND METHODS

Most of the experimental methods followed have been given in chapter II. Specific details about each experiment carried out have been given in the legends to figures.

RESULTS AND DISCUSSION

ISOLATION AND PURIFICATION OF DIFFERENT FRAGMENTS OF S1

Protein S1 was cleaved both enzymatically and chemically to isolate different fragments of S1. Mild digestion of protein S1 with trypsin was performed as described earlier (Suryanarayana and Subramanian, 1979) to generate two fragments namely, N-terminal fragment (from amino acid residues 1 to 171) and central and C-terminal fragment (from amino acid residues 172 to 557). This fragment (S1F1) contains all the four contiguous internal homologous repeat sequences (R1–R4).

Protein S1 was cleaved at its reactive SH group (Cys-349) as described earlier (Suryanarayana and Subramanian, 1984) to generate two fragments namely, S1F4 fragment (from amino acid residues 1 to 348) containing N-terminal ribosome binding domain and two of the four repeating homologous sequences and C-terminal fragment (from amino acid residues 349 to 557) containing the repeats R3 and R4. This fragment is similar to F2b reported by Kimura *et al* (1981).

The isolation and purification of the fragments have been described in

Fig. 4.1: SDS-polyacrylamide gel electrophoretic analysis of purified *E.coli* S1 polypeptide fragments.

Lane 1-6: *E.coli* S1 (0.25 μg), S1F1 (0.5 μg), S1F4 (1.0 μg), C-terminal fragment (2.0 μg), N-terminal fragment (1.0 μg) and standard proteins (phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, lysozyme).

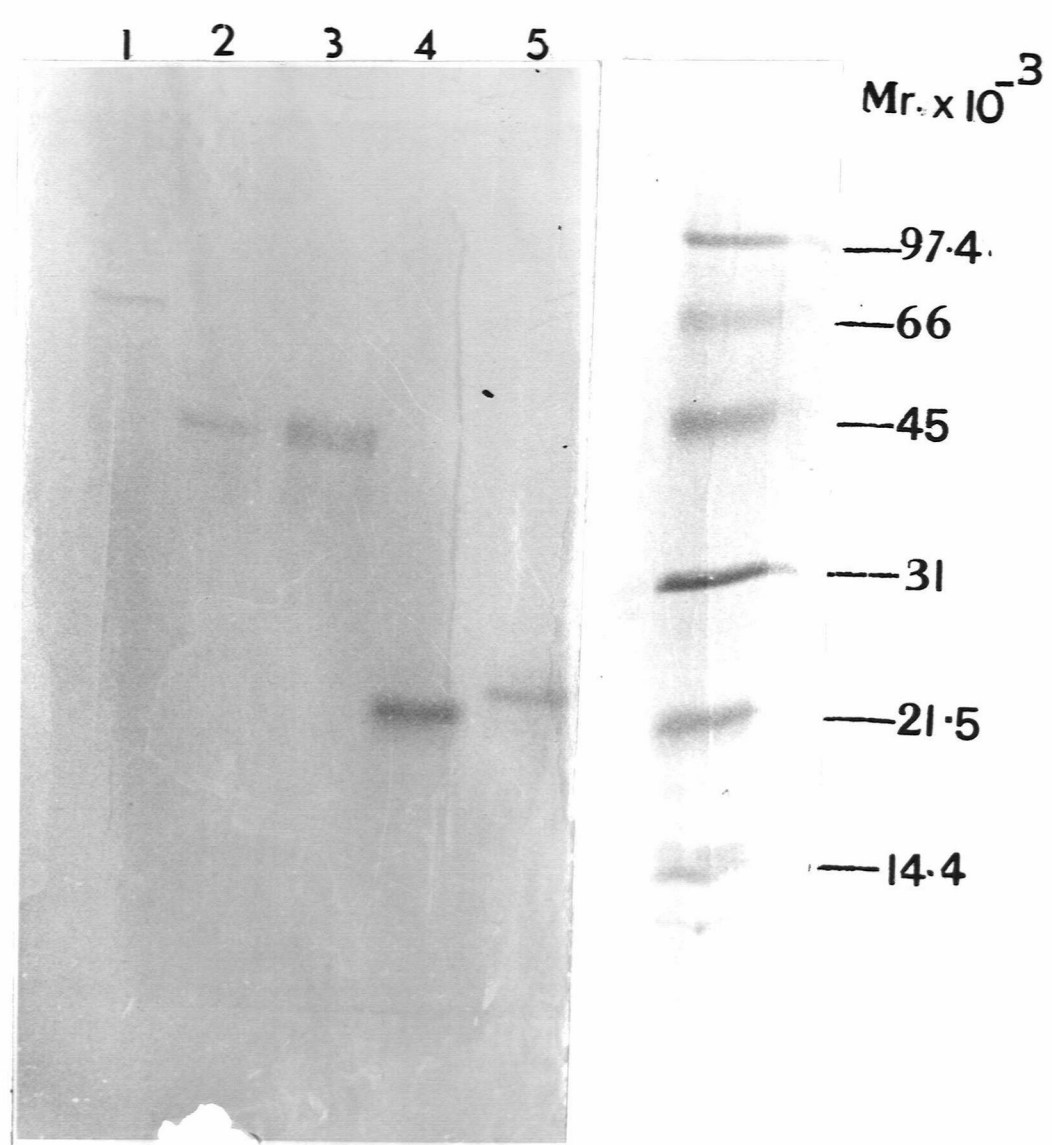


FIG 4-1

chapter II. The purity of the isolated fragments was checked by electrophoresing on 18% SDS-Polyacrylamide gel. Results obtained are presented in Fig. 4.1. In all the cases, protein fragments were highly purified and gave single bands. The nucleic acid (ssRNA and ssDNA) binding of the isolated fragments were carried out in the present study.

BINDING OF S1F4, N-TERMINAL AND C-TERMINAL FRAGMENTS TO POLY (U)-SEPHAROSE

Binding of S1F4, N-terminal and C-terminal fragments of S1 to poly (U) was measured using poly (U)-Sepharose 4B. Protein S1 binds strongly to poly (U)-Sepharose even in the presence of high concentration of salt (1 M NH_4Cl) and is released only when eluted with 7 M urea (Suryanarayana and Subramanian, 1979).

S1F4 fragment was passed through a small column of poly (U)- Sepharose 4B and the column was washed successively with 0.1 M, 0.25 M, 0.5 M, 1.0 M, 2.0 M NH_4Cl and finally with 1.0 M NH_4Cl /7M urea buffer. Results from this experiment are summarized in a histogram (Fig. 4.2 A) which shows that S1F4, like S1 is released from the column only by 1.0 M NH_4Cl /7M urea buffer. This experiment thus confirms that S1F4 retained the active centers of S1 responsible for strong RNA binding activity. S1F4, bound to the poly (U)- Sepharose, did not elute even in the presence of 2 M NH_4Cl indicating that, like intact S1, S1F4 binding to poly (U) is also salt insensitive. The salt insensitive nature of S1F4 suggests that, the major RNA binding site is retained in S1F4 and that the removal of C-terminal repeats R3 and R4 have no apparent effect on the binding of the protein to poly (U). Similarly, strong binding of S1F1 to poly (U) has been shown earlier (Suryanarayana and Subramanian, 1979)

4.2 (A): Binding of S1F4 to poly (U)-Sepharose 4B.

S1F4 (10.0 μ g) was passed through a poly (U)-Sepharose column (0.2 ml) equilibrated with TKM10 buffer. The column was washed successively with 0.8 ml of 0.1 M, 0.25 M, 0.5 M, 1.0 M, 2.0 M NH_4Cl and finally with 1.0 M NH_4Cl /7 M urea, all in TKM10 buffer. Aliquots from each fraction were electrophoresed on SDS– polyacrylamide slab gel (Reddy and Suryanarayana, 1988). Intensity of the protein bands were plotted in arbitrary units.

(B): Binding of C-terminal and N-terminal fragments to poly (U)-Sepharose.

C-terminal, N-terminal fragments (each 10.0 μ g) were passed through a 0.2 ml of poly (U)-Sepharose equilibrated with 10 mM Tris-HCl, pH 7.6, 20 mM NH_4Cl , 10 mM Mg acetate, 7 mM 2- mercaptoethanol. The column was eluted as described in Fig 4.2 (A) and fractions were analysed by SDS-PAGE.

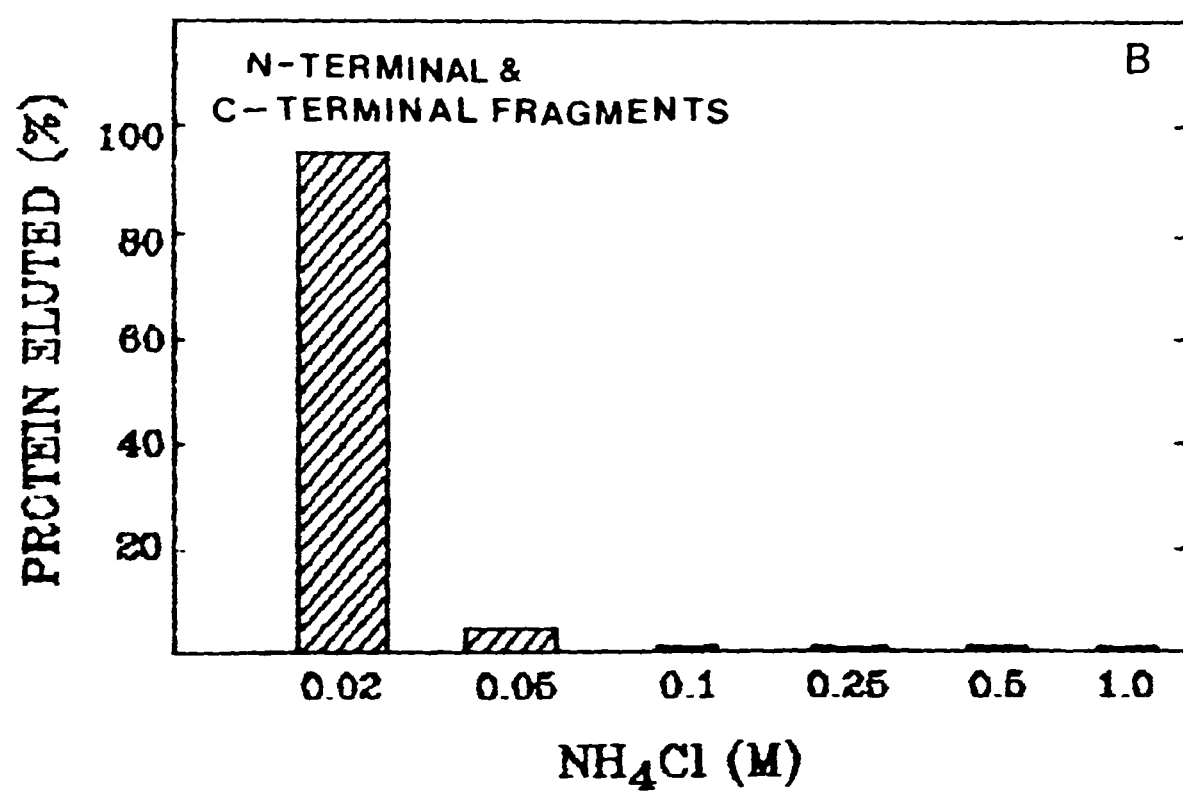
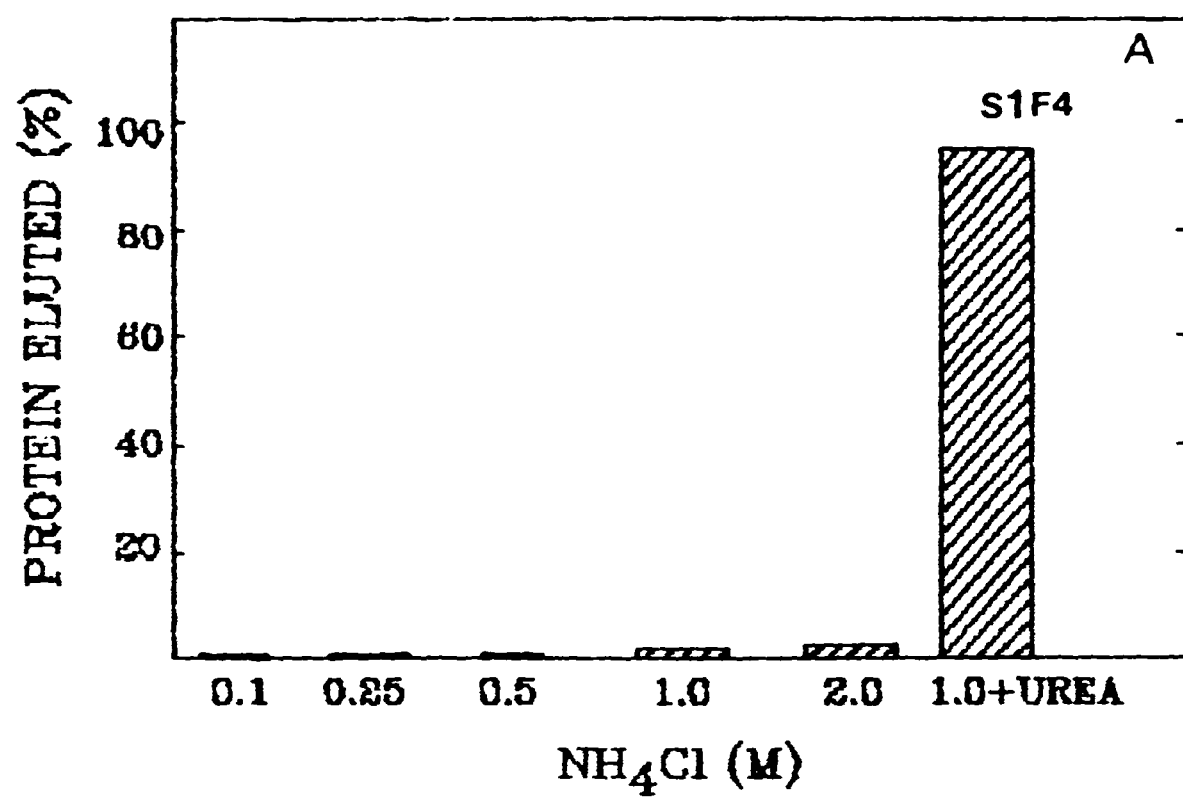


Fig. 4.2

The N-terminal and C-terminal fragments were also passed through small columns of poly (U)-Sepharose separately and the columns were eluted as before. Both the N-terminal and C-terminal fragments did not bind to poly (U)-Sepharose and were eluted from the column in the 0.02 M NH_4Cl buffer (Fig. 4.2 B). The results indicate that N-terminal fragment (from amino acid residues 1 to 171) and C-terminal fragment possessing R3 and R4 repeat sequences apparently do not have binding sites for poly (U).

BINDING OF PROTEIN S1 TO M13 ssDNA

Binding of protein S1 to M13 ssDNA was analyzed using gel mobility shift assay. The assay was carried out as described in chapter II. Complexes were formed by incubating fixed amounts of closed circular M13mp7 ssDNA with varying amounts of protein S1 at 37 °C for 10 min in buffer containing 22 mM NaCl and electrophoresed in agarose gels. The results obtained are presented in Fig. 4.3 (A). The binding of protein to DNA resulted in the retardation or lower migration compared to free DNA. The mobility decreased with increasing amount of protein added.

Binding of S1 to M13 ssDNA was also carried out at high salt conditions. In this case, complexes were formed by incubating M13 ssDNA with protein in buffer containing 0.29 M NaCl and electrophoresed as described in chapter II. The results obtained are presented in Fig. 4.3 (B). From our results it is clear that S1.M13 ssDNA complexes were formed even at high salt (0.29 M NaCl). However the extent of retardation was less when compared with binding of S1 to ssDNA at low salt concentration. At saturation of 6 μg S1/0.5 μg DNA, the binding site size corresponds to approximately 15 nucleotides per one S1 molecule, which is same as that reported for RNA binding (the calculation is

based on the value of $1 A_{260} \text{ ssDNA} = 33 \mu\text{g/ml} = 0.1 \text{ mM}$ in nucleotides). This value corresponds very well with that reported in the literature (Thomas *et al* 1978). However, Draper and von Hippel (1978a) reported a site size of 5 for the binding of polydeoxynucleotides to the site I.

BINDING OF PROTEIN S1 TO λ DNA

Binding of S1 to phage λ DNA was also analyzed using gel mobility shift assay. Complexes were formed by incubating linear double stranded phage λ DNA with excess S1 in buffer containing 22 mM NaCl at 37 °C for 10 min and electrophoresed (for details see chapter II). Binding of S1 to M13 ssDNA was also carried out as a control. The results obtained are presented in Fig. 4.4. As expected S1 bound to M13 ssDNA and electrophoretic mobility of complex formed was retarded (lane 3). There was no difference in the electrophoretic mobility of free λ DNA (lane 2) and λ DNA incubated with S1 (lane 4). This shows that S1 does not bind to λ DNA or the complex does not show any difference in mobility.

Binding of S1 to single stranded DNA in the presence of double stranded DNA was carried out to check whether S1 binding to single stranded DNA is affected by double stranded DNA. For this we have added M13 ssDNA to protein S1 either before or after incubation with λ DNA (lanes 5 and 6). When both M13 ssDNA and λ DNA were incubated with S1, only M13 ssDNA.S1 complexes were formed (lanes 5 and 6) indicating low or no affinity of S1 to double stranded DNA.

ig. 4.3: Binding of S1 to M13 ssDNA.

Electrophoresis of complexes of S1– single stranded M13mp7 DNA in 0.5% agarose. Complexes were formed by incubating M13 ssDNA (0.5 μ g) with increasing amounts of protein S1 at 25 °C in

(A): 10 mM Tris-HCl, pH 7.6, 0.1 mM Na₂EDTA, 22 mM NaCl.

(B): 10 mM Tris-HCl, pH7.6, 0.1 mM Na₂EDTA, 0.29 M NaCl.

In both cases electrophoresis was performed using low salt running buffer (20 mM Tris, pH 7.8, 0.4 mM sodium acetate and 0.2 mM Na₂EDTA).

Lane 1 : M13 ssDNA.

Lanes 2-8: M13 ssDNA incubated with 0.5, 1.0, 2.0, 4.0, 6.0, 7.0 and 8.0 μ g of S1 respectively.

Fig 4.4: Binding of S1 to λ DNA.

Complexes were formed by incubating DNA with protein S1 in 10 mM Tris-HCl, pH 7.6, 0.1 mM Na₂EDTA, 22 mM NaCl at 25°C and were electrophoresed on 0.5% agarose gel.

Lane 1: M13 ssDNA (0.1 μ g).

Lane 2: λ DNA (0.3 μ g).

Lane 3: M13 ssDNA (0.1 μ g) incubated with 2 μ g of S1.

Lane 4: λ DNA (0.3 μ g) incubated with 10 μ g of S1.

Lane 5: 0.1 μ g of M13 ssDNA first incubated with 2 μ g of S1 followed by λ DNA (0.1 μ g).

Lane 6: 0.1 μ g of λ DNA first incubated with 10 μ g of S1 followed by M13 ssDNA (0.1 μ g).

g. 4.5: Binding of S1 to M13 ssDNA in the presence of poly (U).

M13 ssDNA (0.5 μ g) was incubated with S1 before or after the addition of poly (U) in 10 mM Tris-HCl, pH 7.6, 0.1 mM Na₂EDTA, 22 mM Nacl at 25°C. The complexes formed were electrophoresed on a 0.5% agarose gel and the bands were visualized after staining with ethidium bromide.

Lane 1: M13 ssDNA (0.5 μ g).

Lane 2: S1 (4 μ g) incubated with M13 ssDNA (0.5 μ g).

Lane 3: S1 (4 μ g) was incubated with 2 μ g poly (U) and then with M13 ss DNA (0.5 μ g).

Lane 4: S1 (4 μ g) was incubated with 0.5 μ g M13 ss DNA and then with poly (U) (2.0 μ g).

Lane 5: protein S1 (4.0 μ g).

Lane 6: poly (U) (2 μ g).

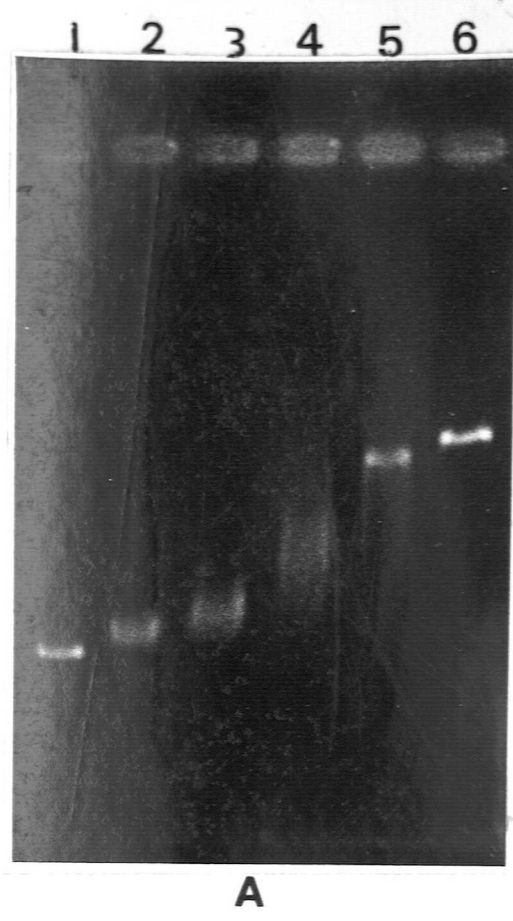


FIG 4-3

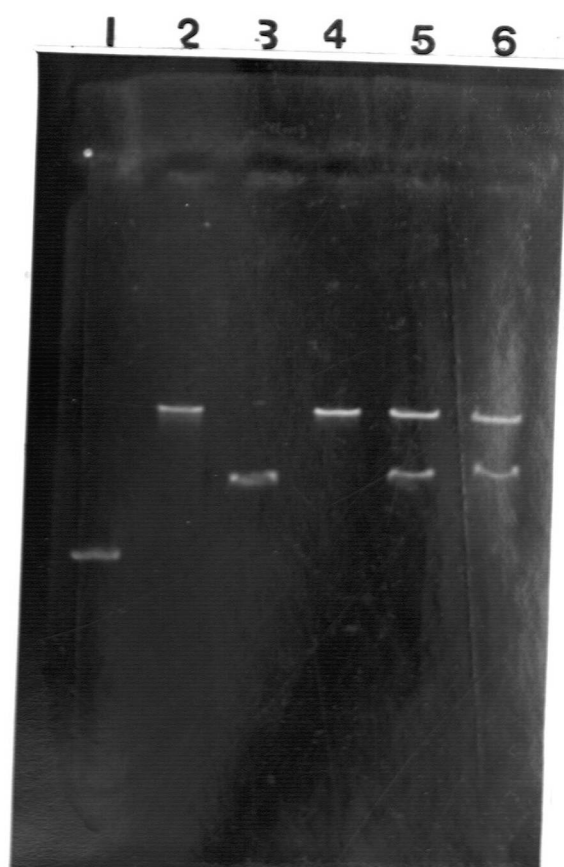


FIG 4-4

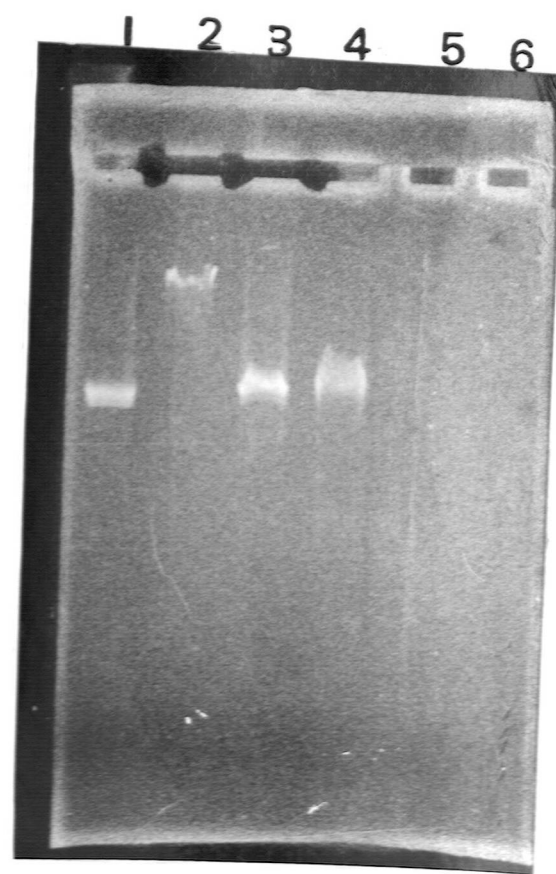


FIG 4-5

BINDING OF M13 ssDNA TO S1 IN THE PRESENCE OF POLY (U)

Binding of S1 to M13 ssDNA was carried out in the presence of poly (U) and the gel retardation of complexes formed were analyzed by agarose gel electrophoresis. Complete experimental details are given in the legend to Fig. 4.5. In the absence of poly (U), S1 bound to M13 ssDNA and the complex showed retarded migration when compared to free M13 ssDNA (Fig. 4.5 lane 2). When Poly (U) and M13 ssDNA were incubated together with protein S1 there was no retardation of M13 ssDNA (Fig. 4.5 lane 3). We have added poly (U) to protein S1 either before or after incubation with M13 ssDNA. In both cases electrophoretic mobility retardation of DNA was not observed. Addition of poly (U) to M13.ssDNA S1 complex results in the dissociation of complex (Fig. 4.5 lane 4). Thus there was no binding of S1 to M13 ssDNA in the presence of poly (U) regardless of the order of addition of poly (U) to protein. The results indicate stronger affinity of S1 to poly (U) and ssDNA is competed out by poly (U).

BINDING OF DIFFERENT FRAGMENTS OF S1 to M13 ssDNA

We have also tested binding of M13 ssDNA to isolated, purified fragments of S1 to identify which of the fragments contain the denatured DNA binding site. Binding of individual fragments of S1 to M13 ssDNA was measured by mixing the fragments with M13 ssDNA and after incubation the complexes formed were electrophoresed in agarose gels (see chapter II for details). The results obtained are presented in Fig. 4.6 (A-E).

Fig. 4.6 (A & B) shows the binding of M13 ssDNA by S1F4. The electrophoretic mobilities of complexes formed between M13 ssDNA and S1F4

Fig. 4.6: Binding of various polypeptide fragments of S1 to M13 ssDNA.

M13 ssDNA (0.5 μg in A and 0.1 μg in B–E) was incubated with increasing amounts of polypeptide fragments of S1 in 10 mM Tris-HCl, pH 7.6, 0.1 mM Na_2EDTA , 22 mM NaCl at 25 ° C. The complexes formed were electrophoresed on 0.5% agarose gels.

(A): Binding of S1F4.

Lane 1 : M13 ssDNA (0.5 μg).

Lanes 2-6: 0.5 μg of M13 ssDNA was incubated with 0.5, 1.0, 2.0, 4.0, and 6.0 μg of S1F4 respectively.

(B) Binding of S1F4.

Lanes 1,6: M13 ssDNA (0.1 μg).

Lanes 2-5: 0.1 μg of M13 ssDNA incubated with 1.0, 2.0, 4.0, and 6.0 μg of S1F4 respectively.

(C) Binding of C-terminal fragment.

Lane 1 : M13 ssDNA (0.1 μg).

Lanes 2-6: 0.1 μg of M13 ssDNA was incubated with 1.0, 2.0, 3.0, 4.0, and 6.0 μg of C-terminal fragment.

(D) Binding of S1F1.

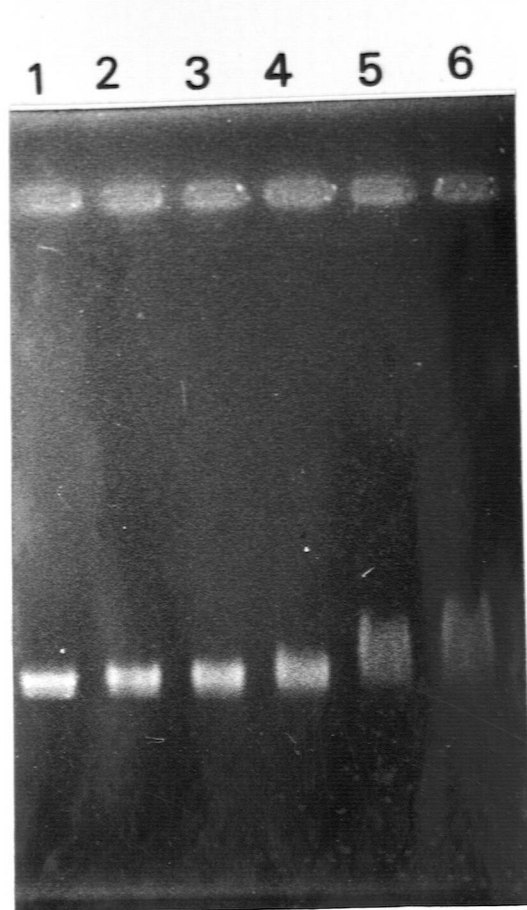
Lane 1 : M13 ssDNA (0.1 μg).

Lanes 2-6: 0.1 μg of M13 ssDNA was incubated with 1.0, 2.0, 3.0, 4.0, and 6.0 μg of S1F1.

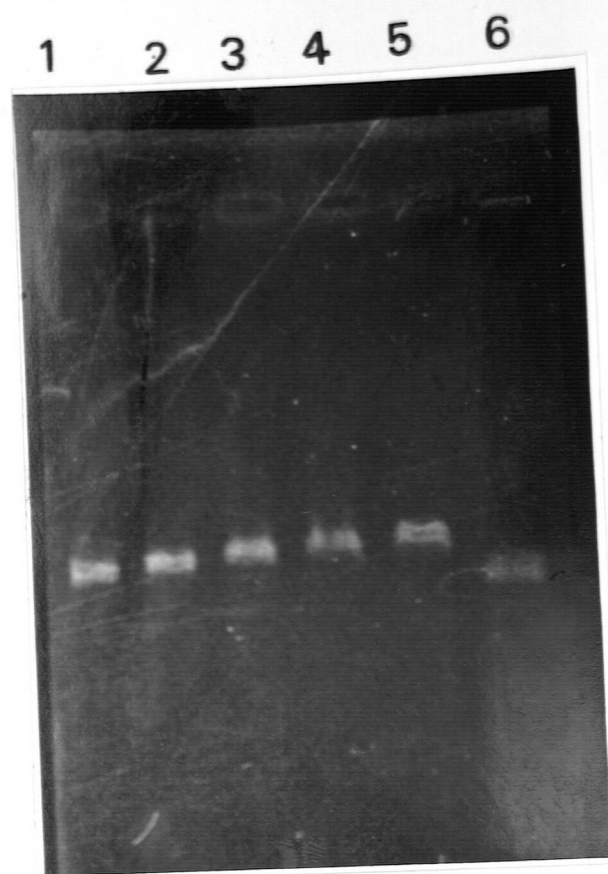
(E) Binding of N-terminal fragment.

Lane 1 : M13 ssDNA (0.1 μg).

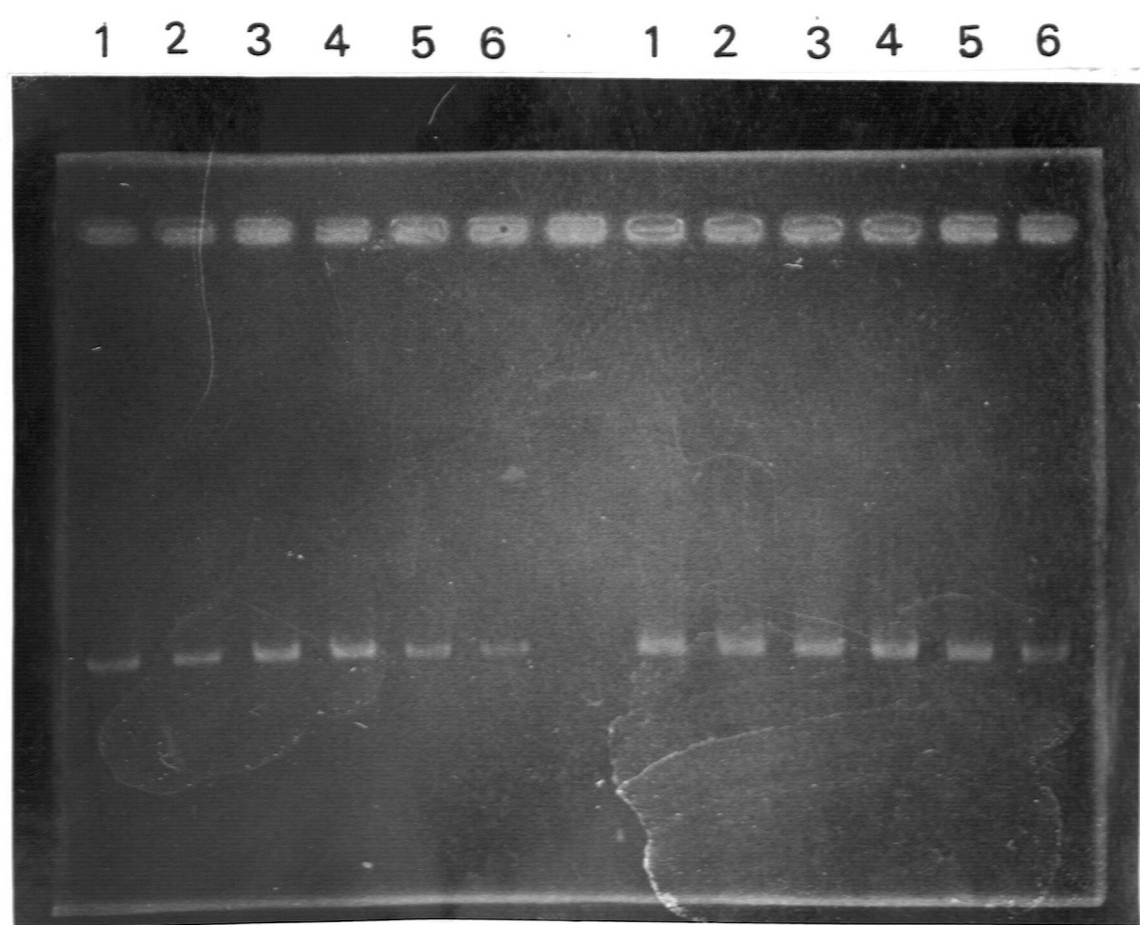
Lanes 2-6: 0.1 μg of M13 ssDNA was incubated with 1.0, 2.0, 3.0, 4.0, and 6.0 μg of N-terminal fragment.



A

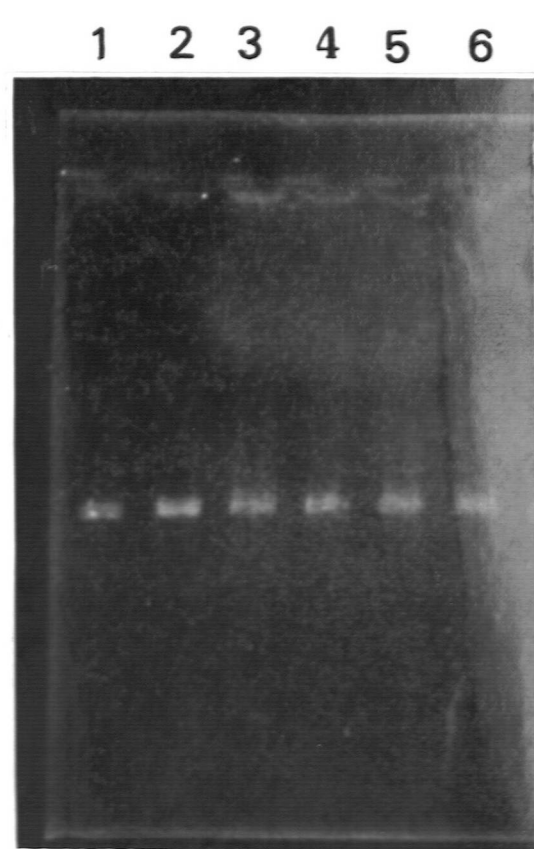


B



C

1 2 3 4 5 6



D

Fig. 4.6

1 2 3 4 5 6

E

are significantly retarded when compared with free M13 ssDNA. The gel mobility of complexes significantly decreased with increasing amount of fragment. This indicates that, S1F4 binds to single stranded DNA. However the mobility shift observed with M13 ssDNA.S1F4 complexes was similar to those formed with S1 at low concentration of the protein. The results indicate that, S1F4 while retaining ssDNA binding site of S1, has lost some other sites responsible for high affinity interactions of S1 to ssDNA or that the retardation of the complexes may be less than S1.DNA complexes retaining the same affinity.

The results shown Fig. 4.6 (C, D, and E) are from agarose gel electrophoresis of C-terminal fragment, S1F1 and N-terminal fragments incubated with M13 ssDNA respectively. No appreciable shift in mobility of M13 ssDNA was observed when incubated with these fragments. So the denatured DNA binding site of protein S1 is not retained in these fragments.

BINDING OF N-TERMINAL FRAGMENT TO 30S SUBUNITS

The N-terminal fragment of S1, which we have used in our studies was obtained with trypsin cleavage of protein S1. This fragment is like F2a isolated by CNBr cleavage except the terminal 22 amino acid residues are missing. We have tested whether the N-terminal fragment isolated by trypsin cleavage is functionally equivalent to F2a. For this, we have carried out binding of N-terminal fragment to S1 depleted *E.coli* 30S subunits using DEAE cellulose chromatography.

The experiment was performed by mixing N-terminal fragment with S1 depleted 30S subunits and chromatographed through a small column of

fig. 4.7: Binding of N-terminal fragment to S1 depleted *E.coli* 30S subunits.

Four A_{260} units of *E.coli* 30S subunits devoid of S1 were incubated with 18 μg of N-terminal fragment in TKM10 buffer at 37°C for 10 min. The reconstituted 30S ribosomes were passed through 1 ml DE-52 column equilibrated with TKM10 buffer. The column was washed with ten bed volumes of equilibrating buffer. The 30S subunits bound to the column were eluted with equilibrating buffer containing 0.6 M ammonium chloride. To detect the binding of N-terminal fragment, 30S subunits eluted from the column (0.1 A_{260} units) were electrophoresed and blotted with anti *E.coli* S1. Binding of protein S1 to S1 depleted 30S ribosomes was also performed as above as a control.

Lane 1: 30S subunits devoid of S1.

Lane 2: 30S subunits lacking S1 incubated with N-terminal fragment.

Lane 3: 30S subunits lacking S1 incubated with protein S1.



FIG 4-7

DE-52 previously equilibrated with 0.2 M NH_4Cl . The 30S subunits bound to DE-52 was eluted with 0.6 M NH_4Cl buffer (for details see chapter II). The 30S subunits were then analyzed by SDS- PAGE and blotted with antibodies to *E.coli* S1 to look for the bound N-terminal fragment. Binding of protein S1 to S1 depleted 30S subunits was also carried out as a control. The results obtained are presented in Fig. 4.7. Like S1, N-terminal fragment also bound to 30S subunits.

Lack of binding of S1F1 to ssDNA was unexpected since, S1F1 retains the major nucleic acid binding domain S1 (Suryanarayana and Subramanian, 1979). Control experiments of S1F1 binding to poly (U)-Sepharose showed strong binding of S1F1 to poly (U) like S1 and S1F4. The results indicate that, the domain that is involved in ssDNA binding may be lost after tryptic cleavage or that N-terminal portion of the molecule may influence the ssDNA binding indirectly. Binding of S1F1 to denatured DNA was previously studied using radiolabelled heat-denatured SV 40 DNA by Millipore filtration. S1F1 binds to DNA much less efficiently than poly (U). The unwinding or helix-destabilizing properties of S1 were not observed with S1F1 either by CD spectroscopy or electron microscopy (Thomas *et al* 1979).

Draper and von Hippel (1978a,b) reported that S1 binds to denatured DNA and RNA through site I (non specific) and only RNA through site I-I (specific). Interestingly S1 binds to several nucleic acid coupled matrices with varying affinities. S1 binds to poly (rC)-cellulose (Carmichael, 1975) and poly (U)-Sepharose (Carmichael, 1975; Surryanarayana and Subramanian, 1979) even in the presence of high concentration of salt and is released from the column with salt plus urea only. On the other hand S1 bound to poly (rA)-cellulose and calf thymus denatured DNA-cellulose was eluted with

1.0 M NH_4Cl . This differential binding may be due to the structural differences in the polynucleotides and that both types of polynucleotides may bind at a single site with differing affinities. The ordered structures such as polydeoxynucleotides may interact with S1 only through electrostatic interaction and the random coiled structured poly (U) and to some extent poly (C) may involve additionally a hydrophobic component. Results of competition experiment (Fig. 4.5) also argue for a single site at which poly (U) and ssDNA bind. If site I is saturated with ssDNA and then poly (U) is added, (should bind at the available site II), one would have expected retarded mobility of M13 ssDNA.

Like F2a, N-terminal fragment also bound to 30S subunits. F2a bound to poly (U)-Sepharose was eluted from the column by 0.5 to 1.0 M NH_4Cl (Giorginis and Subramanian, 1980). However, the N-terminal fragment showed no binding to poly (U)-Sepharose. These results indicate that the 22 amino acid residues at the C-terminus of F2a, which are missing in the N-terminal fragment may contribute along with the homologous repeat R1 in binding to RNA and denatured DNA.

Lack of binding of the C-terminal fragment to polydeoxyribonucleotides or polyribonucleotides raises a question whether the R3 and R4 repeats at all have a nucleic acid binding domain?. It was already shown that removal of the last repeat does not at all impair the function of the protein as in the case of M1S1. However one conclusion that can be drawn from the present studies is that the C-terminal fragment containing R3 and R4 repeat sequences does not apparently contribute to the binding of S1 to either poly (U) or denatured DNA and that the two polynucleotide binding sites of S1, if present, are located within the region that corresponds to amino acid residues 190 to

348. However studies of Suryanarayana and Subramanian (1984) on S1F4 and those of Schnier *et al* (1986) on deletion mutants of rps A gene suggest an important unidentified role for the repeat sequence R3. This function may be a regulatory role connected with protein synthesis other than direct binding of RNA. Alternatively this region may have a sequence specific nucleic acid binding domain (Shine–Dalgarno sequence ?), which has not been tested in the present study. The binding properties of different fragments of S1 to poly (U) and denatured DNA are summarized in table 4.1.

Combining the results of chapter III and chapter IV some important conclusions can be drawn regarding the amino acid functional groups involved in nucleic acid binding of S1. The location on S1 of the different amino acid residues that were modified vis-a-vis the four homologous sequences is given in Fig. 4.8. There are two histidine residues in R1 and R2 in almost identical position. Such distribution is not seen in R3, and R4 has no histidine residue. Similarly there is a single tyrosine residue in each of the repeat sequences. However only R1 and R2 have this residue at approximately same location with respect to start of the repeat sequences. Work presented in this chapter also showed importance of only R1 and R2 repeats in the binding of S1 to polyribo or polydeoxyribonucleotides. The C-terminal fragment containing R3 and R4 does not show any apparent affinity to nucleic acids. The results from both chemical modification and binding of different fragments of S1 to nucleic acids suggest that S1 binds to both polyribo and polydeoxyribonucleotides through a single site located in the region overlapping the C-terminal part of ribosome binding domain and the R1 and R2 repeat sequences.

Fragments	Poly (U) binding	ssDNA binding	Ribosome binding
S1	+	+	+
S1F1	+	—	— ^a
S1F4	+	+	+
N-terminal fragment (tryptic cleavage)	—	—	+
C-terminal fragment	—	—	— ^b

Table. 4.1. Summary of binding of different fragments of S1 to poly (U), ssDNA and ribosomes.
a taken from Suryanarayana and Subramanian (1979)
b taken from Giorginis and Subramanian (1980)

F2a

R1|R2

R3|R4

Location of histidine residues

F2a

R1|R2

R3|R4

Location of tyrosine residues

F2a

R1|R2

R3|R4

Locaation of lysine residues

F2a

R1|R2

R3|R4

Location of arginine residues

Fig. 4.8

Chapter V

PROTEIN S1 IN ARCHAEOBACTERIA

Extensive work has been carried out on the structural and functional characterization of ribosomal protein S1 from *E.coli*. S1-like proteins have been detected in several eubacteria (reviewed in chapter I). There are very few reports on the characterization of S1-like proteins from organisms other than eubacteria. So far no major study was undertaken to look for S1-like proteins in archaeobacteria. Schnier and Faist (1985) studied whether the structural gene *rps A* (coding for S1) is present in *Halobacterium halobium* by southern blot technique. They obtained hybridization under less stringent conditions and hence they could not conclude whether the gene was present or absent in *H.halobium*.

The characterization of S1-like proteins in bacteria where it is present is helpful in understanding how the protein has evolved during evolution. S1 protein may be considered as a chemotaxonomic character and may be useful in the determination of phylogenetic relationship among bacteria. In this chapter ribosomes from bacteria belonging to two families of archaeobacteria (thermoacidophiles and extreme halophiles) have been analyzed for the presence of protein S1 by sensitive western blotting technique. Antibodies raised against ribosome binding and nucleic acid binding domains of *E.coli* S1 were used in blotting experiments to test for the presence of S1 homologues in these bacteria. A brief introduction will be presented in the following pages about archaeobacteria.

Archaeobacteria have been recognized as phylogenetically distinct from other bacteria (eubacteria) and eukaryotes (Woese, 1981; Fewson, 1986). Woese *et al* (1990) basing on the ribosomal RNA sequence comparisons

(Fox *et al* 1980) classified archaebacteria as a separate kingdom (Archaea).

Archaea consist of two sub groups namely

1. Crenarchaeota—includes extensively sulphur dependent extreme thermophiles.
2. Euryarchaeota—includes all the other archaebacteria viz., methanogens and extreme halophiles.

METHANOGENS

These are well adapted to earth's early atmosphere which is rich in hydrogen and carbon dioxide. These archaebacteria appear to be ancient. The organisms are obligatory anaerobes and reduce CO_2 to methane.

HALOPHILIC BACTERIA

Halobacteria are obligatory halophiles and grow on saturated brines. They accumulate even high amount of intracellular salts. These are mostly aerobic and all of them are chemoheterotrophic and require 3–4 M NaCl for their growth.

THE SULPHUR-DEPENDENT ORGANISMS

These are found in extraordinary habitats such as hot acid springs and submarine volcanic areas. Some are capable of novel anaerobic chemolithotrophic metabolism using H_2 and CO_2 and elemental sulphur.

UNIQUE PROPERTIES OF ARCHAEOBACTERIA

The cell envelopes of archaebacteria are completely different from those of eubacteria. Some of the archaebacteria viz., thermoplasma, methanoplasmas have no cell walls. Pseudomurein is well characterized component of

archaebacterial envelopes. A cell wall polymer isolated from methanosarcina has been named methanochondroitin and resembles the chondroitin of eukaryotes. Glycerol ethers predominate the archaebacterial glycerol lipids and these are dextrorotatory. The membranes in archaebacteria are lipid monolayers because of the presence of tetraethers. Thermoplasma and Sulfolobus have lipid monolayers that are more stable at the high temperatures.

A small non-ribosomal 7S RNA has been found in all the major groups of archaebacteria. Some of the archaebacterial mRNAs have no Shine-Dalgarno sequences even though the 16S rRNA has the appropriate 3' terminal poly pyrimidine sequence. The methanogens possess a number of coenzymes such as coenzyme M, cofactor F and factor F which are exclusively confined to these organisms. Both RNA and protein components of archaebacterial ribosomes have many unique properties. It is suggested that archaebacteria are most ancient and adapted to extreme environments that prevailed on the primitive earth about 3.5×10^6 years ago.

MATERIALS AND METHODS

Details of the experimental methods used are presented in the general materials and methods section (chapter II).

RESULTS AND DISCUSSION

IMMUNOBLOTTING OF CRUDE CELL LYSATES OF *S.acidocaldarius* AND *H.halobium* USING ANTIBODIES TO *E.coli* S1 AND ITS FRAGMENTS

Crude cell lysates of *S.acidocaldarius* and *H.halobium* were electrophoresed

Fig. 5.1 (A): SDS-polyacrylamide gel electrophoretic pattern of cell lysates from archaeobacteria.

- Lane 1 : *E.coli* protein S1 (1.0 μ g).
- Lane 2 : *S.acidocaldarius* cell lysate (from 150 μ l mid logarithmic cell culture).
- Lane 3 : *H.halobium* cell lysate (from 250 μ l mid logarithmic cell culture).
- Lane 4 : Standard proteins (bovine serum albumin, ovalbumin, glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase, trypsinogen and α lactalbumin).

(B): Immunoblotting of cell lysates of *S.acidocaldarius* and *H.halobium* using
Anti *E.coli* S1 (Lanes 1-3)
Anti F2a (Lanes 4-6)
Anti S1F1 (Lanes 7-9)

- Lanes 1,4,7: *E.coli* protein S1 (25 ng).
- Lanes 2,5,8: *S.acidocaldarius* cell lysate (from 150 μ l mid logarithmic cell culture).
- Lanes 3,6,9: *H.halobium* cell lysate (from 250 μ l mid logarithmic cell culture).

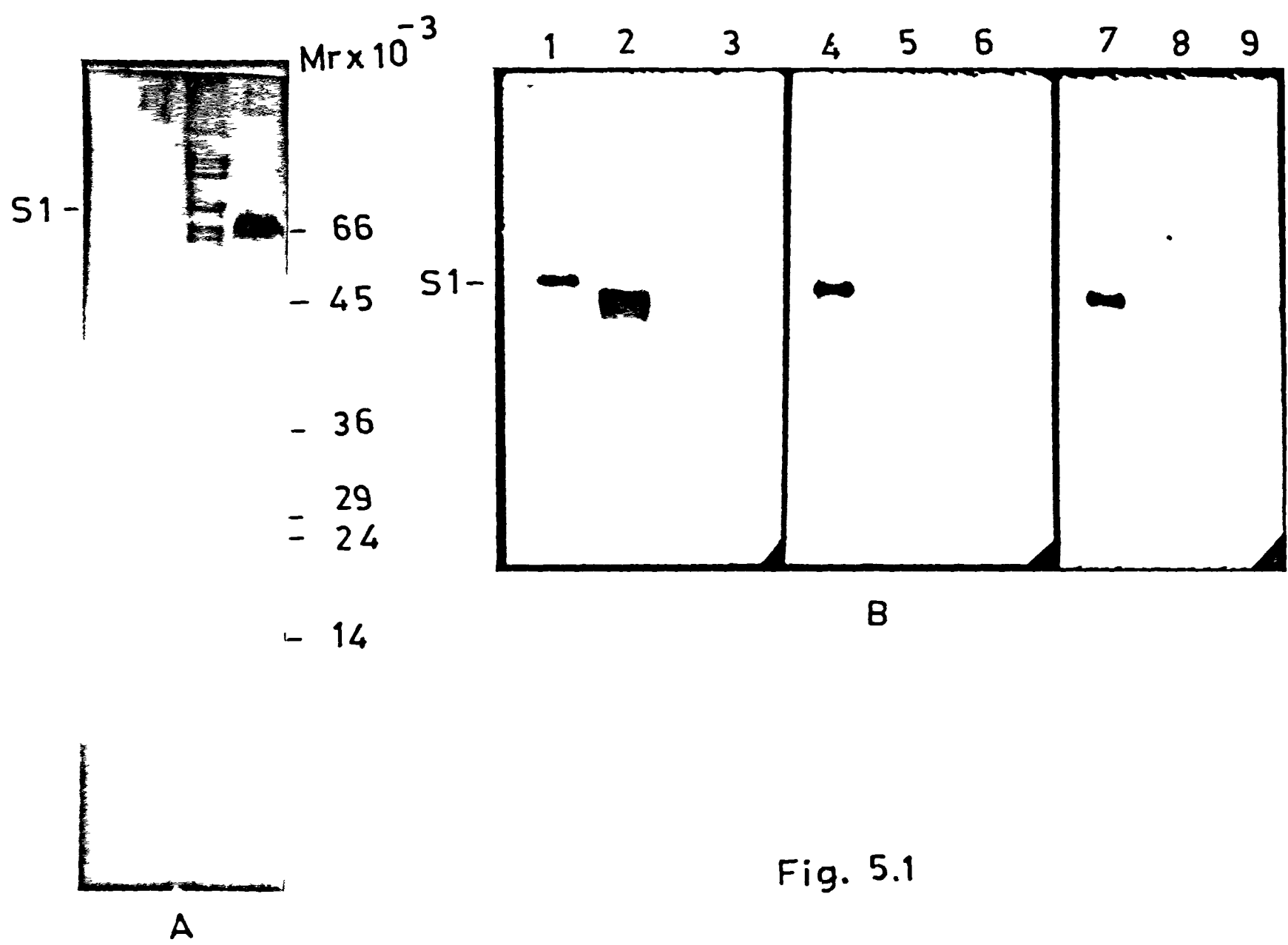


Fig. 5.1

on SDS–poly acrylamide gels. The proteins from polyacrylamide gels were transferred onto nitrocellulose sheets. The membrane was incubated with the antibodies to *E.coli* S1 and its fragments as described in chapter II. The blot was developed using peroxidase conjugated anti rabbit IgG. The results are given in Fig. 5.1 (B). As expected purified protein S1 from *E.coli* gave a strong cross reaction with all the antisera (lanes 1, 4 and 7). *S.acidocaldarius* crude cell extract had a protein with a migration slightly lower than *E.coli* S1 (61 kDa) that cross reacted with anti–S1 (lane 2). However this protein cross reacted very weakly with anti–S1F1 and showed no cross reaction with anti–F2a. A protein of molecular weight 100 kDa in *S.acidocaldarius* showed weak cross reaction with both anti–S1 (lane 2) and anti–S1F1 (lane 8). This protein did not cross react with anti–F2a (lane 5). On the other hand *H.halobium* crude extract had two proteins of molecular weight 95 kDa and 90 kDa that cross reacted with only anti–F2a (lane 6).

IMMUNOBLOTTING OF RIBOSOMES OF *S.acidocaldarius* AND *H.halobium* USING ANTIBODIES TO *E.coli* S1 AND ITS FRAGMENTS

70S ribosomes from *S.acidocaldarius* and *H.halobium* were also tested by immunoblotting. The results obtained are shown in Fig. 5.2 (B). The immunological cross reactivity of the ribosomes was similar to that of crude cell lysates. *S.acidocaldarius* ribosomes had a protein with a molecular weight 100 kDa that cross reacted weakly with anti–S1 in addition to the 61 kDa protein. (lane 2). Both the 100 kDa and 61 kDa proteins of *S.acidocaldarius* ribosomes did not show any cross reaction with anti–F2a (lane 5). *H.halobium* ribosomes had two proteins (95 kDa and 90 kDa) that cross reacted with anti–F2a (lane 6). The results indicate the presence of S1–like proteins in these archaebacteria that show differential cross reactivity with antibodies to the two structural domains of *E.coli* S1.

5.2 (A): SDS–polyacrylamide gel electrophoretic pattern of archaeobacterial 70S ribosomes.
Proteins were stained with silver as described in chapter II.

Lane 1 : *E.coli* S1 (1.0 μ g).
Lane 2 : *S.acidocaldarius* 70S ribosomes (0.25 A_{260} units).
Lane 3 : *H.halobium* 70S ribosomes (0.25 A_{260} units).
Lane 4 : Standard proteins (myosin, β -galactosidase, phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase).

(B): Immunoblotting of ribosomes of *S.acidocaldarius* and *H.halobium* using
Anti *E.coli* S1 (Lanes 1-3)
Anti F2a (Lanes 4-6)
Anti S1F1 (Lanes 7-9)

Lanes 1,4,7: *E.coli* S1 (25 ng).
Lanes 2,5,8: *S.acidocaldarius* 70S ribosomes (0.1 A_{260} units).
Lanes 3,6,9: *H.halobium* 70S ribosomes (0.1 A_{260} units).

(C): Immunoblotting of *S.acidocaldarius* 70S ribosomes.
Antisera used were anti S1 in lanes 1-2, anti F2a in lanes 3-4 and anti S1F1 in lanes 5-6.

Lanes 1,3,5: *E.coli* protein S1 (25 ng).
Lanes 2,4,6: *S.acidocaldarius* 70S ribosomes (0.3 A_{260} units).
Antisera used were anti S1 in lanes 1–2, anti F2a in lanes 3–4 and anti S1F1 in lanes 5–6.

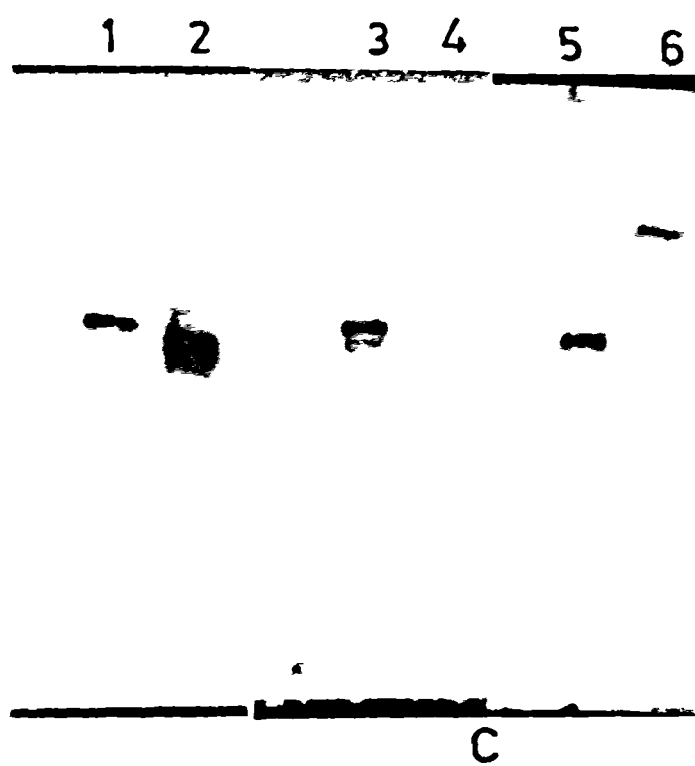
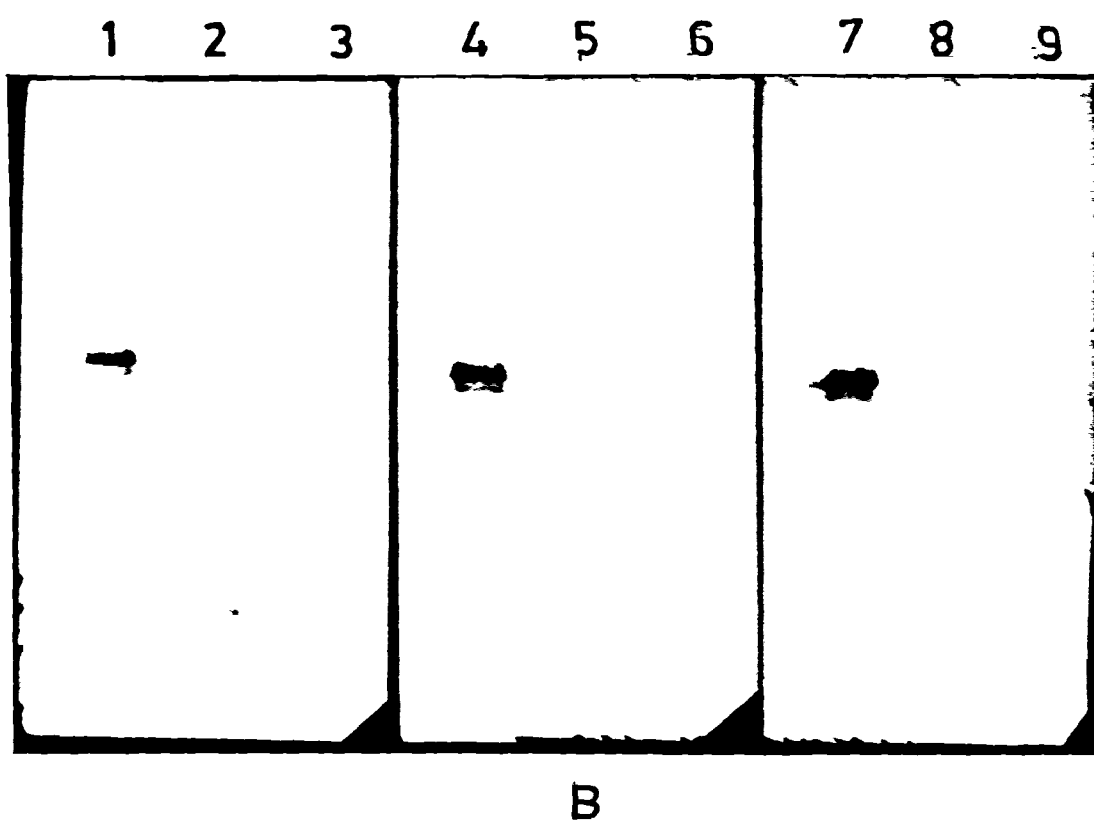


Fig. 5.2

No cross reaction was detected with anti-F2a even when higher amounts of *S.acidocaldarius* ribosomes were used in the blotting experiment (Fig. 5.2 (C) lane 4), whereas the extent of cross reaction of 100 kDa and 61 kDa proteins with anti-S1F1 has improved (lane 6). This result suggests the observation that S1-like protein(s) in *S.acidocaldarius* show immunological homology to the nucleic acid binding region of *E.coli* S1. Presence of two bands with molecular weight (100 kDa and 61 kDa) suggest that 61 kDa protein may be the proteolytic product of 100 kDa or that the 61 kDa protein might be aggregating under the conditions of sample preparation (see later).

IMMUNOBLOTTING OF CRUDE CELL LYSATES AND RIBOSOMES OF HALOBACTERIA WITH ANTIBODIES TO F2a FRAGMENT OF S1

The results obtained in our blotting experiments led us to the conclusion that the protein detected in *H.halobium* shares some antigenic determinants with that of N-terminal fragment of S1. We have searched for S1-like proteins in other species of halophiles viz., *H.cutirubrum* and *H.salinarium*. We have isolated the crude cell lysates as well as ribosomes from these organisms and blotted with antibodies to F2a. The results obtained are shown in Fig. 5.3. Results were similar in both crude cell lysates and ribosomes. The anti-F2a IgG reacted with two proteins of molecular weight 95 kDa and 90 kDa in the case of *H. halobium* (Fig. 5.3. lanes 2, 5) and a single protein of molecular weight 100 kDa in the case of other two halophiles viz., *H. cutirubrum* and *H.salinarium* (Fig. 5.3 lanes 3 to 4 and 6 to 7).

BINDING OF *E.coli* S1 to RIBOSOMES OF *S.acidocaldarius* and *H.salinarium*

We studied the binding of *E.coli* S1 to ribosomes of *S.acidocaldarius* and *H.salinarium*. Binding of S1 to S1 depleted *E.coli* ribosomes was carried out as a positive control. The experiment was performed by incubating protein S1

Fig. 5.3: Immunoblotting of halobacterial cell lysates and 70S ribosomes using anti F2a.

Lane 1 : S1 (25 ng).

Lanes 2-4: Cell lysates from *H.halobium*, *H.cutirubrum* and *H.salinarium* respectively (from 250 μ l mid logarithmic cell cultures).

Lanes 5-7: 70S ribosomes from *H.halobium*, *H.cutirubrum* and *H.salinarium* respectively (0.2 A_{260} units).

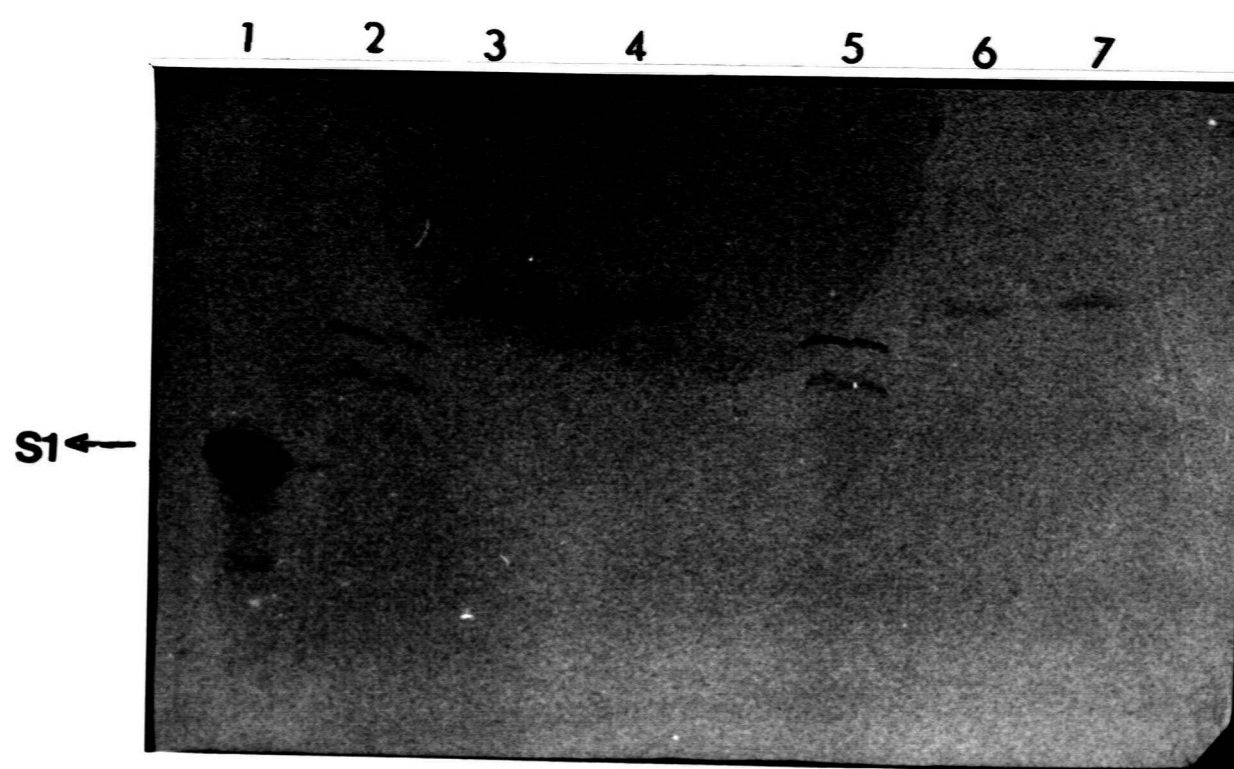


FIG 5.3

Fig. 5.4 (A): Binding of S1 to archaebacterial 70S ribosomes.

10 A₂₆₀ units of 70S ribosomes of *E.coli* (S1 depleted), *S.acidocaldarius*, *H.salinarium* were incubated with 45 µg of *E.coli* protein S1 in TKM10 buffer and incubated at 37°C for 10 min. Binding of S1 to *H.salinarium* 70S ribosomes was performed at both low salt (0.2 M NH₄Cl) and high salt (1.5 M NH₄Cl). The reaction mixtures were then layered over 4 ml of 15% sucrose in TKM10 buffer and pelleted at 50,000 rpm for 5 h at 4°C. Pelleted ribosomes (0.2 A₂₆₀) were electrophoresed and blotted with anti-*E.coli* S1.

Lane 1 : *E.coli* protein S1 (12 ng).

Lane 2 : S1 depleted *E.coli* 70S ribosomes.

Lane 3 : S1 depleted *E.coli* 70 S ribosomes incubated with *E.coli* S1.

Lane 4 : *S.acidocaldarius* 70S ribosomes.

Lane 5 : *S.acidocaldarius* 70S ribosomes after incubation with *E.coli* S1.

Lanes 6-7: 70S ribosomes of *H.salinarium* at low salt (0.2 M NH₄Cl) and high salt (1.5 M NH₄Cl) respectively.

Lanes 8-9: 70S ribosomes of *H.salinarium* after incubation with *E.coli* S1 at low salt (0.2 M NH₄Cl) and high salt (1.5 M NH₄Cl) respectively.

(B): SDS-polyacrylamide gel electrophoretic pattern of *S.acidocaldarius* ribosomes recovered from sucrose top centrifugation.

Gels were stained with silver as described in chapter II.

Lane 1 : *E.coli* protein S1 (1.0 µg).

Lane 2 : *S.acidocaldarius* 70S ribosomes (0.2 A₂₆₀ units).

Lane 3 : *S.acidocaldarius* 70S ribosomes pelleted through sucrose cushion (0.2 A₂₆₀ units).

(C): A part of the enlarged portion of Fig. 5.4 (B). The arrow indicates the missing protein.

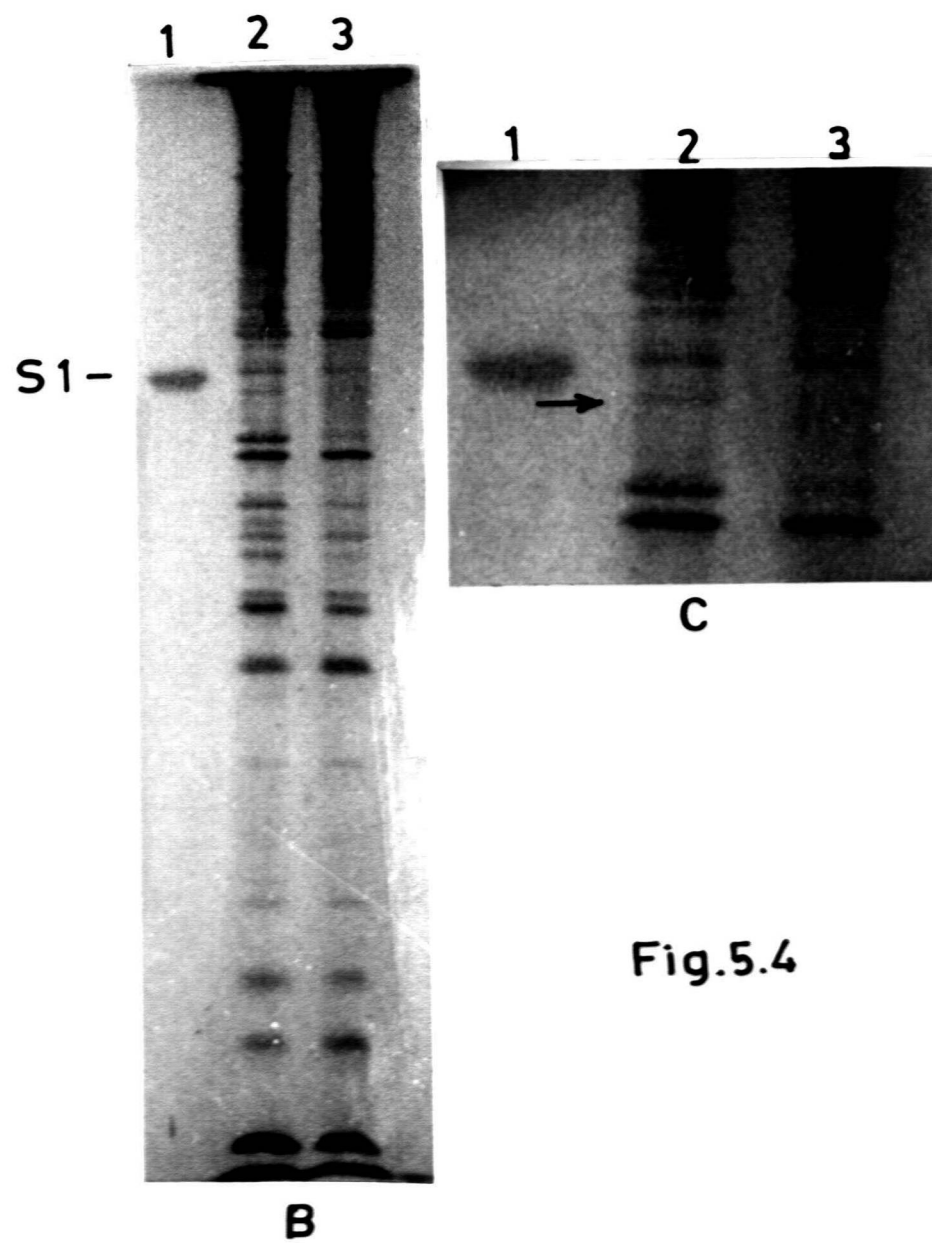
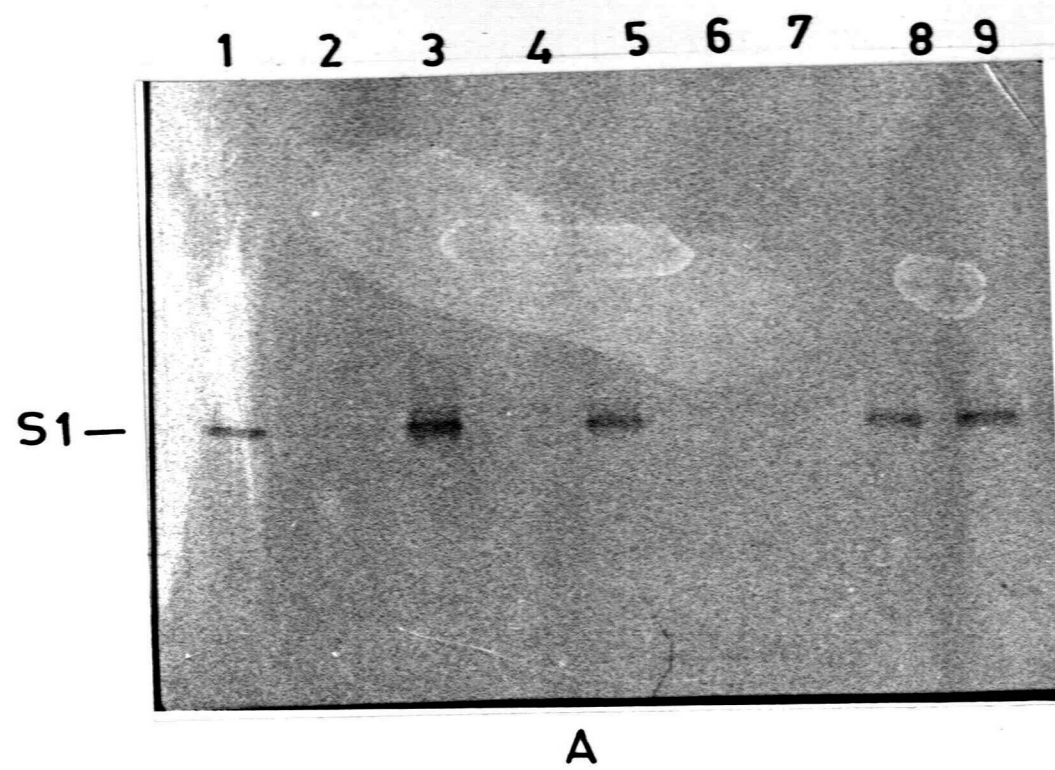


Fig.5.4

with different ribosomes and centrifuging through sucrose gradient to pellet the ribosomes. The ribosomes were then analyzed by SDS polyacrylamide gel electrophoresis and blotted with antibodies to *E.coli* S1. The results are presented in Fig. 5.4 (A). *E.coli* S1 was able to bind to all the three ribosomes viz., S1 depleted *E.coli* ribosomes, *S.acidocaldarius* and *H.salinarium* ribosomes. Since the halobacteria grow in high salt environments we carried out binding of S1 to halobacterial ribosomes at low salt (0.2 M NH_4Cl) and high salt (1.5 M NH_4Cl) conditions. S1 was able to bind to halobacterial 70S under both the conditions (lanes 8 and 9). The two cross reacting proteins could not be detected in *S.acidocaldarius* ribosomes recovered from sucrose centrifugation with antibodies to *E.coli* S1. The reasons for this: (1) the cross reactive proteins may be getting stripped off from the ribosomes in the conditions we have used for binding or (2) the cross reactive proteins of *Sulfolobus* could be loosely associated with the ribosomes and get dissociated when centrifuged through sucrose gradients. SDS-PAGE of the ribosomes recovered from sucrose density centrifugation (Fig. 5.4 (B)) indicated that the cross reactive 61 kDa protein was missing in these ribosomes (lane 3).

EFFECT OF TEMPERATURE ON SAMPLE PREPARATION FOR SDS-PAGE of *S.acidocaldarius* RIBOSOMES

We have studied the effect of temperature on the sample preparation of *S.acidocaldarius* ribosomes for SDS-PAGE and subsequent immunoblotting. Samples for electrophoresis were prepared by treating with SDS at three different temperatures

1. at 31 °C for 15 min.
2. at 65 °C for 15 min.
3. at 100 °C for 2 min.

Fig. 5.5: Effect of temperature on sample preparation of *S.acidocaldarius* ribosomes for SDS-PAGE.

Samples were prepared by treating with SDS sample buffer at different temperatures, electrophoresed and blotted with anti *E.coli* S1.

37°C for 15 min (Lanes 1-2)

65°C for 15 min (Lanes 3-4)

100°C for 2 min (Lanes 5-6)

Lanes 1,3,5: *E.coli* protein S1 (80 ng).

Lanes 2,4,6: *S.acidocaldarius* 70S ribosomes (0.2 A₂₆₀ units).

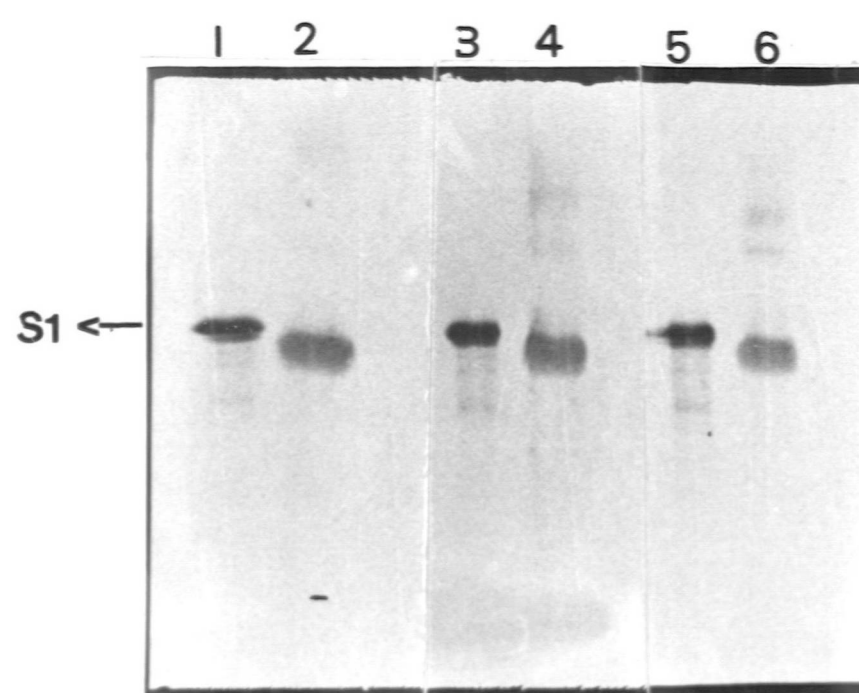


FIG. 5.5

The ribosome samples were then electrophoresed and blotted with antibodies to S1. The results are given in Fig. 5.5. When the sample was prepared by heating with SDS at 65 °C and 100 °C, we have observed high molecular weight protein bands at about 100 kDa position that cross reacted with antibodies to S1 (lanes 4 and 6). The preparation of the sample at room temperature resulted in a cross reaction with a single protein (61 kDa). These studies indicate that the cross reactive band with 100 kDa molecular weight seen in Fig. 5.1 and 5.2 may correspond to artefact band generated due to sample preparation at high temperature and that *S.acidocaldarius* ribosomes have a single cross reactive protein with molecular weight of 61 kDa.

IMMUNOBLOTTING OF RIBOSOMAL SUBUNITS OF SULFOLOBUS WITH ANTIBODIES TO *E.coli* S1

In order to see which of the subunits has the S1 cross reactive protein, ribosome subunits were isolated for immunoblotting experiments. The 70S ribosomes were dissociated into 30S and 50S subunits by two different methods. The first method was usual centrifugation through sucrose density gradients. These subunits were referred as gradient subunits. A preparative sucrose density gradient profile of the isolated subunits is given in Fig. 5.6 (A). The second method was the one developed by Kirillov *et al* (1978). In this method ribosomal subunits were separated by hydrophobic chromatography on Sepharose-4B. These subunits were referred as column subunits. The elution profile of the subunits isolated by this method is given in Fig. 5.6 (B). The one-dimensional gel pattern of the subunits isolated by the two methods is given in Fig. 5.7. Immunoblotting was performed with 30S and 50S subunits using S1 antiserum. (Fig. 5.8).

As expected *Saccharolobus* ribosomes cross reacted with antibodies to

fig. 5.6 (A): Separation of *S.acidocaldarius* ribosomal subunits by density gradient centrifugation.

100 A_{260} units of *S.acidocaldarius* ribosomes in 10 mM Tris-HCl, pH 7.6, 0.5 mM magnesium acetate, 0.5 M NH_4Cl were centrifuged through 10-30% linear sucrose gradient at 40,000 rpm for 2 h in a VTi-50 Beckman rotor. The optical density of the fractions were measured at 260 nm after suitable dilution. The peaks around fractions 5 and 15 represent 50S and 30S subunits respectively.

(B): Separation of *S.acidocaldarius* ribosomal subunits by hydrophobic chromatography on Sepharose 4B.

S.acidocaldarius ribosomes (3000 A_{260}) in 20 mM Tris-HCl, pH 7.6 and 1.5 M ammonium sulfate were passed through Sepharose 4B and eluted with linear reverse salt gradient (1.25 M to 0.02 M ammonium sulfate). The absorbance of each fraction (3.0 ml) was measured at 260 nm. The peak around fractions 70 and 90 represent 30S and 50S subunits respectively.

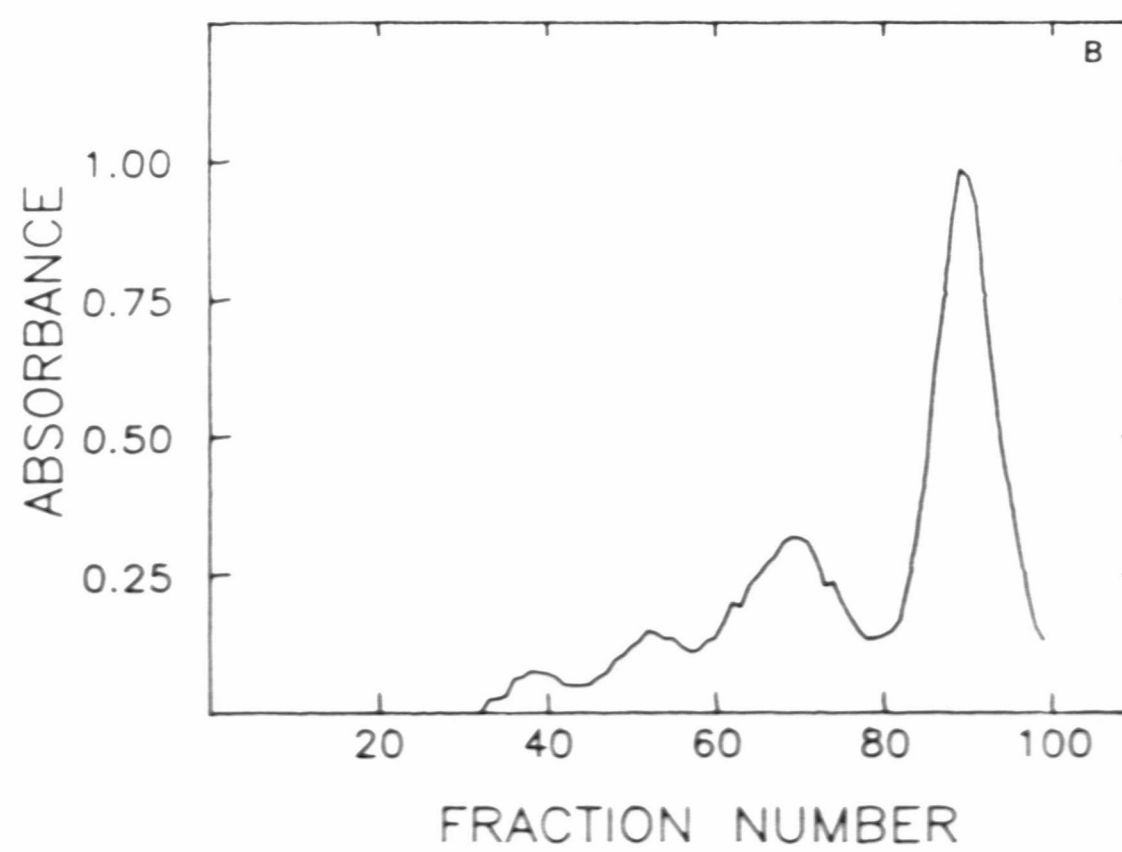
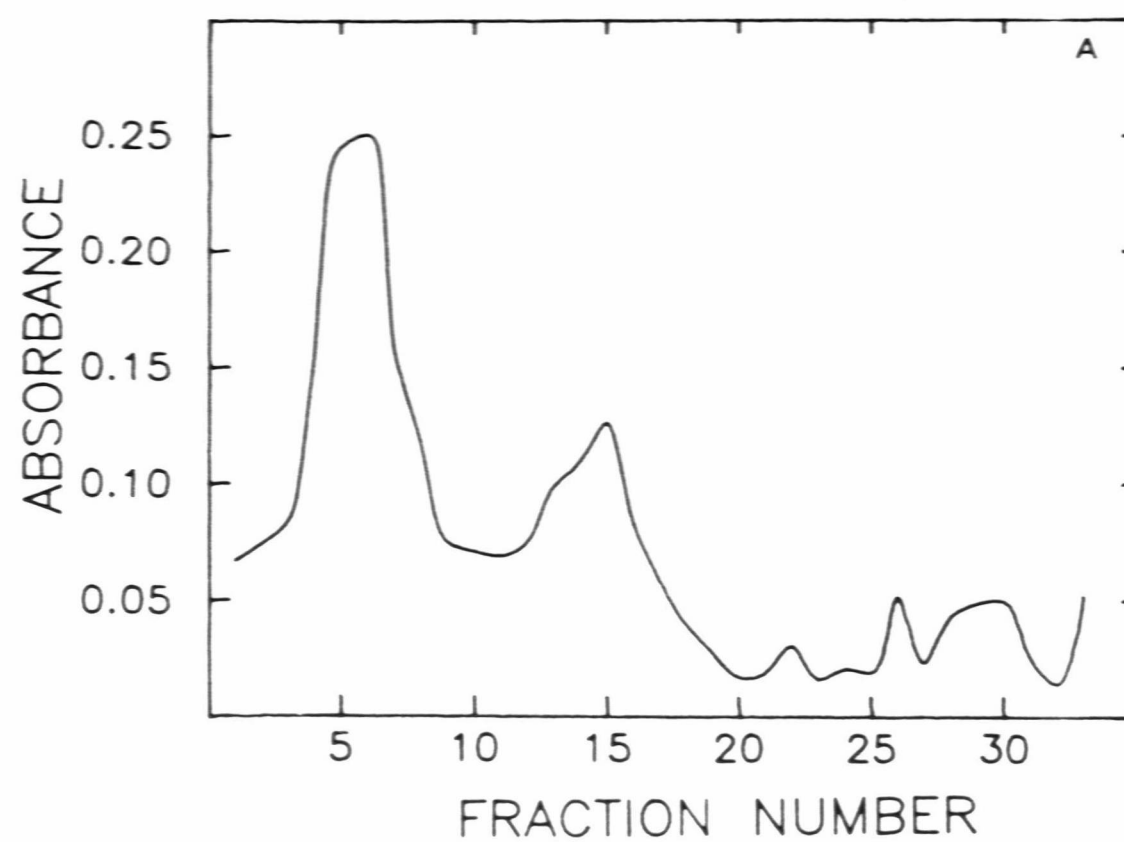


Fig. 5.7: SDS-polyacrylamide gel electrophoretic analysis of *S.acidocaldarius* ribosomal subunits.

Lane 1 : *E.coli* protein S1 (1.0 μg).

Lane 2 : *S.acidocaldarius* 70S ribosomes (1.0 A_{260} units).

Lanes 3-4: *S.acidocaldarius* 30S and 50S ribosomal subunits isolated by density gradient centrifugation (60.0 μg).

Lane 5 : Density gradient top fraction (20.0 μg).

Lanes 6-7: *S.acidocaldarius* 30S and 50S ribosomal subunits isolated by hydrophobic chromatography on Sepharose 4B (60.0 μg).

Lane 8 : Molecular weight standard proteins from top to bottom are phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor and lysozyme.

Fig. 5.8: Immunoblotting of *S.acidocaldarius* ribosomal subunits using anti *E.coli* S1.

Lanes 1,6: *E.coli* protein S1 (10 ng).

Lanes 2,7: *S.acidocaldarius* 70S ribosomes (0.1 A_{260} units).

Lanes 3,4: *S.acidocaldarius* 30S and 50S subunits isolated by density gradient centrifugation (0.05 A_{260} units).

Lane 5 : Density gradient top fraction (20.0 μg).

Lanes 8-9: *S.acidocaldarius* 30S and 50S ribosomal subunits isolated by hydrophobic chromatography on Sepharose 4B (0.05 A_{260} units).

E.coli S1 (lanes 2 and 7). No cross reaction was observed either with 30S or 50S subunits isolated by sucrose gradient method (lane 3 and 4). However, the cross reactive protein could be seen in gradient top fraction (lane 5). On the other hand, the usual cross reaction given by *S.acidocaldarius* ribosomes could be detected in the 30S subunit isolated by hydrophobic chromatography (lane 8). This indicates that the cross reactive protein is the constituent of 30S subunits and it gets dissociated from the subunits or 70S ribosomes during density gradient centrifugation.

The results presented in this section show that both thermoacidophilic and halophilic archaebacteria contain proteins immunologically homologous to *E.coli* S1. S1-like protein in *S acidocaldarius* (SaS1) has a molecular weight of 61kDa and that in halobacteria (HS1) is about 100 kDa.

Immunological cross reactivity using antisera to the two structural domains of *E.coli* S1 showed that SaS1 is structurally related only in the nucleic acid binding domain (the central and C terminal region) and HS1 is homologous only in the ribosome binding domain (the N terminal region) of *E.coli* S1. These results suggest differential conservation of the two domains in these archaebacteria.

Presence of two species of S1 like protein in *Halobacterium* with molecular weight 85 kDa and 90 kDa could be due to proteolytic cleavage of a 100 kDa species found in the other two halobacterial cells (*Halobacterium* and *H. cutirubrum*). Binding of F₂ S1 to both types of ribosomes from *S acidocaldarius* and Halobacteria indicates that these ribosomes probably have conserved the binding sites for *E.coli* S1. However, lack of cross reactivity of SaS1 with anti F2a ribosome binding domain and the observed binding of

E.coli S1 to *S.acidocaldarius* ribosomes may also be due to binding of *E.coli* S1 to *S.acidocaldarius* ribosomes through RNA binding domain. Similarly, the S1 binding to halobacterial ribosomes in the presence of high salt (1.5 M NH_4Cl) suggests that *E.coli* S1 either binds to these ribosomes through the nucleic acid binding domain (which binds RNA in a salt insensitive manner) or that the N-terminal ribosome binding domain has the potential to bind halobacterial ribosomes in a salt insensitive manner. Further experiments are needed to be carried out, by isolating S1-like proteins from these organism and carrying out binding studies with *E.coli*, *S acidocaldarius* and Halobacterial ribosomes, to draw any firm conclusions.

It is interesting to mention about the immunological cross reactivity of S1-like proteins isolated from other eubacteria reported in the literature. S1 homologues from *Azotobacter vinelandii* (Muralikrishna and Suryanarayana, 1987a) and *Micrococcus luteus* (Muralikrishna and Suryanarayana 1987b) showed greater immunological homology in the nucleic acid binding domain and the ribosome binding domain respectively. Although weak cross reactions were noticed in the other regions in S1 protein from these two bacteria. Similarly Mikulík *et al* 1986 have shown that S1 from *Streptomyces aureofaciens* was immunologically homologous to *E. coli* S1 only in the nucleic acid binding region of the molecule. The chloroplast ribosomal protein S1 (CS1) has a central core with three degenerate homologous repeats, one of which shows greater homology with the first homologous repeat sequence in the nucleic acid binding domain of *E. coli* S1.

Nucleotide sequence of the structural genes coding for S1-like proteins are available in the literature for two eubacteria viz, *Proteobacteria* sp. Schnier and Faist, 1985 and *Leishmania* *et al* 1988. In both these cases

greater homology was observed in the region corresponding to the nucleic acid binding domain. From these results and our own, it appears that in most organisms that have ribosomal protein S1 homologues, the nucleic acid binding domain is more highly conserved than the ribosome binding domain during evolution of the organisms. The exception being the halobacteria where the ribosomes contain protein which is homologous only to the ribosome binding domain of *E.coli* S1.

It is tempting to speculate that S1 gene might have evolved through fusion of two genes, one coding for a ribosomal protein (ribosome binding domain) and another coding for a general RNA binding protein (with RNA binding motifs) during the course of evolution.

Chapter VI

SUMMARY

CHAPTER I

A brief introduction about the ribosome structure in bacteria and a detailed account of literature review on ribosomal protein S1 were presented in this chapter. The structural and functional features revealed by physical and bio-chemical studies have been mentioned. Nucleic acid binding properties of protein S1, its functional role in protein synthesis and in RNA phage replication, its interaction with the 30S ribosome have also been presented. A brief account of S1- like proteins isolated and characterized from other eubacteria are also given. Objectives and scope of the present investigation have also been mentioned in this chapter.

CHAPTER II

Details of the experimental methods used in this work have been presented in this chapter.

CHAPTER III

This chapter is on the results obtained with chemical modification of *E.coli* ribosomal protein S1. Histidine, arginine, tyrosine, lysine and cysteine residues of protein S1 were modified with diethyl pyrocarbonate (DEPC) and rose bengal dye (RB), 2,3-butanedione (diacetyl), tetranitromethane (TNM), pyridoxal 5-phosphate (PLP) and N-ethylmaleimide (NEM) respectively. The effect of chemical modification on the function of protein S1 was studied using following assays. 1. Nucleic acid (ssRNA and ssDNA) binding. 2. Ribosome binding. 3. Stimulation of poly (U) directed *in vitro*

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translation. The UV absorption spectra of histidine (DEPC) modified S1, visible absorption spectra of tyrosine (TNM) modified S1 and fluorescence spectra of lysine (PLP) modified S1 showed the presence of modified histidine (N-ethoxyformyl histidine), tyrosine (3-nitrotyrosine) and lysine (ϵ -(5-phosphopyridoxyl)lysine) residues respectively.

Histidine (RB) modification did not result in the loss of ssRNA (poly (U)) binding but effected the salt insensitivity of the nucleic acid binding. Lysine modification resulted in the complete loss of poly (U) binding of the protein. Other modifications had no effect on the ssRNA binding of protein S1. ssDNA binding of S1 was inactivated with the modification of histidine (RB) and lysine residues. Tyrosine modification of S1 also effected ssDNA binding but to a lesser extent. Other modifications had no effect on ssDNA binding of S1.

Modification of histidine residues of protein S1 and its functional fragment (S1F4) with RB resulted in loss of ribosome binding activity. Tyrosine modification also resulted in substantial loss (~ 50%) of ribosome binding. All other modifications did not effect the ribosome binding of protein S1.

Modification of histidine residues of protein S1 with DEPC resulted in substantial loss (75%) of functional activity in polyphenylalanine synthesis, where as the modification with RB resulted in complete inactivation of poly (U) dependent protein synthesis. Arginine and tyrosine modifications of protein S1 have no effect on the activity of the protein to stimulate protein synthesis. Lysine modified S1 was able to stimulate poly (U) directed protein synthesis despite the fact that the modification resulted in the loss of ssRNA

binding as measured using poly (U)–Sephadex.

In summary histidine and lysine residues of S1 play an important role in the binding of S1 to nucleic acids. Histidine and to some extent tyrosine residues play an important role in the binding of S1 to ribosomes. Modification of either single (Cys-349) or both cysteine residues (Cys-349 and Cys-292) of protein S1 with NEM have no effect on the nucleic acid or ribosome binding. The inactivity of histidine (RB) modified S1 in poly (U) dependent polyphenylalanine synthesis could be due to loss of ribosome binding activity.

CHAPTER IV

In this chapter polypeptide fragments of *E.coli* S1 namely S1F1 (from amino acid residues 172–557), S1F4 (amino acid residues 1–348), N-terminal fragment (amino acid residues 1–171) and C-terminal fragment (amino acid residues 349–557) were isolated and their nucleic acid binding properties were studied.

S1F4 showed strong ssRNA binding activity like S1 and S1F1. N terminal fragment and C terminal fragment did not show RNA binding. Protein S1 did not bind to double stranded DNA (λ DNA). No binding of protein S1 to ssDNA was observed in the presence of poly (U) indicating that S1 has strong affinity to poly (U) and ssDNA is competed out by poly (U). Of all the fragments studied, only S1F4 has shown appreciable ssDNA binding by agarose gel retardation. From our results it is most likely that ssDNA binding site of S1 may be overlapping with C terminal part of the ribosome binding

domain and the major RNA binding domain comprising homologous repeat R1 and R2.

CHAPTER V

A brief introduction about archaeobacteria was presented in this chapter. Ribosomes from bacteria belonging to two families of archaeobacteria, a thermoacidophile (*Sulfolobus acidocaldarius*) and extreme halophiles (*Halobacterium halobium*, *H. cutirubrum* and *H. salinarium*) were analyzed for the presence of S1-like proteins using sensitive western blotting technique. Antibodies raised against whole *E. coli* S1 and its functional domains viz., ribosome binding (F2a) and nucleic acid binding (S1F1) domains were used in the present study.

The anti S1 IgG reacted with a protein of 61 kDa from *S. acidocaldarius* which shows temperature dependent aggregating property. This protein failed to cross react with antiserum raised against ribosome binding domain (anti F2a) and cross reacted weakly with antiserum raised against nucleic acid binding domain (anti S1F1). Ribosomes of halobacterium species cross reacted with anti F2a only. The anti F2a IgG reacted with two proteins in the case of *H. halobium* (95 kDa and 90 kDa) and a single protein in the case of *H. salinarium* and *H. cutirubrum* (100 kDa). *E. coli* protein S1 was able to bind to both *S. acidocaldarius* and *H. salinarium* ribosomes. *E. coli* protein S1 showed binding to halobacterial ribosomes in both high and low salt concentrations. Our results indicate that cross reactive protein of *S. acidocaldarius* is the constituent of 30S subunit and it gets dissociated from the subunits or 70S ribosomes during centrifugation through sucrose gradients.

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RIBOSOMAL PROTEIN S1 IN ARCHAEABACTERIA

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Ribosomal protein S1 plays an essential role in the translation of mRNA and is directly involved in the process of mRNA recognition and binding by the ribosomes. A number of bacterial species have been examined for the presence of a protein homologous to Escherichia coli protein S1. The results of these studies indicate that protein S1 is absent from Bacillus subtilis and some other related Gram positive bacteria but is present in most of the Gram negative bacteria tested.

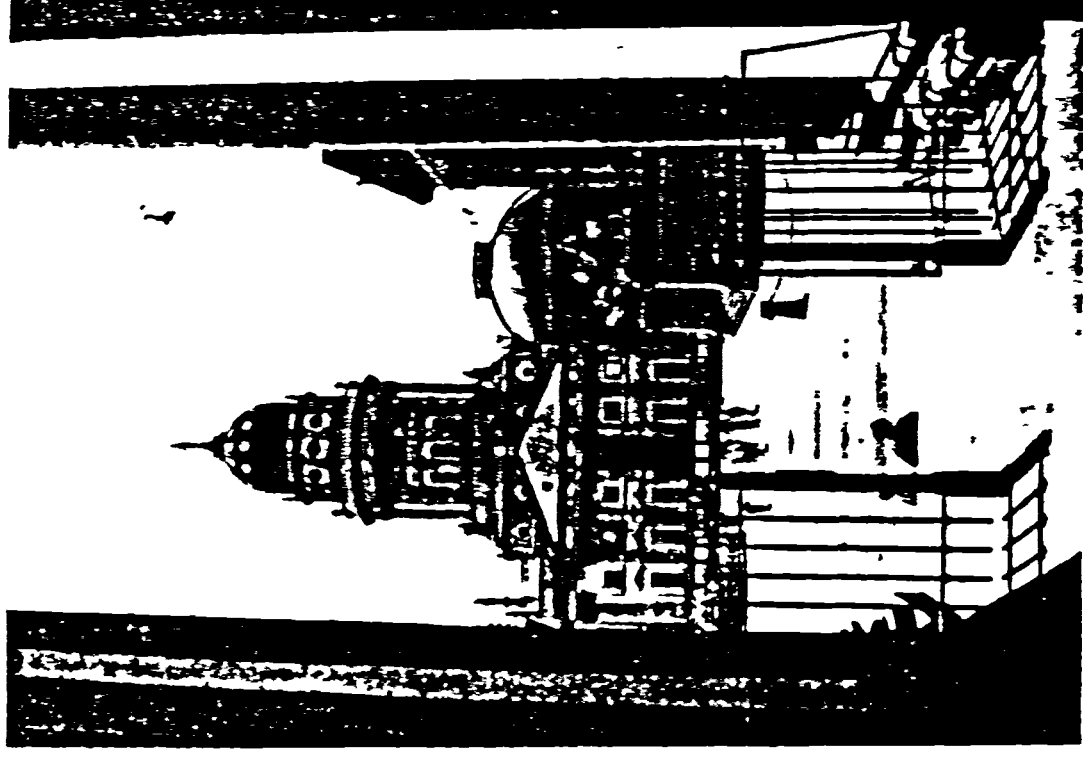
In the present study electro immunoblotting was performed to detect ribosomal protein S1 homologues in cell extracts and isolated ribosomes of thermophilic and halophilic archaeobacteria. Antisera to E. coli protein S1, the N-terminal ribosome binding domain of the protein and the central and C terminal nucleic acid binding domain of the protein were used. The results show that the acidothermophilic archaeobacterium Sulfolobus acidocaldarius contains a protein with a molecular weight of about 100,000 that cross reacts with only the anti serum to the nucleic and acid binding domain but not with anti ribosome binding domain. On the other hand, the halophilic archaeobacteria Halobacterium cutirubrum and Halobacterium salinarum contain a protein with a molecular weight of about 90,000 that cross reacts strongly with the anti ribosome binding domain and weakly with anti-nucleic acid binding domain. The results indicate differential conservation of the structural/ functional domains of protein S1 in archaeobacteria and throw light on the evolution of S1-like proteins and the relatedness of archaeobacteria to eubacteria.

ABSTRACT BOOK

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