DNA BINDING PROTEINS OF THE ARCHAEBACTERIAL CHROMATIN : ISOLATION AND CHARACTERIZATION OF HISTONE-LIKE PROTEINS FROM *SULFOLOBUS ACIDOCALDAR1US*

A THESIS SUBMITTED FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

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July, 1988

ENROLMENT NO. H L- 1477

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This is to certify that I, T. Raghavendar Reddy have carried out the research work embodied in the present thesis under the guidance of Dr. T. Suryanarayana, for the full period prescribed under Ph.D. ordinances of the University.

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

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ACKNOWLEDGEMENTS

It gives me immense pleasure to record my deep sense of gratitude to Dr. T. Suryanarayana for introducing me to the fascinating field of research in Molecular Biology. His constant encouragement and valuable suggestions helped me to gain insight into the subject. My sincere thanks to him for his care and advice.

I am grateful to Prof. P.S. Ramamurthy, former Dean, School of Life Sciences and Prof. K. Subba Rao, Dean, School of Life Sciences for extending all necessary facilities for the work.

I wish to thank Prof. P.R.K. Reddy, Dr. Ch.R.K. Murthy and Dr. Mohan C. Vemuri for their encouragement and interest during the course of this work.

I am very thankful to Dr. T. Malathi, Nizam's Institute of Medical Sciences, Hyderabad for her help and affection shown towards me during my stay in the University.

I wish to extend my sincere thanks to Dr. Chatterji, Laxma Reddy and Chenchal Rao of CCMB, Hyderabad, for their help in fluorescence spectra, amino acid analysis and CD spectra respectively.

I wish to thank my colleagues Dr. P. Murali Krishna, at present at University of Florida, USA, Mr. K. Srinivas and Mr. K. Ravi for their cooperation and help in carrying out this work. I wish to thank all my friends, faculty members and non-teaching staff for extending their cooperation during my stay in the University.

Financial assistance from University Grants Commission and Council of Scientific and Industrial Research, India is gratefully acknowledged.

I wish to express my deepest gratitude and respect to my parents, and Dr. B.T. Sreenivasa Chary for their constant encouragement and support throughoul my academic life.

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ABBREVIATIONS

A_{260} and A_{280}	absorbance at 260 nm and 280 nm
Bistris	bis[2-Hydroxyethyl] iminotris-[hydroxy methyl) methane
bp	base pairs
CD	circular dichroism
CPM	counts per minute
cm	centimeter
CM-	Carboxy methyl-
DNase	deoxyribonuclease
DTT	dithiothreiotol
EDTA	ethylene diamine tetra acetate
FITC	fluorescein isothiocyanate
g	Centrifugal field (number times gravity)
h	hour
IEF	Iso electric focussing
min	minutes
Mr	molecular mass
NEPHGE	Nonequilibrium pH gradient electrophoresis
NP-40	Nonidet P-40 (nonionic detergent)
poly(dA).poly(dT)	Synthetic double stranded polynucleotides containing repeating sequences of Adenine and Thymine base pairs.
poly r(I.C)	Synthetic double stranded polynucleotides containing repeating sequences of Inosine and Cytosine base pairs.
Poly (U)	poly ribouridylic acid
PMSF	phenylmethylsulfonyl fluoride

POPOP	1,4-bis[5-phenyl-2-oxazolyl] benzene
PPO	2,5-Diphenyloxazole
RNase	ribonuclease
RPM	revolutions per minute
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulphate
SRID	single radial immunodiffusion
Tris	tris[hydroxylmethyl] aminomethane
tRNA	transfer ribonucleic acid
TEMED	N, N, N,' N'-tetramethylethylene-diamine

Chemicals

Double stranded DNA-cellulose, Calf thymus DNA, NP-40, Spermidine-HC1, Poly r(I-C), Poly(dA).poly(dT), Tris, Alumina, DNase-I, RNase-A, Bovine serum albumin, Ovalbumin, Chymotrypsinogen, Myaglobin, Cytochrome-C, Coomassie blue R-250, Ethidium bromide, Phenylmethylsulfonyl fluoride, Sucrose, Dimethyl suberimidate, Agarose, PPO, POPOP and calf thymus histones were purchased from Sigma Chemical Company, USA. Poly (U)-Sepharose-4B, Sephacryl S-200 were procured from Pharmacia fine chemicals, Sweden. Acrylamide, N-N-methylene bis acrylamide and SDS were purchased from Serva Company, West Germany. Triethanolamine was purchased from Merck, West Germany. Uniformly labelled (methyl-H) thymidine was from Bhabha Atomic Research Center, Bombay, India. TEMED, 2-mercaptoethanol were obtained from Sisco Research Laboratories, Bombay, India. Complete and incomplete Freund's adjuvants, Bactotryptone, Yeast extract, Casamino acids were purchased from Difco Laboratories, Detroit, Michigan, USA. CM-cellulose was purchased from Whatman, England. Nitrocellulose sheets and discs were obtained from Millipore, France. Anti-rabbit IgG (goat) was from Miles Laboratories, USA. Ampholines pH 5-7, 7-9 and 9-11 were from LKB and pH 3-10 was purchased from Pharmacia. All the other chemicals used in this study were rf analytical grade available locally.

CHAPTER - I

INTRODUCTION

The present thesis deals with the structure of the acidothermophilic archaebacterial chromatin (nucleoid) and the characterization of histone-like DNA-binding proteins that are associated with the intracellular DNA. Hence a brief account of histone-like DNA binding proteins from prokaryotes will be presented. This will be followed by an introduction about archaebacteria and the molecular biological aspects of these organisms.

The basic unit of eukaryotic chromatin is the nucleosome (Kornberg, 1974), which consists of a stretch of DNA wound around a protein core (Isenberg, 1979). The core of the nucleosome is a histone octamer of composition $(H2A)_2$ $(H2B)_2$ $(H3)_2$ $(H4)_2$ (McGhee and Felsenfeld, 1980; Igo-Kemenes et al., 1982).

In prokaryotes the intracellular DNA is in a condensed state called nucleoid or bacterial chromatin. RNA, protein and probably cell membrane might be involved in the stabilization of this highly organized structure (See for review, Pettijohn, 1982). Several classes of DNA binding proteins have been isolated from prokaryotic organisms chiefly Escherichia coli which effect the helical structure of DNA (review Geider and Hoffmann Berling, 1981). Among the histone-like proteins that have been isolated the DNA binding protein II (Mr. 9500) which is one of the most abundant proteins in Escherichia coli has been extensively studied. (See Review, Drlica and Rouviere Yaniv, 1987). The protein is localized in the nucleoid and has been shown to form nucleosomal organization when complexed with duplex DNA (Rouviere-Yaniv and Kjeldgaard, 1979, Broyles and Pettijohn, 1986). This protein has been variously referred to as HU (Rouviere-Yaniv and Gros, 1975), HD (Berthold and Geider, 1976) and NS (Suryanarayana and Subramanian, 1978). The protein exists in two molecular forms, NS1 and NS2 or HU-α and HU-β . Protein HU has been shown to stimulate transcription of DNA

(Rouviere-Yaniv and Gros, 1975) and inhibit transcription of other DNAs (Berthold and Geider, 1976). The protein has also been implicated in the replication of ori C locus of JE. coli (Dixon and Kornberg, 1984). Several other physicochemical properties of the protein have been studied (See GuaiarEi et al., 1986; Paci et aL, 1984; Dijk and Reinhardt, 1986).

Other histone-like proteins from E. coli

Apart from HU, other histone-like DNA binding proteins have also been reported (Table 1.1). The primary structure of integration host factor (IHF) involved in site specific recombination (Nash and Robertson, 1981) is related to the HU protein. It also wraps DNA around it. Three other proteins considered to be histone-like have been isolated. Of these, protein H is the most histone-like DNA binding protein and it has amino acid composition similar to eukaryotic histone H2A (Hubscher et aL, 1980). It was isolated as an activity that inhibits DNA synthesis in vitro. H1 is another small, abundant, neutral DNA binding protein (Cukier-Kahn et ah, 1972). The protein binds strongly to DNA and is able to compact it (Spassky and Buc, 1977 and Spassky et ah, 1984). H1 protein has been shown to be a component of nucleoid (B1, Varshavsky et al., 1977). Another heat stable, acid soluble protein that binds DNA has been isolated which interacts with RNA polymerase and is probably encoded by fir A gene (Lathe et al., 1980). Proteins homologous to JE. coli protein NS are reported from a variety of prokaryotes and their properties have been studied (Drlica and Rouviere-Yaniv 1987, Dijk and Reinhardt, 1986).

Studies on bacterial nucleoid

The bacterial chromosome (nucleoid or chromatin) is released from cells by exposing them to non-ionic detergents in the presence of 1M NaCl or 5 mM spermidine and can be isolated as a rapidly sedimenting condensed

TABLE - 1.1

Histone-like proteins from E. coli

Protein type	Approximate molecular weight
HU (NS, HD, DBP II)	9,300
IHF (α, β)	10,000-11,000
H	28,000
H1	15,500
Fir A protein	17,000

structure (Stonington and Pettijohn, 1971; Worcel and Burgi, 1972; 1974; Kornberg et al., 1974). By electron microscopy it has been shown that the bacterial DNA from gently lysed E. coli is organized into a 12 nm filament having an axial repeat of about 13 nm (Griffith, 1976). The nucleoid DNA has been shown to be in a highly supercoiled state (Materman and Van Gool, 1978). By partial digestion of E. coli chromosomal DNA with endogenous nucleases, DNA fragments bound to protein have been isolated (Varshavsky et aL, 1977). Digestion with micrococcal nuclease resulted in the formation of 120 bp fragments (Varshavsky et aL, 1977). Topological and electron microscopic investigations indicated that bacterial DNA is organized into a wrapped chromatin-like structure. However, no clear biochemical definition for bacterial chromatin is available (see Drlica and Rouviere-Yaniv, 1987 and Pettijohn, 1982). It has been shown, that several different protein components are associated with the nucleoid including RNA polymerase core enzyme, DNA polymerases, several DNA binding proteins and outer and inner membrane proteins both by two dimensional gel analysis and by functional assays (Stonington and Pettijohn, 1971; Portalier and Worcel, 1976; Moriya and Hori, 1981; Moriya et aL, 1981; Lossius et aL, 1984; Gaziev et aL, 1986). Several of the DNA binding proteins of the nucleoid have also been isolated (Yamazaki et al., 1984). In a similar study Hoick and Kleppe, Hoick et aL, (1985, 1987) have analyzed the DNA and RNA-binding proteins of chromatin from E. coli by 2-D polyacrylamide gel electrophoresis. Their study failed to detect the protein HU in the isolated DNA-protein complexes. Folded chromosomes were also isolated from Bacillus subtilis (Guillen et aL, 1978) and Streptomyces hygroscopicus (Sarfert et aL, 1983) and their protein composition analyzed. NS1/NS2 homologues have been isolated and characterized from these organisms as well.

Archaebacteria

The archaebacteria have recently been recognized as a phylogenetically separate group of organisms that are distinct from other bacteria (eubacteria) and from the eukaryotes (See for review, Woese, 1981; Fewson, 1986). Some of their biochemical properties are unique, others are shared with eubacteria or with eukaryotes. Archaebacterial envelops, lipids and membranes, transcriptional and translational machinery, enzymes and cofactors, all show certain unusual features.

The variety of Archaebacteria: There is a diverse array of morphological forms of archaebacteria. The G + C content of their DNA varies over a wide range (27-61%). Some are anaerobes, some aerobes and many of the species are able to grow at extreme conditions of acidity, temperature or salinity.

The archaebacteria can be divided into 3 classes (Table 1.2).

Methanogens: Methanogens are obligate anaerobes that reduce CO.- to methane.

Halophilic bacteria: The halophilic bacteria include some species that grow best in media containing 2.5 - 5.2 M brine and are responsible for the water reddening observed during the preparation of salt from sea water.

The sulphur-dependent organisms: Many but not all of which are thermoacidophilic. These have such distinct properties that a few workers believe that they should be separated out as a fourth kingdom, the "Eocytes".

Various lines of evidence support the view that the methanogens and the halophiles constitute one branch of the archaebacterial whilst most of the sulphur-metabolising organisms are in a separate branch. From the known 16S rRNA (or 18S rRNA) sequences the evolutionary branching of eubacteria, archaebacteria, and eukaryotes has been inferred and the

TABLE - 1.2

Examples of Archaebacteria

I. **Methanogenic bacteria**	II. **Halophilic bacteria**	III. Sulphur-dependent and thermoacidophilic bacteria
Methanobacterium Thermoautotrophicum	Halobacterium	Thermoplasma acidophilum
Methanobacterium formicicum	Halobacterium salinarium	Sulfolobus acidocaldarius
Methanobrevibacter ruminantium	Halobacterium cutirubrum	Sulfolobus brierleyi
Methanothermus fervidus	Halococcus morrhuae	Sulfolobus solfataricus
Methanococcus vannieliii		Thermoproteus tenax
Methanomicrobium mobile		Thermofilum pendens
Methanogenium cariaci		Thermococcus celer
Methanospirillum hungatei		Pyrodictium occultum
Methanosarcina barkeri		Thermodiscus maritimus
Methanothrix soehgenii		

phylogenetic relationship of the organisms was presented (Fox et al., 1980). See Fig. 1.1.

Archaebacterial biochemistry and molecular biology: There are both Gram-positive and Gram-negative archaebacteria and many of them look like eubacteria under the microscope. The pseudomurein is a particularly well characterized component of archaebacterial envelops. The sugar talose is not known to occur in nature except in archaebacteria. Cell envelops lack peptidylglycan and contain certain ether rather than ester linked lipids (De Rosa et ah, 1986; Kandler and Konig 1985, Langworthy 1985). In some respects, it is the lipid composition and membrane structure that separate the archaebacteria most distinctly from eubacteria and eukaryotes.

Different molecular biological approaches are being made to study these archaebacteria (review Dennis, 1986). Using 16S (small ribosome sub-unit) RNA sequences as a phylogenetic index, Woese and his collaborators have provided convincing evidence suggesting that evolution has not been a progression from simple prokaryotes to the more complex eukaryotes. (Fox et ah, 1980; Pace et al., 1986; Woese and Fox, 1977; Woese et al., 1983). Eukaryotic and sulphur-dependent archaebacterial rRNAs are highly modified, whilst the rRNAs of eubacteria and the other archaebacteria have much lower levels of modification. Archaebacterial 5S rRNA appears to have both eubacterial and eukaryotic features. The sulphur-dependent archaebacteria, like eukaryotes have triphosphorylated 5'-termini whereas the methanogens and halophiles like the eubacteria, have a 5'-terminal monophosphate. Archaebacterial tRNA contain a great variety of modified bases, including thiolated nucleosides. Initiation of protein synthesis seems to occur via methionyl-tRNA as in eukaryotes rather than via N-formyl methionyl tRNA (Bayley and Morton, 1978).

Fig. 1.1 Unrooted phylogenetic tree constructed from completed sequences of 16S (or 18S) rRNAs. An approximate indication of evolutionary time is provided by the lengths of the lines which indicate sequence distances (the bar corresponds to 0.1 mutational events per sequence position (The diagram was taken from Fewson, 1986).

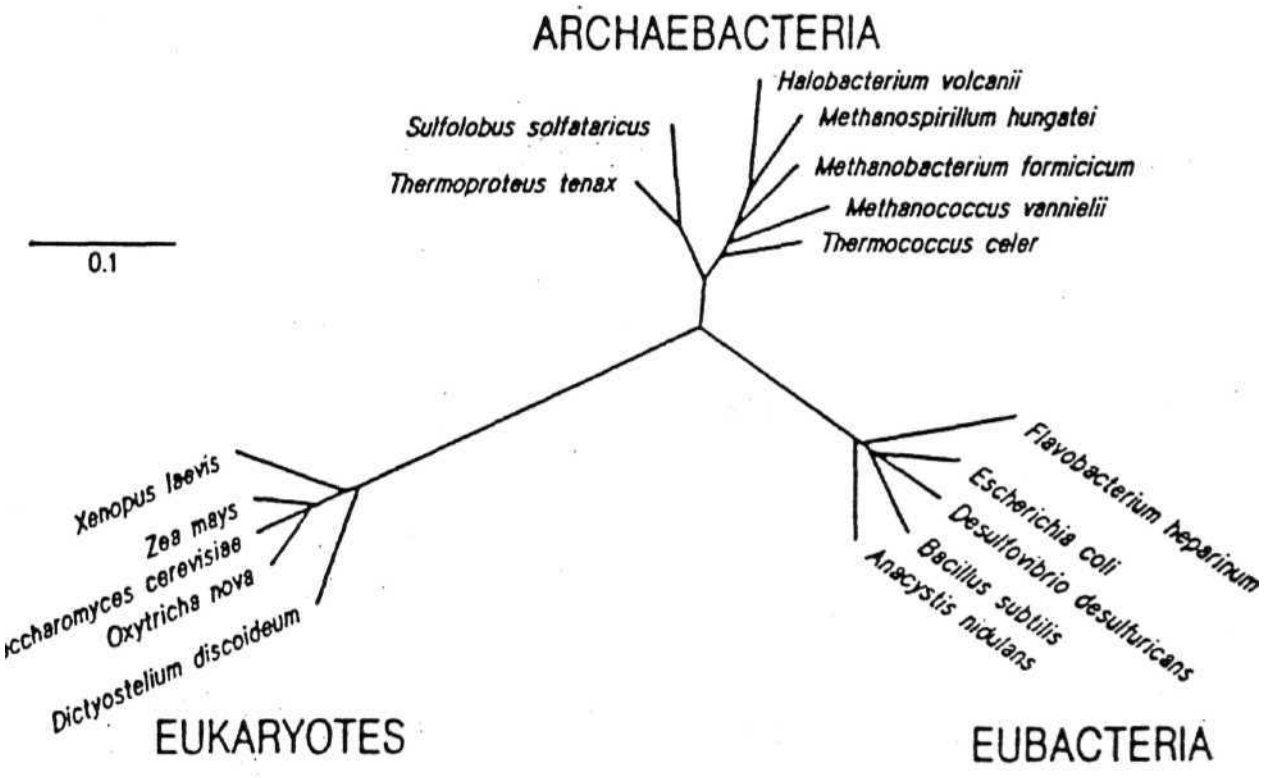

ARCHAEBACTERIA
Halobacterium volcanii
Methanospirillum hungatei
Methanobacterium formicicum
Methanococcus vannielii
Thermococcus celer
Sulfolobus solfataricus
Thermoproteus tenax
0.1
Xenopus laevis
Zea mays
Saccharomyces cerevisiae
Oxytricha nova
Dictyostelium discoideum
Flavobacterium heparinum
Escherichia coli
Desulfovibrio desulfuricans
Bacillus subtilis
Anacystis nidulans
EUKARYOTES
EUBACTERIA

Fig.1.1

Archaebacterial ribosomes like those of eubacteria are smaller (70S) than the cytoplasm ribosomes of eukaryotes (80S). This division is exemplified by many differing characteristics including the morphological structures of the ribosome subunits as visualized by electron microscope (Lake et ah, 1984; Stoffer and Stoffer Meilicke, 1986) and by the protein content of ribosomes. Ribosomes from the halophilic branch in general have a eubacterial like protein content with few or no proteins greater than 30,000 daltons in mass whereas ribosomes from the sulphur dependent acido-thermophilic branch have a much higher eukaryote-like protein content with many proteins greater than *30,000* daltons in mass (Cammarano et aL, 1986). The amino acid sequences of at least some archaebacterial ribosomal proteins exhibit more amino acid sequence homology with their eukaryotic equivalents than with their eubacterial equivalents (Matheson, 1985). Some of the archaebacterial ribosomal proteins appear to resemble those of eubacteria, but the sequences of protein 'A' from the few archae-bacteria examined resemble sequences of eukaryotic protein 'A' more than the equivalent L7/L12 of E. coli (Matheson and Yaguchi, 1982). Archaebacterial ribosomes lack binding sites for eubacterial 70S antibiotic inhibitors such as chloramphenicol or streptomycin but do have binding sites for eukaryotic 80S inhibitors such as anisomycin (Schmid and Bock, 1982). The 16S rRNA gene from Desulfurococcus mirabilis has been shown to contain a single intron (Kjems and Garrett, 1985). All the archaebacterial RNA polymerases are insensitive to rifampicin (which inhibits initiation in eubacteria) and streptolydigin (which inhibits elongation in eubacteria) and to cc -amanitin. Silybin stimulates transcription by RNA polymerase from thermoacidophilic bacteria and by eukaryotic RNA polymerase I (Gropp et aL, 1986). RNA polymerases from a great variety of archaebacteria have been purified and

these show features such as subunit composition, immunological relationships and antibiotic sensitives like eukaryotic polymerases (Schnabel et al., 1983). There are long polyadenylated sequences in Sulfolobus RNA (presumably mRNA) similar to those in eukaryotes. Archaebacteria contain an elongation factor (EF2) which is ADP ribosylated by diphtheria toxin (Kessel and Klink, 1980). The aminoacyl tRNAs binding factor (EF-Tu) of Sulfolobus is completely insensitive to pulvomycin and kirromycin that inhibit eubacterial EF-Tu (Cammarano et al., 1982). It has been shown that the activity of type I topoisomerase of Sulfolobus is low compared with the three other topoisomerases and one of the type II topoisomerase (Reverse gyrase) was able to introduce positive superhelical turns (Kikuchi and Asai, 1984). See Table 1.3 for the summary of the properties of archaebacteria.

Histone-like proteins from archaebacteria: Reports on the archaebacterial nucleoid structure and histone-like proteins are scanty. Searcy and his collaborators have isolated and sequenced a histone-like DNA binding protein (HTa) from Thermoplasma acidophilum (Stein and Searcy, 1978; Searcy and Stein, 1980; Delange et al., 1981; Searcy, 1982). The amino acid composition of this protein shows significant homology to eukaryotic histones, as well as to the DNA binding protein HU of E. coli. Searcy suggests that the protein HTa is more like eukaryotic histones than eubacterial DNA binding proteins, because it condenses DNA into globular particles containing a core of four HTa subunits and a 40 bp loop of DNA. It also has been shown that HTa binds tightly to DNA and protects it from nuclease digestion (Stein and Searcy, 1978; review Searcy, 1986).

Presence of small, basic proteins in the isolated DNA-proteins complexes has been reported in several eu- and archaebacteria by electrophoresis. Antibodies to histone-like protein (HU) of E. coli cross-reacted

TABLE - 1.3

Properties of Archaebacteria

Unique to archaebacteria	Similar to eukaryotes	Similar to eubacteria
1. Many 16S rRNA sequences	1. Initiator tRNA is methionine	1. Prokaryotic cellular organization
2. Pseudomurein	2. RNA polymerase subunit structure	2. 70S ribosomes containing 16S, 23S and 5S rRNAs
3. Ether linked lipids with branched aliphatic chains	3. Elongation factor EF-2 sensitive to diphtheria toxin	3. Ribosomal RNA gene organization
4. Certain structural features of 5S rRNA and tRNAs	4. Ribosomal 'A' protein amino acid sequence	4. Occurrence in vivo of restriction and modification and restriction endonucleases
	5. Introns in some tRNA and rRNA genes	
	6. CCA termini of tRNAs not coded in tRNA genes	
	7. DNA polymerase sensitive to aphidicolin	
	8. RNA polymerase insensitive to rifampicin and streptolydigin	
	9. Sensitivity to protein synthesis inhibitors, e.g. anisomycin	
	10. Hybrid ribosomes with eukaryotic subunits	

with a protein in 13 species of eubacteria. However, antibodies to the histone-like protein (HTa) of Thermoplasma reacted only with the corresponding extract, but not with those of other archaebacteria e.g. Sulfolobus, Thermoproteus, Methanobacterium, Methanococcus and Methanosarcina (Thomm et ah, 1982). Small basic, DNA-binding proteins have also been isolated from the archaebacteria Sulfolobus solfataricus, Methanosarcina barkeri and Sufolobus acidocaldarius. A small DNA binding protein (Mr 7000) from S. solfataricus has been sequenced and it is shown to contain very high lysine content (Kimura et al., 1984). Chartier et ah (1985) isolated a protein HMb as an acid soluble low molecular weight (14.5 KDa) protein from M. barkeri. The physico-chemical properties of the proteins suggest that the protein is unlike the eubacterial HU type of protein. Two major 'chromosomal' proteins have been isolated and characterized from (sheared DNA protein complexes of) S. acidocaldarius by Green et al. (1983) in their search for isolating proteins that might stabilize DNA against thermal denaturation. However, the proteins (Mr. 36,000 and 14,500) could not afford protection to DNA against thermal denaturation to any appreciable extent. In a separate study several types of DNA binding proteins from S. acidocaldarius have been isolated with molecular weights of 10,000, 8000 and *7000* (Grote et ah, 1986) The proteins have been shown to contain several subspecies. The physico-chemical properties of these proteins have been studied (Dijk and Reinhardt, 1986). The interaction of these proteins with DNA has been studied by electron microscopy. In all the above cases DNA binding proteins have been isolated from cell extracts of these organisms, their association with nucleoid has not been documented. In no study rapidly sedimenting nucleoid like structures (as in the case with E. coli) from gently lysed cells have been isolated.

OBJECTIVES AND SCOPE OF THE PRESENT INVESTIGATION

Sulfolobus acidocaldarius, an extreme acidothermophilic archaebacterium was chosen to analyze its chromatin structure and composition. This is because phylogenetic data indicated that this archaebacterium is more closely related to eukaryotes than eubacteria, evolving as a separate line of descent from the progenote (Woese, 1981 and Fewson, 1986). Moreover based on the ribosome morphology by electron microscopy it has been suggested that acidothermophiles may be ancestors to eukaryotes, the eocytes (see Lake, 1984). Furthermore, the organism grows at extreme environmental conditions; 75°C and pH 3.0. Study of the organism would throw light on the unique features observed in this organism and comparison of its properties with those of eubacteria and eukaryotes would help in understanding the biochemical evolution of archaebacteria.

S. acidocaldarius lacks rigid cell wall (Brock et al., 1972) and exists presumably in osmotic equilibrium with its environment. So the intracellular salt concentration is expected to be low (Green et ah, 1983). Since low ionic strength, high temperature of growth (75°C) and a low (G + C) content of the DNA would destabilize DNA, it is speculated that compensatory factor(s) may be present to stabilize the intracellular DNA. Such factors should be associated with the intracellular DNA. Hence, we wanted to isolate the archaebacterial nucleoid (chromatin) to study its properties and protein com - position and look for factors responsible for the thermal stability of the DNA. We show that the nucleoid of this organism contains four acid soluble histone-like DNA binding proteins. Three of these proteins strongly protect the DNA against thermal denaturation. The physicochemical and nucleic acid binding properties of these proteins have been studied.

The present study opens up a new approach to study the archaebacteria. Comparison of the histone-like proteins of the archaebacterium with eubacterial histone-like proteins and eukaryotic histones will help in understanding the evolutionary relatedness of the organisms as well as the evolution of the histone-like proteins. Another interesting aspect is the interaction of histone-like proteins with nucleic acids. The proteins show DNA binding and helix stabilizing properties and hence can be used as model system for the study of protein nucleic acid interaction and the mechanism of helix stabilization. Furthermore, knowledge of the properties of the archaebacterial chromatin structure and histone-like proteins may serve as a model for better understanding the radical difference in chromatin organization between eubacteria and eukaryotes.

CHAPTER - II

IDENTIFICATION AND PURIFICATION OF HISTONE-LIKE PROTEINS FROM S. ACIDOCAL DARIUS

The isolation of chromatin (nucleoid) from the archaebacterium Sulfolobus acidocaldarius was undertaken in order to analyze its composition as well as look for histone-like proteins. This chapter deals with the growth conditions of the archaebacterium and the isolation of chromatin. The chromatin was analyzed by electrophoresis for its protein composition. Methods for purification of four acid soluble DNA binding proteins of the archaebacterial chromatin have been described.

MATERIALS AND METHODS

Bacterial strains: The strains used in this study were Sulfolobus acidocaldarius strain DSM 639 obtained from Deutche Sammulung Von Mikroorganismen, Gottingen, West Germany and Escherichia coli A19 strain from the laboratory of Dr. A.R. Subramanian, Max-Planck Institute for Molecular Genetics, Berlin, West Germany.

Bacterial growth: Sulfolobus acidocaldarius was grown at 75°C for 40 to 48 hrs with vigorous aeration in a medium containing 0.1% yeast extract, 0.1% Bacto tryptone 0.5% casaminoacids, 0.1% glucose, 0.02% NaCl, 0.13% $(NH_4)_2SO_4$, 0.03% KH_2PO_4, 0.025% $MgSO_4$, *0.07%* $CaCl_2$ and 4 ml of 1M H_2SO_4 to adjust the pH to 3.0 (Kikuchi and Asai, 1984). The growth was followed by measurement of the absorbance at 650 nm, every 6 hrs (Fig. 2.1). The bacterial cells were harvested after neutralizing the culture with 1M Tris-base (4 to 5 ml/litre). The yield of the cells was about 1.5 g/litre (wet weight) culture. The cell pellets were stored frozen at -80°C.

E. coli was grown in enriched medium at 37°C with aeration (Minks et ah, 1978). The mid logarithmic phase cultures were chilled and harvested by centrifugation and stored frozen at -80°C.

Isolation of nucleoid (chromatin): Nucleoid from S. acidocaldarius was isolated by a procedure described for E. coli (Stonington and Petti john 1971;

Fig. 2.1 Growth curve of *Sulfolobus* acidocaldarius

The bacteria was grown for 40 to 48 hours at 75°C and absorbance at 650 nm was measured as described in Materials and Methods.

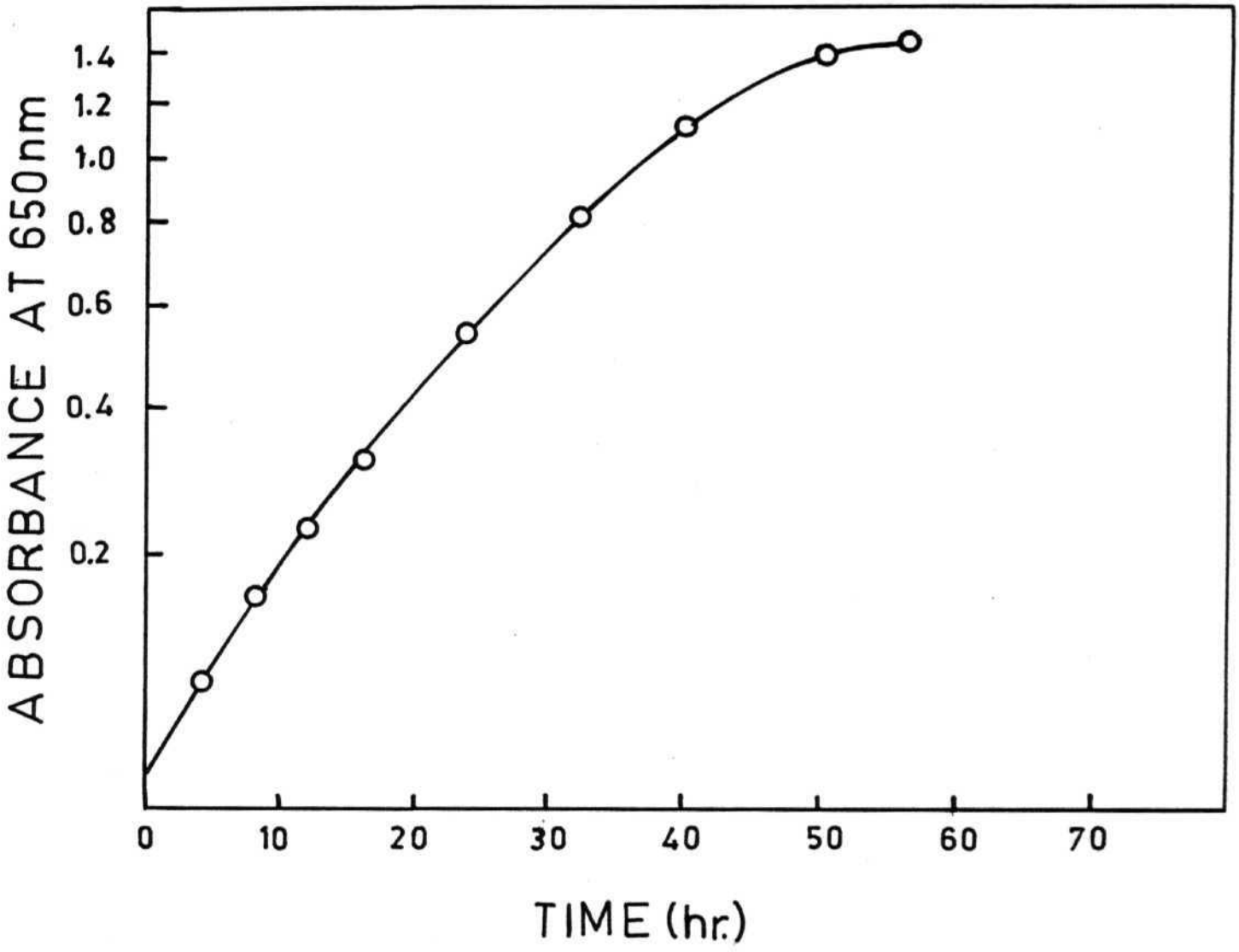
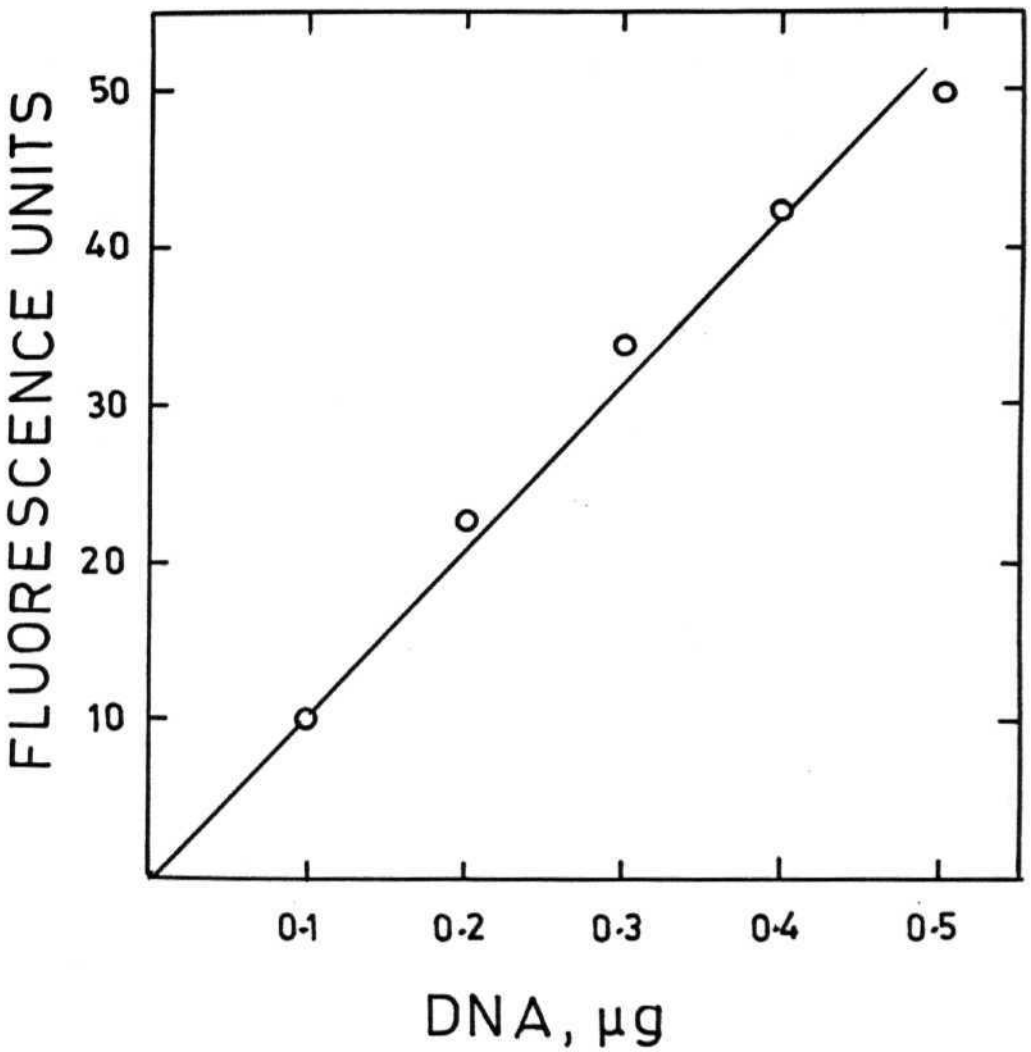

ABSORBANCE AT 650nm
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
10
20
30
40
50
60
70
TIME (hr.)

Fig.2.1

Kornberg et al., 1974) with some modifications. Freshly harvested cells (2 g) were suspended in 4 ml of 10 mM Tris-HCl pH 7.6, 100 mM NaCl and were lysed by the addition of 4 ml of 10 mM Tris-HCl pH 7.6 containing 1% NP-40, 2 mM spermidine-HCl, 10 mM Na_2-EDTA and incubated at 10°C for 30 min. The lysate was centrifuged at 1000 x g for 5 min. The cleared viscous lysate was layered on 15 to 50% linear sucrose gradients in 10 mM Tris-HCl pH 7.6, 3 mM $MgCl_2$ and centrifuged at 10000 x g for 20 min. The gradient was fractionated by collecting 2 ml fractions and analyzed by SDS-polyacrylamide gel electrophoresis (Thomas and Kornberg, 1975). The chromatin was diluted with buffer and centrifuged at 15000 x g. The chromatin pellet was dissolved in reduced volume of buffer containing 20 mM Tris-HCl pH 7.6, 150 mM KC1, 1 mM Na_2-EDTA and 6 mM 2-mercaptoethanol to obtain a DNA concentration of 320 ug/ml. The absorption at 260 nm of the fractions was measured and the DNA was estimated by ethidium bromide fluorescence assay.

Estimation of DNA: The concentration of DNA in chromatin fractions was determined according to Morgan et aL, (1979). DNA was assayed by exploiting the enhanced fluorescence of ethidium intercalated into duplex regions. Increasing amounts of calf thymus DNA (0.1 ug, 0.2 ug, 0.3 ug, 0.4 ug and 0.5 ug) were added to 1 ml of buffer containing *0.5* ug/ml ethidium bromide, 5 mM Tris-HCl pH 8.1, 0.5 mM Na_2-EDTA and the fluorescence emission was measured at 600 nm (excitation at 525 nm) with the maximum slit width in a Hitachi Spectrofluorimeter. Nucleoid fractions *(50* ul) were added to 1 ml of buffer and the fluorescence was measured as before. The amount of DNA in nucleoid fractions was calculated from the standard graph (Fig. 2.2).

Fig. 2.2 Standard curve of DNA estimation by ethidium fluorescence assay using calf thymus DNA.



Fig·2·2

Nucleic acid and protein determination; DNA in isolated nucleoid was determined according to Burton (1956), and RNA by the orcinol method (Ceriotti, 1955). Protein concentrations were determined by the Folin reagent (Lowry et ah, 1951) using bovine serum albumin as standard for total nucleoid protein.

SDS-polyacrylamide gel electrophoresis: Polyacrylamide gel electrophoresis in the presence of SDS was performed using 18% gel slabs as described by Thomas and Kornberg (1975). The ratio of acrylamide to bisacrylamide was 30 : 0.3. The 18% separating gel was in *0.75* M Tris-HCl pH 8.8, 0.002 M Na_2-EDTA, and 0.1% SDS and the stacking gel was of 5% polyacrylamide in 0.08 M Tris-HCl pH 6.8, 0.002 M Na_2-EDTA and 0.1% SDS. Protein samples were treated with 0.1% SDS and 1% 2-mercaptoethanol at 65°C for 15 min. The slabs were run at 170 volts for 3 1/2 hrs with electrode buffer comprised of 0.05 M Tris, 0.38 M glycine and 0.1% SDS .

The gels were removed after the run and washed with 7.5% acetic acid for 30 min, and stained with 0.1% coomassie blue R-250 in *50%* methanol and 7.5% acetic acid for 4 hrs. The gels were destained with a solution containing 5% methanol and *7.5%* acetic acid.

IEF and NEPHGE two dimensional gel electrophoresis: Total nucleoid proteins were analyzed by two dimensional gel electrophoresis as described by O'Farrell, (1975) and O'Farrell et aL, (1977). The total proteins from nucleoid was extracted by incubating at 37°C for 2 hrs with DNase I and RNase A *50* ug/ml each in the presence of 0.5 mM PMSF and 10 mM $MgSO_4$. The extracted proteins were dialyzed against 8 M urea containing 6 mM 2-mercaptoethanol and clarified at 15000 x g for 15 min.

Isoelectric focussing: It was performed as described by O'Farrell (1975) with some modifications. Here we have avoided using the agarose to seal

the one dimension gel to stacking gel of second dimension gel slab. All the protein samples were prepared in lysis buffer containing 9.5 M urea, 2% (w/v) NP-40, 2% Ampholines (comprised of 1.6% pH range 5 to 7 and 0.4% pH range 3 to 10) and 5% 2-mercaptoethanol.

One dimensional electrophoresis (IEF): Polyacrylamide gel electrophoresis in presence of 2% Ampholines was performed using gel mixture composed of 4% acrylamide/bisacrylamide. The gels were prerun at 200 V for 15 min, 300 V for 30 min, and 400 volts for 30 min. Samples were applied on the top of the gels and electrophoresed at 400 volts for 12 hrs and 800 volts for 1 hr using 0.01 M H_3PO as lower electrode buffer and 0.02 M NaOH as upper electrode buffer. The gels were removed and equilibrated with equilibration buffer.

Second dimension: After the run in the first dimension, the gels were removed and placed 1.5 cm above 11.75% polyacrylamide/bisacrylamide gel slab in 0.55 M Tris-HCl pH 8.8 and 0.12% SDS. A stacking gel of 4.75% polyacrylamide/bisacrylamide in 0.125 M Tris-HCl pH 6.8 and 0.1% SDS was formed between the first dimensional gel and the slab gel. Electrophoresis in the second dimension was performed at 15 mA for 2 hr and 20 mA for another 2 1/2 hr. The running buffer contained 0.025 M Tris base, 0.192 M glycine and 0.1% SDS. After the electrophoresis the slab gel was removed and stained by the silver staining method.

Nonequilibrium pH gradient electrophoresis: The stock solutions and most procedures were as described by O'Farrell (1975 and 1977). The lower reservoir was filled with 0.02 M NaOH and upper reservoir was filled with 0.01 M H_3PO_4. Electrophoresis was performed for 3 hr and 10 min at 400 V with the cathode on the bottom and the anode on the top.

Measurement of pH: The one dimension IEF or NEPHGE gel was cut into 1 cm sections. The gel pieces were placed in water and after 16 hrs at room temperature the pH was measured with a pH meter.

Silver staining: Colour silver staining of the gels was performed as described by Adams and Sammons (1981), Sammons et ah (1981) with some modifications. 0.1% $CuSO_4$ was included in fixation solution to remove the ampholines.

Acid-urea gel electrophoresis: It was performed as described by Mets and Bogorad (1974).

First dimension: Protein samples were prepared in 6 M urea, 0.01 M Bis-tris acetic acid pH 4.0, 10% (w/v) glycerol. Basic fuschin was used as a marker dye. Purified protein samples were placed over the 4% polyacrylamide gels and were subjected to electrophoresis at 110 V for 6 hr at room temperature. The upper electrode buffer was 0.01 M Bistris-acetic acid pH 4.0 and the lower electrode buffer 0.18 M potassium acetate-acetic acid pH 5.0. After the run, the gels were removed and processed for second dimension.

Second dimension: The first dimension gels were placed *0.5* cm above 10% gel slabs in a multiple slab gel (10 cm x 10 cm) electrophoresis apparatus. The space between the gel and the slab was filled with a stacking solution containing 4% acrylamide and allowed to polymerize. Electrophoresis (15 mA per slab, 7 hr) in the second dimension was performed at 15°C with upper electrode buffer of *0.07* M Bristris, 0.07 M 2 (N-morpholino) ethane sulfonic acid pH 6.5, 0.016% thioglycolic acid and 0.02% SDS and lower electrode buffer of 0.028 M Bristris-HCl pH 6.75. After the electrophoresis the slab gels were washed in *7.5%* acetic acid for 2 hrs. The gels were then stained with 0.1% coomassie blue R-250 in *50%* methanol and 7.5% acetic acid overnight and destained with a solution containing 5% methanol and *7.5%* acetic acid.

Isolation of acid soluble proteins; The concentrated nucleoid or post-ribosomal supernatant was dialyzed against 0.2 M H_2SO_4 for 16 hrs at 4°C. The precipitated protein was removed by centrifugation at 15000 x g for 15 min and the clear supernatant (acid extract of nucleoid) was dialyzed against the buffer containing 20 mM Tris-HCl pH 7.6, 150 mM KC1, 1 mM Na_2-EDTA and 6 mM 2-mercaptoethanol.

DNA-cellulose chromatography: 2 g of calf thymus native double stranded DNA-cellulose was swollen in 20 mM Tris-HCl pH 7.6, 150 mM KCl, 1 mM Na_2-EDTA and 6 mM 2-mercaptoethanol for 2 hr and packed into a column of 5 ml bed volume (1.2 cm x 4.5 cm) and equilibrated with the same buffer. The acid extract of nucleoid was passed through the column and the protein was eluted by stepwise increasing KCl salt concentration (see Results Section).

Carboxymethyl cellulose chromatography: Preswollen CM-cellulose was suspended in buffer containing 20 mM Tris-HCl pH 7.6, 100 mM KCl 1 mM Na_2-EDTA and 6 mM 2-mercaptoethanol and packed into a column (0.9 cm x 6.5 cm) and equilibrated with the same buffer. The protein pools from 0.3 M and 0.6 M KC1 eluate of DNA-cellulose column chromatography were dialyzed separately against the column equilibrating buffer and passed through the column separately. The column was eluted with stepwise increasing KC1 salt concentration.

Isolation of ribosomes, supernatant fraction (S100) and NH_4Cl wash; These were isolated according to Minks et a]., (1978). All operations were carried out at 0-4°C. Cells were ground with double the weight of alumina until soft and sticky, and extracted with the buffer (3 ml/g) containing 20 mM Tris-HCl pH 7.6, 200 mM KCl, 10 mM magnesium acetate, 6 mM 2-mercapto-ethanol and 2 ug DNase per 3 ml (RNase free). The crude cell extract obtained after centrifugation at 30000 x g for 30 min, was recentrifuged at 100,000

x g for 4 hrs (in Beckman L8-80 Ti 60 rotor) to pellet the ribosomes. The upper two thirds the supernatant (S-100) was collected. Ribosomal pellet was rinsed once with the above buffer and suspended in the same buffer containing 1 M NH_4Cl. The ribosomes in 1 M NH_4Cl were pelleted by centrifugation and the supernatant (NH_4Cl wash) was collected. The ribosomal pellet was dissolved in 10 mM Tris-HCl pH 7.6, 50 mM KCl, 10 mM magnesium acetate and 1 mM DTT containing 15% glycerol and stored frozen at -80°C.

Binding of proteins to native (H^3) DNA: E. coli was labelled with (H^3-methyDthymidine as described by Mahler (1967), and the DNA was isolated according to Marmur (1961). The specific activity of the DNA was 8530 CPM per ug. Binding of proteins to (H^3)-DNA was carried out as described by Labonne et al., (1983) with some modifications. Binding mixture (100 ul) contained 20 mM Tris-HCl pH 7.6, 150 mM KCl, 3 ug native (H^3)-DNA, 20 ug ovalbumin and *50* ul of CM-cellulose column fractions. After incubation at 30°C for 10 min the assay mixture was diluted with 1 ml buffer (same salt composition as assay mixture) and filtered through nitrocellulose filters (previously boiled for 10 min in 20 mM Tris-HCl pH 7.6 and 6 mM 2-mercaptoethanol). The filters were washed with additional 1 ml buffer, dried and counted for radioactivity in Beckman LS-1800 liquid scintillation counter.

Concentration of the purified proteins; The purified proteins were dialyzed against 20 mM Tris-HCl pH 7.6, *50* mM KC1, 1 mM Na_2-EDTA and 6 mM 2-mercaptoethanol and passed through a small CM-cellulose column (1 ml bed volume) which was equilibrated with the same buffer. Finally the protein was eluted with the buffer containing 0.5 M KCl salt. Protein containing peak fractions were pooled and immediately dialyzed against 1 mM Tris-HCl pH 7.6 and 50 mM NaCl.

RESULTS

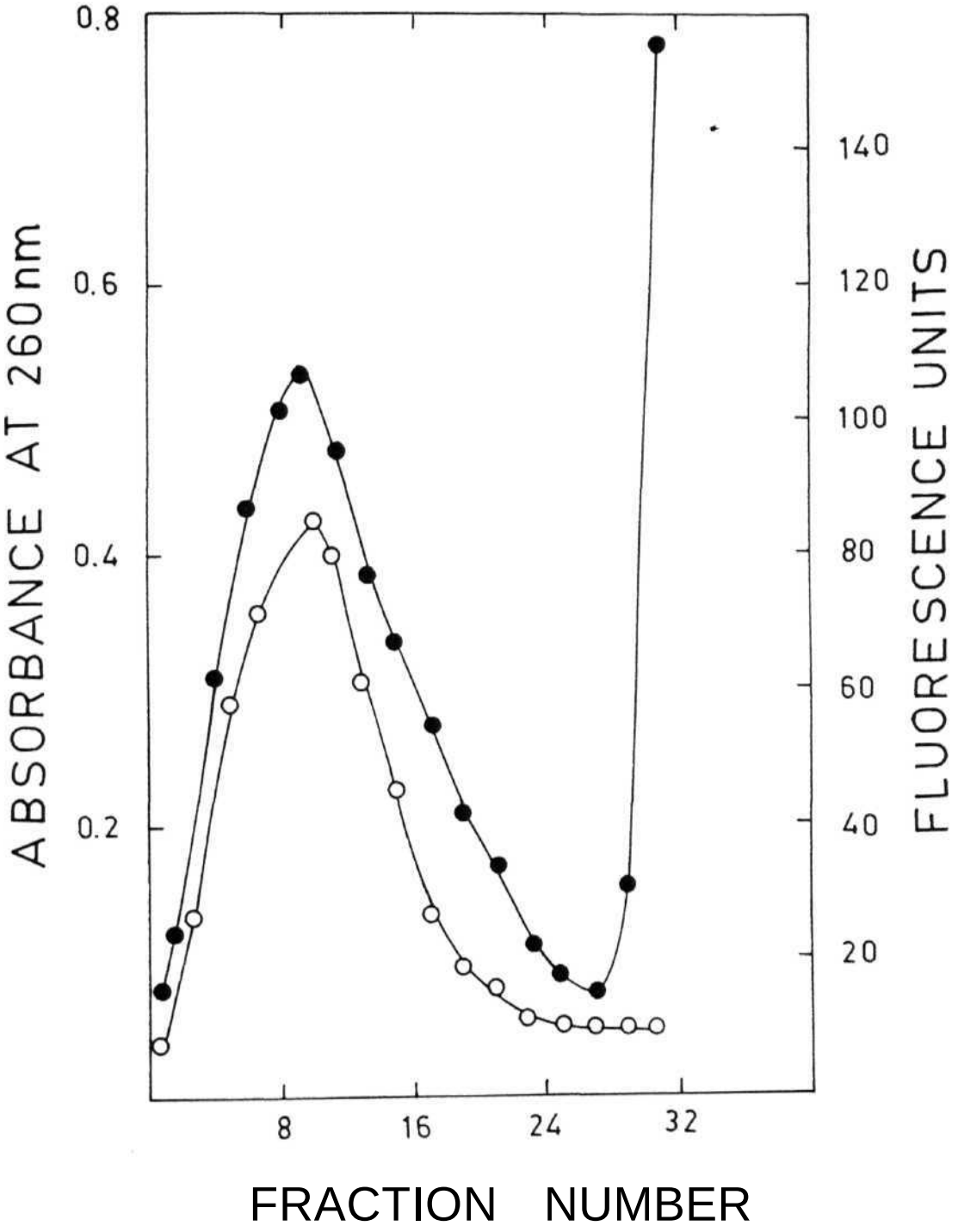
Isolation of nucleoid; Gentle lysis of S. acidocaldarius using nonionic detergents and centrifugation of the lysate through sucrose gradients separates the rapidly sedimenting DNA-protein complex, nucleoid. Fractions from the gradient were analyzed for DNA, by UV absorption and eithidium bromide fluorescence assay (Fig. 2.3). The procedure described for the isolation of nucleoid from E. coli can be successfully employed for the isolation of rapidly sedimenting nucleoid from this archaebacterium. Although there are different methods for chromatin isolation from E. coli viz. Spermidine nucleoid, NaCl nucleoid etc. we have used only the spermidine nucleoid method because NaCl method is known to dissociate some of the DNA binding proteins.

The relative amounts of DNA, RNA and protein in the nucleoid were estimated and were found to be in the ratio of 1 : 0.2 : 7 (w/v).

Gel electrophoretic analysis of nucleoid proteins: Fractions from the nucleoid gradient were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 2.4). The fractions corresponding to nucleoid peak (lanes 6-14) although contains several proteins in the molecular weight range from 8000 to 1,50,000 the most abundant proteins were the low molecular weight proteins (molecular weight 8000 to 12000). We have further analyzed the proteins present in the nucleoid by 2-dimensional polyacrylamide gel electrophoresis according to O'Farrell under three different conditions of 1st dimension electrophoresis viz., IEF pH 5 to 7, NEPHGE pH 3 to 10 and pH 7 to 11 (Fig. 2.5 A, B and C). There were several acidic and neutral proteins in the nucleoid fractions in the molecular weight range 10,000 to 1,00,000 (Fig. 2.5 A), NEPHGE gels (Fig. 2.5 B and C) show presence of several basic proteins in the pI range from 8 to 9.5. Out of the basic proteins the lowmoiecular weight proteins were found to be abundant. Some of the proteins that are present in nucleoid

Fig. 2.3 Sucrose density gradient profile of nucleold from S. acidocaldarius lysate.

Bacterial cells were lysed as described in the Materials and Methods section and the clear lysate was layered on to sucrose gradients and centrifuged as described there in. Fractions (2 ml) were collected from the top by aspiration. The fractions were analyzed for ultraviolet absorption (-●-) by diluting aliquots (0.1 ml) with 10 mM Tris-HCl (pH 7.6)/3mM magnesium acetate, DNA content (-O-) in the fractions was determined, using 15 ul samples, by ethidium fluorescence assay; 10 fluorescence units correspond to 0.1 ug of DNA. Fraction 31 corresponds to the top of the gradient.


0.8
0.6
0.4
0.2
ABSORBANCE AT 260nm
140
120
100
80
60
40
20
FLUORESCENCE UNITS
8
16
24
32
FRACTION NUMBER

Fig·2·3

Fig- **2.4** SDS- polyacrylamide gel electrophoretic analysis of sucrose gradient fractions of nucleold.

Samples (50 ul) of alternate fractions were treated with 1% SDS and 1% 2- mercaptoethanol at 65°C for 10 minutes and electrophoresed in 18% gels.

Lane 1, cell lysate; lanes 2 to 15, correspond to sucrose gradient fractions 31, 29, 25, 23, 19, 15, 13, 11, 9, 8, 6, 4, 2 and 1 respectively. Lane 16, Molecular weight standard proteins (Bovine serum albumin, Ovalbumin, Myoglobin, Cytochrome-C).

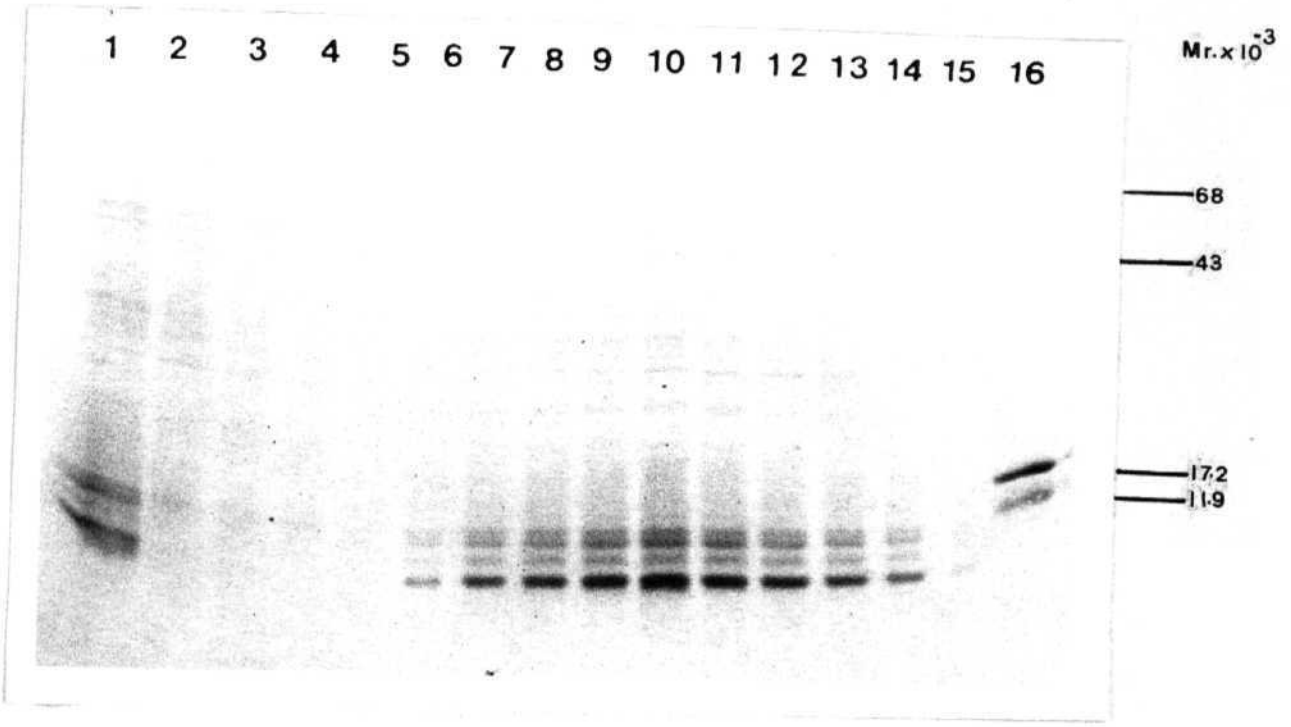


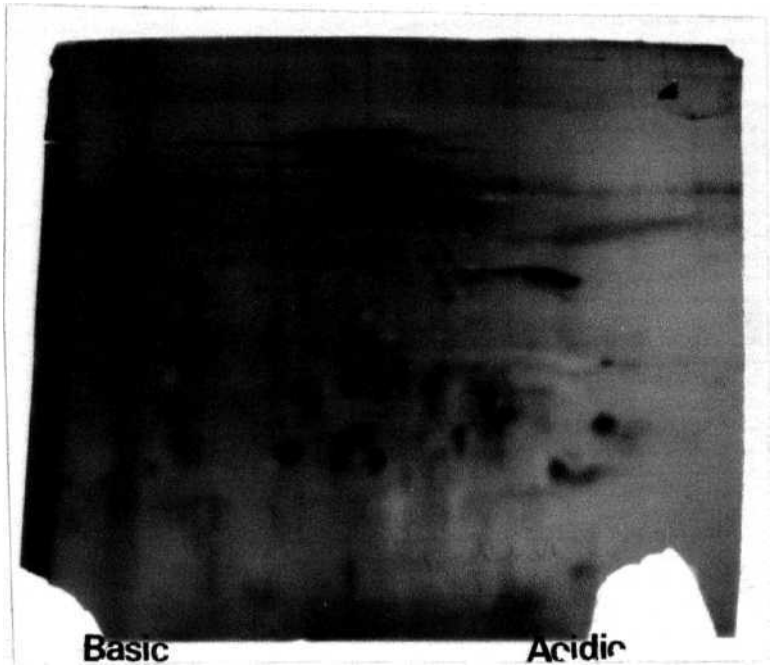
Fig. 2.4

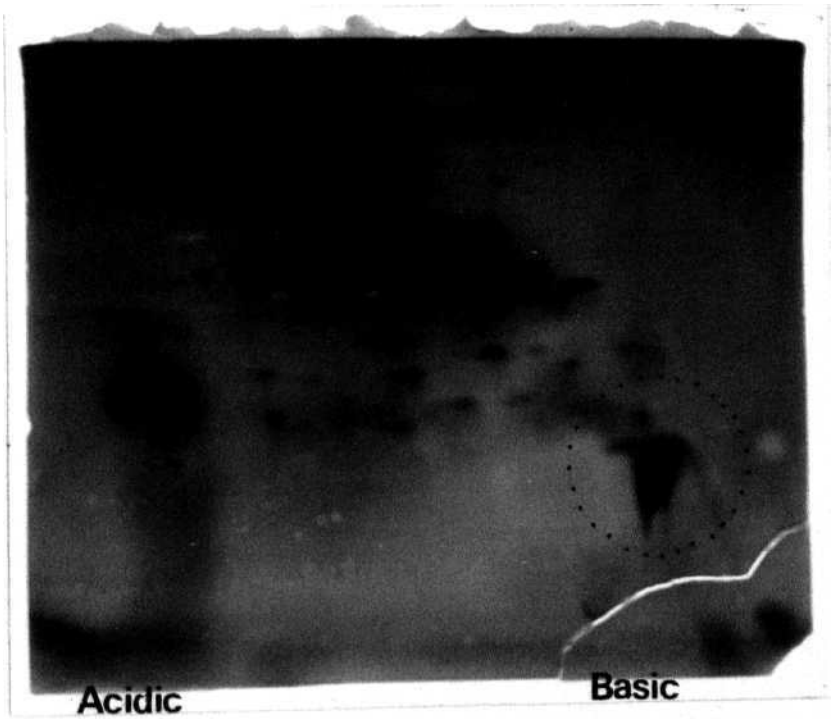
Fig. *2.5* Two-dimensional electrophoretic patterns of nucleoid proteins.

Electrophoretic conditions are described in the text. Nucleoid protein (100 ug) were prepared in the lysis buffer containing 9.5 M Urea, 2% (w/v) NP-40, 2% Ampholines (comprised of 1.6% pH range *5-7* and 0.4% pH range 3-10) and *5%* 2-mercaptoethanol.

(A) IEF pH *5-7*, (B) Nonequilibrium pH gradient electrophoresis, pH 3-10, (C) Nonequilibrium pH gradient electrophoresis pH 7-11.

The position of histone-like proteins is shown with a broken circle.





B

Fig. 2.5

fraction may not be truly nucleoid proteins. These may be membrane proteins because the method of isolation of the nucleoid does not exclude the contamination of membrane.

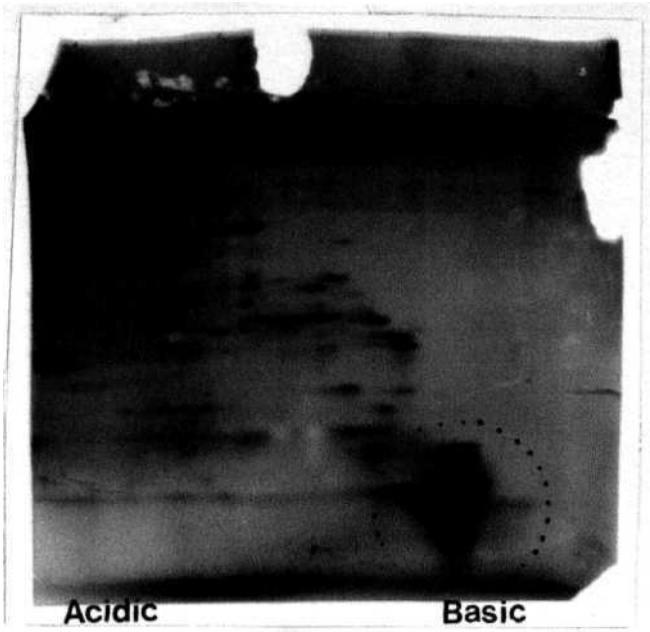
Presence of histone-like proteins in the nucleoid: Electrophoretic analysis of the nucleoid fraction showed the presence of low molecular weight proteins in abundance. In analogy with eukaryotic chromatin we treated the nucleoid with dilute acid to look for the presence of acid soluble proteins. Treatment of the nucleoid with dilute acid (0.2 M H_2SO_4) results in the precipitation of most of the proteins. The acid extract contains four low molecular weight polypeptides as analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 2.6). The four proteins have been named as HSNP-A, DBNP-B, HSNP-C and HSNP-C' based on their DNA binding properties (HSNP-Helix stabilizing nucleoid protein, DBNP-DNA binding nucleoid protein, see Chapter IV). The amount of acid soluble proteins of nucleoid was estimated to be 90% of DNA. Two dimensional electrophoresis of the acid extract of the nucleoid under NEPHGE conditions showed the presence of very low amounts oi a few other proteins of high molecular weight (Fig. 2.7A and B). Since these low molecular weight proteins are acid soluble and basic and are associated with archaebacterial chromatin it was thought that these low molecular weight proteins may correspond to histone-like proteins. Therefore we developed purification procedures for the isolation of these proteins.

Purification of acid soluble proteins of the nucleoid: Since the proteins are associated with the nucleoid and presumably with the intracellular DNA. We used the DNA-cellulose column chromatography for the purification of the four proteins.

DNA-cellulose chromatography: Concentrated nucleoid (2 ml) obtained from 1 g of S. acidocaldarius (see Methods Section) was dialyzed against

Fig. 2.6 SDS-polyacrylamide gel electrophoretic analysis of nucleoid proteins

Lane 1, 30 ug protein of nucleoid peak fraction from sucrose gradient; lane 2, 12 ug protein of acid extract of the nucleoid; lane 3, 40 ug protein of the acid extract of Post-ribosomal supernatant; lane 4, Molecular weight standard proteins (Bovine serum albumin 68,000; Ovalbumin, 43,000; Chymotrypsinogen, 25,600; Myoglobin, 17,200; Cytochrome C, 11,900 and E.coli protein NS, 9,300).



C

Fig·2·5

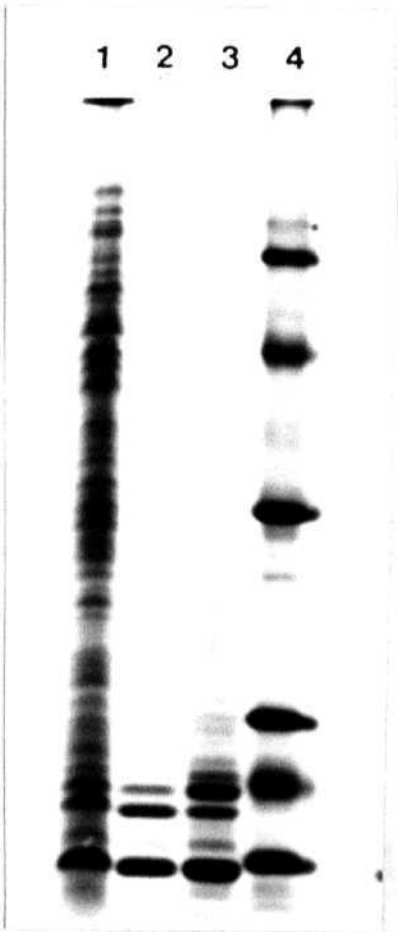
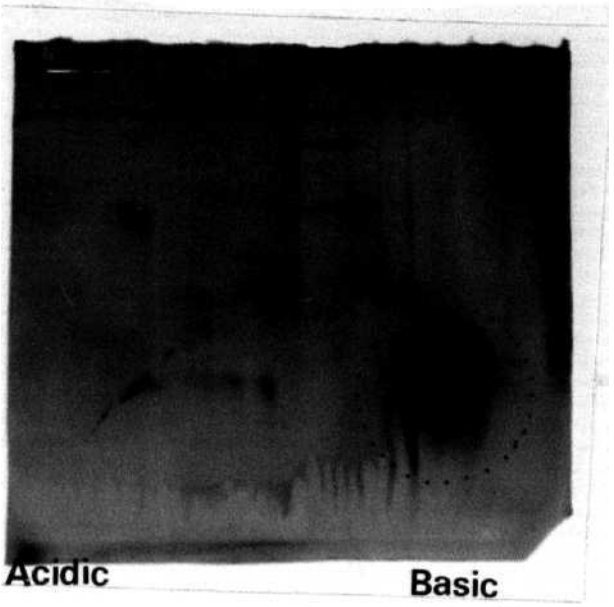
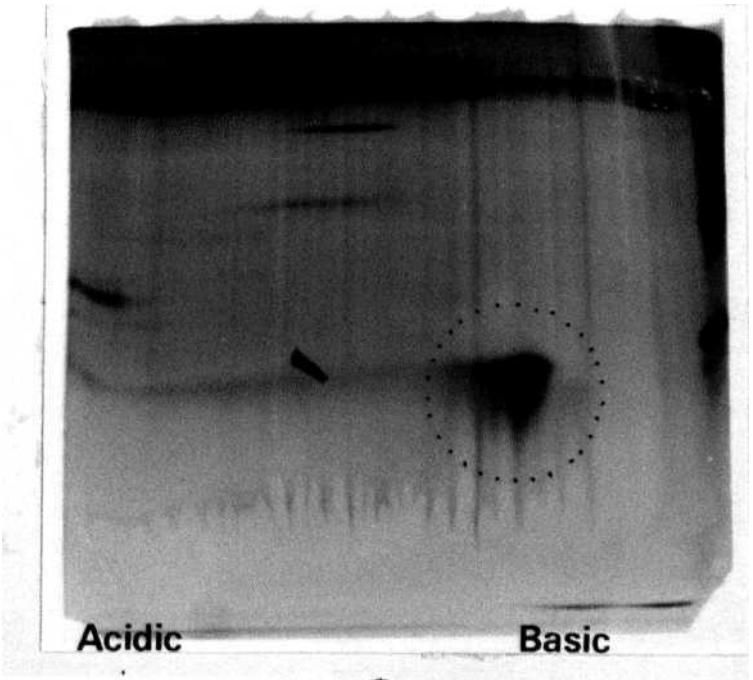


Fig-2-6

Fig. 2.7 Two-dimensional gel electrophoresis of acid extract of nucleoid proteins

(A) NEPHGE pH 3-10 (B) NEPHGE pH 7-11. The low molecular weight basic proteins are shown by broken circle.





B

Fig. 2.7

0.2 M H_2SO_4 as described. The clear acid extract after centrifugation was dialyzed against DNA-cellulose column buffer (20 mM Tris-HCl pH 7.6, 150 mM KCl, 1 mM Na_2-EDTA, and 6 mM 2-mercaptoethanol) and passed through a DNA (calf thymus, native) cellulose column (1 ml bed volume) equilibrated with the above buffer. The column was washed successively with buffers of increasing KCl concentration (0.15 M, 0.3 M, 0.6 M and 2 M). Fractions of 1 ml each were collected. The fractions were analyzed for protein by Lowry method (1951). No protein was eluted with 0.15 M and 2 M salt concentration. The peak fractions of 0.3 M and 0.6 M salt concentration eluates were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 2.8). The protein denoted as HSNP-A and HSNP-C' were eluted together in 0.3 M salt buffer and DBNP-B and HSNP-C were eluted with 0.6 M salt buffer. Both these fractions (0.3 M and 0.6 M eluate) were further chromatographed on CM-cellulose.

Carboxymethyl cellulose chromatography: Pooled 0.3 M and 0.6 M eluates obtained from DNA-cellulose column were separately chromatographed on CM-cellulose columns. The 0.3 KCl eluate was diluted to bring the salt concentration to 0.1 M and passed through CM-cellulose column (1 ml bed volume) equilibrated with 20 mM Tris-HCl pH 7.6, 100 mM KCl, 1 mM Na_2-EDTA and 6 mM 2-mercaptoethanol. The column was washed in a stepwise manner with the equilibrating buffer, 0.25 M KCl buffer and 0.4 M KCl buffer. Peak fractions from each salt elution were analyzed by SDS-polyacrylamide gel electrophoresis. No protein was eluted with 0.1 M salt elution. The proteins HSNP-A and -C were eluted with 0.25 M KCl with HSNP-A eluting in the earlier fractions and HSNP-C eluting in the later fractions (Fig. 2.9). The individual fractions containing each protein were pooled separately and concentrated. The 0.6 M eluate from the DNA-cellulose chromatography

Fig. 2.8 SDS-polyacrylamide gel electrophoretic analysis of fractions obtained after DNA-cellulose chromatography.

Acid extract of nucleoid was passed through the DNA-cellulose column and the fractions were collected as described in Methods. 50 ul of peak fractions were treated with sample buffer as described in legend to Fig. 2.4.

Lane 1, acid extract of nucleoid; lanes 2-6, 0.3 M KC1 buffer eluate peak fractions; lanes 7-11, 0.6 M KC1 buffer eluate peak fractions. The bands corresponding to the nucleoid proteins are indicated.

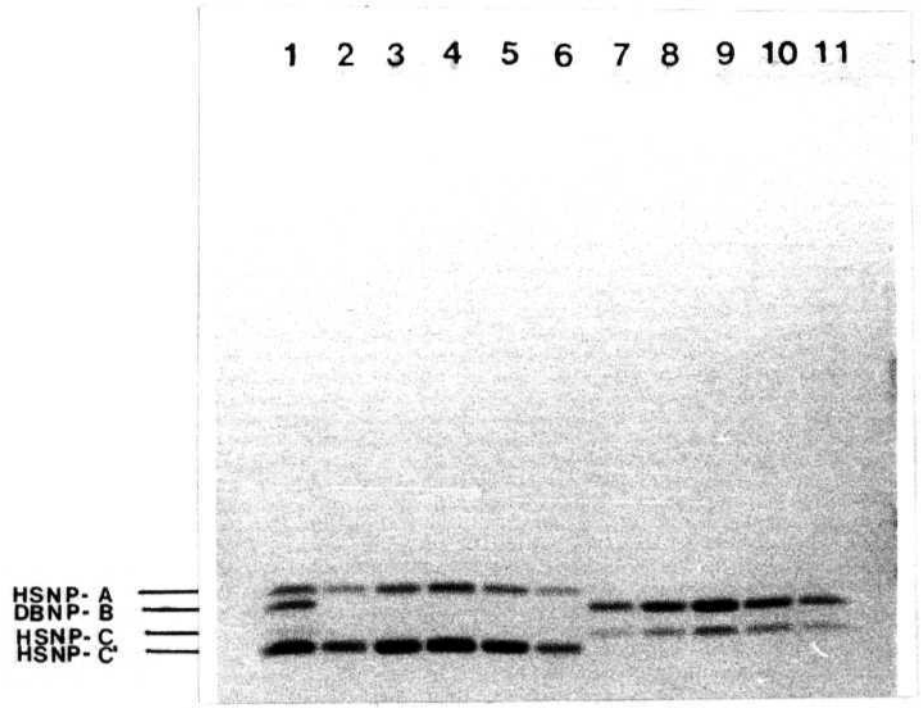


Fig. 2.8

Fig. 2.9 SDS-polyacrylamide gel electrophoresis of fractions obtained after CM-cellulose chromatography. 0.3 M KCl eluate of DNA- cellulose column was passed through CM-cellulose as described in Methods section.

Lane 1, 0.3 M eluate of DNA-cellulose column; lanes 2-5, the early fractions of 0.25 M KCl buffer eluate; lanes 6-8, the middle fractions of 0.25 M KC1 buffer eluate; lanes 9-12, the later fractions of 0.25 M KCl buffer eluate. The protein bands corresponding to HSNP-A and HSNP-C' are indicated.

Fig. 2.10 SDS-polyacrylamide gel electrophoretic analysis of fractions obtained after CM-cellulose chromatography

0.6 M KCl eluate of DNA-cellulose column was passed through a CM-cellulose as described in Methods section.

Lane 1, 0.6 M eluate of DNA-cellulose column; lanes 2-6, peak fractions of 0.25 M KC1 buffer; lanes 7-12, peak fractions of 0.4 M KCl buffer. Positions of DBNP-B and HSNP-C are indicated.

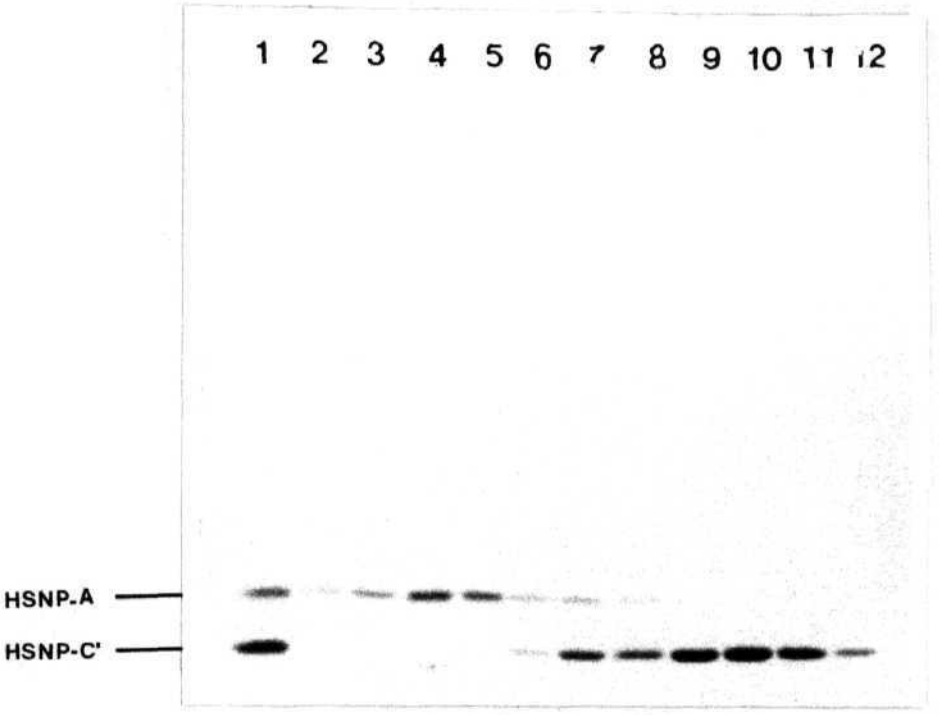


Fig. 2.9

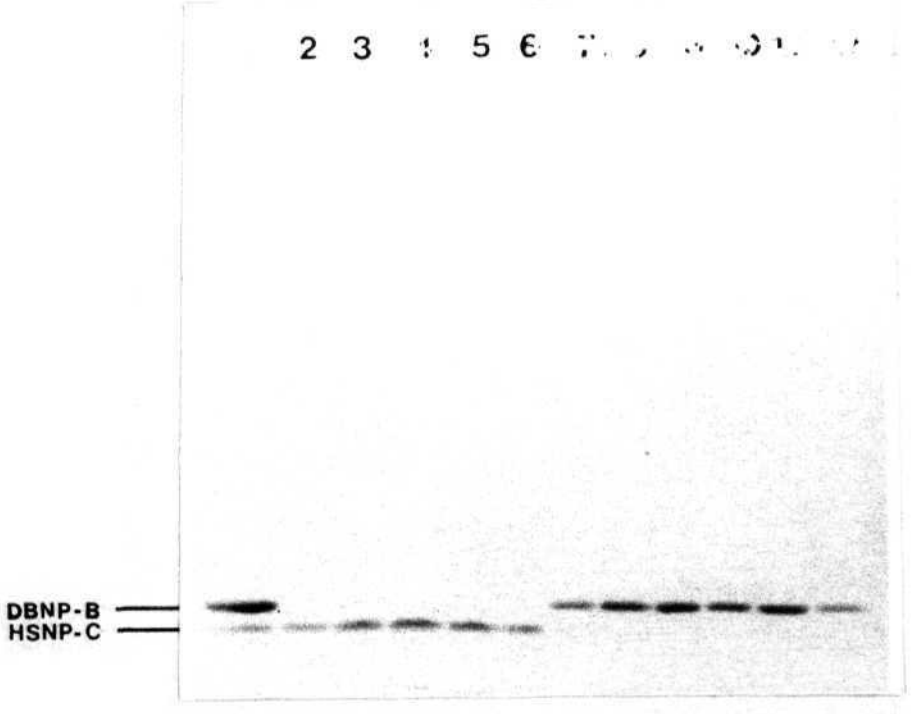


Fig.

was also chromatographed on a CM-cellulose column exactly as described above for 0.3 M KCl eluate. The protein HSNP-C was eluted in 0.25 M salt buffer whereas DBNP-B was eluted in 0.4 M salt buffer (Fig. 2.10). The peak fractions were pooled and concentrated. In the case of HSNP-A and HSNP-C rechromatography on CM-cellulose was performed to remove any trace cross contamination. The proteins were found to be highly purified and gave a single band on SDS-polyacrylamide gel electrophoresed (Fig. 2.11). Purification of HSNP-A, DBNP-B, HSNP-C and HSNP-C from post-ribosomal supernatant: In order to purify reasonably large amounts of these proteins and to avoid repeated nucleoid isolation for purification of the proteins we also developed purification of these proteins from acid treated post-ribosomal supernatant (S-100). We have used DNase treated cell extracts for the preparation of S-100 fraction. Treatment of S-100 with 0.2 M H_2SO_4 was carried out as described in methods section. The acid treatment results in the precipitation of appreciable amounts of the protein. The acid extract of post-ribosomal supernatant contains several high molecular weight polypeptides apart from the abundant low molecular weight proteins (Fig. 2.6 lane 3). The acid extract of S-100 was also analysed by O'Farrell 2-dimensional electrophoresis (Fig. 2.12 A, B and C). The NEPHGE gel patterns (Fig. 2.12 B & C) showed the presence of low molecular weight proteins in abundance.

The acid treated post-ribosomal supernatant (1 ml) was passed through a DNA-cellulose column (1 ml bed volume). The column was eluted in a step wise fashion with buffers containing increasing salt concentrations exactly as described above for DNA-cellulose chromatography of acid extract of nucleoid. The elution pattern obtained was very much like that obtained with nucleoid acid extract. HSNP-A and HSNP-C were eluted together with 0.3 M salt buffer, DBNP-B and HSNP-C were eluted with 0.6 M salt

Fig. 2.11 SDS-polyacrylamide gel electrophoretic analysis of purified nucleoid proteins.

Lane 1, Molecular weight standard proteins (Bovine serum albumin, 68,000; Ovalbumin, 45,000; Chymotrypsinogen, 25,600; Myoglobin, 17200; Cytochrome-C, 11,900 and E.coli protein NS, 9,300); lanes 2-5, 5 ug each of purified HSNP-A, DBNP-B, HSNP-C and HSNP-C' respectively.

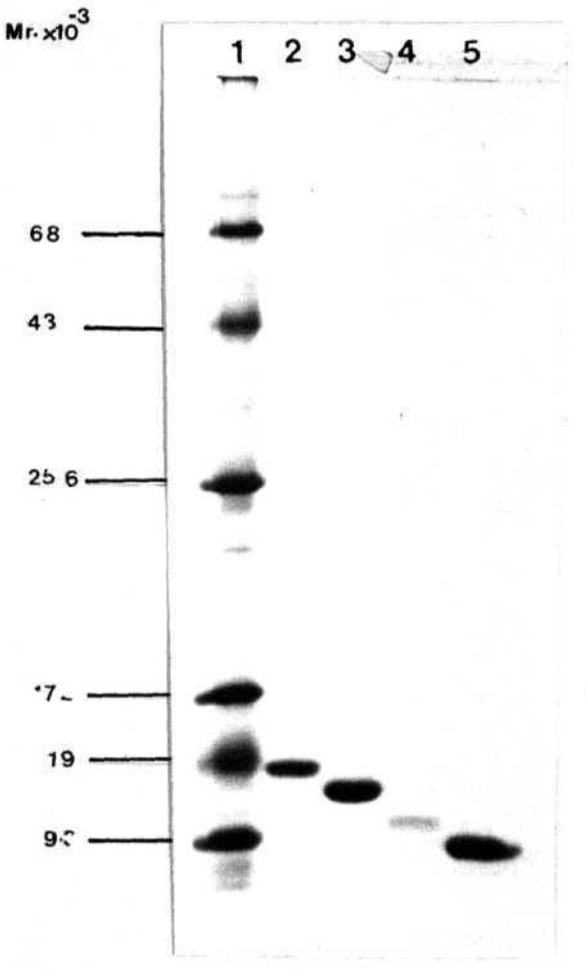
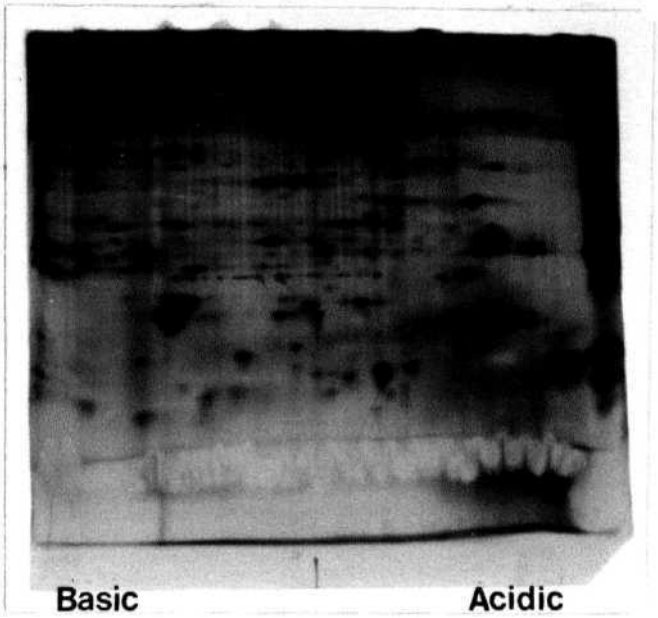


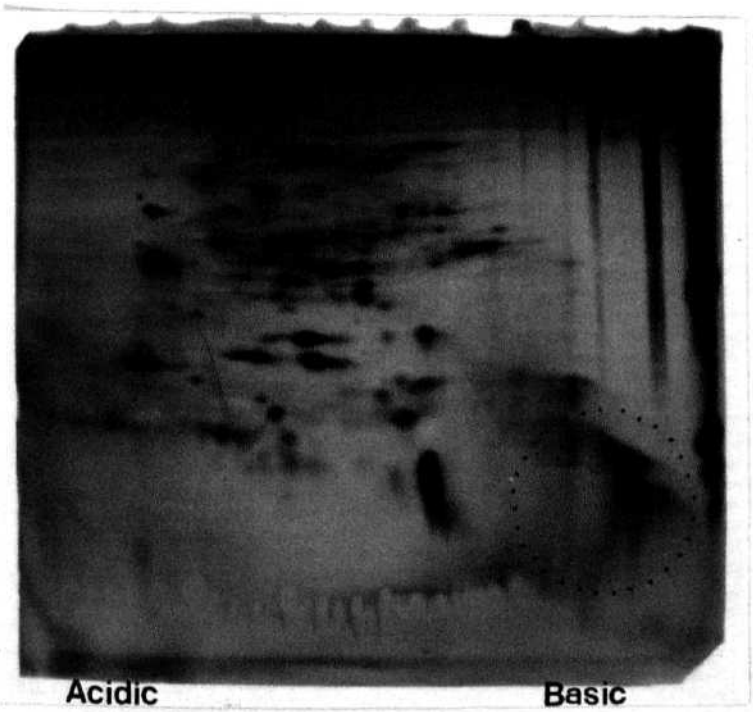
Fig. 2.11

Fig. 2.12 Two-dimensional gel analysis of acid treated Post-ribosomal supernatant. Samples were prepared as described in the legends to Fig. 2.7.

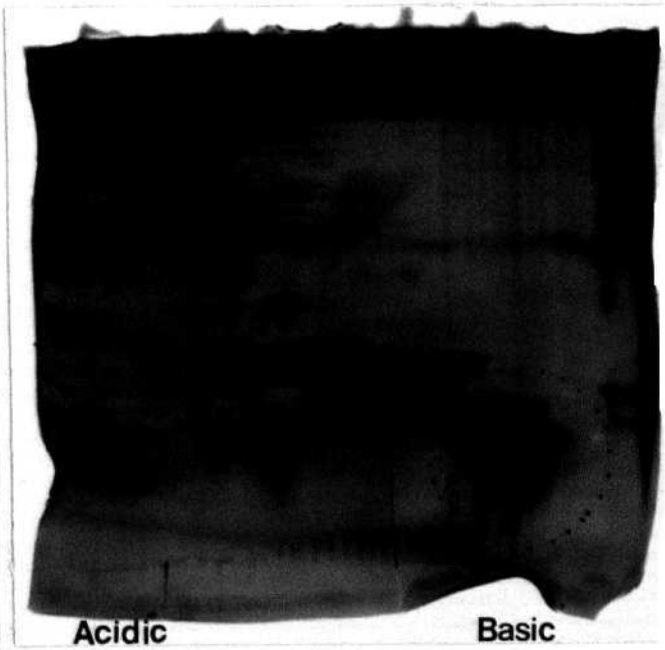
(A) IEF pH 5-7, (B) and (C) NEPHGE pH 3-10 and pH 7-11 respectively. The position of nucleoid proteins is shown by broken circle.



A



B



C

Fig·2·12

buffer (Fig. 2.13). These two salt eluates from DNA-cellulose chromatography were further chromatographed on two CM-cellulose columns to separate the individual proteins. Pure proteins of HSNP-A, HSNP-C', DBNP-B and HSNP-C were obtained (Fig. 2.14 A and B). The salt concentrations required for the elution were same as before (see Fig. 2.9 and 2.10).

Single step separation of the four proteins by CM-cellulose chromatography

In order to purify the proteins starting from large amounts of post-ribosomal supernatant another purification procedure was developed. In this the chromatographic steps were reversed i.e. first CM-cellulose followed by DNA-cellulose chromatography. 10 ml of acid treated S-100 was passed through a CM-cellulose column of 20 ml bed volume (1.2 cm x 16 cm) equilibrated with 0.05 M KCl containing 20 mM Tris-HCl pH 7.6, 1 mM Na_2-EDTA and 6 mM 2-mercaptoethanol and the column was washed successively with 0.05 M, 0.1 M, 0.2 M, and 0.4 M salt buffer. Fractions (3 ml) were collected. The fractions were analyzed for protein absorption at 280 nm and DNA binding by filtration technique (Fig. 2.15). The protein profile showed four protein peaks (Peaks I to IV) out of which three showed binding to DNA. Electrophoretic analysis (Fig. 2.16 A and B) showed the presence of pure HSNP-A (Lane 6-8) in I peak (0.2 M salt), HSNP-C (Lane 10-15) in II peak (0.2 M salt), a mixture of HSNP-C and C (Lane 2-9, Fig. 2.16 B) in III peak (0.2 M salt) and the DBNP-B (Lane 10-12, Fig. 2.16 B) in IV peak (eluted with 0.4 M). Three proteins (HSNP-A, C and DBNP-B) are already obtained in pure forms by this chromatography itself, as can be seen from the electrophoresis of the corresponding peak fracr tions, peaks I, II, IV (Fig. 2.17 A and B). Proteins HSNP-C and C present in the peak III (Fig. 2.17 A lane 4) were further resolved by DNA-cellulose chromatography. HSNP-C was eluted with 0.3 M salt and HSNP-C with 0.6 M salt (Fig. 2.17 B).

Fig. 2.13 SDS-polyacrylamide gel electrophoretic analysis of fractions obtained after DNA-cellulose chromatography of acid treated Post-ribosomal supernatant (S-100).

Acid extract of Post-ribosomal supernatant was passed through a DNA-cellulose column and the column was eluted as described in Methods. 50 ul of peak fractions were treated with sample buffer as described in the legend to Fig. 2.4 and electrophoresed.

Lanes 1 and 16, Molecular weight standard proteins (as in Fig. 2.11); lane 2, Acid treated Post-ribosomal supernatant; lane 3-5, Proteins eluted by 0.15 M KC1 peak fractions; lanes 6-10, 0.3 M KCl peak fractions; lanes 11-15, 0.6 M peak fractions. The position of nucleoid proteins are shown by arrows.

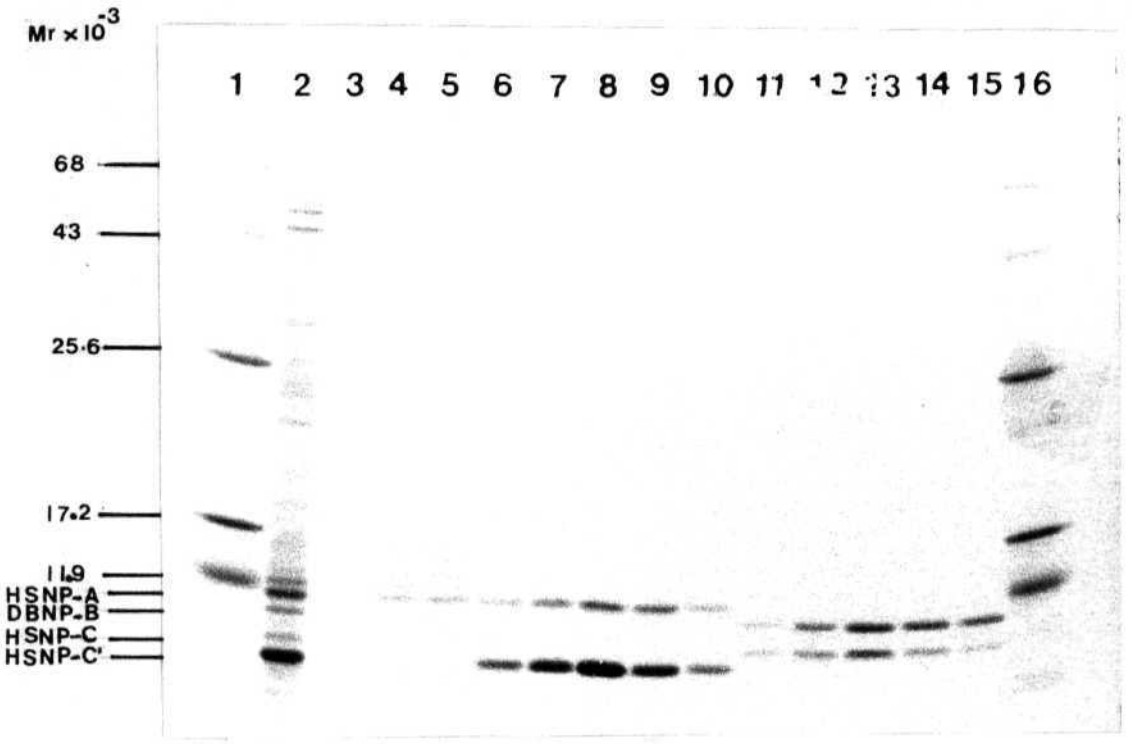

Mr x 10-3
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
68
43
25·6
17·2
11·9
HSNP-A
DBNP-B
HSNP-C
HSNP-C'

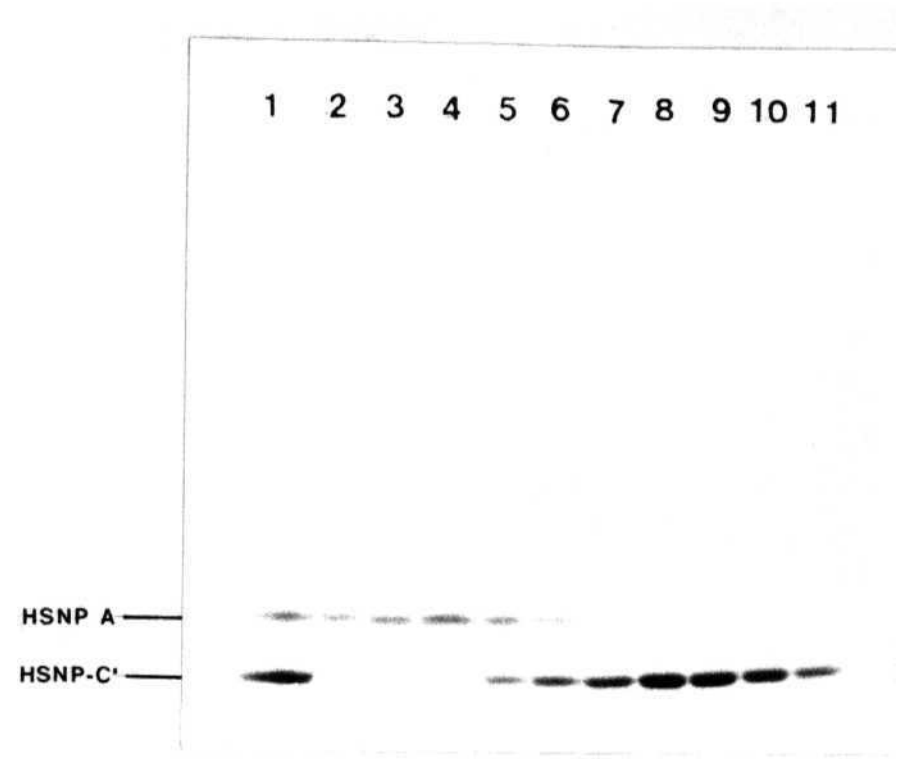
Fig. 2·13

Fig. 2.14 SDS-polyacrylamide gel electrophoresis of fractions obtained after CM-cellulose chromatography.

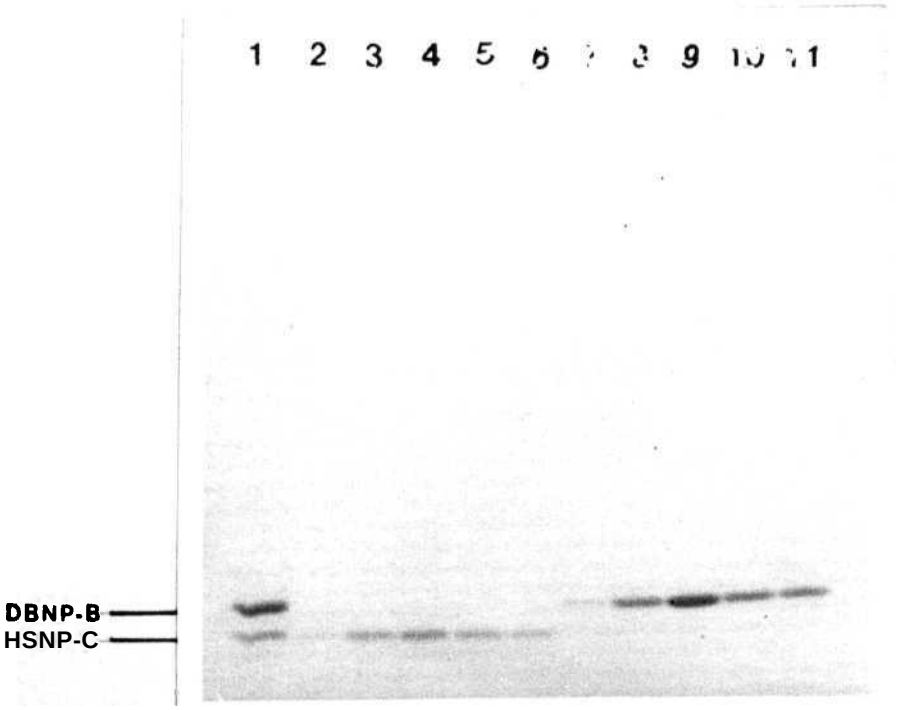
(A) 0.3 M eluate of DNA-cellulose column (B) 0.6 M eluate of DNA-cellulose column were obtained after chromatography of S.100 acid extract as in Fig. 2.13, were passed through CM-cellulose as described in Methods section.

(A) Lane 1, 0.3 M eluate of DNA-cellulose column; lanes 2-4, early fractions of 0.25 M KC1 buffer containing HSNP-A; lanes 5 and 6 middle fractions of 0.25 M KC1 buffer; lanes 7-11, later fractions of 0.25 M KC1 buffer containing HSNP-C'.

(B) Lane 1, 0.6 M eluate of DNA-cellulose column; lanes 2-6, peak fractions of 0.25 M KCl buffer containing DBNP-B; lanes 7-11, peak fractions of 0.4 M KCl buffer containing HSNP-C.



A



a

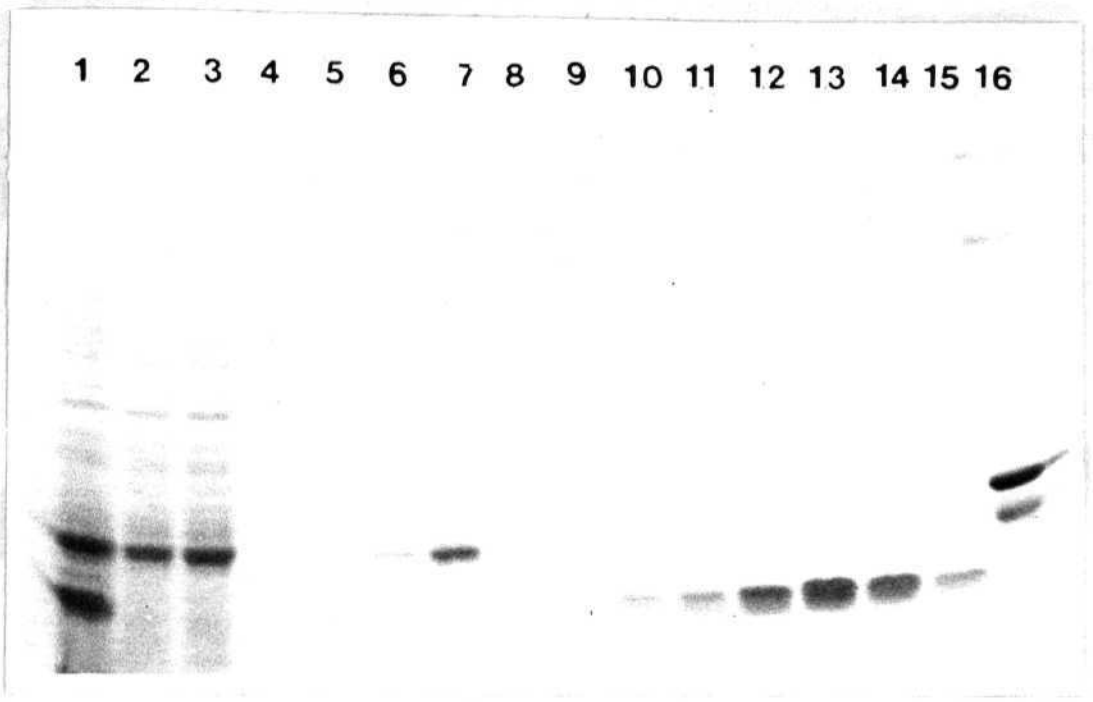
Fig.2.14

Fig. 2.15 CM-cellulose chromatography of acid treated Post-ribosomal supernatant. The chromatography was carried out as described in the text using 10 ml of S-100 acid extract. The column was eluted with increasing KCl salt concentration as indicated. The fractions were analyzed for protein absorbance at 280 nm ( ) and native (H)-DNA binding ( ). Binding of (H) DNA was measured by nitrocellulose filtration technique (see Materials and Methods).

Fig. 2.16 SDS-polyacrylamide gel electrophoretic analysis of fractions obtained after CM-cellulose chromatography of acid treated Post-ribosomal supernatant. 30 ul peak fractions from the CM-cellulose chromatography was performed as in Fig. 2.15 were treated with sample buffer and electrophoresed.

(A) Lane 1, acid treated post-ribosomal supernatant; lanes 2 and 3, 0.05 M KCl buffer fractions; lanes 4 and 5, peak fractions of 0.1 M KCl buffer; lanes 6-15, fractions of 0.2 M KC1 eluate corresponding to peak I and II fractions (see Fig. 2.15); lane 16, Molecular weight standard proteins (Bovine serum albumin, Ovalbumin, Myoglobin and Cytochrome-C).

(B) Lane 1, acid treated post ribosomal supernatant; lanes 2-9, later fractions of 0.2 M KCl eluate corresponding to peak III in Fig. 2.15; lanes 10-13, peak fractions of 0.4 M KCl eluate corresponding to peak IV in Fig. 2.15; lane 14, Molecular weight standard proteins (as in A).



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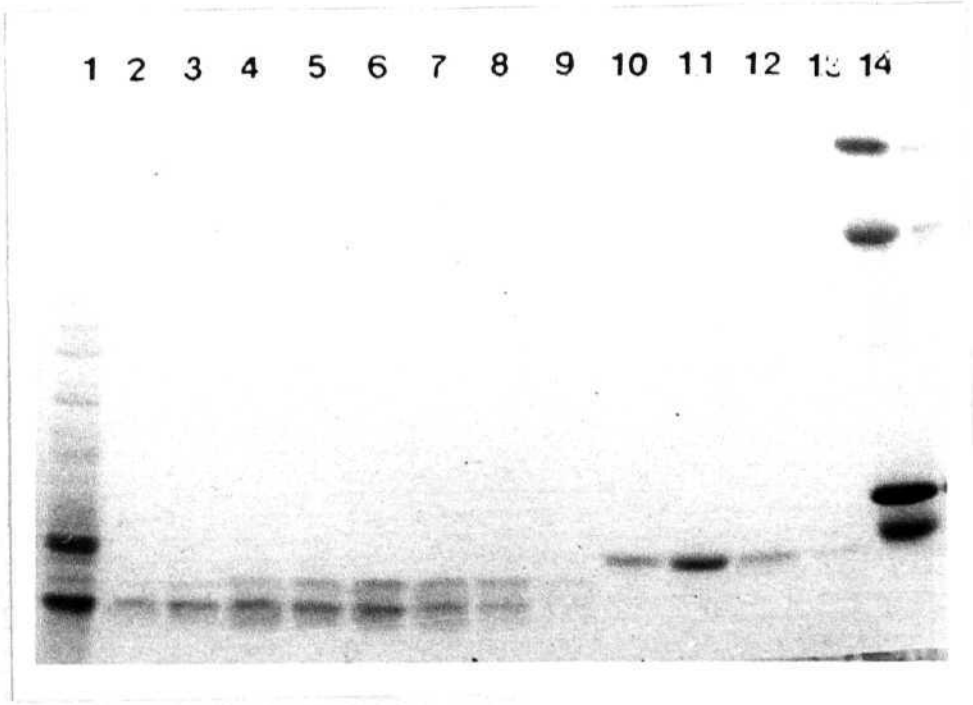


B

Fig. 2.16

Fig. 2.17 SDS-polyacrylamide gel electrophoretic analysis of pooled peak fractions obtained after CM-cellulose chromatography of acid treated post ribosomal supernatant.

(A) Lane 1, acid treated post-ribosomal supernatant; lanes 2,3,4 and 5 correspond to peaks I, II, III and IV of figure 2.15 respectively; lane 6, Molecular weight standard proteins (as in Fig. 2.16 A).

(B) SDS-polyacrylamide gel electrophoretic analysis of fractions from DNA-cellulose chromatography of peak HI (Fig. 2.15). The protein was eluted by stepwise increasing KCl salt concentration. In all the cases 50 ul of sample is treated with sample buffer. Lane 1, peak III fraction (Fig. 2.15); lanes 2-5, peak fractions of 0.3 M KCl eluate containing HSNP-C; lanes 6-9, peak fractions of 0.6 M KCl eluate containing HSNP-C; lane 10, Molecular weight standard proteins (as in 2.16 A).

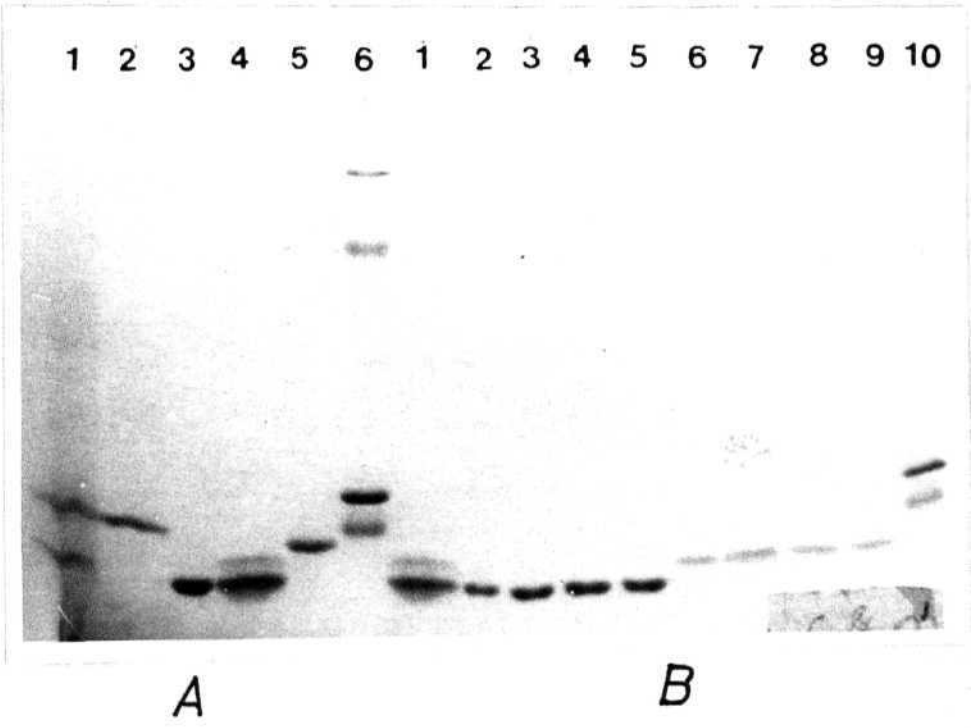


Fig. 2.17

All the three methods of purification gave rise to highly purified proteins. SDS-polyacrylamide gel electrophoretic analysis of the purified proteins showed a single band for each protein. The purified proteins were analyzed by 2-dimensional polyacrylamide gel electrophoresis as per Mets and Bogorad (Mets and Bogorad, 1974). Each protein gave a single spot on electrophoresis (Fig. 2.18). The yield of the proteins by different methods of purification was estimated. Recovery of the four proteins from the acid treated nucleoid and post-ribosomal supernatant was estimated. The amounts of these protein obtained from 2 ml concentrated nucleoid (1 g /cell) were HSNP-A (80 ug), DBNP-B (200 ug), HNSP-C (60 ug) and HSNP-C[1] (350 ug). The amounts of proteins obtained from acid treated S-100 (from 1 g . cell) 0.1 mg, HSNP-A; 0.3 mg, DBNP-B; 0.075 mg, HSNP-C and 0.5 mg HSNP-C Approximately similar amounts of these proteins were obtained from acid treated post ribosomal supernatant irrespective of the sequence of the two chromatographic steps (Table 2.1).

DISCUSSION

The nucleoid (or chromatin) isolated from gently lysed S. acidocaldarius cells contain four acid soluble proteins in abundant amounts as analyzed by gel electrophoresis. We have observed that S. acidocaldarius cells can be easily lysed by dilute nonionic detergents. This is probably because of the absence of rigid cell wall structure (Brock et al., 1972). The procedure described for the isolation of nucleoid from E. coli can be successfully employed for the isolation of rapidly sedimenting nucleoid from this archaebacterium. Nucleoid (chromatin) has not been isolated and studied from any archaebacterium. This study is the first in this aspect. We have not used any further purification of the nucleoid because of the possibility of removal of some proteins which are genuinely associated with DNA (Moriya and Hori, 1981). Protein content of the nucleoid was found to be quite high. The ratio of

Fig. 2.18 Two-dimensional electropherograms of purified nucleoid proteins. Electrophoresis was performed as per Mets and Bogorad (1974).

(A) Total nucleoid proteins (DNase treated), 120 ug;
(B) Mixture of purified nucleoid proteins, 20 ug;
(C) HSNP-A, 5 ug;
(D) DBNP-B, 5 ug;
(F) HSNP-C, 5 ug;
(F) HSNP-C, 5 ug;

The position of the proteins are shown by arrows in B.

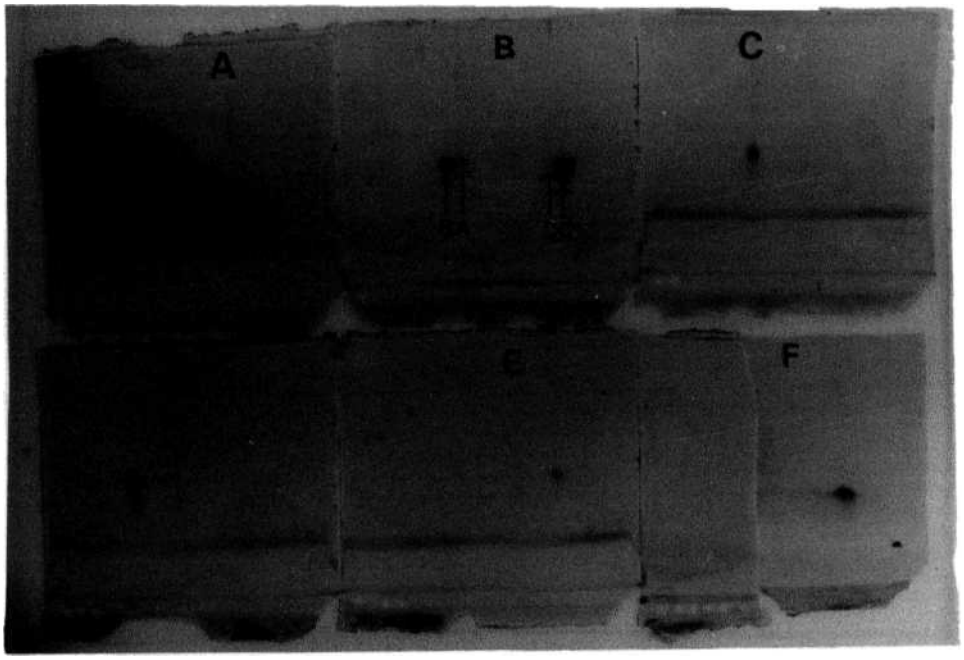


Fig.2.18

TABLE - 2.1

Recovery of nucleoid proteins after purification

Material		Yield of purified proteins per gram cells			
		HSNP-A	DBNP-B	HSNP-C	HSNP-C'
Nucleoid (acid-treated)	DNA-cellulose followed by CM-cellulose chromatography	0.08 mg	0.2 mg	0.06 mg	0.35 mg
Post-ribosomal supernatant (acid treated)	-do-	0.1 mg	0.3 mg	0.075 mg	0.5 mg
Post-ribosomal supernatant (acid-treated)	CM-cellulose followed by DNA-cellulose chromatography	0.16 mg	0.21 mg	0.1 mg	1 mg

nucleic acid (DNA + RNA) to protein is 1.2:7. Relatively high content of protein is present in the S. acidocaldarius chromatin that we have isolated than in the DNA. protein complexes isolated by Green et al (1983). Higher protein content may be due to membrane contamination in the nucleoid preparation or because the nucleoid was not washed with high salt (1M NaCl). Furthermore, Green et al (1983) did not isolate intact nucleoid but isolated sheared DNA. Protein complexes by gel filtration. The nucleoid proteins were analyzed by SDS-polyacrylamide gel electrophoresis. The nucleoid contains proteins with molecular weights in the range of 9000 to 100,000. The acidic and neutral proteins predominate in the nucleoid. The basic proteins were fewer than acidic and neutral proteins. Out of the basic proteins the low molecular weight proteins are present in abundance accounting upto more than 60% of the total amount of basic proteins (see Figx 2.5). Since proteins similar to histones are expected to be basic and are therefore acid soluble, we treated the nucleoid with dilute acid. The acid extract contains very few proteins as analyzed by gel electrophoresis. The amount of the low molecular weight proteins (the four proteins) in the acid extract was found to be 80-90%. These proteins have been denoted as HSNP-A, DBNP-B, HSNP-C and HSNP-C'. These are basic proteins with pI in the range 8.5 to 10.

The four proteins have been purified to homogenity by employing DNA-cellulose and CM-cellulose column chromatographies. Because of their acid-soluble basic nature and DNA binding property, the proteins could be purified relatively easily from the nucleoid. Post-ribosomal supernatant isolated from DNase treated cell extracts also contains abundant amounts of these proteins. Purification of the proteins from acid treated nucleoid yields only small amounts of these proteins, because the starting material (nucleoid)

is in small amounts (each time isolated from 2 g of cells). In order to obtain large quantities of the purified proteins and to avoid repeated nucleoid isolation the four proteins were isolated from acid treated post-ribosomal supernatant which contains large amounts of these proteins. The salt concentration required for the elution of these proteins on DNA-cellulose is probably an indication of their strong affinity to DNA. DBNP-B and HSNP-C which are eluted with salt concentration of 0.4 to 0.6 M are found to bind native DNA efficiently (see Chapter VI). However, the elution pattern of these proteins on CM-cellulose at pH 7.6 is different from that expected from their electrophoretic migration under acidic pH conditions. HSNP-C and HSNP-C migrate faster than DBNP-B at pH *5.0* electrophoresis. CM-cellulose chromatography of the proteins show stronger binding of DBNP-B than HSNP-C and HSNP-C This may be because of a pH dependent variation in the ionization of charged groups in the proteins. The purification of three of the four proteins can be achieved in a single step by chromatography of S-100 acid extract on CM-cellulose. The elution pattern of the proteins suggest interaction of the proteins with each other. HSNP-C is eluted as single large peak when S-100 acid extract was chromatographed on CM-cellulose (Fig. 2.15 peak II). Part of HSNP-C also eluted in the peak III along with HSNP-C indicating possible interaction of HSNP-C and **HSNP-C with each** other. The purification procedures yielded good amounts of DBNP-B and HSNP-C (Table 2.1).

CHAPTER - III

PHYSICO-CHEMICAL AND IMMUNOCHEMICAL PROPERTIES OF THE HISTONE-LIKE PROTEINS

Histone-like proteins from variety of eubacteria have been purified and characterized. Particularly DNA binding protein HU(NS) has been extensively studied. In contrast very few reports are there in the literature regarding the isolation and characterization of histone-like proteins from archae-bacteria (see Introduction). In this chapter several properties of the purified histone-like proteins from S. acidocaldarius are described. The properties studied include immunochemical and physicochemical properties.

MATERIALS AND METHODS

Antisera: Antibodies to purified proteins HSNP-A, DBNP-B, HSNP-C and HSNP-C' were raised in rabbits and Ouchterlony Immunodiffusion assays were performed as described by Stoffler and Wittmann (1971). About 200 ug of protein was emulsified with Freund's complete adjuvant and injected subcutaneously into rabbits at multiple sites. After four weeks, booster injections each of 100 ug of protein in Freund's incomplete adjuvant were given subcutaneously at 4th, 5th and 6th weeks. Rabbits were bled after 3rd booster injection through the pinna vein. Antiserum was collected after centrifugation of **the** clotted blood.

Immunodiffusion: Agarose (1.5%) was dissolved in 0.9% NaCl and 0.019 M Na-barbital buffer pH 8.4, Py heating in a boiling waterbath for 45 min. This was poured into immunodiffusion plates or on microscopic slides to a height of 3 mm and allowed to cool at room temperature. After cutting the wells, the sample and antisera were placed in them. The diffusion was allowed to take place for 6-12 hrs at room temperature. The immunodiffusion plates were photographed against dark background with scattering light. In some cases the immunodiffusion gel plates were extensively washed with 0.9% NaCl and finally with water. The gel plates were dried, stained with coomassie blue and destained.

FITC-labelling of anti-rabbit IgG: It was done according to Nargessi and Landon (1981). Anti-rabbit IgG (Goat), 2 ml (14.0 mg/ml) was passed through a Sephadex G-25 column (1.2 x 20 cm) equilibrated with sodium carbonate buffer pH 8.7. To the anti-IgG peak pool (6.8 ml) from the G-25 column, 0.68 ml of FITC (2 mg/ml in the above buffer) was added dropwise at room temperature (28°C) and left for 2 hrs. The unreacted FITC in : FITC-conjugated anti-IgG was removed by passing through a G-25 column (1.2 x 20 cm) equilibrated with phosphate buffered saline (0.02 M sodium phosphate pH 7.4, 0.15 M NaCl). Yellow coloured protein fractions were pooled. The concentration of FITC-conjugated anti-IgG was 2 mg/ml.

Immunoblotting: It was done according to Towbin et al (1979) with minor modifications. Purified nucleoid proteins (5 ug each) and a mixture of the four proteins were electrophoresed according to Thomas and Kornberg (1975). The proteins were transferred electrophoreticaliy to nitrocellulose sheets (0.45 um pore size) using the electrode buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol pH 8.3). The electroblots were soaked in immunoblotting incubation buffer (IBIB) (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% bovine serum albumin, 0.02% sodium azide) for 5 hrs and washed three times (3 x 15 min each) with IBIB buffer containing *0.05%* NP-40 detergent. Each sheet was incubated separately in 10% antiserum to nucleoid proteins in IBIB buffer containing *0.05%* NP-40. Then the blotted sheets were incubated in FITC-conjugated anti-rabbit IgG at 0.3 mg/ml in IBIB buffer containing 0.05% NP-40. After incubation for 6 hrs at room temperature the blot was washed with IBIB buffer devoid of bovine serum albumin and photographed with a camera under long-wave UV light with an yellow filter.

Single Radial Immunodiffusion: Agarose (1.5%) was dissolved in 0.9% NaCl and 0.019 M Na-barbital buffer pH 8.4, by heating in a boiling water bath for 45 min. When the temperature of agarose solution reduced to 50°C, antiserum was added to 5% (to final conc.) and mixed thoroughly. This was poured into immunodiffusion plates and allowed to cool at room temperature. After cutting the wells, the protein was placed in the wells. The diffusion was allowed to take place for 6-12 hrs at room temperature and the plates were observed for circular precipitation rings around the well. These were photographed against dark background with scattering light.

The amount of the protein was calculated by the formula:

$$\text{ug of protein} = (\text{Precipitation ring diameter})^2 - (\text{well diarneter})^2$$

Molecular weight determination; Purified proteins (5 ug each) were electrophoresed in 18% polyacrylamide gels in the presence of SDS (Thomas and Kornberg, 1975). Standard proteins used were BSA 68000; ovalbumin 43000; chymotrypsinogen 25600; myoglobin 17200; cytochrome C 11900; and E. coli NS 9300.

Gel filtration on Sephacryl S-200: The chromatography was performed at 40°C. The protein solutions (100 ug each) were passed through sephacryl S-200 (1.2 cm x 71 cm) column equilibrated with 10 mM Tris-HCl pH 7.6 and 500 mM NaCl. The column was eluted with the equilibrating buffer and 0.5 ml fractions were collected and analyzed by 18% polyacrylamide **gel** electrophoresis.

Cross linking of proteins with dimethyl suberimidate: The purified nucleoid proteins were freed of salt by precipitation with 5 volumes of acetone and the protein pellet was dissolved in 0.1 M triethanolamine-HCl pH 8.5 and was allowed to react with 6 mg/ml dimethyl suberimidate at room temperature, 50°C and 75°C. Protein concentrations were in the range of 4 to 8 mg/ml in a final volume of 8 ul. After 4 1/2 hrs of reaction the

cross linked products were analyzed by 18% polyacrylamide gel electrophoresis in the presence of SDS.

Amino acid analysis: Purified protein (each 40 ug) was hydrolyzed with 6 N HC1 at 110°C for 72 hrs in evacuated tube and hydrolysates were analyzed in a LKB automatic amino acid analyzer.

Absorption and fluorescence spectra: The absorption and fluorescence spectra of the four purified nucleoid proteins were recorded at 25°C in Hitachi spectrophotometer and Hitachi spectrofluorimeter. The protein solutions were in 1 mM Tris-HCl pH 7.4 and *50* mM NaCl.

Circular dichroism: Circular dichroism spectra of the purified nucleoid proteins (in 1 mM Tris-HCl pH 7.4, 20 mM NaCl) were recorded in a Jasco-20 scanning spectropolarimeter. The instrument was calibrated using an aqueous solution of d-10 camphorsulfonic acid. The spectropolarimeter was continuously purged with dry 99.8% nitrogen before and during the experiment. Cell of 0.1 cm path length was used. All CD spectral data were plotted as mean residue ellipticity (0) using the formula:

$$\text{Molar ellipticity } (\Theta)_M = \frac{(\Theta) \text{ obs (in degrees x 100 x MRW)}}{c \times l}$$

where Θ obs = ellipticity observed in degrees, c = concentration of protein sample in grams/ml, 1 = path length in cm, MRW = mean residue molecular weight.

The units $[\Theta]$ are deg cm^2 $decimole^{-1}$.

Fourth derivative spectra: Fourth derivative spectra of the purified proteins (in 1 mM Tris-HCl pH 7.4, 20 mM NaCl) were recorded in a Hitachi dual wavelength double beam spectrophotometer (model 557) at 25°C.

RESULTS

Immunological studies: Antibodies to individual purified nucleoid proteins (HSNP-A, DBNP-B, HSNP-C and HSNP-C') have been raised in rabbits

as described in methods. Ouchterlony immunodiffusion tests were performed to test cross-reaction of the antibody to one protein with the other three proteins in order to see any cross-reaction. The results presented in figure 3.1 show that each antibody cross reacted with its corresponding antigen and did not cross-react with the other three proteins indicating that the four nucleoid proteins are antigenically distinct. Essentially similar results were obtained with increasing amounts of antigens and antibodies. However, interesting results were obtained when the cross-reactivity of the antibodies were tested after electroimmunoblotting. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed with individual purified proteins and mixture of the four proteins. After electrophoresis the proteins were electrophoretically transferred from gel to nitrocellulose membrane. The blotted nitrocellulose sheets were treated individually with antisera to the four proteins. Cross reactivity was detected with the help of FITC-labelled anti-rabbit IgG (see Methods for details). Anti HSNP-A and anti DBNP-B reacted with the corresponding antigens (Fig. 3.2 lanes 1 and 3) and did not cross react with the remaining three proteins (lanes 2 and 4). However antibodies to HSNP-C and HSNP-C not only cross reacted with their corresponding antigens but also showed cross reaction with two other proteins. HSNP-C antibodies showed strong cross reaction with DBNP-B and HSNP-C apart from HSNP-C (Fig. 3.2, lanes 5 and 6). HSNP-C antibodies also showed similar cross reaction with other proteins. But in this case cross reaction was noticed with HSNP-A and HSNP-C apart from HSNP-C (Fig. 3.2, lanes 7 and 8). Inability to detect cross reaction in immunodiffusion may be due to formation of soluble complexes of anti HSNP-C and anti HSNP-C with **DBNP-B and HSNP-C and HSNP-A** and HSNP-C respectively (see Discussion).

Fig. 3.1 Ouchterlony immunodiffusion test for the purified nucieoid proteins. The central wells contained 20 ul each of antiserum to HSNP-A (top left), DBNP-B (top right), HSNP-C (bottom left) and HSNP-C' (bottom right). Wells 1-4 contained 5 ug each of HSNP-A, DBNP-B, HSNP-C and HSNP-C respectively.

Fig. 3.2 Immunoblotting of individual purified proteins and mixture of purified nucleoid proteins using antibodies to purified proteins. In each case 5 ug of purified protein and 20 ug of mixture of purified nucleoid proteins were electrophoresed using 18% gel slabs and immunoblotting was performed as described in Methods section.

Lane 1, HSNP-A; lanes 2,4,6 and 8 mixture of purified nucleoid proteins; lane 3, DBNP-B; lane 5, HSNP-C; lane 7, HSNP-C.

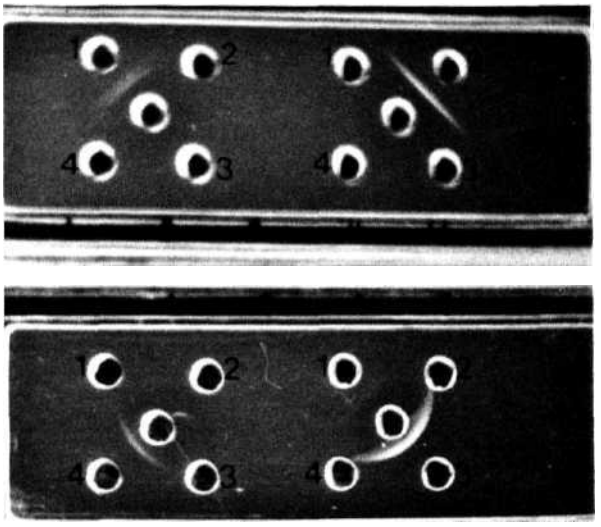


Fig.3.1

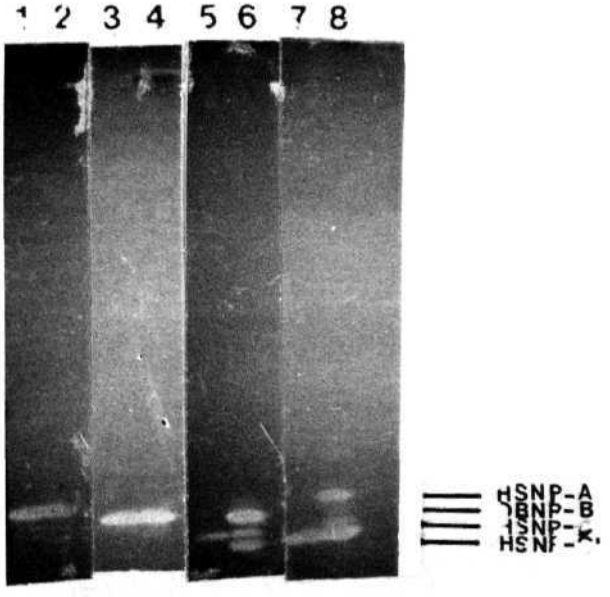


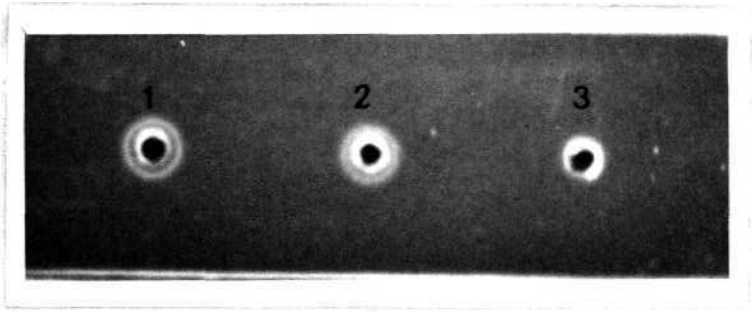
Fig.3.2

Quantitation of the histone-like proteins in different cell fractions: The amounts of these proteins in cell extract, post-ribosomal supernatant (S-100) and 1 M NH_4Cl wash of the ribosomes were determined with the help of single radial immunodiffusion (SRID). The cell fractions were obtained from DNase treated cell extracts. The results presented in Figure 3.3 A, C, E and G show that all the four proteins present in the cell extracts are distributed between S-100 and NH_4Cl wash to different extents. The amounts of these proteins in the three fractions has been quantitated by performing single radial immunodiffusion with the known concentrations of the pure proteins individually (Fig. 3.3 B, D, F and H and Table 3.1). In the cell extract HSNP-A was present at a concentration of 260 ug/ml corresponding to about 800 ug of this protein per g of cells (wet weight). This amount was distributed between S-100 and NH_4Cl wash in the ratio 2:1. The concentration of DBNP-B, HSNP-C and HSNP-C' in cell extracts was found to be 1100 ug, 600 ug and 1200 ug respectively. The distribution of DBNP-B between S-100 and NH_4Cl was found to be equal. In the case of HSNP-C more of the protein was present in post-ribosomal supernatant (three times than in NH_4Cl wash). Very small amount of (about 10 per cent) HSNP-C was detected in NH_4Cl wash (see also Figure 3.4 A to D). These results point out that the affinities of the proteins to bind to ribosomes is different.

Presence of these proteins in isolated nucleoid was also demonstrated by immunodiffusion test. The Figure 3.5 shows cross reaction of nucleoid with antisera to HSNP-A, DBNP-B, HSNP-C and HSNP-C indicating their presence in the nucleoid.

Molecular weight determination: Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed to determine the denatured molecular weight of these proteins. The mobilities of the four proteins were compared

Fig. 3.3 Single radial immunodiffusion (SRID) for the estimation of HSNP-A, DBNP-B, HSNP-C, and HSNP-C. Cell extract, Post-ribosomal supernatant and 1 M NH_4Cl ribosomal wash were isolated as described in Methods section of Chapter II. Immunodiffusion plates were prepared with agar containing antibodies to four purified proteins. Gels in A and B contained antiserum to HSNP-A; C and D contained antiserum to DBNP-B; E and F contained antiserum to HSNP-C; G and H contained antiserum to HSNP-C. The wells 1 to 3 of gels in A, E and G contained 10 ul each of cell extract, S-100 and 1 M NH_4Cl wash respectively. The gel in C contained 12 ul each of cell extract, S-100 and 1 M NH_4Cl wash in the wells marked 1 to 3. The wells 1 to 5 in B, D, F and H contained 1 ug, 2 ug, 4 ug, 6 ug and 8 ug of purified HSNP-A (B); DBNP-B (D); HSNP-C (F) and HSNP-C (H)



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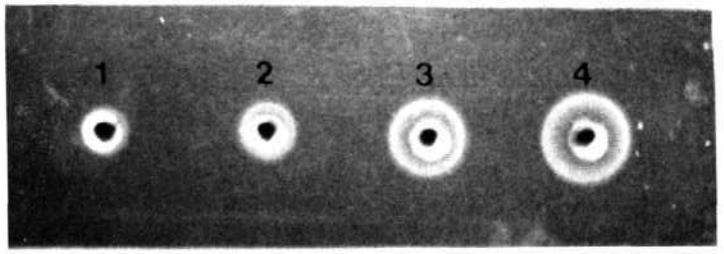

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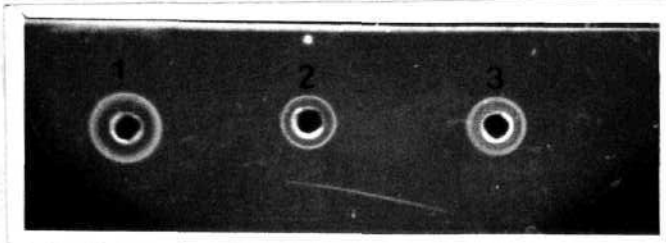

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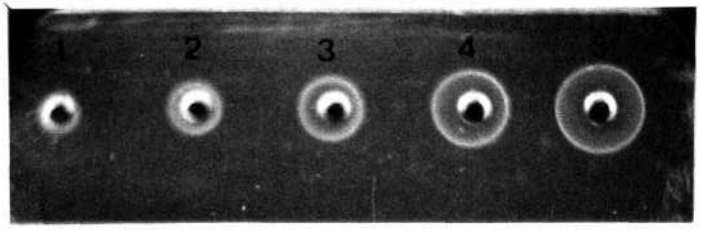

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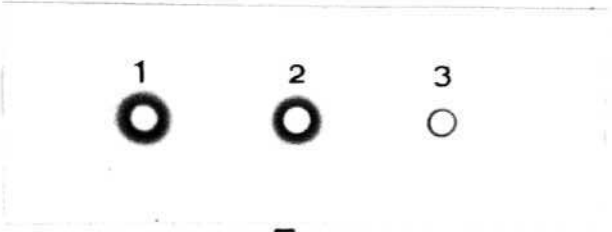
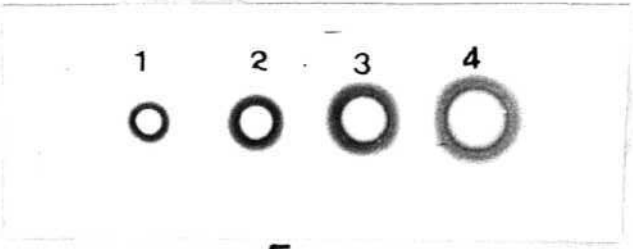
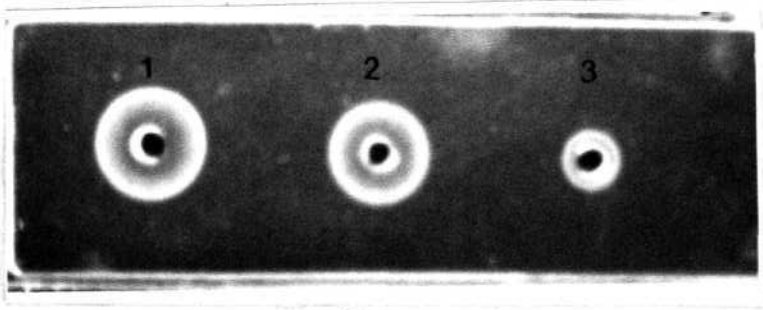


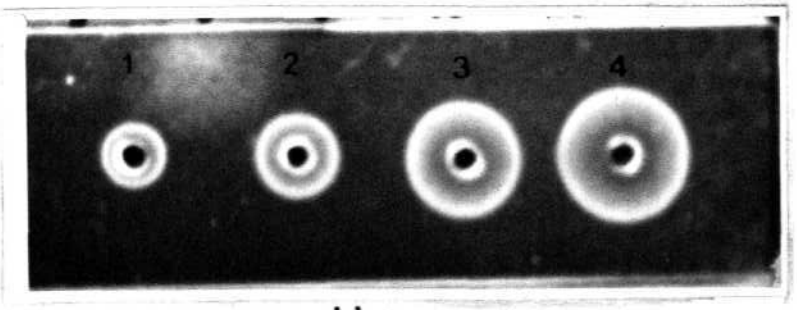
E



F



G



H

Fig·3·3

Fig. 3.4 Calibration curve for estimation of purified nucleoid proteins in cell extract ▲, Post-ribosomal supernatant, 1 M NH_4Cl ribosomal wash ●.

(A) HSNP-A (B) DBNP-B (C) HSNP-C and (D) HSNP-C'. Open circles correspond to known amounts of purified proteins.

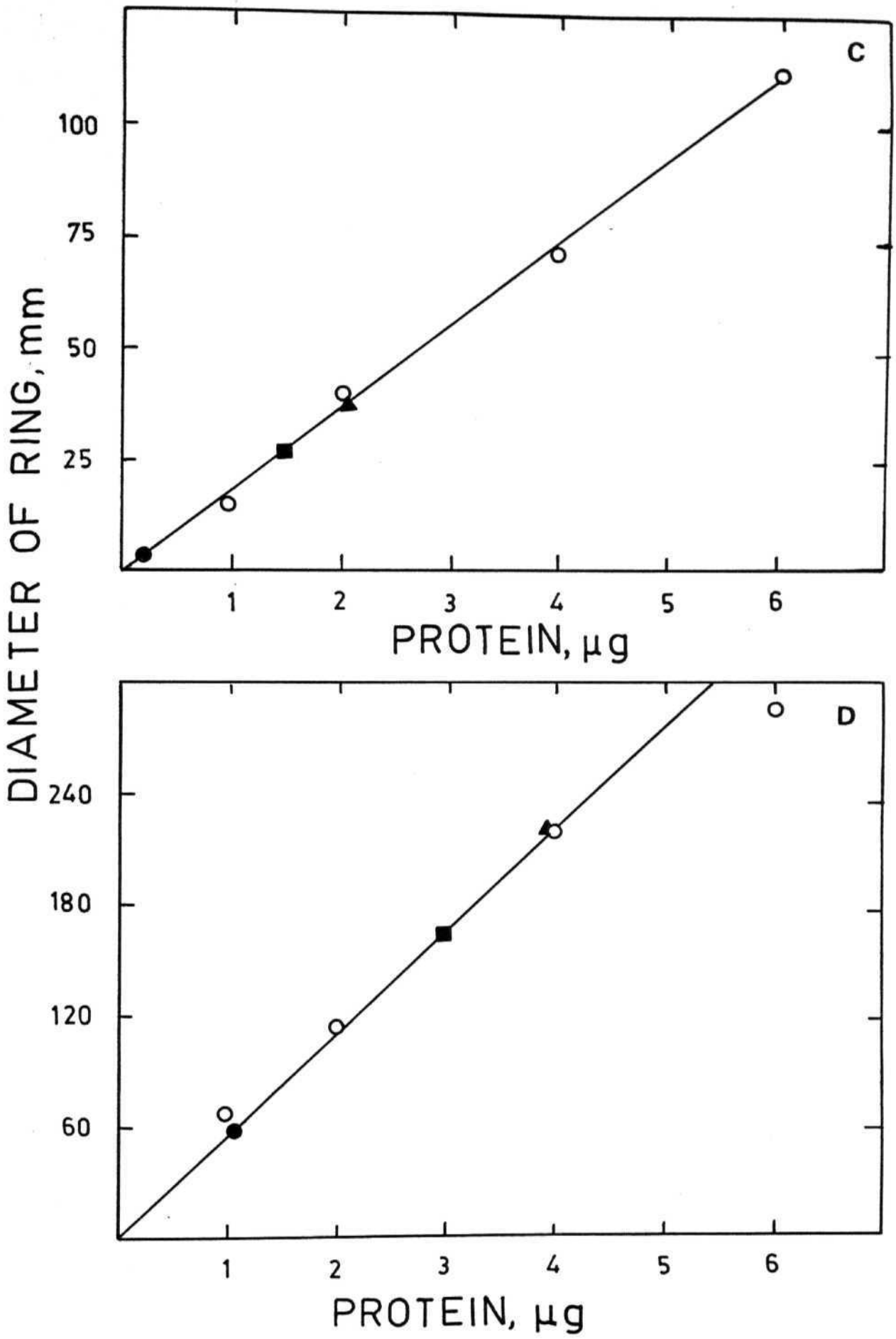

C
100
75
50
25
DIAMETER OF RING, mm
1
2
3
4
5
6
PROTEIN, µg
D
240
180
120
60
1
2
3
4
5
6
PROTEIN, µg

Fig.3.4

TABLE - 3.1

Estimation of nucleoid proteins in different cell fractions by single radial immunodiffusion

Cell fraction	Amount of Protein per gram of cells			
	HSNP-A	DBNP-B	HSNP-C	HSNP-C'
Cell extract (S-30)	0.8 mg	1.1 mg	0.6 mg	1.2 mg
Post-ribosomal supernatant (S-100)	0.50 mg	0.56 mg	0.45 mg	0.9 mg
1M NH_4Cl wash	0.30 mg	0.54 mg	0.06 mg	0.3 mg

Fig. 3.5 Ouchterlony immunodiffusion test for the nucleoid proteins.

The central well contained 120 ug protein of nucleoid fraction from sucrose gradient. Outer wells 1-4, contained 20 ul each of antiserum to HSNP-A, DBNP-B, HSNP-C and HSNP-C respectively.

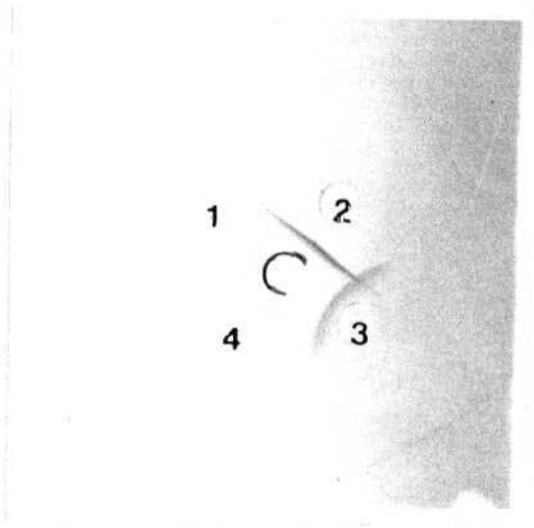


Fig.3.5

with those of the standard proteins. The plot in Figure 3.6 gave molecular weights of 12000; 11500; 10500; and 9000 for HSNP-A, DBNP-B, HSNP-C and HSNP-C respectively (Figure 2.11).

Aggregation properties of histone-like proteins; In order to determine the native molecular weight as well as to see whether these proteins exist in solution as aggregates, we performed gel filtration and cross linking studies. Purified proteins in 10 mM Tris-HCl pH 7.6 and *500* mM NaCl buffer were individually passed through Sephacryl S-200 column equilibrated with the above buffer and eluted with the same buffer. The elution volume for each protein was determined after SDS-polyacrylamide gel electrophoresis of the column fractions, HSNP-A, DBNP-B and HSNP-C were eluted with elution volumes of *55.5* ml, 56.5 ml and *57.5* ml corresponding to molecular weights of 25000; 23000 and 20000 respectively indicating dimeric aggregation behaviour of HSNP-A, DBNP-B and HSNP-C in solution (Fig. 3.7). A small fraction of these proteins (15 to 25%) also eluted at volumes corresponding to their monomer molecular weight. However HSNP-C eluted only at elution volume corresponding to its monomeric molecular weight. Cross linking experiments were performed by treating the protein with bifunctional cross-linking agent dimethyl suberimidate asdescribed in Methods at different temperatures. Cross-linked complexes were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 3.8 A to D). In the cases of HSNP-A, DBNP-B and HSNP-C additional band due to the cross linked protein were noticed with molecular weight corresponding to dimers. The cross linked complexes were found to be formed to a greater extent at elevated temperatures (50°C to 75°C). However in the case of HSNP-C we did not detect any cross-linked complex indicating that HSNP-C probably does not dimerize.

Fig. 3.6 Estimation of monomer molecular weights *oi* purified proteins by SDS-polyacrylamide slab gel electrophoresis. Data was obtained from co-electrophoresis of the purified proteins with standard proteins (1) Bovine serum albumin (68000); (2) Ovalbumin (43000); (3) Chymotrypsinogen (25600); (4) Myoglobin (17200) and (5) Cytochrome-C (11900).

HSNP-A, • ; DBNP-B, ▲ ; HSNP-C, • ; HSNP-C', □.

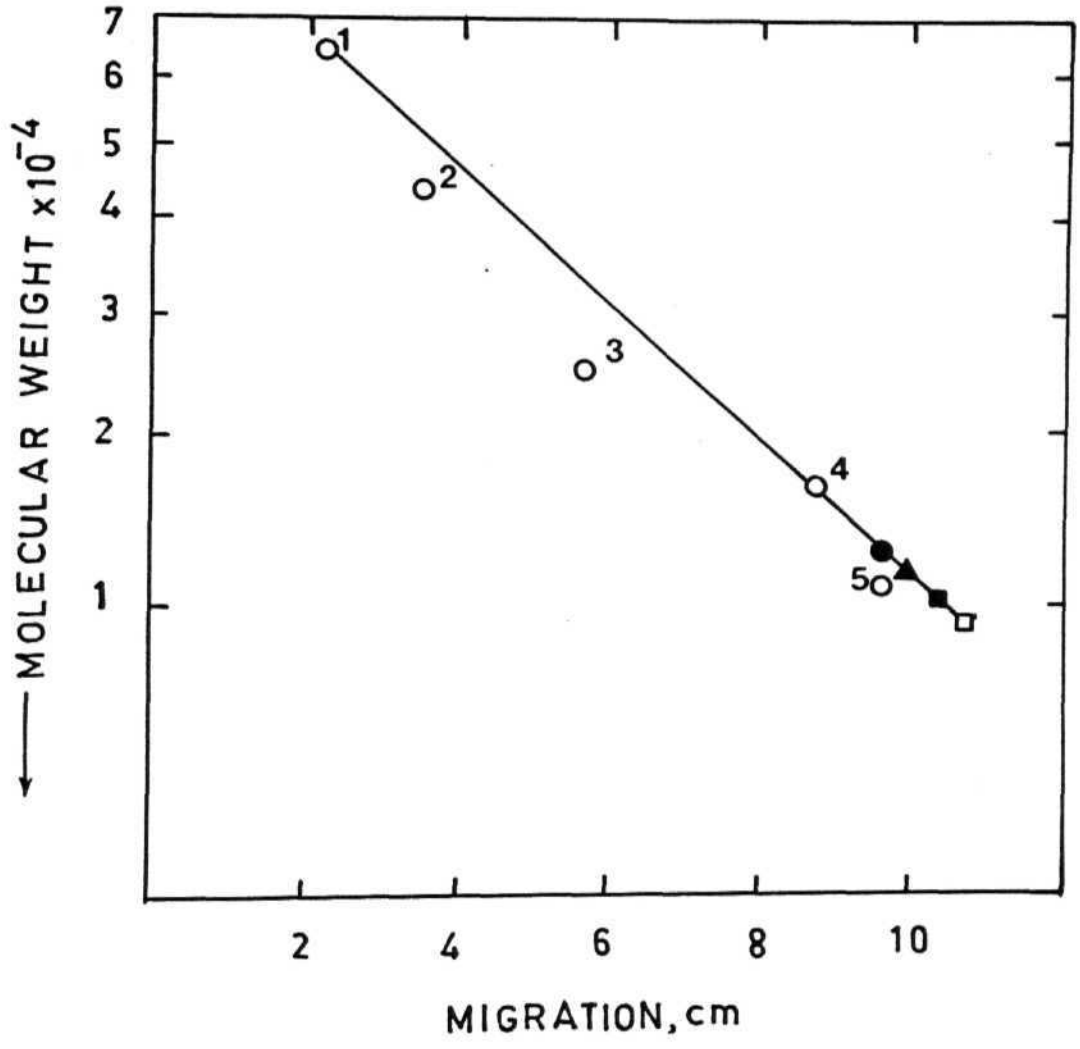


Fig. 3.6

Fig. 3.7 Native molecular weights of purified proteins. Data was obtained from co-chromatography of the purified proteins on a Sephacryl S-200 column with standard proteins (same as described in legends to Fig. 5.6). The elution volumes were determined afterSDS-polyacrylamide gel electrophoresis of the column fractions. Elution volumes of (1) Bovine serum albumin, 44 ml, (2) Ovalbumin 48 ml, (3) Myoglobin, 59 ml are indicated.

HSNP-A, # ; DBNP-B, ▲ ; HSNP-C, • ; HSNP-C', □.

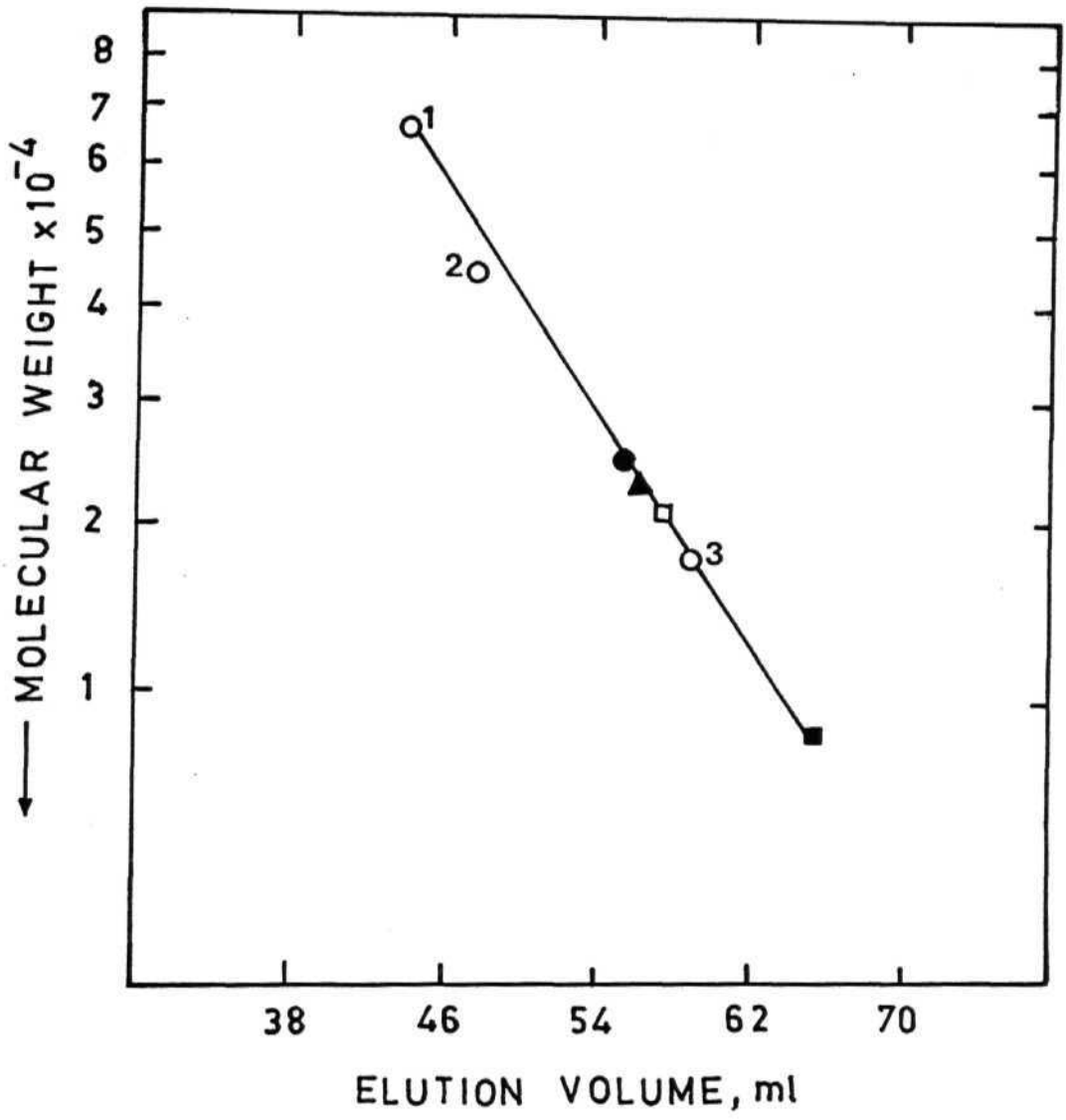

MOLECULAR WEIGHT x10⁻⁴
8
7
6
5
4
3
2
1
1
2
3
38
46
54
62
70
ELUTION VOLUME, ml

Fig.3.7

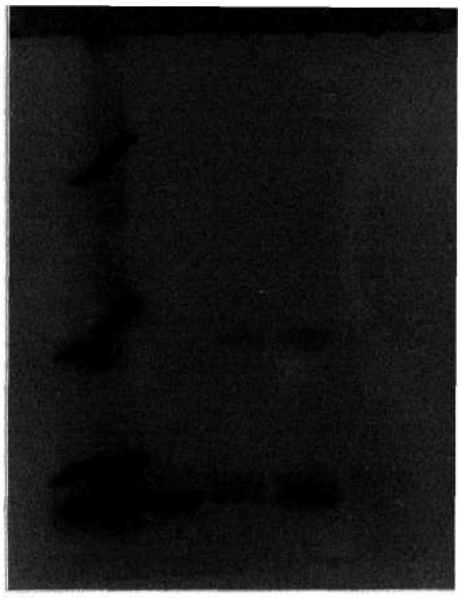
Fig. 3.8 SDS-polyacrylamide gel electrophoretic analysis of HSNP-A, DBNP-B, HSNP-C and HSNP-C' after cross-linking with N,N-Dimethyl suberimidate.

(A) Lane 1, Molecular weight standards (as in Fig. 2.6); lane 2, HSNP-A without cross-linking; lanes 3, 4 and 5, HSNP-A after cross-linking at room temperature, 50°C and 75°C respectively.

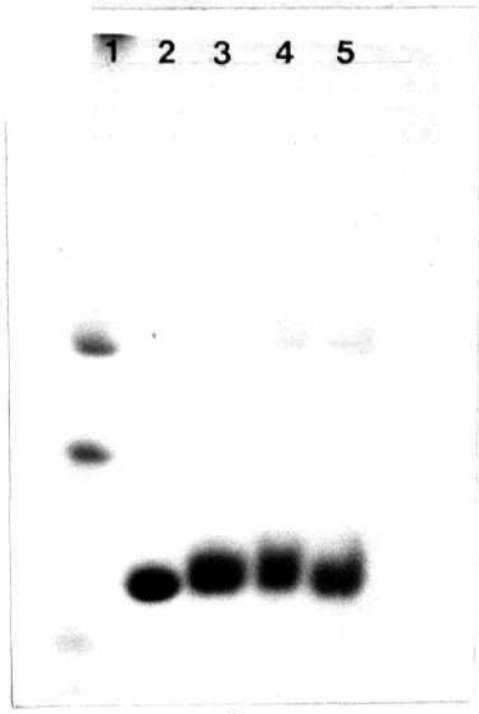
(B) Lane 1, Molecular weight standards (as in Fig. 2.6); lane 2, DBNP-B without cross-linking; lane 3, 4 and 5, DBNP-B after cross linking at room temperature, 50°C and 75°C respectively.

(C) Lane 1, Molecular weight standards (as in Fig. 2.6); lane 2, HSNP-C without cross linking; lane 3, 4 and 5, HSNP-C after cross linking at room temperature, 50°C and 75°C respectively.

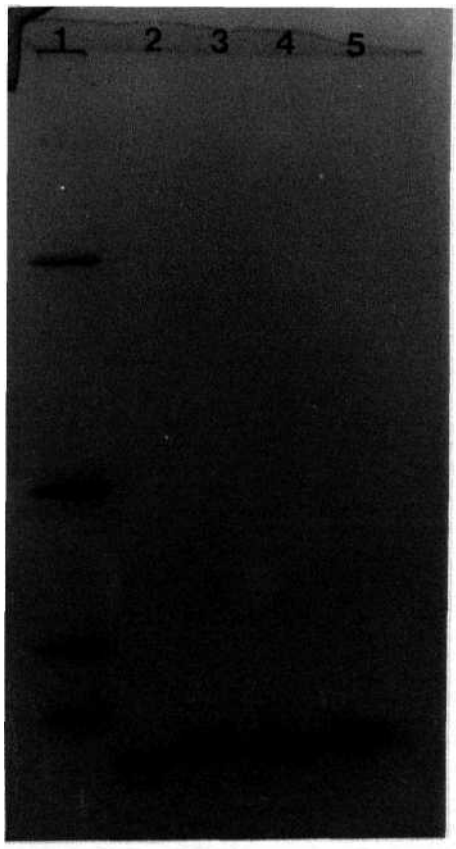
(D) Lane 1, Molecular weight standards (as in Fig. 2.6); lane 2, HSNP-C without cross-linking; lane 3, 4 and 5, HSNP-C after cross-linking at room temperature, 50°C and 75°C respectively.



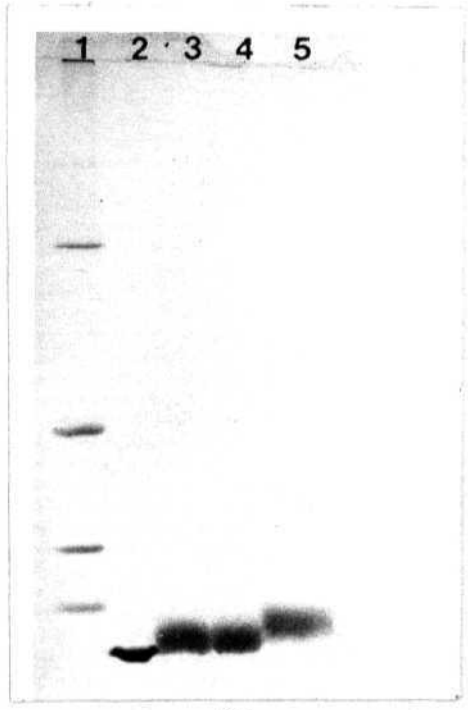
A



B



C



D

Fig 3. 8

Ultraviolet light absorption spectra of the proteins: The U.V. absorption spectra and fourth derivative spectra of the purified proteins were recorded at 25°C as described in Materials and Methods section. All the four proteins gave absorbance peak in the range 260 to 285 nm (Fig. 3.9). HSNP-A and HSNP-C showed absorbance peak in the range 260 nm to 278 nm. DBNP-B and HSNP-C had absorbance peaks in the range from 270 nm to 286 nm. The contributions of aromatic amino acids to U.V. absorption in proteins can be separated with the help of fourth derivative spectrophotometry (Padros et ah, 1984). The fourth derivative spectrum of HSNP-A (Fig. 3.10 A) showed contribution of all the three aromatic amino acids to absorption. In the case of DBNP-B the characteristic tryptophan minimum around 290 nm is missing (Fig. 3.10 B). The spectra of HSNP-C and HSNP-C showed contribution of all the three amino acids to different extents (See Discussion).

Intrinsic fluorescence emission spectra: Fluorescence excitation and emission spectra are recorded for the four purified proteins. The fluorescence emission spectra of HSNP-A, HSNP-C and HSNP-C (Fig. 3.11 B, F and H) are characteristic of the tryptophan emission spectra (max. at 345-348 nm) when excited at 285 nm. In the case of DBNP-B a single fluorescence emission spectrum with a maximum at 302 nm was obtained indicating the absence of tryptophan (Fig. 3.11 D). Both the proteins HSNP-A and HSNP-C gave two kinds of emission spectra depending on the excitation wavelength. Upon excitation at 274 nm the emission spectra showed strong contribution by tyrosine (Fig. 3.11 B and F). This contribution of tyrosine is not visible when excited at 285 nm. HSNP-C showed fluorescence emission spectra characteristic of tryptophan emission. The excitation spectra of the proteins reflect the strong contribution by tyrosine/tryptophan.

Fig. 3.9 U.V. absorption spectra of purified nucleoid proteins. U.V. absorption spectra of proteins was recorded. The protein solutions were in buffer containing 1 mM Tris-HCl, pH 7.4 and 50 mM NaCl.

(A) HSNP-A, 100 ug/ml; (B) DBNP-B, 500 ug/ml; (C) HSNP-C, 75 ug/ml and (D) HSNP-C', 300 ug/ml.

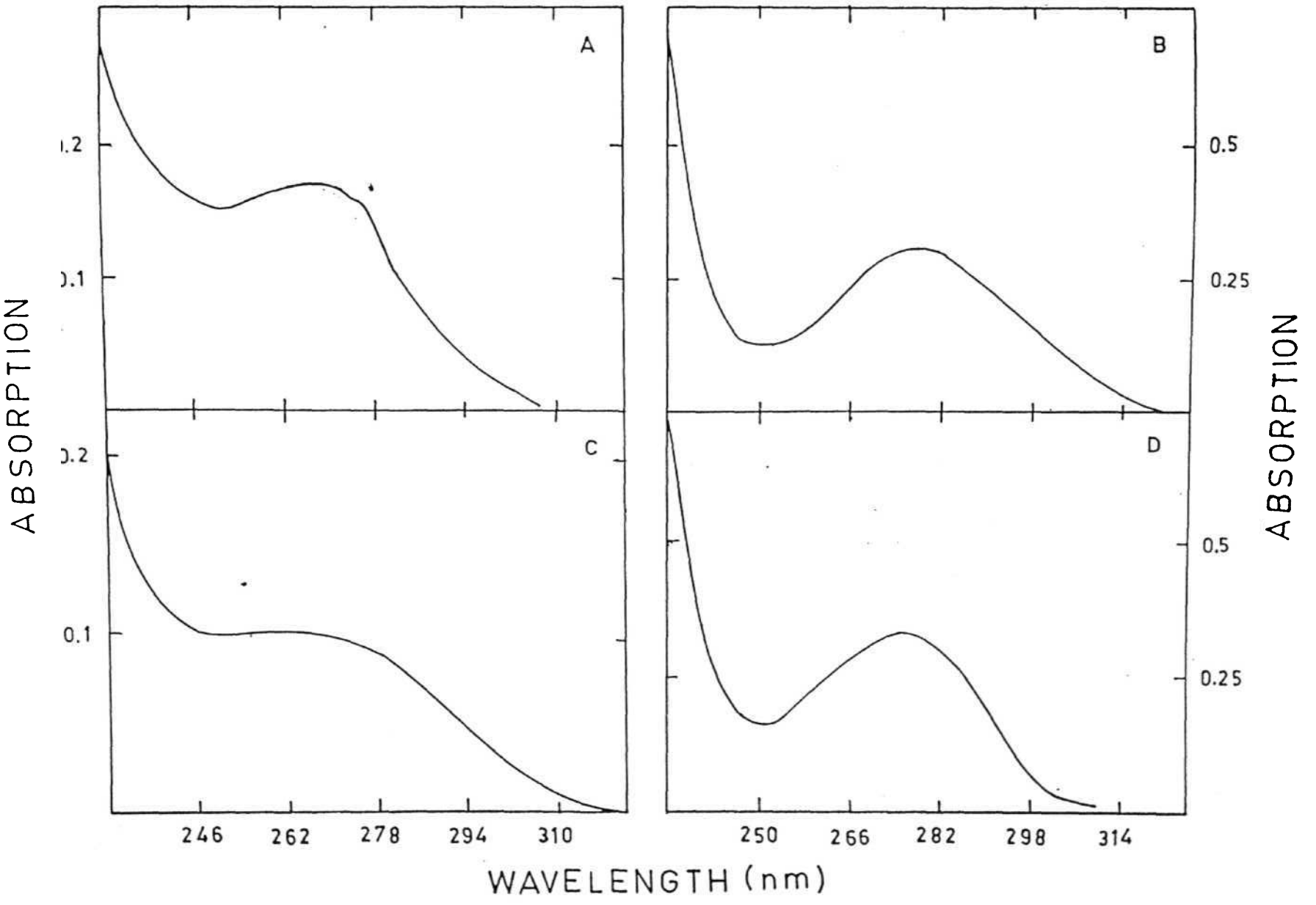

A
B
C
D
ABSORPTION
ABSORPTION
0.2
0.1
0.2
0.1
0.5
0.25
0.5
0.25
246
262
278
294
310
250
266
282
298
314
WAVELENGTH (nm)

Fig.3·9

Fig. 3.10 Fourth derivative spectra of purified nucleoid proteins. Fourth derivative spectra of proteins was recorded as described in the text.

(A) H5NP-A, 100 ug/ml; (B) DBNP-B, 500 ug/ml; (C) HSNP-C, 75 ug/ml and (D) HSNP-C', 300 ug/ml.

Fig. 3.11 Fluorescence spectra of purified nucleoid proteins. Excitation and emission spectra of these proteins were recorded in Hitachi spectrofluorimeter as described in the text. 50 ug/ml each of HSNP-A, DBNP-B, HSNP-C and HSNP-C' were in the buffer 1 mM Tris-HC1 pH 7.4 and 50 mM NaCl.

(A) Excitation spectrum of HSNP-A.
Emission at 302 nm, •; Emission at 350 nm, A.

(B) Emission spectrum of HSNP-A
Excitation at 274 nm, Δ; Excitation at 285nm, O.

(C) Excitation spectrum of DBNP-B.
Emission at 302 nm, #.

(D) Emission spectrum of DBNP-B
Excitation at 274 nm, O.

(E) Excitation spectrum of HSNP-C
Emission at 302 nm, ●; Emission at 350 nm, ▲.

(F) Emission spectrum of HSNP-C
Excitation at 274 nm, A ; Excitation at 285 nm, O.

(G) Excitation spectrum of HSNP-C
Emission at 350 nm, ●.

(H) Emission spectrum of HSNP-C[1]
Excitation at 285 nm, O.

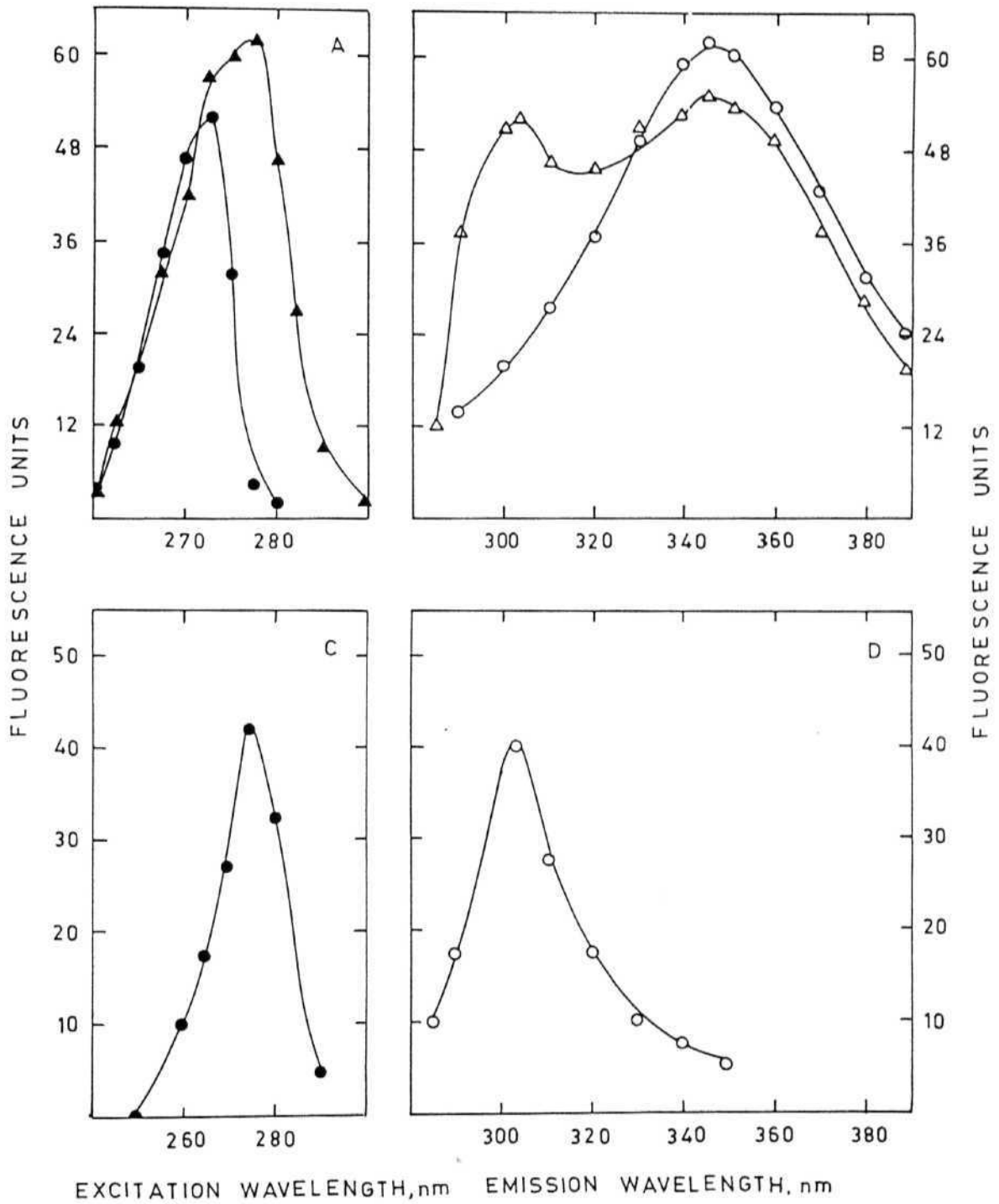

A
B
C
D
FLUORESCENCE UNITS
FLUORESCENCE UNITS
EXCITATION WAVELENGTH, nm
EMISSION WAVELENGTH, nm
60
48
36
24
12
50
40
30
20
10
270
280
300
320
340
360
380
260

Fig. 3.11

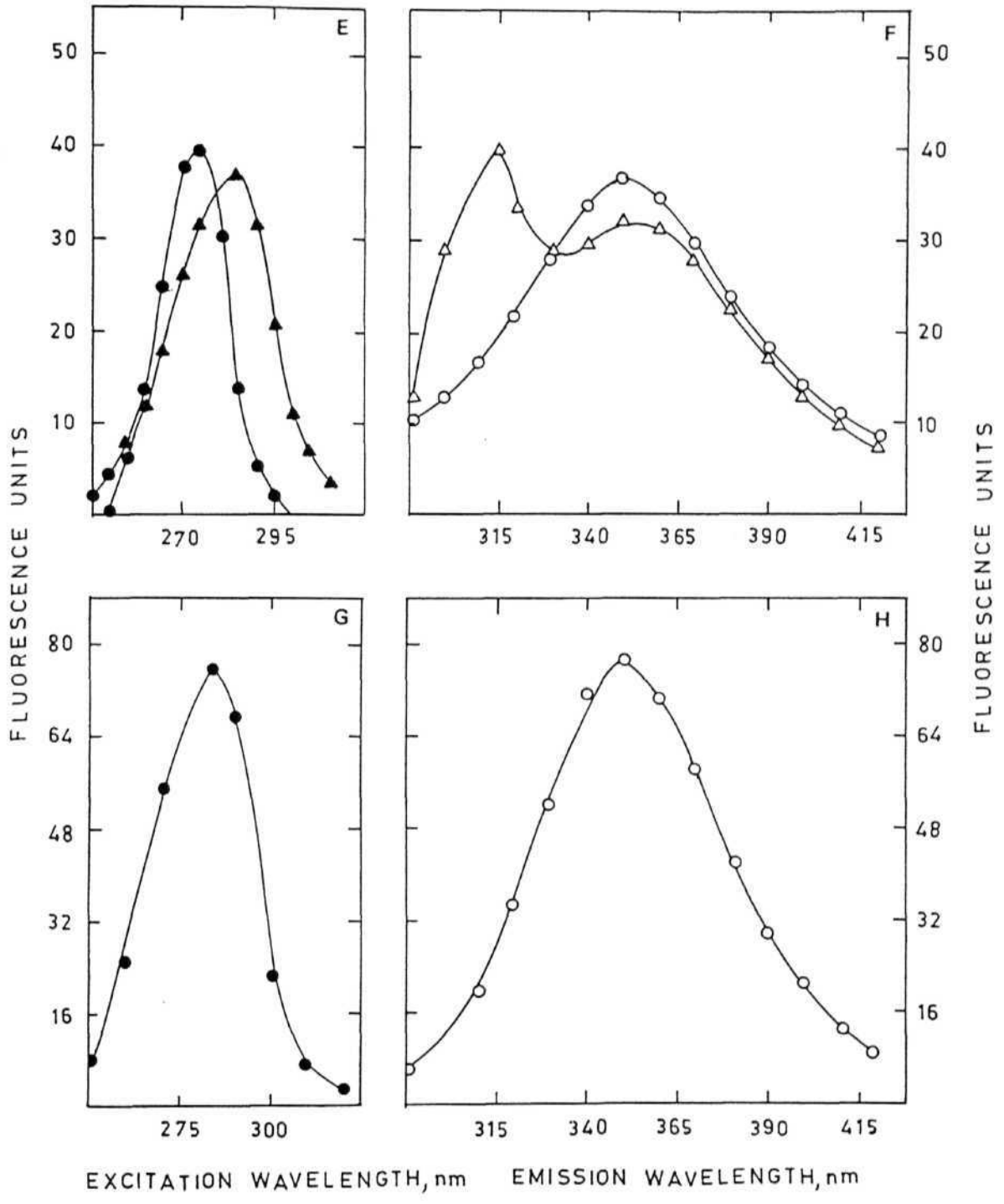

E
F
G
H
50
40
30
20
10
80
64
48
32
16
270
295
315
340
365
390
415
275
300
FLUORESCENCE UNITS
FLUORESCENCE UNITS
EXCITATION WAVELENGTH, nm
EMISSION WAVELENGTH, nm

Fig.3.11

Circular dichroic spectra of the proteins: DNA binding protein II (NS) has a well defined secondary structure. The CD spectra of the DNA binding proteins from S. acidocaldarius were recorded in a Jasco spectropolarimeter at 25°C as described in Materials and Methods section. HSNP-A, DBNP-B and HSNP-C' have CD spectra suggestive of presence of moderate amount of α-helix (Fig. 3.12 A, B and D). HSNP-A showed the two characteristic minima at 208 nm and 224 nm for a protein with dominating cc-helical structure and the spectrum resembles that of protein NS from E. coli (Dijk and Reinhardt, 1986). However quantitation of the amount of alpha helix by different methods gave low values (see Table 3.2). Calculation showed that the protein DBNP-B has highest content of ordered structure (Table 3.3). The CD spectrum of HSNP-C, after calculation as per Chen et al (1974), gave a value of 25% α-helix, 22% ß-sheet structure for this protein (Table 3.3). Protein HSNP-C has a CD spectrum with a rather unusual shape and a very low signal intensity and it shows some resemblance of fd gene 5 protein (Day, 1973).

Amino acid composition of the nucleoid proteins: The amino acid composition of all the four proteins has been determined and is given in Table 3.4. For comparison the amino acid composition of the calf thymus histones is also given (Table 3.5). The amino acid composition of the histone-like DNA binding proteins isolated from E. coli and some archaebacteria are also presented in Table 3.6. The amino acid composition of the nucleoid proteins (Table 3.4) shows preponderance of basic amino acids. The mol % of basic amino acids is in the range of 20-30%. One striking aspect in the composition is the absence of methionine in all the four proteins. Proline is either absent (HSNP-A and HSNP-C) or is present only in very small amounts (DBNP-B and HSNP-C). All the four proteins contain phenylalanine and tyrosine. Fluorescence emission spectra indicate the presence of tryptophan also in **HSNP-A, HSNP-C** and **HSNP-C. However, DBNP-B does not contain tryptophan.**

Fig. 3.12 CD. spectra of purified nucleoid proteins

CD. spectra of the proteins were recorded in 1 mM Tris-HCl pH 7.4 and 20 mM NaCl using 0.1 cm path length cuvette.

(A) HSNP-A, 100 ug/ml; (B) DBNP-B, 500 ug/ml; (C) HSNP-C, 75 ug/ml and (D) HSNP-C', 300 ug/ml.

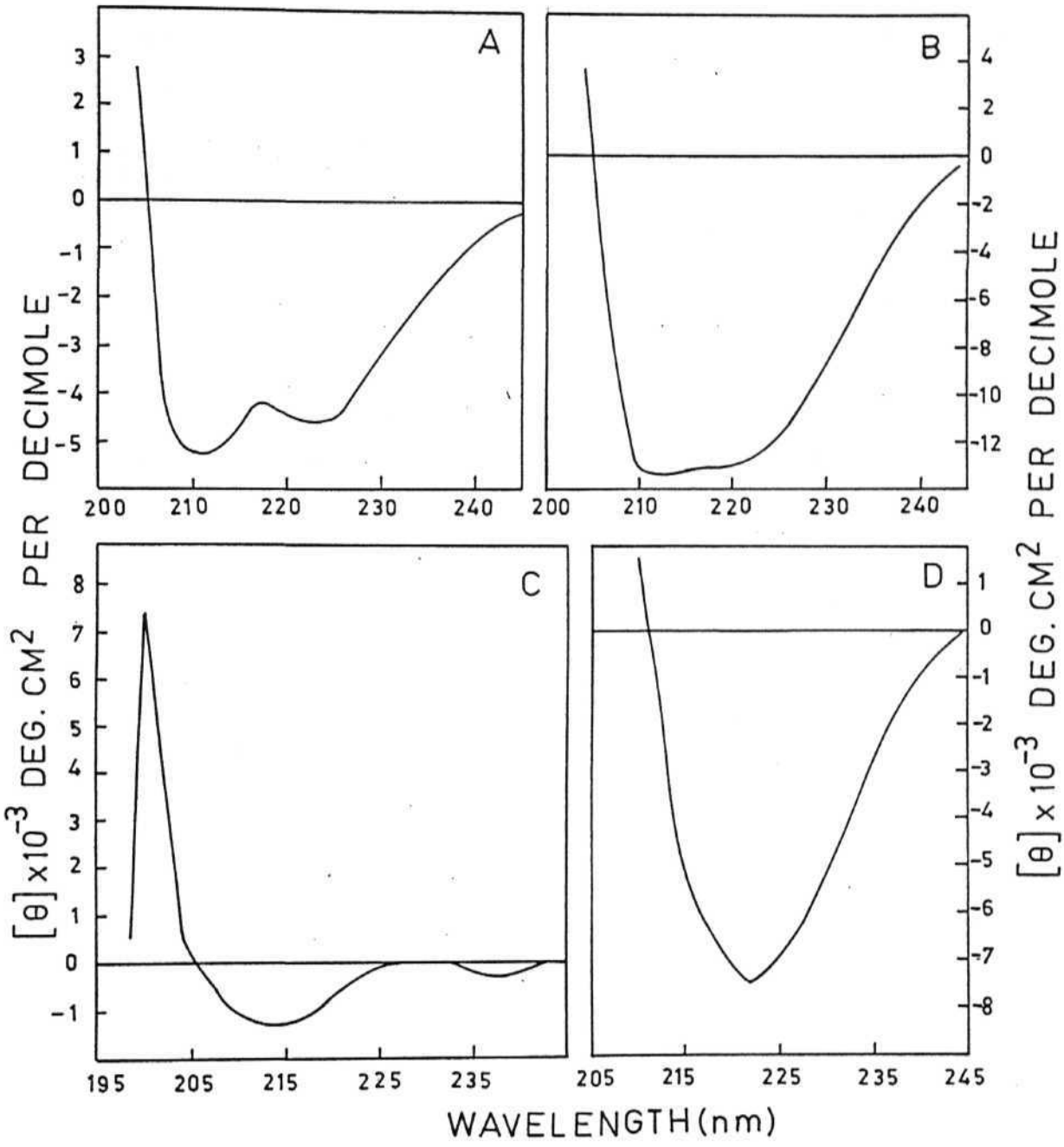


Fig. 3.12

TABLE - 3.2

Helicity of the nucleoid proteins obtained by evaluation of the CD spectra

Protein	Helix[a] (%)	Helix[b] (%)
HSNP-A	7	4
DBNP-B	34	20
HSNP-C	9	negative value
HSNP-C'	17	negative value

a) Calculated according to Chen et al (1972) based on the molar residue ellipticities of the proteins at 222 nm $[\Theta]_{222}$ as per the equation $[\Theta]_{222}$ = -30,300 fH-2340.

b) Calculated according to Greenfield and Fasman (1969) based on the molar ellipticities of the proteins at 208 nm, $[\Theta]_{208}$ nm.

TABLE - 3.3

The distribution of secondary structure in the nucleoid proteins obtained by evaluation of CD spectra

Protein	α-helix (%)	β sheet (%)	Remainder (%)
HSNP-A	17	7	76
DBNP-B	43	23	34
HSNP-C	2	13	85
HSNP-C'	25	22	53

(a) The calculations were according to Chen et al (1974) based on the values of fH, fβ and fR for five reference proteins (Yang, 1974). The equations in the form $[\Theta]_\lambda$ = fH.xH + fB.xB + fR.xR were solved for f values in the wavelength range from 225 nm to 240 nm simultaneously by Gauss-Seidel method. The xH, xB and xR are the reference values that would be obtained if the protein molecule were made up of segments of pure helix (H), B sheet (β) and unordered or remainder forms (R). The fs are the fractions of the helix, β and remainder forms of a protein molecule which is obtained from the CD-spectrum of the protein molecule. In the table fs were expressed as % values.

TABLE - 3.4

Amino acid composition of **S.** acidocaldarius nucleoid proteins

Amino acid residue	Protein			
	HSNP-A (mol%)	DBNP-B (mol %)	HSNP-C (mol %)	HSNP-C' (mol %)
Asx	11.7	7.5	5.0	9.3
Thr	6.2	6.2	3.7	4.5
Ser	3.9	7.4	2.5	3.9
Glx	13.7	10.6	10.6	12.9
Pro	0.0	0.2	0.5	0.0
Gly	8.4	8.7	13.1	9.7
Ala	7.0	8.2	2.5	7.2
Val	3.9	13.4	10.1	11.1
Met	0.0	0.0	0.0	0.0
He	6.2	9.9	1.7	1.6
Leu	12.6	8.0	11.2	5.5
Tyr	1.7	0.2	0.5	0.9
Phe	1.0	1.2	4.3	3.4
His	1.0	0.0	1.7	0.0
Lys	19.2	9.9	23.8	23.4
Are	3.6	8.8	9.0	6.5
Total	100.0	100.2	100.2	99.9
% Basic amino acids	23.8	18.7	34.5	29.9
% Asx + Glx	17.9	13.7	8.7	13.8
Lys/Arg	5.3	1.1	2.6	3.6

Asx = Asn + Asp

Glx = Gln + Glu

TABLE - 3.5

Amino acid composition of calf thymus histones*

Amino acid residue	Protein				
	H1 (mol %)	H2A (mol %)	H2B (mol %)	H3 (mol %)	H4 (mol %)
Asx	2.5	6.2	4.8	4.2	5.2
Thr	5.6	3.9	6.4	6.8	6.3
Ser	5.6	3.1	11.2	3.6	2.2
Glx	3.7	9.3	8.0	11.6	6.9
Pro	9.2	3.9	4.8	4.6	1.5
Gly	7.2	10.9	5.6	5.4	14.9
Ala	24.3	13.2	10.4	13.3	7.7
Val	5.4	6.2	7.2	4.4	8.2
Met	0.0	0.0	1.6	1.1	1.0
Ile	1.5	4.7	4.8	5.3	5.7
Leu	4.5	12.4	4.8	9.1	8.2
Tyr	0.9	2.3	4.0	2.2	3.8
Phe	0.9	0.8	1.6	3.1	2.1
His	1.0	3.1	2.4	1.7	2.2
Lys	26.8	10.9	16.0	10.0	11.4
Arg	1.8	9.3	6.4	13.0	12.8
Total	99.9	100.2	100.0	99.4	100.1
% Basic amino acids	28.6	23.3	24.8	24.7	26.4
% Asx + Glx	6.2	9.3	12.8	15.8	12.1
Lys/Arg	14.9	1.2	2.5	0.8	0.9

*Data from Mayes and Johns (1982) and Rizzo et al., (1985)

TABLE - 3.6

Amino acid composition of histone-like proteins from prokaryotes

Amino acid residue	E. coli			Archaebacteria			
	NS1[a]	NS2[a]	H1[b]	HSa[c]	7KD[d]	HTa[e]	HMb[f]
	mol %	moJ %	mol %	mol %	mol %	mol %	mol %
Asx	9.5	8.0	9.0	9.3	9.7	5.6	10.5
Thr	5.5	7.1	a6.7	3.8	4.2	5.6	4.4
Ser	5.1	4.2	4.6	8.4	3.6	7.8	3.5
Glx	7.7	12.3	19.3	11.0	12.6	13.3	13.2
Pro	2.2	2.2	2.3	3.1	1.4	3.3	6.5
Gly	9.1	7.6	4.6	9.7	4.0	6.7	6.9
Ala	19.7	17.2	11.0	7.2	4.5	7.8	6.5
Val	8.4	9.0	4.8	4.6	9.7	8.9	7.1
Met	1.2	0.9	2.4	3.1	3.6	1.1	2.3
Ile	7.1	5.3	4.2	4.2	1.6	7.8	4.2
Leu	5.9	7.3	10.3	14.9	4.7	3.3	3.6
Tyr	0.0	0.0	2.0	2.9	4.5	1.1	0.0
Phe	3.1	3.0	0.8	1.8	4.1	5.6	4.2
His	0.0	0.9	0.0	0.2	0.0	0.0	2.2
Lys	10.1	11.5	8.9	10.4	23.2	15.6	16.0
Arg	5.6	3.6	8.1	5.4	8.7	6.7	9.0
Total	100.2	100.1	99.0	99.9	100.1	100.2	100.1
% Basic amino acids	15.7	16.0	17.0	16.0	31.9	22.3	27.2
% Asx + Glx	17.2	20.3	28.3	20.3	22.3	18.9	23.7
Lys/Arg	1.8	3.2	1.1	1.9	2.7	2.3	1.8

a) From Suryanarayana and Subramanian (1978).

b) From Laine and Sautiere (1984).

c) Protein HSa isolated from S. acidocaldarius DNA. protein complexes (Green et al., 1983). Tryptophan was excluded and mol.% recalculated.

d) 7KD protein isolated from Sulfolobus solfataricus. The data was calculated from the amino acid sequence. Tryptophan was not included (Kimura et ah, 1984).

e) Protein HTa (T. acidophilum) composition was calculated from the amino acid sequence (De Lange et al., 1981).

f) Chromosomal protein HMb from M. barkeri. The data from Chartier et al. was recalculated excluding tryptophan and the composition in mol % was given.

Histidine is present in low amounts in HSNP-A and HSNP-C and is absent from DBNP-B and HSNP-C[1]. The proteins are relatively rich in dicarboxylic amino acids, glycine, valine and leucine. The amino acid composition of these proteins show both similarities and differences with eukaryotic histones and prokaryotic histone-like proteins (see Discussion).

DISCUSSION

Immunochemical and physico-chemical properties of the purified nucleoid proteins have been studied. Antibodies to purified proteins have been used to study cross-reaction of each antiserum with the four proteins. Immunodiffusion results indicated that the four nucleoid proteins are antigenically distinct. However cross-reactions of antibodies to HSNP-C and HSNP-C[1] with other proteins was noted by electroimmunoblotting. HSNP-C antiserum cross-reacted with DBNP-B and HSNP-C apart from its cognate antigen. Similarly HSNP-C antiserum showed cross-reaction with proteins HSNP-A and HSNP-C. These results point out the formation of soluble antigen-antibody complexes of anti-HSNP-C with DBNP-B and HSNP-C and anti HSNP-C with HSNP-A and HSNP-C. This may be the reason for not seeing the cross-reaction by immunodiffusion. Furthermore, one or two antigenic determinant(s) in HSNP-C or HSNP-C may be present in DBNP-B and HSNP-C or HSNP-A and HSNP-C respectively. This site may well be the DNA binding site in these proteins which is immunogenic in only HSNP-C and HSNP-C along with other antigenic determinants. Lack of cross-reaction of anti-HSNP-A and anti-DBNP-B with the other proteins may be due to lack of antibodies to common determinants in the antisera to these proteins. At this juncture it is worth mentioning about a recent report by Magnus and Lattman (1983) where it was shown that histones H2A and H3 are homologous to lambda **repressor** and Cro proteins in 22 residue segments implicated in DNA binding.

Similarly primary structural homology between lac repressor and lambda Cro protein in the same region has been observed (Matthews et al., 1982). The amounts of the four nucleoid proteins in different cell fractions have been determined by single radial immunodiffusion. We could not determine the copy number of protein per cell because the bacterial cell number in the cultures could not be determined. The proteins DBNP-B and HSNP-C' were found in the ribosome fractions in appreciable amounts indicating stronger affinity of these proteins to RNA as well. The proteins with monomer molecular weights of 12,000 for HSNP-A, 11,500 for DBNP-B, 10,500 for HSNP-C and 9000 for HSNP-C were found to show interesting aggregation behaviour except HSNP-C. Gel filtration and cross-linking experiments showed temperature dependent dimerization of HSNP-A, DBNP-B and HSNP-C. The results of cross-linking experiments indicated that the equilibrium of monomer to dimer aggregation under the experimental condition is more towards monomer formation or that the bifunctional reagent was not effective at high temperature of incubation (50°C) because the yield of dimer species was found to be very low. However, gel filtration experiments on Sephacryl S-200 showed that a large proportion *(50%)* of the proteins HSNP-A, DBNP-B and HSNP-C were eluted at elution volumes corresponding to dimer molecular weight. Ultra violet spectra and their fourth derivative spectra of HSNP-A and HSNP-C show dominant phenylalanine / tyrosine absorption and those of DBNP-B and HSNP-C show dominant tyrosine/tryptophan absorption. In the case of HSNP-C the fourth derivative spectrum clearly shows the dominant phenylalanine contribution to absorption as indicated by the strong absorption bands in the region 245 nm to 265 nm. The fourth derivative spectrum of DBNP-B showed lack of tryptophan in this protein.

The fluorescence spectra of HSNP-A and HSNP-C upon excitation at 274 nm show strong tyrosine contribution. Such strong contribution to emission by tyrosine in a tryptophan containing protein is unusual since proteins with a single tryptophan typically exhibit an emission characteristic of tryptophan. However, excitation at 285 nm abolishes the emission peak at 302 nm (due to tyrosine) resulting in emission spectrum (max. 346 nm) characteristic of tryptophan. HSNP-C which also contains larger content of phenylalanine and tyrosine does not show such a behaviour. The emission spectrum, characteristic of tryptophan, of this proteins is unchanged when excited at 274 nm and 285 nm. The emission spectra of DBNP-B which contains fewer tyrosine (probably less than phenylalanine) is characteristic of tyrosine emission with emission A max at 302 nm. That the protein does not contain tryptophan is indicated by no shift in the emission spectrum even when excited at 285 nm.

Circular dichroic spectra of the four proteins suggest presence of ordered secondary structural elements in all the four proteins. The spectra have the unusual property of intersecting the base line around 205-210 nm (usually 200 nm). Although HSNP-A showed a CD spectrum characteristic of an α-helical protein estimation of α-helix content in this protein gave low values. This may be because of the low signal intensity observed for this protein and also because of the uncertainty associated with structure prediction based on CD measurements. The CD spectrum DBNP-B showed that this protein has high content of ordered secondary structure. The CD spectrum of HSNP-C is found to be characteristic of a protein with β-sheet structure with a single minimum around 220 nm. The similarity of the CD spectrum of HSNP-C with that of gene 5 protein of phage fd may suggest that this proteins may be an all β-sheet protein. The CD spectra of some low molecular weight DNA binding proteins isolated from S. acidocaldarius are available

in the literature (Dijk and Reinhardt, 1986). For example the CD spectrum of a 7000 molecular weight protein (protein 7e) was very much like that of HSNP-C. Similarly the spectrum of protein 10b showed resemblance to that of DBNP-B. The protein 8a of Dijk and Reinhardt had a CD spectrum similar to that of HSNP-C. The proteins 7e, 10b and 8a described by Dijk and Reinhardt may correspond to HSNP-C, DBNP-B and HSNP-C. Further work is needed to establish the identify among these proteins. The amino acid compositions show certain unique features such as absence of methionine, very low content or absence of proline. The basic amino acids content of these proteins is very much like that in eukaryotic histones except for DBNP-B which has relatively lower content of these amino acids. However the lysine content of HSNP-A, HSNP-C and HSNP-C is relatively higher than most of the eukaryotic histones (H2A, H2B, H3 and H4) and is like that in histone H1. The amino acid composition of DBNP-B shows some similarities with the composition of H2A. Similarly, HSNP-C composition shows resemblance to the composition of H4 in several amino acids. However clear differences are also present in the content of a few amino acids e.g. the alanine content in the nucleoid proteins is very much lower than that in histones. Overall the amino acid compositions of these archaebacterial nucleoid proteins show a few similarities with eukaryotic histones. Comparison of the amino acid compositons of the nucleoid proteins with histone-like proteins from E. coli and some archaebacteria reveal certain similarities. For example the overall composition of protein H1 from E. coli shows similarities with that of HSNP-A of S. acidocaldarius. However the lysine/arginine ratio for these proteins is quite different. Protein composition of H1 also shows some similarities to the composition of DBNP-B, particularly the lysine/arginine ratio is found to be the same for these two proteins. The amino acid composition of NS1/NS2 shows

similarities with that of HSNP-C' exlcuding the content of alanine which is lower in HSNP-C. There are also similarities in the composition of the proteins HSNP-C and HSNP-C. Among the archaebacterial histone-like DNA binding proteins isolated, the amino acid composition of 7 KDa protein isolated from Sulfolobus solfataricus shows strong similarities with the composition of HSNP-C. The 7 KDa protein could well be the counterpart of HSNP-C in this strain of Sulfolobus. Other archaebacterial histone like proteins, HSa from Sulfolobus acidocaldarius, HTa from T. acidophilum and HMb from M. barkeri show only superficial similarities in their amino acid composition to the nucleoid proteins studied in the present investigation. In fact protein HSa has been isolated from S. acidocaldarius as a DNA binding protein from sheared DNA protein complexes, in order to look for proteins that stabilize DNA against thermal denaturation (Green et ah, 1983). However protein HSa could not stabilize DNA against thermal denaturation. Among the four proteins we have purified from the nucleoid of S. acidocaldarius DBNP-B which binds strongly to DNA, does not stabilize DNA against thermal denaturation (see Chapter IV). Hence based on the molecular weight of HSa (reported Molecular weight 14500), its acid soluble nature, DNA binding property without stabilizing the DNA, the protein HSa may correspond to DBNP-B we have studied. However the amino acid compositions of DBNP-B and HSa do not show any striking similarities except in the contentsof lysine, arginine, aspartic acid, serine, glutamic acid, glycine and alanine.

CHAPTER - IV

NUCLEIC ACID BINDING PROPERTIES OF THE HISTONE-LIKE PROTEINS

The purified histone-like proteins from Sulfolobus acidocaldarius have been further studied for their nucleic acid binding properties. As already mentioned in the introduction S. acidocaldarius grows at 75°C optimally. The organism lacks rigid cell wall and exists presumably in osmotic equilibrium with its environment. Therefore intracellular concentration is expected to be low (Green et al., 1983). Since low ionic strength as well as high temperature destabilize the DNA, factors may be present to stabilize the intracellular DNA with low G + C content. The four basic acid soluble proteins present in the nucleoid have been tested for their ability to protect DNA against thermal denaturation. Binding of these proteins to polynucleotides has also been studied. The results presented in this chapter show that three of the histone-like proteins HSNP-A, HSNP-C and HSNP-C strongly stabilize the DNA against heat denaturation. Hence these proteins are called helix stabilizing nucleoid proteins (HSNP). The fourth protein although binds strongly to DNA, does not show helix stabilizing property. Hence this protein has been referred as DNA binding nucleoid protein-B (DBNP-B).

MATERIALS AND METHODS

Binding of purified proteins to DNA-cellulose: 0.2 ml of preswollen DNA cellulose column was packed and equilibrated with 20 mM Tris-HCl pH 7.6, 50 mM KCl, 1 mM Na$_2$-EDTA and 6 mM 2-mercaptoethanol. Purified protein (each 10 ug) was loaded on the column and eluted with increasing KC1 concentration in a stepwise manner (0.05 M, 0.15 M, 0.3 M, 0.5 M and 2 M). The protein fractions were spotted on Whatmann 3 MM filter paper strip (pre-soaked with 7.5% acetic acid), stained with 0.1% coomassie blue in 50% methanol, 7.5% acetic acid and destained with the solution containing 5% methanol and 7.5% acetic acid.

Binding of purified proteins to [H] DNA: E. coli DNA was labelled with [H -methyl] thymidine described in Mahler (1967) and the DNA was isolated according to Marmur (1961). The specific activity of the DNA was 8530 CPM per ug. Binding of these proteins to [H] DNA was carried out as described by Labonne et al., (1983) with some modifications. This was already described in Chapter II.

Binding of proteins to poly (U): 0.2 ml of preswollen poly (U)-Sepharose (Pharmacia) was packed into a column and equilibrated with 10 mM Tris-HCl pH 7.6 and 100 mM • KCl. 10 ug of each of the purified protein was loaded onto the column and the column was washed with buffers containing increasing KCl concentration (0.1 M, 0.2 M, 0.5 M and 1 M). The protein was quantitated after spotting the fractions on Whatmann 3 MM filter paper (presoaked with *7.5%* acetic acid), staining with 0.1% coomassie blue in *50%* methanol, *7.5%* acetic acid and destaining with a solution containing 5% methanol and *7.5%* acetic acid.

Binding of purified proteins to ribosomes: Five A_{260} of S. acidocaldarius ribosomes were incubated with 15 ug each of HSNP-A, DBNP-B, HSNP-C HSNP-C' at 37°C for 15 min in buffer of 10 mM Tris-HCl, pH 7.6, *50* mM KC1, 10 mM magnesium acetate and 7 mM 2-mercaptoethanol. The mixture was chilled on ice and layered over a 4 ml 10 to 30% sucrose gradients in the above buffer and centrifuged at 54000 rpm (SW 60 rotor) for 90 min. Fractions of 0.2 ml were collected and analyzed by Ouchterlony immunodiffusion.

Thermal denaturation of DNA: DNA from E. coli and S. acidocaldarius was isolated according to Marmur (1961). Thermal denaturation profiles of DNA in the absence and presence of proteins were obtained by heating different DNAs in 300 ul of 1 mM Tris-HCl pH 7.2 and 25-50 mM NaCl

or 1 mM Tris-HCl pH 7.2 and 1 mM Na -EDTA. The rate of heating was 1°C per min. Increase in absorbance at 260 nm was measured continuously in a Gilford spectrophotometer (model 250) equipped with a thermoprogrammer (model 2527). The m. ling curves were simultaneously recorded. Before heating has started, protein was added to DNA, gently mixed and incubated for 5 min at the starting temperature. Separately buffer blank and protein solutions were also heated to see any temperature dependent variation in absorbance, .hich was found to be negligible.

RESULTS

Binding of purified proteins to DNA-cellulose: The fact that these proteins are associated with nucleoid and could be purified by DNA-cellulose chromatography suggested that these proteins have affinity to DNA. Ability of the purified proteins to bind native DNA-cellulose and the strength of the binding was examined by passing 10 ug of each protein through a small DNA-cellulose column and eluting with buffer of increasing salt concentrations. DBNP-B bound strongly to native DNA-cellulose and could be eluted completely with *0.5* M salt concentration. HSNP-A under similar conditions eluted with buffer containing 0.3 M KCl. HSNP-C also bound strongly to DNA-cellulose but protein started eluting out at 0.3 M salt concentration where about 10% of the input was eluted and the remaining 80% of the protein was eluted with *0.5* M KCl concentration. HSNP-C' binding was found to be weaker than the other three proteins. The protein in the absence of 2-mercaptoethanoJ was eluted partly (27%) with 0.15 M KCl concentration, and remainder of the protein (67%) was eluted with 0.3 M KCl salt. The extent of binding of HSNP-C was found to be increased in the presence of 2-mercaptoethanol. Only 10% of protein was eluted with 0.15 M salt, and the majority of the protein (85%) was eluted with 0.3 M salt buffer (Table 4.1).

TABLE - 4.

Binding of purified nucleoid proteins to native DNA-cellulose

Salt concentration for elution	per cent protein eluted				
	HSNP-A	DBNP-B	H5NP-C	HSNP-C'	
				-Me	+Me
0.05M	.	.	.	.	-
0.15M	-			27%	10%
0.30M	90%	-	10%	67%	85%
0.50M	-	95%	80%		
2.00M	-	-	-	-	-

Table 4.1 Binding of purified proteinsto native DNA cellulose.

DNA-cellulose column (0.2 ml) is packed and equilibrated with *0.05* M KCl buffer. Each purified protein (10 ug) was passed through the column and eluted with 5 bed volumes of buffer containing increasing KCl concentration (0.05 M, 0.15 M, 0.3 M, *0.5* M and 2 M) in a stepwise manner. Fractions (0.1 ml) were collected. The protein eluted in different salt concentrations was estimated as described in the text.

Me. 2-mercaptoethanol

Binding of purified proteins to [H 3 DNA: Binding of individual proteins to (H)-DNA was measured by mixing the protein with (H)-DNA (native and denatured) and after incubation, DNA-protein complexes were retained on nitrocellulose filters and the radioactivity was measured (Figure 4.1 A and B) (see Materials and Methods for details). HSNP-A bound native DNA strongly and about 80% of the input radioactivity was retained on the filter in the presence of 8-10 ug protein. However HSNP-A showed weak binding to denatured DNA. DBNP-B bound equally efficiently to both native and denatured DNAs. Greater than 90% input DNA was retained on the filter in the presence of 0.8 to 1 ug of this protein. In the case of HSNP-C, substantial binding of both native and denatured DNA was also observed and the binding curves were sigmoidal. However, HSNP-C, which is one of the most abundant proteins of the nucleoid, did not show any binding to either native or denatured DNA. We have not detected any binding of HSNP-C by this assay, even up to a protein/DNA ratio of 13.

Binding of purified proteins to RNA: Binding of purified proteins to poly(U) was measured (Table 4.2) by passing purified proteins individually through a small poly(U)-sepharose column. In each case the salt concentration required for the elution of the proteins was determined as described in Methods section. Almost all of HSNP-A was eluted with buffer of 0.2 M KCl conc. HSNP-C showed weak binding and protein started eluting with the equilibration buffer (salt conc. 0.1 M). About 70% of HSNP-C was eluted at a salt concentration of 0.2 M KCl and very little of the protein eluted with 0.5 M KCl buffer. Both DBNP-B and HSNP-C bound strongly to poly(U)-sepharose, as indicated by their elution with 0.5 M to 1 M KCl buffer. The binding characteristics of the proteins to poly(U)-sepharose are similar to those of binding to DNA-cellulose column except that there is small

Fig. 4.1 Binding of nucleoid proteins to native and denatured *E. coli* ^{3}H DNA. Binding of (^{3}H) DNA to nucleoid proteins was measured by nitrocellulose filtration technique (see Materials and Methods of Chapter II).

(A) Binding to native DNA; (B) Binding to denatured DNA HSNP-A, Δ ; DBNP-B, • ; HSNP-C, O and HSNP-C' □ .

TABLE - 4.2

Binding of purified nucieoid proteins to Poly (U)-Sepharose

Salt concentration for elution	Per cent protein eluted			
	HSNP-A	DBNP-B	HSNP-C	HSNP-C
0.1M	-	-	-	20%
0.2M	90%	-	-	71%
0.5M	-	70%	90%	3%
1.0M	-	25%		

Table 4.2: Binding of purified proteins to Poly (U)-Sepharose. Poly (U)-Sepharose (0.2 ml) is packed and equilibrated with .0.1 M KCl containing buffer. Each purified protein (10 ug) was passed through the column and the column was eluted with 5 bed volumes buffers of increasing KCl concentrations (0.1 M, 0.2 M, 0.5 M and 1 M). Fractions of 0.1 ml were collected. The protein eluted in different salt concentrations was estimated as described in the text.

decrease in the affinity of the proteins except DBNP-B to poly (U) as indicated by lower salt concentrations required for the elution of the proteins from poly (U)-Sepharose column.

E. coli NS protein (HU) was known to bind ribosome. In fact Suryanarayana and Subramanian (1978) isolated NS protein from native 30S ribosomal subunits of E. coli. This binding of protein NS is most probably due to its interaction with rRNA. We have also checked the binding of S. acidocaldarius nucleoid proteins to ribosomes. Five A_{260} units of ribosomes (1 M NH_4Cl washed) from S. acidocaldarius were mixed with 10 ug of each of the purified protein, (approximately 10 fold molar excess) incubated at 37°C for 15 min and centrifuged through sucrose gradient (see Methods Section). Binding of the proteins was detected by immunodiffusion test (Fig. 4.2) of the ribosome peak obtained from the sucrose gradient. Both DBNP-B and HSNP-C were found to bind strongly to ribosome. HSNP-A and HSNP-C also bind to ribosome, but the extent of binding was found to be less as indicated by the presence of free protein at the top of the gradient.

Thermal denaturation of DNA and protection by the isolated proteins; We performed thermal denaturation studies using different DNAs, and tested the influence of these proteins on DNA melting. The Tm of S. acidocaldarius DNA in 0.025 M NaCl was found to be 70.5°C (Fig. 4.3). Addition of HSNP-A dramatically increased the Tm, and with increasing amounts of protein there was increase in the Tm of DNA. Very little melting of the DNA was observed even at 94°C at a protein to DNA ratio (w/w) of 1:1 (Fig. 4.3 A). Similar results were obtained with the addition of HSNP-C (Fig. 4.3 C) and HSNP-C (Fig. 4.3 D), although the extent of stabilization was less than that obtained with HSNP-A. A slightly higher amount of HSNP-C

Fig. 4.2 Ouchterlony immunodiffusion test for the binding of the nucleoid proteins to ribosomes. Five A_{260} units of 1 M NH_4Cl washed ribosomes were incubated with 15 ug each of purified HSNP-A, DBNP-B, HSNP-C and HSNP-C, and centrifuged through sucrose gradient solution as described in the text. Central well contained ribosome peak fraction from sucrose gradient; Outer wells 1-4, contained 20 ul each of antiserum to HSNP-A, DBNP-B, HSNP-C and HSNP-C respectively.

Fig. 4.2

Fig. 4.3 Effect of nucleoid proteins on the melting profiles of S. acidocaldarius DNA. Varying amounts of nucleoid proteins were added to 10 ug of DNA and incubated at the starting temperature for 5 min. before heating at a rate of 1°C per min. A_{260} increase was continuously recorded. Panels A, B, C and D are with HSNP-A, DBNP-B, HSNP-C and HSNP-C' respectively.

No proteins, o ; 2 ug, Δ ; 5 ug, • ; 10 ug, D ; 20 ug, ▲ .

(Protein to DNA ratio of 2:1) was needed to observe protection comparable to that obtained with HSNP-A. HSNP-C showed a strong stabilizing effect and the Tm was shifted by +21.5°C in the presence of the protein (HSNP-C/ DNA ratio of 1:1). DBNP-B although showed strong binding to native and denatured DNA, did not show such a stabilizing effect under these conditions (Fig. 4.3 B). Melting studies of native nucleoid were also performed. When an amount of nucleoid corresponding to 20 ug DNA was used there was hardly any melting, as indicated by the small increase in A_{260}The melting curve obtained was similar to that obtained with S. acidocaldarius DNA in the presence of HSNP-A (Protein : DNA ratio, 1:1, Fig. 4.3 A).

In figure 4.4 thermal denaturation profiles of calf thymus DNA (A), E. coli DNA (B) and poly(dA).poly(dT) (C) were given. In these cases also we observed similar effects of these proteins when added in increasing amounts. Denaturation profiles with maximum amounts of protein added were presented. HSNP-C was found to exert a maximum stabilizing effect with all the three DNAs. An increase in Tm of 20-25°C was obtained in the presence of the protein. HSNP-C and HSNP-A behaved similarly with both calf thymus and E. coli DNA. Increases in Tm by 15 and 17.5°C of E. coli (DNA/Protein 1:1 w/w) and 15 and 14°C of calf thymus DNA (DNA/ Protein 1:2 w/w) were obtained with HSNP-C and HSNP-A respectively. HSNP-C was relatively less effective with poly(dA).poly(dT) than HSNP-C and HSNP-A. DBNP-B however could not stabilize poly(dA).poly(dT) (Fig. 4.4 C). DBNP-B gave a small but significant increase in Tm (4°C) of E. coli DNA. However this increase was very much lower than the increase with other three proteins. The protection by these proteins was found to be highly specific to DNA. Double stranded RNA such as poly r(I.C) was not at all stabilized against thermal denaturation (Fig. 4.5).

Fig. 4.4 Effects of nucleoid proteins on the melting profiles of (A) Calf thymus DNA, (B) E. coli DNA, (C) poly(dA).poly(dT). Melting profiles were obtained as described in Materials and Methods and legend to Fig. 4.3. Only those melting profiles with the maximum amount of each protein added are given. Calf thymus DNA used was 20 ug and E. coli DNA and poly(dA).poly(dT) 10 ug each. In (A) 40 ug of each protein and in (B) and (C) 20 ug of each protein was used.

No protein, o ; HSNP-A, • ; DBNP-B, ▲ ; HSNP-C; • ; HSNP-C, X.

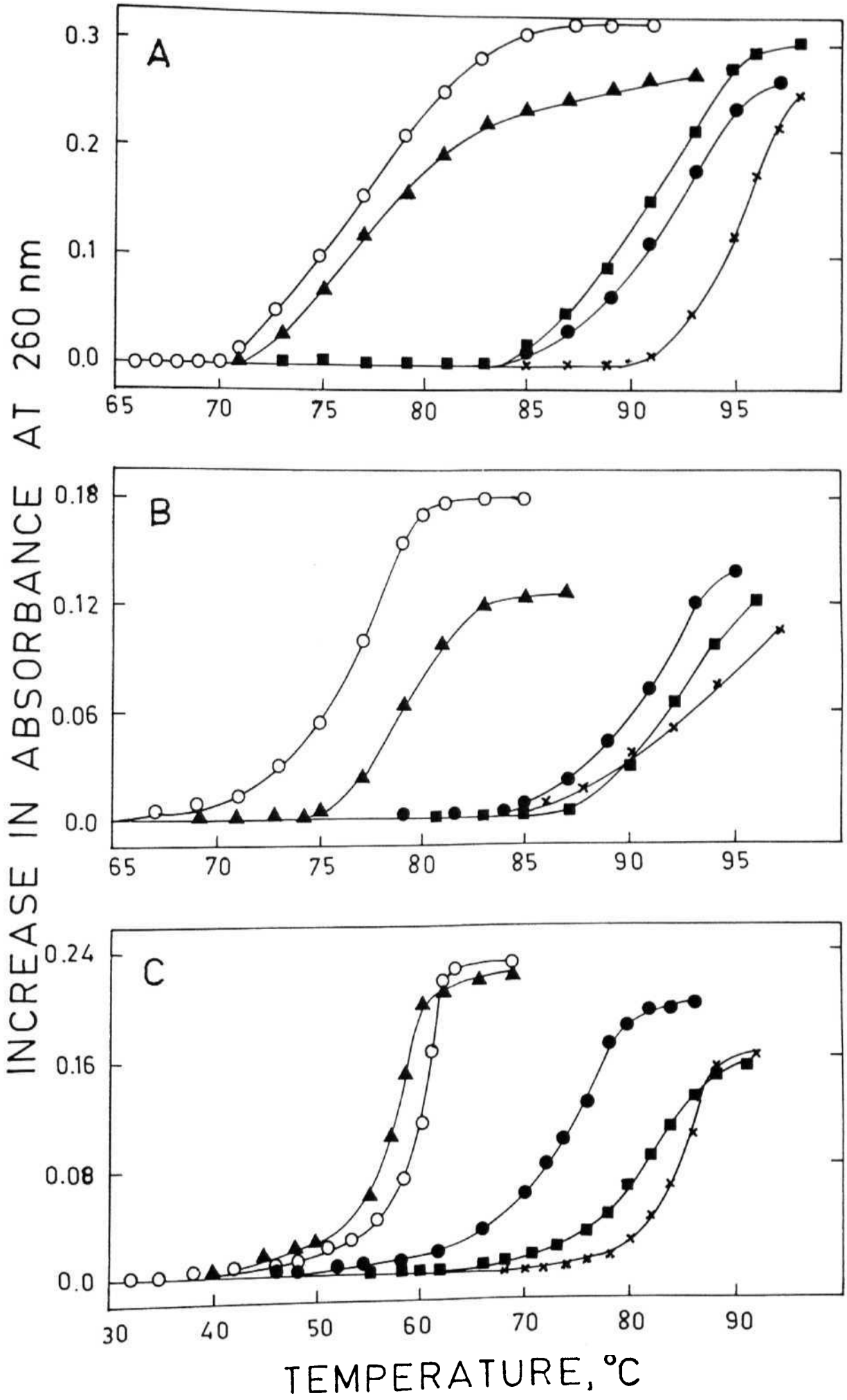

A
B
C
0.3
0.2
0.1
0.0
0.18
0.12
0.06
0.24
0.16
0.08
65
70
75
80
85
90
95
30
40
50
60
INCREASE IN ABSORBANCE AT 260 nm
TEMPERATURE, °C

Fig-4-4

Fig. 4.5 Effect of nucleoid protein on the melting profile of poly r(I.C). Melting profiles were obtained as described. The amount of poly r(I.C) used in each experiment was 10 ug. The amount of each protein added was 20 ug.

No protein, ○ ; HSNP-A, •; DBNP-B, ▲ ; HSNP-C', ●; HSNP-C, X .

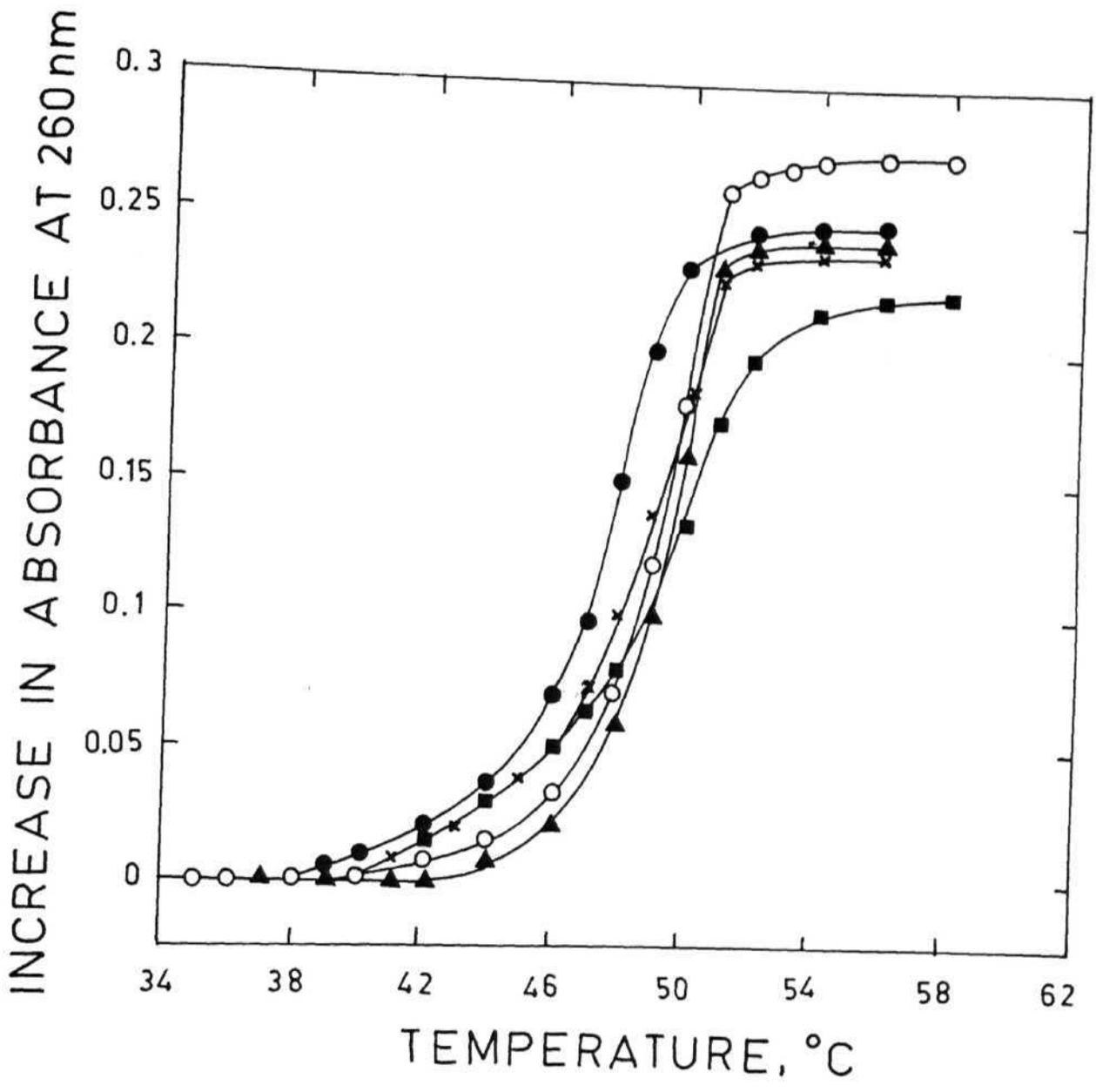

INCREASE IN ABSORBANCE AT 260nm
0.3
0.25
0.2
0.15
0.1
0.05
0
34
38
42
46
50
54
58
62
TEMPERATURE, °C

Fig.45

We have also tested the stabilizing effect of E. coli histone-like protein NS under the same conditions (Fig. 4.6). As reported previously (Miano et al., 1982) E. coli NS protein showed only a marginal stabilizing effect with an increase in Tm of 4°C.

The data obtained from melting studies of different DNAs were plotted to show the increase in Tm with the increasing amounts of protein added (Fig. 4.7 A, B, C and D). When the three proteins HSNP-A, HSNP-C and HSNP-C were added to calf thymus DNA (Fig. 4.7 A), Sulfolobus DNA (Fig. 4.7 B), poly(dA).poly(dT) (Fig. 4.7 C) and E. coli DNA (Fig. 4.7 D) there was an increase in Tm of DNA with increasing protein to DNA ratio. However differences were noted in the extent of stabilization of different DNAs with the three proteins. DBNP-B did not show any stabilizing effect as indicated by no increase in Tm of S. acidocaldarius DNA and poly(dA).poly(dT). However addition of DBNP-B showed small increase in the Tm of calf thymus and E. coli DNAs. At a weight ratio DBNP-B to DNA of 2 an increase of 4 to 5°C in the Tm of calf thymus DNA and E. coli DNA was observed. We have also tested the influence of the proteins on the thermal stability of E. coli DNA when added in combination. Results presented in Figure 4.8 A and B show that when the proteins were added in the combinations of two proteins, there was increase in the Tm of the DNA. The Tm increase observed when combination of two proteins containing DBNP-B and any of the other three proteins i.e., HSNP-A and DBNP-B, HSNP-C and DBNP-B. HSNP-C' and DBNP-B was found to be additive of Tm increases obtained with the individiual proteins. For example, when 5 ug of each of HSNP-A and DBNP-B were added together an increase in Tm of 7.5°C was observed. The corresponding Tm increases obtained with 5 ug of HSNP-A and DBNP-B when added alone were 5°C and 2°C respectively (Fig. 4.7 D). Similarly the other

Fig. 4.6 Effect of NS protein on the melting profile of *E. coli* DNA. Melting profiles were obtained as described. The amount of *E. coli* DNA used in this experiment was 10 ug.

No protein, X; 20 ug NS, •.

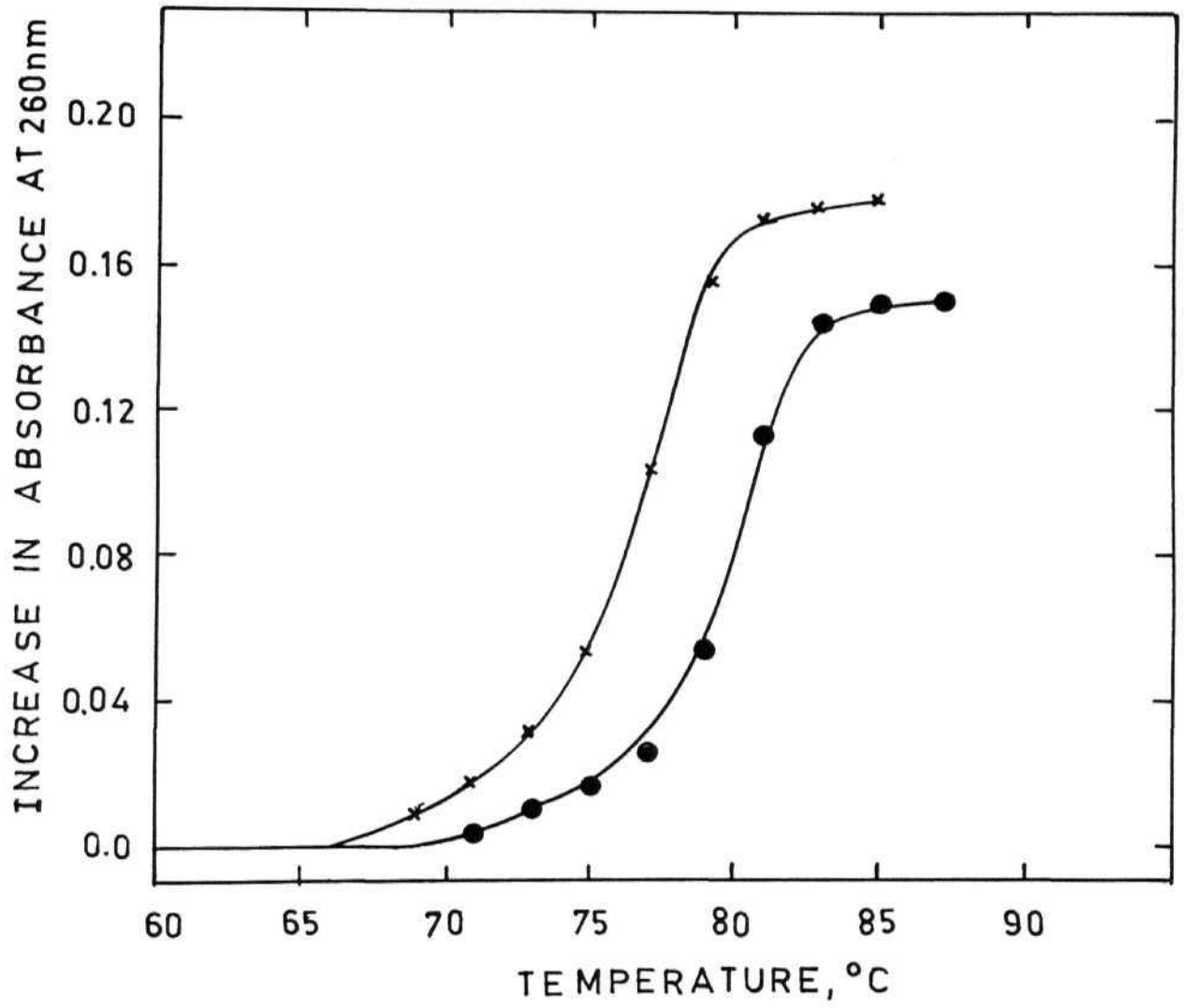

INCREASE IN ABSORBANCE AT 260nm
0.20
0.16
0.12
0.08
0.04
0.0
60
65
70
75
80
85
90
TEMPERATURE, °C

Fig46

Fig- 4.7 The Tm increase of different DNAs in the presence of nucleoid proteins. (A) Calf thymus DNA, (B) S. acidocaldarius DNA, (C) poly(dA).poly(dT), (D) E. coli DNA.

HSNP-A, A ; DBNP-B, D ; HSNP-C', • ; HSNP-C, O .

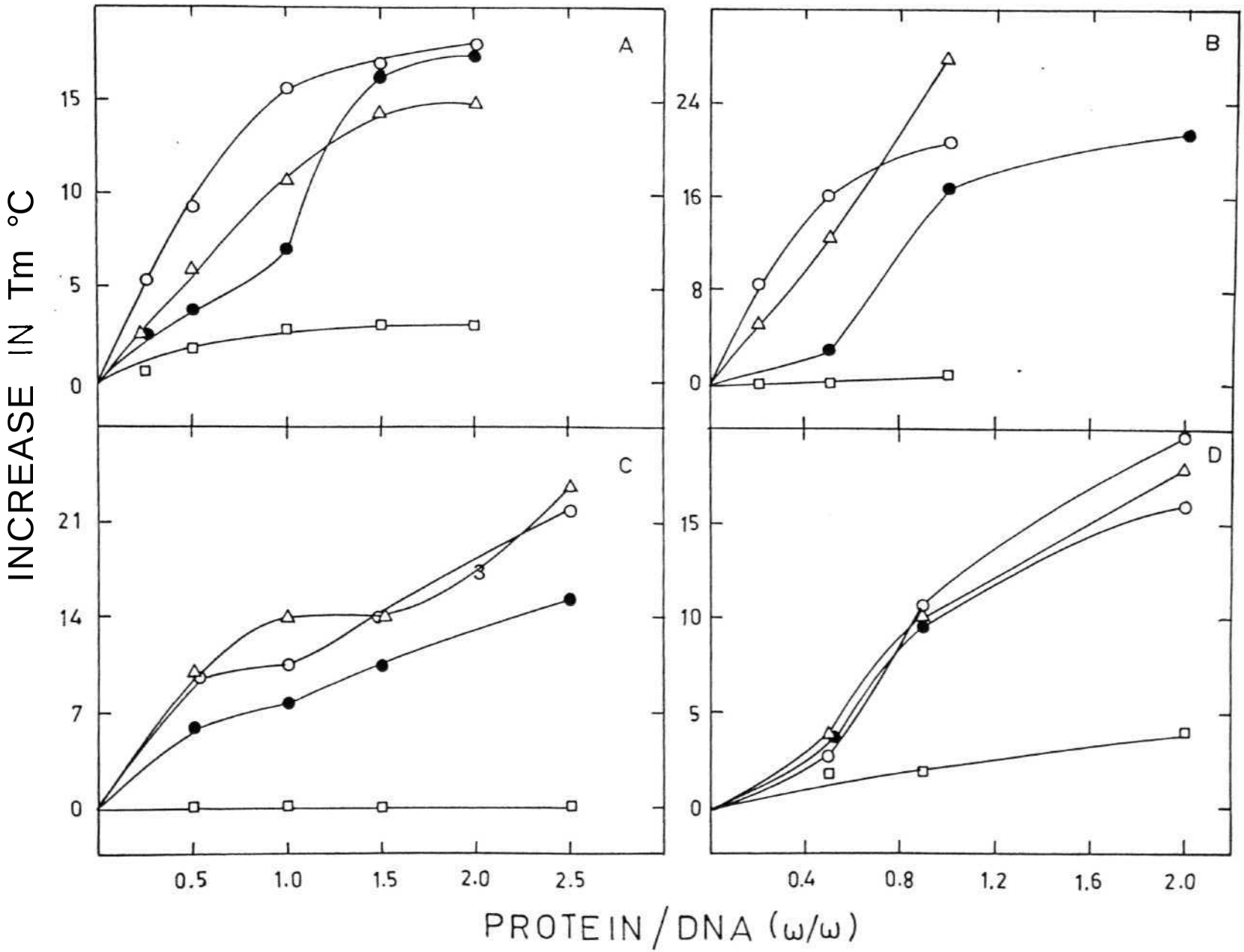

INCREASE IN Tm °C
A
B
C
D
15
10
5
0
24
16
8
0
21
14
7
0
15
10
5
0
0.5
1.0
1.5
2.0
2.5
0.4
0.8
1.2
1.6
2.0
PROTEIN / DNA (ω/ω)

Fig. 4.7

two protein mixtures containing DBNP-B and HSNP-C or DBNP-B and HSNP-C' also showed Tm increases suggestive of additive effect of these proteins (Fig. 4.8B). It was already observed that DBNP-B protected the DNA to a lesser extent (Fig. 4.7D). However when any of the two protein combinations of HSNP-A, HSNP-C and HSNP-C were added the Tm increase obtained was more than the mere additive of Tm's obtained with individual proteins. For example when 5 ug of HSNP-A was added along with 5 ug of HSNP-C or HSNP-C, the Tm increase obtained was found to be 12.5°C (Fig. 4.8A). But the increases in Tm observed with 5 ug of HSNP-A, HSNP-C and HSNP-C when added alone were 5°C, 3°C and 4.5°C respectively. So if the proteins were showing the additive effect one would expect a Tm increase of only 8 to 9°C. Similarly the proteins HSNP-C and HSNP-C when added together the Tm increase (11.5°C) obtained (Fig. 4.8B) was more than the addition of the Tms obtained with corresponding amounts of individual proteins. In Figure 4.9 the melting profile of S. acidocaldarius DNA in the presence of a mixture of HSNP-A, HSNP-C and HSNP-C (5 ug each) is presented along with that of free DNA. The combination of the three proteins had a strong stabilizing effect on the DNA. A Tm increase of 23°C was obtained when all the three proteins were added together.

Thermal denaturation studies in the presence of very low salts: We have performed thermal denaturation of calf thymus DNA, E. coli DNA and poly(dA).poly(dT) in the presence of 1 mM Tris-HCl pH 7.6 and 1 mM Na_2-EDTA and studied the effect of addition of these proteins (Fig. 4.10). Under these conditions also all the proteins including DBNP-B showed strong stabilizing effect. Calf thymus DNA under this salt concentration condition showed a Tm of 57°C which increased to about 65°C in the presence of 20 ug of either HSNP-A or DBNP-B and about 74°C with 20 ug of HSNP-C or HSNP-C

Fig. 4.8 Effect of addition of two nucleoid proteins together on the melting profiles of E. coli DNA. E. coli DNA used was 10 ug and melting profiles were obtained as described.

A) No protein, X; 5 ug each of HSNP-A and DBNP-B, Δ; 5 ug each of HSNP-A and HSNP-C, • ; 5 ug each of HSNP-A and HSNP-C, • .

(B) No protein, X; 5 ug each of HSNP-C and DBNP-B, ▲; 5 ug each of HSNP-C and DBNP-B, O ; 5 ug each of HSNP-C and HSNP-C, ■.

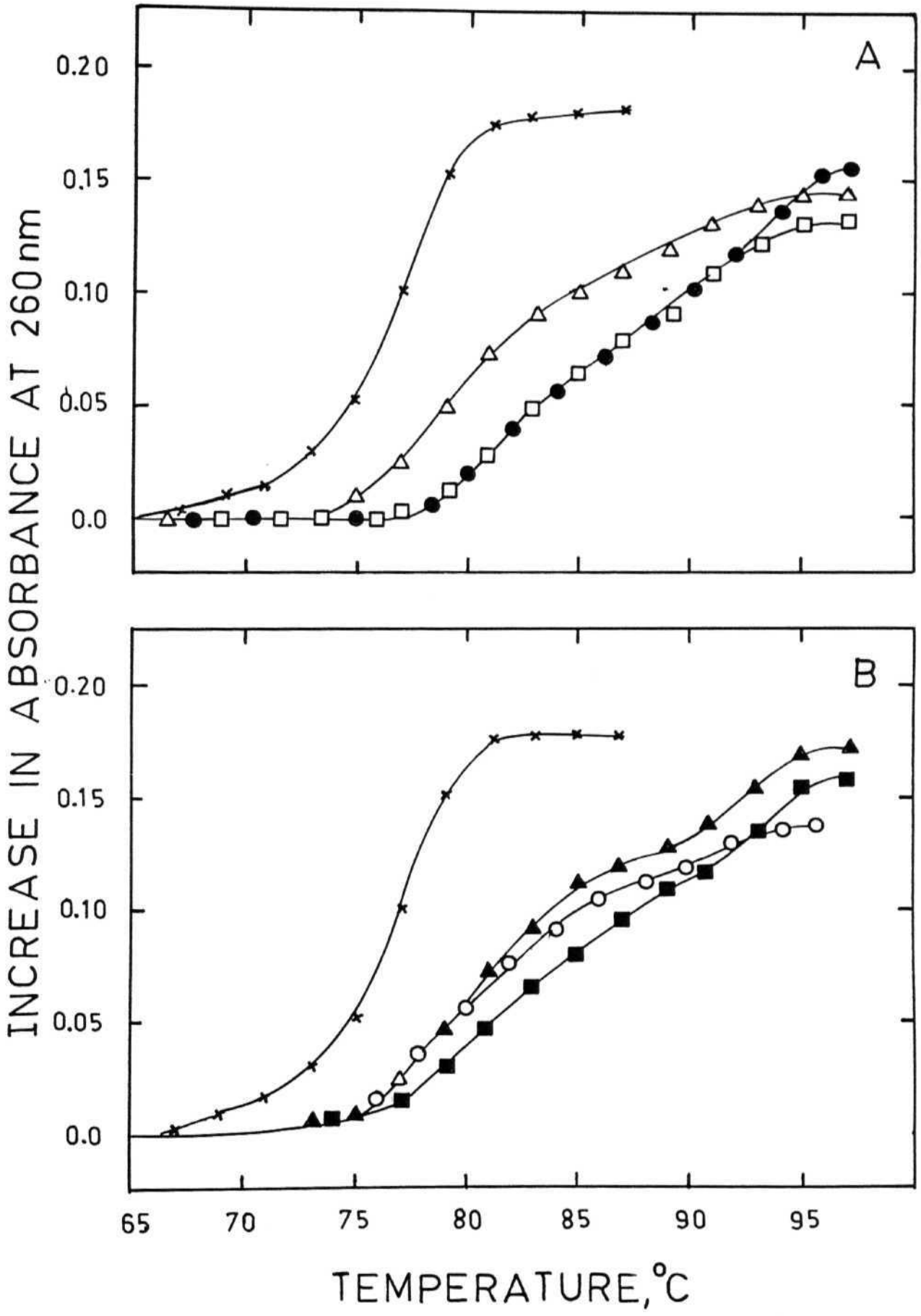

A
B
0.20
0.15
0.10
0.05
0.0
INCREASE IN ABSORBANCE AT 260nm
65
70
75
80
85
90
95
TEMPERATURE, °C

Fig.4.8

Fig. 4.9 Effect of addition of the three helix stabilizing nucleoid proteins together on the melting profile of S. acidocaldarius DNA. S. acidocaldarius DNA used was 10 ug and melting profiles were obtained as described.

No protein, O ; 5 ug each of HSNP-A, HSNP-C and HSNP-C[1], •.

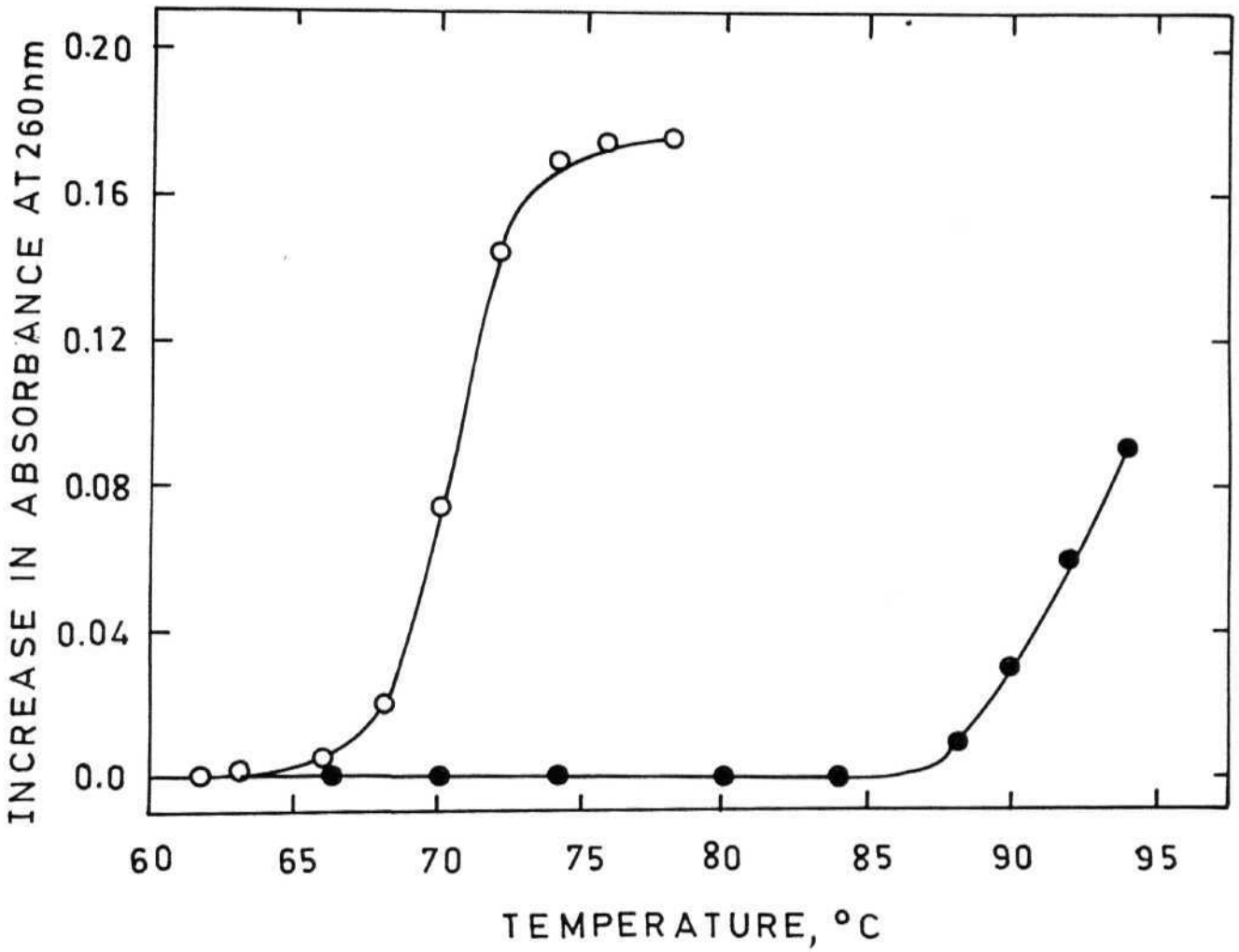

INCREASE IN ABSORBANCE AT 260nm
0.20
0.16
0.12
0.08
0.04
0.0
60
65
70
75
80
85
90
95
TEMPERATURE, °C

Fig49

Fig. 4.10 Effect of nucleoid proteins on the melting profiles of (A) Calf thymus DNA (B) E. coli DNA (C) poly (dA). poly(dT). Melting of the DNAs was performed in buffers containing 1 mM Tris-HCl pH 7.6, 1 mM Na_2-EDTA. Only those melting profiles with the maximum amounts of each protein added (25 ug) are given.

Calf thymus DNA used was 20 ug O ; E. coli DNA, Δ; and poly(dA).poly(dT), □; 10 ug each;

HSNP-A, *A* ; DBNP-B, ■ ; HSNP-C, X; HSNP-C', ● .

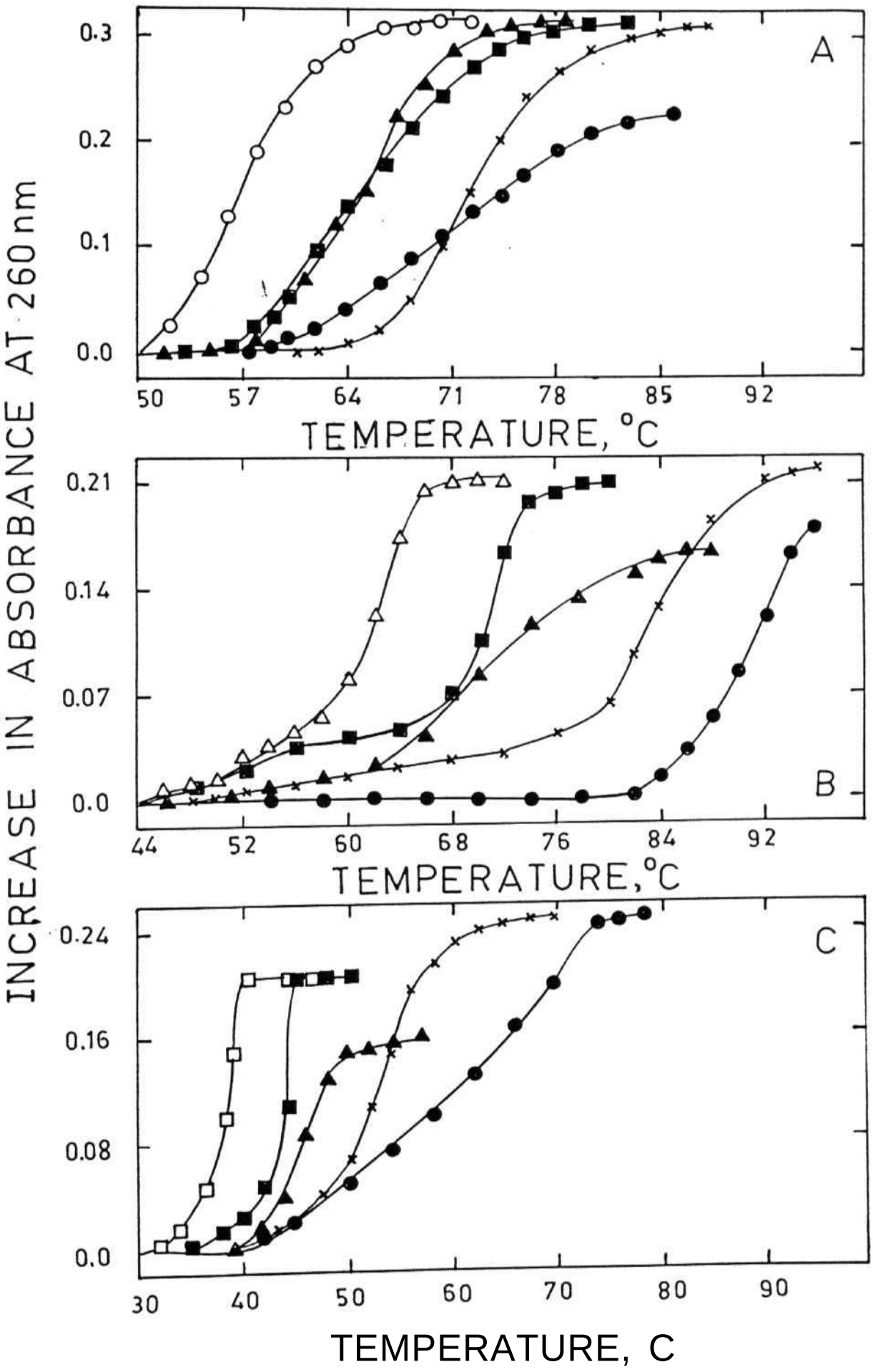

INCREASE IN ABSORBANCE AT 260 nm
0.3
0.2
0.1
0.0
A
50
57
64
71
78
85
92
TEMPERATURE, °C
0.21
0.14
0.07
0.0
B
44
52
60
68
76
84
92
TEMPERATURE, °C
0.24
0.16
0.08
0.0
C
30
40
50
60
70
80
90
TEMPERATURE, C

Fig.4.10

(Fig. 4.10 A). Similarly experiments were performed with E. coli DNA in the presence of individual proteins added at a protein to DNA ratio of 2 (w/w). A maximum stabilizing effect resulting in the increase in the Tm of E. coli DNA by 31°C was observed with HSNP-C' (Tm of E. coli DNA alone 61°C). With the other three proteins the Tm of E. coli DNA increased by 9°C to 22°C (Fig. 4.10 B). Similarly with poly(dA).poly(dT) an increase in Tm by 21°C was observed by the addition of HSNP-C over the Tm of poly(dA). poly(dT) alone (37.9°C). Proteins HSNP-A, DBNP-B and HSNP-C also stabilized poly(dA).poly(dT) with Tm increases in the range of 6 to 14°C (Fig. 4.10 C). Under low salt conditions E. coli DNA was maximally protected by these proteins. These data are summarized in Table 4.3.

DISCUSSION

The results presented in this chapter show that the nucleoid proteins HSNP-A, DBNP-B, HSNP-C and HSNP-C bind nucleic acids and three proteins HSNP-A, -C and -C bind native DNA and strongly protect it against thermal denaturation. The binding of the proteins to DNA was measured by DNA-cellulose chromatography of the purified proteins and by radioactive DNA binding by filtration technique. The binding properties differed from one protein to another. DBNP-B showed strong binding to both native and denatured DNAs. Both the proteins DBNP-B and HSNP-C bound to DNA-cellulose column and could be eluted with high salt. Protein HSNP-C was eluted in the later fractions of 0.3M salt whereas DBNP-B was eluted only with 0.5M salt indicating that the binding of DBNP-B to DNA was stronger than that of HSNP-C. HSNP-A and HSNP-C binding to DNA-cellulose was weaker as indicated by lower salt concentration (0.15M to 0.3M) required for their elution. All the proteins showed binding to RNA (e.g. poly U). The affinity of binding of these proteins to poly(U) was similar to their binding to DNA as indicated

TABLE - 4.3

The increase in Tm of different DNAs in the presence of nucleoid proteins under low salt conditions

Protein added	Calf thymus DNA Tm	E. coli DNA Tm	poiy(dA).poly(dT) Tm
No protein	61°C	57°C	37.9°C
HSNP-A (20 ug)	70°C	65°C	44.0°C
DBNP-B (20 ug)	73°C	64°C	47.0°C
HSNP-C (20 ug)	83°C	72°C	52.0°C
HSNP-C' (20 ug)	91°C	75°C	58.0°C

Table 4.3 Melting profiles of DNA in the absence and presence of the proteins were obtained as described. The buffer used was **1 mM Tris·HCl pH 7.6 and 1 mM Na_2-EDTA.**

by the salt concentrations required for their elution. The binding to native and denatured DNA by DBNP-B as measured by filtration techniques showed saturation at protein to DNA ratio of 0.5 (w/w), indicating binding to 23 bp native DNA/protein molecule (monomer) or 46 bases of denatured DNA per protein molecule. Strong binding of HSNP-C to both native and denatured DNAs was observed; the binding curves were sigmoidal, indicating cooperative binding of HSNP-C to DNA. We have also tested the binding of HSNP-C to DNA in the presence of phenylmethylsulphonyl fluoride, a protease inhibitor to rule out the possibility of obtaining sigmoidal binding curve because of contamination of low levels protease in DNA preparation (Pestana et al., 1978). We found that essentially identical sigmoidal binding curves were obtained in the absence and presence of PMSF. These results indicate that the binding of HSNP-C to DNA is cooperative. At saturation, binding of 4.35 bp of native DNA per protein molecule (HSNP-C) was indicated. HSNP-A showed strong binding only to native DNA and very weak binding to denatured DNA. HSNP-C showed no binding to either native or denatured DNA by this assay. However, HSNP-C binds to native DNA cellulose; this binding is promoted by 2-mercaptoethanol. These results indicate that HSNP-C DNA complexes may not be retained on the nitrocellulose filters or that the complexes readily dissociate during filtration and washing. DNA binding properties of the proteins was also tested at elevated physiological temperatures. There were no appreciable differences in the DNA binding properties.

The proteins also bind to ribosomes as was observed in the case of _E. coli NS protein (Suryanarayana and Subramanian, 1978). Binding to ribosomes by these proteins indicate that the proteins have affinity to the exposed RNA on the ribosome.

Three of the four proteins, HSNP-A, -C and -C' showed strong helix stabilizing effect. Even at 25-50 mM NaCl concentration the proteins protected DNA against thermal denaturation. At a weight ratio of 1-2 (protein/DNA) there was very little melting of the DNA, even at 90°C. The amount of these proteins, which are the only acid soluble proteins, is 90% of the weight of DNA in the nucleoid. Therefore, the observed protection of the protein is physiologically significant. The proteins stabilize all types of DNA (prokaryotic, eukaryotic and synthetic). However the inability of the proteins to protect double stranded RNA (poly I.C) indicates that the stabilizing effect is specific for DNA. As mentioned in the introduction, low intracellular salt concentration and high growth temperature would have a destabilizing effect on the intracellular DNA of this organism of low G + C content, about 40% (Zillig et ah, 1980). The DNA in isolated nucleoid of S. acidocaldarius does not appreciably melt when heated, even up to 95°C. Our results point out that the proteins, HSNP-A, HSNP-C and HSNP-C' may be responsible for the stabilization of S. acidocaldarius DNA at the optimal growth temperature of the organism (75°C).

Thermal denaturation studies were also performed at high and very low salt concentrations. The proteins afford strong protection to DNA at all the salt concentrations (0 mM, 25 mM, 50 mM and 100 mM NaCl) tested indicating strong stabilizing effect of these proteins. Under very low salt concentration (1 mM Tris-HCl, 1 mM EDTA) conditions all the four proteins including DBNP-B stabilized DNA against thermal denaturation; with HSNP-C exerting maximum stabilizing effect. The results of protection of DNA when mixture of proteins were added indicated synergistic effect of these proteins in protecting DNA against thermal denaturation as indicated by increased Tm by 5°C over and above the Tm that would expected of an additive effect.

These results clearly point out the strong and specific stabilizing effect of these three proteins. Although eubacterial histohe -like protein (NS or HU) condenses the duplex DNA into nucleosomal structure it could not protect DNA against thermal denaturation to any appreciable extent. An increase in Tm of E. coli DNA of about 4°C was observed when protein NS was added at protein/DNA ratio (w/w) of 2-4 (our results and those of Miano et al., 1982). A small DNA binding protein from the Thermus thermophilus has been purified which was shown to effect marginally the melting profile of DNA (Zierer et al., 1986). The histone-like protein (HTa) from T. acidophilum showed a stabilizing effect on DNA against thermal denaturation (Searcy , 1975; Stein and Searcy, 1978). However, the organism growth temperature is 59°C, the temperature at which DNA would not start to melt appreciably even in the presence of low salt (e.g., 25 mM NaCl). The authors suggested that protein HTa may protect DNA when the organism is transiently exposed to elevated temperatures (say 80°C). A histone-like protein (HSa) from S. acidocaldarius was isolated in order to look for a stabilizing factor against thermal denaturation of DNA (Green et al., 1983). These authors concluded that the problem of how S. acidocaldarius keeps its DNA double stranded remained unsolved. The protein HSa probably may correspond to DBNP-B (see Chapter III). So among the DNA binding proteins isolated till now from eubacteria and archaebacteria, the proteins HSNP-A, HSNP-C and HSNP-C' are unique in their helix stabilizing property, which is physiologically very important.

CONCLUSIONS

The nucleoid or the chromatin of Sulfolobus acidocaldarius, an acidothermophilic organism with an optimum growth temperature of 75-80°C has been isolated and its protein components analyzed in order to look for factors that can stabilize the DNA duplex structure. The nucleoid contains four acid soluble, basic, low molecular weight proteins in abundance. The purified proteins have been characterized with respect to physico-chemical properties and DNA binding properties. Three proteins have been named as Helix stabilizing nucleoid proteins (HSNP-A, HSNP-C and HSNP-C') and one protein as DNA binding nucleoid protein (DBNP-B). Immunochemical properties of the proteins show that the proteins are distinct and yet show certain similarities in part of their structure. CD spectra of the proteins suggest that the proteins contain ordered secondary structural elements. These proteins contain phenylalanine and tyrosine and/or tryptophan which contribute to fluorescence and absorption spectral properties of the proteins. Comparison of the amino acid composition of the proteins showed similarities as well as differences with eukaryotic histones and prokaryotic histone-like proteins. All the four proteins bind to native and denatured DNAs to different extents with differing binding properties. Three of the proteins HSNP-A, HSNP-C and HSNP-C specifically stabilize double stranded DNA during thermal denaturation and increase the Tm of DNA by about 25°C. These nucleoid proteins resemble eukaryotic histones in several respects, viz., (i) association with intracellular DNA, (ii) acid solubility, (iii) low molecular weight, (iv) basic nature (v) partly in amino acid composition and (vi) in the protection of DNA against thermal denaturation (Ohlenhusch et ah, 1967). HSNP-A, HSNP-C and HSNP-C showed the strong helix stabilizing effect at a protein to DNA ratio of 1-2. The amount of these proteins is 90% of the weight of DNA

in the nucleoid. Therefore, the observed protection by the proteins is physiologically significant. As mentioned already in the introduction and elsewhere, low intracellular salt concentrations and high growth temperature could have a destabilizing effect on the intracellular DNA of this organism with a low G + C content of 40%. The results presented here indicate that the proteins HSNP-A, HSNP-C and HSNP-C may be responsible for the stabilization of the intracellular DNA. Furthermore, the proteins may also show DNA condensing properties because of their similarities with eukaryotic histones and eubacterial histone-like proteins helping the compaction of intracellular DNA. Study on the archaebacterial histones and their comparison with eubacterial histone-like proteins and eukaryotic histones will aid in understanding the evolutionary relatedness and divergence of the three primary kingdoms of organisms. Better understanding of the packing mechanisms of DNA in prokaryotes will require a more detailed description of the composition of bacterial chromatin and the interaction between its components. The present thesis is the first step in this direction. Further work on the mechanism of interaction of these proteins with DNA resulting in DNA duplex stabilization would be interesting and worthwhile. Studies on archaebacterial chromatin similar to those on the eukaryotic chromatin and its comparison with eubacterial chromatin will also help in understanding the radical difference in the organization of chromosomal DNA in eukaryotes and prokaryotes.

SUMMARY

Chapter I: In this introductory chapter a brief account of the DNA binding proteins isolated from prokaryotes has been presented. A literature review on Archaebacteria, and their molecular biological aspects were also given. This was followed by a brief account on the DNA binding proteins from different archaebacteria. Objectives and scope of the present investigation have also been mentioned in this chapter.

Chapter II: In this chapter, the nucleoid isolated from S. acidocaldarius has been analyzed for total nucleoid protein composition by SDS-polyacrylamide gel electrophoresis and two-dimensional polyacrylamide gel electrophoresis. The nucleoid contains several proteins with high molecular weight, but the predominant proteins are low molecular weight proteins (molecular weight 9000 to 12000) which are highly basic. Acid extract of nucleoid contain four low molecular weight basic proteins. These four proteins were purified by DNA-cellulose chromatography and CM-cellulose chromatography.

Chapter III: The four purified proteins were characterized with respect to physico-chemical properties. Immunochemical studies were also performed. Immunodiffusion studies indicated that the four proteins HSNP-A, DBNP-B, HSNP-C and HSNP-C are antigenically distinct. However cross reaction of anti HSNP-C with DBNP-B and HSNP-C; anti HSNP-C with HSNP-A and HSNP-C have been observed by western blotting. The amounts of these proteins in different cell fractions has been quantitated. The results indicate that HSNP-C and DBNP-B were present in larger amounts than HSNP-C and HSNP-A. Molecular weights of these four nucleoid proteins have been determined by SDS-polyacrylamide gel electrophoresis. The molecular weight calculated from the mobilities for these proteins were HSNP-A, 12000; DBNP-B, 11500; HSNP-C, 10500; HSNP-C', 9000. Native state of these proteins in

solution have been determined by gel filtration and by bifunctional cross linking agent, dimethyl suberimidate. The three proteins HSNP-A, DBNP-B and HSNP-C' wee found to aggregate as dimers in solution. Amino acid composition of these proteins have been analyzed and the basic nature of these proteins was reflected by the presence of high lysine content. Absorption spectra, fourth derivative absorption spectra and fluorescence spectra of the proteins have been presented. The data suggest preponderance of phenylalanine and tyrosine over tryptophan. The CD measurements indicated that the proteins have ordered secondary structural elements like helix and B-sheet structures.

Chapter IV: In this chapter data is presented on the binding properties of four purified nucleoid proteins to nucleic acids. All the four proteins were found to bind DNA and RNA to different extents. We have performed thermal denaturation studies using different DNAs and tested the influence of these proteins on DNA melting. HSNP-A, C and C' strongly stabilized different DNAs (S. acidocaldarius; E. coli; poly(dA).poly(dT) and calf thymus) against thermal denaturation. In the presence of these proteins the Tm of DNA was raised by 18-30°C. DBNP-B was not found to exert appreciable stabilizing effect on the DNAs. Double stranded RNA such as poly r(I.C) was not at all stabilized against thermal denaturation, indicating that these proteins are highly specific to DNA in the protection against heat denaturation.

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Biochimica et Biophysica Acta 949 (1988) 87-96
Elsevier

BBA 91774

Novel histone-like DNA-binding proteins in the nucleoid from the acidothermophillic archaebacterium *Sulfolobus acidocaldarius* that protect DNA against thermal denaturation

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(Received 8 July 1987)

Key words: Nucleoid; DNA binding protein; Histone-like protein; Helix stabilization; (Archaebacteria)

DNA of acidothermophilic archaebacterium *Sulfolobus acidocaldarius* has a base composition of about 40 mol% G + C content. A low intracellular salt concentration has been inferred for this organism. These features and the high optimal temperature of growth (75 °C) would have a destabilising effect on the helical structure of the intracellular DNA. Hence, the nucleoid of this organism has been isolated in order to analyse its proteins composition and to identify any protein factors responsible for stabilisation of the organism's DNA at its growth temperature. The acid-soluble fraction of the nucleoid contains four low-molecular-weight basic proteins. The four proteins have been purified to homogeneity and antibodies to these proteins have been raised in rabbits. Immunodiffusion results suggest that the proteins are antigenically distinct. Three proteins (A, C and C) stabilise different double-stranded DNA during thermal denaturation and increase T_m of DNA by about 25 C°. These proteins are referred to as helix-stabilising nucleoid proteins (HSNP). Protein B (referred to as DNA-binding nucleoid protein, DBNP-B) does not show helix-stabilising effect. None of the four proteins stabilises double-stranded RNA. The four proteins bind to native and denatured DNA to different extents as measured by DNA-cellulose chromatography and [^{3}H]DNA binding by filtration. We suggest, based on the DNA binding, histone-like and helix-stabilising properties, that the intracellular function of these proteins is to prevent strand separation of DNA at the optimal temperature of growth (75 °C).

Introduction

Eukaryotic DNA is packaged into chromatin structure with the help of the basic proteins, histones [1-3]. The amount of research investigating the eukaryotic chromosome structure is exceedingly higher in comparison with prokaryotic chromosome structure. Recently, attention has been focused to understand the structure of prokaryotic chromosome (nucleoid). RNA, proteins and the cell envelope might be important in stabilising and maintaining this highly organised structure [4]. In *Escherichia coli* at least three histone-like proteins have been found. Of these, the most abundant is HLP-II, which exists in two molecular forms, HLP-IIa and HLP-IIb [4,5]. This protein has been isolated in different laboratories as HU [6], HD [7] and NS1/NS2 [8] and has also been shown to be associated with the nucleoid [9]. The protein binds duplex DNA and forms a nucleosome-like structure [10]. Structurally homologous proteins have been isolated from a number of cubacteria [11], including *Thermus thermophilus* [12]. It is also reported that the interaction of *E. coli* HU with

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DNA results in the formation of nucleosomal structure with altered DNA helical pitch [13].

The archaebacteria comprise one of the three primary kingdoms of cellular organisms. A variety of evidence suggests that this group is distinct from the rest of prokaryotes (the eubacteria), as well as from eukaryotes, and has evolved from the progenote as a third line of descent [14,15]. Reports on the archaebacterial nucleoid structure and histone like proteins are scanty. A small basic DNA-bound protein was first detected in large amounts in *Thermoplasma acidophilum* [16,17]. The protein HTa has been shown to condense DNA into nucleosome like structure [18] and has been reported to have partial amino-acid sequence homology to eukaryotic histones H2A, H3 and *E. coli* protein HU [19]. Small basic DNA-binding proteins have also been isolated from the archaebacteria *Sulfolobus solfataricus* [20], *Methanosarcina barken* [21] and *Sulfolobus acidocaldarius* [22]. However, their association with nucleoid has not been documented. Presence of histone like proteins in the isolated DNA-protein complexes has been reported in several eu- and archaebacteria by electrophoresis. The archebacterial proteins did not show any immunological relatedness to eubacterial DNA-binding protein HU [23]. In all these cases, the physiological role of these proteins is not known.

We have chosen *Sulfolobus acidocaldarius* to analyse its nucleoid structure. *S. acidocaldarius* lacks a rigid cell wall [24] and exists presumably in osmotic equilibrium with its environment. Therefore intracellular salt concentration is expected to be low [22]. Since low ionic strength as well as high temperature (optimal growth at 75 °C) destabilise DNA, compensatory factor(s) may be present to stabilise the intracellular DNA. Therefore, in order to understand the nucleoid protein composition and structure as well as to find the factor(s) responsible for the stabilisation of the organism's DNA, we isolated the nucleoid from gently lysed cells. In this report we show that the nucleoid of *S. acidocaldarius* contains four basic, acid-soluble proteins, all of which bind to DNA but three of which strongly protect DNA against thermal denaturation. We would like to call these proteins helix-stabilising nucleoid proteins (HS-NP).

Materials and Methods

Bacterial growth. Sulfolobus acidocaldarius strain DSM 639 was obtained from the Deutsche Sammulung von Mikroorganismen, Gottigen, F.R.G. It was grown at 75 °C for 40 h with vigorous aeration in a medium containing 0.1% yeast extract, 0.1% casamino acids, 0.1% glucose, 0.02% NaCl, 0.13% $(NH_4)_2SO_4$, 0.03% KH_2PO_4, 0.025% $MgSO_4$, 0.007% $CaCl_2$ and 4.0 ml 1 M H_2SO_4 to adjust pH to 3.0 [25]. Bacterial cells were harvested after neutralising the culture with] M Tris base (4.0 to 5.0 ml/1). The yield of the cells was about 1.5 g (wet weight) per litre culture. The cell pellets were stored frozen at — 80 ° C. For nucleoid isolation, freshly harvested cells were used.

Isolation of nucleoids. Nucleoids from *S. acidocaldarius* were isolated by a procedure described for *E. coli* [26,27] with some modifications. Cells (2 g) were suspended in 4.0 ml 10 mM Tris-HCl (pH 7.6)/100 mM NaCl and were lysed by the addition of 4.0 ml 10 mM Tris-HCl (pH *1.6)/]%* Nonidet P-40 (Shell)/2 mM spermidine-HC1/10 mM Na_2EDTA and incubated at 10 °C for 30 min. The lysate was centrifuged at 1000 X *g* for 5 min. The cleared lysate was layered on 15-50% linear sucrose gradients in 10 mM Tris-HCl (pH 7.6)/3 mM magnesium acetate and centrifuged at $10\,000 \times g$ for 20 min. The white opalescent band of nucleoid was visible at two-thirds distance from the top of the tube (Fig. 1). The gradient was fractionated by collecting 2-ml fractions. The fractions were analysed for ultraviolet absorption and DNA estimation by the ethidium fluorescence assay [28].

Isolation of acid-soluble nucleoid proteins. Peak fractions of the nucleoid from the sucrose gradients were pooled and centrifuged at 15 000 X g for 30 min. The nucleoid pellet was dissolved in 10 mM Tris-HCl (pH 7.6)/150 mM KC1/7 mM 2-mercaptoethanol and dialysed against 0.25 M H_2SO_4 for 12 h at 4 ° C. The precipitated protein was removed by centrifugation and the clear supernatant (acid extract of nucleoid) was dialysed against 10 mM Tris-HCl (pH 7.6)/150 mM KC1/7 mM 2-mercaptoethanol. This fraction contained the four acid-soluble nucloid proteins. The proteins were separated by chromatography on DNA-cellulose (native calf thymus) and further

purified by CM-cellulose chromatography. We have also developed a purification procedure for these proteins directly from dilute-acid-treated post-ribosomal supernatants by a single chromatographic step on CM-cellulose. Details of the purification of these proteins will be published elsewhere (Raghavendar Reddy and Suryanarayana, unpublished data).

Measurement of [^{3}H]DNA binding to isolated proteins. E. coli DNA was labelled with [*methyl*-^{3}H]thymidine as described in Ref. 29 and the DNA was isolated according to Ref. 30. The specific activity of the DNA was 8350 cpm per μg. Binding of the proteins to [^{3}H]DNA was carried out as described [31] with some modifications. Binding mixture (100 μl) contained 20 mM Tris-HC1 (pH 7.6), 150 mM KC1, 6 mM 2-mercaptoethanol, 3 μg native or denatured [^{3}H]DNA, 20 μg ovalbumin and the indicated amounts of nucleoid proteins. After incubation at 30 °C for 10 min the assay mixture was diluted with 1 ml buffer (same salt composition as assay mixture) and filtered through nitrocellulose filters (previously boiled for 10 min in Tris-HCl (pH 7.6)/6 mM 2-mercaptoethanol). The filters were washed with additional 1.0 ml buffer, dried and counted for radioactivity in Beck man LS-1800 liquid scintillation counter.

Thermal denaturation of DNA. Thermal denaturation profiles of DNA in the absence and presence of protein were obtained by heating DNA in 300 μl mM Tris-HCl (pH 7.2)/25-50 mM NaCl. Absorbance measurements at 260 nm were done in a Gilford Spectrophotometer (Model 250) equipped with a thermoprogrammer (Model 2527). The rate of heating was 1 C° per min. The melting curves were recorded simultaneously. Before heating was started, protein was added to DNA, gently mixed and incubated for 5 min at the starting temperature. Separately, buffer blank and protein solutions were also heated to observe any temperature-dependent variation in absorbance, which was found to be negligible.

Electrophoresis. Polyacrylamide gel electrophoresis in the presence of SDS was performed using 18% gel slabs as described in Ref. 32. The ratio of acrylamide to bisacrylamide was 30 : 0.3. Acid-urea polyacrylamide gel electrophoresis was performed in *4%* cylindrical gels as in Ref. 33.

Antibodies to nucleoid proteins. Antibodies to purified nucleoid proteins were raised in rabbits and OuchterJony immunodiffusion tests were performed as described before [34].

Nucleic acid and protein determination. DNA in isolated nucleoid was determined according to Burton [35], and RNA by the orcinol method [36]. Protein concentrations were determined by the folin reagent [37] using bovine serum albumin as standard for total nucleoid protein and *E. coli* protein NS1/NS2 [8] for purified nucleoid proteins.

Materials. Calf thymus native DNA-cellulose. Calf thymus native DNA, poly(dA)-poly(dT) and nuclease-free sucrose were purchased from Sigma, U.S.A. DNA from *S. acidocaldarius* was isolated as described in Ref. 30.

Results

Purification of the acid-soluble nucleoid proteins

Gentle lysis of *S. acidocaldarius* using nonionic detergents and centrifugation of the lysate through sucrose gradients separates the rapidly sedimenting DNA-protein complex, nucleoid (Fig. 1). The relative amounts of DNA, RNA and protein in the nucleoid were found to be in the ratio of 1 : 0.2 : 7 by weight, respectively. The nucleoid has several polypeptides associated with it as shown by polyacrylamide gel electrophoresis in the presence of SDS (Fig. 2a). Dilute acid treatment of the nucleoid results in the precipitation of most of the proteins. The acid extract contains four polypeptides as analysed by SDS-gel electrophoresis (Fig. 2). The amount of acid-soluble proteins in the nucleoid was estimated to be 90% of the weight of DNA. We tried to separate the four proteins by double-stranded DNA-cellulose chromatography. The column was developed by stepwise elution with increasing salt concentration. The two proteins, HSNP-A and HSNP-C′, were eluted with 0.3 M KC1 buffer. The DBNP-B and HSNP-C were eluted with 0.6 M KG buffer. CM-cellulose chromatography of the 0.3 M KC1 eluate was performed to resolve HSNP-A and HSNP-C′. Similarly, the 0.6 M KC1 fraction was chromatographed on CM-cellulose to separate DBNP-B and HSNP-C. We have also developed purification of all the four proteins in a single step di-

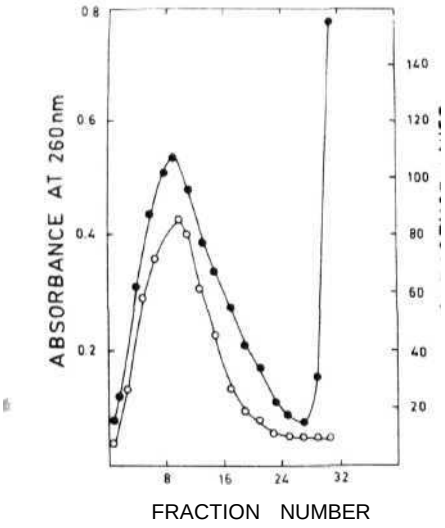

Fig. 1. Sucrose density gradient profile of nucleoid from *S. acidocaldarius* lysate. Bacterial cells were lysed as described in the Materials and Methods section and the cleared lysate was layered on to sucrose gradients and centrifuged as described therein. Fractions (2 ml) were collected from the top by aspiration. The fractions were analysed for ultraviolet absorption (•) by diluting aliquots (0.1 ml) with 10 mM Tris-HCl (pH 7.6)/3 mM magnesium acetate. DNA content (○) in the fractions was determined, using 50 μl samples, by the ethidium bromide fluorescence assay. 10 fluorescence units corresponded to 0.1 μg of DNA.

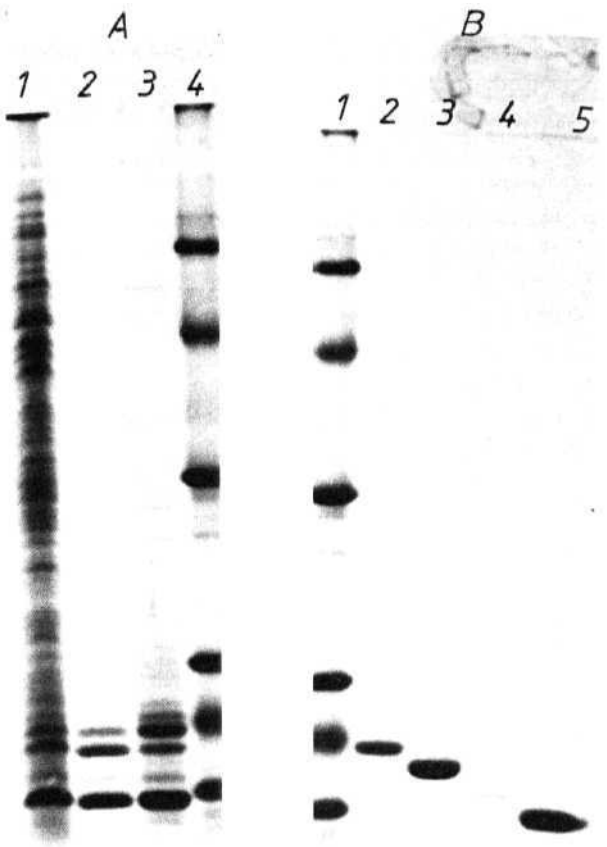

Fig. 2. SDS-polyacrylamide gel electrophoretic analysis of nucleoid proteins. (A) Lane 1, 30 /xg protein of nucleoid peak fraction from sucrose gradient; lane 2, 12 μg protein of acid extract of the nucleoid; lane 3, 40 μg protein of acid extract of post ribosomal supernatant; lane 4, molecular weight standard proteins (bovine serum albumin, ovalbumin, chymotrypsinogen, myoglobin, cytochrome *c* and *E, coli* protein NS). (B) Lane 1, Molecular weight standard proteins as in (A); Lanes 2-5, 5 μg each of purified HSNP-A, DBNP-B, HSNP-C and HSNP-C', respectively.

rectly from acid-treated post ribosomal supernatant (Fig. 2a) by chromatography on CM-cellulose. Details of the purification of these proteins and their physicochemical properties will be published separately (Raghavendar Reddy and Suryanarayana, unpublished data).

Some properties of the purified proteins

Fig. 2b shows the polyacrylamide gel analysis of purified proteins. The molecular weights calculated from the mobilities for these proteins are HSNP-A, 12000; DBNP-B, 11 500; HSNP-C, 10 500; and HSNP-C, 9000. Acid-urea polyacrylamide gel electrophoresis showed that each protein migrated as a single band. HSNP-C and -C migrated as the most basic proteins. The relative abundance of these proteins in the nucleoid and cell extract was determined by single radial immunodiffusion. HSNP-C and DBNP-B were the most abundant, and HNSP-C and -A were present in lesser amounts. Fig. 3a shows immunodiffusion results of the cross-reaction of nucleoid with antisera to HSNP-A, DBNP-B, HSNP-C and HSNP-C. In all cases precipitation lines were formed, indicating the presence of these proteins in the nucleoid. Immunodiffusion tests were also performed to observe the cross-reaction of antibody to one protein with the other three proteins. The results in Fig. 3b shows that each antibody cross-reacted with its corresponding antigen and did not cross-react with other three

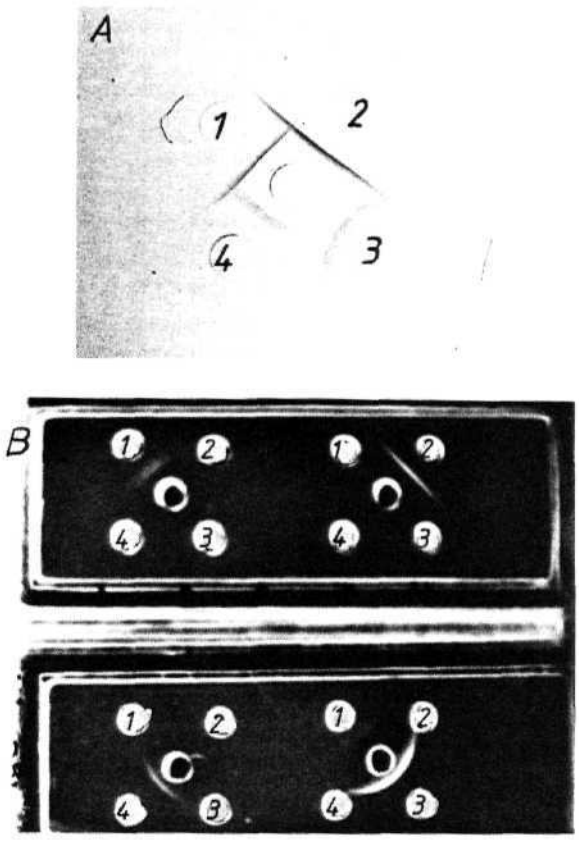

Fig. 3. Ouchterlony immunodiffusion test for the nucleoid proteins. (A) The central well contained 120 μg protein of nucleoid fraction from sucrose gradient. Outer wells 1-4 contained 20μl each of antiserum to HSNP-A, DBNP-B, HSNP-C and HSNP-C, respectively. (B) The central wells contained 20 μl each antiserum to HSNP-A (top left), DBNP-B (top right), HSNP-C (bottom left) and HSNP-C (bottom right). Wells 1-4 contained 5 μg each of HSNP-A, DBNP-B, HSNP-C′ and HSNP-C, respectively.

proteins, indicating that the four nucleoid proteins are antigenically distinct.

Binding of [^{3}H]DNA to purified proteins

The fact that these proteins were associated with the nucleoid and could be purified by DNA-cellulose chromatography suggested that these proteins have affinity to DNA. The binding of individual proteins to DNA was measured by mixing the proteins with *E. coli* [^{3}H]DNA and after incubation, DNA-protein complexes were retained on nitrocellulose filters and the radioactivity was determined (Fig. 4a, b). HSNP-A bound native DNA strongly and about 80% of the input radioactivity was retained on the filter in the presence of 8-10 μg protein. However, HSNP-A showed weak binding to denatured DNA. DBNP-B bound equally efficiently to both native and denatured DNA. Greater than 90% input DNA was retained on the filter in the presence of 0.8 to 1 μg of this protein. In the case of HSNP-C. substantial binding of both native and denatured DNA was also observed and the binding curves were sigmoidal. However, HSNP-C, which is one of the most abundant proteins of the nucleoid, did not show any binding to either native or denatured DNA. We have not detected any binding of HSNP-C by this assay, even up to a protein/DNA ratio of 13. HSNP-C, however, bound to DNA-cellulose, and this binding was observed only in the presence of 2-mercaptoethanol.

Thermal denaturation of DNA and protection by the isolated proteins

We performed thermal denaturation studies using different DNAs, and tested the influence of these proteins on DNA melting. The T_m of *S. acidocaldarius* DNA at 0.025 M NaCl was found to be 70.5°C (Fig. 5). Addition of HSNP-A dramatically increased the T_m, and with increasing amounts of protein there was increase in the T_m of DNA. Very little melting of the DNA was observed even at 94°C at a protein to DNA ratio (w/w) of 1:1 (Fig. 5a). Similar results were obtained with the addition of HSNP-C (Fig. 5c) and HSNP-C (Fig. 5d), although the extent of stabilisation was less than that obtained with HSNP-A. A slightly higher amount of HSNP-C (protein to DNA ratio of 2:1) was needed to observe protection comparable to that with HSNP-A. HSNP-C showed a strong stabilising effect and the T_m was shifted by +21.5 C° in the presence of the protein (HSNP-C′/DNA ratio of 1 : 1). DBNP-B, although showing strong binding to native and denatured DNA, did not show such a stabilising effect under these conditions (Fig. 5b). Melting studies of native nucleoid were also performed. When an amount of nucleoid corresponding to 20 μg DNA was used there was hardly any melting, as indicated by the small increase in A_{260}. The curve obtained was similar to that obtained with *S. acidocaldarius* DNA in

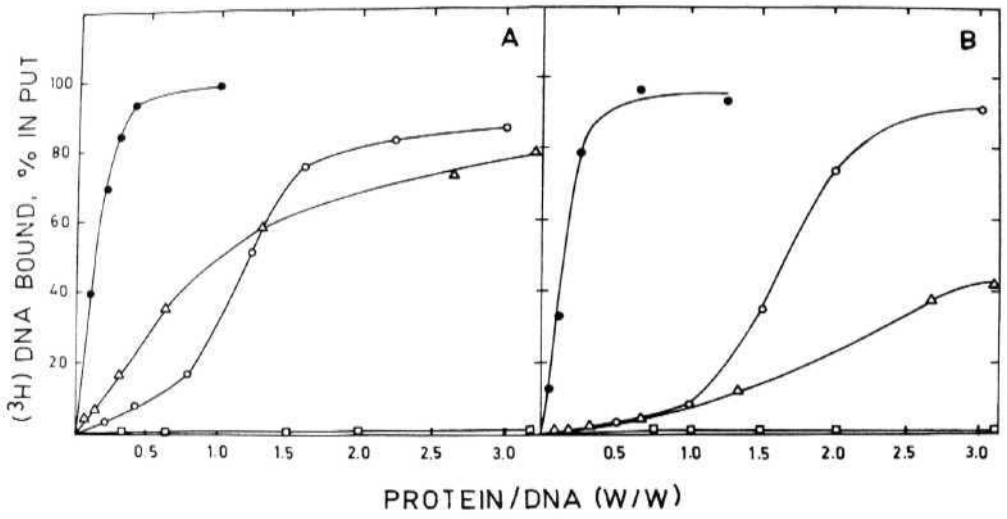

Fig. 4. Binding of nucleoid proteins to native and denatured *E. coli* [^{3}H]DNA. Binding of [^{3}H]DNA to nucleoid proteins was measured by nitrocellulose filtration technique (see Materials and Methods). (A) Binding to native DNA; (B) binding to denatured DNA. A, HSNP-A; •, DBNP-B; ○, HSNP-C and □, HSNP-C′.

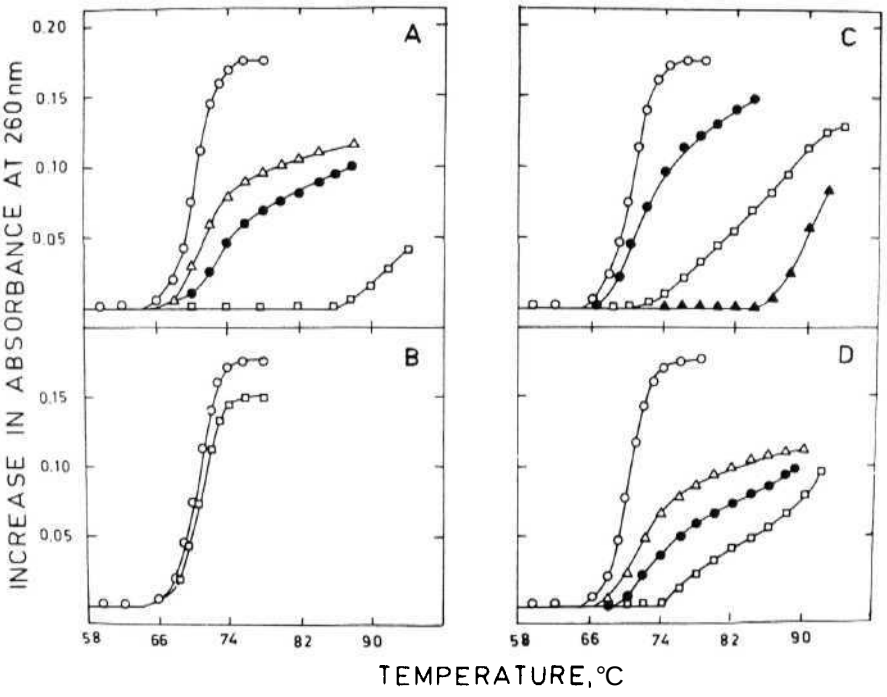

Fig. 5. Effect of nucleoid proteins on the melting profiles of *S. acidocaldarius* DNA. Varying amounts of nucleoid proteins were added to 10 μg of DNA and incubated at the starting temperature for 5 min before heating at a rate of 1 C° per min. A_{260} increase was continuously recorded. Panels A, B, C and D are with HSNP-A, DBNP-B, HSNP-C and HSNP-C, respectively. ○, No proteins; A, 2 μg; •, 5 μg; □, 10 μg; •, 20 μg.

the presence of HSNP-A (protein : DNA ratio, 1 : 1, Fig. 5a).

In Fig. 6 thermal denaturation profiles of calf thymus DNA (A) *E. coli* DNA (B) and poly(dA)-poly(dT) (C) were given. In these cases also we observed similar effects of these proteins when added in increasing amounts. Denaturation profiles with maximum amounts of proteins added are presented. HSNP-C was found to exert a maximim stabilising effect with all the three DNAs. An increase in T_m of 20-25 C° was obtained in the presence of the protein. HSNP-C and A behaved similarly with both calf thymus and *E. coli* DNA. Increases in T_m by 15 and 17.5 C° of *E. colt* (DNA/protein, 1 : 1, w/w) and 15 and 14 C° of calf thymus DNA (DNA/protein, 1:2, w/w) were obtained with HSNP-C and HSNP-A, respectively. HSNP-C was relatively less effective with poly(dA)-poly(dT) than HSNP-A and HSNP-C. DBNP-B, however, could not stabilise poly(dA)-poly(dT) (Fig. 6c). DBNP-B gave a small but significant increase in T_m (4C°) of *E. coli* DNA. However, this increase was very much lower than the increase with other three proteins. The protection by these proteins was found to be highly specific to DNA. Double-stranded RNA such as poly(I • C) was not at all stabilised against thermal denaturation (Fig. 7).

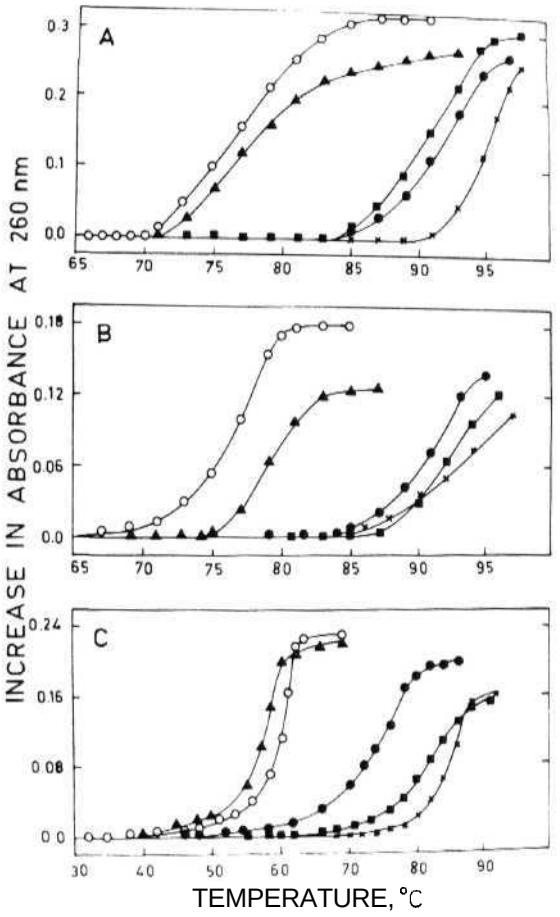

Fig. 6. Effects of nucleoid proteins on the melting profiles of (A) calf thymus DNA, (B) *E. coli* DNA and (C) poly(dA)-poly(dT). Melting profiles were obtained as described in Materials and Methods and legend to Fig. 5. Only those melting profiles with the maximum amount of each protein added are given. Calf thymus DNA used was 20 μg and *E. coli* DNA and poly(dA)• poly(dT), 10 μg each. In (A) 40μg of each protein and in (B) and (C) 20 μg of each protein was used. ○, No protein; •, HSNP-A; A, DBNP-B, •, HSNP-C; X, HSNP-C.

We have also tested the influence of these proteins at different salt concentrations. At higher salt concentration (e.g., 0.1 M NaCl) there was still a significant shift in melting curves and no melting of DNA was observed at a protein/DNA ratio of 2. In the absence of NaCl, the extent of protection was significantly higher and DBNP-B also showed a greater stabilising effect. When the proteins were added in combination we observed an additive effect (results not shown).

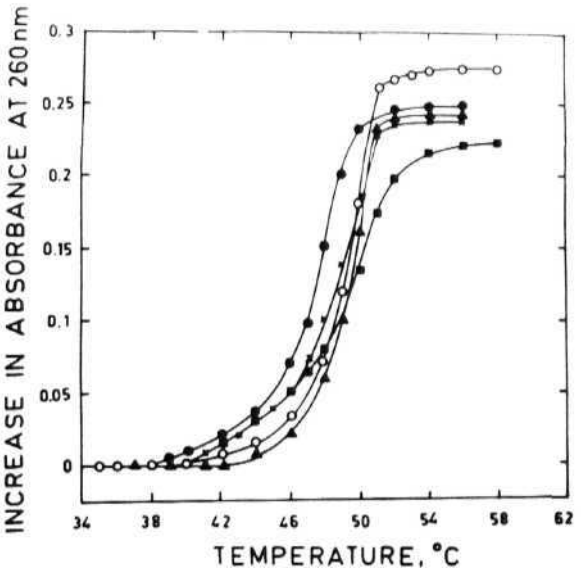

Fig. 7. Effect of nucleoid protein on the melting profile of poly(rI)·poly(rC). Melting profiles were obtained as described. The amount of poly(rI)poly(rC) used in each experiment was 10 μg. ○, No protein; •. 20 μg HSNP-A; A, 20 μg DBNP-B; •. 20 μg HSNP-C; x, 20 μg HSNP-C.

Discussion

Nucleoid isolated from gently lysed *S. acidocaldarius* cells has four acid-soluble proteins associated with it. We have observed that *S. acidocaldarius* cells are very easily lysed by dilute nonionic detergent solutions. This is probably because of absence of rigid cell wall structure [24]. The procedure described for the isolation of nucleoid from *E. coli* can be successfully employed for the isolation of rapidly sedimenting nucleoid from this archaebacterium. We have not used any further purification of the nucleoid because of the possibility of removal of some proteins which are genuinely associated with DNA. The proteins HSNP-A, -C and -C and DBNP-B have all been obtained in homogeneous form and results using antibodies to these proteins suggest that these proteins are antigenically distinct. In the native state, DBNP-B behaves as a dimer, HSNP-C as a monomer. Ami no-acid analysis data on these proteins indicate similarities with eukaryotic histones. HSNP-A, HSNP-C and HSNP-C, which strongly stabilise DNA, have very high lysine content (19-24 mol%). Detailed physicochemical properties of the proteins will be published elsewhere (Raghavendar Reddy and Suryanarayana, unpublished data). DNA-protein complexes isolated from several arachaebacteria and eubacteria have been analysed by electrophoresis, and the presence of two species of histone-like proteins of M_r 9300 and 6200 in *S. acidocaldarius* has been reported [23]. The isolation of small basic DNA binding proteins has been reported from other arachaebacteria, *Sulfolobus solfataricus* [19] and *Methanosarcina barkeri* [20]. However, their relation to the proteins we described here is at present not known.

All the four proteins of *S. acidocaldarius* showed some DNA-binding property as measured by DNA-cellulose chromatography and radioactive DNA binding. The binding properties differed from one protein to another. DBNP-B showed strong binding to both native and denaturated DNA. It also showed strong binding to RNA (e.g., poly(U)). The binding to native and denaturated DNA showed saturation at protein to DNA ratio of 0.5 (w/w), indicating binding to 23 bp native DNA per protein molecule (monomer) or 46 bases of denatured DNA per protein molecule. Strong binding of HSNP-C to both native and denatured DNAs was observed; the binding curves were sigmoidal, indicating cooperative binding of HSNP-C to DNA. At saturation, binding of 4.35 bp of native DNA per protein molecule and 6.25 bases of denatured DNA per protein molecule was indicated. HSNP-A showed strong binding only to native DNA and very weak binding to denaturated DNA. HSNP-C showed no binding to either native DNA or denatured DNA by this assay. Binding was, however, observed to native DNA-cellulose in the presence of 2-mercaptoethanol. In the absence of 2-mercaptoethanol, the protein bound weakly to DNA-cellulose. The helix-stabilising effect of this protein also suggests that this protein binds native DNA. These results indicate that HSNP-C DNA complexes may not be retained on nitrocellulose filters or that the complexes readily dissociate during filtration and washing. We have also tested binding of these proteins at elevated physiological temperature (70 °C). There was no appreciable difference in the DNA-binding properties.

Three of the four proteins, HSNP-A, -C and -C, showed a strong helix-stabilising effect. Even at 25 50 mM NaCl concentration the proteins protected DNA against thermal denaturation. At weight ratio of 1 2 (protein/DNA) there was very little melting of the DNA, even at 90 °C. The amount of these proteins, which are the only acid-soluble proteins, is 90% of the weight of DNA in the nucleoid. Therefore, the observed protection of the proteins is physiologically significant. As mentioned in the introduction, low intracellular salt concentrations and high growth temperature could have a destabilising effect on the intracellular DNA of this organism of low $G + C$ content, about 40% [38]. The DNA in isolated nucleoid of *S. acidocaldarius* does not appreciably melt when heated, even up to 95 °C. Our results indicate that the proteins, HSNP-A, HSNP-C and HSNP-C, may be responsible for the stabilisation of *S. acidocaldarius* DNA at the optimal growth temperature of the organism (75 °C). Although eubacterial histone-like protein (NS or HU) condenses the duplex DNA into a nucleosomal structure, it could not protect the DNA against thermal denaturation to any appreciable extent. An increase in the T_m of *E. coli*

DNA of 4 C° was observed when protein NS was added at protein/DNA ratio (w/w) of 4 (Ref. 39 and our unpublished results). A small DNA-binding protein from the thermophilic eubacterium *Thermus thermophilus* has been purified which has been shown to affect marginally the melting profile of DNA. A histone-like protein (HTa) from *T. acidophilum* showed a stabilising effect on DNA against thermal denaturation [16,17]. However, the organism's optimum growth temperature is 59 °C, the temperature at which DNA would not start to melt appreciably even in the presence of low salt (e.g., 25 mM NaCl). The authors suggested that the protein HTa may protect the DNA when the organism is transiently exposed to elevated temperatures (80 °C). A histone-like protein (HSa) from *S. acidocaldarius* was isolated in order to look for a stabilising factor against thermal denaturation of the organism's DNA. However, HSa did not protect DNA against thermal denaturation [22]. The authors in Ref. 22 concluded that the problem of how *S. acidocaldarius* keeps its DNA double-stranded remained unsolved (see also Ref. 15). The protein they studied could well be the DBNP-B we have reported here. So, among the DNA-binding proteins isolated till now from eubacteria and archaebacteria, the proteins HSNP-A, HSNP-C and HSNP-C′ arc unique in their helix-stabilising property, which is physiologically very important. Future work on the mechanism of interaction of these proteins with DNA resulting in DNA duplex stabilisation would be interesting and worthwhile.

Furthermore, the proteins reported here resemble eukaryotic histones in several respects, viz., (i) association with intracellular DNA, (ii) acid solubility, (iii) low molecular weight, (iv) high basicity (HSNP-C and C′), (v) amino-acid composition, and (vi) in the protection of DNA against thermal denaturation [40]. Preliminary results of an immunodot-binding assay (results not shown) showed a strong reaction of DBNP-B antibodies, and a weak reaction of HSNP-C and -C′ antibodies with a mixture of calf thymus histones (Raghavendar Reddy and Suryanarayana unpublished data). Further work is in progress to identify the histones homologous to *S. acidocaldarius* nucleoid proteins by electroimmunoblotting.

Acknowledgements

We thank Professor K. Subba Rao, Dean, School of Life Sciences, for encouragement and support. T.R.R. is a Junior Research Fellow in a research scheme sponsored by the University Grants Commission (UGC), New Delhi. We thank the UGC for financial assistance.

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