Characterisation and nucleic acid binding properties of a helix stabilising nucleoid associated DNA binding protein HSNP-C from the thermoacidophilic archaeon *Sulfolobus acidocaldarius*

Thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

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Dedicated to the memory
of my father

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Certificate

This is to certify that the thesis entitled **Characterisation and nucleic acid binding properties of a helix stabilising nucleoid associated DNA binding protein, HSNP-C from the thermoacidophilic archaeon, *Sulfolobus* *acidocaldarius*** is based on the results of the work done by Ms **Francina Celestina** for the degree of **Doctor of Philosophy** under my supervision. This work has not been submitted for any degree or diploma of any University or Institution.

Prof. A. R. Reddy
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DECLARATION

I hereby declare that the work presented in the present thesis entitled, **Characterisation and nucleic acid binding properties of a helix stabilising nucleoid associated DNA binding protein, HSNP-C from the thermoacidophilic archaeon *Sulfolobus acidocaldarius is*** entirely original and was carried out by me under the guidance **of Prof. T. Suryanarayana,** Department **of** Biochemistry, University of Hyderabad, Hyderabad, INDIA. I also declare that this has not been submitted before for the award of degree or diploma from any University or Institution.

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Acknowledgements

I wish I'd get the right word to express the enormous amount of respect, gratitude and the overwhelming fullness in the pursuit of knowledge, I owe to **my** mentor, ideal and guide, **Prof. T. Suryanarayana, but** for whose kind co-operation, patience and endurance, this thesis would have been an impossible task. Being a perfectionist to the core, he has led me triumphantly through the meticulously knit path of knowledge flanked by hardwork and determination towards the grandeur of success. I am truly indebted to him for all that he has done during the tenure of my Ph.D.

I wish to thank **Prof.** A. **R. Reddy,** Dean, **Prof. K. Subba Rao, Prof. P. R. K. Reddy** and **Prof. N. C. Subrahmanyam,** former Deans, School of Life Sciences for providing the required facilities for my Ph.D program. I also owe my sincere thanks to **Dr. A.** S. **Raghavendra, Dr. Kolluru V. A. Ramaiah, Dr. Mohan C. Vemuri, Prof. Radhakrishna Murthy** and **Prof. R. P. Sharma,** for their timely help, inhesitant and kind co-operation in some of my most crucial moments.

I owe my heartfelt thanks also to **Prof. T. Malati** (NIMS) for the kind hospitality and affectionate concern shown towards me during the tenure of my Ph.D. I wholeheartedly acknowledge the constant encouragement and the genuine concern of **Syamala teacher** and **Mr. C. Vivekanandam.**

I gratefully acknowledge the help provided by **Dr. Lurz,** Max Planck Institute, Germany, in carrying out electron microscopic studies.

I wish to thank my colleagues, **Dr. T. Raghavendar Reddy, Dr. K. Sreenivas** and **Dr. K. Ravi,** for helping me in the initial stages. I also thank **Srinivas, Nirmala, Srividya, Sivakumar** and **Prem Kumar** for their lively company and kind co-operation in the lab.

I am deeply touched by the sincere and wholehearted affection of my beloved friends particularly, **Rampalli Srinivas, Sarada, Padma Sree, Madhavi, Savithri and Manju.** 1 have benfited enormously living in the warmth and light of their friendship that helped me move triumphantly towards the path of success. I also wish to extend my thanks to **Naresh, Venkat, Kasyapa** and **Karthi** for their help. I wish to specially thank **Mr. Pattabhiraman** for helping me in my necessities.

Finally, I think it hardly needs to be mentioned that I would have been crippled if I hadn't received the immense amount of moral support, encouragement and the timely assistance from my beloved **family members,** who have gone through each **pain that I** felt and every tension I've experienced and have wholeheartedly encouraged me in all my endeavours.

Financial assistance from **University Grants Commission, New Delhi,** is gratefully acknowledged.

Francina Celestina

ABBREVIATIONS

nm	nano meters
bp	base pairs
cm	centimeter
CM-	Carboxy methyl-
DEAE-	Diethyl aminoethyl-
DFDNB	Difluoro dinitro benzene
DMS	Dimethyl suberimidate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dsDNA	double stranded DNA
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetate
g	Centrifugal field (number of times g)
HSNP-	Helix stabilising nucleoid protein -
HCHO	formaldehyde
IgG	Immunoglobulin G
Mr	Molecular mass
kDa	kilo daltons
NP-40	Nonidet P-40 (non ionic detergent)
O.D	optical density
poly (U)	poly ribouridylic acid

poly (A)	poly riboadenylic acid
PMSF	phenyl methyl sulfonyl fluoride
RNase	Ribonuclease
RPM	revolutions per minute
R_f	distance migrated relative to the solvent front
SDS	sodium dodecyl sulphate
Tris	tris (hydroxy methyl) aminomethane
TEMED	N, N, N', N'-tetramethylethylenediamine
UV	Ultra violet

CHEMICALS

Double stranded DNA-cellulose, Calf thymus DNA, NP-40, Spermidine-HCl, Alumina, DNAse-I, RNAse-A. Bovine serum albumin, Ovalbumin, Cytochrome-c, Coomassie **brillaint** blue R-250, Ethidium bromide, Phenyl methyl sulfonyl fluoride, Sucrose, Dimethyl suberimidate, **Agarose,** calf thymus histones were purchased from Sigma Chemical Company, USA. Poly (U) **sepharose,** Poly (A)-sepharose, Sephacryl **S-200,** Sephadex G-100 were procured from Pharmacia Fine chemicals, Sweden. Acrylamide, N-N methylene bis acrylamide were purchased and SDS were purchased from Serva Company, West Germany. Triethanolamine was purchased from Merck, West Germany. Tris was from Spectrochem. Bombay and Glycine from Merck/Qualigens, Galxo laboratories. TEMED, 2-mercaptoethanol were from Sisco research laboratories. Bombay. Complete and incomplete freund's adjuvants, **bactotryptone,** yeast extract, casamino acids were purchased from Difco laboratories, Detroit, Michigan, USA. CM-cellulose, DEAE-cellulose were purchased from Whatman, England. Nitro-cellulose sheets were procured from Millipore, France. Anti-rabbit IgG (goat), lambda DNA were from Bangalore Genei, India. All other chemicals used in the course of this study were of local analytical grade.

Chapter-1
INTRODUCTION

INTRODUCTION

The present thesis deals with the helix stabilising nucleoid associated protein, ***HSNP-C*** from *Sulfolobus acidocaldarius* and its role in the condensation and organisation of genomic DNA of the organism DNA compaction in eukaryotes is essentially achieved with the help of histones H2A, H2B, H3 and H4 forming nucleosome structures. However, no such defined structures, have been reported from any of the prokaryotic organisms (archaea or eubacteria) so far. Except for HTa from *Thermoplasmaacidophilum*, which compacts around 40 bp of DNA into globular structures (Searcy and DeLange. 1980), not much is known about DNA-protein interactions with respect to compaction of DNA at the genomic level Hence, our study of ***HSNP-C*** and its interaction with DNA may help in understanding the strategy that is involved in the mechanisms of DNA packaging in *Sulfolobus acidocaldarius* in particular and *archaea* in general

The concept that unicellular prokaryotes have given rise to more and more complex multicellular eukaryotes (Woese and Fox. 1977). was contradicted by Woese and his collaborators on the basis of 16S rRNA sequence phylogenetic index Henceforth, organisms have been categorised into three distinct kingdoms/genomic lineages viz.. *eubacteria*, *eukarya* and the new group *archaea* (Woese *el ai,* 1983; 1990). Each of these kingdoms is believed to have arisen independently from an ancestral progenote *Archaea* (previously known as archaebacteria), as a third form of descent from the ancestral progenote. has revolutionised our concept of prokaryote-eukaryote relationships However, efforts are being made to study the archaeal characteristics so as to understand the nature of relationships between the different categories of living organisms

Diversity within the Archaeal kingdom:

Within the archaeal kingdom there is a diversification into three major groups They are the methane producing *methanogens*, **the** sulphur dependent *thermoacidophiles* and the extreme *halophiles*. All **methanogens,** isolated, grow best near neutral pH These are abundant in mesophilic and **thermophilic** environments The most **extremely** thermophilic species, *Methanococcujanesch,* has a growth optimum at 86 °C (Jones ***et*** *ai,* 1984). The lowest temperature reported so far is 20 °C for *Methanogenium cariaci* (Romesser ***et*** *ai,* 1979). No psychrophilic methanogens have been reported so far These organisms are obligate methane producers and comprise two classes of organisms The second group of organisms, the thermoacidophiles, grow in extremes of temperatures and pH Extensive 16S rRNA-DNA cross hybridisation studies showed that *Sulfolobales* and *Thermoplasmales* form a distinct second branch of the archaeal urkingdom (Tu *et ai.* 1982) They have such distinct properties that a few people believe that they should be separated out as the fourth kingdom-the *eocytes* (Lake *et ai,* 1984). typically metabolizing sulphur by chemically reducing it with hydrogen to **form** hydrogen sulphide (Lake, 1989). The ubiquity of **the** thennophilic characteristics and their predominance as the most primitive and the most slowly evolving branch of the archaeal tree have led to the proposal that thermophily may be the ancestral character of archaea (Wheelis *et ai,* 1992). The third group of organisms, halophiles. are predominantly responsible for the reddening and rotting of salted meat and fish Only two major classes have been recognised in the case of halophiles *viz.*, the rod shaped/pleiomorphic isolates which include halobacteria and the coccoid types which include halococci *Halobacteria* at present include ten species (Oren, 1983) Bacteria of the genus *Halobacteriacea* are obligatory halophiles that survive only in extremely high salt

concentrations by accumulating even higher amounts of intracellular salts (Christian and Walther. 1962) It is through these organisms that we have been introduced to both the unique lipids of archaea and the simplest of the biological photosystems *viz.,* the bacterial rhodopsins or the purple membrane systems The bacteriorhodopsins along with another retinal based halorhodopsin pigment (hR) present in the halobacterial membrane have revealed new ways of transducing the energy of light through biological membranes These organisms grow optimally in the temperature range of 40-45 °C No growth has been reported below 10 °C or above 50 °C so far (Larsen. 1987) In some places it is evident that halobacteria and methanogens are related from the evolutionary point of view.

As revealed by the rRNA chronometer. the archaea form a coherent phylogenetic unit that is equivalent **in** status to eubacteria. An intron was cited in the 23S rRNA gene of the arch **aeon** *Desulfovibrio mobilis* (Kjems and Garrett. 1985) However, these organisms show a great number of resemblances towards each other than with the organisms of the other two kingdoms viz., *eubacteria* and *eukarya.* Based on the similarities and differences **with** respect to eukarya/eubacteria. Wheelis *el al.* (1992), have divided archaea further into two kingdoms viz. the *euryarchaeota* that encompasses all methanogens, extreme halophiles and several lineages of sulphur metabolizing thermophiles. and the second kingdom. *crenarchaeota,* that include only a few sulphur metabolising thermophiles Infact. archaea in terms of superficial resemblance tend to incline towards the prokaryotic class but in the true sense. they possess quite a few characteristics reminiscent of the eukaryotes However. they invariably possess certain characters which are not shared by either of the two kingdoms

General Molecular Features of Archaea:

There are both gram positive as well as gram negative groups in archaea One of **the** distinguishing features separating archaea from the rest is their cell wall chemistry characterised by the lack of peptidoglycan in the cell walls such as **in** *Methanosarcina barkeri* which mainly contain D-galactosamine. D-glucuronic acid, D-glucose and acetic acid. The phosphate and sulphate content is negligible (Kandler and Hippe. 1978; Fox ***et*** *ai,* 1980). The common cell wall components such as muramic acid/peptidoglycan were never detected so far from any of the members of the archaea Instead. the archaeal cell walls are characterised by their pronounced structural diversity in the chemistry of cell wall envelopes With a few exceptions such as *Thermoplasma* and *Mcihanoplasma* that lack the presence of any kind of cell wall envelopes (Darland *el ai,* 1970. Rose and Pirit. 1981), all other archaea show several profiles of cell envelopes which have a strong characteristic feature of not having murein **in** their cell **wall** components Instead, they possess psuedomurein which is a non-sulphated acidic heteropolysaccharide in their cell wall components (Schleifer and Kandler. 1972) Archaeal lipids are characterised by the presence of isoprenoid, hydro-isoprenoid hydrocarbons and isopranyl glycerol ether lipids

Genomic structure:

Archaea exhibit a great diversity in their chromosomal **G+C** content. For instance, methanogens span a range of 27-61 mol % **G+C** (Balch *et ai,* 1979) Other groups within the archaeal kingdom such as *Thermoproteales*, *Sulfolobales*, *Thermoplasmales*etc., show G+C content from 46-60 mol% G+C (Zillig *et ai.* 1982). Certain species of archaea were reported to contain considerable amounts of satellite DNA with relatively low G+C content in their genome (Bayley and

Morton, 1978) The RNA polymerases from archaea show similarity more towards the eukaryal types Mechanisms that control **DNA** superhelicity in **halophilic** archaea appear quite similar to those of bacteria and their plasmids are negatively supercoiled as compared to the thermoacidophilic archaea (Sioud *et ai.* 1988) Presence of putative **introns** in t-RNA genes (Kaine *et ai.* 1983), and absence of ribothymidine in the Tv|/C loop of t-RNA (Best. 1978) have been reported in archaea. Antibiotics like kirromycin and pulvomycin which affect amino-acyl t-RNA binding to ribosome in eubacterial systems are ineffective as inhibitors of poly phenylalanine synthesis in *Sulfolobus solfataricus* (Friedman. 1985).

Genes encoding the two subunits of DNA topoisomerase II resembling bacterial gyrases have been isolated from the euryarchaeon *Haloferax* sp. (Holmes and Smith, 1991). All the hyperthennophilic archaea contain a particular ATP dependent DNA topoisomerase I. a reverse gyrase. which can introduce positive supercoils in DNA molecules (Kikuchi and Asai. 1984; Boutier *et ai.* 1990) Moreover, plasmids from these archaea are relaxed at growth temperatures **of** these organisms (Charbonnier *et al.*, 1992) Recently, a DNA topoisomerase II from a hyperthermophilic archaeon *Sulfolobus shibitae* was purified to homogeneity This enzyme was found to be ATP as well as Mg^{++} dependent and to possess the capacity of relaxing both negatively and positively supercoiled DNA It also showed supercoiling activities reminiscent of eukaryotes (Bergerat *et ai,* 1994). DNA topoisomerase I (DNA topoisomerase V) from another hyperthermophilic archaeon. *Methanopyrus kandleri,* has been isolated that relaxes positively supercoiled DNA (Slesarev *et ai,* 1993). An α–like DNA polymerase was purified to homogeneity from the thennoacidophilic archaeon *S. solfataricus* that has a protease hypersensitive site which gets partially cleaved

during the purification procedure due to the action of endogenous proteases (Rossi *et al.*, 1986).

Archaeal ribosomes, like those of eubacterial ones. are smaller (70S) than that of cytoplasmic ribosomes of eukaryotes (80S) (Lake *et* ***ai,*** 1984; Stoffler and Meilicke, 1986). Ribosomes from *euryarchaeotes* resemble eubacteria in having few or no **r-proteins** greater than 30 kDa, whereas the ribosomes from *crenarchaeotes* have a much higher eukaryote-like protein content with many proteins greater than 30 kDa in mass (Cammarano ***et*** *ai,* 1986). Small subunits from archaea show **similarity** towards eukaryotic subunits. i.e.. they contain a feature on the head of the subunit. known as *"**the** archaebacterialbill"* that is absent **in** eubacteria The ribosomal subunits from the *Sulfolobus* genus differ slightly from the others **in** having a small "bulb" present at the base of the subunit (Lake ***et*** *ai,* 1982) The archaeal ribosomal proteins also exhibit more amino acid sequence homology with their eukaryotic counterparts (Matheson, 1985). Comparative studies on two ribosomal proteins from the large subunit indicate that eubacterial proteins resemble their archaeal counterparts Sulphur dependent archaeal rRNAs resemble those of eukaryotes in being highly modified as compared to much lower levels of eubacterial r-RNA modifications 5S r-RNA from archaea possess both eubacterial and archaeal features. However, they resemble eukaryotes **in** most of the ways. The sulphur dependent thermoacidophiles in contrast to halophiles and methanogens have triphosphorylated 5' **termini** instead of 5' terminal monophosphates. Initiation of protein synthesis occurs via non-formylated methionyl t-RNA as is prevalent **in** eukaryotes (White and Bayley. 1972; Bayley and Morton. 1978) The DNA polymeiase gene from an aichaeon *Thermococcus littoralis* was cloned and was shown to contain two intervening sequences that form one continuous open

reading frame **with** three polymerase exons (Francine *el al.*. 1992) **Eukaryotic** rRNA genes are inferred to have evolved from the eocytes; a group of **extremely** thermophilic sulphur metabolising anucleate cells (Lake, 1989). Eocytic rRNA operons contain a 16S **RNA,** which lack a t-RNA in its spacer region and is followed by a 23S RNA The 5S RNA is elsewhere on the chromosome as in the case of eukaryotes in general (Gerbi, 1985). A dna K (hsp 70) homologue from *H. marismortui* has been cloned and sequenced. DNA K and its homologues of the 70 kDa heat-shock protein family are highly conserved molecular chaperones found in many species belonging to each of the three phylogenetic domains of the archaeal kingdom (Gupta and Singh. 1992) Another archaeal protein MC1 was isolated which is able to promote the circularization of shoil DNA fragments by T4 DNA ligase (Hodges-Garcia *el al.*, 1989. Lame *el al.,* 1991). Protein MC1 binds DNA as a monomer in a non co-operative way and can also protect DNA against thermal denaturation (Chattier *el al.,* 1989) One more interesting feature is regarding the 70 kDa protein produced as a response to heat shock **in** animals This protein (70 kDa). was found to be absent in *V. acidocaldarius* as a response of heat shock as in the case of other animals Instead, the expression of three different proteins of 66, 64 & 22 kDa molecular masses that were normally present were either increased under heat stress (>80 °C) or decreased under cold stress (60 °C) (Macario and Macario. 1994) Elongation factor **(EF-2)** is ADP ribosylated in archaea (Pappenheimer *el al.*. 1983)

Very little is known about the archaeal RNAses as of today Recently, two RNAses P (8.5 kDa and a pI of 10.1) have been identified and characterised, one from *S. solfataricus* and another from *Haloferax volcanii* (Dan **et** *al.,* 1992). Protein p3, a ribonuclease. was also isolated from *S. solfataricus* (Fusi *el al.*, 1993). The complete **primary** structure of the protein revealed close similarity to a

class of small proteins previously isolated from *S. acidocaldarius* and 5. *solfataricm* and identified as DNA binding proteins (Choli *et ai,* 1988a. 1988b). Further investigations revealed that the enzyme that was produced by a synthetic p2-encoding gene expressed in *E. coli* was endowed with RNAse activity and was catalytically indistinguishable from the native enzyme (Fusi *et ai.* 1995a). Furthermore, no alteration in the thermal **stability** was observed with the complete preservation of activity upto a temperature of 80 °C (Fusi *et ai,* 1995b)

Another RNAse from the extreme thennophile *S. acidocaldahus* was isolated by Kulms *et al.* (1995), which is a small 7 kDa protein and is characterised by posessing RNAse activity in the absence of histidine at the site of **activity.** A similar protein was detected from the archaeon *S. solfataricm* (Fusi *et ai,* 1993)

DNA **binding** proteins:

Cells produce a variety of proteins which help to manipulate the helical structure of DNA in addition to **the** function of **re-structuring** the physical **form** of DNA as a part of the vital processes of the cell. Of these, the enzymatically inert proteins such as histones/non-histones. serve to organise the structure of the genetic material in eukaryotes These proteins bind non-specifically **(irrespective** of the nucleotide sequence) to DNA in a stoichiometric manner (Geider and Hoffman-berling. 1981) and bring about changes in the structural form of the genomic DNA A major problem encountered in the case of prokaryotic cells is regarding the packaging of large DNA molecules. simultaneously allowing it to be biologically active Although it is clear that prokaryotes have organised structures of intracellular DNA, the nature of the organisation is far from clear (Ussery *et al.*, 1994). Owing to the less structured organisation and lack of genetic data that can

possibly give an edge **to** the investigation of the prokaiyotic genome organisation. our understanding of the prokaiyotic chroinatin **structure** has lagged behind that *of* the eukaryotes

The fundamental metabolic activities **occurring** inside cells of living systems. that can otherwise be referred to as vital processes, such as packaging. replication, recombination, transcription and restriction are undoubtedly based upon protein-DNA and protein-protein interactions In order to study the mechanisms involved in DNA-protein interactions, that play a major role in the structural organisation of genomic DNA and related functions. a thorough knowledge about DNA binding proteins is essential Majority of these DNA binding proteins are basic proteins known as histoncs in the case of eukaryotes and histone-like proteins **in** the case of prokaiyotic organisms On the other hand. proteins with greater affinity to ssDNA are easily distinguished from those **with** stronger affinity to dsDNA Proteins from the former category are variously viewed as DNA unwinding proteins (Sigal *et ai.* 1972). DNA extending proteins (Abdel Monam *el ai.* 1977). DNA melting proteins (Jensen and Von Hippel. 1976). single stranded DNA binding proteins (SSBs) (Meyer *el ai.* 1979) and helix destabilising proteins (Alberts and Sternglanz. 1977) Apart from these, the presence of a few acidic proteins has also been reported to be associated **with** DNA

Histone-like proteins, (HLPs), could possibly share some properties with the non histone proteins HMG1 and HMG2. which also change the helical structure of DNA (Javaherian *el ai,* 1978) On the **contrary,** there is another group of proteins known as helix destabilizing proteins. **(HDPs),** which binds only to single stranded DNA (Rouviere-Yaniv and Yaniv, 1979). *E. coli* SSB and T4 gp 32 proteins, which can remove intra-strand folding thus favouring re-annealing of

DNA, come under this category (McEntee, 1985). T4 gp 32 is analogous to *E. coli* SSB, in stimulating quite a few reactions of RecA *invitro* (Egner *el a/.,* 1987) and is required for the replication of T4 DNA.

Some of the proteins that are essentially involved in molecular reactions are those that promote renaturation. recombination and aggregation of DNA molecules. These processes go via certain inevitable intermediate steps ultimately leading to replication, repair and genetic recombinations in target cells. Proteins participating in such reactions have been reported from both prokaryotes and eukaryotes. On the basis of their ATP utilisation, they have been classified as ATP dependent and ATP independent types (Eggleston and Kowalczykowski, 1991) RecA protein from *E. coli* is the best example for a protein. which is involved in the pairing and strand exchange reactions of DNA molecules, mediated by the three strand intermediate in the process of DNA recombinational repair (Radding. 1991, West. 1992)

Histone-Like proteins from Prokaryotes:

The genome in prokaryotes may be smaller than in eukaryotes but in terms of precise organisation or efficient functioning. they are no less than their eukaryotic counterparts (Pettijohn. 1988) Unlike eukaryotic histones, bacterial histone-like proteins or "chromatin-associated-proteins", as designated by Ussery *et al.* (1994). have not been shown to interact as a unit with DNA to form complexes analogous to nucleosomes.

Eukaryotic and prokaryotic organisms are strikingly distinct in terms of the ease with which chromatin can be isolated. There is as yet no standard and effective procedure to isolate chromatin from prokaryotes contrary to the easy

isolation as in the case of the eukaryotes. This in turn obscures the certainty as to which of the proteins are instrumental, if any, in packaging bacterial DNA However, the intracellular DNA was found to exist in a nucleoid form achieved by the interaction with two or more DNA binding basic proteins and RNA (Pettijohn. 1982; 1988). The original procedure for the isolation of nucleoid was developed in the early 1970s by Stonington and Pettijohn, (1971). Worcel and Burgi, (1972) This procedure relies on the lysis of cells in the presence of sufficient counterions to stabilise DNA charge-charge repulsion. Initially, 1 M NaCl was used for the nucleoid isolation, but later experiments showed that polyamines could stabilise DNA at lower concentrations This meant that 5 mM spermidine could be used in the place of 1 M NaCl (Kornberg *el a/.,* 1974). The spermidine concentrations approximate those that are found *invivo* (Tabor and **Tabor.** 1985) The rapidly sedimentable condensed **DNA-protein** complexes are released from cells by treating with non ionic detergents in the presence of salt and spermidine (Stonington and Pettijohn. 1971; Worcel and **Burgi.** 1972; Kornberg *el al.,* 1974). Subsequent studies have led to the general conclusion that RNA does not play **any** important role in the organisation of the nucleoid. It was also inferred that the presence of RNA in high salt prepared nucleoids should be regarded as preparation artefacts (Sloof *el ai,* 1983; Kleppe *et ai,* 1984). RNA associated **with** the nucleoid was found to be exclusively nascent RNA (Schmid, 1988). An intensive study into the nature of the RNA molecules revealed that the number of *invivo* domains of supeicoiling is not diminished by rifampicin treatment of cells. Thus it was established that the nascent RNA does not stabilise nucleoid domains found *invivu* (Sinden and Pettijohn. 1981)

Although the structural and compositional details of bacterial nucleoid is not yet well defined, there is compelling evidence that bacterial DNA exists in a

compacted state attributed to the presence of atleast three proteins that bind to DNA (Rouviere-Yaniv and **Yaniv,** 1979; **Pettijohn,** 1982; Geider and Hoffman-Berling, 1981).

Out of the three proteins from *E. coli,* that have been identified as potential DNA binding proteins. the best characterised component is a small basic protein that is ubiquitously distributed **in** eubacteria **viz.,** HU or DBP-II **with** Mr 10.000 (Pettijohn, 1982). HU protein exists as a hetero-dimer and is variously referred to by different people as H2 (Cukier Kahn *et al.*, 1972), HD (Spassky *et al.,* 1984). BH2 (Varshavsky *et al.*, 1977) or NS (Suryanarayana and Subramanian, 1978). This protein is composed of two polypeptides NS1 and **NS2** whose primary structures display 69% homology HU has the ability to compact DNA. It has been reported that HU has the **ability** to form bead-like structures along the relaxed. circular simian virus 40 DNA. in the presence of a nicking closing **enzyme** (Rouviere-Yaniv and **Yaniv.** 1979) It has also been shown to stimulate transcription of A. DNA (Rouviere-Yaniv and **Gros,** 1975) The protein has been implicated in the replication of ori C locus of *E. coli* (Dixon and Kornberg. 1984) Topological assays have been used to study the wrapping of DNA by HU and also to study the co-operativity of HU-DNA interactions (Broyles and Pettijohn. 1986) In prokaryotes. sufficient amount of work has been carried out on the ternary and quaternary structures of proteins, such as NS (NS1 and NS2). Cross-linking experiments have indicated that the aggregated state of NS is the tetramer or possibly the octamer (Lammi *at al.,* 1984), with the basic functional unit consisting of heterotypic dimers (Paci *et al.,* 1984; Rouviere-Yaniv and Kjeldgard, 1979). As already mentioned earlier. the HU protein from *E. coli* exists as a dimer in solution and this dimer can be visualised as a lobster shaped structure having long arms that bind to DNA. The dimer is wedge shaped and it has been suggested

that 8-10 dimers could wrap **DNA** into a nucleosome-like structure having 80-100 bp/ turn (Bjornsti *el* ***ai,*** 1986) HU protein has been reported to influence DNA structure that results in differential recognition by regulatory proteins (Flashner and Grella. 1988). A similar protein HB has been purified from *Bacillus globigii.* The binding of the HB protein to nucleic acid is non-specific and co-operative (Imbert *et al.*, 1982).

IHF, integration host factor, essential for integration of bacteriophage λ has also been classified as histone-like protein mainly because it shares ammo acid sequence homology with HU (Nash and Robertson. 1981) Recently, it has been reported that despite similarities in structure. HU and IHF form different protein-DNA complexes with short DNA fragments (Bonnefoy and Rouviere-Yaniv. 1991). H protein (Mr 28 kDa) is estimated to be present in 30.000 copies/cell It shows resemblances **with** the eukaryotic H2A with which it also shows immunological cross **reactivity** (Bjornsti *el ai,* 1986) It was **first** recognised through its **activity** as an inhibitor of DNA synthesis *in vitro* (Hubscher *et ai,* 1980).

Another protein H1, a neutral protein with a monomeric mass of 16 kDa was detected in several forms viz.. H1a, H1b and H1c which differ in their isoelectric point. It has also been called B1 or H-NS (Bakae\. 1981). This protein was originally isolated from the high salt ribosomal wash

H-NS (also called H1) was identified as a major component of the *E. coli* nucleoid which has been implicated in the condensation of the chromosome and in the organisation of the prokaryotic nucleoid structure It has no sequence homology to other well characterised nucleoid associated proteins such as HU and IHF. H-NS protein contains many charged amino acids but overall, is a neutral protein composed of 137 amino acids (Pon *el ai,* 1988). It exists in solution

predominantly as a homo-dimer **(Falconi** *et al,* 1988) Although H-NS is characterised as a relatively non-specific DNA binding protein. its noticeable feature among other nucleoid proteins. is to exhibit a preference for curved DNA sequences (Yamada *et al,* 1990; Bracco *et al,* 1989). The transcriptional inhibition by H-NS is thought to result from its binding to the promoters which often contained curved DNA sequences (Owen-Hughes *et al,* 1992; Ueguchi *et al.,* 1993; Yoshida *et al.,* 1993). Ueguchi and Mizuno, (1993) have demonstrated that H-NS can selectively inhibit transcription of *Pro U* by *E. coli* RNA polymerase *invitro* suggesting the direct function of H-NS as a transcriptional repressor affecting early steps of transcription initiation (Ueguchi *et al.,* 1993). Inspite of such interesting properties of H-NS no structural infonnation is known upto date The roles of these proteins *invivo* are now clouded by their inter-connections with DNA topoisomerases and perhaps with each other (Ussery *et al,* 1994).

Extensive studies on the biological functions of H-NS demonstrated that it plays a significant role in the regulation of many apparently unlinked genes on the *E. call* chromosome and generally represses the expression of a variety of proteins at the level of transcription. either by binding to DNA directly or through changes in DNA topology (Hulton *et al.,* 1990. Gorannson *et al.,* 1990. Ueguchi *et al..* 1993; Ueguchi and Mizuno. 1993; Lucht *el al.,* 1994) Recently, H-NS was shown to be implicated in the post transcriptional regulation of the expression of the rpoS gene (Takefumi *et al.,* 1995)

Yet another protein is HLP-1 (Varshavsky *et al,* 1977; Lathe *et al,* 1980) of 17 **kDa** monomeric mass which is probably coded by the **fir** A gene (Schaffer and Zillig. 1973). The Fir A gene product is known to interact with RNA polymerase and is speculated to be involved in transcription. It is speculated that

the 17 kDa protein is a part of a scaffold structure, or in some other way alone or together with other proteins might be responsible for maintaining the independent domains of supercoiling observed *in vivo* (Sinden and Pettijohn, 1981)

It is a point to note that even bacteriophages encode histone-like proteins The best studied example is TF1, a protein isolated from *B. subtilis* after infection with phage SPO1. The structural gene for the transcription factor (TF1) of *B. subtilis* phage SPO1 has recently been sequenced and found to code for a 99 a.a. long protein which is 50% homologous to *B. stearothermophilus*NS It is believed that the function of TFl is to organise SPO1 DNA into a chromatin-like structure (Greene *et al.*, 1984)

Histone-Like proteins **of** Archaea:

Reports on archaeal **histone-like** proteins are still very scanty. In general, several of the archaeal DNA binding proteins show almost no resemblances to any of the proteins from the other two kingdoms Among the archaeal **histone-like** proteins studied so far. HTa from *Thernwplasma acidophilum* has been successfully characterised to a considerable extent. The protein remained bound to DNA in ionic concentrations upto 0 7 M NaCl which is more than ten times the physiological intracellular ionic concentrations of about 0.05 M K^+ (DeLange *et al.*, 1981). HTa from ***Thernwplasma*** *acidophilum* was known to condense DNA into globular structures wrapping around 40 bp of DNA. Cross-linking studies have revealed that tetramers of HTa are known to exist in the nucleoprotein preparations. HTa stabilises DNA against thermal denaturation and the effect could be as high as 40 ^{o}C (Searcy, 1986; Stein and Searcy, 1978), the protection being attributed to the presence of 5 Phe residues that appear to be buried in the hydrophobic protein core (Searcy, 1986). This protein shows significant homology

to eukaryotic histones in terms of the ammo acid composition. Electron microscopic studies reveal globular structures of nucleoprotein complexes of 5-6 nm (in diameter) involving HTa. In addition to sequence homology, the protein HTa resembles eukaryotic histones in that it protects associated DNA Nuclease protection studies indicated that the protein in the tetrameric fonn protects around 40 bp of DNA confinned by both protein cross-linking studies and measurements of **the** ratio of protein to DNA (Searcy and Stein, 1980). Moreover this protein was found to have properties similar to HU of *E. coli* and the protein has been localised in the intracellular DNA by immunochemistry (Bohnnann *et al.,* 1990) Several histonc-like proteins have been detected by electrophoresis in **the** isolated nucleoprotein complexes from archaea by Thomm *et ai* (1982)

A DNA binding protein with a molecular weight of 14.757. HMb. from *Methanosarcina barkeri* was isolated by Chartier *et al* (1985) Another protein from *Methanosarcina* sp. CH 7155 of 11 kDa was isolated which was shown to protect DNA against **thermal** denaturation. It resembles MC1 of *Methanosarcina barkeri.* Two basic proteins with Mr 14,000 (HSa) and 36.000 (NHSa) were detected from *Sulfolobus acidocaldarius* by Green *et al.* (1983), which did not show any specific DNA protection properties However. 5% of the DNA was found to be protected against nuclease digestion in the presence of these two proteins. This was followed by the discovery of a set of several small basic and abundant DNA binding proteins with molecular masses in the range of 7, 8 and 10 kDa from the same organism by Grote *et al.* (1986). These proteins were categorised on the basis of molecular weight and their elution pattern from an ion-exchange column (CM-Sepharose CL6B column). Proteins (7 kDa) exhibit micro-heterogeneity owing probably to gene amplification mediated evolution (Choli *et ai,* 1988). Lurz *et ai* (1986), have carried out election microscopic studies of the

complexes of 7, 8 and 10 kDa proteins and showed that they bind to both double stranded and single stranded DNA to form compact structures. However, except for amino acid sequencing and electron microscopic studies, no other structural and functional properties of the proteins have been so far reported One of the recent reports about the DNA binding proteins from archaea include the identification of a 7 kDa protein from *S. solfataricus* with an amino acid constitution made up of mostly lysine and glutamic acid residues. The protein was able to protect DNA against thermal denaturation and DNAse 1 digestion in a dose dependent manner (Faraone and Farina. 1995). It has also been reported that 7 kDa protein of *S. solfataricus* can be ADP-ribosylated *invitro.* The archaea] ADP-ribosylation reaction was found to be time, temperature and NAD dependent, simultaneously proving to be thermostable The acceptor proteins were modified by ADP-ribose both enzymatically, (via ADP-ribosylating enzymes) and by chemical attachment of free ADP-ribose (Faraone-Manella *et ai,* 1995).

Another DNA binding protein HMf. from *Methanothermus fervidus* was shown to compact double stranded DNA into structures reminiscent of eukaryal nucleosomes (Sandman *el ai,* 1990) Although most of the proteins have been shown to compact DNA in a manner **similar** to that of eukaryotic systems, compelling evidences for concurrently conserved nucleoprotein structures **with** a consistence equivalent to chiomatosomes of eukaiyotes has not been documented as yet However so far, quite a few archaeal proteins such as MC1 from *M. barken* (Imbert *et al.*, 1988), and HMf from *M. fervidus* have been shown to clearly bind. wrap and compact DNA molecules *inviiro.* These proteins have been shown to be localised with the genomic DNA *invivo* (Sandmann *el* ***ai,*** 1990). But in contrast to negative toroidal wrapping of DNA on eukaryotic nucleosomes and in HU-DNA complexes, binding of HMf to DNA results in positive supeicoiling

of DNA HMf binding increases the helical **periodicity** of DNA molecules to approximately 11 bp/turn and that DNA molecules in the nucleosome-like structures are constrained in positive toroidal supercoils Based on the mass of HMf needed to cause a change in the linking number and electron microscopy, it was concluded that HMf-DNA structure contains between 90 and 150 bps of DNA wrapped in 1.5 positive toroidal supercoils around a core of four HMf molecules (Musgrave *el al.*, 1991). Another protein SaRD from *S. aadocaldanus* possessing RNAse activity with pH optima between 6.5-7.0 at 65 °C was isolated. This protein was reported to have DNA binding properties also (Kulms *et al.,* 1995). It is reported to show similarity to a 7 kDa and a 8.5 kDa RNAse isolated from *S. aadocaldanus* (Fusi *et al.,* 1993). However, the protein did not show any oligomeric aggregate formations *invitro*.

Nucleoid from the thermoacidophilic archaeon *S. aadocaldanus,* was isolated for the first time by Reddy and Suryanarayana. (1988; 1989), and subsequently four nucleoid associated acid soluble DNA binding proteins were purified to homogeneity Three out of four proteins isolated. HSNP-A (12 kDa). HSNP-C (9 kDa) and ***HSNP-C'*** (8 kDa). protect DNA against thermal denaturation The fourth protein DBNP-B (11 kDa). now referred to as *Renaturase* only binds **strongly** to single and double stranded DNA The protein aggregates nucleic acids and promotes renaturation of complementary single stranded DNA Proteins HSNP-A. HSNP-C & ***HSNP-C'*** from *S. aadocaldanus* have been recently reported to be present exclusively on the ribosome-free genomic DNA of the cell. In contrast DBNP-B which binds to single and double stranded DNA and RNA *invitro* was found to be located predominantly in the cytoplasm in *S. aadocaldanus* and therefore is unlikely to be responsible for DNA compaction and genome organisation (Bohrmann *et al.,* 1994).

Role of histone-like proteins in DNA condensation:

Reconstitution experiments suggest that chromatin structure is a dynamic one. The alignment of histones H2A. H2B. H3 and H4 have been demonstrated precisely with respect to each other X-ray and neutron scattering studies have shown that chromatin has an organised structure and dominant periodicities of 110, 55, 37 and 27 °A The core is a flat particle of dimensions of about 110 x 110 x 57 °A, somewhat wedge shaped and strongly divided into two layers consistent with DNA being wound into about $1^3/_4$ turns of a flat superhelix of a pitch of about 28 °A (Finch *et al.,* 1977).

No related work has been reported so far from prokaryotes and archaea including the thermoacidophilic archaeon *Sulfolobus acidocaldarius.* Although a few of the archaeal histone-like proteins have been characterised. their role in DNA compaction is not known. However, it is known that helix stabilising nucleoid proteins HSNP-A and ***HSNP-C*** are co-localised along with the genomic DNA. Electron microscopy of the .V *acidocaldarius* chromatin released from ruptured nucleoids in the presence of potassium glutamatc and polyamines revealed the presence of spherical structures. reminiscent to nucleosomes (Bohrmann *et al.*. 1994) These structures were found to be similar to those previously observed in the case of chromatin from *T. acidophilum* (Bohrmann *et al.,* 1990), and *Halobacterium salinarium* (Shioda *et al.,* 1989) The binding of HMf or ***HSNP-C*** to double stranded DNA molecules *in vitro* causes an increase in the melting temperature of the DNA (Reddy and Suryanarayana. 1988; Krzycki *et al.,* 1990). Apart from these. another DNA binding protein (HMt) from *Methanobacterium thermoautotrophicum,* was purified and characterised that has virtually identical properties to that of HMf (Boutier *et al.,* 1990) In addition to protecting DNA against thermal denaturation, these proteins may also protect the

genomes from heat denaturation during their growth at high temperatures The possible role of these histone-like proteins in the compaction of intracellular DNA needs further investigation.

Objectives and Scope of the present Investigation :

Sulfolobus acidocaldarius, a crenarchaeote. was chosen as a system to study the DNA-protein interactions involved in and responsible for the effective condensation as well as efficient functioning of the DNA in this **thermophilic** organism. The unusual conditions required for the growth of this archaeon suggests the presence of certain intracellular factors or components that help the organism to survive under the extreme conditions which otherwise prove fatal for the rest of the organisms

Sulfolobus acidocaldarius, a thermoacidophilic archaeon was first isolated by Brock *et al.* (1972) from acid hot springs at Yellowstone National park The cells are generally spherical in nature with unusual cell wall structures devoid of peptidoglycan. It is unusual in growing optimally at pH 3.0 and temperature 75 °C. The DNA base composition (CsCl gradient centrifugation) was found to 41 mol % **G+C** content **(Zillig** *el* ***al.*** 1980).

Although several histone-like DNA binding proteins **have** been isolated and characterised from prokaryotes. their role in structural organisation of the intracellular DNA is yet to be documented. Hence, in an attempt to understand **the** thermal stability of intracellular DNA and its organisation, four DNA binding proteins have been isolated from the organism from our laboratory (Reddy and Suryanarayana, 1988. 1989). Out of the four nucleoid associated DNA binding proteins isolated from *Sulfolobus acidocaldarius*, HSNP-A. HSNP-C, ***HSNP-C'*** protect DNA against thermal denaturation while the fourth protein DBNP-B. inspite of its strong binding to both double stranded and single stranded DNA, does not show such a property. Since ***HSNP-C'*** is the most abundant of all **the** four proteins associated **with** the nucleoid. it implies that the protein could be

responsible for some very important functions concerned with the organisation of the genetic material. This aspect has been substantiated on the basis of the electron microscopic studies by Bohrmann *et al.* (1994). which show that these helix stabilising nucleoid proteins (HSNP-A and *HSNP-C'*) are exclusively localised on the genomic DNA Hence. we have attempted to study the interactions of *HSNP-C'* and DNA, using a variety of biochemical and biophysical approaches such as fluorescence and absorption spectroscopy, electron microscopy, gel retardation. DNA melting analysis, chemical modification of the specific aminoacid residues of the protein, etc..

The results obtained from the present study indicate the possible role of the protein in the condensation of intracellular DNA as well as the forces responsible for the interaction of the protein with DNA The results also pave the way for work on the mechanism of action of the protein and its role along with other proteins in the organisation of intracellular DNA

Chapter-2
MATERIALS & METHODS

MATERIALS AND METHODS :

Cell strains used in the present study :

Sulfolobus acidocaldarius DSM **639,** *Sulfolobus solfataricus* DSM 1616, *Thermoplasma acidophilum* and *Halobacterium halobium* were obtained from Deutsche Sammulung Von Mikroorganismen, Gottingen, **Germany;** *Escherichia coli* A19 was obtained from the laboratory of Dr. A. R. Subramanian, Max Planck Institute, Berlin, Germany; and *Bacillus stearothermophilus* from NCIM, Pune, India.

Growth of cell cultures:

Sulfolobus acidocaldarius was grown according to Kikuchi and Asai. (1984) in a medium containing 0.1% yeast extract, 0.1% tryptone, 0.1% casaminoacids, 0.1% glucose. 0 02% NaCl, **0.13%** $(NH_4)_2SO_4$. 0 025% $MgSO_4$. 0.03% KH_2PO_4 and 0.007% $CaCl_2$ adjusted to pH **3.0** with 10 N H_2SO_4 at a temperature of 75 °C. The growth of cells was arrested at the mid logarithmic phase (40-48h) by neutralising the culture with 1 M Tris base. The cells were harvested thereafter by centrifugation at 7000 RPM (in a GS-3 rotor of a Sorvall RC5C centrifuge) for 15 minutes The cell pellets were finally suspended **in** 10 mM Tris-Cl (pH 7.6), 50 mM KCl, 10 mM magnesium acetate and 7 mM mercaptoethanol ($TKM_{10}Me_7$), centrifuged at 8000 RPM for 30 minutes and the final pellets were stored frozen at -80 °C until further use *Sulfolobus solfataricus* was grown at 75 °C in pH 4.5 medium according to Grote *et al.* (1986) *Thermoplasma acidophilum* was grown at 59 °C in pH 1.7 according to Searcy

and Stein. (1980). *Bacillus stearothermophilus* was grown at 59 °C in an enriched medium supplemented with $MnCl_2$ (990 μg/ml) at pH 7.0 (Struck *et al.*, 1986)

Escherichia coli was grown in a medium containing 0 5% sodium chloride. 0.2% yeast extract and 1% tryptone adjusted to a pH of 7.2 with 2 N NaOH 50% glucose (separately autoclaved). was added to a final concentration of 0.5% to the medium just before inoculation The medium was inoculated with 1% inoculum culture and allowed to grow at 37 °C for 4-5 hours (Minks *et al.*, 1978). The cell growth was arrested with the direct addition of ice. The cells were collected by centrifugation and cell pellets stored frozen until further use

Halobacterium halobium was grown in a medium containing 4 31 M sodium chloride (25% w/v); 0.5% tryptone, 0.5% yeast extract, 0.02% calcium chloride and 20% magnesium sulphate with vigorous aeration at 37 °C. The cells were harvested after a growth of 40-50 hours (identified by a characteristic pink colour) by centrifugation at 6.000 RPM (Sorvall SS-34) for 15 minutes The cell pellets were washed in basal salt solution containing 25% NaCl. 0 3% sodium citrate, 0.2% KCl, 2% $MgSO_4$ and the final cell pellet stored frozen until further use.

Isolation of nucleoids from different organisms:

Nucleoid from *Sulfolobus acidocaldarius*: Nucleoid from *S. acidocaldarius* was isolated according to the procedure of Reddy and Suryanarayana, (1988; 1989). Cells were suspended in buffer (2 ml/g) containing 10 mM Tris-Cl (pH 7.4). 100 mM NaCl and lysed with the addition of 4 ml of lysis buffer containing 10 mM Tris, 1% NP-40, 2 mM spermidine trichloride, 10 mM Na_2EDTA The lysate was spun on a 15-50% sucrose gradient at 15,000 RPM for 20 minutes. The nucleoid band, seen at two-thirds the distance from the top of the tube. was

collected, diluted with buffer and centrifuged at 18,500 RPM for 30 minutes The viscous pellet obtained was suspended in dilute buffer and stored at -20 °C until further use. Nucleoids from *S. solfataricus* and *T. acidophilum* were also isolated according to the procedure of Reddy and Suryanarayana. (1989)

Nucleoid from ***Escherichiacoli:*** *Escherichia coli* nucleoid was isolated according to the procedure of Stonington and Pettijohn, (1971) and Kornberg, (1974). 2-2.5g cells were suspended in buffer containing 50 mM Tris and 50 mM Na_2EDTA The cells thus suspended were lysed with 4 mg lysozyme and by the addition of buffer containing 1% N'P-40. The nucleoid band was collected by centrifuging the lysate on 15-50% sucrose gradient at 10,000 RPM for 30 minutes Nucleoid from *Bacillus stearothermophilus* was also isolated according to the procedure of Stonington and Pettijohn, (1971).

Nucleoid from ***Halobacteriumhalobium:*** *Halobacterium halobium* nucleoid was isolated for the first time in our laboratory. Cells were suspended in (2 ml/g) buffer containing 10 mM Tris-Cl (pH 7.6), 3 M KCl, 100 mM $(NH_4)_2SO_4$, 1 mM β-mercaptoethanol, 1 mM Na_2EDTA, 5% sucrose and lysed by the addition of 4 ml of lysis buffer containing 10 mM Tris-Cl, 3 M **NaCl,** 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 1 mM β-mercaptoethanol, 1 mM Na_2EDTA, 0.1% NP-40 amidst constant stirring and incubated at room temperature for 15-20 minutes. The viscous lysate was layered on 5-40% sucrose gradients containing 10 mM Tris-Cl (pH 7.6), 3 M NaCl, 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 1 mM β-mercaptoethanol, 1 mM Na_2EDTA and centrifuged at 11,500 RPM for 50 minutes. A viscous opalescent band was seen at about one-fourths the length of the tube (from the top) The gradient was fractionated and analysed for nucleic acid.

protein content and DNA-protein complexes (nuclcoid) containing fractions were collected, diluted with buffer and centrifuged at 13,000 RPM. The pellet was suspended in buffer containing 100 mM **KC1,** 10 mM Tris-Cl (pH 7.6), 1 mM PMSF and stored frozen until further use.

Protein purification protocols:

Isolation of S-100: Cells were ground with double the weight of alumina till a soft and sticky paste of the cell lysate was obtained Alternatively, the cells were lysed by the addition of Triton-X-100 and NP-40 to the cell suspension to a final concentration of 0.1% and 0.5% respectively The extract was prepared in 20 mM Tris-Cl (pH 7.6), 50 mM KCl, 10 mM Mg acetate and 7 mM β-mercaptoethanol The suspension was centrifuged at 30,000 X g for 15 minutes and the supernatant (S-30) obtained, was subjected to centrifugation at 1,00,000 X g for 4 1/2 hours. The resulting supernatant was the starting material (S-100). for the purification of helix stabilising proteins.

Isolation of acid soluble proteins: The supernatant obtained after pelleting ribosomes (S-100), was subjected to dialysis against 0.2 M H_2SO_4 for 16 hours The precipitating proteins were removed after centrifugation and the clear supernatant was dialysed against 20 mM Tris-Cl (pH 7.6), 150 mM KCl, 1 mM Na_2EDTA and 7 mM P-mercaptoethanol for 20 hours This acid extract predominantly contains four basic proteins viz., HSNP-A; DBNP-B; HSNP-C & HSNP-C which were further purified by ion exchange column **chromatography.**

Purification of HSNP-C from S-100 acid extract: HSNP-C was purified to homogeneity according to the procedure of Reddy and Suryanarayana, (1988) with

some minor modifications. The clear dialysed S-100 acid extract was loaded on to a column of CM-cellulose previously equilibrated with the same buffer The column was then eluted with a linear gradient of 75-250 mM KC1 containing buffer. The alternate fractions were analysed for the presence of protein by electrophoresis and measurement of absorbance at 280 nm HSNP-C' containing peak fractions were pooled and re-chromatographed on a smaller column of CM-cellulose.

Sample concentration: The pooled fractions were diluted with buffer devoid of KC1 (-KC1 buffer) so as to obtain a final concentration of 50 mM KCl. This was then loaded on to the column and eluted with 500 mM KC1 containing buffer. The protein containing peak fractions were collected, dialysed against 10 mM Tris (pH 7.6), 10 mM sodium acetate (pH 5.0) and stored frozen until further use.

Sepharose CL 6B chromatography :

This was performed exactly as described by Grote *et al.* (1986) for the purification of 7d protein. The S-100 (post ribosomal supernatant) was dialysed against buffer containing 20 mM NaCl; 10 mM sodium phosphate (pH 7.5). Then the sample was loaded on the column previously equilibrated with the same buffer and eluted with a linear gradient of 50-500 mM NaCl in 0.01 M sodium phosphate buffer (pH 7.5). Fractions were analysed by SDS-PAGE using 15% gels (Laemmli, 1970).

SDS-polyacrylamide gel electrophoresis:

Electrophoresis was carried out according to Laemmli. (1970) on 15% polyacrylamide gels. The ratio of acrylamide to bisacrylamide was 30:0.8 percent

The resolving gel contained 0.375 M Tris-Cl (pH 8.8), 0.1% SDS and the stacking gel was in 0.125 M Tris-Cl (pH 6.8), 0.1% SDS; acrylamide concentration was 5% in the upper gel and 15% in the resolving gel. The protein samples to be electrophoresed were treated with 0.1% SDS. 1% β-mercaptoethanol, 0.05 M Tris (pH 6.8), 2.5 mM Na_2EDTA and 0.01% bromophenol blue at 65 ^oC for 15 minutes before loading on to the gel The electrode buffer used for electrophoresis contained 0.05 M Tris, 0.38 M glycine and 0.1% SDS. The voltage was maintained at 60 V till the sample entered the resolving gel and later raised to 120 V till the end of the run The electrophoresis was terminated after the indicator dye, bromophenol blue reached the bottom of the gel. Gels were removed and fixed with 7.5% acetic acid for 15 minutes, stained in 0.1% Coomassie brilliant blue R-250 in 50% methanol and 7.5% acetic acid The gels were then destained in 7.5% acetic acid containing 5% methanol.

Silver staining of gels: Silver staining was done according to the procedure of Blum *et al.* (1987). The gels were fixed in 7.5% acetic acid, 20% methanol containing 0 05% formaldehyde for 6-8 hours. This was followed by a brief incubation of the gels in 50% ethanol and 30% ethanol for 30 minutes and 20 minutes **respectively.** Gels were then pie-treated **with** 0.02% sodium thiosulfate for 1 minute, briefly rinsed with water for 1 minute (as mentioned earlier) and impregnated with 0.2% $AgNO_3$ in 0.075% formaldehyde for 20 minutes. The gels were briefly washed with water to dispense with the excess of $AgNO_3$ and the colour development was performed in an alkaline medium containing 6% Na_2CO_3, 0.05% formaldehyde and 0.0004% sodium thiosulfate for a period of 5-10 minutes. The gel was finally washed with water (3 times. 20 seconds each time) and stored in 50% methanol containing 12% acetic acid.

Western blotting:

The procedure was according to Towbin *et al.* (1979) Proteins were separated on a 15% SDS-polyacrylamide gel (Laemmli, 1970), and transferred on to nitro-cellulose membranes (0.45 μm pore size) electrophoretically for a period of 3-5 hours. The transfer buffer employed contained 25 mM Tris. 192 mM glycine, 0.1% SDS and 20% methanol at pH 8.3 After the completion of transfer, the blots were air dried briefly and incubated in the blocking buffer (10 mM Tris-Cl (pH 7.6), 150 mM NaCl 0.1% Tween-20) for 2 hours. The blots were then incubated in the cognate **primary** antibody (blocking buffer containing 0.1% anti-HSNP-C IgG) **for** 2 hours After the incubation, **the** blots were washed for 1 hour with the blocking buffer containing 0.5% N'P-40 (with three changes of buffer) The blots were finally incubated with the secondary antibody **(anti-rabbit** IgG from goat coupled to horse radish peroxidase at a dilution of 1:500) This was followed by thorough washing of the blots for 1 hour with three buffer changes at intervals of 20 minutes The final washing was carried out in 10 mM **Tris-Cl** (pH 7.6), 150 mM NaCl For colour development, a freshly prepared solution of 3 mg/ml 4-chloro-1-naphthol was added (to the blot in 10 mM Tris-Cl. 150 mM NaCl), to a final concentration of 0.3 mg/ml in the presence of 0.03% H_2O_2

Raising antibodies to pure *HSNP-C'*:

Antibodies to purified HSNP-C were raised in a healthy rabbit. About 200 μg of protein was emulsified with Fruend's complete adjuvant and injected subcutaneously into the rabbit at multiple sites After four weeks. booster doses were injected subcutaneously every week till the sixth (4^{th}, 5^{th} & 6^{th}) week, the emulsification being made with Fruend's incomplete adjuvant for each booster dose. The rabbit was bled after the third booster injection through the pinna vein.

Blood collected from the rabbit was kept at room temperature for 2 hours and then left at 4 °C for 12-16 hours. The clot was removed by centrifugation at 6000 RPM for 15 minutes The supernatant obtained was recentrifuged at 10.000 RPM for 30 minutes The supernatant (antiserum) obtained was incubated at 56 °C for 30 minutes. followed by centrifugation at 10.000 RPM The supernatant was collected and stored frozen in aliquots

Isolation of IgG: Antiserum was fractionated by $(NH_4)_2SO_4$. IgG fraction was obtained from 50-60% $(NH_4)_2SO_4$ saturation, dialysed against phosphate buffered saline and loaded on to a DEAE-cellulose. The column was eluted with the same buffer and fractions (2 ml) were collected The protein content was analysed by measuring absorbance at 280 nm. Peak fractions were pooled. IgG was precipitated with three-fifths volume of saturated $(NH_4)_2SO_4$ and centrifuged at 8,000 RPM for 40 minutes The final pellet was suspended in phosphate buffered saline, dialysed against the same buffer and stored frozen in small aliquots

Agarose gel electrophoresis:

The DNA and nucleoprotein complexes were analysed on 0.45-0 85% agarose gels. Electrophoresis was carried out at 2.6-2 8 V/cm for 4-5 hours. The gels were then stained in 0.5 μg/ml ethidium bromide in electrophoresis buffer and viewed under UV illumination

Gel retardation assay :

Gel retardation assays of HSNP-C'-DNA complexes were performed under two different salt conditions The reaction volume was 20 μl containing 20 mM Tris-Cl (pH 7.8). 0.4 mM sodium acetate and 0.2 mM Na_2EDTA (Lohman *el al.*,

1986). The binding reaction was performed at 25 °C for 30 minutes Sample buffer containing 15% glycerol; 0.1% BPB was added to each of the tubes and the samples electrophoresed on 0.45% agarose gels The electrophoresis was carried out at 2.6-2.8 V/cm for 5 hours in buffer containing 20 mM Tris-Cl (pH 7 8). 0.4 mM sodium acetate and 0.2 mM EDTA for 4-5 hours at 2.6-2 8 **V/cm.** The gels were stained in 0.5 µg/ml ethidium bromide at 4 °C and viewed under UV illumination.

Isolation of DN A from *Sulfolobus acidocaldarius* :

DNA from *S. acidocaldarius* was isolated from freshly harvested cells according to the procedure of Hempstead, (1990) **with** some minor modifications. Freshly collected cell pellets from 50 ml well-grown cultures were suspended in 100 µl of 50 mM Tris-CI (pH 7.6), 10 mM EDTA after two washes with the same buffer. The cell pellet was then stored frozen at -80 °C for a period of 1 hour followed by rapid thawing at 55 °C Proteinase K and SDS to a final concentration of 0.1 mg/ml and 0.1% respectively. were added to the cell suspension and the mixture was incubated at 55 °C for another 15 minutes. RNAse A (55 jag/ml in 0.1 M sodium acetate (pH 4 8); 0.3 mM EDTA) was added to the above mixture and incubated at 37 °C for 1 hour. On completion of the incubation. 5 M potassium acetate (100 µl) was added to a final concentration of 0.5 M and incubated on ice for 30 minutes. The above contents were centrifuged at 11,000 RPM and the supernatant was extracted with equal volume of $CHCl_3$:isoamyl alcohol (24:1) at 25 °C for 15 minutes. The two phases were separated by centrifugation. The aqueous phase was re-extracted with $CHCl_3$:isoamyl alcohol. The final aqueous phase was precipitated with one-tenth volume of 3 M sodium acetate (pH 4.8) and two volumes of 95% ethanol. The DNA was spooled on to a

thin glass rod or on a micro-tip. washed briefly by immersion in 70% ethanol. air dried and dissolved in 100 µl of 10 mM Tris-Cl (pH 8 0). 1 mM EDTA.

Cross-linking studies :

Protein cross-linking experiments were performed with three different reagents viz, formaldehyde, (HCHO) (Jackson. 1978), dimethyl suberimidate (DMS), (Thomas and Kornberg. 1975) and difluorodinitrobenzene (DFDNB) at three different temperatures.

Formaldehyde cross-linking: HSNP-C (40 µg) was acetone precipitated to free the proteins of other buffer salts. The pellet was dissolved in 18 µl of 10 mM TEA (pH 7.6); 50 mM NaCl. Neutral HCHO (2 µl of 1 M concentration) was added to the protein sample and incubated at 37 °C, 50 °C and 65 °C for a duration of 1 hour. The reaction was terminated with 50% trichloroacetic acid (final concentration of 10%) followed by an incubation on ice for 30 minutes The suspension was then centrifuged and the precipitate was washed with acetone. The pellet was then dissolved in 1 X electrophoresis sample buffer containing 0.1% SDS but devoid of (3-mercaptoethanol/DTT and electiophoiesed on 15% SDS-polyacrylamide gels (Laemmli. 1970).

Dimethyl suberimidate and Difluoro **dinitro** benzene cross **linking:** HSNP-C (50 µg/assay) was acetone precipitated and the protein pellets obtained after centrifugation were dissolved in 100 mM sodium borate (pH 8.0) containing 100 mM NaCl (Davies and Stark. 1970). DMS or DFDNB was added to a final concentration of 0.2 mg/ml or 0.1 mg/ml respectively. The samples were incubated at 37 °C, 50 °C and 65 °C for 15 minutes. At the end of the 15th minute the

reactions were terminated by the addition of 50% TCA to a final concentration of 10% followed by incubation on ice for 30 minutes The suspension was then centrifuged, and the precipitate was washed with acetone (to remove all traces of TCA) and dissolved in sample buffer containing 0 1% SDS The samples were then electrophoresed on 15% SDS-polyacrylamide gels (Laemmli, 1970). Cross-linking with DFDNB was also perfonned at pH 9 0 under similar conditions

Gel Filtration chromatography :

Pre-swollen sephadex G-100 was deareated and packed into a thin long column of 40 ml bed volume and equilibrated with 20 mM Tris-Cl (pH 7.6), 50 mM KCl, 1 mM EDTA containing 7 mM β-mercaptoethanol. Native or DFDNB cross-linked HSNP-C (each 200 |ig) was loaded on to the column separately and eluted with the same buffer. Fractions (1 ml) were collected from the column at a flow rate of 2 ml/ hour Alternate fractions were analysed for the presence of protein by both SDS-polyacrylamide gels and fluorescence intensity measurements with excitation at 285 nm and emission at 350 nm Simultaneously, protein markers viz., bovine serum albumin (66 kDa). ovalbumin (44 kDa). and cytochrome-C (12 kDa) were also chromatographed on the column for reference purposes The molecular weights of the **oligomeric** HSNP-C protein aggregates eluting from the column were detennined with respect to standard proteins.

Binding of HSNP-C to immobilized nucleic acid columns :-

Poly U sepharose: poly A sepharose. dsDNA-cellulose and ssDNA-cellulose were packed into different columns and equilibrated with 20 mM Tris (pH 7.6); 10 mM NaCl; 1 mM EDTA and 7 mM β-mercaptoethanol HSNP-C (50 μg) was loaded on each of these four columns at a flow rate of 0.6 ml/hour. The

columns were subsequently washed with 10 mM NaCl containing buffer and eluted with a step wise gradient of 100-500 mM NaCl Fractions (0.1 ml) were collected and analysed for the presence of protein by both SDS-PAGF and fluorescence intensity measurements.

Flourescence titrations of HSNP-C':

Fluorescence measurements of all protein samples were carried out in a FP-777 Jasco spectrofluorimeter The measurements were performed in 10 mM Tris-Cl (pH 7.0) and 20-50 mM NaCl at 25 ^{o}C Excitation and emission band widths were 5 nm and 10 nm respectively (For ethidium bromide linked experiments, the band width was 1.5 nm for excitation and 20 nm for emission) After each addition of titrant to protein, the contents were mixed gently and allowed to stand for 30-40 seconds. The observations were recorded after the fluorescence signal was stabilised Average of the three readings, within an interval of 60 seconds were taken for each **titration** point The fluorescence units (arbitrary units) were corrected for dilution. We have checked for inner filter effects (if any) in our experimental conditions which were found to be negligible

Direct Titrations: This is one of the approaches employed for the study of **the** interaction of the protein with nucleic acid. In this case the ligand (protein) was added to the lattice (nucleic acid) and the fluorescence of both bound and free protein was measured. To a fixed amount of DNA in 1 ml of reaction buffer. aliquots (2 μl) of HSNP-C were added and fluorescence readings were recorded simultaneously The excitation wavelength and emission wavelength were held constant at 285 nm and 350 nm respectively for all fluorescence measurements.

Reverse Titrations: Aliquots of nucleic acid or other quenchers were added to a fixed concentration of HSNP-C (10 fig) taken in 1 ml of reaction buffer (10 mM Tris-Cl (pH 7.0). 50 mM sodium chloride) and the decrease in fluorescence intensity was measured The initial protein fluorescence was taken to be 100% and all other measurements were made with reference to the initial fluorescence of the protein Titrations were also performed with different mononucleotides.

Displacement of DNA bound ethidium by *HSNP-C':*

Fluorescence emission spectrum of ethidium bromide (400 ng) in 1 ml of 10 mM Tris-Cl (pH 7.6); 50 mM NaCl was recorded with excitation at 480 nm and emission in the range of 500-660 nm To this 4 μg of calf thymus DNA was added and the fluorescence spectrum of ethidium bound DNA was recorded. Emission spectra were recorded after each addition of 8 μg HSNP-C to ethidium DNA complexes. The excitation and emission slit widths were set at 1.5 nm and 20 nm respectively. The concentration of ethidium displaced was calculated from the measurements of the absorbance at 480 nm using molar absoiption coefficient of 5700.

Thermal melting studies:

Thermal denaturation profiles of calf thymus DNA in the absence (control) and presence of HSNP-C were obtained by heating the samples in 400 μl of 10 mM sodium cacodylate (pH 7.0), 1 mM EDTA. 10 mM NaCl in a PU 8700 spectrophotometer equipped with a thennoprogrammer (model PU 8764). Increase in absorbance at 260 nm was monitored and melting curves were recorded simultaneously. The rate of heating was 2 °C rise/min Prior to heating. HSNP-C

was added to DNA, gently mixed and incubated for 5 minutes at the stalling temperature. Buffer blanks and protein solutions were heated separately to check for temperature dependant variations in absorbance which were found to be negligeble

Effect of different concentrations of magnesium acetate, spermidine and sodium chloride on the melting of DNA in the presence of different amounts of protein were also studied.

DNA aggregation:

DNA aggregation by HSNP-C was studied by the extent of light scattering monitored by the increase in absorbance at 320 nm by spectrophotometric measurements.

Increasing amounts of HSNP-C were added to a fixed amount of DNA (2 μg) in 1 ml of reaction buffer containing 10 mM sodium acetate, 1 mM Na_2EDTA. The formation of DNA-HSNP-C aggregates was assayed by the measure of increase in absorbance at 320 nm The experiment was performed at four different temperatures viz . 35 °C, 45 °C, 55 °C and 65 °C in order to standardise the optimal conditions for DNA aggregation Influence of Mg^{++} and high salt concentrations on the **formation** of DNA-protein complexes also were assayed.

DNase digestion of DNA-HSNP-C complex:

Increasing amounts of HSNP-C were incubated **with** 200 ng of λ DNA (in 20 μl reaction volume) in 20 mM Tris-Cl (pH 7.8); 0.4 mM sodium acetate and 0.2 mM Na_2EDTA at pH 7.8, in the presence of 75-100 mM NaCl for 30 minutes. $CaCl_2$ and $MgCl_2$ were added to a final concentration of 0.5 mM and 5.0 mM

respectively. DNase I (4 μg) was added to the above reaction mixture and incubated at 37 °C for 15 minutes. The reaction was terminated with the addition of Na_2EDTA to a final concentration of 10 mM. The sample was loaded onto an agarose gel (0.8%) and electrophoresed The gel was stained in ethidium bromide and visualized under UV illumination

Spectrophotometric assay of DNAse I:

Native Calf thymus DNA (10 μg) in 400 μl of 10 mM Tris-Cl pH 7 4, 25 mM $MgCl_2$; 20 mM NaCl; 1 mM EDTA was incubated with 1.0 μg pancreatic DNAse I in spectrophotometric cuvettes in the presence and absence of HSNP-C'. The increase in absorbance at 260 nm was monitored for 30 minutes

Electron Microscopy of HSNP-C'-dsDNA complexes :

Electron microscopy of HSNP-C'-dsDNA complexes was canied out by Dr Lurz, Max-Planck Institute for Molecular Genetic. Berlin. Germany. The preparation of the nucleoprotein complexes and their processing was canied out exactly as described by Lurz *et al.* (1986) RSF 1010 plasmid DNA was incubated with different amounts of HSNP-C in buffer (10 mM TEA-C1, (pH 7.6), 50 mM KG, 2.5 mM DTT and 0 5 mM Na_2EDTA) at 37 °C for 15 minutes The samples were fixed with glutaraldehyde (0 2% final concentration) The fixed nucleloprotein complexes were adsorbed on to mica for 1 minute. followed by 1 minute staining in 2% aqueous uranyl acetate. washed twice in water and air-dried,

Chemical modification of HSNP-C:

HSNP-C was treated with different modifying reagents that selectively modify the amino acid residues to assess the amino acid functional groups

involved in its interaction with **DNA.** Five different modifications were carried out, two specific for lysine and one each for arginine, tyrosine and tryptophan, the details of which are given below.

Reductive methylation of lysine: The reductive methylation of lysine was carried out with sodium borohydride in the presence of fonnaldehyde (Means and Feeney. 1968). HSNP-C (0.5 mg) in 1 ml of 0.2 M sodium borate (pH 9.0) was treated with freshly prepared sodium borohydride (40 mg/ml) to a final concentration of 50 μg and incubated for 10 minutes on ice. Five consecutive additions of 5 μl each of 18% HCHO were made at **intervals** of 5 minutes At the end of the 30th minute. **the** reaction mixture was transferred into a dialysis tubing and dialysed exhaustively against 10 mM Tris-Cl (pH 7.6) containing 1 mM PMSF and stored frozen until further use.

Quantification of the modification of the reductive methylation of lysine:
HSNP-C (200 μg) was taken in 5.7 N HC1 and left overnight (constant boiling) in the presence of 5% phenol (aq). The hydrolysate obtained was spotted on Whatman No. 3 filter paper which was previously dipped and dried in 0 1 M Na_2EDTA (pH 7.0). The samples on the Whatman filter papers were subjected to descending chromatography for 16-18 hours at 21 °C (Stewart 1963). The buffers used for the puipose contained m-cresol (88%):phenol:borate buffer in the ratio of 190:165:45 (v/v). The amino acid spots were visualised with 0.1% ninhydrin reagent in 95% ethanol:glacial acetic acid:collidine **(50:15:2).**

Pyridoxal Phosphate modification of lysine: This modification was performed according to the method of Ohsawa and Gualerzi, (1981). HSNP-C (1 mg) in 1 ml

of 20 mM TEA; 10 mM KCl was incubated with a final concentration of 1 mM pyridoxal phosphate for 12 minutes at 37 ^{o}C. At the end of the twelfth minute, 0.5 mg equivalent of $NaBH_4$ was added to the above mixture and incubation was continued on ice for 10 minutes to ensure complete reduction of the available lysine residues. It was then exhaustively dialysed against 10 mM Tris-Cl (pH 7.6) containing 1 mM PMSF and stored frozen until further use.

Diacetyl (1,3, butanedione) modification of arginine: The protocol for arginine modification was adopted from Rohrbach and Bodley, (1977). HSNP-C' (1 mg) in 1 **ml** of 20 mM TEA buffer (pH 8.0) was reacted with diacetyl reagent at a final concentration of 4 mM. The incubation was carried out at 37 °C for 30 minutes and the reaction mixture was dialysed exhaustively against 100 mM sodium borate (pH 9.0) containing 2 mM β-mercaptoethanol.

TNM modification for tyrosine: Tyrosine modification of HSNP-C was performed according to Cheng and Pierce, (1972). Commercially available TNM stock (8.35 M) was diluted to 100 mM with methanol and the diluted stock was used for further reactions. HSNP-C (1 mg) in 1 ml of 0.1 M sodium phosphate at pH 7.0; 0.001 M Na_2EDTA was reacted with a final concentration of 20 mM TNM and incubated at 21 °C for 60 minutes. The reaction was terminated with the addition of P-mercaptoethanol to a final concentration of 140 mM and the reaction mixture was dialysed exhaustively against 1% ammonium carbonate.

BNPS-skatole modification for tryptophan: 2-(2-nitrophenylsulfenyl)-3-methyl-3-bromo indoline (BNPS-skatole) in 50% aqueous acetic acid, was **the** reagent

used to modify tryptophan residues of HSNP-C according to Fontana, (1972). The protein (1 mg/ml in 70% acetic acid) was reacted with BNPS-skatole (final concentration of 0.34 mg/ml) and stirred in the dark for 15 minutes. The reaction was terminated with a final concentration of 0 04% β-mercaptoethanol and the reaction mixture was dialysed against 10 mM Tris-Cl (pH 7.6) containing 1 mM PMSF.

Chemical and proteolytic cleavage of HSNP-C:

CNBr treatment of HSNP-C: HSNP-C (1 mg) was dissolved in 0.5 ml of 75% formic acid and treated with 6 mg of cyanogen bromide. The mixture incubated in the dark for 48 hours The reaction mixture was then lyophilised and the protein dissolved in 50 mM sodium acetate (pH 5 0). The protein sample was loaded on to a column of CM-cellulose pre-equilibrated with the same buffer and eluted with a linear gradient of 50-400 mM sodium acetate (pH 5.0). Fractions (2 ml) were collected and analysed for protein content both by SDS-polyacrylamide gel elecrrophoresis and absorbance at 280 nm.

Tryptic cleavage of HSNP-C and purification of tryptic core by chromatography: Tryptic cleavage of HSNP-C was carried out according to procedure of Suryanarayana and Subramanian, (1979). TPCK-trypsin was dissolved in 10 mM TEA (pH 8.0) 20 µl of 0.5 mg/ml TPCK-trypsin stock was added to HSNP-C (1 mg) and incubated at 0 °C/37 °C for 2 hours The reaction was terminated with the addition of soyabean trypsin inhibitor (10 times the concentration of TPCK-trypsin) followed by incubation at 0 °C for 30 minutes The protein was pelleted with the addition of 50% trichloroacetic acid to a final concentration of 10% The protein pellet was dissolved in 10 mM Tris-Cl (pH

7.6); 20 mM KCl and loaded on a CM-cellulose column equilibrated with the same buffer The column was then eluted with a linear gradient of 75-250 mM KC1 and fractions (2 ml) were collected The fractions were then analysed by SDS-polyacrylamide gel electrophoresis and absorbance at 280 nm The peak fractions were pooled. concentrated and dialysed against buffer containing 10 mM Tris-Cl (pH 7.6); 1 mM PMSF and stored frozen in aliquots

Chapter-3
RESULTS

RESULTS

Purification of *HSNP-C'*:

HSNP-C', an 8 kDa DNA binding protein associated with the nucleoid of *Sulfolobus acidocaldarius* was purified according to the procedure of Reddy and Suryanarayana, (1989). However, a few selective modifications resulted in better yield of the protein. As mentioned in the materials and methods section, CM-cellulose column was equilibrated with 50 mM KCl containing buffer on to which the crude HSNP-C sample was loaded. This was eluted with a linear gradient of 75 mM to 250 mM KC1 in Tris-buffer. Alternate fractions were electrophoresed on a 15% SDS-polyacrylamide gel according to Laemmli, (1970) Fig.1A shows the chromatographic separation of two helix stabilising nucleoid proteins, HSNP-A eluting at 100 mM KC1 concentration followed by HSNP-C at 180 mM KCl The pure HSNP-C peak fractions were pooled and diluted to a final concentration of 50 mM KCl with Tris containing buffer devoid of KC1. This was loaded once again on a smaller column of CM-cellulose pre-equilibrated with buffer containing 50 mM KCl. The concentrated protein was then eluted as a sharp peak with high salt (500 mM KCl). The peak fractions were pooled, dialysed against 10 mM Tris-Cl (pH 7.6) and analysed for protein by SDS-PAGE (fig 1B). The samples were finally estimated for the protein content (Lowry *el al.*, 1951) and stored frozen in aliquots until further use.

Purity was checked by silver staining of the gel which revealed a single band of HSNP-C and the molecular mass was determined to be 8 kDa (fig. 2). The yield of the protein was calculated to be 2.78 mg HSNP-C'/10 g wet cells.

Fig. 1A:SDS-PAGE analysis of fractions obtained from CM-cellulose chromatography of acid extract of the post-ribosomal supernatant.

Alternate fractions from the column were treated with SDS sample buffer and electrophoresed on 15% SDS-polyacrylamide gels.
Lane 1: Control protein (pure HSNP-C)
Lane 2: S-100 (post-ribosomal supernatant) acid extract.
Lanes 3-5: Fraction numbers 12, 14, 16 respectively.
Lanes 8-12: Fraction numbers 22, 24, 26, 28, 30 resp., all of them containing HSNP-C.

Fig. 1B: SDS-PAGE analysis of HSNP-C' peak fractions obtained from a smaller CM-cellulose column.

Lanes 1-4: Fraction numbers 3, 4, 5, 6 containing concentrated HSNP-C as detected previously by measuring absorbance at 280 nm.

FIGURE 1

Fig. 2: Silver staining of HSNP-C after SDS-PAGE

Pure protein (HSNP-C) was dialysed against low ionic strength buffer (10 mM Tris-Cl (pH 7.6)/10 mM sodium acetate (pH 6.0)
Lane 1: Pure HSNP-C, 20 ng.
Lane 2: Molecular weight markers (bovine serum albumin, 68 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa; trypsin inhibitor 20 kDa and cytochrome-C-12 kDa.)

Fig. 3: Immunoblot analysis of different amounts of HSNP-C (native).

Increasing amounts of HSNP-C (native), was electrophoresed (without β-mercapto ethanol / DTT treatment) on 15% polyacrylamide gels, electrophoretically tiansfened on to nitrocellulose membranes and probed with anti-HSNP-C IgG and peroxidase conjugated anti-rabbit IgG
Lanes 1-6: 40, 80, 120, 150, 180, 200 μg HSNP-C respectively.
Lane 10: HSNP-C, 40 μg treated with 1% SDS.

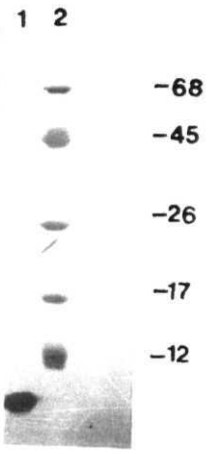


FIGURE 2

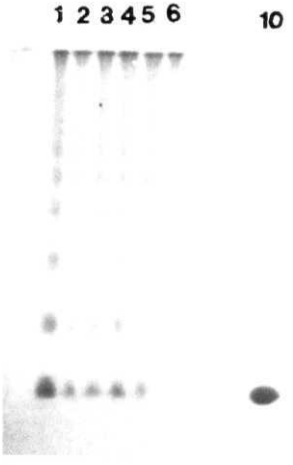


FIGURE 3

Aggregation properties of HSNP-C':

Immunoblotting of the purified protein showed cross-reactive bands corresponding to the position of dimers, trimers, tetramers and higher oligomers indicating that the protein exists as multimeric aggregates in solution (fig. 3).

Protein cross-linking: Cross-linking was used as a technique to study the aggregation behaviour of HSNP-C. HSNP-C has a tendency to exist in aggregated states as indicated by the experiment in fig. 3. Hence, to understand the quaternary structure of the protein, cross-linking experiments were performed with bifunctional cross-linking agents such as DMS and DFDNB and also with zero level cross-linker, HCHO, at three different temperatures.

Formaldehyde cross-linking produced a series of bands on electrophoresis corresponding to dimers, trimers, tetramers, pentamers and hexamers with progressively decreasing intensities (fig. 4A). At very high concentrations of cross-linking reagents as well as longer times of incubation, very large multimeric aggregates were formed which failed to enter the polyacrylamide gel. Cross-linking with DMS and DFDNB revealed the formation of well-defined higher aggregate forms of the protein with molecular weights in the range of 200 K apart from small amounts of dimers and trimers (fig. 4B). Analysis of cross-linked products obtained with DMS and DFDNB by electrophoresis on 10% gels (fig. 4C) showed the presence of four distinct bands with molecular weights of about 165,000; 150,000, 135,000 and 120,000. These results indicate that HSNP-C exists in solution as multimeric aggregates. Absence of control HSNP-C was due to migration of the protein outside the gel.

In addition to these, cross-linking of HSNP-C with DFDNB at pH 9.0 in 100 mM sodium borate was also performed, which showed an intense band

Fig. 4A: SDS-PAGE of formaldehyde cross-linked complexes of HSNP-C

Cross-linking with formaldehyde was canied out as described in the text in pH 7.4 buffer at different temparatures and the cross-linked complexes were analysed by electrophoresis on 15% gels.
Lanes 1-3: Cross-linking performed at 35 °C, 50 °C, and 65 °C respectively;
Lane 4: HSNP-C control;
Lane 6: Molecular weight markers(bovine serum albumin, 68 kDa; Ovalbumin, 45 kDa; Chymotrypsinogen, 26 kDa; myoglobin, 17 kDa and cytochrome C, 12 kDa).

Fig. 4B: SDS-PAGE of cross-linked complexes of HSNP-C obtained in pH 8.6 buffer.

Lane 1: Untreated HSNP-C
Lane 2: Molecular weight markers as in Fig. 1
Lanes 3-5: Cross-linking with HCHO at 65 °C, 50 °C and 37 °C respectively;
Lanes 6-8: Cross-linking with DMS at 65 °C, 50 °C and 37 °C respectively;
Lanes 9-1 l:Cross-linking with DFDNB at 65 °C, 50 °C and 37 °C respectively.

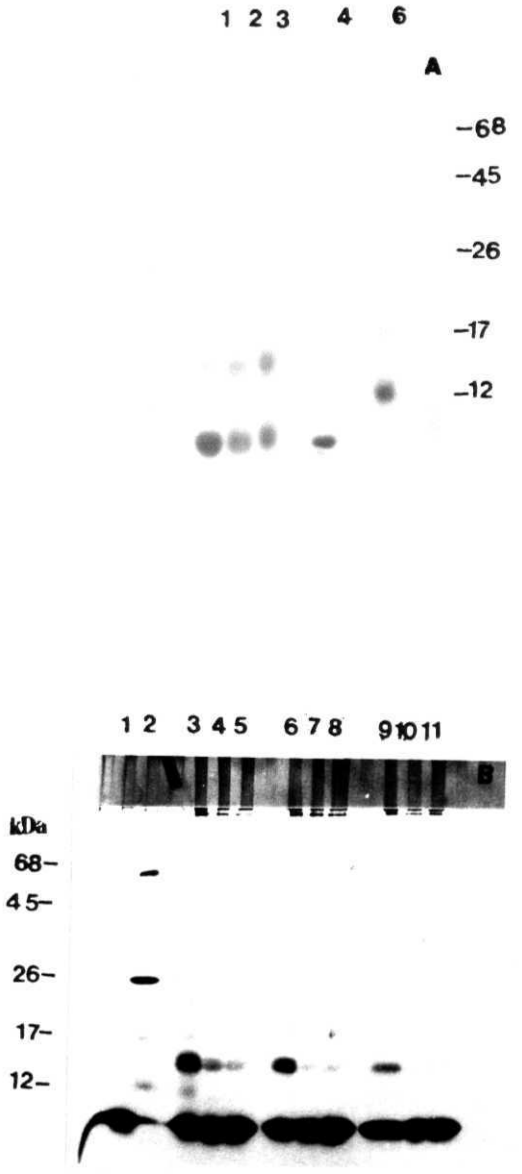


FIGURE 4

Fig. 4C: SDS-PAGE analysis of cross-linked complexes on 10% gels

Cross-linked complexes were analysed by electrophoresis on 10% gels.
Lane 1: Molecular weight markers (myosin, 205 kDa; beta-galactosidase, 116 kDa; Phosphorylase a, 97 kDa; bovine serum albumin, 68 kDa),
Lanes 2-4: Cross-linking at 65 °C, 37 °C and 50 °C with DMS
Lanes 5-7: Cross-linking at 65 °C, 37 °C and 50 °C with DFDNB.

Fig. 4D: SDS-PAGE analysis of HSNP-C cross-linked with DFDNB at different temperatures (37, 50 and 65 °C) in a buffer maintained at pH 9.0.

Lanes 1-3: HSNP-C cross-linked at 37 °C; 50 °C and 65 °C respectively
Lane 4: HSNP-C (control)

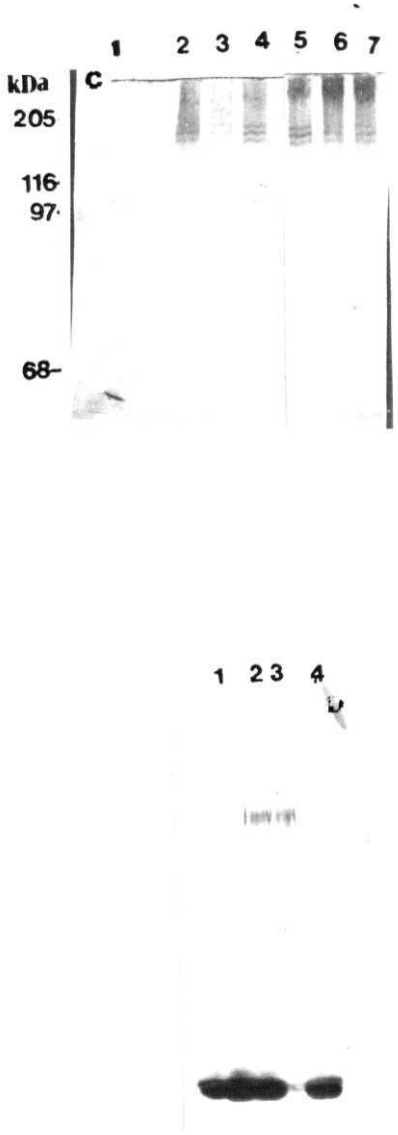


FIGURE 4

corresponding to molecular weight of octameric aggregation of the protein apart from the higher molecular weight aggregates which barely entered the gel (fig. 4D).

Gel-filtration chromatography: Aggregation properties of HSNP-C were also studied by gel-filtration chromatography. A column of sephadex G-100 (40 ml bed vol.) was equilibrated with buffer containing 20 mM Tris-Cl (pH 7.6), 50 mM KC1, 1 mM Na_2EDTA and 7 mM β-mercaptoethanol. Native or cross-linked HSNP-C was loaded and the column was eluted with the same buffer. Small volume fractions were collected simultaneously. Similarly, molecular weight marker proteins were also chromatographed. Aggregates were eluted at volumes corresponding to 142 kDa, 128 kDa, 78 kDa and 30 kDa in the case of cross-linked protein. Aggregates corresponding to 68 kDa and 28 kDa were predominantly observed in the case of native protein (fig. 5).

Nucleic acid binding properties of HSNP-C:

Several techniques have been employed to study the interaction between HSNP-C and DNA involving fluorescence titrations, binding affinity of the protein to immobilised nucleic acid matrices, thermal melting analyses, DNA aggregation, electron microscopy and other assays.

Binding affinities to immobilised nucleic acids: Strength of binding of HSNP-C to nucleic acids was assessed by affinity chromatography on nucleic acid matrices. HSNP-C was chromatographed on small columns of ssDNA-, dsDNA-celluloses and poly (U) and poly (A) sepharoses (fig. 6) The concentration of salt required to elute the bound protein in each case was determined. The results

Fig. 5: Gel-filtration chromatographic analysis of HSNP-C' in native and cross-linked states.

HSNP-C (200 μg) was loaded on to a column of Sephacryl S-200 (40 ml) and eluted with equilibration buffer. Fractions were collected and analysed by measuring fluorescence emission intensities with Exc at 285 nm.

A: Elution profile of HSNP-C (native protein 200 μg) from the column
B: Elution profile of HSNP-C (200 μg) cross-linked with DFDNB.
C: Elution profile of molecular weight markers, viz., bovine serum albumin (68 kDa); ovalbumin (45 kDa) and cytochrome-C (12 kDa)

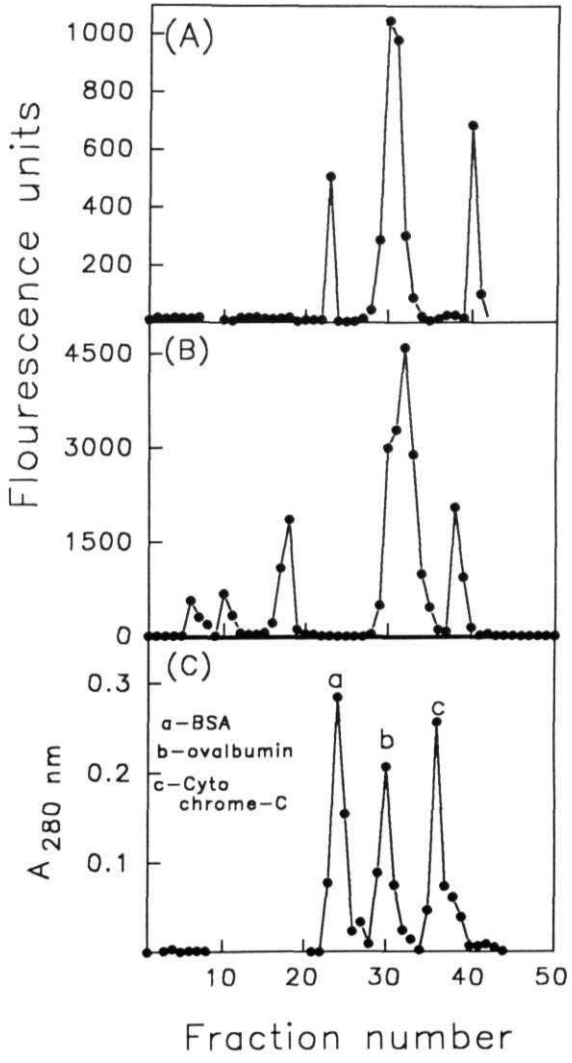

(A)
(B)
(C)
Flourescence units
1000
800
600
400
200
4500
3000
1500
0
0.3
0.2
0.1
A 280 nm
a
b
c
a–BSA
b–ovalbumin
c–Cyto chrome–C
10
20
30
40
50
Fraction number

FIGURE 5

Fig. 6: Binding of HSNP-C' to immobilised nucleic acid matrices was carried out as described in the text.

Elution profiles of HSNP-C from (A) dsDNA-cellulose; (B) ssDNA-cellulose; (C) Poly (U)- sepharose and (D) Poly (A)-sepharose

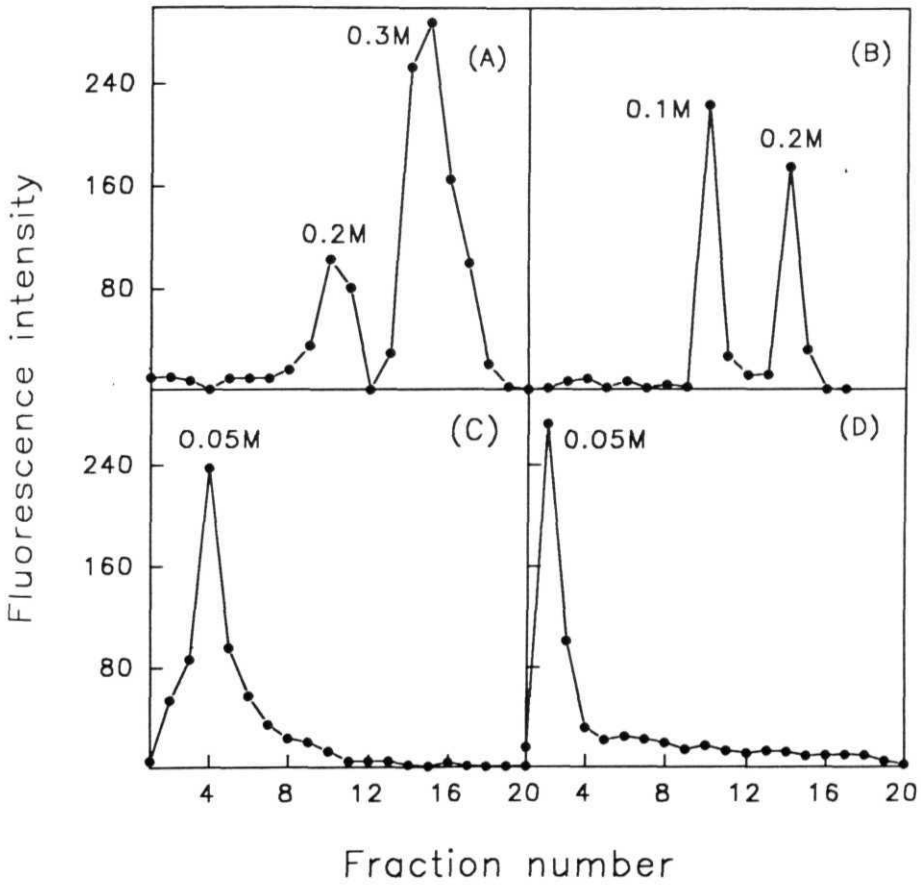

0.3M
(A)
(B)
0.1M
0.2M
0.2M
240
160
80
0.05M
(C)
0.05M
(D)
240
160
80
4
8
12
16
20
4
8
12
16
20
Fluorescence intensity
Fraction number

FIGURE 6

indicate that HSNP-C binds strongest to dsDNA (with elution requiring 300 mM NaCl) and reasonably strongly to ssDNA (eluting at 250 mM NaCl) The protein showed very weak binding to RNA (the protein was eluted in the break-through fractions). The elution of the protein on dsDNA-cellulose/ssDNA-cellulose showed the presence of two distinct peaks eluting at two different salt concentrations indicating the presence of two different forms of the protein which may differ in the extent of methylation of lysine residues *(see results on chemical modifications of HSNP-C)*. The protein probably exists in two forms differing in the extent of methylation of the lysine residues.

Gel retardation assays: The binding of HSNP-C to dsDNA was also analysed by agarose gel electrophoresis This technique can also be used to observe the distribution of protein on DNA The elecrrophoresis through agarose gel separates DNA molecules according to the amount of HSNP-C bound to each DNA molecule. Free DNA migrates fastest, and DNA with increasing amount of protein bound migrates progressively slower. The other advantage of this technique is that the complexes formed are frozen as they enter agarose gel and no redistribution of the bound protein to different DNA molecules can occur Two such experiments in which *X* DNA was mixed and incubated with increasing amount of HSNP-C at low (20 mM) and high (220 mM) concentrations of NaCl before elecrrophoresis are shown in fig. 7A & B At both salt concentrations, electrophoretic pattern indicates co-operative mode of binding of HSNP-C. The complexes formed at low salt showed a broad smear, from the position of free DNA to DNA fully saturated (fig. 7A, lane 4) which is indicative of non-random distribution of the protein among the DNA molecules and intermediate co-operativity in the binding mode. However, there was a dramatic and very large increase in the co-operativity of

Fig. 7: Gel mobility shift analysis of HSNP-C'-DNA complexes.

Lambda phage DNA (0.2 μg) was incubated with increasing amounts of HSNP-C in 20 mM NaCl (A) or 220 mM NaCl (B) buffer before electrophoresis on 0.8% agarose gels.

Lane 1: λ DNA in the absence of protein (control)

Lanes 2-6: λ DNA incubated with 0.2, 0.4, 0.6, 0.8 and 1.0 μg HSNP-C respectively.

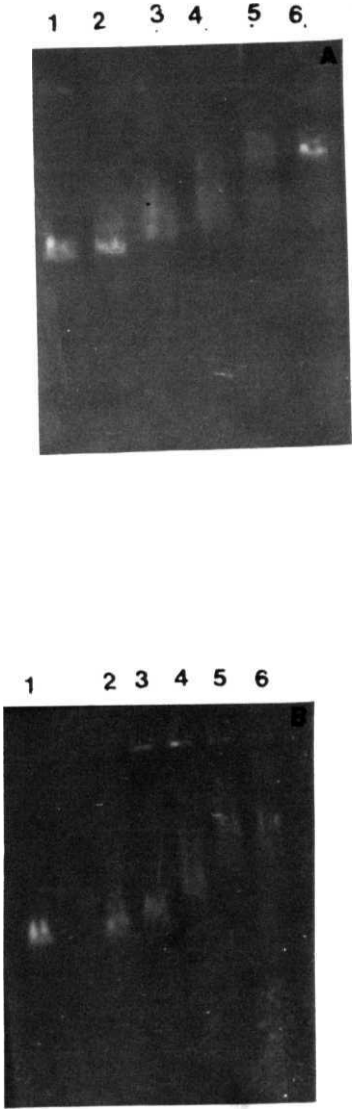


FIGURE 7

binding of the protein to dsDNA at high salt as in fig. 7B, as indicated by the presence of two sharp bands representing DNA that was fully saturated with protein retained in the well of the agarose gel (fig. 7B, lane 3) and free DNA at low binding density (large excess of nucleic acid). At higher protein (fig. 7B, lane 4, 5) half saturated DNA molecules were also formed as indicated by the appearance of bands with intermediate mobility. These complexes could be similar to those formed at saturation at low salt (fig. 7A, lane 5).

In the case of M13 ssDNA, maximum retardation was observed with 0.4 μg HSNP-C'. Although retardation in mobility was observed, the magnitude was lesser as compared to dsDNA (data not shown).

Fluorescence titration studies: Protein-nucleic acid interactions can be studied by changes in the intrinsic fluorescence of the protein Upon binding to nucleic acids, the fluorescence of aromatic amino acids is quenched, if these residues form a part of the binding site or are in the vicinity of the binding site This property can be used to quantitate the binding of proteins to nucleic acids and to obtain thermodynamic parameters of the interaction. Such fluorescence titration data are useful in determining the (i) strength of ligand(protein)-lattice(nucleotide) interactions as given by the binding constants, (ii) binding site size of the protein on the nucleic acid and (iii) binding mode of the protein to nucleic acid.

Fluorescence titrations for the study of these interactions were carried out by two approaches. In the first approach (reverse titrations), nucleic acid was added to the protein and the resultant decrease in fluorescence was measured. In the second approach (direct titrations) protein was added to the nucleic acid and the fluorescence of both bound and free protein were measured. Thermodynamic binding parameters were determined using Scatchard formulation as given by

McGhee and Von Hippel, (1974) and by a non-Scatchard approach according to Schwarz and Watanabe, (1983).

HSNP-C' was excited at 285 nm and the fluorescence was measured at an emission wavelength of 350 nm. Addition of double stranded DNA to HSNP-C causes quenching of the protein fluorescence. Such titrations were performed at different salt concentrations (fig 8A). At low salt concentration (20 mM NaCl) the binding is tight and essentially stoichiometric. At higher DNA/protein ratios saturation in binding was reached as indicated by a plateau at and above a ratio of 8 nucleotides per protein monomer The low salt titration curve was used to determine the binding site size, 'n', the average number of nucleotides bound by a protein molecule (monomer). The ratio of DNA to protein at the intersection point of the initial and final slope of the titration curve corresponds to the site size. A site size of 4.4 was obtained for dsDNA. The titration curves obtained at higher salt concentration showed decreased extent of quenching indicating weak binding at these salt concentrations The fluorescence titrations do not indicate co-operativiry in the binding of HSNP-C to dsDNA. Data were analysed by the equation 10 of McGhee and Von Hippel, (1974). Binding constant "K" was determined from the data points after determining the free concentration of protein (Lf) and the binding density (v moles of ligand bound per mole nucleotide). In the case of HSNP-C, the ratio of Qobs/Qmax was taken to be equal to bound ligand/total ligand, where Qmax was obtained by the low salt titration curve (72%). The calculation of v and Lf was according to Bujalowski and Lohman, (1987). Scatchard plots of v vs Lf of the data of binding of HSNP-C to dsDNA at 20 mM and 150 mM NaCl are given in fig 9. The intercept on extrapolation of the curve at y axis gives the intrinsic binding constant "K" for the non-co-operative binding interaction. The Scatchard plots are typical of non-co-operative binding

Fig. 8: Quenching of intrinsic fluorescence of HSNP-C' with dsDNA at different concentrations of salt

(A): HSNP-C **(1.5** μM) in **1 ml** of buffer was titrated with increasing concentration of dsDNA. The fluorescence intensity was measured after each addition

o-o 20 mM NaCl.
•-• 50 mM NaCl.
A-A 150 mM NaCl
v-v 200 mM NaCl

(B): Reverse titration of HSNP-C with *S. acidocaldanus* DNA and CT DNA at 20 and 200 mM NaCl.

o-o at 20 mM NaCl (CT DNA)
•-• at 20 mM NaCl *(S.* ***acidocaldanus*** DNA)
A-A at 200 mM NaCl (CT DNA)
v-v at 200 mM NaCl (5. *acidocaldanus* DNA)

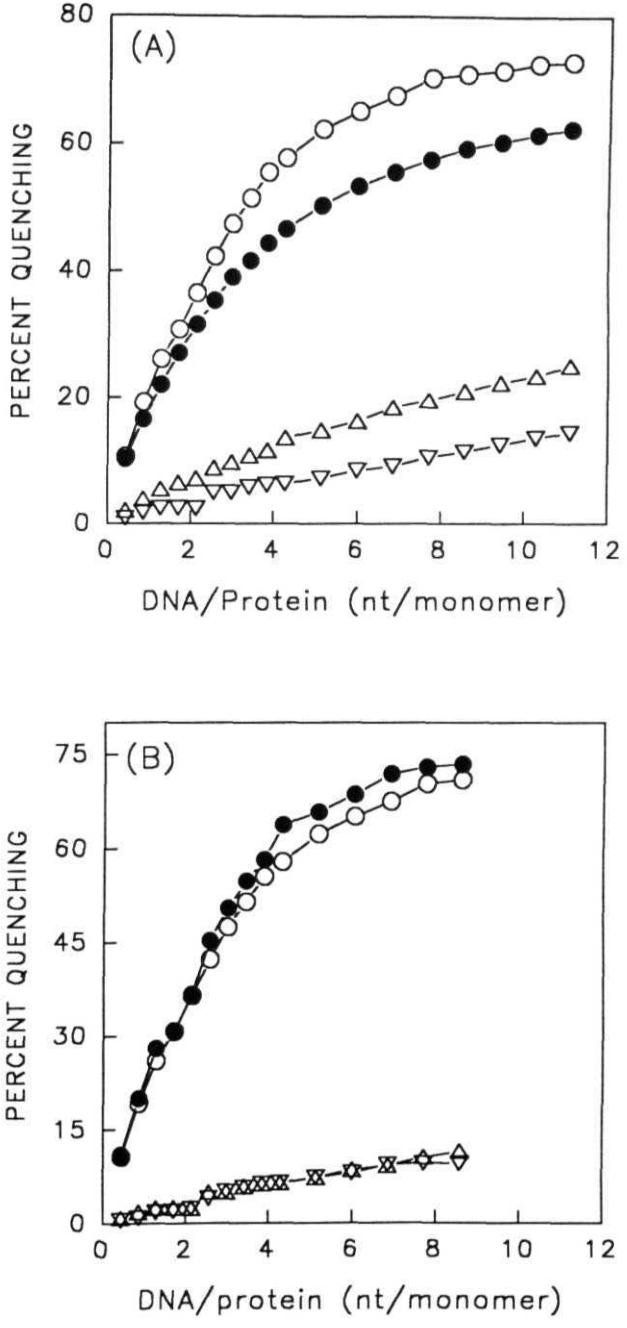

(A)
PERCENT QUENCHING
80
60
40
20
0
0
2
4
6
8
10
12
DNA/Protein (nt/monomer)
(B)
PERCENT QUENCHING
75
60
45
30
15
0
0
2
4
6
8
10
12
DNA/protein (nt/monomer)

FIGURE 8

with n > 2 i.e., they are markedly convex down and shallow as v increases (McGhee and Von Hippel, 1974). The intrinsic binding constants, "K", at 20 mM and 150 mM NaCl were 4 x 10^6 M^{-1} and 4.5 x 10^5 M^{-1} respectively. Essentially similar results were obtained when titrations were performed with *S. acidocaldanus* DNA (fig. 8B).

Thermodynamic binding parameters of the interaction at 20 mM NaCl were obtained by non-Scatchard approach (Schwarz and Watanabe, 1983; Watanabe and Schwarz, 1983) for the binding of large ligands to long lattice structure like nucleic acids. In this case, direct titrations were made by adding HSNP-C to *S. acidocaldarius* DNA (fig 10A and B) The initial straight line of this type of titration was extrapolated to the saturation plateau. At saturation a linear asymptote was reached. This asymptote was parallel to the straight line for the titration without DNA. The intercept of the saturation asymptote with the initial straight line gives the site size, 'n' equal to 4.1 for dsDNA Similar 'n' value was obtained when titrations were performed with 5 |ig DNA. The degree of saturation is determined from the difference between the titrations in the presence and absence of DNA, denoted by 'y' in fig. 10. In order to obtain correct normalisation, it is divided by 'y_α', the distance between the saturation asymptote and the parallel straight line for the titration in the absence of DNA. On the other hand, the observed fluorescence intensity was taken to be proportional to the free protein concentration because we neglected the contribution of the bound protein to fluorescence intensity, since the quenching efficiency is high (greater than 0.7). The degree of saturation, 6 is plotted as a function of the free protein concentration (fig. 11A). Inspection of fig. 11A revealed low but definite co-operativity in the mode of binding. The equation, $\{(2\theta-1)/\sqrt{[\theta(1-\theta)]} = V(q7n)\ (Kc-1)\}$ could be used

Fig. 9: Scatchard plot of the data obtained in fig. 8

Scatchard plot of the data points obtained in fig. 7 for titrations of HSNP-C' with dsDNA at 20 mM and 150 mM NaCl. The data points were extrapolated to the y-axis by 2° regression. The intercept on the y-axis gave a value of 4 x 10^6 M^{-1} at 20 mM NaCl and 4.5 x 10^5 M^{-1} at 150 mM NaCl.

0-0 Data points obtained when titrations were performed at 20 mM NaCl.

•"• Data points obtained when titrated at 150 mM NaCl.

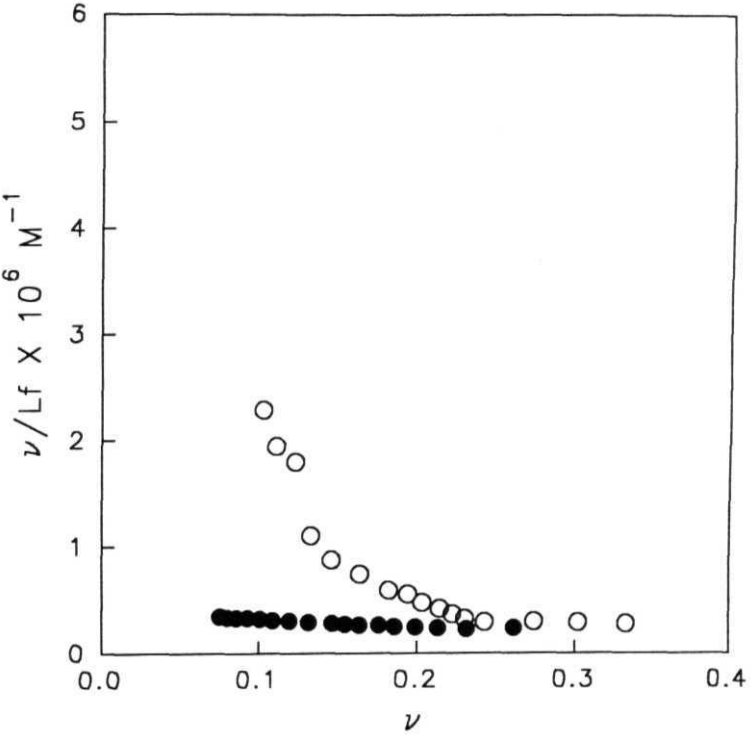


FIGURE 9

Fig. 10: Direct fluorescence titrations of HSNP-C'.

Fluorescence measurements were made by adding increasing amounts of HSNP-C (2 μl) to constant amount of dsDNA in 1 ml of buffer. Details of the analysis are described in the text.
Titrations were performed with:
(A) 6 μM nucleotide dsDNA
(B)15 μM nucleotide dsDNA

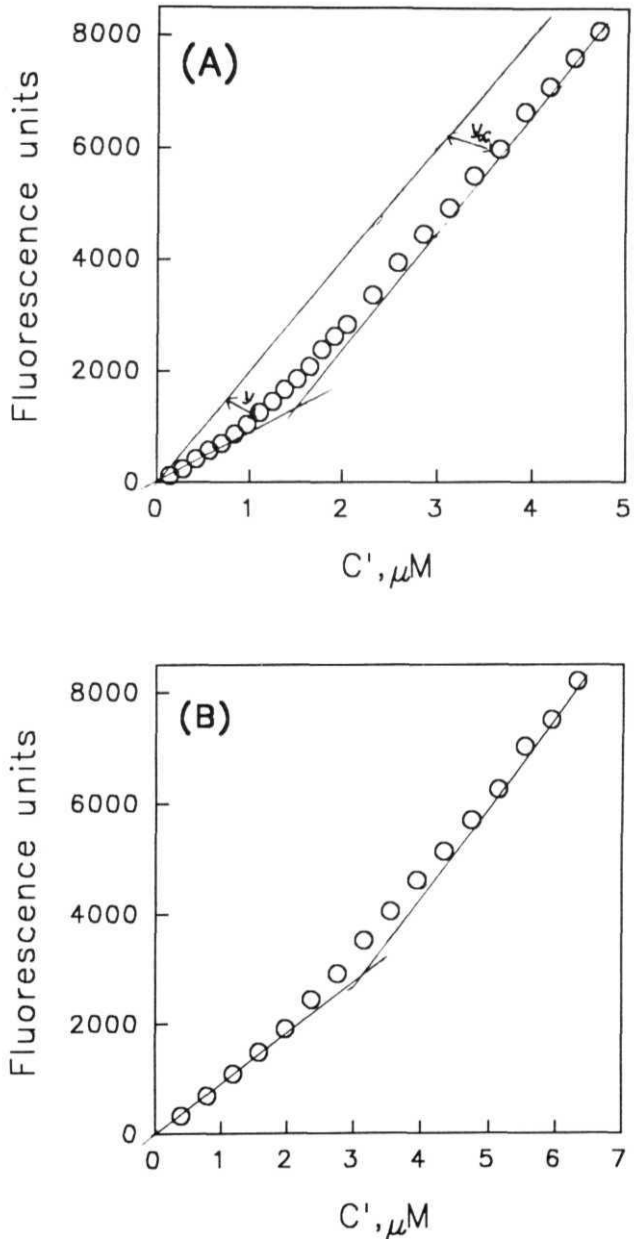

(A)
Fluorescence units
8000
6000
4000
2000
0
0
1
2
3
4
5
C', μM
(B)
Fluorescence units
8000
6000
4000
2000
0
0
1
2
3
4
5
6
7
C', μM

FIGURE 10

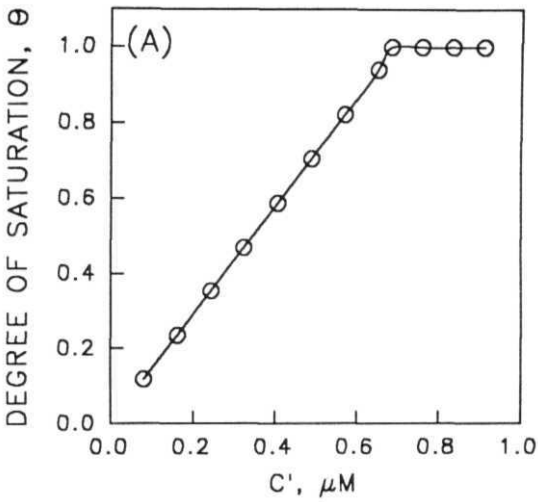
as a good approximation to the binding behaviour around [G = 1/2] where "q" is the co-operativity parameter; "K", the binding constant for the co-operative growth of continous sequences of bound ligand, and c the free protein concentration. A plot of $[(2\theta-1)/\sqrt{[\theta(1-\theta)]}$ versus the free protein concentration is given in fig. 11(B). The value of "K" was evaluated from the reciprocal of the intercept on the abscissa. The value of "q" was estimated from the linearly extrapolated intercept on the ordinate ($= -\sqrt{(q/n)}$) using the value of 'n'. We have obtained from such an analysis, values of "K" of 2.8 x 10^6 M^{-1} and "q" of 2.8 for dsDNA. However, this "q" value is very low for co-operatively binding proteins *(see discussion).* The binding parameters of HSNP-C determined by both the methods are given in table 1.

Fluorescence titrations of HSNP-C were also performed with different nucleic acids such as ssDNA, poly (U) and poly (A). HSNP-C showed weak interaction as indicated by lower extent of quenching (6-12%) to RNA (polyA and polyU). Although, ssDNA quenched the HSNP-C fluorescence, the extent of quenching was much lower than that observed with dsDNA (fig. 12).

Salt titrations: Salt titrations are generally performed to find out the strength of binding between protein and DNA. To a fixed amount of protein, increasing concentration of DNA was added and the increase in quenching was recorded till a saturation point was attained. At this stage aliquots of 4 M NaCl were added directly to the nucleoprotein complexes and the increase in fluorescence intensity was measured (fig 13). The concentration of salt where the maximum fluorescence was recovered was taken to be the concentration of salt required to dissociate the nucleoprotein complexes. More than 80% of the initial fluorescence of the protein was recovered with a NaCl concentration of about 200 mM.

Fig. 11: Plots for the evaluation of "K" and "q" for HSNP-C'

Data of titrations performed with 6 μM nucleotide dsDNA (Fig. 10 A) were used.
(A): Plot of degree of saturation 0, versus concentration of free HSNP-C.
(B): Plot of (20-l)/V[0 (1-0)] versus free concentration of HSNP-C


(A)
DEGREE OF SATURATION, θ
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.2
0.4
0.6
0.8
1.0
C', μM

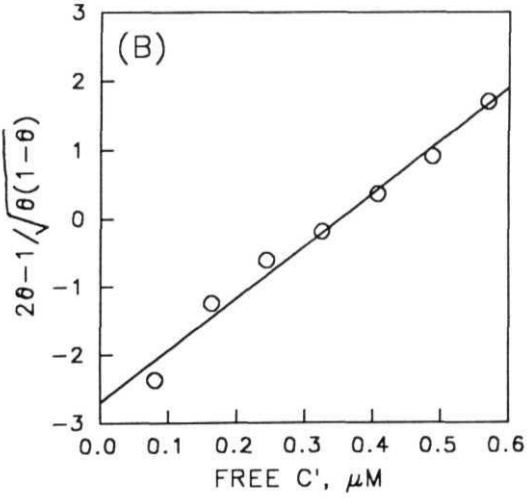

(B)
$2\theta - 1/\sqrt{\theta(1-\theta)}$
3
2
1
0
−1
−2
−3
0.0
0.1
0.2
0.3
0.4
0.5
0.6
FREE C', μM

FIGURE 11

Table 1

Thermodynamic binding parameters of HSNP-C to dsDNA at 20 mM NaCl

Binding site size "n"	Binding constant "K"	Method of analysis
4.4	**4.0 x** 10^6 M^{-1}	McGhee and Von Hippel (1974)
4.1	2.8 **x** 10^6 M^{-1}	Schwaiz and Watanabe (1983)

Fig. 12: Reverse titrations of **HSNP-C'** with different nucleic acids

o-o ssDNA
•-• poly (U)
V-V poly (A)

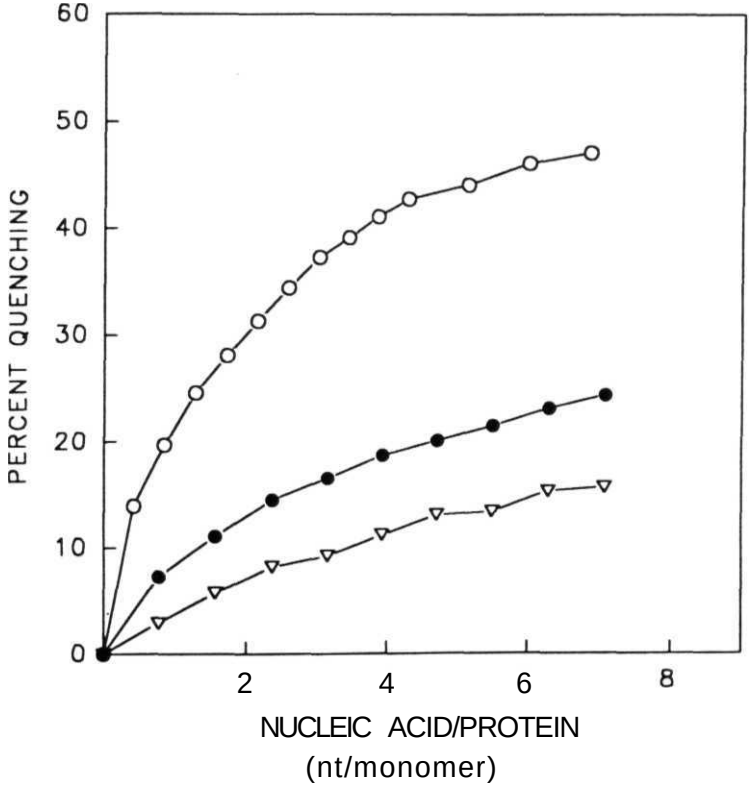

60
50
40
30
20
10
0
PERCENT QUENCHING
2
4
6
8
NUCLEIC ACID/PROTEIN
(nt/monomer)

FIGURE 12

Fig. 13: Back titrations of the DNA-HSNP-C' complex with NaCl.

HSNP-C' was titrated with dsDNA till the saturation point was attained. At this stage, 4 M NaCl (in aliquots) was added to the nucleoprotein complexes and the increase in fluorescence emission was measured.

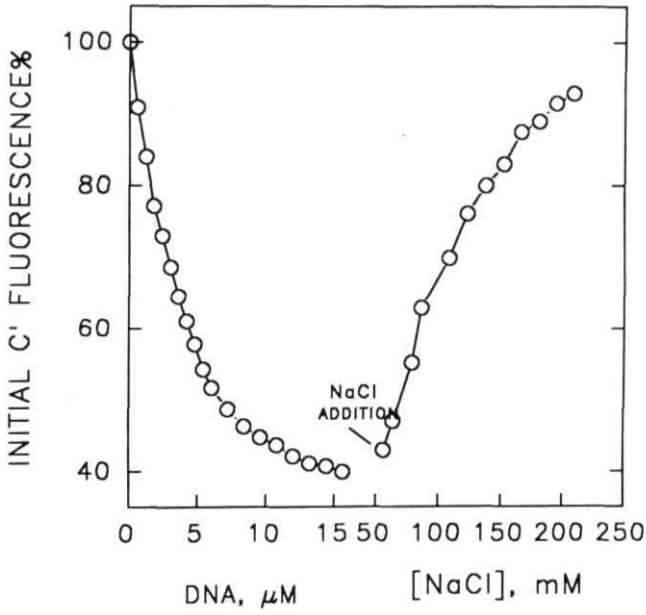

INITIAL C' FLUORESCENCE%
100
80
60
40
NaCl
ADDITION
0
5
10
15
50
100
150
200
250
DNA, μM
[NaCl], mM

FIGURE 13

However, at this salt concentration, substantial binding of HSNP-C' occurs to dsDNA as determined by affinity chromatography (fig. 6A). Probably, the fluorescence titrations do not truly reflect the strength of binding of the protein to nucleic acids *(see discussion).*

Electron microscopy of DNA-HSNP-C' complexes: Electron microscopy of HSNP-C'-RSF 1010 DNA (dsDNA) complexes formed at protein to DNA ratios (w/w) of 0:1, 1.5:1, 3.5:1 and 7:1 are shown in fig. 14: A, B, C and D respectively. At low protein to DNA ratio (upto 3.5), clusters of bound protein on the DNA could be seen (fig. 14: B and C). With increasing protein to DNA ratio (> 7 and higher), the single clumps of protein on the DNA were compacted into one or two central DNA-protein clusters with small loops of free DNA Such clumped structures were also formed with single stranded ϕx 174 DNA (fig. 15: A, B, C and D). The binding to single stranded DNA appears to be non-specific as indicated by the presence of protein aggregates at several sites on the ssDNA

Similarity of HSNP-C to 7d protein :

The structures formed by HSNP-C **with** DNA as studied by electron microscopy resemble those formed by the low molecular weight basic DNA binding protein designated as 7d (Lurz *et al.*, 1986) This indicates that HSNP-C may be similar to 7d protein.

In order to confirm the similarity between 7d and HSNP-C, 7d was purified according to the procedure of Grote *et al.* (1986) Protein 7d containing fractions were electrophoresed on a 15% SDS-polyacrylamide gel (fig 16A). The gel was immunoblotted against anti-serum directed to HSNP-C. Immunoblot of the gel in fig. 16B, with anti-HSNP-C IgG, showed cross-reaction with 7d protein. 7d

Fig. 14: Electron microscopy of **HSNP-C'-dsDNA complexes:**

The experiment was performed by Dr. Lurz of Max Planck Institute for Molecular Genetics, Berlin, Germany. HSNP-C' complexes were formed with RSF 1010 plasmid DNA (8.7 kbp) at different ratios of DNA/ protein (w/w) as given below: (A) 1:0; (B) 1:1:5; (C) 1:3:5 and (D) 1:7.

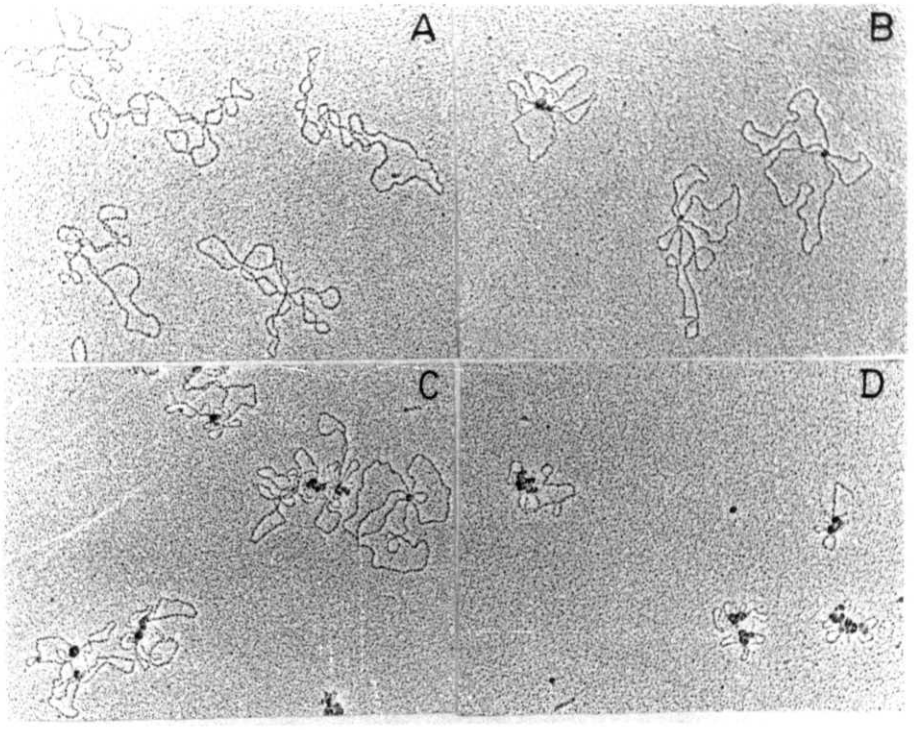


FIGURE 14

Fig. 15: Electron microscopy of HSNP-C'-ssDNA complexes:

HSNP-C' complexes were formed with ϕX-174 ss DNA at ratios (DNA/protein,w/w) as mentioned below:
(A) 1:0; (B) **1:1:5;** (C) 1:3:5 and (D) 1:7.

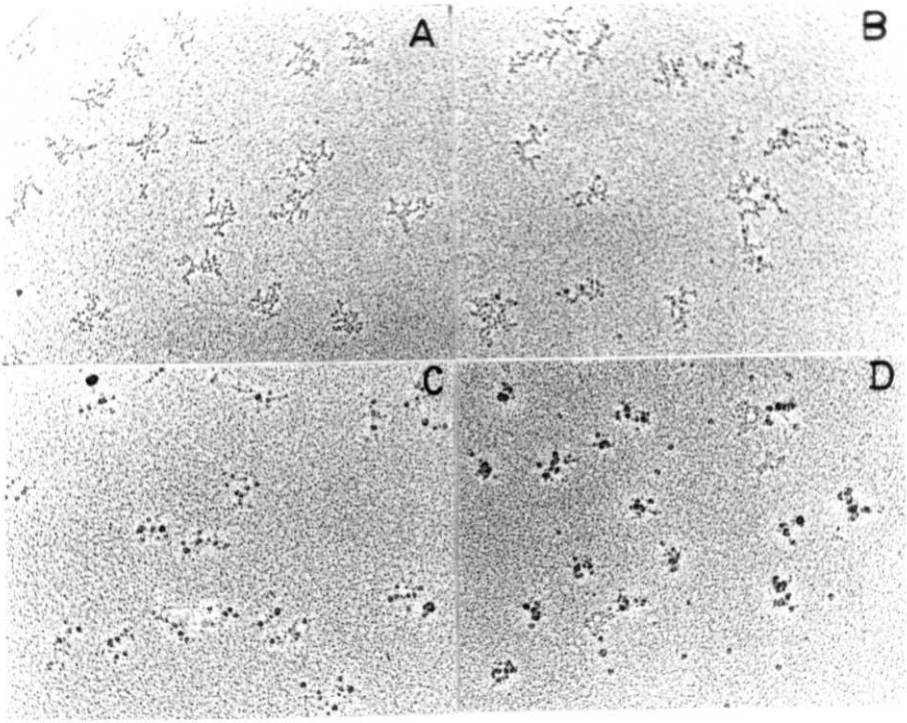


FIGURE 15

Fig. 16A: SDS-PAGE analysis of fractions obtained from CM-sepharose CL6B column chromatography.

Chromatography was performed as described by Grote *et al.*, (1986). S-100 acid extract was loaded on the column and eluted with a linear salt gradient. Alternate fractions were analysed for the presence of protein by SDS-PAGE.
Lane 1: sample loaded
Lane 2: flow through
Lanes 3-18: Alternate fractions obtained from the column.

Fig. 16B: Immunoblot analysis of the above fractions

Proteins from the lower part of the gel in fig. 16A, in the region corresponding to 7d (fractions 11-13) were transferred on to nitrocellulose membranes. The nitrocellulose membrane was probed with anti-HSNP-C and peroxidase conjugated anti-rabbit IgG.
Lane 1: HSNP-C' (control).
Lanes 2-4: Fractions 11-13 respectively

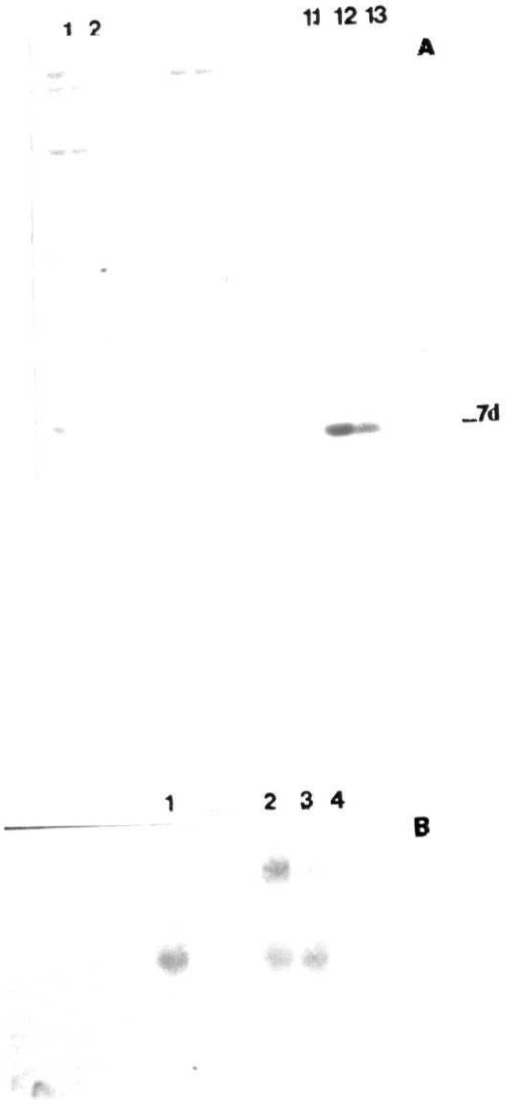


FIGURE 16

protein also showed the formation of higher aggregates such as dimers, trimers etc. as observed in the case of HSNP-C These results indicate that the two proteins are similar if not identical. The work carried out on 7d protein available in the literature include purification, amino acid sequencing and electron microscopic studies of its interaction with DNA Apart from these, no other functional characterisation of the protein has been reported as yet.

Binding of *HSNP-C'* to different mononucleotides:

From the amino acid sequence available in the literature regarding the 7d protein of Grote *et al.* (1986), we have identified an interesting motif (which has not been reported) that is similar to the phosphate binding motif (P-loop sequence) of GTP/ATP binding proteins This phosphate binding motif viz, G-X-X-X-X-G-K (TS) is a common motif in the GTP and ATP binding proteins. In the case of 7d or HSNP-C, a sequence, Gly-Lys-Thr-Gly-Arg-Gly-Ala-Val-Ser-Glu-Lys, was identified between residues 37 to 47. This prompted us to study the interaction of HSNP-C with different nucleotides.

Fig. 17 shows the fluorescence titration curves with different nucleotides. Maximum quenching (28%) was observed with GTP compared to the other nucleotides viz, ATP, CTP, dTTP and dATP. These results are suggestive of the presence of a nucleotide binding domain characteristic of GTP binding proteins However, other nucleotides also bound to HSNP-C with varying affinities as indicated by lower extents of quenching of the intrinsic fluorescence of HSNP-C. The titration curves were biphasic with all the nucleotides tested

In order to distinguish the nucleotide binding domain from the nucleic acid binding domain, step wise titrations with dsDNA and GTP were performed

Fig. 17: Fluorescence titrations of HSNP-C with different mononucleotides

o-o GTP
●-● dTTP
v-v dATP
□-□ CTP
♥-♥ ATP

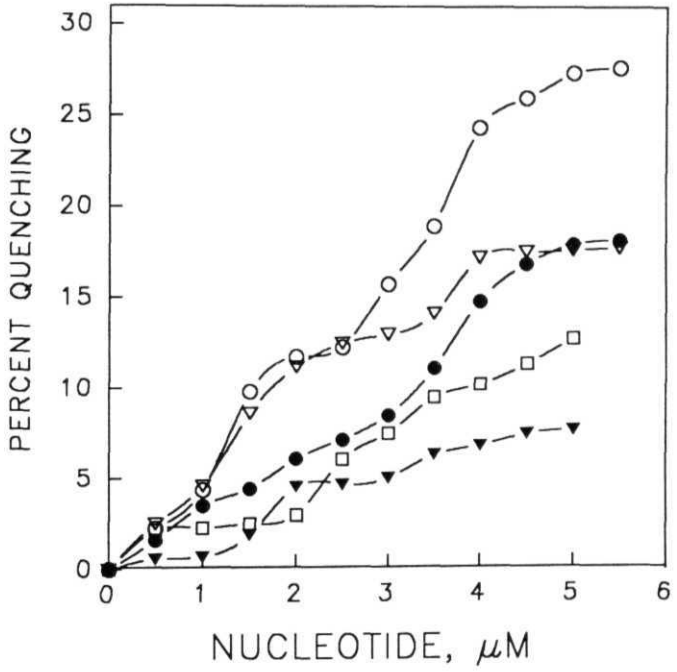


FIGURE 17

Titrations with DNA were performed till saturation in quenching was reached Subsequently, titration was done with GTP. Titrations were also performed first with GTP and then with DNA Results presented in figs. 18A & B show that the intrinsic fluorescence of HSNP-C was quenched upto its normal value when titrated with DNA. Addition of GTP brought about a further detectable quenching (~ 15%) in the fluorescence of the protein. Similar results were obtained when titrated first with GTP and then with DNA. The results clearly indicate that the nucleic acid binding domain is distinct from the nucleotide binding domain on the protein. The results also suggest heterogeneity in the binding of nuclelotides as indicated by biphasic curves when titrated with GTP alone

Fluorescence study with artificial quenchers:

The concentration of salt (178 mM), required to dissociate the nucleoprotein complexes as indicated by reverse titrations was much less than the concentration of NaCl required to elute HSNP-C bound to dsDNA-cellulose column. This prompted us to study the exposure/availability of rryptophan residues in HSNP-C for quenching. Fluorescence in proteins is also quenched by small molecular weight ligands such as acrylamide and iodide Titration with these have been earlier used to assess the extent of exposure of aromatic amino acid in proteins (Omar and Schleich, 1981). Fig 19A & B show the fluorescence quenching curves of HSNP-C with increasing concentrations of acrylamide and KI in the presence of different concentrations of NaCl. The extent of fluorescence quenching decreased with increasing salt concentrations. These results suggest that the aromatic amino acid residues in HSNP-C become buried or unavailable for quenching as a result of conformational changes in the protein at high concentrations of salt.

Fig. 18: Stepwise fluorescence titrations of HSNP-C' were performed with dsDNA and GTP

HSNP-C (10 μg) was titrated as follows:
(A)-first with dsDNA and then with GTP;
(B)-first with GTP and then with dsDNA.

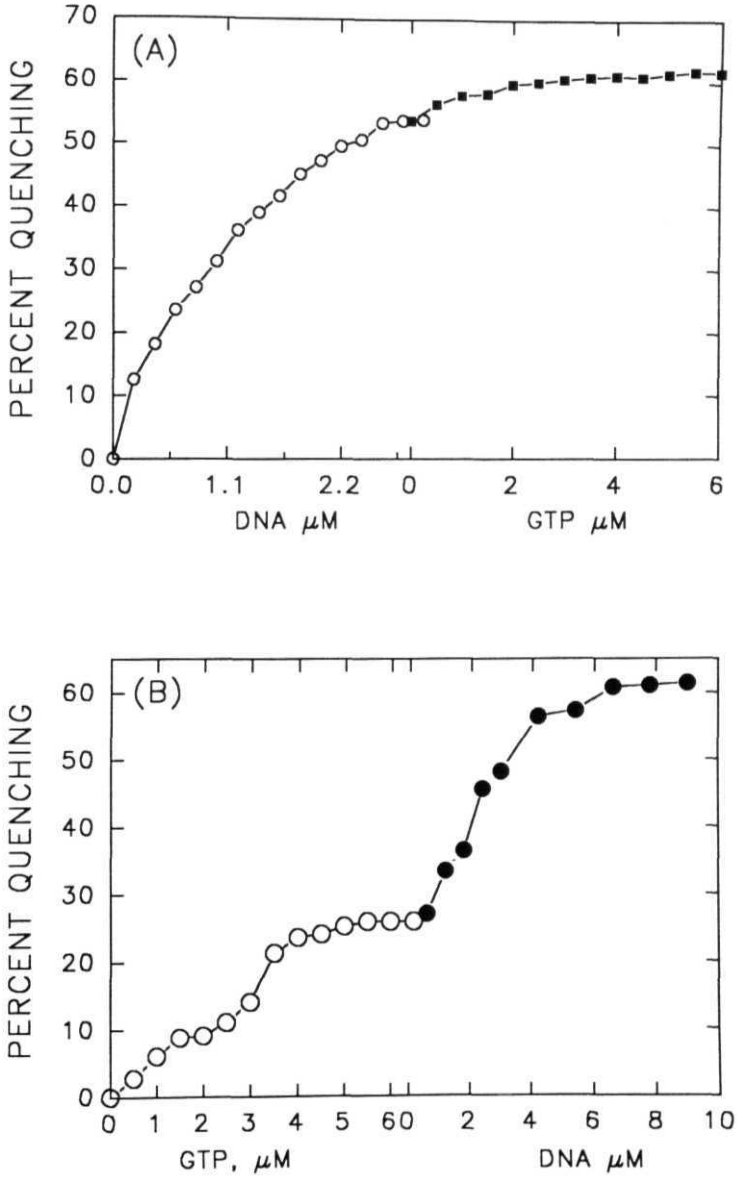

(A)
PERCENT QUENCHING
70
60
50
40
30
20
10
0
0.0
1.1
2.2
0
2
4
6
DNA μM
GTP μM
(B)
PERCENT QUENCHING
60
50
40
30
20
10
0
0 1 2 3 4 5 60 2 4 6 8 10
GTP, μM
DNA μM

FIGURE 18

Fig. 19A: Quenching of the intrinsic fluorescence of HSNP-C by acrylamide

HSNP-C (1.1 (iM) was titrated with increasing concentrations of acrylamide at different conditions of salt.

o-o 20 mM NaCl
•-• 50 mM NaCl
Δ-Δ 150 mM NaCl
V-V 200 mM NaCl

Fig. 19B: Quenching of the intrinsic fluorescence of HSNP-C by Kl

HSNP-C **(1.1** μM) was titrated with increasing concentrations of Kl at different NaCl concentrations

o-o 20 mM NaCl
•-• 50 mM NaCl
A-A 150 mM NaCl
V-V 200 mM NaCl

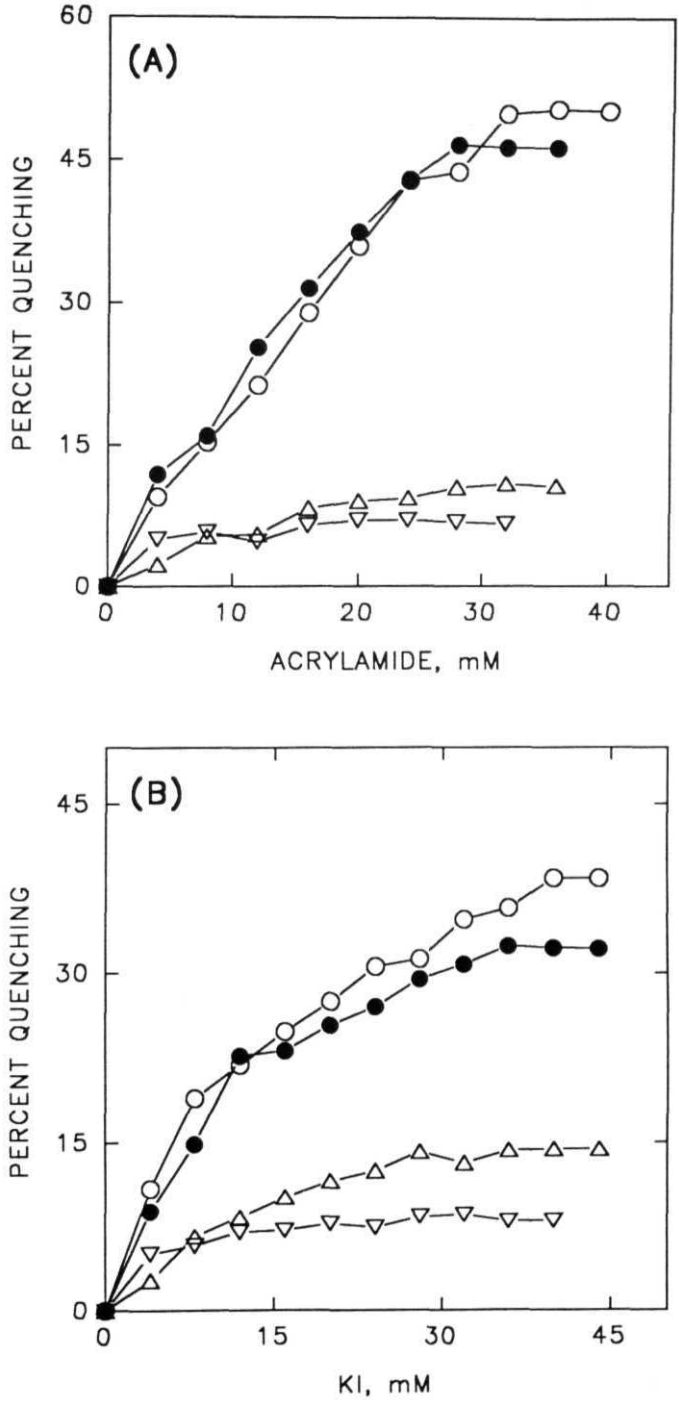

(A)
PERCENT QUENCHING
60
45
30
15
0
0
10
20
30
40
ACRYLAMIDE, mM
(B)
PERCENT QUENCHING
45
30
15
0
0
15
30
45
KI, mM

FIGURE 19

Fluorescence emission spectra of HSNP-C titrated with acrylamide (fig. 20A) and KI (fig. 20B) at maximum ligand concentration in the presence of increasing NaCl concentrations are given in fig 20A & B. The quenching caused by KI and acrylamide gradually decreased with increase in salt concentrations.

Fluorescence emission spectra of HSNP-C in different salt concentrations were also recorded. There is a gradual decrease in the intensity of intrinsic fluorescence of HSNP-C with the increase in the concentration of salt in the medium (fig. 21A).

Effect of Pi concentration on emission **spectrum of *HSNP-C*:** Fig 22 shows the fluorescence emission spectra of HSNP-C in the presence of different concentrations of Pi. Increase in the Pi concentration resulted in the decrease in the intensity of fluorescence spectrum All the spectral curves treated with Pi showed an isosbestic point at 405 nm These results indicate that phosphate forms a specific complex with HSNP-C.

Thermal melting studies:

HSNP-C strongly stabilises DNA against thermal denaturation (Reddy and Suryanarayana, 1989). Since monovalent, divalent and polyvalent cations such as polyamines are also known to strongly protect DNA against thermal denaturation, experiments were carried out to see the effect of salt concentration on the stabilisation of DNA by HSNP-C.

Thermal denaturation profiles of DNA and protection by HSNP-C in the presence and absence of spermidine are given in fig 23A &B. Tm curves were obtained at different protein/DNA mole ratios. At maximum protein/DNA mole ratio tested (i.e., 5), an increase in the Tm of 15 degrees centigrade was obtained.

Fig. 20A: Fluorescence emission spectra of HSNP-C titrated with acrylamide at different salt concentrations

HSNP-C was titrated with acrylamide as in fig. 19A at different NaCl concentrations. Fluorescence emission spectia of acrylamide-HSNP-C' at saturation were recorded.
Spectrum 1: HSNP-C control
Spectra 2-5: acrylamide-HSNP-C complex at 20, 50, 150, 200 mM NaCl respectively.

Fig. 20B: Fluorescence emission spectra of HSNP-C titrated with KI at different salt concentrations

Fluorescence emission spectra at each salt concentration of Kl-HSNP-C at saturation were recorded.
Spectrum 1: HSNP-C control
Spectra 2-5: Kl-HSNP-C complex at 20, 50, 150, 200 mM NaCl respectively.

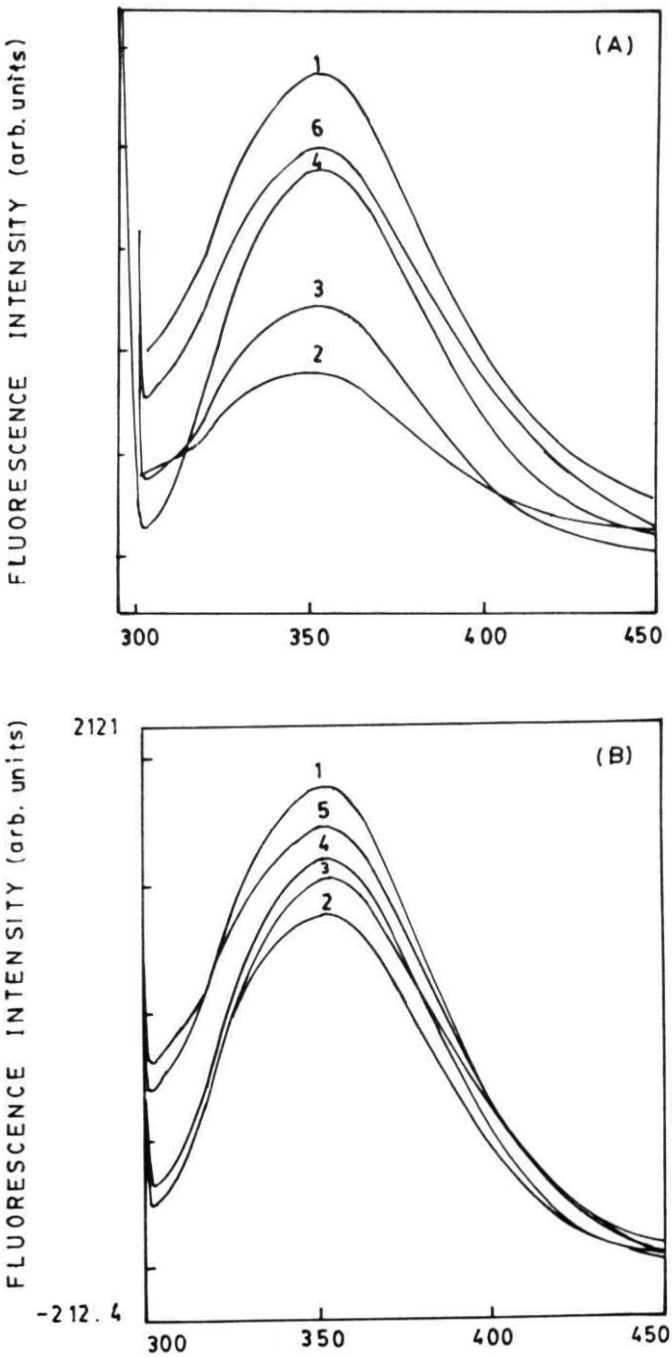

(A)
FLUORESCENCE INTENSITY (arb. units)
1
6
4
3
2
300
350
400
450
(B)
2121
-212.4
5

Figure 20

Fig. 21: Fluorescence emission spectra of HSNP-C' in different salt concentrations

HSNP-C' (10 μg) was incubated for 10 minutes in the reaction buffer and the spectrum was recorded with Exc. at 285 nm and Em between 300 and 450 nm. The concentration of salt in the reaction buffer was varied and spectra recorded separately in each case.
Spectra 1-6: recorded at 20, 50, 100, 200, 300 and 400 mM NaCl respectively

Fig. 22: Effect of Pi on the emission spectra of **HSNP-C**

The concentration of Pi in the reaction buffer was varied and spectra recorded as mentioned in the legend to the fig.21
Spectra 1-7: recorded at 0, 20, 50, 100, 200, 300 and 400 mM Pi concentration respectively

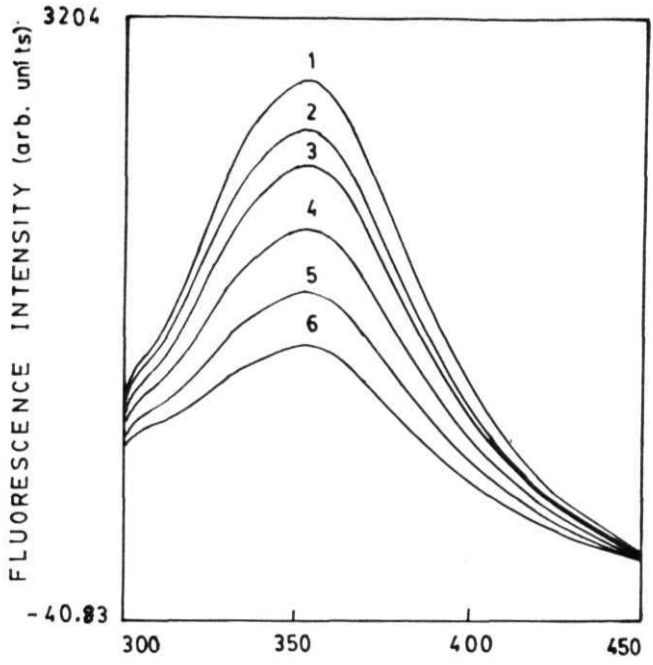


Figure 21

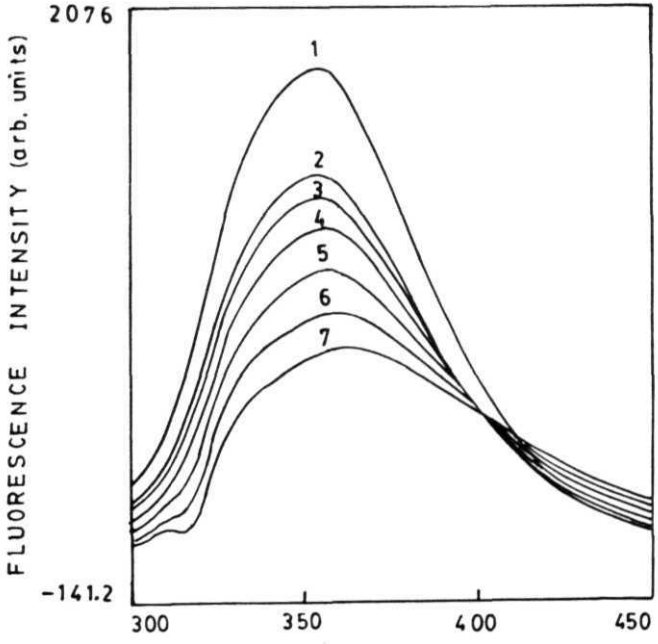


Figure 22

Fig. 23: Thermal denaturation of DNA and protection by HSNP-C'

Thermal denaturation profiles of DNA were obtained in the absence (A) and presence (B) of spermidine. The Tm curves were obtained at different protein to DNA ratios. Increase in A_{260} was plotted against the rise in temperature in °C. HSNP-C was incubated with dsDNA at different protein/DNA ratios before denaturation at the starting temperature.

••• protein/DNA = 0
0-0 protein/DNA = 1
♥-♥ protein/DNA = 2
V-V protein/DNA • 5

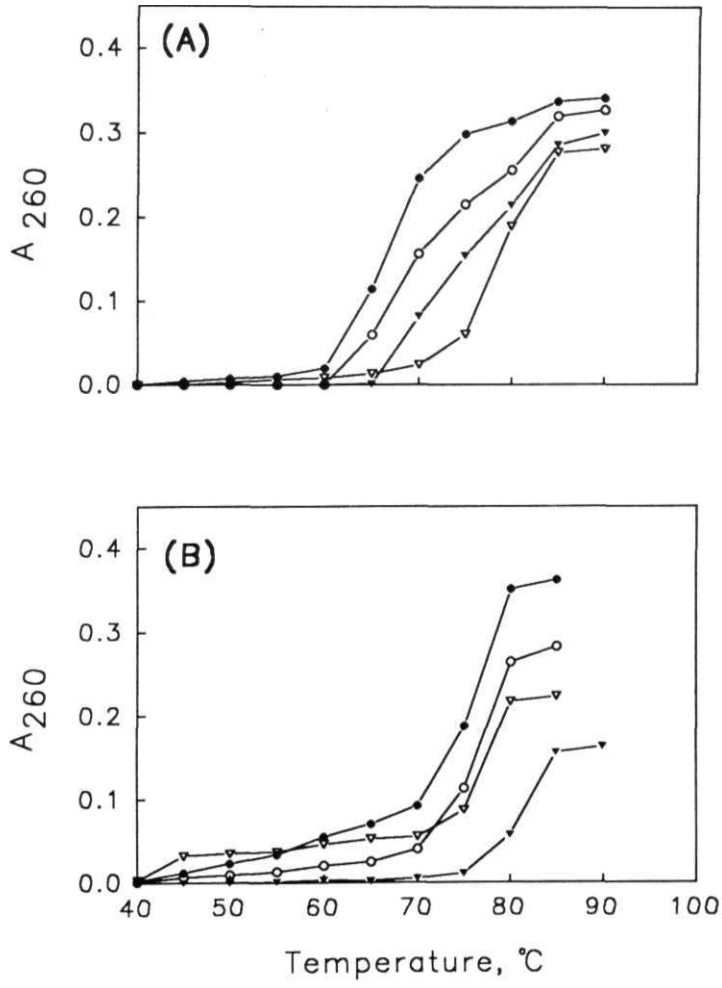


FIGURE 23

Thermal denaturation in the presence of spennidine and other cations (at moderate concentrations) showed an increase in Tm by 5-8 degrees centigrade. Addition of HSNP-C under these conditions resulted in an increase of Tm by a further 16-18 degrees centigrade. These results suggest that HSNP-C protects DNA even in the presence of polyvalent cations.

DNA aggregation by *HSNP-C'*:

HSNP-C aggregates DNA at high concentration (protein/DNA >5). Formation of DNA-protein aggregates was studied by light scattering monitored as increase in absorbance at 320 nm. DNA aggregation by HSNP-C was carried out at different temperatures and pH. Results presented in fig 24 show that at a protein to DNA ratio greater than 4, there was aggregation of DNA as indicated by the increase in O.D at 320 nm. Aggregation was found to be optimum at 35 °C. However, appreciable aggregation was also noted at other temperatures.

Protection of DNA against DNAse I hydrolysis by *HSNP-C':*

DNAse I digestion of dsDNA in the presence and absence of HSNP-C was studied by spectrophotometry by measuring increase in absorbance at 260 nm with time of incubation (fig 25). Increasing amounts of HSNP-C progressively protected DNA against DNAse I digestion as indicated by the declined rate and the extent of increase in A_{260}. We could not see the effect of addition of higher amounts of the protein as there was aggregation of DNA-protein complexes.

Protection of DNA by HSNP-C against DNAse 1 digestion was also analysed by agarose gel electrophoresis. HSNP-C protected λ DNA against

Fig. 24: Formation of DNA protein aggregates studied by light scattering method

DNA aggregation by HSNP-C at different conditions of temperature were studied by measuring the increase in absorbance at 320 nm.

●-● assay performed at 25 °C

O-O at 35 °C

A-A at 45 °C

▽-▽ at 55 °C

□-□ at 65 °C

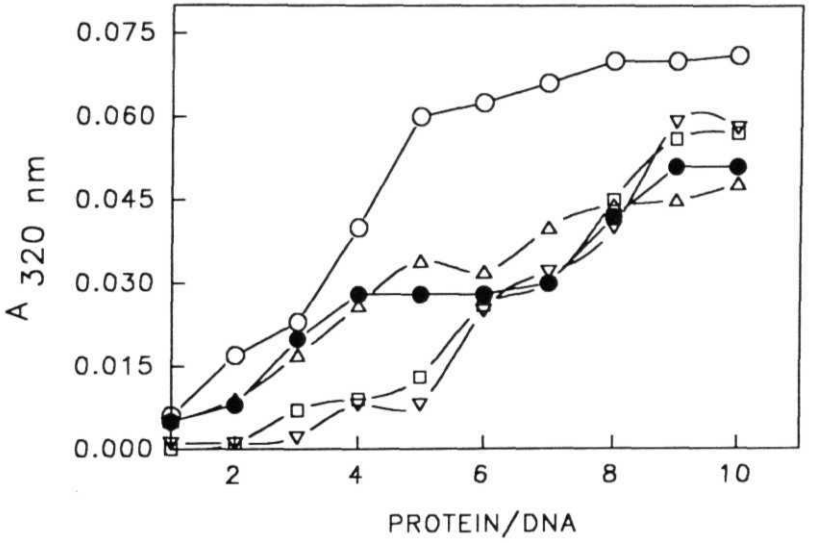


FIGURE 24

Fig. 25: Effect of HSNP-C' on the action of DNAse I.

Native calf thymus native DNA (10 μg) was incubated with different amounts of protein and then subjected to pancreatic DNAse I (0.5 μg) treatment. Increase in A_{260} was recorded at 30 °C for 60 min.

o-o No HSNP-C
•-• 20 μg HSNP-C
v-v 40 μg HSNP-C

Fig. 26: Protection of λ DNA against DNAse I digestion by HSNP-C

λ DNA (200 ng) in different vials was incubated with different amounts of HSNP-C at 37 °C for 30 minutes. DNAse I (2 μg) was added to each of the samples and incubated at 37 °C for 15 minutes. At the end of the 15th minute the samples were treated with EDTA (10 mM), deproteinized with 1% SDS, loaded on 0.8% agarose gels electrophoresed at 26 V for 4 hours.

Lanes 1-5: *X* DNA incubated with 1 μg; 800 ng; 600 ng; 400 ng and 200 ng HSNP-C respectively before DNAse I digestion.
Lane 6: *X* DNA digested with DNAse I
Lane 7: λ DNA (control)

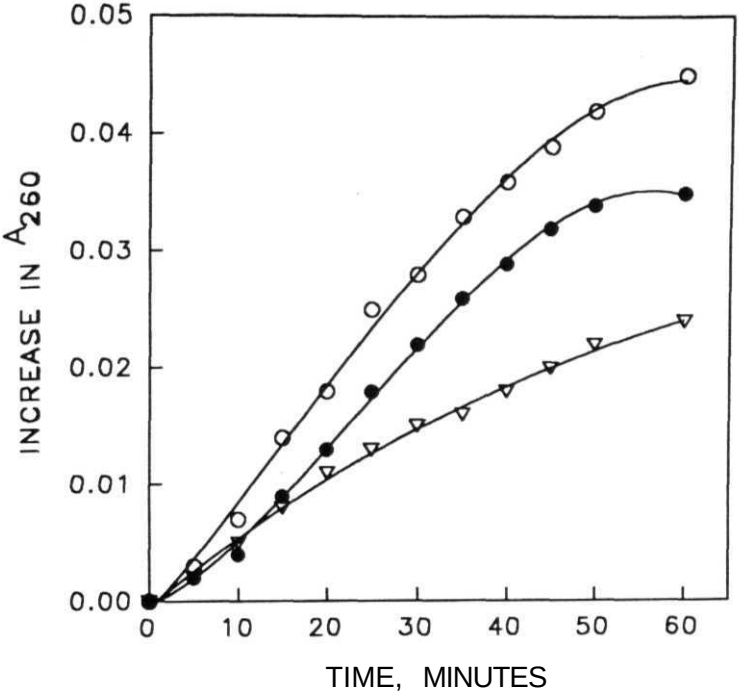


FIGURE 25

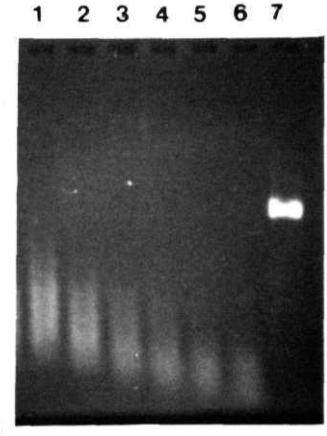


FIGURE 26

DNAse I hydrolysis with increasing concentration as indicated by the increase in the size of heterogenous size fragments (fig 26)

Displacement of DNA-bound EtBr by *HSNP-C'*:

Ethidium, free in solution shows low fluorescence emission at 595 nm when excited at its absorption maximum i.e., 480 nm. The fluorescence of ethidium increases several fold when incubated with dsDNA and this increase is due to stacking of ethidium by intercalation into dsDNA (Lepecq and Paoletti, 1967). HSNP-C was added to this ethidium-DNA complex and the fluorescence emission spectrum was recorded after each addition (10 μg) (fig. 27). A gradual decrease in fluorescence intensity is seen after each addition indicating the release of DNA bound ethidium. DNA complexes formed after the addition of increasing amounts of HSNP-C are shown in fig. 28

CHEMICAL MODIFICATION OF *HSNP-C'* AM) THE EFFECT ON ITS INTERACTION WITH DNA:

Chemical modifications of specific amino acid residues in proteins are widely used to assess the involvement of amino acid functional groups in the active site of enzymes and also in assessing their role in protein-nucleic acid interactions. With the help of chemical modifications as a tool, the role of specific amino acids in nucleic acid-protein interactions can be assessed. The common amino acid functional groups implicated in the interaction of proteins with nucleic acids are arg. lys contributing to electrostatic interactions and phe, tyr, trp contributing to hydrophobic and intercalating interactions. The effect of chemical

Fig. 27: Displacement of intercalated ethidium from DNA by HSNP-C.

Fluorescence emission spectia of ethidium bromide, intercalated and displaced from dsDNA were recorded. The excitation was at 480 nm and emission scan was obtained between 500-660 nm.

Spectrum 1: free ethidium bromide (400 ng) in solution

Spectrum 2: ethidium bromide in the presence of DNA.

Spectrum 3-8: obtained as a consequence of addition of 1 μM; 2 μM; 3 μM, 4 μM, 5 μM, 6 μM HSNP-C respectively.

Fig. 28: Data from Fig. 28 was plotted again taken % relative fluorescence intensity as y-axis and HSNP-C concentration as x-axis.

o-o Ovalbumin

•-• HSNP-C.

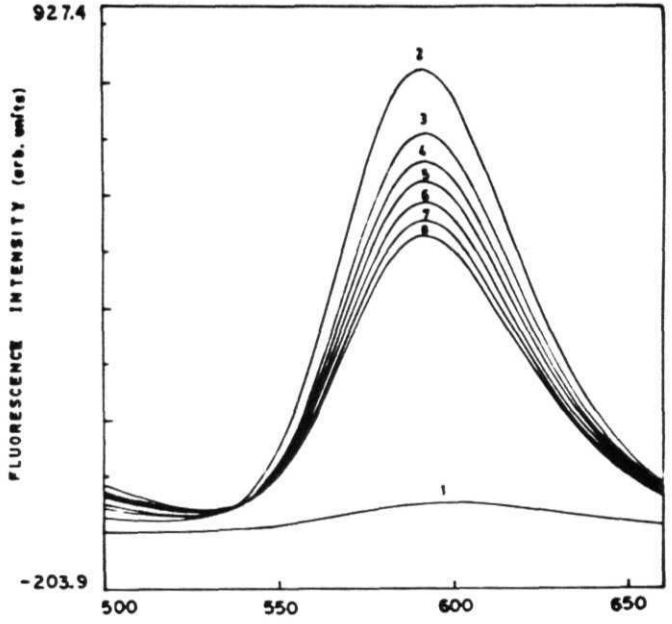


FIGURE 27

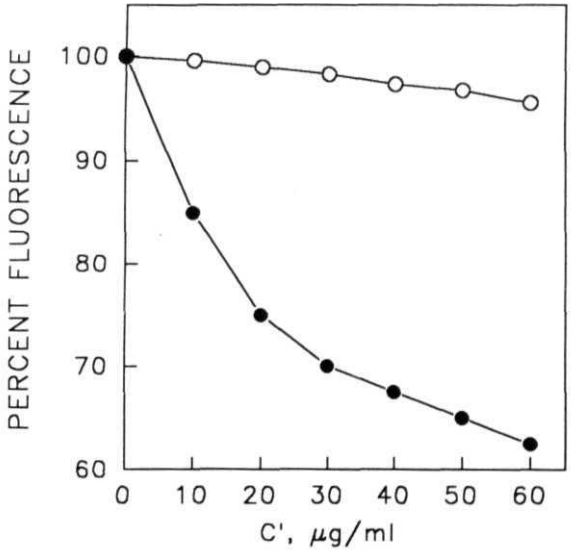


FIGURE 28

modifications of HSNP-C was tested on its interactions with nucleic acid to assess the amino acyl functional groups involved and to understand the mechanism of helix stabilisation by HSNP-C.

Since basic amino acids are generally implicated in DNA-binding by several of the basic proteins like histones, that result in the condensation of DNA. lys and arg in HSNP-C were modified Lysine was modified by reductive methylation in the presence of formaldehyde (HCHO) and reductive alkylation in the presence of pyridoxal phosphate (PLP), while arg was modified with 2,3, butanedione commonly known as diacetyl. Apart from these aromatic amino acids tyr and trp were also modified using tetranitromethane (TNM) and BNPS-skatole respectively.

Reductive methylation of *HSNP-C'* using HCHO: Selective alkylation of amino groups of lysine was achieved by exposure of protein in alkaline medium to low concentrations of formaldehyde and borohydride (Means and Feeney, 1968) The presence of methylated lysines could be identified by descending chromatography (lysine-R_f = 0.183; ε-N-monomethyl lysine-R_f = 0.38-0.44) Buffers used were m-cresol:88% phenol: borate buffer in the ratio 190:165:45 The reaction mechanism can be summarised as follows:-

$$R\text{-}NH_2 + HCHO \underset{+H_2O}{\overset{-H2O}{<======>}} R\text{-}N{=}CH_2 \xrightarrow{NaBH4} R\text{-}NH\text{-}CH3$$

$$R\text{-}NH\text{-}CH_3 + HCHO \underset{+H_2O}{\overset{-H_2O}{<==========>}} R\text{-}(CH_3)N^{+}{=}CH_2 \xrightarrow{NaBH_4} (RNCH_3)_2$$

Different colours were obtained for different derivatives Normal lysines gave a blue-grey colour whereas the derivative of the modified lysine gave **a** blue-violet colour. Rf value was characteristic of monomethyl residues (DeLange *et ai,* 1969)

Reductive methylation using PLP: This modification was carried out according to Ohsawa and Gualerzi, (1981). Site specific chemical modification *of* proteins with pyridoxal phosphate has been used widely to inactivate phosphate binding enzymes (Strausbauch and Fischer, 1970; Piszkiewicz *et ai,* 1977). The scheme of the reaction is given in fig 29A The ε-NH_2 group of lysine forms a schiffs base with the aldehyde group of pyridoxal phosphate which is stabilized by reduction with $NaBH_4$ to form E-5'-phosphopyridoxyl lysine It has a maximum intensity of fluorescence with a characteristic λmax of 325 nm (fig. 29B). The absorbance peak in the case of modified lysine residues (pyridoxyl-lysine) also shifted from 278 nm to 325 nm in the visible absorption spectrum as compared to the control (fig 29C). The calculation suggests that about 6 out of 13 residues of lysines were modified ($\varepsilon_{325} = 9710\ M^{-1}$ for PLP-lys).

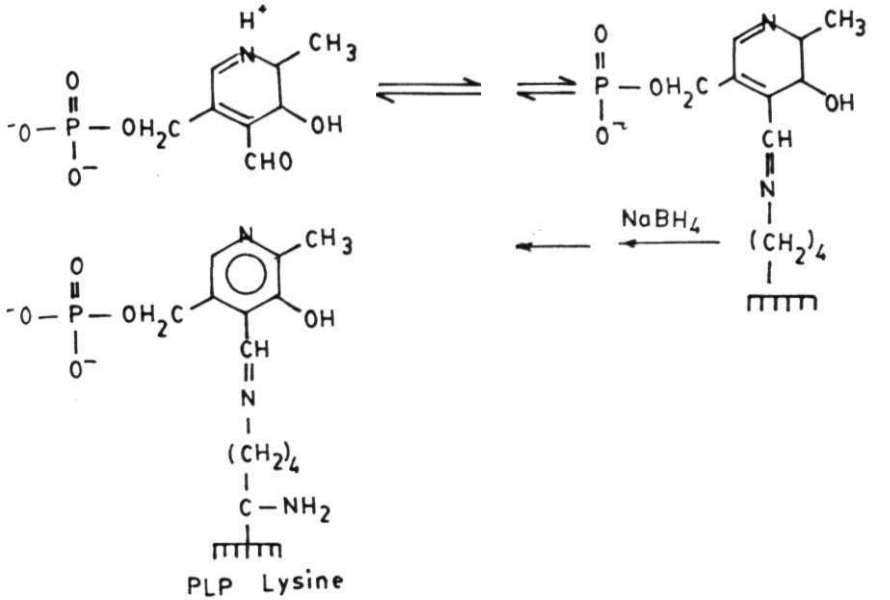
Modification **of Arginine:** Yankeelov *et ai,* in 1968 have reported that the trimer of 2,3 butanedione (I) as well as the dimer (II) are the reactive forms of this reagent as shown in fig. 30A. Riordan, (1973), has reported that the monomer of 2,3 butanedione inactivates carboxypeptidase as effectively as the trimer. One interesting feature of his study showed that 0.05 M borate buffer medium enhances the rate of modified arginines, the probable reason being attributed to the formation of a cyclic borate ester following the initial condensation of the guanidino group with 2,3 butanedione. Using this reagent, several enzymes acting on phosphate containing substrates were shown to have arginyl residues at their

Fig. 29A: Mechanism of the reaction that occurs during the modification of lysine residues of **HSNP-C'** using pyridoxal phosphate resulting in the formation of pyridoxyl-lysine in the protein.

Fig. 29B: Absorption spectra of **pyridoxyl-HSNP-C'**

Spectrum 1: Control HSNP-C
Spectrum 2: lys-(PLP) modified HSNP-C

(A)


PLP Lysine
NaBH4

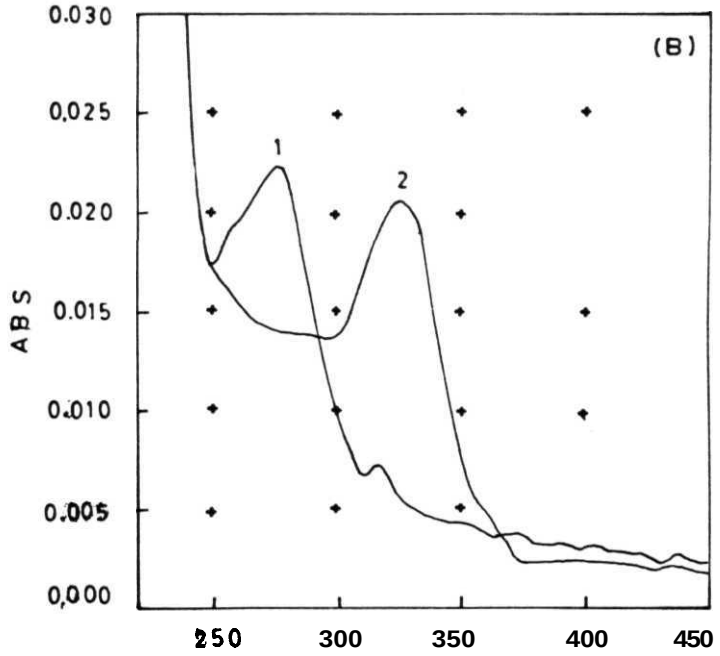

(B)
ABS
0.030
0.025
0.020
0.015
0.010
0.005
0.000
250
300
350
400
450
1
2

Figure 29

Fig. 29C: Fluorescence spectra of pyridoxyl-HSNP-C'

Spectrum 1: control HSNP-C'
Spectrum 2: lys-(PLP) modified HSNP-C

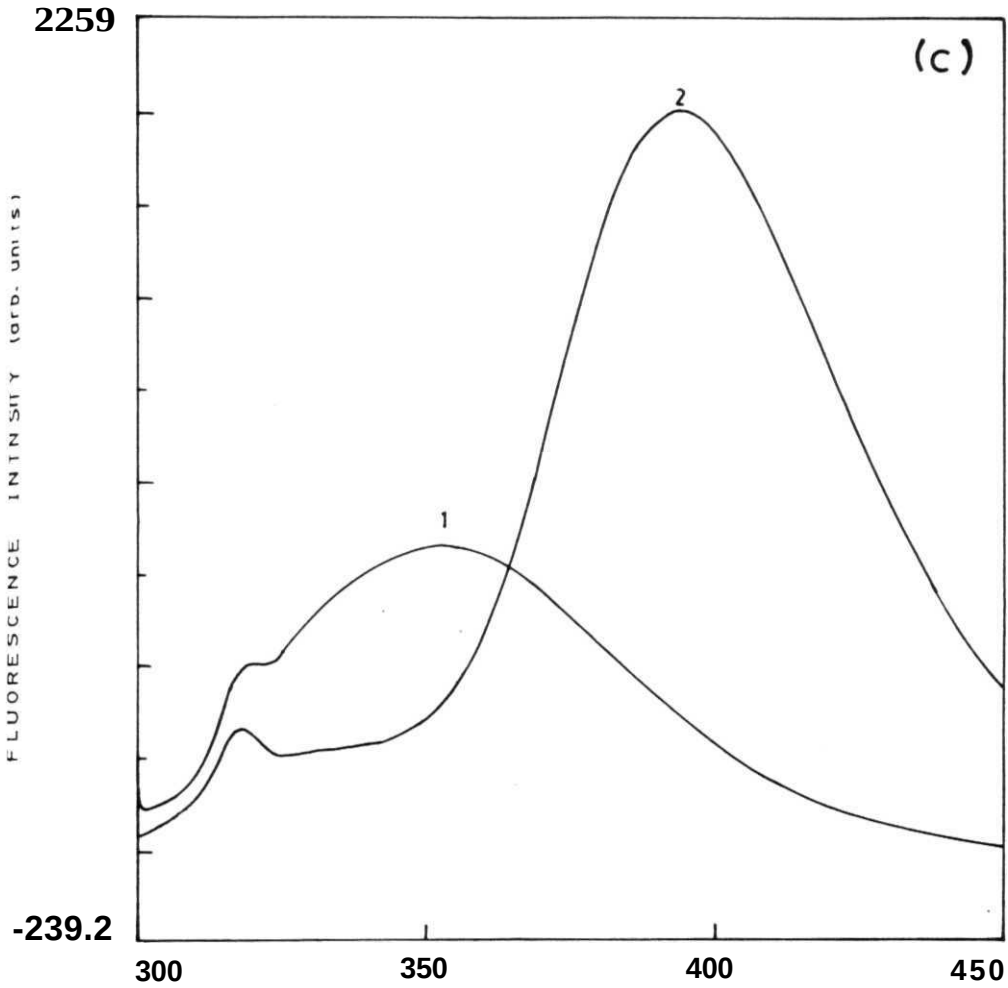
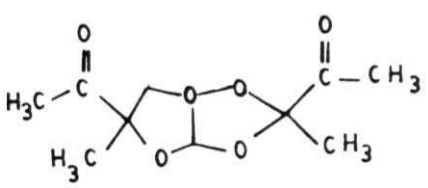

2259
(c)
2
1
FLUORESCENCE INTENSITY (arb. units)
-239.2
300
350
400
450

Figure 29

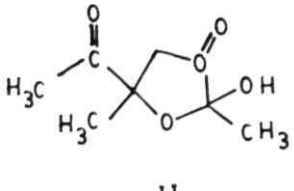
Fig. 30A: Represents the active forms of the reagent, 2, 3, butanedione (diacetyl). The trimer (I) as well as the dimer (II) of 2, 3, butanedione are reactive forms of the reagent..

Fig. 30B: Mechanism of modification of arginine by 2, 3, butanedione reagent. A cyclic borate ester is formed following the condensation of guanidinium group with 2, 3, butanedione.

(A)



I



II

(B)

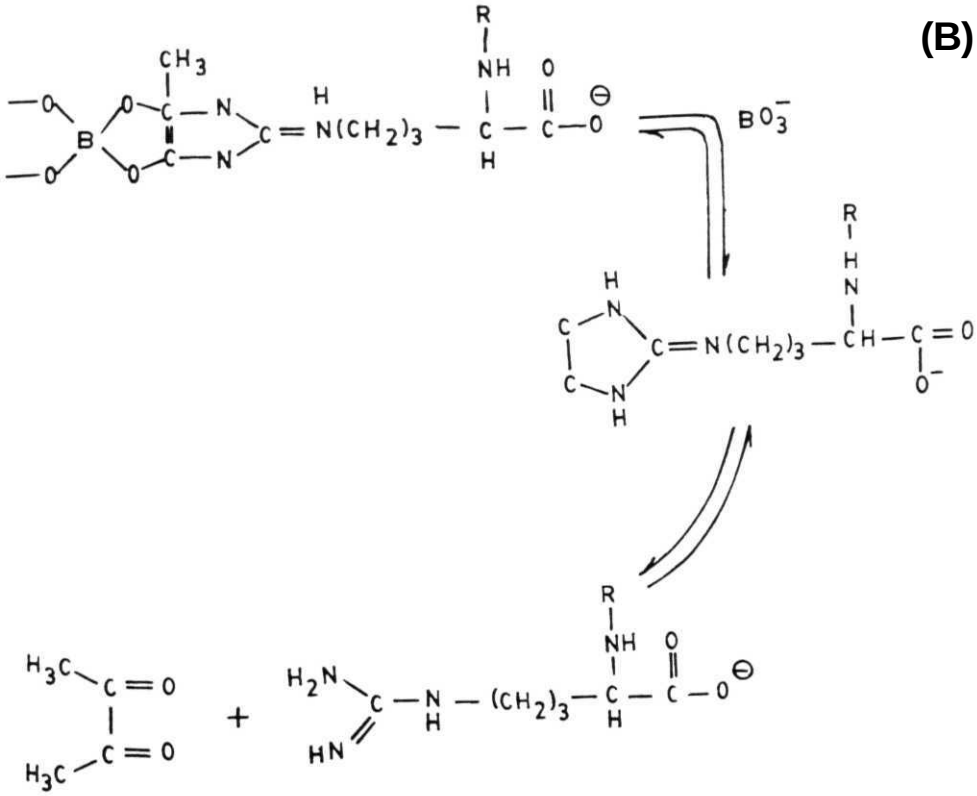


Figure 30

active sites (alcohol dehydrogenase, alkaline phosphatase etc.). The mechanism of the modification is given in fig. 30B.

Modification of tyrosine residues: There are two most widely used procedures for tyrosine modification, viz., nitration and iodination. Nitration of tyrosyl residues with TNM is now one of the most frequently attempted modification reactions for native proteins (Riordan and Sokolovsky, 1971). The procedure for modification of HSNP-C was according to Cheng and Pierce, (1972). The mechanism of the reaction is given in fig. 31A.

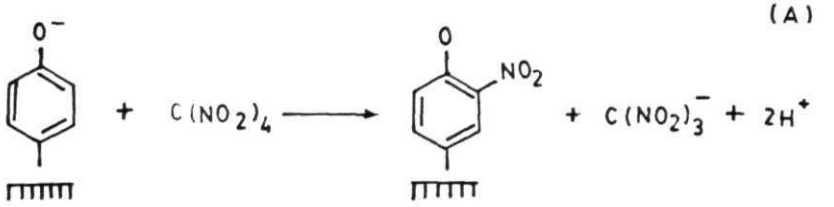
The visible absorption spectrum of the control and modified protein was measured in a solution buffered at pH 9.0 Absorption spectrum (fig 31B), shows a peak at 350 nm in the case of modified protein which is characteristic of the formation of the mid-product, nitroform anion, which eventually leads to the formation of nitro-tyrosine. From the extinction coefficients of nitro-tyrosine (ε_{428} = 4200), it was inferred that both the tyrosines in HSNP-C are modified.

Modification of tryptophan residues: At low reagent to protein tryptophan ratios, in 50% aqueous acetic acid, BNPS-skatole reacts selectively with trp residues converting these to oxindole derivatives (fig. 32) (Fontana and Seoffone. 1972). BNPS-skatole quantitatively oxidises the indole ring of tryptophan and the sulphur atom of methionine. Since methionine sulfoxide is reduced to methionine afterwards, it is possible to obtain a protein derivative selectively modified at tryptophan residues.

Fig. 31 A: Mechanism of nitration of tyrosyl residues of proteins with TNM.

Fig. 31B: Absorption spectra of Tyr-modified HSNP-C'

A characteristic peak was seen during the progress of the modification reaction at a wavelength of 350 nm characteristic of the presence of nitroform derivative of the tyrosyl residue eventually leading to the formation of nitro-tyrosyl residues of the protein.
Spectrum 1: control HSNP-C
Spectrum 2: tyr-(TNM) modified HSNP-C


(A)
O⁻
+ C(NO2)4
O
NO2
+ C(NO2)3⁻ + 2H⁺

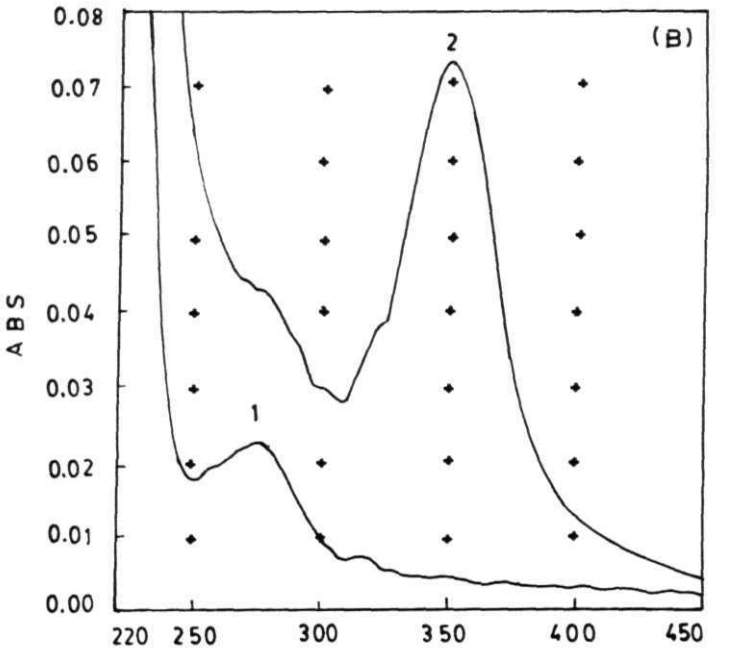

(B)
ABS
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
220
250
300
350
400
450
1
2

Figure 31

Fig. 32: Mechanism of modification of tryptophan residues in the protein brought about by the reagent, BNPS-skatole (2, (2-nitrophenyl sulfenyl)- 3-methyl-3-bromoindolamine).

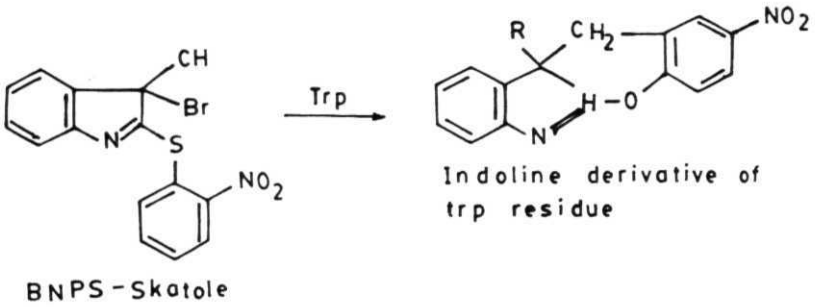

CH
Br
N
S
NO2
Trp
R
CH2
NO2
H-O
N
Indoline derivative of
trp residue
BNPS-Skatole

Figure 32

HSNP-C' modified by different reagents was electrophoresed on SDS-PAGE. No cross-linked or degradative products were observed as a consequence of the modifications (fig 33).

Fluorescence spectra of modified proteins:

From the amino acid composition as previously determined (Reddy and Suryanarayana, 1988), HSNP-C is calculated to contain 2 phe, 2 tyr and 1 trp residues. Modification of aromatic amino acids (tyr and trp), had drastically abolished the characteristic intrinsic fluorescence of HSNP-C. As depicted in fig. 34, a decrease in the intensity of the fluorescence was observed as a function of modifications which could probably mean that these modifications possibly bring about some changes in the conformation of the protein Lysine, arginine modifications resulted in drastic reduction in the intensity of fluorescence emission (50-60%). The modified proteins were later tested for their interactions with DNA

Effect of chemical modification of HSNP-C on nucleic acid binding properties:

Effect of chemical modification on the interaction of HSNP-C to nucleic acids was studied by affinity chromatography on DNA-cellulose matrices; DNA mobility shift assays; DNA aggregation and protection of DNA against thermal denaturation.

Effect of modification on the binding of HSNP-C to DNA-cellulose: Results of the binding of the modified HSNP-C to dsDNA- and ssDNA-cellulose are shown in fig. 35 and fig. 36 respectively. Lysine modified HSNP-C bound to both single and double stranded DNA with difference in the elution pattern of the

Fig. 33: SDS-PAGE analysis of modified proteins

Each of the protein samples (20 μg) was electrophoresed on 15% SDS polyacrylamide gels after treating with sample buffer containing 1% mercapto ethanol.

Lanes 1-3: Lys-(HCHO) modified HSNP-C', lys-(PLP) modified HSNP-C' and arg-(diacetyl) modified HSNP-C respectively.

Lanes 5, 6: Tyr-(TNM) modified HSNP-C[1], trp-(BNPS-skatole) modified HSNP-C respectively.

Lane 9: Unmodified HSNP-C (control).

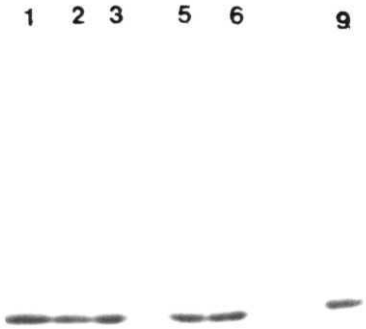


FIGURE 33

Fig. 34: Fluorescence emission spectra of HSNP-C modified by different reagents

Fluorescence emission spectra of HSNP-C, control or modified (10 μg) in 1 ml of buffer (10 mM Tris-Cl pH 7.6; 1 mM Na_2EDTA) was recorded between 300-450 nm (excitation at 285 nm).
Spectrum 1: control HSNP-C
Spectrum 2: lysine modified (HCHO) HSNP-C
Spectrum 3: lysine modified (PLP) HSNP-C
Spectrum 4: arginine modified (Diacetyl) HSNP-C
Spectrum 5: tryptophan modified (BNPS-skatole) HSNP-C
Spectrum 6: tyrosine modified (TNM) HSNP-C

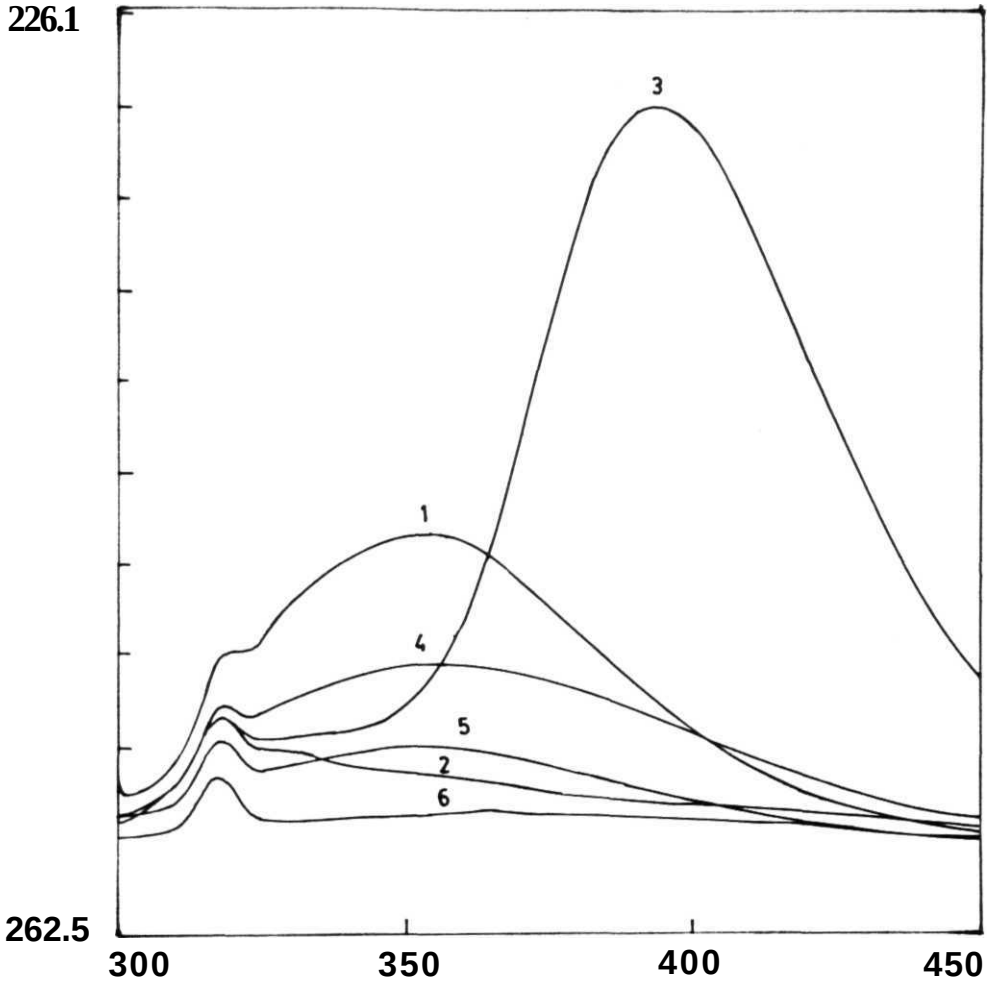


Figure 34

two forms. Unmodified HSNP-C' was eluted in two peaks (0.2 M and 0.3 M NaCl) from dsDNA-cellulose and (0.1 M and 0.2 M NaCl) from ssDNA-cellulose. In the case of lysine modified protein (by formaldehyde and PLP), large increase in the fraction eluting with 0.2 M NaCl was observed Similar elution profile was obtained by chromatography on ssDNA-cellulose also However, the salt concentration required was lower than that in the case of dsDNA-cellulose. In the case of arginine and tryptophan modifications, all the protein eluted with 0.2 M NaCl from both dsDNA-cellulose and ssDNA-cellulose indicating a marginal reduction in the affinity of HSNP-C to dsDNA. Arginine modified protein bound to ssDNA more strongly than the rest (elution with 0.3 M NaCl). However, tyrosine modification of HSNP-C resulted in the total loss of affinity to both dsDNA- and ssDNA-cellulose.

Effect of modification on the thermal protection of DNA by HSNP-C: HSNP-C strongly protects DNA against thermal denaturation. Modified proteins were also tested for their capacity to protect DNA against thermal denaturation (fig. 37). Modification of lysine and arginine residues resulted in greater protection of DNA by the protein. Tyrosine modification resulted in the substantial loss in the activity of the protein to protect DNA. Trp modification also resulted in the loss of ability of the protein to protect DNA. Melting curves obtained at protein/DNA ratios of 5.0 with different modified HSNP-C are given in fig. 37. The results are summarised in Table 2

Effect of modification on DNA aggregation: Tyr modification resulted in drastic reduction in the ability of protein to aggregate DNA (fig. 39). In the case of trp modification, there was only a marginal decrease in the aggregation of DNA.

Fig. 35: Effect of chemical modification of HSNP-C on the binding affinity to dsDNA-cellulose column.

(A)-(F): Elution profiles of HSNP-C (control), lys-(HCHO) modified HSNP-C, lys-(PLP) modified HSNP-C, arg-(diacetyl) modified HSNP-C, tyr-(TNM) modified HSNP-C and trp-(BNPS-skatole) modified HSNP-C respectively from dsDNA-cellulose column.

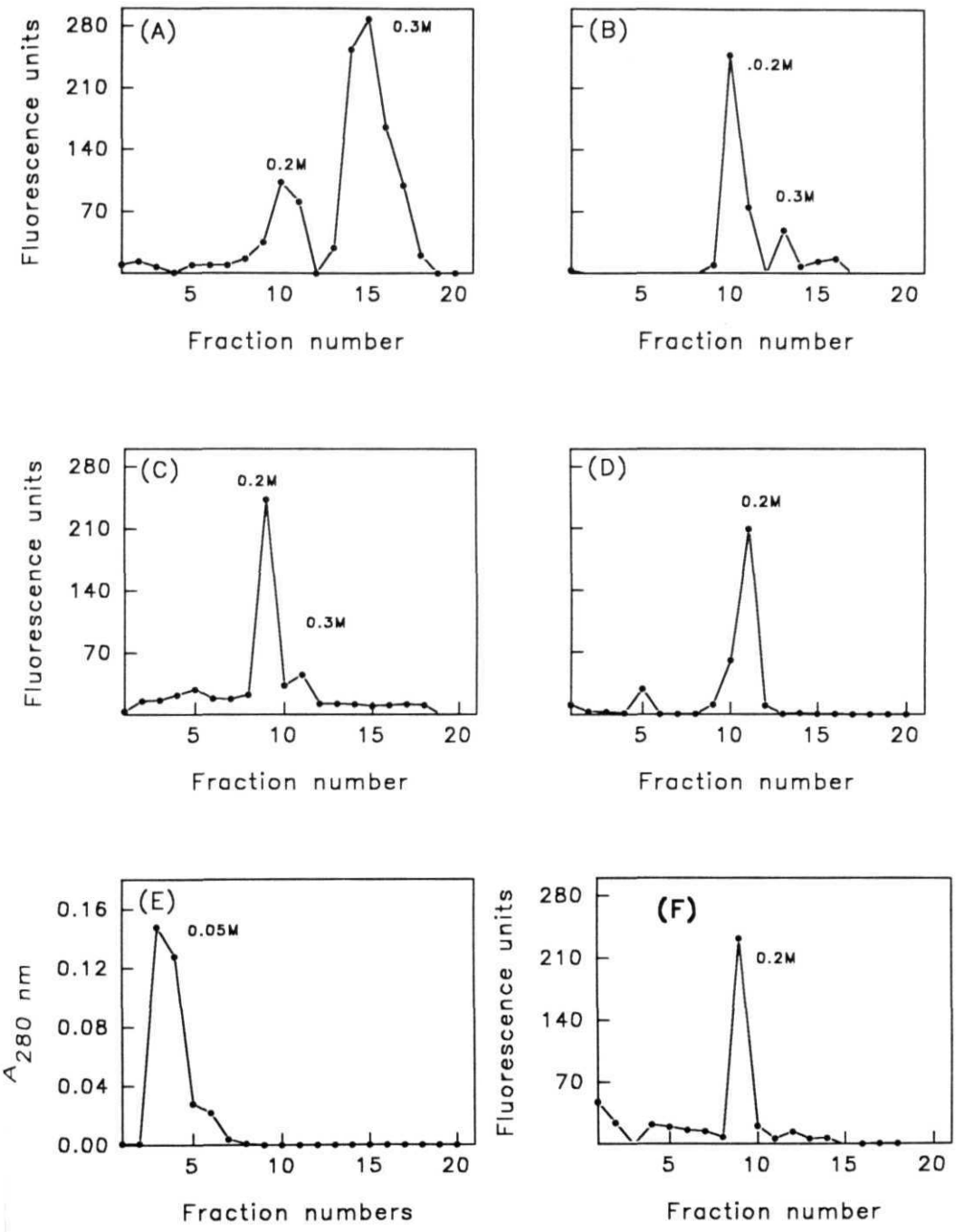

(A)
0.3M
0.2M
280
210
140
70
Fluorescence units
5
10
15
20
Fraction number
(B)
.0.2M
0.3M
5
10
15
20
Fraction number
(C)
0.2M
0.3M
280
210
140
70
Fluorescence units
5
10
15
20
Fraction number
(D)
0.2M
5
10
15
20
Fraction number
(E)
0.05M
0.16
0.12
0.08
0.04
0.00
A280 nm
5
10
15
20
Fraction numbers
(F)
0.2M
280
210
140
70
Fluorescence units
5
10
15
20
Fraction number

FIGURE 35

Fig. 36: Effect of chemical modification of HSNP-C' on the binding affinity to ssDNA-cellulose

(A)-(F): Elution profiles of HSNP-C (control), lys-(HCHO) modified HSNP-C, lys-(PLP) modified HSNP-C, arg-(diacetyl) modified HSNP-C, tyr-(TNM) modified HSNP-C and trp-(BNPS-skatole) modified HSNP-C respectively from ssDNA-cellulose column.

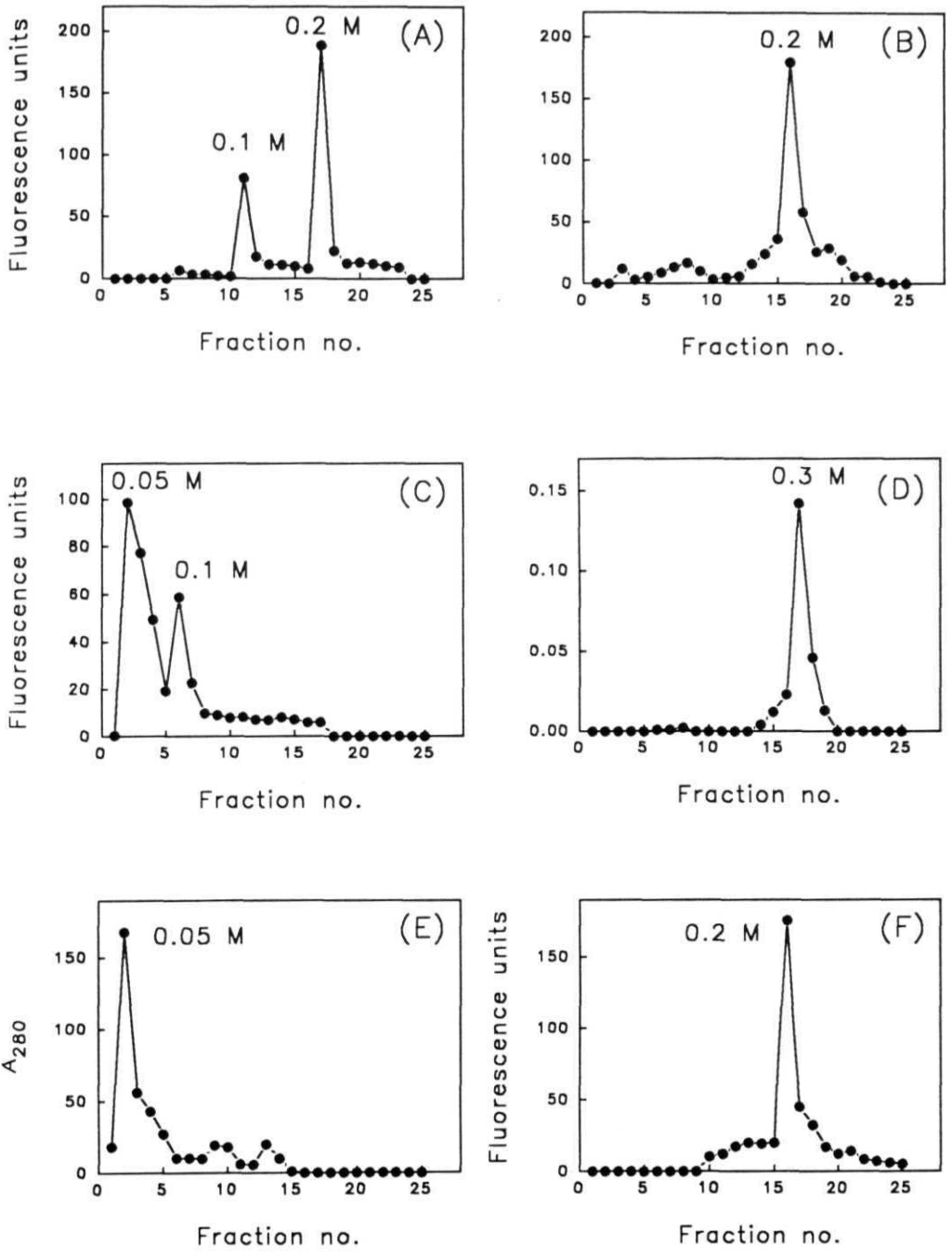

Fluorescence units
0.2 M
(A)
0.1 M
Fraction no.
0.2 M
(B)
Fraction no.
Fluorescence units
0.05 M
(C)
0.1 M
Fraction no.
0.3 M
(D)
Fraction no.
A_{280}
0.05 M
(E)
Fraction no.
Fluorescence units
0.2 M
(F)
Fraction no.

FIGURE 36

Fig. 37: Effect of chemical modification of **HSNP-C'** on the protection of **dsDNA** against thermal denaturation

(A)-(F): Thermal denaturation profiles of dsDNA in the presence of HSNP-C' (control), lys-(HCHO) modified HSNP-C, lys-(PLP) modified HSNP-C, arg-(diacetyl) modified HSNP-C tyr-(TNM) modified HSNP-C and trp-(BNPS-skatole) modified HSNP-C, respectively.

••• protein/DNA = 0
o-o = 1
♥-♥ = 2
V-V = 5

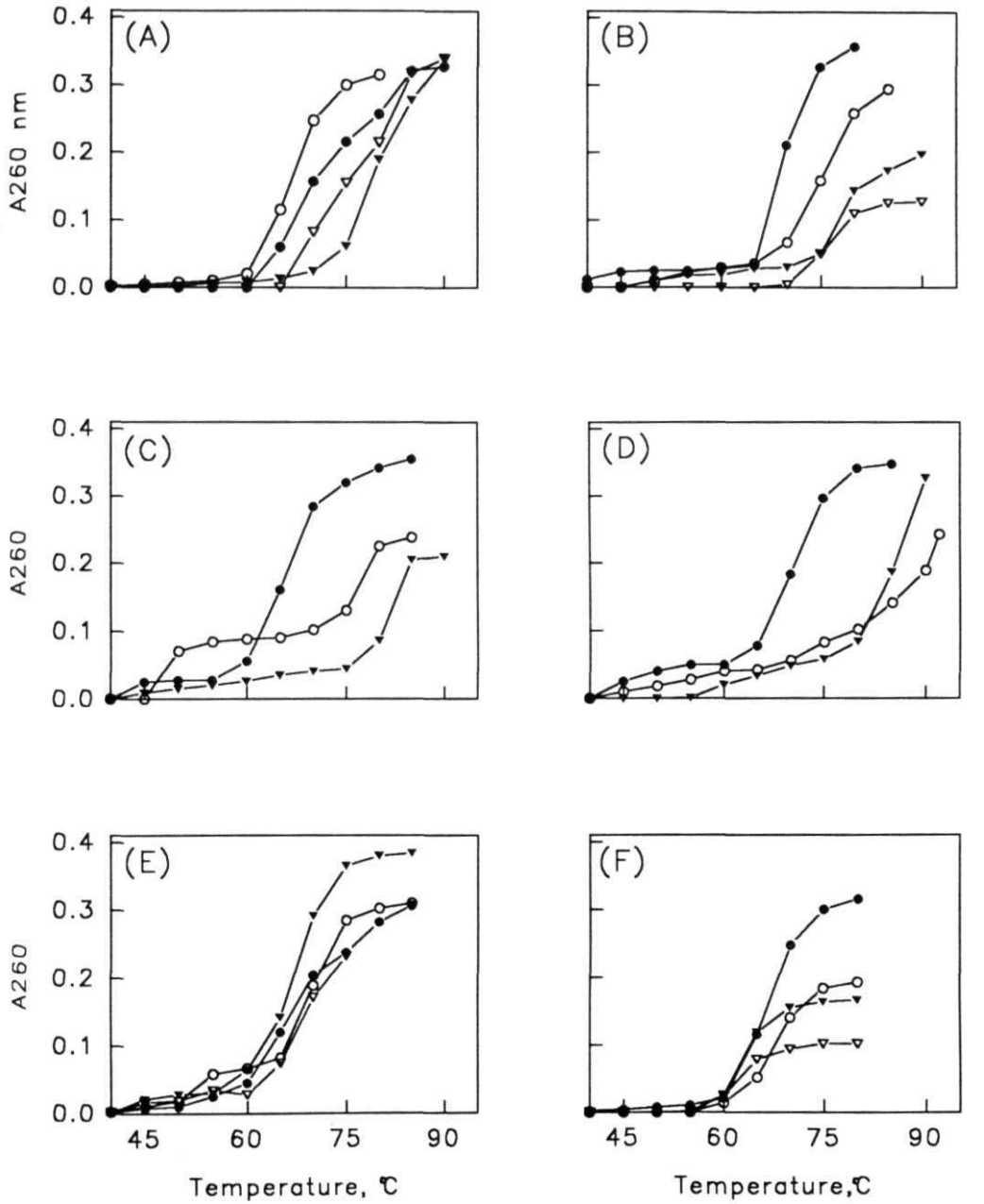

(A)
(B)
(C)
(D)
(E)
(F)
A260 nm
A260
0.0
0.1
0.2
0.3
0.4
45
60
75
90
Temperature, ℃
Temperature,℃

FIGURE 37

Fig. 38: Melting profiles obtained with *S. acidocaldarius* DNA **and the** protection offered by different modified proteins at **protein/DNA** = 5

•"• in the absence of protein
o-o HSNP-C' (control)
♥-♥ lys-(HCHO) modified HSNP-C
V-V lys-(PLP) modified HSNP-C
□-□ arg-(Diacetyl) modified HSNP-C
•-• tyr-(TNM) modified HSNP-C
A-A trp-(BNPS-skatole) modified HSNP-C

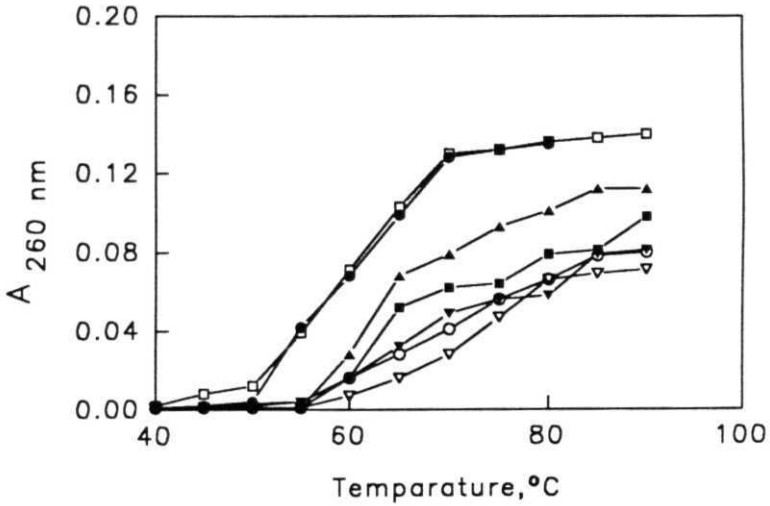


FIGURE 38

Fig. 39: Effect of chemical modification on DNA aggregation by HSNP-C'

DNA aggregation assay by control as well as each of the modified HSNP-C samples was carried out as given in the text.

o-o control HSNP-C
•-• lys-(HCHO) modified HSNP-C
V-V lys-(PLP) modified HSNP-C
♥-♥ arg-(diacetyl) modified HSNP-C
□-□ tyr-(TNM) modified HSNP-C
• ■ trp-(BNPS-skatole) modified HSNP-C

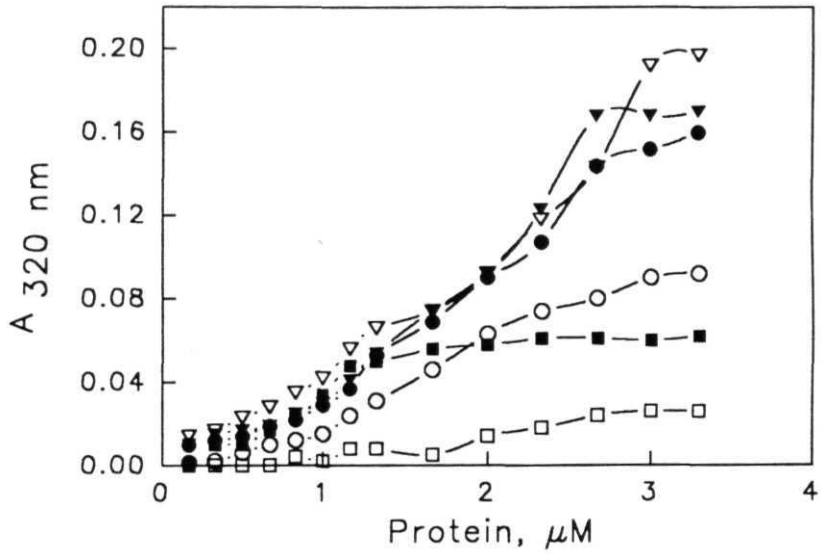


FIGURE 39

Table 2

Nature of modification	**Extent of increase in Tm in the presence of HSNP-C' at protein/DNA = 2**	**Extent of increase in Tm in the presence of HSNP-C' at protein/DNA = 5**
None	13.2	16.0
HCHO modified **(Lys)**	13.0	16.9
PLP modified (Lys)	13.0	17.8
Diacetyl modified (Arg)	**10.4**	**14.4**
TNM modified (Tyr)	**2.3**	**2.0**
BNPS modified (Trp)	**5.0**	**6.8**

However modification of lysine and arginine residues resulted in a dramatic increase in DNA aggregation by HSNP-C'

Chemical modification and DNA mobility shift: As shown earlier, HSNP-C binding results in the electrophoretic mobility retardation of DNA during agarose electrophoresis. Interesting results were obtained when modified proteins were tested for their ability to bind to dsDNA (fig. 40). Modification of lysine and arginine residues resulted in increased retardation of DNA and the DNA-protein complexes formed sharp bands at the slots of agarose gels. There was a total loss in the activity of HSNP-C modified with TNM to retard DNA. However, trp modified HSNP-C was also unable to retard DNA as effectively as unmodified HSNP-C. DNA formed a diffuse smear indicating loss of strong binding in trp modified HSNP-C.

Cleavage of *HSNP-C'* with TPCK-Trypsin and CNBr :

In an attempt to locate the nucleic acid binding domain on the protein, the protein was subjected to proteolytic cleavage with TPCK-trypsin and cyanogen bromide. Exhaustive treatment with TPCK-trypsin resulted in the production of an ~ 6000 kDa DNA binding fragment which was stable to further proteolysis. The primary structure or the amino acid sequence of the 7d protein as reported in the literature shows lysyl clusters in the terminal region. Out of the 13 lysine residues present, 8 residues are located in the first twenty amino acid residues and the remaining five in the central and C-terminal portion of the molecules (43 residues). This result indicates that the protein exists in a highly compacted state and internal lysine and arginine residues are not available for cleavage The protein was

Fig. 40: Gel mobility shift assay of modified **HSNP-C'.**

X DNA (200 ng) was incubated with 1 μg of HSNP-C (contTol or modified), before electrophoresis on 0.6% agarose electrophoresis
Lane 1: Unmodified HSNP-C
Lane 2: trp-(BNPS-skatole) modified HSNP-C
Lane 3: tyr-(TNM) modified HSNP-C
Lane 4: arg-(diacetyl) modified HSNP-C
Lane 5: lys-(PLP) modified HSNP-C
Lane 6: lys-(HCHO) modified HSNP-C

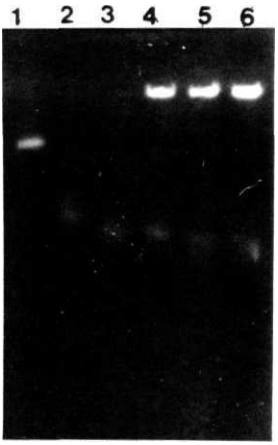


FIGURE 40

resistant to cleavage with CNBr at the two methionine residues that are located at positions 28 and 56.

HSNP-C' was completely converted to a tryptic core by 25 minutes which was resistant to the action of trypsin even upto six hours (fig. 41). The tryptic resistant core along with the control HSNP-C were electrophoresed on 15% SDS-PAGE (fig. 42). The tryptic resistant core was purified by CM-cellulose chromatography (fig. 43), and was tested for functional activity. It was found to posess similar multimeric aggregation and other DNA binding properties (fig 45). There was no detectable difference in the cross-linking pattern of the tryptic core compared to the control (fig. 44).

The tryptic resistant core of HSNP-C was tested in all the functional assays. The binding affinities of the tryptic core to dsDNA-/ssDNA-cellulose columns; the capacity to protect DNA against thermal denaruration and the property to aggregate dsDNA are all retained in the tryptic core (fig. 45: A, B, C and D).

Immunological homologous proteins in archaea and eubacteria :

Nucleoid samples from six organisms, both from archaea as well as eubacteria, *Sulfolobus acidocaldarius*, *Sulfolobus sofataricus*, *Thermoplasma acidophilum*, *Halobacterium halobium*, *Escherichia colt* and *Bacillus stearothermophilus* were electrophoresed and immunoblotted against anti-HSNP-C to look for the presence of immunologically homologous proteins in these organisms. In the case of *E. coli* nucleoid, only one protein (8.5 kDa) showed cross-reaction with anti-HSNP-C. A related thermophile, *S. solfatancus,* contained an immunologically homologous protein of HSNP-C. Nucleoids from

Fig. 41: SDS-PAGE of HSNP-C digested with TPCK-trypsin

HSNP-C was digested with TPCK-trypsin (1:100) for different time intervals. The activity of trypsin was terminated by the addition of soyabean trypsin inhibitor as given in the text.
Lane 1: HSNP-C + trypsin at zero time
Lanes 2-12: HSNP-C + trypsin after 5, 10, 15, 20, 25, 30, 60 minutes and 2, 3, 4, 6 hours respectively.

Fig. 42: SDS-PAGE analysis of HSNP-C (control) and the resistant tryptic core
Lane 1: HSNP-C control
Lane 2: tryptic resistant core

1 3 5 7 9 fl

FIGURE 41

1 2

FIGURE 42

Fig. 43: Purification pattern of tryptic core of HSNP-C'

The tryptic resistant core of HSNP-C' was purified on a CM-cellulose column and eluted with a linear KCl gradient. The figure shows the pattern of control as well as that of the tryptic core.
•-• elution profile of tryptic core
o-o elution profile of control HSNP-C

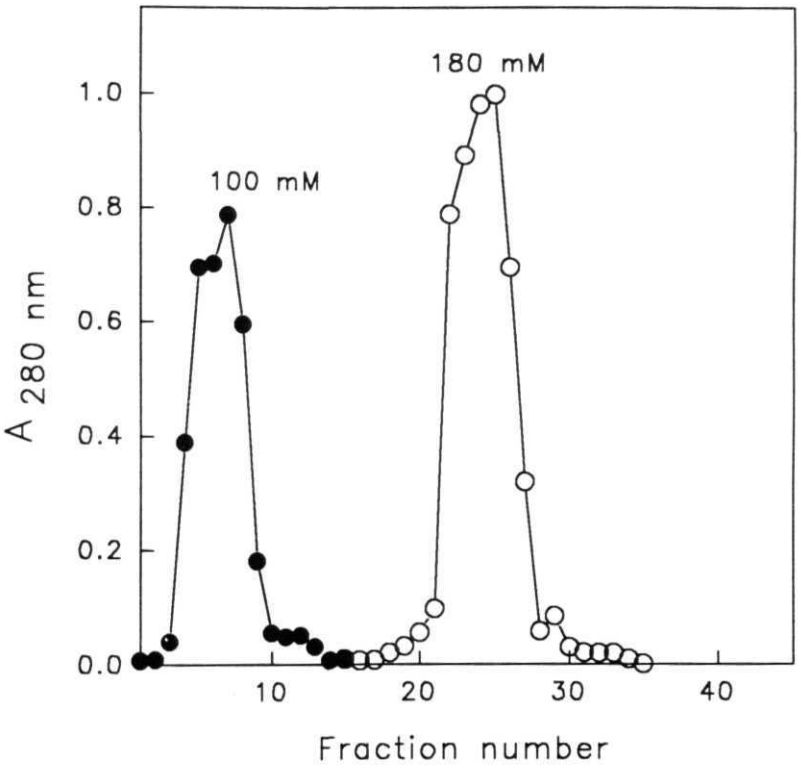

180 mM
100 mM
1.0
0.8
0.6
0.4
0.2
0.0
A 280 nm
10
20
30
40
Fraction number

FIGURE 43

Fig. 44: SDS-PAGE analysis of cross-linked products.

The tryptic core of HSNP-C' was cross-linked with HCHO, DMS and DFDNB at 65 °C.
Lane 1: HCHO cross-linked products
Lane 2: DMS cross-linked products
Lane 3: DFDNB cross-linked products.

FIGURE 44

Fig. 45: Interaction of **tryptic** core with **dsDNA** and **ssDNA**

Binding affinities of the tryptic core to dsDNA/ssDNA-cellulose and it's effect on the thermal protection and DNA aggregation were studied in the same manner as that of the control protein.

A : elution profiles from dsDNA-cellulose column

B: from ssDNA-cellulose column

C : protection of DNA against thermal denaturation (•"• -No protein; v-v - protein/DNA=2; ♦-♦- protein/DNA=3)

D: DNA aggregation at different P/D ratios.

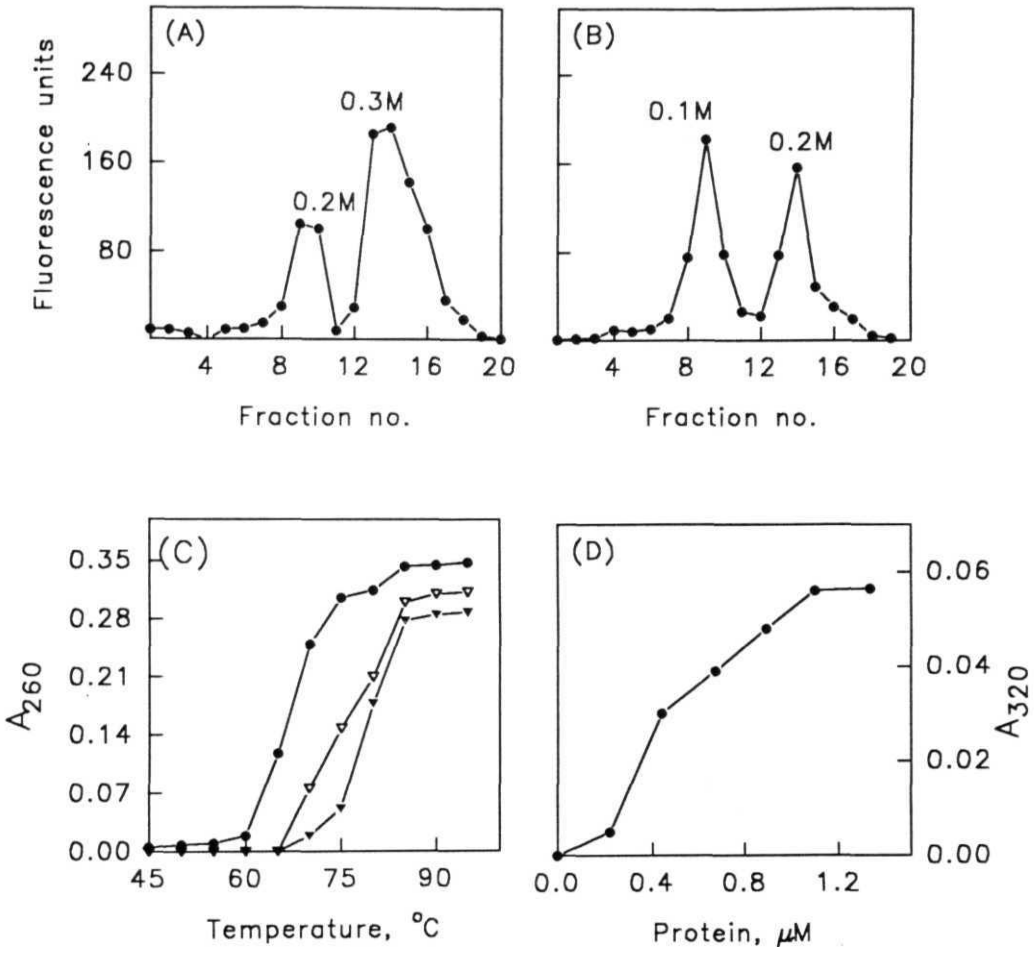

(A)
Fluorescence units
240
160
80
0.3M
0.2M
4
8
12
16
20
Fraction no.
(B)
0.1M
0.2M
4
8
12
16
20
Fraction no.
(C)
A_{260}
0.35
0.28
0.21
0.14
0.07
0.00
45
60
75
90
Temperature, °C
(D)
0.06
0.04
0.02
0.00
A_{320}
0.0
0.4
0.8
1.2
Protein, μM

FIGURE 45

Fig. 46: Immunological homology between HSNP-C and related proteins from organisms.

Immunoblot analysis of electTophoresed nucleoid samples from different organisms

Lane 1: **S.** *acidocaldarius*
Lane 2: *B. stearothermophilus*
Lane 3: *E. coli*
Lane 4: 7. *acidophilum*
Lane 5: ***H.*** *halobium*
Lane 6: **5.** *solfataricus*

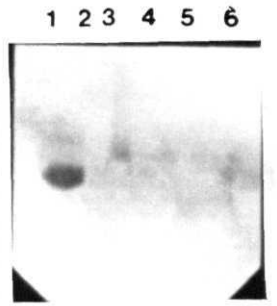


FIGURE 46

all other organisms did not show coss-reaction indicating the absence of HSNP-C like protein in these organisms.

Chapter-4
DISCUSSION

DISCUSSION

The packaging of DNA into chromatin in eukaryotes requires several levels of organisation, from the nucleosome structure to the 30 nm fibre (Wolffe, 1992). On the other hand in bacteria, the assembly of DNA in the nucleoid conformation is probably simpler with free supercoils and compaction with histone-like proteins (De Lange *et al*, 1981; Forterre *et al*, 1993). Differences in the gross anatomy of the nuclear material viz., the presence of multiple, paired and distinct chromosomes and a nuclear membrane were historically the primary basis for the separation of organisms into eukaryotes (eukarya) and prokaryotes (archaea and bacteria).

The archaeal kingdom includes those organisms which survive under conditions that normally are non-congenial for the rest. *S. acidocaldarius*, the organism of the present investigation is categorised into the sulphur-dependant thermoacidophilic class and more specifically into the 'crenarchaeotes' (Woese *et al*, 1990). Although, archaea morphologically resemble bacteria, they share biochemical and genetic properties with eukaryotes and are suggested to posess nucleosome-like structures. The chromosome structure with respect to thermal stability from this organism has been widely investigated in order to understand the stability of DNA in these hyperthermophiles (Forterre *et al*, 1993). A chromosome-like organisation of DNA in these microorganisms definitely implies the presence of specific proteins that could probably be involved in the nucleosomal assembly and conformational changes related to archaeal DNA metabolism. However, no definite scheme for the organisation of archaeal or bacterial DNA has been worked out.

Work from our laboratory has identified four DNA binding proteins from the nucleoid of *S. acidocaldarius*. Three of these proteins, HSNP-A, -C and C' protect DNA against thermal denaturation (Reddy and Suryanarayana, 1988,1989). Our present study is aimed at understanding the role of HSNP-C' in the structural organisation of DNA in this organism, since the protein is localised within the nucleoid sub-domains *invivo* (Bohrmann et al., 1994), which essentially implies that the protein performs a significant role in genomic organisation and function.

HSNP-C is one of the most abundant DNA binding proteins in *S. acidocaldarius* The protein strongly protects DNA against thermal denaturation and it is localised exclusively on the intracellular compacted DNA structures, the nucleoid (Bohrmann *et al.,* 1994). HSNP-C is very similar to the 7 kDa group of proteins reported earlier (Grote *et al.,* 1986). We have investigated on the structure of the protein and its interaction with nucleic acids to understand its physiological role. We have characterised the protein in terms of nucleic acid binding properties and its inherent existence *invivo.*

HSNP-C' with monomer molecular weight of about 8 kDa exists as a multimeric aggregate in solution as indicated by the cross-linking studies. Detection of different forms of aggregates viz., dimers, trimers, tetramers. pentamers etc., with zero length cross-linkers such as formaldehyde indicate that the monomer unit has several contacting sites for individual monomers to interact. The largest distinct aggregates that are identified by cross-linking studies are of 120 kDa, 135 kDa, 155 kDa and 165 kDa molecular mass possibly containing about 16-22 monomer units. Longer periods of cross-linking produced very large

aggregates with greater than 180 kDa molecular mass, which failed to enter the gel during electrophoresis. These large multimeric aggregates may be formed by the polymers of aggregates with molecular weight greater than 120 kDa. Cross-linking of HSNP-C and DNA complexes indicated that the protein forms very compact structures in the presence of DNA also. The aggregation of the protein is more prominent at elevated temperatures (> 65 C) indicating that the physiological state of the protein is a homopolymeric aggregate. Precise estimation of the basic unit of aggregation and its composition requires further experimentation. The aggregation behaviour of this protein is very novel and to our knowledge, no other DNA binding protein forms such homo-polymeric compact structures. Although RecA protein forms polymeric aggregates, these structures are filamentous both in the presence of DNA and its absence (Roca and Cox, 1990). Cross-linking of HSNP-C with DMS at pH 8.0, resulted in the formation of a set of aggregates ranging from dimers to structures with apparent molecular weight of ~ 170,000. However, cross-linking with the same reagent at pH 9.0 resulted in the formation of an octameric aggregate (Mr. 68,000). These results are similar to those observed when histones were cross-linked by DMS at pH 8.0 and pH 9.0 (Thomas and Kornberg, 1975; Hyde and Walker 1975). Further experimentation is required to analyse the structure of the nucleoprotein complex of HSNP-C and dsDNA.

The protein binds strongly to dsDNA and relatively weakly to ssDNA. It has no affinity for RNA. Chromatography on DNA-cellulose column revealed two forms of the protein eluting at two salt concentrations. Results on the chemical modification of the protein by reductive methylation of lysines indicate that the strongly bound form can be converted to the one eluting with 0.2 M NaCl. Hence, the two forms of HSNP-C may differ on the basis of the extent of methylation of

lysine residues in the protein. It will be of interest to see the effect of methylation of HSNP-C' on its interaction with DNA.

Fluorescence titrations, generally, are used to analyse co-operative binding characteristics of most of the nucleic acid binding proteins which bind linearly along the polynucleotide chain at the adjacent sites (Schwarz and Watanabe, 1983; Kowalczykowski *et ai,* 1986, Bujalowski and Lohman, 1987a; 1987b). We have employed fluorescence titrations to analyse the interaction of HSNP-C with DNA. Quenching of the intrinsic fluorescence of HSNP-C was measured as a function of the increasing concentration of DNA at different salt concentrations. Data from fluorescence titrations performed at low salt concentrations (20 mM) where the binding is tight and stoichiometric, were used to determine the site size "n" of the protein. A binding site size of about 4 nucleotides per monomer was obtained by both the approaches (Scatchard approach as given by McGhee and Von Hippel, 1974 and the non-Scatchard approach by Schwarz and Watanabe, 1983). A weak co-operativity in binding is indicated by the sigmoidal type of inflection in the binding curve. The binding behaviour is independent of the DNA concentration implying that the interactions of DNA molecules themselves (e.g. aggregation etc.) do not interfere with the binding process of the protein to DNA (Schwarz and Watanabe, 1983). The degree of saturation, 0, was determined from the difference between the titrations in the presence and absence of DNA. In order to obtain the correct normalisation it was divided by y_{α}, the distance between the saturation asymptote and the parallel straight line for the titration in the absence of DNA. The mobility shift assays and electron microscopic studies clearly indicate that HSNP-C binds highly co-operatively to dsDNA. Fluorescence titrations, however, indicated very low co-operativity. This may be due to unusual binding

characteristics of the protein which forms clumped compact structures. It has already been reported by us that the complexes of HSNP-C and DNA are not retained after filtration on nitro-cellulose membranes (Reddy and Suryanarayana, 1988), unlike the case with most of the DNA binding proteins. The ability of the protein to protect DNA against DNAse 1 hydrolysis and to release DNA bound ethidium also may be manifestations of the compact structures formed when the protein is bound to DNA.

Mobility shift assays were performed with protein-DNA complexes obtained after incubating λ DNA with increasing concentrations of HSNP-C at two different salt concentrations (20 mM and 220 mM) before electrophoresis. Both the conditions show co-operative mode of binding of the protein. The complexes at low salt show a broad smear indicative of the non-random distribution of the protein among the DNA molecules and intermediate co-operativity in the binding mode. A very large increase in the co-operativity of binding was observed at high salt concentrations indicated by the presence of sharp bands representing DNA that was fully saturated with protein, retained in the slots along with free DNA at low binding density.

Salt titrations are generally performed to test the strength of binding of protein to nucleic acid. Interestingly, the concentration of salt required to recover part of the initial HSNP-C fluorescence (which generally indicates dissociation of the DNA protein complex) was found to be lower than that required for the elution of the protein from dsDNA-cellulose column. One of the reasons for the decrease in fluorescence quenching during salt titration could be due to the non-availability of the aromatic amino acids in the active site at high concentration of salt.

Titrations have been earlier used to assess the extent of exposure of aromatic amino acids in proteins (Omar and Schleich, 1981). Fluorescence titrations performed with artificial quenchers such as KI and acrylamide at different concentrations of salt indicated that the decrease in the extent of quenching of the fluorescence of HSNP-C' at high salt concentration is because of non-availability of the aromatic amino acid residues which may be due to conformational changes in the protein at high salt concentrations. Hence, the recovery of fluorescence by salt titration of HSNP-C-DNA complexes may be due to decrease in quenching of protein fluorescence by DNA at high salt (150 to 200 mM NaCl) and not due to dissociation of the complex at these salt concentrations. This is substantiated by the mobility retardation of DNA at high salt concentration (220 mM NaCl) and by affinity chromatography.

Electron microscopic studies on the complexes of HSNP-C with dsDNA (RSF 1010) and ssDNA (ϕx174) showed the formation of well defined structures. At low protein/DNA ratios (less than 3:1 w/w) clusters of bound protein on the DNA could be observed. With increasing protein/DNA ratios single clumps of protein on the DNA were compacted more into one central protein-DNA cluster surrounded by loops of free DNA. At very high protein/DNA ratio i.e., 10, free DNA was scarcely seen, but instead very large DNA-protein aggregates were formed. Single stranded DNA also formed similar structures (fig. 14 & 15: A, B, C, D). These structures resemble those that were reported with 7d by Lurz *et al.* (1986)

A phosphate binding motif similar to the GTP/ATP binding proteins (G-X-X-X-X-G-K (ST) was located in HSNP-C between 37 to 47 residues (Gly-Lys-

Thr-Gly-Arg-Gly-Ala-Val-Ser-Glu-Lys) indicative of a nucleotide binding domain on the protein. Results on the fluorescence titrations of HSNP-C' with different nucleotides showed maximum quenching by GTP as compared to other nucleotides, viz., ATP, CTP, dTTP, dATP. However other nucleotides also bound to HSNP-C but with varying affinities. The titration curves were biphasic with all the nucleotides tested. Stepwise titrations involving dsDNA and GTP with HSNP-C clearly indicate a distinct site for nucleotide binding, apart from the nucleic acid binding domain on the protein. This could suggest other additional roles possibly played by HSNP-C in addition to compacting the genomic DNA involving GTP. The role of GTP in the interaction of HSNP-C to DNA needs further study.

Electron microscopic pictures of complexes of HSNP-C with DNA resemble those obtained with protein 7d. These results indicate that protein 7d (Ssa 7d and Sso 7d) and HSNP-C are very similar if not identical. Earlier, only the sequence of 7d type of proteins (Kimura *et al.*, 1984; Choli *et ai,* 1988a and b) and interaction with DNA studied by electron microscopy (Lurz *et ai,* 1986) were reported in the literature. Sequence analysis of these 7 kDa group of proteins also showed that 7a; 7b and 7d differ only in the extent of methylation of the lysine residues (Choli *et ai,* 1988a). Very recently, a DNA binding protein isolated from *Sulfolobus solfataricus* similar to 7 kDa proteins in N-terminal sequence was shown to be ADP-ribosylated by homologous cell extracts (Kulms *et ai,* 1995). A protein, SaRD, with N-terminal sequence very similar to 7 kDa proteins with Mr. of 9 kDa was isolated from *S. acidocaldarius* that exhibits both DNA binding activity (it strongly protects DNA against thermal denaturation like HSNP-C) and shows RNase activity. At present, the significance of RNase activity of SaRD is

not very clear. Preliminary, results with HSNP-C showed no associated RNase activity. A model for the DNA binding surface of the Sso 7d from 5. *solfataricus* has been worked out from NMR spectroscopy (Baumann *et ai,* 1995) which suggest that the protein binds at the major groove with contacts made by lysines situated at the central portion of the molecule (positions 20, 21, 27, 39, and 48) and tyr7, ser46 and trp23. This study also suggests that the N-terminal and C-terminal lysines (lys4, lys6, lys60, lys62 and lys63) that are mono-methylated are exposed to the solvent.

It has already been reported that HSNP-C strongly protects DNA against thermal denaturation (Reddy and Suryanarayana, 1989). Monovalent, divalent as well as polyvalent cations such as polyamines are also known to strongly protect DNA. HSNP-C protects DNA even in the presence of these polyamines, which indicates that other hydrophobic/hydrogen bonding interactions also play a role in addition to the electrostatic interactions in the stabilisation of DNA.

In order to understand the interactions involved in the binding of HSNP-C to DNA, effect of chemical modification of specific amino acids in the protein was studied. Modified protein was tested for DNA binding and the ability to protect DNA against thermal denaturation and DNA aggregation. Lysine modification (both by formaldehyde and pyridoxal phosphate) slightly lowered the affinity of the protein whereas arginine modification had no effect. The modified lysines are probably those that are located at the N and C-terminal ends of the protein (corresponding to those methylated *invivo).* The internal lysines involved in the DNA binding as inferred from NMR spectroscopy could be buried in the hydrophobic regions and are probably not available for modification. This inference is also supported by lack of cleavage by trypsin at these internal lysine

residues (see below). One of the interesting consequence of the lysine modification is the increased ability of the modified protein to protect DNA against thermal denaturation and/or the stabilisation of the protein itself at high temperature. However, tyrosine modification resulted in total loss of the DNA binding activity of the protein and consequently the protein failed to protect DNA against thermal denaturation. Tryptophan modification also resulted in similar effect as tyrosine modification but the effect was less pronounced. These results suggest that hydrophobic and probably H-bonding interactions are the major interactions through which the protein binds to DNA and that the electrostatic interactions, through lysine (and to a less extent arginine), are established subsequently resulting in the stabilisation of the HSNP-C'-DNA complex. Our results give chemical evidence to the model proposed for the interaction by Baumann *et al.* (1995). Furthermore, the increased ability of lysine and arginine modified HSNP-C to protect DNA against thermal denaturation suggest importance of hydrophobic interactions This is also supported by the observed capacity of the protein to protect DNA even in the presence of mono and polyvalent cations The effect of chemical modification on aggregation of DNA by HSNP-C parallels with that of protection against thermal denaturation. Lysine and arginine modified HSNP-C aggregate DNA more efficiently as indicated by mobility shift assay and light scattering experiments. However, the presence of trp in the vicinity and the significant involvement of tyr residues in the nucleic acid binding domain of the protein has been clearly shown in the present study.

Proteolytic cleavage of the protein with TPCK-Trypsin and CNBr revealed the compact nature of the protein. Tryptic cleavage resulted in the formation of a protein core of Mr. 6.5 kDa. This tryptic core retained DNA binding and other

consequent effect such as protection of DNA against thermal denaturation and DNA aggregation. Lack of cleavage by CNBr also suggest hydrophobic environment in the central region of the molecule involved in the DNA binding

Electron microscopic data, nucleic acid binding characteristics, aggregation behaviour of the protein and the reported exclusive localisation of HSNP-C' on intracellular nucleoid (Bohrmann *et al.*, 1994)) suggest that the protein *in vivo* may be involved in the condensation of DNA protecting it against thermal denaturation and may be acting as a packaging protein. The structures formed by this protein also suggest that it may be involved as a scaffold protein for creating folded domains of intracellular DNA. DNA binding proteins which act as such scaffold proteins although implicated in the organisation of bacterial nucleoid or eukaryotic chromatin have not been identified. Hence HSNP-C (protein 7d) may be the first such protein to be identified as the scaffold protein.

Chapter-5
SUMMARY & CONCLUSIONS

Summary and Conclusions

Chapter 1: Introduction

This chapter deals with the introductory aspects of the biological kingdom in general, but with special emphasis on archaea (previously referred to as the archaebacteria). These organisms are grouped into a third novel kingdom comprising of organisms that are specially adapted to living in the extremeties of temperature, pH and salinities. In addition to these, the various aspects of DNA binding proteins such as histone-like proteins from the three kingdoms are also presented in this chapter.

Chapter 2: Materials and Methods:

This chapter provides information about the chemicals, materials and methodologies used during the course of the thesis. Apart from these, it provides sufficient details regarding the procedures and protocols made use of in the present study along with minor modifications if any. The present task was accomplished with the help of various techniques such as fluorescence titrations, mobility shift assays, studies on binding affinities to immobilized nucleic acid matrices, Tm studies, DNA aggregation, electron microscopy and chemical modification studies.

Chapter 3: Results

HSNP-C', an 8 kDa DNA binding protein is associated with the ribosome free domains of genomic DNA in the cell. The abundant protein was purified from *S. acidocaldarius* to homogeneity as analysed by SDS-PAGE followed by silver staining. HSNP-C exists in solution as a multimeric aggregate as analysed by cross-linking studies as well as immunoblotting studies. The nucleic acid binding

properties of the protein were studied by fluorescence titrations, affinity chromatography on nucleic acid matrices, electron microscopy, DNA aggregation and protection of DNA against thermal denaruration. The protein binds to dsDNA with a site size of 4 and a binding constant "K" of 4 $x10^6 M^{-1}$ at 20 mM NaCl. It has weak affinity to single stranded DNA and no affinity to RNA. HSNP-C also binds to mononucleotides through a site which is probably distinct from the dsDNA binding site. The protein exists in atleast two distinct forms which differ in the extent of methylation of lysine residues. Electron microscopic studies suggest that the protein forms compact structures with folded dsDNA domains.

Chemical modification studies suggest importance of aromatic amino acids in the binding of protein to dsDNA and that the electrostatic interactions stabilise the binding of the protein. Chemical modification studies and proteolytic digestion also suggest very compact structure of the protein which is resistant to proteolytic enzymes and modifying reagents.

Chapter 4: Discussion

Cross-linking studies using both bifunctional cross-linkers (DMS and DFDNB) as well as zero level cross-linkers revealed the tendency of the protein, HSNP-C to exist as multimeric aggregates in solution. The protein has a very compact structure and binds strongly and co-operatively to dsDNA. Electron microscopic studies also strengthen the view that HSNP-C forms compact structures with DNA suggesting the condensation of intracellular DNA of the organism. HSNP-C was found to aggregate DNA and protect DNA against thermal denaruration. The protection offered by HSNP-C against thermal denaturation in the presence as well as absence of polyvalent cations suggests the involvement of hydrophobic/hydrogen bonding interactions in the stabilisation of

DNA in the organism

Chemical modification studies yielded information suggesting the involvement of tyr and trp in the nucleic acid binding domain of the protein. Our study gives evidence for the model proposed for Sso 7d by Bauinann *et al.* (1995). This is the first detailed study on the nucleic acid binding properties of the protein which has the capacity to condense DNA into compact structures.

Chapter-6
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Indian Journal of Biochemistry & Biophysics
Vol. 32, December 1995, pp. 447-455

Nucleic acid binding properties of a helix stabilising nucleoid protein from the thermoacidophilic archaeon *Sulfolobus acidocaldarius* that condenses DNA into compact structures*

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Received 29 January 1995; revised 28 August 1995; accepted 7 September 1995

Helix stabilising nucleoid protein (HSNP-C) from an acidothermophilic archaeon *Sulfolobus acidocaldarius* has been characterised with respect to interaction with nucleic acids by gel retardation assay, binding to nucleic acid columns, fluorescence titrations and electron microscopy. The protein exists in solution as very large multimeric aggregates as indicated by cross-linking studies. The protein binds strongly and co-operatively to double stranded DNA. Electron microscopy of the complexes of the protein with DNA shows compact structures suggesting that the protein condenses DNA.

Intracellular DNA in bacteria is condensed into an organised structure called the nucleoid or bacterial chromatin which is stabilised by proteins and RNA and probably by membrane (see for reiews, ref. 1 and 2). Several classes of DNA binding proteins which affect the helical structure of DNA[3,4] have been isolated from prokaryotes, chiefly *Escherichia coli*. Proteins HU (NS) and H1 (H-NS) are well characterised and are prokaryotic histone-like proteins[5,6].

There are very few reports concerning intracellular organisation of DNA and the histone-like proteins in archaea (archaebacteria) in general and thermoacidophilic archaea in particular. **Thermoacidophiles** are considered to be ancestral phenotypes of archaea, the Crenarchaeyotes[7] which constitute the third kingdom of organisms[8]. A DNA binding protein HTa has been isolated and characterised which binds tightly to DNA and protects it from nuclease digestion[9,10,11]. Presence of several small basic proteins in isolated nucleoids from eu**bacteria** and archaea was demonstrated by electrophoresis. The proteins from archaea were reported to be immunologically distinct from those of eubacteria[12]. Several types of DNA binding proteins were isolated from the thermoacidophilic archaeon *Sulfolobus acidocaldarius* DSM1616 with M_r of 10 kDa and 7 kDa (ref. 13). The interaction of these proteins with ssDNA and dsDNA has been studied by electron microscopy[14]. The 7 kDa group contains 5 proteins which are closely related in amino acid sequence (7a, 7b, 7c, 7d and 7e) (ref. 15, 16). Another 7 kDa protein has been isolated from *Sulfolobus solfataricus* and sequenced[17]. However, in all the above cases, the association of these proteins with intracellular DNA has not been documented.

We have been working on the histone-like DNA binding proteins of the thermoacidophilic organism, *Sulfolobus acidocaldarius* and identified four low molecular weight DNA binding proteins associated with the archaebacterial chromatin. Three of the proteins named as helix stabilising nucleoid proteins (HSNP-A, C and -C) protect DNA against thermal denaturation. The fourth protein, the DNA binding nucleoid protein (DBNP-B), which is one of the most abundant proteins does not show helix stabilising property[18,19]. Detailed work on the nucleic acid binding properties of DBNP-B has indicated that it binds to single stranded DNA (ssDNA), double stranded DNA (dsDNA) and RNA with stronger affinity to ssDNA and distorts the helical structure of polynucleotide[20,21]. It aggregates ssDNA and dsDNA and promotes the renaturation of complementary single stranded DNA[21].

In the present study, the helix stabilising nucleoid protein (HSNP-C) which is one of the most abundant cellular protein has been characterised with respect to it's aggregation properties and nucleic acid binding properties. We also show that it is very similar, if not identical, to protein 7d reported by Grote *et al*[13]. Recent results of our collaborative work showed that this protein is pre-

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dominantly localised, within distinct nucleoid subdomains as shown by immunogold-electron microscopy[22]. Our results suggest that HSNP-C′ may participate in the condensation of intracellular DNA.

Materials and Methods

Dimethyl suberimidate, difluoro-dinitrobenzene, ethidum bromide, dsDNA cellulose and ssDNA cellulose were purchased from Sigma chemical company, U.S.A. Pancreatic DNAse I, poly (A) and poly (U) were procured from Boehringer Mannheim, Germany, Poly (A)- and poly U Sepharose 4B were obtained from Pharmacia, Sweden. Phage lambda DNA was purchased from Bangalore Genei, India.

Sulfolobus acidocaldarius DSM 639 (obtained from Duetsche Sammulung von Mikro-organismen, Germany) was grown at 75°C for 40-48 hrs with vigorous aeration as described before[18]. The cells were harvested after neutralising the culture with 1 *M* Tris solution. Cell pellet thus obtained was processed further or stored at - 80°C till further use. DNA was isolated according to the procedure described[23]. M13mp7 ssDNA was isolated using *Escherichia coli* JM109 as the host[24]. Concentrations of dsDNA, ssDNA were determined spectrophotometrically using the molar absorption coefficients of 6.5 x l()^3 and 8.7 x]0^3 (ref. 25) and of poly (A) and poly (U) using 10.3 x 10^3 and 9.2 x 10^3 (ref. 26) at 260 nm respectively. Protein concentration was determined by the Folin reagent[27] using bovine serum albumin as standard. HSNP-C was purified to homogenity from *S. acidocaldarius* post-ribosomal supernatant according to the procedure described previously[19]. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed[28] on 15% polyacrylamide gels. Gels were fixed in 7.5% acetic acid, stained in Coomassie blue R-250 in 50% methanol and 7.5% acetic acid and destained in 7.5% acetic acid containing 5% methanol.

Cross-linking studies

Protein cross-linking experiments were performed with three different reagents viz., formaldehyde[29], dimethyl suberimidate (DMS)[30] and difluoro-dinitro-benzene (DFDNB) at three different temperatures. Purified protein (100 µg each) was acetone precipitated and dissolved in 50 m*M* triethanolamine-HCl, pH 7.6, 50 m*M* NaCl or 50 m*M* triethanolamine-HCl, pH 8.3, 50 *m*M NaCl. The concentrations of the reagents HCHO, DMS and DFDNB were 0.1 M; 0.1 mg/ml and 0.1 mg/ml respectively. Samples were incubated at 35°C, 50°C and 65°C for one hour in the case of treatment with HCHO and 10 min in the case of treatment with DMS and 20 minutes in the case of treatment with DFDNB. The reactions were terminated with the addition of trichloroacetic acid to a final concentration of 10%. Precipitated proteins were dissolved in the sample buffer devoid of mercaptoethanol and electrophoresed on 15% SDS-polyacrylamide gels.

Binding of HSNP-C" to immobilised nucleic acid matrices

Pre-swollen dsDNA cellulose, ssDNA-cellulose, poly (U)-Sepharose and poly (A)-Sepharose were packed into different columns (0.2 ml each) and equilibrated with 20 m*M* Tris-HCl pH 7.4, 50 m*M* NaCl, 1 m*M* Na_2-EDTA. HSNP-C (50 µg) was loaded to each of these columns and eluted with 0.1, 0.2, 0.3, 0.4 and 0.5 M NaCl containing buffers. The fractions were analysed for protein content by fluorescence measurements and SDS-PAGE.

Electron microscopy of HSNP-C′-dsDNA complexes

Electron microscopy of HSNP-C -dsDNA complexes was carried out by Dr. Lurz, Max-Planck Institute for Molecular Genetic, Berlin, Germany[14]. RSF 1010 plasmid DNA was incubated with different amounts of HSNP-C in buffer (10 m*M* TEA-C1, (pH 7.6), 50 m*M* KC1, 2.5 m*M* DTT and 0.5 m*M* Na_2-EDTA) at 37°C for 15 min. The samples were refixed with glutaraldehyde (0.2% final concentration). The fixed nucleoprotein complexes were adsorbed onto mica for 1 min, followed by 1 min staining in 2% aqueous uranyl acetate, washed twice in water and air-dried.

Gel mobility shift assays

Gel mobility shift analysis of HSNP-C′ nucleic acid complexes was carried out exactly as described[31]. The reaction was carried out in 30 µl volume in 10 m*M* Tris-HCl (pH 7.0), 0.01 m*M* Na_2-EDTA buffer containing 20 or 220 m*M* NaCl. HSNP-C′-nucleic acid complexes were formed at different protein to nucleic acid ratios and 2 µl loading dye (50% v/v) glycerol, 0.04% (w/v) bromophenol blue) was added to each 30 ul sample and applied onto agarose gel slots. Electrophoresis was carried out on 0.6% agarose gel in 20 m*M* Tris-HCl (pH 7.8), 0.4 *mM* sodium acetate, 0.2 m*M* EDTA. The gels were stained 0.5 µg/ml ethidium bromide and photographed under uv illumination.

Fluorescence titrations

Direct **titrations** were performed by adding small volumes (2 μl) of the ligand (protein) soluton **to** constant amount of lattice (dsDNA) and the fluorescence emission of the protein was measured. In this mode of titrations, initially the quenched fluorescence of the bound protein was measured and after saturation, the fluorescence intensities of both the bound and free protein were measured. These data were analysed by a non-**Scatchard** general approach[32,33] to arrive at the binding parameters. Details are given in the results section. All fluorescence measurements were obtained using JASCO FP-777 spectrofluorimeter. The measurements were performed in 10 m*M* Tris-HCl, 1 m*M* DTT and 20 m*M* NaCl at room temperature (25°C). The band width was 5 nm and 10 nm for the excited and emitted light respectively. After each addition of nucleic acid to protein, the reactions were mixed gently and left undisturbed for 60 sec. The fluorescence readings were recorded after another 60 sec, the time needed for the stabilisation of the fluorescence signal **Three** readings (at 10 s intervals) were noted for each titration point and average of these readings wee used for the analysis. The fluorescence intensity measured in arbitrary units was corrected lor dilution during titrations. In our experimental conditions, the inner filter effect was found to be insignificant.

Protection of DNA from DNAse I digestion by HSNP-C

The sensitivity of HSNP-C-dsDNA complexes to DNAse I enzyme was followed spectrophotometrically, in PU8740 UV/visible Philips spectrophotometer. The reaction was carried out in a cuvette (600 μl reaction volume) in 10 m*M* Tris-HCl (*p*H 7.6), 50 m*M* NaCl, 5 m*M* $MgCl_2$ and 1 m*M* DTT buffer. HSNP-C in increasing amount's was mixed with 5 μg of calf thymus DNA and incubated at 37°C for 15 min. DNAse I (0.5 *jug)* was added directly to the reaction mixture in the cuvettes and the hyperchromicity at A_{260} nm was recorded. The increase in absorbance was plotted against time.

Displacement of DNA bound ethidium bromide by HSNP-C

Calf thymus DNA (4.8 μg) was added to 1 ml buffer (10 m*M* Tris-HCl *p*H 7.6, 50 m*M* NaCl) in a cuvette containing 400 ng ethidium and the fluorescence emission spectrum was recorded. Prior to **that, the** emission spectrum of ethidium was also recorded. Small aliquots (2-3 μl) of protein solution were added to the reaction mixture in the cuvette, mixed gently and the fluorescence emission spectra were recorded. Samples were excited at absorption maximum of ethidium (480 nm) and the emission spectrum was recorded between 500 nm and 660 nm. The excitation and the emission slit widths were set at 1.5 nm and 20 nm respectively. The concentration of ethidium was evaluated from measurements of the absorbance at 480 nm using molar absorption coefficient of 5700.

Results

HSNP-C exists as an oligomeric aggregate in solution

The protein has a molecular mass of about 68 kDa. Cross-linking with formaldehyde produced a series of bands corresponding to dimer, trimer, tetramer, pentamer and hexamer with progressively decreasing intensity (Fig. 1). Cross-linking with DFDNB and DMS resulted in the formation of aggregates with M_r in the range of 200,000 kDa apart from dimers and small amounts of trimers (Fig. 2A). Cross-linking for longer period of time produced higher molecular weight aggregates

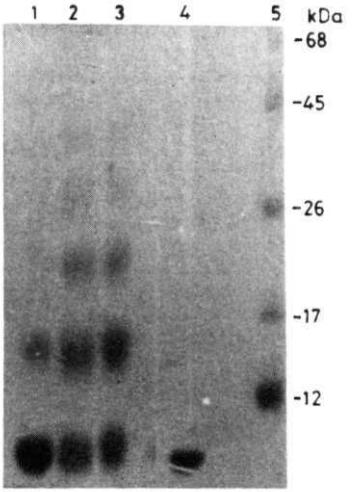

Fig. 1— SDS-PAGE of formaldehyde cross-linked complexes of HSNP-C [Cross-linking was carried out as described in the text in pH 7.4 buffer at different temperatures and cross linked complexes were analysed by electrophoresis on 15% gels. Lanes 1 to 3. cross-linking at 35°C, 50°C, and 65°C respectively; lane 4, HSNP-C control; and 5, mol wt markers bovine serum albumin, 68 kDa; ovalbumin, 45 kDa; chymotrypsinogen, 26 kDa; myoglobin, 17 kDa and cytochrome c, 12 kDa].

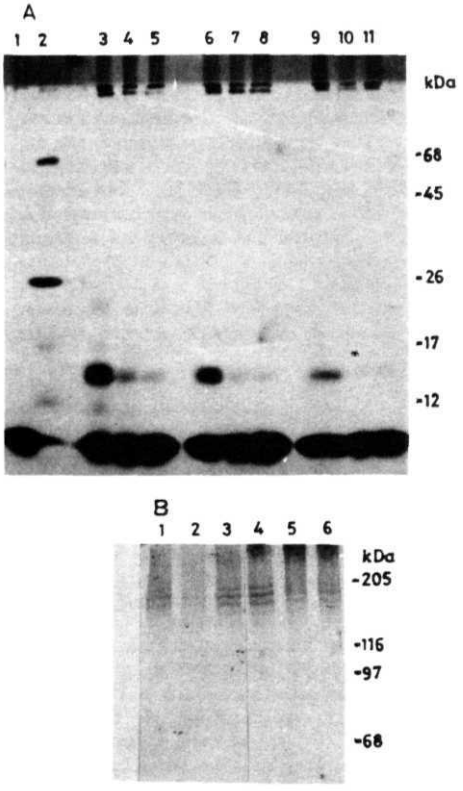

Fig. 2-SDS-PAGE of cross-linked complexes >f HSNP-C′ obtained in *p*H 8.6 buffer. [(A): Electrophoresis using 15% gels. Lane 1. untreated HSNP-C; lane 2 to 4, cross-linking with formaldehyde at 65°C. 50°C and 37°C respectively; lanes 5 to 7, cross-linking with DMS at 65°C, 50°C and 37°C respectively; lanes 8 to 10, cross-linking with DFDNB at 65°C, 50°C and 37°C respectively. Mol wt markers are as in Fig. 1. (B): Cross-linked complexes were analysed by electrophoresis on 10% gels. Lanes 1 to 3, cross-linking at 65°C, 37°C and 50°C with DMS; and lanes 4 to 6, cross-linking at 65°C, 37°C and 50°C with DFDNB. Positions of mol wt marker proteins are shown : myosin, 205 kDa; beta-galactosidase, 116 kDa; phosphorylase a, 97 kDa; bovine serum albumin, 68 kDa]

some of which did not enter the gel and remained in the stacking gel. Analysis of cross-linking products obtained with dimethyl suberimidate and DFDNB by electrophoresis showed presence of 4 distinct bands with M_r about 165,000, 150,000, 135,000 and 120,000 (Fig. 2B). These results indicate that the protein forms multimeric aggregates in solution.

Binding of HSNP-C′ to immobilised nucleic acid columns

Strength of the binding of HSNP-C to dsDNA- and ssDNA-cellulose, poly (U)- and poly (A)-Sepharose (Fig. 3) was assessed by the salt concentration required for the elution of the protein from the nucleic acid columns. The protein bound strongly to dsDNA and a minor fraction was eluted with 0.2 *M* NaCl and a major fraction eluted at 0.3 *M* NaCl. Similar results were obtained with ssDNA although the affinity was less than that of dsDNA. The protein did not bind to either to poly (U) or poly (A) indicating very weak or no binding to RNA.

Fluorescence titration studies

Thermodynamic binding parameters of the interaction of 20 m*M* NaCl are obtained by non-Scatchard approach[32,33] for the binding of large ligands to long lattice structure like nucleic acids. In this case, direct titrations were made by adding HSNP-C to *S. acidocaldarius* DNA (Fig. 4A and

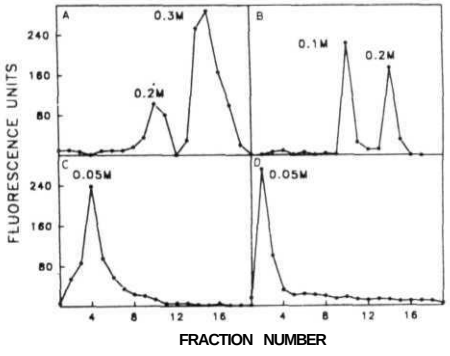

Fig. 3—Binding of HSNP-C to immobilised nucleic acid columns. [(A): dsDNA-Cellulose (B): ssDNA-Cellulose (C): poly (U)-Sepharose (D): poly (A)-Sepharose]

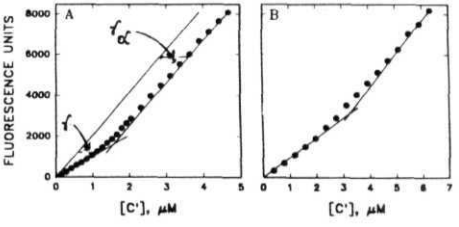

Fig. 4—Direct fluorescence titrations of HSNP-C [Increasing amounts of HSNP-C were added to constant amount of DNA and titrations were performed with 6 μM (A) and 15 μM (B) nucleotide dsDNA. Details of the analysis are described in the text]

B). The initial straight line of this type of titration was extrapolated to the saturation plateau. At saturation a linear asymptote was reached. This asymptote was parallel to the straight line for the titration wjthout DNA. The intercept of the saturation asymptote with the initial straight line gives the site size, n is equal to 4.1 for dsDNA. Similar n value was obtained when titrations were performed with 5 μg DNA. The degree of saturation is determined from the difference between the titrations, in the presence and absence of DNA, denoted by y in Fig. 4. In order to obtain correct normalisation, it is divided by y^{∞}, the distance between the saturation asymptote and the parallel straight line for the titration in the absence of DNA. On the other hand, the observed fluorescence intensity was taken to be proportional to the free protein concentration because we neglected the contribution of the bound protein to fluorescence intensity, since the quenching efficiency is high (greater then 0.7). The degree of saturation, θ is plotted as a function of the free protein concentration (Fig. 5A). There is low but definite co-operativity in mode of binding. The equation, $(2\theta-1)/\sqrt{[\theta(1-\theta)}\sqrt{(q/n)}\ (Kc-1)$ could be used as a good approximation to the binding behaviour around $\theta = 1/2$ where q is the co-operativity parameter; K, the binding constant for the co-operative growth of continuos sequences of bound ligand, and r, the free protein concentration. A plot of $(2\theta-1)/\sqrt{[\theta(1-\theta)]}$ *versus* the free protein concentration is given in Fig. 5B. The value of K is evaluated from the reciprocal of the intercept on the abscissa. The value of q is estimated from the linearly extrapolated intercept on the ordinate $(= -\sqrt{(q/n)})$ using the value of n determined beforehand. We obtained from such an analysis, values of K of 2.8 x 10^6 M^{-1} and q of 2.8 for dsDNA. However, this q value is very low for co-operatively binding proteins (see discussion).

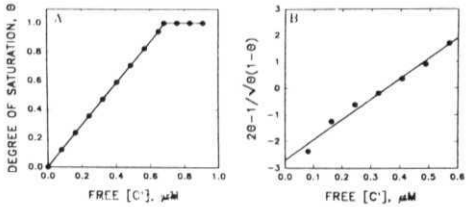

Fig. 5—(A): Plot of degree of saturation θ, *versus* concentration of free HSNP-C. [Data of Titrations performed with 6 μM nucleotide dsDNA (Fig. 4A) were used. (B): Plot of $(2\theta - 1)/\sqrt{[\theta(1 - \theta)]}$ *versus* free concentration of HSNP-C for evaluation of K and q. See text for details. Conditions as given in (A) with 6 μM nucleotide dsDNA.]

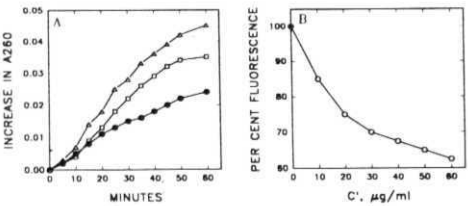

Fig. 6-(A): Effect of HSNP-C on the action of DNAse I. [The assay was carried out as described in the text. Calf thymus native DNA (10 μg) was incubated with 0 μg (A); 20 μg (•) and 40 μg (•) HSNP-C and then treated with 0.5 μg of pancreatic DNAse I. Increase in A_{260} was recorded at 30°C for 60 min. (B): Displacement of intercalated ethidium bromide from DNA by HSNP-C . Data obtained after addition of the protein were plotted as per cent fluorescence intensity *versus* protein concentration]

Protection of DNA against DNAse I hydrolysis and release of DNA bound ethidium by HSNP-C

DNAse 1 digestion of dsDNA in the presence and absence of HSNP-C was studied by spectrophotometry by measuring increase in hyperchromicity with time of incubation (Fig. 6A). Increasing amounts of HSNP-C progressively protected DNA against DNAse I digestion as indicated by declined rate and extent of increase in hyperchromicity. We could not see the effect of addition of higher amounts of the protein as there was aggregation of DNA-protein complexes.

Ethidium, free in solution shows low fluorescence emission at 595 nm when excited at it's absorption maximum i.e., 480 nm. The fluorescence of ethidium increases several fold when incubated with dsDNA and this increase is due to stacking of ethidium by intercalation into dsDNA[14]. HSNP-C was added to this ethidium-DNA complex and the change in ethidium fluorescence after each addition (10 ug) was recorded (Fig. 6B). A gradual decrease in fluorescence intensity is seen after each addition indicating the release of DNA bound ethidium.

Mobility shift of DNA complexed with HSNP-C

The binding of HSNP-C to dsDNA was also analysed by agarose gel electrophoresis. This technique can also be used to observe the distribution of protein on DNA. The electrophoresis through agarose gel separates DNA molecules according to the amount of HSNP-C bound to each DNA molecule. Free DNA migrates fastest, and DNA with increasing amount of protein bound migrates

progressively slower. The other advantage of this technique is that the complexes formed are frozen as they enter agarose gel and no redistribution of the bound protein to different DNA molecules can occur. Two such experiments in which lambda DNA was mixed and incubated with increasing amount of HSNP-C at low (20 m*M*) and high (220 m*M*) concentration of NaCl before electrophoresis are shown in Fig. 7. At both salt concentration, electrophoretic pattern indicates co-operative mode of binding of HSNP-C. The complexes formed at low salt show broad smear, from the position of free DNA to DNA fully saturated (Fig. 7A, lane 4) which is indicative of non-random distribution of the protein among the DNA molecules and intermediate co-operativity in the binding mode (Fig. 7A). However, there was a dramatic and very large increase in the co-operativity of binding of the protein to dsDNA at high salt (Fig. 7B) as indicated by the presence of two sharp bands representing DNA that was bound to the protein retained in the well of the agarose gel (Fig. 7B, lane 3) and free DNA at low binding density (large excess of nucleic acid). At higher protein (Fig. 7B, lane 4, 5) half saturated DNA molecules were also formed as indicated by the appearance of bands with intermediate mobility. These complexes could be similar to those formed at saturation at low salt (Fig. 7A, lane 5).

Electron Microscopy of the complexes of HSNP-C′ and DNA:

Electron microscopy of HSNP-C′-RSF 1010 DNA (dsDNA) complexes formed at protein to DNA ratio (w/w) of 0:1, 1.5:1, 3.5:1 and 7:1 are shown in Fig. 8A, B, C and D respectively. At low protein to DNA ratio (upto 3.5) clusters of bound protein on the DNA could be seen (Fig. 8B and C). With increasing protein to DNA ratio (> 7 and higher) the single clumps of protein on the DNA were compacted into one or two central DNA-protein clusters with small loops of free DNA. Such clumped structures were also formed with single stranded $\phi\times$ 174 DNA (Fig. 9A, B, C and D). However the binding to ssDNA seems to be non-specific.

Discussion

HSNP-C is one of the most abundant DNA binding proteins in *S. acidocaldarius*. The protein strongly protects DNA against thermal denaturation[18,19] and it is localised exclusively on the intracellular compacted DNA structures, the nucleoid[22]. HSNP-C is very similar to 7 kDa group of proteins reported earlier (ref. 13, see below). We have investigated the structure of the protein and it's interaction with nucleic acids to understand it's physicological role.

HSNP—C′ with monomeric molecular mass of about 8 kDa exists as a multimeric aggregate in solution as indicated by the cross-linking studies. Detection of different forms of aggregates viz., dimers, trimers, tetramers, pentamers etc., with zero length cross-linkers such as formaldehyde indicate that the monomeric unit has several contacting sits for individual monomers to interact. The largest

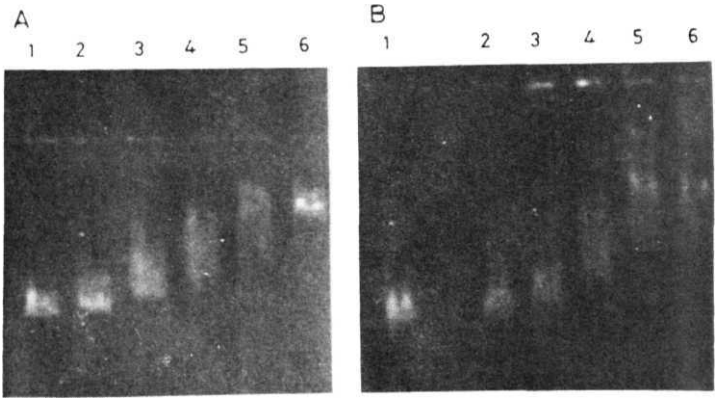

Fig 7—Gel mobility shift analysis of HSNP-C′-DNA complexes. (A): Lambda phage DNA (0.2 μg) was incubated with increasing amounts of HSNP-C in 20 m*M* NaCl (A) or 220 m*M* NaCl (B) buffer before electrophoresis on 0.8% agarose gels. In both cases, lane 1, lambda DNA incubated in the absence of protein; lanes 2 to 6 lambda DNA incubated with 0.2, 0.4, 0.6, 0.8 and 1.0 *fig*, HSNP-C′ respectively.]

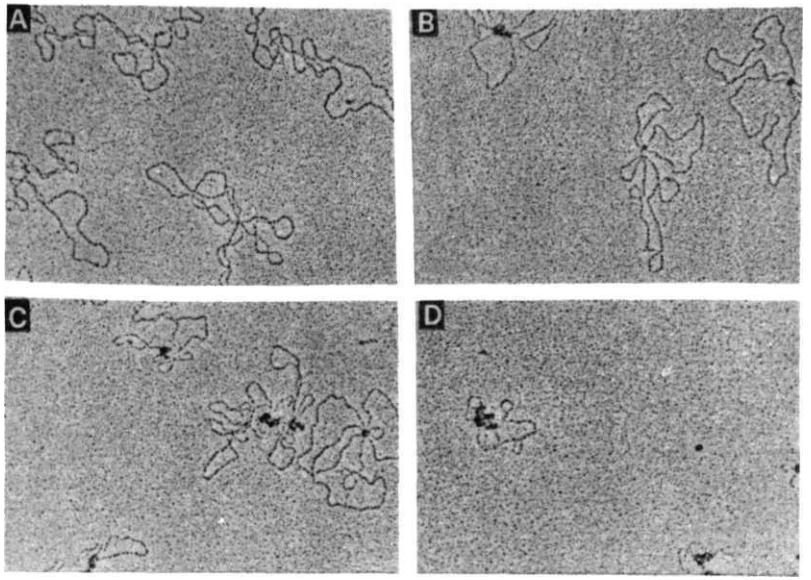

Fig. 8—Electron microscopy of HSNP-C'dsDNA complexes. [HSNP-C' complexes formed with RSF 1010 plasmid DNA (8.7 kbp) at ratios (DNA to protein, w/w) of (A), 1:0; (B), 1:1.5; (C), 1:3.5 and D 1:7.]

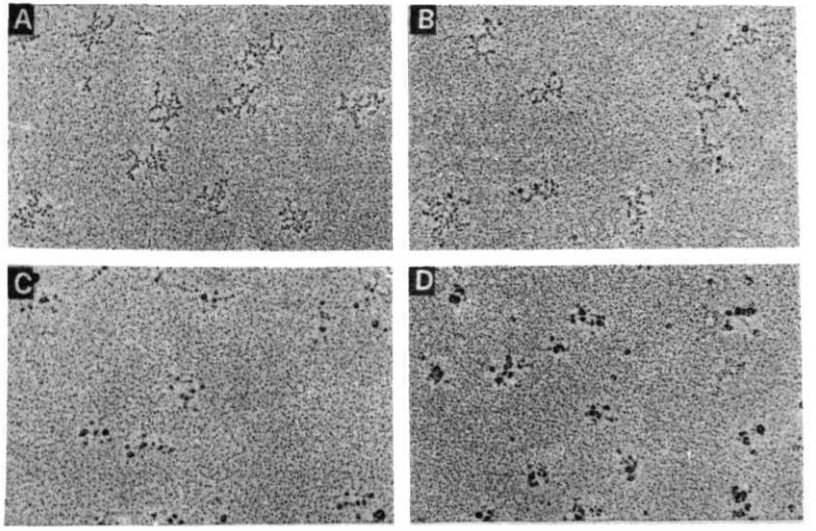

Fig. 9—Electron microscopy of HSNP-C ssDNA complexes [c. nplexes formed with ϕ X-174 ssDNA at ratios (DNA to protein, w/w) of (A), 1:0, (B), 1:1.5, (C), 1:3.5 and (D), 1:7.]

distinct aggregates that are identified by cross-linking studies are of 120, 135, 155 and 165 kDa and possibly containing about 16-22 monomeric units. Longer periods of cross-linking produced very large aggregates with M_r greater than 120 kDa. Cross-linking performed in HSNP-C and DNA complexes also indicated that the protein forms very compact structures with DNA. The aggregation of the protein is more prominent at elevated temperatures (>65°C) indicating that the physiological state of the protein is a homopolymeric aggregate. Precise estimation of the basic unit of aggregation and it's composition requires further experimentation. The aggregation behaviour of this protein is quite novel and to our knowledge no other DNA binding protein forms such homopolymeric compact structures. Although Rec A protein forms polymeric aggregates, these structure are filamentous both in the presence of DNA and it's absence[35].

The protein binds strongly to dsDNA and relatively weakly to ssDNA. It has no affinity for RNA. Chromatography on DNA-cellulose column revealed two forms of the protein eluting at two salt concentrations. Results on the chemical modification of the protein by reductive methylation of lysines indicate that the strongly bound form can be converted to the one eluting with 0.2 *M* NaCl (Celestina and Suryanarayana, manuscript in preparation). Hence the forms of HSNP-C may differ in the extent of methylation of lysine residues in the protein. Sequence analysis on 7d type of proteins[31] also showed that the 7a, 7b and 7d differ only in the extent of methylation of the lysine residues. The mobility shift assays clearly indicate that HSNP-C binds highly co-operatively to dsDNA. This is also supported by the elector) microscopic studies. Fluorescence titrations, however indicated very low co-operativity. This may be due to unusual binding characteristics of the protein which forms clumped compact structures. We had already reported that the complexes of HSNP-C and DNA are not retained after filtration on nitro-cellulose membranes[18] unlike the case with most of the DNA binding protein. Generally fluorescence titrations are used to analyse co-operative binding characteristics of most of the nucleic acid binding proteins which bind linearly along the polynucleotide chain at the adjacent sites[32,36,37,38].

Electron microscopic pictures of complexes of HSNP–C′ with DNA resemble those obtained with protein 7d (ref. 14) of *S. solfataricus* indicating similarity between 7d and HSNP-C. Protein 7d purified in our laboratory exactly as described by Grote *et al.* and cross-reacted with HSNP-C antibodies with immunological identity reaction in immuno-diffusion gels. These results indicate that protein 7d and HSNP-C are very similar, if not identical. At low ratio of protein to DNA (≤ 3.5) a single small cluster of the protein is visible (Fig. 8B&C) which may represent the basic aggregate of HSNP-C.

The binding is co-operative with increasing protein concentration as indicated by binding at adjacent sites with the formation of central core of protein. At very high ratio (>7), large clumps of protein and DNA form the core with loops of free DNA. The binding site size, *n* of 4 nucleotides per monomer determined by fluorescence titrations will be consistent with the ability of the protein to envelope large regions of DNA in the central DNA-protein core. The ability of the protein to protect DNA against DNAse I hydrolysis and to release DNA bound ethidium may be manifestations of the compact structures formed when the protein is bound to DNA. These results and the reported exclusive localisation of HSNP-C on intracellular nucleoid[22] suggest that the protein *in vivo* may be involved in the condensation of DNA and may be acting as a packaging protein. The structures formed by this protein also suggest that it may be involved as a scaffold protein for creating folded domains of intracellular DNA. DNA binding proteins which act as such scaffold proteins in the organisation of bacterial nucleoid or eukaryotic chromatin have not been identified[39].

Acknowledgements

We thank Council of Scientific and Industrial Research (CSIR) for financial asistance in the form of research project. Ms. F. Celestina was awarded a fellowship by UGC during the period of this work.

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