Biochemical characterization of nucleoid and nucleoid associated DNA binding protein HSNP A from thermophilic archaeon *Sulfolobus acidocaldarius.*

A thesis submitted for the degree of
Doctor of Philosophy

In

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Dedicated to the memory of
my late grand parents.....

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Declaration

I hereby declare that the work presented in this thesis entitled **"Biochemical characterization of nucleoid and nucleoid associated DNA binding protein HSNP A from thermophilic archaeon *Sulfolobus acidocaldarius.*"** is entirely original and was carried out by me in the Department of Biochemistry, University of Hyderabad, under the supervision of **Prof. T. Suryanarayana. I** further declare that to the best of my knowledge this work has not been submitted before for the award of degree or diploma from any University or Institution.

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Certificate

This is to certify that this thesis entitled **"Biochemical characterization of nucleoid and nucleoid associated DNA binding protein HSNP A from thermophilic archaeon *Sulfolobus acidoocaldarius."*** Submitted by **Ms. Zeenath Fathima** for the degree of **Doctor of Philosophy** to the University of Hyderabad is based on the studies carried out by her under my supervision. This work has not been submitted for any degree or diploma of any University or Institution.

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Abbreviations

A_{260}, A_{280}, A600	Absorbance at 260, 280 and 600 nm respectively
BNPS-skatole	2-(2-nitrophenylsulfenyl)-3-methyl-3-bromo indoline
Bp	base pairs
BSA	Bovine serum albumin
CM	Carboxy methyl
DMS	Dimethyly suberimidate
DNA	Deoxyribonuclease
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
ds DNA	double stranded DNA
EDTA	Ethylene diamine tetra acetate
g	Centrifugal field
HCHO	Formaldehyde
HSNP	Helix stabilizing nucleoid protein
kDa	kilo Daltons
mM	milli molar
NP-40	Nonidet P-40
PLP	Pyridoxal phosphate
Poly A	Poly deoxy riboadenylic acid
Poly U	Poly ribourydylic acid
RNA	Ribonucleic acid
RNase	Ribonuclease
RPM	Revolutions per minute
SDS	Sodium dodecyl sulfate
ss DNA	single stranded DNA
TEMED	N, N, N', N'-tetremethylethylenediamine
Tris	tris (hydroxyl methyl) aminomethane
UV	Ultraviolet

Contents

Introduction

Introduction

The work carried out in the present dissertation deals with the biochemical characterization of the nucleoid from the thermoacidophilic crenarchaeote *Sulfolobus acidocaldarius* and the helix stabilizing nucleoid protein, HSNP A.

A brief account of the archaea, their molecular biological properties, the chromatin organization in archaea, the proteins associated with archaeal nucleoid, the cell cycle and the associated biochemical variations observed is given in the following pages.

Archaea: a brief introduction:

Domain archaea comprise an evolutionary lineage of prokaryotes only distantly related to bacteria (Woese and Fox, 1977; Woese et al., 1978; Woese et al., 1990). Archaea from geo and hydrothermal habitats include the most thermophilic organisms known (Stetter, 1999), they represent the shortest and deepest branches in the universal tree of life and exhibit features formerly thought to be uniquely eukaryotic. Therefore studies of thermophilic archaea provide insights into mechanisms of thermal stability and the fundamental evolutionary relationships among cellular organisms.

Archaeal features can be classified as bacteria-like, eukaryotic-like or unique to the archaea. As much as a third of the archaeal genome consists of genes found uniquely in archaea (Woese and Gupta, 1981).

General features of the Archaea

Morphology: Phenotypically, the Archaea are a more like Bacteria. Most are small (0.5-5 microns) rods, cocci, spirilla, and filaments. Many of them grow as clusters or aggregates. Archaea generally reproduce by fission, like Bacteria and most Eukarya. The genomes of Archaea are generally 2- 4Mbp in size, similar to Bacteria. However, majority of Archaea are thermophilie - in fact, many are *extremely* thermophilic. They

are also either autotrophic or sulfur respirers. Like eukaryotes, some Archaea have abundant histone-like proteins and the DNA is packaged in the form of nucleosomes.

Cell envelopes: The Archaeal cells are generally surrounded by complex and diverse cell envelopes with glycoprotein containing S-layers and cytoplasmic membranes containing tetraether lipids. Archaeal membrane lipids are unique ether-linked (not ester-linked) glycerol derivatives comprising a glycerol diether with two C_{20} chains and diglycerol tetra ether with two C40 chains. Unsaturations in the lipid chain are generally conjugated (those of Bacteria & eukaryotes are unconjugated). 40-carbon lipids are ether-linked to glycerol at ends, and so form lipid monolayers rather than bilayers, with polar groups at each end. This unique structure increases the chemical and thermal stability of the membrane and may reduce excess fluidity of the membrane at extremely high temperatures. Archaea are incapable of adapting to temperature changes by altering the degree of unsaturation or chain length of membrane lipids like the mesophiles. Increase in growth temperature has been shown to increase the frequency of cyclopentane rings in *Sulfolobus solfataricus* and *Thermoplasma acidophilum*. These lipids can be used as chemical signatures for the identification of Archaea in a sample (Kandler and Kŏnig, 1985).

Archaea are further classified into three sub domains, the Euryarchaeota, the Crenarchaeota and the Korarchaeota. The Korarchaeota have been deduced from small subunit rDNA sequence data, they have not been isolated or cultivated as yet. (Barns et al., 1996).

Members of the Euryarchaeota include the methanogens and halophiles. Methanogenic archaea use three compounds as carbon sources, methanol, acetate and CO2 -type substrates and produce methane by reducing these compounds and oxidizing H_2. Methanogens cannot tolerate O_2 and are thus found in anoxic environments like swamps.

The halophilic archaea include aerobes. These organisms are found in salty or alkaline environments, some require saturated salt solutions for growth. All of the extreme halophilic archaea are chemoorganotrophic.

Some members of the Euryarchaeota are hyperthermophiles and can grow in extremely acidic environments (<pH 2.0).

Members of the Korarcheaota were initially based on recovered rDNA sequences from volcanic hot springs. They have been detected in mixed cultures using fluorescent DNA probes. These organisms probably have similar metabolisms to other Archaea from extreme environments. The Korarcheaota branch near the division between bacteria, archaea and eukaryotes

Cultivated crenarchaea are all thermophilic, and most are extremely thermophilic, with optimal growth temperatures above 80 °C. As a group, these are the most thermophilic organisms known that grow between 80° and 100°C; the maximum temperature of growth can be as high as 113°C (*Pyrobolus)*. Most of the hyperthermophilic species have been isolated from hot springs and shallow or deep-sea hydrothermal vents. Some species can live in dilute solutions of hot sulfuric acid; members of the order Sulfolobales grow at pH 1-2. Low temperatures Crenarchaeota have been identified by genetic analysis of environmental samples. rDNA signatures of Crenarchaeota species have been detected in low-temperature environments such as ocean waters (Antarctic Ocean) and terrestrial sediments and soils.

The Crenarchaea are more diverse both phenotypically and phylogenetically than the cultivated species would imply. Analysis of a single sample from Obsidian Pool in Yellowstone revealed the presence of more crenarchaeal species with more phylogenetic range than all of the cultivated Crenarchaea taken together. Crenarchaeal small subunit-rRNA sequences unrelated to cultivated species are commonly isolated from forest soil and lake sediment, suggesting the existence of species that grow in moderate conditions. One species, *Crenarchaeum symbiosum*, which so far has not been grown in culture, is a symbiont of marine sponges

Several novel methods currently in use to detect previously unidentified microbes show archaea to occur widely in nature. Novel archaea have been localized in agricultural (Kudo et al., 1997, Bintrim et al, 1997) and forest soils (Jurgens et al., 1997) in fresh water lake sediments (Schleper, et al., 1997), in coastal water

Comparison between Bacteria, Archaea and Eukaryotes

Attribute	**Bacteria**	**Archaea**	Eukaryotes
Cell structure	prokaryotic	prokaryotic	eukaryotic
DNA	mostly circular	mostly circular	linear chromosomes
Histones present	No	yes	yes
cell wall	peptidoglycan (muramic acid)	muramic acid absent	muramic acid absent
membrane lipids	ester-linked	ether-linked	ester-linked
Ribosomes	70S	70S	80s
Initiator tRNA	N-formylmethionine	methionine	methionine
Introns	no (mostly)	no (mostly)	yes (mostly)
Operons	Yes	yes	no
Plasmids	common	common	rare
RNA polymerase	one (4 subunits)	One (8-12 subunits)	three (12-14 subunits each)
Promoter structure	-10 and -35 (Pribnow box)	TATA box	TATA box
Sensitivity to chloramphenicol, kanamycin and streptomycin	Yes	no	no
Methanogenesis	yes	yes	no
Nitrification	yes	no	no
Nitrogen fixation	yes	yes	no
Chlorophyll-based photosynthesis	yes	no	yes (chloroplasts)
Chemolithotrophy	yes	yes	no
Growth above 80C	yes	yes	no

picoplankton (Delong,1994), in deep waters upto 3000 m of the oceans (Fuhrman et al., 1997) besides the well established extreme environments.

Crenarchaeota metabolism ranges from chemoorganotrophy to chemolithoautotrophy. Most are also acidophilic and autotrophic. Since this phenotype is shared by the most primitive & first branches of the Euryarchaea, and even the Bacteria, it is probably the primitive phenotype of the Archaea as well as the last common ancestor.

The Crenarchaea fall into 2 subgroups: *Thermoproteus* and relatives (7 genera) and *Sulfolobus* and relatives (5 genera).

***Thermoproteus* and related species:** They are strict anaerobes that grow best by sulfur respiration under culture conditions, the more usual growth conditions for these organisms in the wild is autotrophic sulfur reduction i.e., these organisms can either oxidize or reduce sulfur and the switch between growth modes is a distinct developmental switch. These organisms are anaerobic, extreme thermophiles. *Pyrodictium spp* cultures grow optimally at upto 115 °C and are among the most primitive organisms known. Related species are *Desulfurococcus*, *Staphylothermus*, and *Thermodiscus*.

***Sulfolobus* and related species:** These organisms are common, generally predominant, organisms of solfataras, boiling mud pots, and hot acid mine drainage. They are generally facultative microaerophiles, growing best in culture by sulfur oxidation (aerobically or microaerophilically) or sulfur reduction (microaerophilically or anerobically). They are all acidophilic, growing best at pH 1 to 3.5, although a couple of species grow at pH 0. They are also all thermophilic, growing optimally at 75 to 95 °C. These organisms like most acidophiles, are oligotrophic and require small amounts of organics for growth. Organic acids in particular are toxic to acidophiles since they are protonated at the pH's of the growth media (below 3.5), and are therefore uncharged, facilitating them to diffuse freely into the cytoplasm through the lipid membrane. At pH >5.5, the internal pH of the cells, the organic acids ionize, releasing H+. This both acidifies the cytoplasm and decomposes the proton gradient which is harmful to the cell. Related genera are *Metallosphaera*, *Acidianus*, *Desulfurolobus*, and *Stygiolobus*.

Sulfolobus acidocaldarius

Sulfolobus acidocaldarius grows optimally at 80°C and pH 3 and it is a lobed coccus. The lobes are budding scars, left over from budding reproduction, and appear like appendages that hold the cell to the substrate sulfur granules. *Sulfolobus* is a strict microaerophile, first discovered in Moose pool (Yellowstone National Park) where it grows nearly in pure culture at about 10^8 cells per ml. *Sulfolobus spp* have developed as an important model for studies of hyperthermophilic archaea, including cell cycle analyses (Bernander and Poplawski, 1997; Hjort and Bernander, 1999; Poplawski and Bernander, 1997) and development of simple genetic techniques (Grogan, 1989, 1996; Grogan and Gunsalus, 1993; Schleper et al., 1994).

Studies on the cell cycle of *Sulfolobus* demonstrate that the chromosome replication, cell division, nucleoid structure and distribution and cellular growth can be selectively inhibited or uncoupled (Bernander et al., 2000). The post replication stage has been determined to be the preferred cell cycle arrest stage. A method for synchronizing the *Sulfolobus acidocaldarius* cultures has been developed by Hjort and Brenander, 1999. When a stationary phase culture, in which all cells are in the post replication stage of the cell cycle, is diluted into fresh medium, initiation of replication does not occur until the preceeding nucleoid partition and cell division.

No analogue of the ftsZ (filamentation temperature sensitive) gene, whose product initiates bacterial cell division and participates in the constriction process, has been identified in a crenarchaeote.

Archaea differ considerably in their biology from Bacteria and Eukaryotes - at least 30% of their genes have no detectable homologues in the other two domains - they are a rich source of novel enzymes. Moreover, since many of the archaeal homologs of eukaryotic multi-subunit enzymes are monomeric, this greatly extends their applicability to biotechnological processes.

In 2001, The European *Sulfolobus* Project was initiated to screen for and express thermostable enzymes of industrial interest from *Sulfolobus* and other Crenarchaeotes. The purpose of this project is to exploit the expertise of the european laboratories

involved in studies on this organism and to screen for novel enzyme activities both at the genomic level and in an extensive and unique European collection of thermophilic Crenarchaeotes, to develop and refine shuttle vector systems for expressing these proteins in a range of Bacterial and Eukaryotic hosts by modifying the Archaeal genetic signals, to develop a vector-host system for expressing Crenarchaeal enzymes in a *Sulfolobus* host on a large scale, to perform large-scale fermentation to express new crenarchaeal enzymes and to thermoadapt mesophilic enzymes in *Sulfolobus.* The project involves a genomic approach where we exploit the finished genome of *S. solfataricus* (She et al., 2001) and perform low pass sequencing of the genomes of two other diverse and divergent Crenarchaeotes *Sulfolobus acidocaldarius* and *Hyperthermus butylicus.*

Molecular biology of Archaea:

Among the major cellular functions to be characterized in archaea are chromosome replication, nucleoid segregation and cell division, and the coordination of these processes with cellular growth. Analysis of the archaeal genome sequences has revealed the replication, transcription and translation machineries of archaea to be similar to those of eukaryotic organisms, while genes involved in the metabolic processes show more similarity to their bacterial counterparts. For example, the gene products involved in the chromosome segregation show similarity to both eukaryal and bacterial proteins whereas the cell division apparatus is mostly of bacterial type. (Bernander, 1998). Since the archaea cell cycle contains a mixture of features from both eukarya and bacteria they may contribute to a better understanding of the evolution of these processes. There is speculation that the eukaryotes may have originated from a fusion event between a bacterium and an archaeon (Gupta and Golding, 1996; Martin and Muller, 1998; Moreira and Lopez-Garcia, 1998) which implies that the eukaryal cell cycle characteristics may be derived from those of an archaea like ancestor.

Certain features however are characteristic of archaea like their ribosomal RNA sequences, the lipid composition (ether glycerol lipids) and organization (bi or mono layer) of the cytoplasmic membrane and also particular modes of energy metabolism,

such as methanogenesis and bacterio rhodopsin- driven photosynthesis (Jones et al., 1987)

DNA replication machinery; *Sulfolobus solfataricus* has two functional DNA polymerases, PolB1 and PolY1. PolB1 specifically recognizes the presence of deaminated bases hypoxanthine and uracil in the template by stalling polymerization 3-4 bases upstream of these lesions and strongly associates with oligonucleotides containing them (Greagg et al., 1999). PolB1 also stops at 8-oxoguanine and is unable to bypass an abasic site in the template. PolYl belongs to the family of lesion bypass DNA polymerases and readily bypasses hypoxanthine, uracil and 8-oxoguanine, but not an abasic site in the template. Both PolBl and PolYl incorporate aberrant DNA precursors dUTP and dITP (Gruz et al., 2003). The error prone PolYl a homolog of DinB uses a steric gate residue for discriminating against incorporation of ribonucleotides during polymerization. (DeLucia et al., 2003).

An 800 bp intergeneric sequence between the genes encoding Cdc6/Orcl* and methylthioadenosine phosphorylase which is highly conserved in the *Pyrococcous* species has been identified as the origin for replication in this organism (Kelman, 2000). Two active regions of replication have been identified in the chromosome of the hyperthermophilic archaeon *Sulfolobus solfataricus.* Conserved sequences are present within these regions which are recognized by a family of three *Sulfolobus* proteins homologous to Orc 1 and Cdc6 proteins. The two regions are recognized by distinct subsets of these Orc1/Cdc6 homologs (Robinson et al., 2004).

PCNA (proliferating cell nuclear antigen) has been shown to be functionally conserved not only within eukaryotes but also from hyperthermophilic euryarchaeotes to mammals (Henneke et al., 2000). The sliding clamp, PCNA of the archaeon *Sulfolobus solfataricus* P2 is a heterotrimer of three distinct subunits (PCNA1, 2, 3) that assembles in a defined manner. The PCNA heterotrimer, but not the individual subunits, stimulates the activities of DNA polymerase, DNA ligase I and the flap endonuclease (FEN1) of *S.solfataricus.* Unique subunit specific interactions between components of the clamploader, RFC, suggest a model for clamp loading of PCNA (Dionne, I., et al., 2003).

Primase activity of eukaryotes resides within the DNA polymerase a-primase complex (pol a-pri) on the p48 subunit. A p48-like DNA primase from *Pyrococcus furiosus*, *Pfu*p41, preferentially utilizes deoxynucleotides to synthesize DNA fragments upto several kilobases in length with out primers. Reports that a DNA polymerase can be engineered from an RNA polymerase by site specific mutagenesis support the idea that DNA polymerases might have evolved from RNA polymerases. *Pfup4*\ may be an intermediate in such pathways between an RNA polymerase-primase and a DNA polymerase (Bocquier et al., 2001).

Archaeal minichromosome maintenance (MCM) helicase moves along ds DNA even in the absence of a 3' ss DNA overhang (Shin et al., 2003). DNA topology of hyperthermophilic archaea during stress relies on the physical effect of temperature on topoisomerase activity and on the DNA geometry (Lopez-Garcia et al., 1999). Sso MCM has NTPase and helicase activity which acts preferentially on DNA duplexes containing a 5' tail and is stimulated by the single stranded DNA binding protein SSB. (Carpentieri, et al., 2002)

The first member of the DNA polymerase E family has been reported from *Sulfolobus islandicus* (Lipps et al., 2003). ORF904, encoded by a plasmid pRN1 has ATPase, primase and DNA polymerase activity. The primase and polymerase activity are located in the N-terminal half of the protein, which does not share homology with any known DNA polymerase or primase. The ORF904 type of replication enzyme could have evolved independently from the eubacterial and archaeal/eukaryal proteins of DNA replication.

Transcription and translation machinery: The transcriptional/translational machinery in Archaea is generally like those of Bacteria and Eukarya, with 70S (bacterial-sized) ribosomes. Genes are arranged in co-transcribed clusters called operons. Ribosomes recognize translational start sites and bind to the mRNAs directly at 'Shine-Dalgarno' (SD) sequences just like Bacteria. Also like in Bacteria, transcription and translation are coupled - that is, they occur simultaneously, and failure of an mRNA to be translated causes the RNA polymerase to abort transcription. Introns in protein coding genes are ubiquitous in eukaryotes but are

rarely found in prokaryotes. Recent evidence through computational analysis has revealed the presence of introns in protein encoding gene, the Cbf5p (centeromere-binding factor 5) homolog, in the genomes of the archaea, *Sulfolobus solfataricus*, *S. tokodaii* and *Aeropyrum pernix* (Watanabe et al., 2002).

However, in many ways transcription and translation in Archaea is like it is in Eukarya. Although each promoter drives the expression of an entire operon, the promoters are just like eukaryotic RNA polymerase II promoters, and are binding sites for transcription factors (rather than the RNA polymerase itself, as in Bacteria). Archaea have a single RNA polymerase (like Bacteria), but this RNA polymerase is just like eukaryotic RNA polymerase II, and requires the same transcription factors for promoter recognition. Translation is initiated with methionine (like Eukarya), not formyl-methionine (Bacteria). Translation is inhibited by diphtheria toxin, as eukaryal ribosomes, but is not inhibited by most bacterial-translation-inhibiting antibiotics. Hybrid archaeal/eukarya ribosomes are functional where as bacterial/archaeal or bacterial /eukarya ribosomes are not functional.

Cell cycle characteristics of Archaea:

The cell cycle characteristics of archaea share similarities with both prokarya and eukarya. Growth phase dependent modifications of nucleoid size and a long gap period between the termination of chromosome replication and completion of nucleoid partition, similar to the G2 phase in eukaryotic cells were observed in the euryarchaeon *Thermococcus kodakaraensis* (Jeon., et al., 2001) and the crenarchaeon *Sulfolobus acidocaldarius* (Poplawski and Bernander., 1997).The period of visible cell constriction, however was found to be similar to that of prokaryotes. The separation of nucleoids to opposite cell halves before division is similar to a recent model proposed for nucleoid partition in eubacteria (Wheeler and Shapiro., 1997) in which the replication origins become attached to the cell poles before division. During the exponential phase the nucleoids are higly organized but have an unstructured appearance during stationary phase. The structure of *E. coli* nucleoid has been suggested to be highly dependent on the transcriptional activity through coupled transcription and translation of membrane proteins which would anchor transcription

complexes and the associated DNA in the membrane and thereby extend the nucleoid. (Woldringh and Nanninga, 1985; Woldringh et al., 1995). If coupled transcription, translation and membrane insertion also occurs in archaea, the relaxed nucleoid structure in stationary phase may at least in part due to reduced transcriptional activity.

Genome projects have revealed the presence of eukarya -like regulators for cell division, like CDC proteins, in archaea. (Buit, et al., 1996; Kawarabayasi, et al., 1998; Klenk et al., 1997). One of the CDC 48 homologues is shown to be functional in archaeal cells (Jeon et al., 1999). A Cdc6-like factor has been purified and characterized from *Sulfolobus solfataricus*. (De Felice, et al., 2003).

Sulfolobus acidocaldarius exhibits an unusual cell cycle, with a short prereplication period and a long post replication period. Also, in stationary phase the cell rests with two fully replicated chromosomes (Poplawski and Bernander, 1997). The tightly regulated chromosome replication, nucleoid organization, nucleoid partition and cell division have been shown to be inhibited or uncoupled by mutation (Bernander, et al., 2000). The *Sulfolobus* nucleoid shows a high level of organization at exponential phase, it is extended, arc like with clearly visible lobular structures. At stationary phase, cells are more transparent than exponential phase cells nucleoid has more relaxed organization. Nucleoid was observed to occupy more space at stationary phase as compared to exponential phase (Poplawski and Bernander., 1997).

Multiple copies of the chromosome were also observed in *Methanococcus jannaschii* (Malandrin et al., 1999), *Archaeoglobus fulgidus* and *Pyrococcus furiosus*.

Chromosome and extra chromosomal elements of archaea:

The archaeal chromosome is circular consisting of 1.5-4 x 106 bp (Klein and Schnorr, 1984). The G+C content in archaea ranges from 21 to 68 mol%. Genes are arranged in polycistronic operons. Archaeal introns have mostly been observed in tRNA and rRNA genes. Large free introns derived from pre-rRNAs have been observed as stable and abundant circular RNAs in certain Crenarchaeota (Salgia et al., 2003). Introns in

protein encoding genes are ubiquitous in eukaryotic cells, but pre- mRNA splicing is yet to be reported in archaeal and its viral genomes. Recently introns were reported in genes encoding a homolog of eukaryotic Cbf5p (centeromere binding factor 5), a sub unit of a small nucleolar ribonucloeprotein, from *Aeropyrum pernix, Sulfolobus solfataricus* and *Sulfolobus tokodaii* (Watanabe, et al., 2002).

Plasmids isolated from hyperthermophilic archaea, Crenarchaeota *(Thermoproteales* and *Sulfolobales)* and the orders *Thermococcales* and *Thermoplasmales* in the kingdom Euryarchaeota, were found to range in size from 4.5 kb to about 40 kb. Most of them occur in high copy number. These plasmids are from relaxed to positively supercoiled in contrast to the negatively supercoiled state of DNA molecules in mesophiles, bacteria, euryarchaea or eucaryotes. Most are cryptic, pNOB8 is conjugative, the widespread pDL10 alleviates in an unknown way autotrophic growth of its host *Desulfurolobus* by sulfur reduction. The plasmid pTIK4 appears to encode a killer function. pNOB8 has been used as a vector for the transfer of the lac z (beta-galactosidase) gene into a mutant of *S. solfataricus.* The order *Sulfolobales* has around ten plasmids. *Halobacterium salinarium* has three plasmids with covalently closed circular DNA; *Halobacterium halobium* has the plasmid, pHH1. *T. acidophilum* has a 15.2 kb plasmid.

Buit et al (1996) determined the complete 1.66 mega base pair genome sequence of *Methanococcus jannaschii* and its 58 kb and 16 kb extra chromosomal elements. Only 38 % of the predicted protein encoding regions have been assigned cellular roles. Sequencing of the *Sulfolobus solfataricus* genome has shown the clustering of genes by function (Charlebois et al., 1996).

The availability of the complete genome sequence of several archaea and their analysis for frequency, location and phylogeny of archaeal mobile elements has revealed the presence of autonomous insertion sequence elements and the non autonomous miniature inverted repeat element (MITE)-like elements. The number and diversity of elements differs greatly between the genomes. *Sulfolobus solfataricus* P2 and *Halobacterium* NRC-1 are very rich in elements whereas *Methanobacterium*

thermoautotrophicum contains none. The putative *oriC* and *terC* regions act as barriers for the motility of the IS and MITE -like elements in *Sulfolobus solfataricus.*

The genome of the archaeon *Sulfolobus solfataricus* contains atleast four types of short sequence elements lacking open reading frames similar to the eukaryal non autonomous mobile elements -the more conserved, SM1 and SM2 and the less conserved, SM3 and SM4- which together constitute 0.6% of the genome. (Redder et al., 2001).

Viruses of *Sulfolobus* have been assigned to four novel families- Fuselloviridae (viruses SSV1, SSV2 and SSV3), Rudiviridae (SIRV1 and SIRV2), Lipothrixviridae (SIFV) and Guttaviridae (SNDV) - on basis of their unique morphology. None of these viruses are lytic. Fuselloviruses are temperate whereas the other families are present in the host in a carrier state. They specifically infect thermophilic and hyperthermophilic archaea. In fusellovirus lysogens, the viral genome is integrated specifically into the host genome at a tRNA gene, by means of a virally encoded integrase, and is also present as a plasmid copy (Prangishvili et al., 2001).

Chromatin organization in archaea:

Chromatin structure and regulation of gene expression is determined in part by protein - DNA interactions. Histones, nuclear matrix and chromosomal scaffold proteins and enzymes involved in replication, repair and recombination contribute to chromatin structure, function and regulation.

Eukaryotic chromatin consists of DNA -protein complexes, nucleosomes where two turns of DNA are wound around an octamer of histones. The nucleosome core particle consists of 146 bp of DNA (H3+H4)2 and two H2A -H2B dimmers. Nucleosome cores are connected by a linker DNA associated with a single H1 histone molecule.

The organization of the chromatin in prokaryotes is not completely understood. The genome is present as a tightly condensed supercoiled structure, the nucleoid, within the cell (Kornberg et al., 1974; Materman and Van Gool, 1978). Nucleoid has been

shown to be associated with several DNA binding proteins, outer and inner membrane proteins, RNA polymerase core and DNA polymerases (Stonington and Pettijohn, 1971; Worcel and Burgi, 197; Portalier and Worcel, 1976; Moriya et al., 1981; Lossius et al., 1984; Yamazaki et al., 1984; Gaziev et al., 1986;). Folded chromosomes have been isolated from *Bacillus subtilis* (Guillen et al., 1978) and *E.coli* (Worcel and Burgi, 1972).

Chromatin organization in archaea has both eukaryal and eubacterial characterstics. Euryarchaea contain histones that have primary sequences in common with eukaryal nucleosome core histones. They also form a histone fold that facilitates DNA wrapping into nucleosome-like structures. Archaeal nucleosomes comprising an archaeal histone tetramer circumscribed by ~80bp of DNA, similar to the eukaryal tetrasome (H3+H4)2 with ~80bp of DNA have been reported in this organism. (Pereira et al., 1997; Sandman., et al., 1990). Archaeal nucleosomes in *Methanobacterium thermoautotrophicum* and *Methanobacterium fervidus* protect ~ 60 bp of DNA and multiples of 60 bp from micrococcal nuclease digestion. DNA structure which is dependent on DNA sequences directs the assembly of archaeal nucleosomes. The repeat sequence features are similar to the eukaryal nucleosome positioning elements. Repeated sequences like phased oligo (dA) tracts, 5'- (G/C) 3NN (A/T) 3NN-3' and CTG repeats direct both archaeal and eukaryal nucleosome positioning (Bailey and Reeve, 1999; Sandman and Reeve, 1999). HMfB from *Methanobacterium fervidus* selectively incorporates GC, AA and TA dinucleotides at ~10 bp intervals into archaeal nucleosomes. These molecules direct the positioned assembly of archaeal nucleosomes. Fourier analysis also reveals an enrichment of the AA dinucleotide in the eucaryal and euryarchaeal genomes. Histone packaging of the genomic DNA apparently imposes constraints on genome sequence evolution (Bailey, et al, 2000).

The chromatin of *Halobacterium salinarium* consists of regions of DNA associated with protein and regions of DNA free of protein. These regions are interchangeable depending on the phase of growth (Takayanagi et al, 1992). Electron micrographs show the DNA to associate with the proteins tandemnly similar to the primary structure of the eukaryotic chromosome (Shioda et al, 1989). Sheared chromosomes

obtained from the late exponential phase cells resolved into two peaks on a sucrose density gradient. The peak I consists of protein free DNA and peak II consists of rugged fibres consisting of nucleosome like structures. Most of the DNA is in peak I form during exponential phase and in peak II form during stationary phase. The transition between the two forms occurs during the late exponential phase (Takayanagi et al., 1992).

The chromosome in *Sulfolobus acidocaldarius* is highly structured. One to two copies of the chromosome are present during exponential phase and two copies are present during stationary phase. The sheared chromatin of *Sulfolobus acidocaldarius* isolated at exponential phase resolves into two peaks during gel filteration. Peak I consists of high molecular weight aggregates of DNA and proteins. Peak II consists of high molecular weight and an abundance of low molecular weight proteins, mainly HSNP C', DBNP B, HSNP A, along with proteins in the molecular weight range of 45 kDa, 25 kDa and 15 kDa (Jaya 1998). Electron microscopic studies have shown the exponential phase nucleoid to be highly organized with a structured appearance, occupying a relatively small part of the cell. At stationary phase the nucleoid shows a relaxed organization, is unstructured and occupies a larger part of the cell (Poplawski and Brenander, 1997).

Nucleoid associated proteins in archaea:

Four proteins, HU, H-NS, HLP1 and H have been identified as eubacterial histone-like proteins (Drlica and Rouviere-Yaniv, 1987). HU (9 kDa) is a basic protein which stabilizes the DNA against thermal denaturation. It wraps DNA into nucleosome like structures having 8-10 dimers of HU and 275 bp of DNA (Rouviere-Yaniv et al., 1979). H-NS is a neutral protein which binds intrinsically curved AT rich DNA with slight sequence specificity (Zuber et al., 1994; Falconi et al., 1996). It does not wrap DNA but influences the initiation of replication (Katayama et al., 1996), acts as a transcription repressor (Ueguchi et al., 1996) and also plays a role in post transcriptional regulation of rpo-s gene. HLP1 (17 kDa) encoded by Fir A gene interacts with RNA polymerase and is involved in transcription and maintenance of individual domains of supercoiling (Schaffer and Zillig, 1973; Lathe et al., 1980;

Sinden and Pettijohn, 1981). H (28 kDa) is a basic protein which binds DNA, inhibits DNA synthesis in vitro and has an amino acid sequence similar to eukaryotic histones (Hubscher et al., 1980).

Hyperthermophilic archaea grow at extreme conditions of temperature, pH and anaerobicity (Fiaia and Stetter, 1986; Bouthier et al., 1990). However their chromosomal G+C content is less than 40 mol%, *Pyrococcus* (38-45%), *Thermococcus* (38-57%) and *Methanothermus* (33 %). This demonstrates that a high G+C content is not essential for maintaining the stability of the genomic DNA *in vivo* at high temperatures. Many extremophiles produce low molecular weight compounds or compatible solutes to protect macromolecules especially proteins against denaturation by heat, oxidation and dryness. Compatible solutes stabilize the native tertiary structure of the protein, preventing protein aggregation. Mannosylglycerate and Mannosylglyceramide from *Rhodothermus marinus*, cyclic diphosphoglycerate from *Methanothermus fervidus*, di-myo-inosytol phosphate from *Pyrococcus furiosus*, and diglycerol phosphate from *Archaeoglobus fulgidus* are a few examples of such compatible solutes. These solutes also protect the proteins against proteolytic degradation by stabilizing the folded state of the protein thus making the proteolytic sites less accessible to proteases (Bagyan et al., 2003).

Polyamines which are positively charged at physiological conditions stabilize the DNA against thermal denaturation by binding to the negatively recharged phosphate groups (Tomita et al., 1989; Tabor and Tabor, 1984; Morgan et al., 1987). Linear, tertiary branched and quarternary branched polyamines have been detected in hyperthermophilic archaea (Hamana, et al., 1994). High intracellular salt concentration also plays a role in maintaining double stranded DNA. *Methanococcus fervidus* and *Pyrococcus woesei* have 1 to 0.6 M potassium ion concentrations (Scholz et al., 1992; Bowater et al., 1994).

Several small basic proteins have been isolated from archaea which bind to DNA non sequence specifically. They have core domain structure similar to that of eukaryotic histones. Archaeal histones are the structural homologues of the eukaryal nucleosome core histones. Archaeal histones are not present in crenarchaeota (sulfur dependent hyperthermophiles and acidophiles) but are present in most euryarchaeota

(methanogens, halophiles) except *Thermoplasma* (Ruepp, et al., 2000). The combination of histones of the archaeal nucleosomes varies with the growth conditions similar to the changes observed in the histone like proteins in the nucleoids of bacteria at different growth phases (Claret, et al., 1997).

HTa (9.9 kDa) from *Thermus acidophilum*, was the first archaeal histone-like protein identified (Searcy 1975, Stein and Searcy, 1978). It is a basic protein which exists as a tetramer in solution (Searcy and Stein, 1980) and protects DNA against thermal denaturation. It has a greater homology to HU than to histones H2A and H3 (Drilica and Rouviere- Yaniv, 1987; DeLange et al., 1981).

MC1 (Hmb), a 14 kDa abundant histone like protein was isolated from *Methanosarcina* species (Chartier et al., 1988; Chattier et al., 1989). It preferentially binds ds DNA (Culard et al., 1993) and protects DNA against thermal denaturation (Chartier et al., 1988) and radiolysis by fast neurons (Isabelle et al., 1993). It stimulates transcription (Chartier et al., 1989) though it does not form nucleosomes; it preferentially binds negatively supercoiled DNA and bends the DNA upon binding (Teyssier et al., 1996). The amino acid sequence of this protein does not share homology with any of the known histones or histone like proteins.

In *Methanococcus fervidus*, proteins like HMfA and HMfB compact the DNA and protect it against thermal denaturation in vitro. The DNA-binding and nuclease-protection properties of the Hmf histones from the hyperthermophilic archaeon *Methanothermus fervidus* have been shown to be consistent with the formation of nucleosome-like structures (NLS). These proteins bind to DNA molecules as short as 20 bp and form complexes that protect DNA fragments from micrococcal nuclease (MNase) digestion that are 30 bp, approximately 60 bp and multiples of approximately 60 bp in length. DNA structure, determined by DNA sequence plays an important role in positioning and such repeated sequences as phased oligo(dA) tracts, 5'-(G/C)3NN(A/T)3NN-3' and CTG repeats direct both archaeal and eucaryal nucleosome positioning (Sandman et al., 1990; Bailey and Reeve, 1999). HpkA and HpkB from *Pyrococcus kodakaraensis* KOD1 also compact DNA and protect against thermal denaturation (Higashibata et al., 1999).

Methanobacterium formicum has three Hmf like proteins, HFoA1, HFoA2 and HFoA3. They bind and compact DNA into nucleosome like structures similar to those formed by Hmf proteins (Darcy et al., 1995).

Thermococcus species AN1 has a 4.3 kDa histone like protein, HAN1 which protects DNA against thermal denaturation (Ronimus and Musgrave, 1996a). The N-terminus of this protein shares a high degree of homology to the Hmf proteins (Ronimus and Musgrave, 1996b).

Sulfolobus genus does not have histone proteins, but it has a number of small basic and abundant DNA binding proteins ranging from 7 kDa, 8 kDa and 10 kDa which play important roles in DNA compaction, helix stabilization and DNA processing mechanisms (Kimura et al., 1984; Choli, et al., 1988; Reddy and Suryanarayana, 1988; Reddy and Suryanarayana 1989).

The 7 kDa class of proteins comprises five isoforms, 7a to 7e. The 8 kDa and 10 kDa classes of proteins comprise two proteins respectively, 8a, 8b and 10a, 10b. Electron microscopy of the protein-DNA complexes of 7 kDa, 8 kDa and 10 kDa proteins show that they bind ss and ds DNA to form compact structures (Lurz et al., 1986).

The 7d protein (known as Sso 7d, Sac7d, and Ssh7d depending on species of origin) and Alba (known as Sso 10b, Sac10b, and Ssh 10b depending on species of origin) are two highly abundant DNA binding proteins have been characterized. The non-sequence specific DNA binding protein Sso7d protects DNA against thermal denaturation and promotes the renaturation of complementary DNA strands at temperatures above the melting point of the duplex in a strictly homology dependent manner.(Guagliardi, et al., 1997). Sso7d is a 62 residue basic protein. At neutral pH it denatures at about 100 °C. Binding of multiple Sso7d molecules to short DNA fragments induces significant curvature and reduces the stiffness of the complex. It induces negative supercoiling in DNA molecules of any topology- relaxed, positively or negatively supercoiled. It compacts positively supercoiled and relaxed forms of DNA but not negatively supercoiled DNA. It also inhibits the positive supercoiling activity of the thermophile specific enzyme, reverse gyrase (Napoli, et al., 2002).

Family B DNA polymerase (polB1) from the hyperthermophile *Sulfolobus solfataricus* degrades both ss and ds DNA at similar rates in vitro at physiological temperatures. Studies on the effect of the 7 kDa proteins on the polymerization and 3'-5' exonuclease activities of this enzyme showed that they modulate the extension and excision activities thus reducing the cost of proofreading. The 7 kDa proteins inhibit the excision and enhance the extension of matched template primers. They however do not protect the ss DNA from cleavage by polB 1. They also do not affect the proof reading ability of polB 1 and do not inhibit the excision of mismatched primers. The dNTP concentrations required for effective inhibition of 3'-5' exonuclease activity of ploB1 was lowered from 1mM to 50 μM in the presence of the 7 kDa proteins at 65°C (Lou, et al., 2004). So far the 7 kDa proteins have only been observed in *Sulfolobus* species, wherein it is highly expressed and highly conserved.

Archaeal proteins of Sac 10b family are thought to affect the topology of the chromosomal DNA in thermophilic archaea. Ssh 10b constrains negative DNA supercoils in a temperature dependent manner (Xue et al., 2000). Studies have shown that two forms of Ssh 10b homodimers coexist I solution and the slow *cis-trans* isomerization of the Leu61-Pro62 peptide bond accounts for the conformational heterogeneity of the homodimer. The *trans* form (T-form) is dominant at high temperatures and the *cis* form (C-form) at lower temperatures. Both forms have the same DNA binding site but different conformational features which are responsible for the temperature dependent nature of Ssh 10b-DNA interaction. (Cui, et al., 2003). The alba proteins coat the ds DNA without significantly compacting it protecting it from nuclease digestion (Lurz et al., 1986). Recent reports show that Ssh 10b binds with similar affinity to ds DNA, ss DNA and RNA invitro and binds exclusively to RNA in *Sulfolobus shibitae* cells invivo (Guo et al., 2003).

Alba where present, always occurs along with a second chromatin protein, a histone, 7d protein or HU homologue. Archaeal species lacking alba encode histones along with another DNA binding protein, such as MC1 in mesophilic methanogens. This leads to the speculation of interaction or cooperation between more than one type of chromatin proteins to compact DNA fully in archaea (White and Bell, 2002).

Reddy and Suryanarayana, (1988) purified four acid soluble low molecular weight proteins from the nucleoid of Sulfolobus acidocaldarius, HSNP (Helix stabilizing nucleoid proteins) A (12 kDa), C (9 kDa) and C (7 kDa) and DBNP (DNA binding nucleoid protein) B (10 kDa). The HSNP proteins stabilize the DNA helix and protect DNA against thermal denaturation. They are present exclusively on the genomic DNA as revealed by immuno gold labeling electron microscopy of the nucleoid and could be responsible for DNA compaction and genome organization (Bohrmann et al., 1994). HSNP C is identical to Sac 7d (Gauri 1997) and DBNP B is similar to Sac 10b (Sreenivas 1994). DBNPB binds ds and ss DNA strongly but does not protect against thermal denaturation. It forms different types of novel complexes with DNA at different protein to DNA ratios (Sreenivas et al., 1998).

HSNP A strongly binds native DNA and RNA, weakly to denatured DNA and ribosomes. It protects the DNA against thermal denaturation but not RNA.

Growth phase dependent variation in content and levels of proteins associated with nucleoid:

Structural and functional modulations occur in the nucleoid of the prokaryotes and chromatin of eukaryotes during the transition from exponential growth to stationary phase. Cells enter into stationary phase upon sensing an impending saturation level of their population density.

Nucleoid proteins play functional roles besides their structural roles, in the regulation of essential DNA functions such as replication, recombination and transcription. Studies on the composition of these nucleoid associated proteins show that a variation in protein contents during the stationary phase is accompanied by compaction of the genome DNA and silencing of the genome functions.

In *E.coli*, the major DNA binding proteins, Fis, Hu and Hfq during exponential phase are replaced by a single protein, Dps at stationary phase. CbpA, the curved DNA binding protein appears only in the late stationary phase (Ishihama, 1999, Azam et al., 1999).

The observed increase in density of cells at stationary phase has been reported to require the presence of the RNA polymerase RpoS (sigma-S) needed for stationary phase gene transcription (Makinoshima, et al., 2003).

Growth phase dependent expression and degradation of histones has been observed in the thermophilic archaeon *Thermococcus zilligii,* the levels of certain proteins decreased dramatically on entry into stationary phase. The HTz protein could not be detected by late stationary phase (Dinger et al., 2000).

Methanothermus fervidus cells have been shown to contain HmfA and HmfB homodimers and HmfA-HmfB heterodimers. The HMfA/HmfB ratio and the relative amounts of homodimers and heterodimers is growth phase regulated. HmfA is more abundant in exponential phase and HmfB during the stationary phase (Sandman, 1994).

In the present study detailed characterization of *S. acidocaldarius* nucleoid was undertaken to understand the organization of the intracellular DNA in this Crenarchaeote. Studies were also done on HSNP A to characterize its biochemical properties and analyze its DNA binding properties.

Objectives and scope of the present investigation

Intracelluiar DNA in eukaryotes is organized into chromatin with nucleosomal repeat structure formed by the interaction of DNA with basic proteins, histones. In contrast, in eubacteria like *E. coli* the DNA is in condensed state with no clear defined structural organization. In eubacteria, several histone-like proteins have been found associated with intracelluiar DNA with no nucleosomal organization.

Archaea constitute a separate domain of organisms sharing some properties with eukarya and eubacteria and some unique properties of their own. In euryarchaeota the primitive nucleosomal organization is seen in case of *M. fervidus* which has histone-like homologs, Hmf A and Hmf B which interact with DNA forming nucleosomal structure with 80 bp DNA repeat structure. Not much work has been carried out on the chromatin organization in crenarchaeota.

In the present investigation, work has been carried out on the isolation and biochemical characterization of the nucleoid from the crenarchaeote *S. acidocaldarius,* an organism which survives at extreme conditions of temperature (75-80 °C), pH (3.0), has a low G+C content (40%) and also lacks a cell wall.

The composition of nucleic acid and protein from the nucleoid was analyzed. The components involved in the structural organization of the nucleoid were determined and analyzed by employing various biochemical techniques. The results indicate that the nucleoid of *S. acidocaldarius* is similar to eubacteria. The nucleoid was found to occur in two forms, membrane associated form and membrane free nucleoid.

Important results of the present investigation are detection of S-layer protein in the membrane associated nucleoid, observance of abundant RNA which helps formation of nucleic acid protein aggregates and variations observed in the nucleoid organization and composition during stationary phase of growth.

Work has also been carried out on the solution state and DNA binding properties of nucleoid associated protein HSNP A. The results indicate that the protein binds both

single stranded and double stranded DNA. It interacts by coating the DNA strands starting from the free ends.

Further work is necessary to understand mode of interaction of S-layer proteins with nucleoid DNA and the role of RNA and other protein components in the condensed nucleoid structure. Analysis of the interaction between HSNP A and HSNP C' and the subsequent role in nucleoid condensation in vivo needs to be worked out.

Materials and Methods

MATERIALS AND METHODS:

Chemicals used:

Calf thymus DNA, ds DNA cellulose, NP-40, sucrose, spermidine-HCl, alumina, DNase I, RNase A, bovine serum albumin, cytochrome C, ovalbumin, coomassie brilliant blue R-250, ethidium bromide, dimethyl suberimidate, magnesium acetate, BNPS-skatole, pyridoxal phosphate and ampicillin were purchased from Sigma chemical company, U. S. A. Sephacryl S-1000, sephacryl S-200, sephadex G-50, acrylamide and Tris were purchased from Amersham Pharmacia, Sweden. Bisacrylamide and SDS were purchased from Serva Company, West Germany.

Yeast acid extract, tryptone and casaminoacid extract were purchased from Himedia Chemicals, Bombay. Glycine, sodium chloride, ammonium chloride, calcium chloride and urea were purchased from Qualigens, Glaxo laboratories. TEMED and Folin's phenol reagent were purchased from SRL, Bombay and mercaptoethanol from Spectrochem, Bombay.

Sulfolobus acidocaldarius strain DSM 639 was a gift from Prof Rolf Bernander, Dept of Microbiology, Uppsala University, Uppsala, Sweden.

Growth and harvesting:

Sulfolobus acidocaldarius was grown at 75°C for 40- 48 hrs with vigorous aeration in a medium containing 0.1 % yeast extract, 0.1 % tryptone, 0.1 % casein acid hydrolysate, 0.1 % glucose, 0.13 % ammonium sulfate, 0.02 % sodium chloride, 0.03 % potassium dihydrogen phosphate, 0.025 % magnesium sulfate, 0.007 % calcium chloride and pH 2.8 adjusted with sulfuric acid, as described by Kikuchi and Asai(1984). For harvesting, the culture was first neutralized with 1 M Tris base solution to arrest cell growth. The cells were harvested by centrifugation at 3000 rpm at 4°C for 15 minutes. The cell pellet was washed by resuspending it in 10 mM Tris-Cl pH 7.6, 50 mM KC1, 10 mM Magnesium acetate, 7 mM β-mercaptoethanol followed by centrifugation at 5000 rpm at 4°C

Nucleoid isolation:

Nucleoid was isolated as described by Reddy and Suryanarayana (1988) with some modifications. 2 g cell pellet was resuspended in 4 ml of buffer containing 10 mM Tris-Cl pH 7.6, 150 mM KCl. To this, 4 ml of lysis buffer containing 10 mM Tris-Cl pH 7.6, 1 % NP-40, 2 mM spermidine –HCl, 10 mM sodium EDTA pH 8 was added and incubated at 10°C for 1 hr. The lysate was centrifuged at 1500 rpm at 4°C for 10 minutes. The supernatant was collected and loaded on to a 30 % sucrose cushion and centrifuged at 10,000 rpm at 4°C for 1 hr. The nucleoid pellet was resuspended in 2-3 ml of buffer containing 20 mM Tris-Cl ph 7.6, 150 mM KC1, 2 mM sodium EDTA and 6 mM [3- mercaptoethanol.

MNase **digestion:**

This was performed following the procedure of Owen-Hughes and Workman (1996). Nucleoid (lμg) was incubated with 1 unit of MNase in 20mM Tris-Cl pH 8.8, 50mM NaCl, 50mM $MgCl_2$ and 1mM $CaCl_2$ at 37°C for 1 hour. Reaction was stopped by addition of SDS and EDTA to 1% and 25 mM final concentrations respectively as required.

Sephacryl S-200 column chromatography;

Sephacryl S-200 matrix was packed to a bed volume of 100ml in a column measuring 55 cm x 0.8 cm and equilibrated with nucleoid buffer. Nucleoid (2 ml) was digested with micrococcal nuclease (1unit/10μg DNA) as described above. The sample was dialyzed with 20 mM Tris-Cl ph 7.6, 150 mM KC1, 2 mM sodium EDTA and 6 mM (3- mercaptoethanol buffer and loaded onto the S-200 column equilibrated with the same buffer. Elution was done with 2 bed volumes of the buffer and 3 ml fractions were collected at 24 ml per hour. Fractions collected were analyzed by measuring absorbance at 260 and 280 nm, SDS-PAGE, agarose gel analysis of nucleoprotein complexes, DNA and RNA.

Sephacryl S-1000 column chromatography:

Sephacryl S-1000 matrix was packed to a bed volume of 100ml in a column measuring 55 cm x 0.8 cm and equilibrated with nucleoid buffer. Nucleoid (2 ml) was sonicated and clarified by centrifugation at 1500 rpm at 4°C for 10 mins and loaded

onto the S-1000 column. Elution was done with 2 bed volumes and 3 ml fractions were collected at 24 ml per hour. Fractions collected were analyzed by measuring absorbance at 260 and 280 nm. Fractions were also analyzed by protein estimation, DNA estimation, RNA estimation, SDS-PAGE analysis of protein, agarose gel electrophoresis for DNA and RNA content.

Sucrose density gradient centrifugation:

Peak I and peak II samples were concentrated and dialyzed against 10 mM Tris-Cl (pH 7.6), 1mM sodium EDTA, 50mM NaCl and 7 mM β-mercaptoethanol and 1ml of sample containing 2mg protein was loaded on to 30 ml 15-30% sucrose density gradient in buffer containing 10mM Tris-Cl (pH 7.6), 3mM $MgCl_2$. The samples were centrifuged in Hitachi ultra centrifuge at 40000 rpm for 16 hrs at 4°C. 1ml fractions were carefully collected from the top of the tubes with a pipette and A260 and A280 were measured. Fractions were analyzed by SDS-PAGE and agarose gel electrophoresis.

Formaldehyde fixation:

Formaldehyde fixation of nucleoid was carried out as described by Solomon and Varshavasky (1985). To the nucleoid isolated from 2 g cell pellet, a solution of 11 % formaldehyde, 0.1 M NaCl, 1 mM sodium EDTA was added to a final concentration of 1 % formaldehyde and incubated for 2 hrs at room temperature. Ammonium acetate was added to a final concentration of 0.4 M, to neutralize the formaldehyde. The nucleoid was centrifuged at 6000 rpm at 4°C for 15 mins. The pellet was washed once with and resuspended in nucleoid buffer.

RNase A digestion:

Nucleoid was digested with RNase A (1 µg per gm cells) by incubation at 37°C for 1 hr. Digestion was carried out in nucleoid buffer.

Isolation of S-layer:

S- layer was isolated as described by Grogan (1996). Cells harvested at mid logarithmic phase as were resuspended in buffer A containing 10mM NaCl, 1 mM PMSF, 20 mM magnesium sulfate and 0.5 % sodium lauroylsarcosine. 2µg DNase I

was added per ml suspension and incubated for 20 minutes at 45 °C. The suspension was centrifuged for 10 minutes at 14000 rpm, the supernatant was discarded and the pellet resuspended in buffer A and the above steps repeated. The white layer (S layer) on top of the pellet was resuspended carefully in buffer B containing 10 mM NaCl, 20 mM magnesium sulfate and 0.5 % SDS, incubated for 20 minutes at 45 °C and centrifuged at 14000 rpm for 10 minutes. The supernatant was discarded and the pellet resuspended in buffer B and the above steps were repeated. The white pellet was washed throughly with water and resuspended in water.

ds DNA cellulose column chromatography of S-layer proteins:

The S-layer sample (2 mg) was dialyzed against 20 mM Tris-Cl pH 7.6, I mM sodium EDTA and 150 mM KCl buffer and was loaded on to a 5 ml ds DNA cellulose column equilibrated with the same buffer. The column was washed with 150 mM and 300 mM KC1 containing buffers. 1 ml fractions were collected and analyzed by absorption at 280 nm and SDS-PAGE.

Isolation of ribosomes and post ribosomal supernatant fraction (S-100):

Isolation of ribosomes and post ribosomal supernatant fraction (S-100) was done according to Minks et al., (1978). Cells harvested at midlogarithmic phase were ground with double the weight of alumina until until soft and sticky, extracted with buffer (3 ml/ gm cells) containing 20 mM Tris- Cl pH 7.6, 50 mM KC1, 10 mM magnesium acetate, 7 mM β-mercaptoethanol. 2 µg DNase I (RNase free) was added per gm cells. The lysate was spun at 30,000 x g for 30 mins at 4°C to obtain cell extract (S-30). The S-30 was centrifuged at 1, 00,000 x g for 4 hrs to pellet the ribosomes. S-100 was collected and stored at -80°C. The crude ribosomal buffer was rinsed with above buffer and resuspended in the same buffer containing 1 M ammonium chloride and left overnight at 4°C. The ribosomes were pelleted by centrifugation at 1, 00,000 x g for 4 hrs at 4°C. The supernatant was collected and stored at -80°C. The ribosomal pellet was resuspended in 10 mM Tris-Cl ph 7.6, 50 mM NaCl, 10 mM magnesium acetate, 7 mM β-mercaptoethanol, 10 % glycerol and stored at -80°C.

Acid extraction of basic proteins:

Basic proteins were extracted from post ribosomal supernatant (S-100), 1 M ammonium chloride wash, and nucleoid. One tenth the volume of 2.7 M sulfuric acid was added drop wise to S-100 while stirring on ice. The sample was kept for stirring for 4 hrs on ice and then centrifuged at 10,000 x g at 4°C for 15 mins. The sample was then neutralized by dialysis against nucleoid buffer for 20 hrs and then clarified again by centrifugation at 10,000 x g at 4°C for 15 mins. The clear supernatant was dialyzed again for 20 hrs against nucleoid buffer; pH was checked and then stored at -20°C for future use.

Purification of HSNP A:

CM-52 column chromatography:

20 ml of acid treated S-100 was passed through CM-52 column of 30 ml bed volume (33 cm x 0.8 cm) equilibrated with 0.05 M KC1 containing 20 mM Tris-Cl pH 7.6, 1 mM sodium EDTA and 6 mM β-mercaptoethanol. The column was washed successively with 0.05 M, 0.1 M, 0.15 M, 0.3 M and 0.6 M KC1 containing buffers. 3 ml Fractions were collected and analyzed by absorption at 280 nm and SDS-PAGE.

dsDNA cellulose column chromatography;

The HSNP A containing fractions were pooled and dialyzed against 20 mM Tris-Cl pH 7.6, 1 mM sodium EDTA and 150 mM KC1. The sample was loaded on to a 5 ml ds DNA cellulose column equilibrated with the same buffer. The column was washed with 150 mM and 300 mM KC1 containing buffers. 1 ml fractions were collected and analyzed by absorption at 280 nm and SDS-PAGE.

Gel flltration chromatography of HSNP A:

Sephadex G-50 matrix was packed into a long thin column of 50 ml bed volume, equilibrated with 20 mM Tris-Cl pH 7.6, I mM sodium EDTA and 50 mM KC1 and 7 mM P-mercaptoethanol. HSNP A (500 µg) was loaded on to the column and eluted with the same buffer. Fractions (1ml) were collected and A 280 was measured. Fractions were analyzed by SDS - PAGE. Protein molecular weight markers, BSA (66 kDa), ovalbumin (44 kDa) and cytochrome C (13 kDa) were also chromatographed on

the same column. Elution volumes of these proteins were plotted on a graph to determine the molecular weight of HSNP A in its native state.

Crosslinking studies:

HSNP A crosslinking experiments were performed with formaldehyde and dimethylsuberimidate.

Crosslinking with formaldehyde:

HSNP A cross linking with formaldehyde was performed as described by Jackson (1978) with some modifications. HSNP A in 20 mM Triethanolamine-HCl pH 7.5 or 10 mM sodium acetate pH 5.0 was reacted with 200 mM HCHO at different temperatures (37°C, 55°C and 72°C) for 1 hour. Crosslinking was quenched by addition of TCA to 10% final concentration. Precipitates were collected by centrifugation at 10,000 rpm for 15 mins at 4°C after incubation on ice for 60 mins. Pellet collected was washed with acetone, dissolved in sample buffer containing 0.1% SDS but no β-mercaptoethanol and electrophoresed on 18% SDS-polyacrylamide gel.

Cross linking with dimethylsuberimidate:

HSNP A cross linking with dimethylsuberimidate was done as described by Thomas and Kornberg (1975). HSNP A in 20 mM Triethanolamine pH 8.0 was allowed to react with 0.2mg/ml DMS (in 20 mM TEA pH 8) at 37°C and 72°C for 24 hours. Crosslinking was quenched by addition of TCA to 10% final concentration. Precipitates were collected by centrifugation at 10,000 rpm for 15 mins at 4°C after incubation on ice for 60 mins. Pellet collected was washed with acetone, dissolved in sample buffer containing 0.1% SDS but no P-mercaptoethanol and electrophoresed on 18% SDS-polyacrylamide gel.

Flourescence titrations of HSNP A:

Flourescence titrations of all protein samples were carried out in a FP-77 Jasco spectroflourimeter. The measurements were performed in 10 mM Tris-Cl pH 7.6 and 20-50 mM NaCl at 30°C. Excitation and emission band widths were 5 nm and 10 nm respectively. After each addition of the lattice to the protein, the contents were mixed gently and allowed to stand for 30 seconds. The observations were recorded after the

fluorescence signal stabilized, an average of three readings within an interval of 60 seconds were taken for each titration point.

Thermal denaturation of DNA:

Thermal denaturation profile of calf thymus DNA in the absence (control) and presence of HSNP A were obtained by heating the samples in 500 µl of 20 mM Tris-Cl pH 7.6, 20 mM NaCl and 1mM EDTA in a Thermospectronic spectrophotometer equipped with a thermoprogrammer. Increase in OD at 260 nm was monitored and melting curves were recorded simultaneously. The rate of heating was 2 °C rise per minute. Prior to heating, HSNP A was added to DNA, gently mixed and incubated for 5 minutes at the starting temperature. Buffer blanks and protein solutions were heated separately to check for temperature dependent variations in absorbance which were found to be negligible. Effect of Magnesium on the melting of DNA in the presence of different amounts of protein was also studied.

Displacement of ethidium bromide bound to DNA by HSNP A:

Calf thymus DNA was added to 1 ml buffer containing 10 mM Tris-Cl pH 7.6 and 50 mM NaCl, in a cuvette containing 400 ng ethidium bromide and the fluorescence emission spectrum was recorded. Small aliquots of the protein were added to the reaction mixture, mixed gently and fluorescence emission spectra were recorded. Samples were excited at the absorption maxima of ethidium bromide, 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.

Gel mobility shift assay:

Gel mobility shift analysis of HSNP A- DNA complexes was done as described by Lohman et al., (1986). HSNP A-DNA complexes were allowed to form in 10 mM Tris- HC1 pH 7.6, 1mM sodium EDTA, 50 mM NaCl and 7 mM β-mercaptoethanol at different DNA to protein ratio. Samples were incubated at 37°C and 75°C for 10 - 30 minutes and analyzed by electrophoresis on a 0.8% agarose gel in TAE buffer (40 mM Tris-acetate pH 7.8 and 1 mM sodium EDTA). Gels were stained with 0.5 µg/ml ethidium bromide and visualized under UV illumination.

Chemical modification of HSNP A:

HSNP A was treated with modifying agents that selectively modify the amino acid residues to assess the amino acid functional groups involved in its interaction with DNA. Three modifications were carried out, two specific for lysine and one for tryptophan, the details of are given below.

Reductive methylation of lysine: The reductive methylation of lysine was carried out with sodium borohydride in the presence of formaldehyde (Means and Feeney, 1968). HSNP A (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 % formaldehyde were made at intervals of 5 minutes. At the end of the 30th minute, the reaction mixture was transferred to a dialysis tubing and dialyzed exhaustively against 10 mM Tris-Cl pH 7.6 containing 1 mM PMSF and stored frozen until further use.

Pyridoxal phosphate modification of lysine: This modification was performed according to the method of Ohsawa and Gualerzi, (1981). HSNP A (1 mg) in 1 ml of 20 mM Triethanolamine- HC1 pH 7, 10 mM KC1 was incubated with a final concentration of 1 mM pyridoxal phosphate for 12 minutes at 37 °C. At the end of the 12th minute, 0.5 mg equivalent of sodium borohydride 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 dialyzed against 10 mM Tris-Cl pH 7.6 containing 1 mM PMSF and stored frozen until further use.

BNPS-skatole modification for tryptophan: 2-(2-nitrophenylsulfenyl)-3-methyl-3-bromo indoline (BNPS-skatole) in 50 % acetic acid was the reagent used to modify the tryptophan residues of HSNP A according to Veronese (1972). The protein (1 mg/ml in 70 % acetic acid) was treated with BNPS-skatole (final concentration of 0.34 mg/ml) and stirred in the dark for 15 mins. The reaction was terminated with a final concentration of 0.04 % β-mercaptoethanol and the reaction mixture was dialyzed against 10 mM Tris-Cl pH 7.6 containing 1 mM PMSF.

Isolation of pBS supercoiled DNA:

pBS was isolated as described by Sambrook et al., (1994) from *E. coli* cells. LB plates containing 25 µg/ml ampicillin were streaked with *E. coli* containing pBS plasmid and grown for 24 hrs at 37 °C. A single colony was used to inoculate 25 ml LB medium, supplemented with 1 % glucose and 25 µg/ml ampicillin. The culture was grown with vigorous aeration, at 37 °C to OD 0.3 at 550 nm and used as inoculum. 250 ml LB media was inoculated with 1% of inoculum and grown at 37 ºC for 16 hrs. Cells were harvested by centrifugation at 5000 rpm for 10 minutes at 4 °C. The cell pellet was resuspended in 10 ml of ice cold solution I (50 mM glucose, 25 mM Tri-Cl pH 8.0, 10mM Na_2EDTA pH 8.0). To the cell suspension, 20 ml of freshly prepared solution II (0.2 N NaOH, 1% SDS) was added, mixed rapidly and incubated on ice for 10 minutes. Added 15 ml of ice cold solution III (3 M potassium acetate, 11.5 % glacial acetic acid) mixed gently and stored on ice for 3-5 minutes. The suspension was centrifuged at 12,000 g for 5 minutes at 4 °C and the supernatant was transferred to a fresh tube. Equal volume of phenol: chloroform: isoamylalcohol (24: 1: 1) was added, mixed gently and centrifuged at 12,000 g for 5 minutes at 4 °C. The aqueous phase was collected to it two volumes of absolute alcohol was added. DNA was left to precipitate overnight at -20°C. The DNA pellet was washed with 70 % ethanol and dissolved in TE buffer (10 mM Tris-Cl pH 8.0 and 1 mM sodium EDTA pH 8.0) containing 2 ug/ ml DNase free RNase stored at -80ºC.

Isolation of DNA:

DNA was isolated by phenol chloroform extraction. The salt concentration in the buffer was raised to 1 M. Equal volume of buffer saturated phenol was added to the samples, gently mixed, centrifuged at 10,000 rpm for 15 mins and the upper aqueous phase collected. The organic phase was re- extracted with buffer and the aqueous phases were pooled. The aqueous phase was extracted with phenol: chloroform: isoamylalcohol (24: 1: 1). The aqueous phase was collected and sodium acetate pH 5.0 was added to a final concentration of 2 mM and 2 volumes of absolute alcohol. DNA was left to precipitate overnight at -20°C. The DNA pellet was washed with 70 % ethanol and dissolved in TE buffer (10 mM Tris-Cl pH 8.0 and 1 mM sodium EDTA pH 8.0) containing 2 µg/ ml DNase free RNase and incubated at 30ºC for 1 hour and stored at -80°C.

Isolation of RNA:

RNA isolation was done by LiCl precipitation. The salt concentration of the sample was raised to 1M; equal volume of buffer saturated phenol was added, gently mixed and centrifuged at 10,000 rpm for 15 minutes. The upper aqueous phase was collected and the organic phase was re-extracted with buffer and aqueous phase was pooled. The aqueous phase was extracted with phenol: chloroform: isoamylalcohol (24: 1:1). Upper aqueous phase was collected and sodium acetate pH 5.0 was added to 2 mM and 2 volumes of absolute alcohol and kept at -80°C for 1 hour. The pellet was washed with 70 % ethanol and resuspended in TE buffer pH 8.0. Lithium chloride was added to 2 M, mixed gently and the sample was kept on ice in a cold room, overnight. RNA pellet was collected by centrifugation at 15000 rpm for 15 minutes and dissolved in TE buffer. Add 1.5 vols of 5 M potassium acetate and incubate for 5 hrs at -20°C. Added 2 volumes of absolute alcohol and incubated at -80°C for 1 hour and centrifuge at 15000 rpm for 15 minutes. Washed pellet with 70 % ethanol and resuspended in TE buffer. Stored at -80°C till further use.

Gel electrophoresis of RNA:

RNA was analyzed by electrophoresis on 10 % polyacrylamide gels in the presence of 7 M urea (Marzluff and Huang, 1984). A (30:1) acrylamide: bisacrylamide solution was used. The acrylamide, bisacrylamide, urea solution was made in 1 X TBE. The solution was degassed, polymerized by addition of TEMED and APS and poured. The sample was prepared by mixing 10 µl of RNA with 20 µl of 10 M urea, 0.1 X TBE, 0.01 % bromophenol blue, 0.01 % xylene cyanol and heating at 65°C for 1 hr prior to loading. The gel was run at 180 V for 2 hours in 1 X TBE buffer. The gel was then stained with 0.5 µg/ ml ethidium bromide for 30 mins followed by destaining in water for 1 hr and visualized by UV illumination.

Agarose gel electrophoresis:

DNA and the nucleic acid: protein aggregates were visualized by electrophoresis on 1 % agarose gels. Agarose was melted in 1 X TAE (40 mM Tris-acetate pH 7.8 and 1 mM sodium EDTA) buffer and the gel was cast. Sample was prepared by mixing the DNA with 6 X buffer containing 0.25 % bromophenol blue, 0.25 % xylene cyanol and 30 % glycerol to a final concentration of 1 X. Electrophoresis was done in 1 X TAE

buffer at 40 V till the bromophenol blue dye reached 3 quarters of the gel. The gel was stained with 0.5 μg/ml ethidium bromide for 30 mins, destained with water for 1 hour and visualized by UV illumination.

SDS- polyacrylamide gel electrophoresis:

SDS-PAGE was performed using 18 % gels as described by Thomas and Kornberg (1975). The ratio of acrylamide to bisacrylamide was 30:0.3. The 18 % separating gel was in 0.75 M Tris-Cl pH 8.8, 0.00 2M sodium EDTA and 0.1 % SDS and the stacking gel was of 5 % polyacrylamide in 0.08 M Tris –HCl pH 6.8, 0.002 M sodium EDTA and 0.1 % SDS. Protein samples were treated with 0.1% SDS and 1 % β-mercaptoethanol at 65° C for 15 mins. The slabs were run at 9 W cm for 4 hrs for mini gels and for 10 hrs for standard gels. Electrode buffer used was 0.05 M Tris, 0.38 M glycine and 0.1 % SDS.

Staining of polyacrylamide gels:

Coomassie blue staining:

Gels were fixed for 1 hour in 50:50:10 methanol: water: glacial acetic acid and then stained with coomassie blue R-250 (0.1 % in 50 % methanol, 7.5 % acetic acid) for 1 hour and destained in 5 % methanol, 7.5 % acetic acid.

Silver staining:

Gels were stained according to Blum and Gross (1987). Gels were fixed in 50 % methanol, 12.5 % acetic acid and 0.02% formaldehyde for 1 hour, rinsed with water and washed three times 50 % ethanol for 20 minutes each. Gels were rinsed with water, treated with 0.02 % hypo for 1 minute and washed with water three times for 20 seconds each. Gel was soaked in 0.2% silver nitrate solution containing 0.03 % formaldehyde for 20 minutes. Gel was rinsed with water and developed with 0.2% hypo, 6 % sodium carbonate, 0.02 % formaldehyde for 10 minutes. Reaction was terminated by addition of fixative. Gels were stored in 10 % methanol.

Schiff's reagent staining for glycoprotein detection:

Gels were fixed with fixative solution (40 % ethanol, 7 % acetic acid) for 30 minutes. Gels were washed four times, 30 minutes each wash, with fresh fixative solution. Fixation was continued overnight with fresh fixative solution. Next day, the gels were

washed twice, 30 minutes each wash with fresh fixative solution. Gels were immersed in oxidizing solution (1 % periodic acid, 3 % acetic acid) for 60 minutes. Oxidised gels were washed 10 times, 10 minutes per wash, to remove traces of periodic acid. Immersed the gels in Schiffs reagent (0.46 % pararosaniline and 0.73 % sodium metabisulfite in 0.1 N HCL) in the dark for 60 minutes. Washed the gels three times, 10 minutes per wash, with destaining solution (0.5 % potassium metabisulfite, 3 % acetic acid). Continued washing till all back ground is eliminated. Non specifically stained bands fade completely when stored over night in the dark in destaining solution. Glycoproteins are visible as red-purple bands.

Nucleic acid estimation:

Nucleic acid content was determined according to Morgan et al., (1979). Increasing amounts of calf thymus DNA (0.1 µg, 0.2 µg, 0.3 µg, 0.4 ug, 05 |xg) were added to 1 ml of buffer containing 0.5 µg/ml ethidium bromide, 5 mM Tris-HCl pH 8.1, 0.5 mM sodium EDTA and the fluorescence emission was measured at 600nm (excitation at 525 nm) with the maximum slit width in a JASCO Spectroflourimeter. 50 µl of the sample was added to 1 ml of buffer and the fluorescence was measured as before. The amount of nucleic acid in the sample was calculated from the standard graph.

DNA estimation:

DNA was estimated by diphenylamine method as described by Burton (1956). Increasing amounts of calf thymus DNA (10 µg, 20 µg, 40 µg, 80 ug, 120 µg, 160 µg) were taken in 2 ml water and mixed with 4 ml of diphenylamine reagent containing 0.1 % diphenylamine, 2.5 % sulfuric acid and 97.5 % glacial acetic acid. The samples were kept in a boiling water bath for 10 minutes, cooled and absorbance measured at 595 nm. 1 ml of the sample was diluted with 1 ml of water, mixed with 4 ml of DPA reagent and absorbance measured as before. The amount of DNA in the sample was calculated from the standard graph.

Protein estimation:

Protein concentration was estimated as described by Lowry et al., (1951) with folin-ciocalteau reagent using bovine serum albumin as a standard.

RNA estimation:

RNA was estimated by orcinol method as described by Ceriotti (1955). Increasing amounts of E.coli tRNA (10 µg, 20 µg, 40 µg, 80 µg, 120 µg, 160 µg) were taken in 2 ml water and mixed with 3 ml of orcinol reagent containing per 100ml, 0.1 g ferric chloride, 2.5 ml 6% w/v orcinol in alcohol and 97.5 ml concentrated hydrochloric acid. The samples were kept in a boiling water bath for 20 minutes, cooled and absorbance measured at 665 nm. 1 ml of the sample was diluted with 1 ml of water, mixed with 3 ml of orcinol reagent and absorbance measured as before. The amount of RNA in the sample was calculated from the standard graph.

Electron microscopy of HSNP A- ds DNA, HSNP A-HSNP C'- ds DNA complexes:

Electron microscopy of ds DNA complexes was carried out by Dr. Rudi Lurz, Max Planck Institute for Molecular Genetics, Berlin, Germany. The preparation of the nucleoprotein complexes and their processing was carried out exactly as described by Lurz et al., (1986). pUC 18 ds DNA was digested with *SmaI* and incubated with HSNP A, HSNP C and a mixture of the two proteins in binding buffer (10 mM TEA-Cl pH 7.5, 100 mM KCl, 1 mM EDTA, 5 mM DTE) at 60°C for 15 minutes, respectively. The samples were fixed with glutaraldehyde (0.1 % final concentration) for 15 minutes and adsorbed onto mica after addition of $MgCl_2$ (6.25 mM final concentration) for 1 minute, followed by 1 minute staining in 2 % aqueous uranyl acetate, washed twice in water and air dried.

Results

RESULTS

Biochemical characterization of thermoacidophilic archaeal nucleoid from *Sulfolobus acidocaldarius:*

Growth *of Sulfolobus acidocaldarius:*

Sulfolobus acidocaldarius was grown at 72°C as described in methods section. The growth was followed by measurement of absorbance at 650 nm after every 4 hr intervals (Fig. 1).

Isolation of nucleoid:

Nucleoid from *Sulfolobus acidocaldarius* was isolated according to Reddy and Suryanarayana, (1988) with some modifications. Freshly harvested cells (2 gm) grown up to midlogarithmic phase were gently lysed using non ionic detergents and the lysate was centrifuged through a 30 % sucrose cushion to separate the rapidly sedimenting DNA-protein complex, nucleoid (chromatin). The nucleoid pellet was dissolved in 20 raM Tris- Cl (pH 7.6), 150 raM KCl, 1mM Na_2EDTA and 6 mM β-mercaptoethanol (3ml/ 2gm cells). The absorption spectra of nucleoid isolated on 30 % sucrose cushion (Fig. 2) shows a high amount of protein as indicated by high absorption at 220-230 nm.

Purification of nucleoid by Sephacryl S-1000 column chromatography:

The nucleoid isolated on a 30% sucrose cushion was sheared by sonication and chromatographed on a Sephacryl S-1000 column to further purify it and separate out the membrane and membrane bound fractions if any from the nucleoid as described in the methods section. As seen in Fig. 3, nucleoid resolved into two peaks. Absorption spectra analysis (Fig 4a and 4b) indicates a higher protein to DNA ratio in peak I as compared to peak II. The total amount of protein, DNA and RNA is much higher in peak II than peak I (Fig 5a, 5b and 5c). Peak I has 350 ug of protein, 68 ug RNA and 16 (ig DNA per ml of peak fraction. Peak II has 310 µg protein, 544 µg RNA and 43 µg DNA per ml of peak fraction.

SDS-polyacrylamide gel electrophoresis of peak I and peak II fractions (Fig 6) showed presence of mostly high molecular weight proteins (> 60 kDa) in peak I and proteins ranging from high molecular weight to low molecular weight ~60 kDa to ~7 kDa in peak II. The low

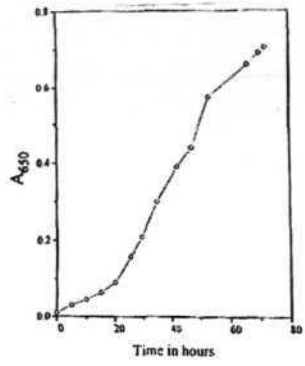


Fig. 1: Growth curve of *Sulfolobus acidocaldarius* strain dsm639

The bacteria were grown up to 75 hours at 72°C and the absorbance was measured at 650 nm.

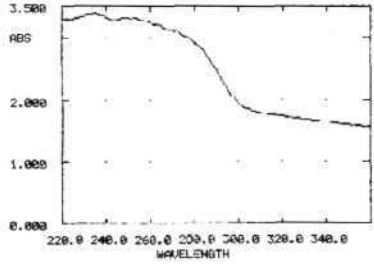


Fig 2: The absorption spectrum of nucleoid isolated on 30 % sucrose cushion.

Absorbance spectrum of nucleoid isolated on 30% sucrose cushion from exponential phase cells and resuspended in 20 mM Tris-Cl pH 7.6, 150 mM KC1, 1mM EDTA pH 8 and 6 mM β-mercaptoethanol buffer were recorded between 220 nm to 350 nm.

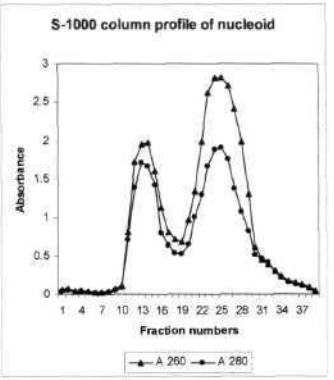
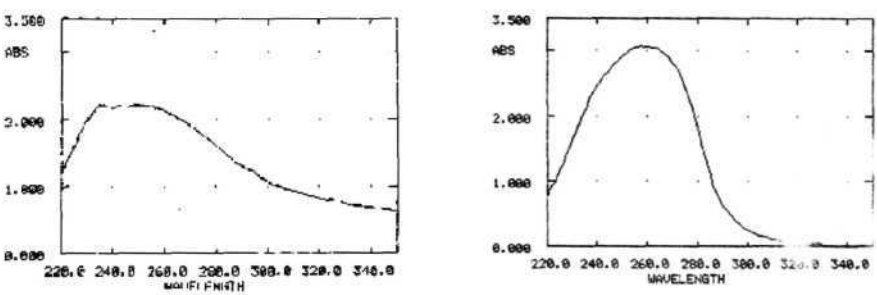


Fig 3: Sephacryl S-1000 column elution profile of *Sulfolobus acidocaldarius* nucleoid isolated from midlogarithmic phase cells.

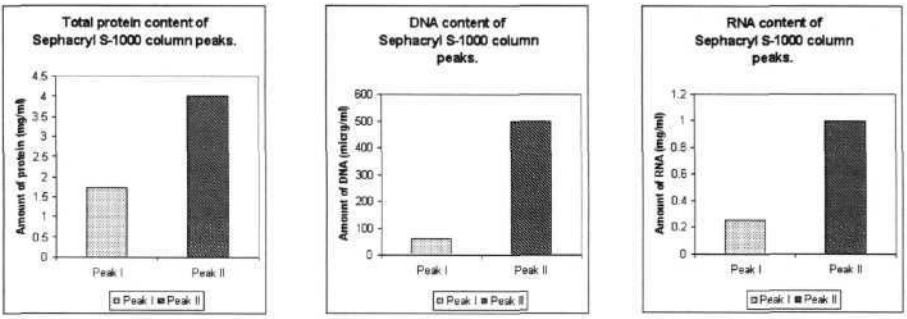
Absorbance at 260 and 280 nm of fractions obtained from the sephacryl S-1000 column chromatography of nucleoid isolated from cells harvested at mid logarithmic phase was measured.

Fig 4: The absorption spectra of peak I and peak II in 20 mM Tris-Cl pH 7.6, 150 mM KC1, lmM EDTA pH 8.0 and 6 mM β-mercaptoethanol buffer were recorded between 220 nm to 350 nm.



a: Absorption spectrum of peak I from sephacryl S-1000 column chromatography of nucleoid isolated from midlogarithmic phase cells.
b: Absorption spectrum of peak II from sephacryl S-1000 column chromatography of nucleoid isolated from midlogarithmic phase cells

Fig 5: The protein DNA and RNA content of the two peaks eluted from sephacryl S-1000 column chromatography of nucleoid from mid logarithmic phase cells.



a b c

a: Protein content of peak I and peak II.
b: DNA content of peak I and peak II.
c: RNA content of peak I and peak II

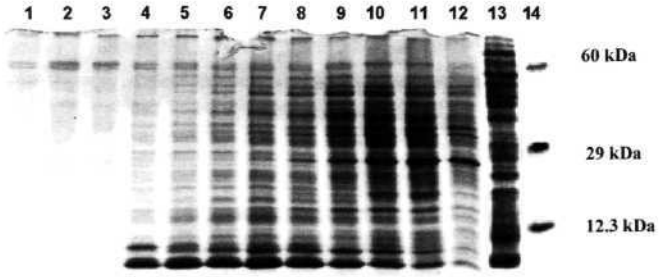


Fig 6: SDS-PAGE analysis of fractions obtained from sephacryl S-1000 column chromatography of nucleoid.

Nucleoid isolated from midlogarithmic phase cells was chromatographed on a sephacryl S-1000 column. Fractions (200 µl) were TCA precipitated and analyzed on an 18 % SDS-polyacrylamide gel followed by coomassie blue staining.
Lane 1-3: Peak 1 fractions 12, 13 and 14 respectively.
Lane 4-12: Peak II fractions 21 to 29 respectively.
Lane 13: Nucleoid (10 µl).
Lane 14: Molecular weight markers (BSA (60 kDa), carbonic anhydrase (29 kDa), cyt C (12.3 kDa).

molecular weight proteins, HSNP A, DBNP B (Sac 10b) and HSNP C' (Sac 7d) are present in abundance.

Nucleoprotein complexes from sephacryl S-1000 column nucleoid fractions were analyzed by agarose gel electrophoresis (Fig 7a) high molecular weight aggregates which were retained in the wells of the gels. Peak II initial fractions show a smear ranging from 5 kbp to 1 kbp. Later fractions show 500 bp and 1 kbp bands along with a smear ~ 10 kbp to 2 kbp. End fractions show a 500 bp band and a smear from 15 kbp to 4 kbp.

Nucleic acids extracted from peak I and peak II were analyzed by agarose gel electrophoresis (Fig 7b) fractions migrate as a smear along with E.coli tRNA. In the fractions of peak I the nucleic acids migrated as a smear of 1 kbp to 3 kbp. High molecular weight nucleic acids are retained in the wells. Peak II fractions migrated as a smear of 200 bp to ~ 2 kbp. Slower migration of nucleic acids as seen in Fig 7b is due to complex formation of nucleic acids with proteins.

Fractions from S-1000 column were treated with RNase before analyzing by agarose gel electrophoresis to assess the nature of DNA in the fractions. DNA from the initial peak I fractions is retained in the wells whereas in the later fractions it migrates as a smear in the ~1 kbp to 3 kbp range (Fig 7c). The initial peak II fractions contained a smear of DNA bands at ~ 500 bp, 1.5 kbp and close to the well (~ 70 kbp) are observed. Part of sample is also retained in the wells. In the later fractions of peak II DNA was mostly of 1 kbp to ~500 bp DNA is observed.

S-1000 column fractions were treated with RNase free DNase I to visualize the RNA present in the nucleoid. As seen in Fig 7d, initial fractions of peak I had RNA that migrates slower than E.coli tRNA. Peak II fractions also contained similar RNA species ranging from ~500 bp. Additional bands seen above this range and in the wells of the gel could be due to the DNA which had not been digested completely and/or high molecular weight RNA.

The results presented indicate that peak II from the sephacryl S-1000 column chromatography of nucleoid has DNA, RNA and associated proteins and forms the major part of the nucleoid and peak I consisting of highly aggregated complex of protein, DNA and RNA the minor part of the nucleoid.

Fig 7: Agarose gel electrophoresis of the nucleoprotein complex, nucleic acids, DNA and RNA of nucleoid, peak I and peak II.

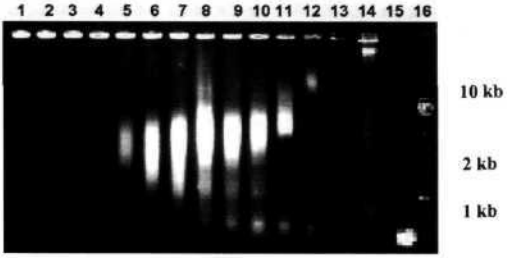


Fig 7a: Nucleoprotein complex profile of nucleoid chromatographed on sephacryl S-1000 column

Fractions (500 µl) from the sephacryl S-1000 column chromatography of nucleoid isolated from the mid logarithmic phase cells were analyzed on a 1.4 % agarose gel.
Lane 1-4: Peak I fractions 12-15.
Lane 5-13: Peak II fractions 20-28.
Lane 14: Nucleoid
Lane 15: *E. coli* tRNA.
Lane 16: Molecular weight markers (1 kb ladder).

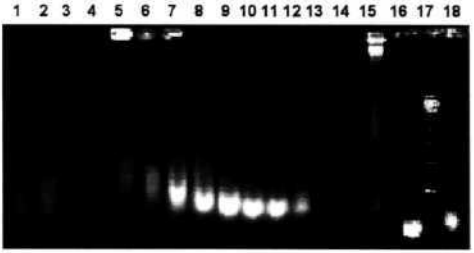


Fig 7b: Nucleic acid profile of nucleoid chromatographed on sephacryl S-1000 column.

Fractions (500 µl) from the sephacryl S-1000 column chromatography of nucleoid isolated from the mid logarithmic phase cells were phenolized precipitated with ethanol and analyzed on a 1.4 % agarose gel.
Lane 1-4: Peak I fractions 12-15.
Lane 5-14: Peak II fractions 20-29.
Lane 15: Nucleoid
Lane 16: *E.coli* tRNA.
Lane 17: Molecular weight markers (1 kb ladder)
Lane 18: *S. acidocaldarius* ribosomes.

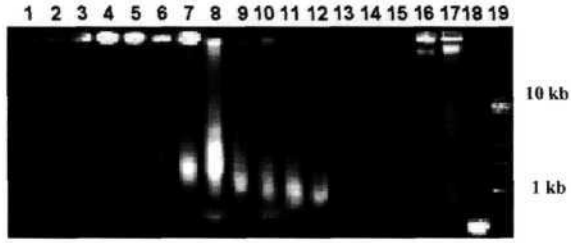


Fig 7c: DNA profile of nucleoid chromatographed on sephacryl S-1000 column.

Fractions (500 µl) from the sephacryl S-1000 column chromatography of nucleoid isolated from the mid logarithmic phase cells were phenolized, precipitated with ethanol, resuspended in TE (pH 8.0) buffer containing RNase and analyzed on a 1.4 % agarose gel.
Lane 1-4: Peak I fractions 12-15.
Lane 5-14: Peak II fractions 20-29.
Lane 15: Nucleoid
Lane 16: *E.coli* tRNA.
Lane 17: Molecular weight markers (1 kb ladder)
Lane 18: *S. acidocaldarius* ribosomes.

Fig 7d: RNA profile of nucleoid chromatographed on sephacryl S-1000 column.

Fractions (500 µl) from the sephacryl S-1000 column chromatography of nucleoid isolated from the mid logarithmic phase cells were phenolized, precipitated with ethanol, resuspended in TE (pH 8.0) buffer containing DNase and analyzed on a 1.4 % agarose gel.
Lane 1-4: Peak I fractions 12-15.
Lane 5-14: Peak II fractions 20-29.
Lane 15: Nucleoid RNA
Lane 16: Nucleoid DNA
Lane 17: Nucleoid
Lane 18: E.coli tRNA.
Lane 19: Molecular weight markers (1kb ladder).

Sucrose density gradient analysis of sephacryl S-1000 column fractions:

In order to ascertain whether RNA detected in the nucleoid preparation is a component of the nucleoid or is a part of ribonucleoprotein complex associated with the genomic DNA peak I and peak II were analyzed by sucrose density gradient centrifugation.

Peak I and peak II components of the nucleoid were pooled, concentrated and resolved on a 15-30 % sucrose density gradient as described in the methods section. Peak I fractions were pooled as the early peak I fractions pool and the later peak I fractions pool.

Sucrose density gradient analysis of sephacryl S-1000 column early peak I fractions pool:

The A260/A280 absorbance profile of the early peak I fractions pool (Fig 8a) shows an almost uniform distribution along the gradient. The pellet and the fractions at around 22% sucrose show a comparatively higher OD.

As seen from the SDS-PAGE analysis of the fractions collected from the gradient (Fig 8b) the gradient top has high molecular weight proteins ~70 kDa to 50 kDa, lanes 1-3, followed by fractions containing abundant HSNP A and HSNP C along with proteins ~ 70 kDa, 50 kDa and 45 kDa, lanes 4-6. The middle fractions of the gradient have abundant proteins in the ~70 kDa to 45 kDa range, lane 7-10. The amount of HSNP A and HSNP C in these fractions is reduced. The bottom fractions of the gradient show proteins in the ~50 kDa range, lanes 11 to 21. The pellet from the gradient has the ~70 kDa protein in abundance along with the ~50 kDa proteins which may be the smaller sub unit of S-layer protein.

As seen in Fig 8c all of the nucleic acid of the early fractions of peak I pool was in the pellet. No DNA or RNA is evident in any other part of the gradient.

These results show that the early fractions of the sephacryl S-1000 column peak I contain high molecular weight DNA in association with RNA, loosely associated with high molecular weight proteins. Histone-like proteins, DBNP B, HSNP A and HSNP C are also present but are not tightly associated with the high molecular weight DNA component. Only the smaller S-layer protein is present along with DNA.

Fig 8: Sephacryl S-1000 column peak I pool (early fractions) analyzed on 15-30% Sucrose density gradient.

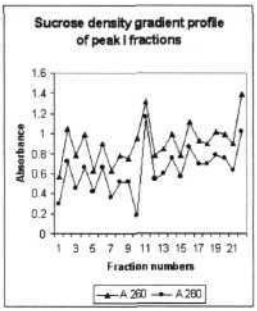


Fig 8a: Sephacryl S-1000 column peak I (early fractions) pool 15-30 % sucrose density gradient profile.

Sephacryl S-1000 peak I fractions were pooled and 100 µg of the protein was fractionated on a 15-30 % sucrose density gradient. 1 ml fractions were collected of the gradient and absorbance at 260 and 280 nm was observed.

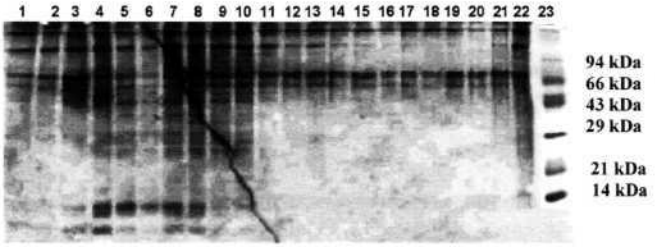


Fig 8b: Protein profile of peak I (early fractions) from nucleoid chromatographed on sephacryl S-1000 resolved on a 15-30% sucrose density gradient.

Peak I fractions 100µg (protein) was resolved on a 15-30% sucrose density gradient as described in the methods section. Fractions (70 µl) from the gradient were electrophoresed on an 18 % SDS polyacrylamide gel and visualized by coomassie blue staining.
Lane 1 -20: Fraction numbers 1 to 20 respectively.
Lane 21: Pellet from gradient.
Lane 22: Nucleoid.
Lane 23: Molecular weight markers.

As seen in Fig 9a, the later fractions of peak I are distributed all along the 15-30% sucrose density gradient. The SDS-PAGE profile as seen in Fig 9b has the 70 kDa protein in abundance in the fractions from the top part of the gradient along with small amounts of HSNP A, DBNP B and HSNP C. The pellet from the gradient has proteins ranging from ~ 70 kDa to 40 kDa along with HSNP A, *C* and DBNP B.

Fractions which show the histone like proteins in the SDS-PAGE profile are seen to have high molecular weight nucleic acid component which is retained in the lanes during agarose gel electrophoresis (Fig 9c). The pellet fraction also is retained in the well.

Sucrose density gradient analysis of sephacryl S-1000 column peak II fractions pool:

The absorbance profile of peak II fractions resolved on the 15-30 % sucrose gradient (Fig 10a) shows most of the sample to be retained at the top. Absorbance decreases sharply towards the middle and gradually till the bottom.

SDS-PAGE analysis of the gradient fractions (Fig 10b) shows an abundance of proteins in the ~ 7 kDa to ~ 60 kDa range. Histone like proteins HSNP C'(Sac 7d) and DBNP B (Sac 10b) are also present in abundance. The middle of the gradient mainly shows proteins in the ~ 70 kDa range. The gradient pellet has the ~70 kDa, ~ 60 kDa, ~ 45 kDa proteins along with HSNP C' and DBNP B.

Agarose gel analysis of the nucleic acid content of the gradient fractions (Fig 10c) shows the initial fractions to contain low molecular weight nucleic acids similar to the RNA observed in the sephacryl S-1000 column fraction analysis (Fig 7e). The pellet from the gradient has the same profile as the nucleoid, but lacks the high molecular weight band which occurs close to, or is retained in, the well.

The results obtained indicate that peak II comprises the nucleoid core and has most of the genomic DNA (in fragments) and RNA along with their associated proteins. A few small RNA molecules and their associated proteins also present could be either fragments of the core disrupted during handling of the sample or could constitute a separate component of peak II.

Fig 8c: Nucleic acid profile of peak I (early fractions) from nucleoid chromatographed on sephacryl S-1000 resolved on a 15-30% sucrose density gradient.

Peak I fractions 100µg (protein) was resolved on a 15-30% sucrose density gradient as described in the methods section. Fractions (200 µl) from the gradient were phenolized, the nucleic acid precipitated with alcohol, electrophoresed on a 1.4% agarose gel and visualized by staining with ethidium bromide.
Lane 1-16: Fraction numbers 1 to 16 respectively.
Lane 17: Pellet from gradient.
Lane 18: Nucleoid.
Lane 19: E.coli tRNA.
Lane 20: Molecular weight markers (1 kb ladder).

Fig 9: Sephacryl S-1000 column peak I pool (later fractions) analyzed on 15-30% Sucrose density gradient.

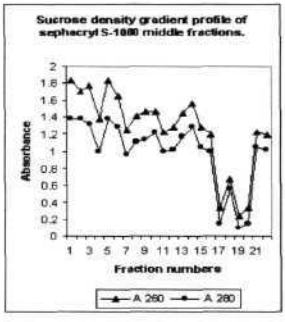


Fig 9a: Sephacryl S-1000 column peak I (later fractions) pool 15-30 % sucrose density gradient profile.

Sephacryl S-1000 fractions between peak I and peak II were pooled and 100 µg of the protein was fractionated on a 15-30 % sucrose density gradient. 1 ml fractions were collected of the gradient and absorbance at 260 and 280 nm was observed.

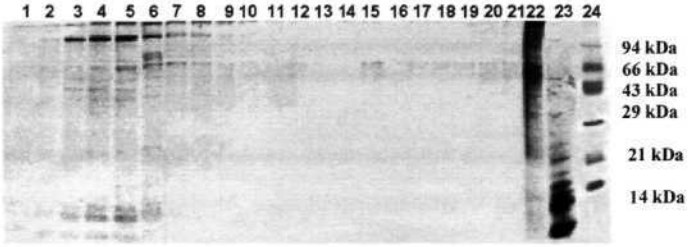


Fig 9b: Protein profile of peak I (later fractions) from nucleoid chromatographed on sephacryl S-1000 resolved on a 15-30% sucrose density gradient.

100µg protein was resolved on a 15-30% sucrose density gradient as described in the methods section. Fractions (70 µl) from the gradient were electrophoresed on an 18 % SDS polyacrylamide gel and visualized by coomassie blue staining.
Lane 1-21: Fraction numbers 1 to 21 respectively.
Lane 22: Pellet from gradient.
Lane 23: Nucleoid.
Lane 24: Molecular weight markers.

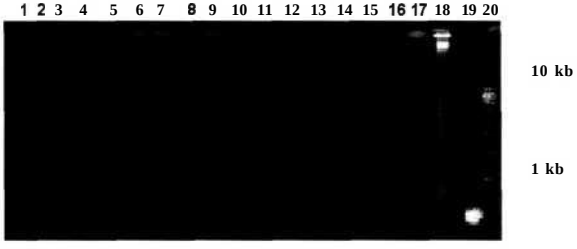


Fig 9c: Nucleic acid profile of peak I (later fractions) from nucleoid chromatographed on sephacryl S-1000 resolved on a 15-30% sucrose density gradient.

100µg protein was resolved on a 15-30% sucrose density gradient as described in the methods section. Fractions (200 µl) from the gradient were phenolized, the nucleic acid precipitated with alcohol, electrophoresed on a 1.4% agarose gel and visualized by staining with ethidium bromide.
Lane 1-16: Fraction numbers 1 to 16 respectively.
Lane 17: Pellet from gradient.
Lane 18: Nucleoid
Lane 19: E.coli tRNA.
Lane 20: Molecular weight markers (1 kb ladder).

Fig 10: Sephacryl S-1000 column peak II pool analyzed on 15-30% Sucrose density gradient.

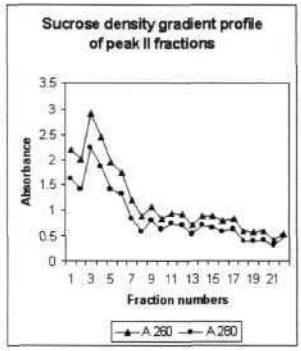


Fig 10a: 15-30 % sucrose density gradient profile of Sephacryl S-1000 column peak II pool.

Sephacryl S-1000 peak II fractions were pooled and 100 µg of the protein was fractionated on a 15-30 % sucrose density gradient. 1 ml fractions were collected of the gradient and absorbance at 260 and 280 nm was observed.

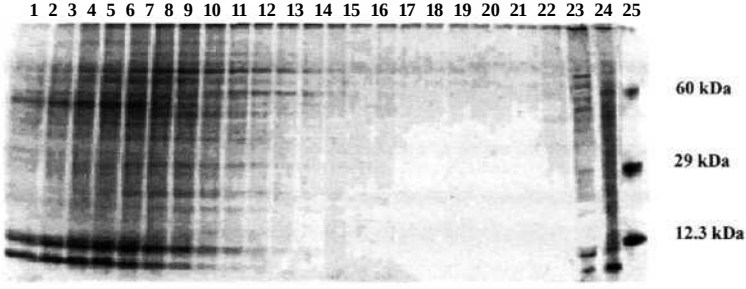


Fig 10b: Protein profile of peak II from nucleoid chromatographed on sephacryl S-1000 resolved on a 15-30% sucrose density gradient.

100µg protein was resolved on a 15-30% sucrose density gradient as described in the methods section. Fractions (70 µl / 140ul from pellet fraction) from the gradient were electrophoresed on an 18 % SDS polyacrylamide gel and visualized by coomassie blue staining.
Lane 1-22: Fraction numbers 1 to 22 respectively.
Lane 23: Pellet from gradient.
Lane 24: Nucleoid.
Lane 25: Molecular weight markers (BSA (60 kDa), carbonic anhydrase (29 kDa), cyt C (12.3 kDa).

RNase digestion of nucleoid:

As seen from fig 7d, RNA is present in substantial amounts in the nucleoid and also apparently plays a role in formation of the high molecular weight aggregates seen at the top of both SDS page and agarose gels. To determine the role played by RNA in the elution profile of the nucleoid on sephacryl S-1000 column, the nucleoid was digested with RNase, as described in the methods section, prior to sephacryl S-1000 column chromatography.

The elution profile of the RNase digested nucleoid on sephacryl S-1000 column (Fig 11a) showed greater resolution of peak I. The peak II showed a shift in the elution pattern and eluted out much later (in the second bed volume).

Peak I of the RNase digested nucleoid has proteins ranging from ~60 kDa to 7 kDa along with histone like proteins which were previously not detected in the untreated nucleoid (Fig 11b) are now visible. High molecular weight aggregates at the top of the gels were also not observed. Level of ~ 70 kDa protein is markedly reduced. Peak II protein profile does not show any variation.

Analysis of nucleoprotein complexes present in the sephacryl S-1000 peak I and peak II fractions (Fig 11c) show peak I fractions to have high molecular weight aggregates retained in the wells in the initial fractions. A smear ~3 kbp to 6 kbp along with a small amount of sample retained in the wells is detected in the later fractions. Peak II fractions range from 1.2 kbp to 10 kbp, a small amount of sample is also retained in the wells.

Formaldehyde cross linking of nucleoid:

Nucleoid sample was treated with formaldehyde to produce DNA-protein, RNA-protein and protein-protein cross-links in the genomic DNA and the RNA and proteins associated with it. Formaldehyde was used as the cross linking reagent since it crosslinks only the proteins which are bound to DNA/RNA. It has no reactivity with the free ds DNA.

Fig 10c: Nucleic acid profile of peak II from nucleoid chromatographed on sephacryl S-1000 resolved on a 15-30% sucrose density gradient.

100µg protein was resolved on a 15-30% sucrose density gradient as described in the methods section. Fractions (200 µl / 400µl from pellet fraction) from the gradient were phenolized, the nucleic acid precipitated with alcohol, electrophoresed on a 1.4% agarose gel and visualized by staining with ethidium bromide.
Lane 1-15: Fraction numbers 1 to 15 respectively.
Lane 16: Pellet from gradient
Lane 17: Nucleoid.
Lane 18: E.coli tRNA.
Lane 19: Molecular weight markers (1kb ladder).

Fig 11: Sephacryl S-1000 column chromatography of *Sulfolobus acidocaldarius* nucleoid isolated from cells at exponential phase and digested with RNase.

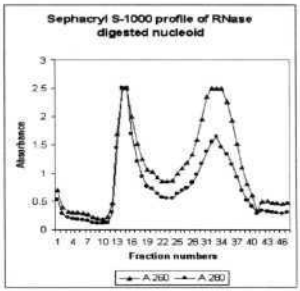


Fig 11a: Sephacryl S-1000 column elution profile of *Sulfolobus acidocaldarius* nucleoid isolated from cells at exponential phase and digested with RNase.

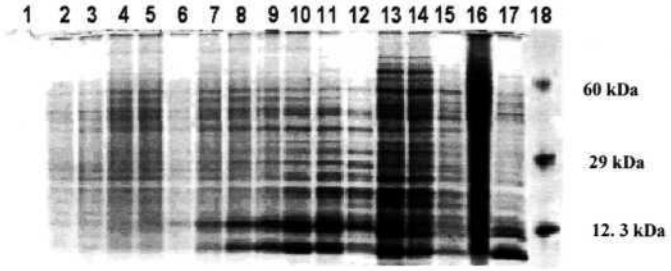


Fig 11b: SDS-PAGE analysis of peak fractions from sephacryl S-1000 column chromatography of nucleoid digested with RNase analyzed by 18% SDS-PAGE
Fractions (200 µl) were TCA precipitated and electrophoresed on an 18 % SDS-polyacrylamide gel followed by coomassie blue staining.
Lane 1-6: Peak I fractions 14-19 respectively.
Lane 7-15: Peak II fractions 25-33 respectively.
Lane 16: Nucleoid.
Lane 17: Nucleoid acid extract.
Lane 18: Molecular weight markers (BSA (60 kDa), carbonic anhydrase (29 kDa), cyt C (12.3 kDa).

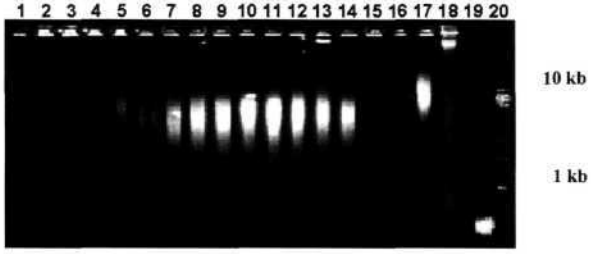


Fig 11c: Nucleoprotein complex profile of nucleoid digested with RNase at exponential phase and chromatographed on sephacryl S-1000 column
Fractions (50 µl) from the sephacryl S-1000 column chromatography of nucleoid isolated from the mid logarithmic phase cells and digested with RNase were analyzed on a 1.4 % agarose gel.
Lane 1-6: Peak I fractions 14-19 respectively.
Lane 7-16: Peak II fractions 24-33 respectively.
Lane 17: Nucleoid digested with RNase.
Lane 18: Nucleoid
Lane 19: E.coli tRNA.
Lane 20: Molecular weight markers (1kb ladder).

Sephacryl S-1000 column chromatography was performed with nucleoid cross linked with formaldehyde and nucleoid cross linked with formaldehyde followed by digestion with RNase.

A. Sephacryl S-1000 column chromatography of nucleoid crosslinked with formaldehyde

The sephacryl S-1000 column elution profile of formaldehyde cross linked nucleoid (Fig 12a) showed a small shoulder at peak I and a broad extended peak at peak II resulting in the merger of peak I and peak II. As seen from the SDS-PAGE analysis of peak fractions (Fig 12b), the initial fractions of peak I have no significant protein content. Peak II from formaldehyde treated nucleoid has all the protein components observed in untreated sample along with a significant band seen at the top/start of the resolving gel indicating formation of cross linked nucleoprotein complex.

The formaldehyde crosslinking is complete, as is evident by the complete retention of the treated nucleoid sample in the well. Phenolization resulted in the precipitation of nucleic acids along with the proteins at the interface hence only the nucleoprotein complexes of the column fractions were analyzed. As seen in Fig 12c the all fractions from peak I and peak II are retained in the wells.

Crosslinking of the nucleoid components (corresponding to peak I and peak II) prior to chromatography on sephacryl S-1000 column confirms both peaks to be components of the nucleoid which are associated in solution and not artifacts resulting from handling during sample preparation.

B. Sephacryl S-1000 column chromatography of nucleoid cross linked with formaldehyde followed by digestion with RNase.

The RNase digestion of formaldehyde crosslinked nucleoid prior to Sephacryl S-1000 column chromatography yielded the characteristic double peak profile (Fig 13a). The elution profile is exactly the same as observed for the RNase digested nucleoid with out cross linking (Fig 11a).

Fig 12: Sephacryl S-1000 column elution of formaldehyde fixed nucleoid

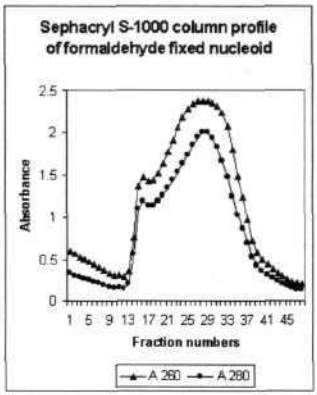


Fig 12a: Sephacryl S-1000 column elution profile of *Sulfolobus acidocaldarius* nucleoid fixed with formaldehyde at exponential phase.

Nucleoid isolated from cells harvested at mid logarithmic phase was fixed with formaldehyde and chromatographed on a sephacryl S-1000 column. Absorbance at 260 and 280 nm of fractions obtained was measured.

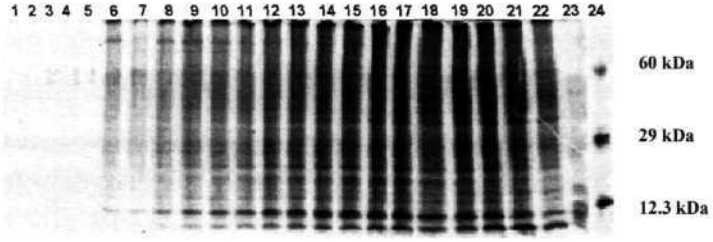


Fig 12b: SDS-PAGE analysis of peak fractions from sephacryl S-1000 column chromatography of formaldehyde fixed nucleoid.

Fractions (200 µl) were TCA precipitated and electrophoresed on an 18 % SDS-polyacrylamide gel followed by coomassie blue staining.
Lane 1-6: Peak I fractions 14-19 respectively.
Lane 7-22: Peak II fractions 20-35 respectively.
Lane 23: Nucleoid acid extract.
Lane 24: Molecular weight markers (BSA (60 kDa), carbonic anhydrase (29 kDa), cyt C (12.3 kDa).

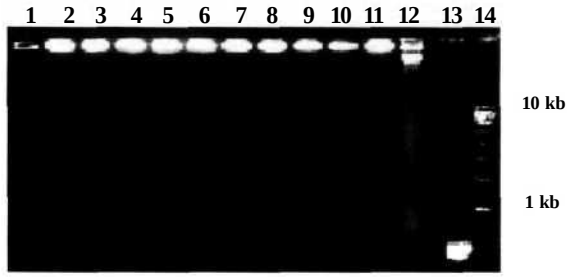


Fig 12c: Nucleoprotein complex profile of formaldehyde fixed nucleoid chromatographed on sephacryl S-1000 column

Fractions (50 µl) from the sephacryl S-1000 column chromatography of nucleoid isolated from the mid logarithmic phase cells and fixed with formaldehyde were analyzed on a 1.4 % agarose gel.

Lane 1: Peak I fraction 18

Lane 2-10: Peak II fraction numbers 20, 22, 24, 26, 28, 29, 31, 33 and 35 respectively.

Lane 11: Formaldehyde fixed nucleoid

Lane 12: Nucleoid.

Lane 13: E.coli tRNA.

Lane 14: Molecular weight markers (1 kb ladder).

Fig 13: Sephacryl S-1000 column elution of formaldehyde fixed nucleoid digested with RNase

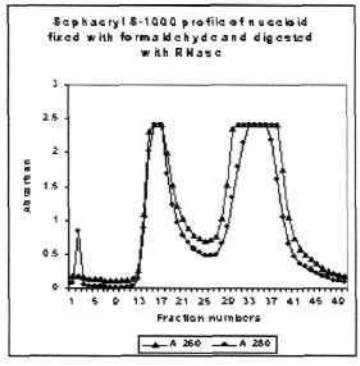


Fig 13a: Sephacryl S-1000 column elution profile of *Sulfolobus acidocaldarius* nucleoid fixed with formaldehyde at exponential phase and digested with RNase.

SDS-PAGE analysis of the column fractions, Fig 13b, showed aggregated complexes at the top of the gel in all fractions. The peak I profile has DBNP B and HSNP C'along with high molecular weight proteins.

Peak I and peak II are distinct components of the nucleoid and RNA plays a role in the occurrence of and in stabilizing these components.

Micrococcal nuclease digestion of nucleoid:

Nucleoid was digested with micrococcal nuclease as described in methods section. As seen in fig 14, both RNA and DNA are present in the nucleoid. The high molecular weight complex retained in the wells was found to have RNA as one of its components. Low molecular weight RNA similar to the E.coli tRNA is also present.

Isolation of MNase digested products of nucleoid by S-200 chromatography:

Sephacryl S-200 column chromatography profile of MNase digested nucleoid has a distinct peak in the initial fractions and a small peak in the later fractions (Fig 15a).
SDS-PAGE analysis (Fig 15b) shows the initial fractions to comprise high molecular weight proteins ~ 60 to 45 kDa and a few proteins of ~ 30 to 20 kDa. DBNP B is also present along with a small amount of HSNP A. The later fractions mainly comprise HSNP C along with HSNP A. A few proteins in the range of ~45 kDa, ~29 kDa and ~20 kDa are also present.
As evident from agarose gel analysis (Fig 15c) the initial fractions of the sephacryl S-200 column comprise nucleoprotein complexes ~500 bp to 1.5 kbp. The later fractions, rich in HSNP C however show very small amount of nucleic acids.

MNase digestion releases HSNP A and HSNP C' from the rest of the nucleoid leaving the undigested nucleoprotein complexes in the first peak.

Electron microscopic studies with HSNP C had shown it to form clusters with DNA i.e., central protein DNA cores with small loops of free DNA. HSNP C' also shows a strong affinity to HSNP A. HSNP C' could therefore be expected to form similar structures in vivo in the nucleoid.

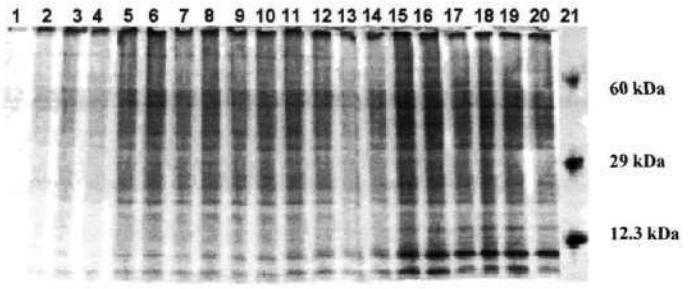


Fig 13b: SDS-PAGE analysis of peak fractions from sephacryl S-1000 column chromatography of formaldehyde fixed nucleoid digested with RNase analyzed by 18% SDS-PAGE

Fractions (200 |il) were TCA precipitated and electrophoresed on an 18 % SDS-polyacrylamide gel followed by coomassie blue staining.
Lane 1-6: Peak I fractions 14-19 respectively.
Lane 7-20: Peak II fractions 20-33 respectively.
Lane 21: Molecular weight markers (BSA (60 kDa), carbonic anhydrase (29 kDa), cyt C (12.3 kDa).

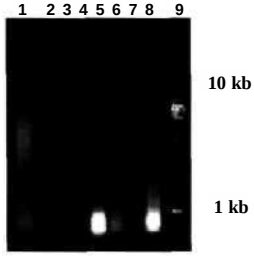


Fig 14: Effect of **Micrococcal nuelease digestion on *S.acidocaldarius* nucleoid.**

Nucleoid (2 µg) was digested with 1 U of MNase and/or 2 µg of RNase and the nucleoprotein complexes and the protein free nucleic acids were analyzed on a 0.8 % agarose gel as described.
Lane 1-3: Nucleo-protein complexes from, nucleoid - digested with MNase, MNase and RNase, only RNase.
Lane 4: Crude nucleoid (control).
Lane 5-7: Deproteinised sample from nucleoid - digested with MNase, MNase and RNase, and only RNase.
Lane 8: Deproteinised nucleoid (control).

Fig 15: Sephacryl S-200 column elution of MNase digested nucleoid.

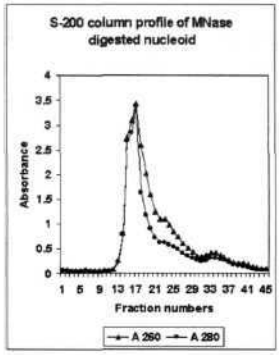


Fig 15a: Sephacryl S-200 column elution profile of *Sulfolobus acidocaldarius* nucleoid digested with micrococcal nuclease at exponential phase.

Absorbance at 260 and 280 nm of fractions obtained from the sephacryl S-200 column chromatography of nucleoid isolated from cells harvested at mid logarithmic phase and digested with micrococcal nuclease was measured.

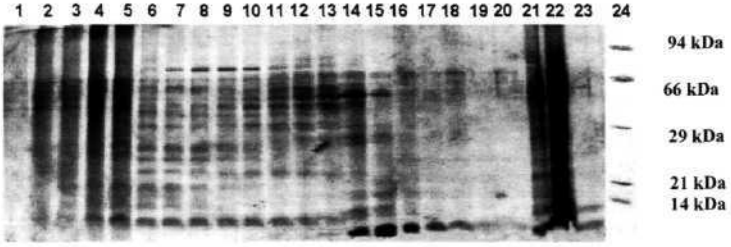


Fig 15b: SDS-PAGE analysis of fractions obtained from sephacryl S-200 column chromatography of nucleoid digested with micrococcal nuclease.

Nucleoid isolated from midlogarithmic phase cells was digested with micrococcal nuclease and chromatographed on a sephacryl S-200 column. Fractions (200 µl) were TCA precipitated and analyzed on an 18 % SDS-polyacrylamide gel followed by coomassie blue staining.
Lane 1-13: Fraction numbers 14-26 respectively.
Lane 14-20: Fraction numbers 28, 30, 32, 33, 34, 35 and 36 respectively.
Lane 21: MNase digested nucleoid.
Lane 22: Crude nucleoid.
Lane 23: Nucleoid acid extract.
Lane 24: Molecular weight markers.

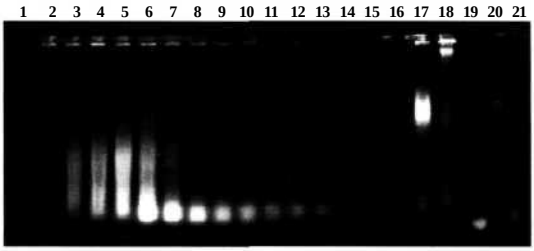


Fig 15c: Nucleoprotein complex profile of nucleoid digested with MNase chromatographed on sephacryl S-200 column

a. Fractions (500 µl) from the sephacryl S-200 column chromatography of nucleoid isolated from the mid logarithmic phase cells and digested with MNase were analyzed on a 0.8 % agarose gel.
Lane 1-15: Fraction numbers 14-26, 28 and 30 respectively.
Lane 16: Molecular weight markers
Lane 17: Nucleoid digested with MNase.
Lane 18: Nucleoid.
Lane 19: *E.coli* tRNA.
Lane 20: Molecular weight markers (1 kb ladder, 10 to 1 kb).
Lane 21: *Sulfolobus* ribosomes.

Studies on growth phase dependent variations in nucleoid.

Genomic DNA associated with structural DNA binding proteins form the nucleoid. Nucleoid proteins play a structural role and functional roles in the regulation of replication, recombination and transcription. These proteins have been shown to vary in their expression and level of expression in a growth phase dependent manner.
A preliminary study has been done on the nucleoid from stationary phase cells.

Sephacryl S-1000 column chromatography of nucleoid from stationary phase cells.

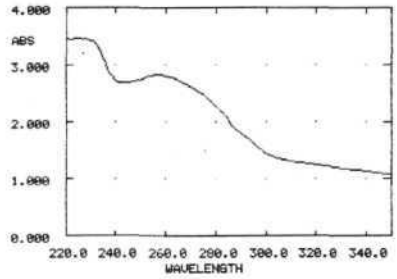
The absorption spectrum of the stationary phase nucleoid (Fig 16a) is distinctly different from that of the exponential phase nucleoid. The elution profile of the stationary phase nucleoid on sephacryl 1 S-1000 column (Fig 16b) shows a shoulder like peak I and a distinct peak II. The protein content in peak II is reduced.

Absorbance spectra (Fig 17a) showed a much reduced amount of nucleic acid in peak 1 than peak II unlike what was observed in case of nucleoid isolated from mid logarithmic phase cells.

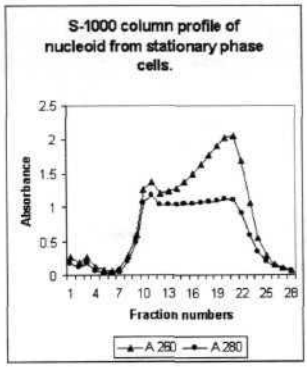
The SDS-PAGE analysis of the sephacryl S-1000 column fractions (Fig 17b) shows peak I to contain proteins ranging from ~50 kDa to 30 kDa. None of the histone like proteins are observed in peak 1. Peak II has DBNP B in greater abundance than HSNP C which is present in lesser amounts than the nucleoid isolated from mid logarithmic cultures. A ~ 66 kDa, a ~ 55 kDa protein and a ~ 30 kDa protein are also observed to be abundant as compared to nucleoid isolated from midlogarithmic phase cells. These proteins could be specifically associating with nucleoid at stationary phase.

Analysis of the nucleoprotein complexes in the column fractions (Fig 18a) shows peak I to contain high molecular weight complexes which are retained in the wells. Peak II complexes are retained in the wells. Early peak II fractions migrate as a smear ~ 500 bp to 1 kbp. The later fractions have smears increase in size of DNA, the last peak II fractions migrate as a smear from the sample well to 500 bp.

Fig 16: Sephacryl S-1000 column chromatography of nucleoid isolated from stationary phase cells.



a

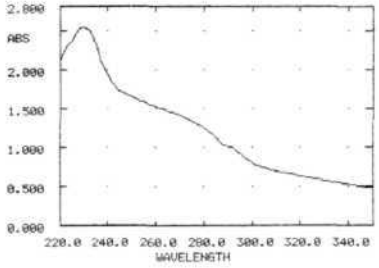


b

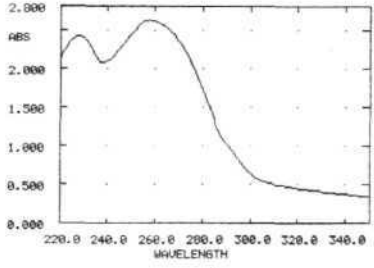
a: Absorbance spectrum of nucleoid isolated on 30% sucrose cushion from stationary phase cells at 220 nm to 350 nm.

b: Sephacryl S-1000 column elution profile of nucleoid isolated from stationary phase cells. Absorbance at 260 and 280 nm of fractions obtained from the sephacryl S-1000 column chromatography of nucleoid isolated from cells harvested at stationary phase was measured.

Fig 17: The absorption spectra of peak I and peak II of nucleoid from stationary phase cells, in 20 mM Tris-Cl pH 7.6, 150 mM KC1, 1mM EDTA pH 8.0 and 6 mM p-mercaptoethanol buffer were recorded between 220 nm to 350 nm.



a



b

a: Absorption spectrum of peak I from sephacryl S-1000 column chromatography of nucleoid isolated from stationary phase cells.

b: Absorption spectrum of peak II from sephacryl S-1000 column chromatography of nucleoid isolated from stationary phase cells.

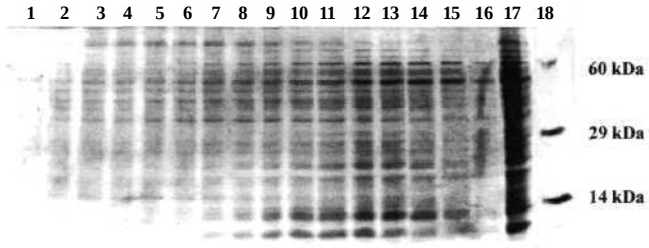


Fig 17b: SDS-PAGE analysis of peak fractions from sephacryl S-1000 column chromatography of nucleoid from stationary phase cells.
Fractions (200 µl) were TCA precipitated and electrophoresed on an 18 % SDS-polyacrylamide gel followed by coomassie blue staining.
Lane 1-4: Peak I fractions 9-12 respectively.
Lane 5-16: Peak II fractions 13-24 respectively.
Lane 17: Nucleoid from stationary phase cells.
Lane 18: Molecular weight markers (BSA (60 kDa), carbonic anhydrase (29 kDa), cyt C (12.3 kDa).

Fig 18: Agarose gel electrophoresis of the nucleoprotein complex and nucleic acids of nucleoid, peak I and peak II from stationary phase cells.

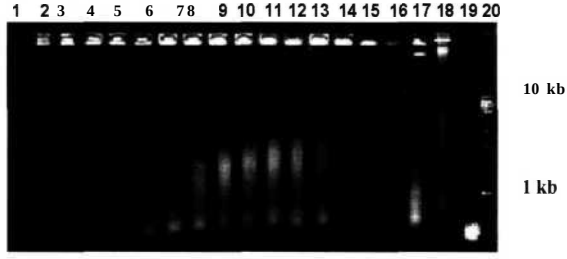


Fig 18a: Nucleoprotein profile of nucleoid from stationary phase cells chromatographed on sephacryl S-1000 analyzed on a 1.4% agarose gel.
Lane 1-4: Peak 1 fractions 9-12.
Lane 5-16: Peak II fractions 13-24.
Lane 17: Nucleoid from stationary phase cells.
Lane 18: Nucleoid from exponential phase cells.
Lane 19: *E.coli* tRNA.
Lane 20: Molecular weight markers (1 kbp ladder).

Agarose gel analysis of the nucleic acid content (Fig 18b) shows peak 1 to have no free nucleic acids. Peak II, shows sample retained in the wells in some fractions and a smear ranging from ~ 4 kbp to ~ 500 bp. The end fractions show a band at ~ 500 bp. Overall the nucleic acid profile in peak II is similar to that of nucleoid isolated from mid logarithmic phase cells.

The nucleoid in stationary phase has a high DNA to protein ratio in peak II. Transcriptional activities are reduced during stationary phase and therefore the levels of RNA associated with the genomic DNA, as also the associated protein levels would reduce. The nucleoid during stationary phase has been reported to show a comparatively more relaxed organization. Low levels of HSNP C, which has been reported to play a role in aggregating the nucleoid and DBNP B which helps in reannealing of DNA could partially be responsible for the relaxed structure of the nucleoid.

Sephacryl S-1000 column profile of nucleoid digested with RNase from stationary phase cells.

The elution profile of the stationary phase nucleoid digested with RNase and chromatographed on sephacryl S-1000 column (Fig 19a) shows two peaks at elution volumes similar to Fig 5.

SDS-PAGE analysis of the column fractions (Fig 19b) shows peak I to have proteins of molecular weight ranging from ~ 94 kDa to ~ 20 kDa. HSNP C is also observed in small amounts. Peak II has high levels of ~66 kDa protein, ~45 kDa protein and ~29 kDa protein. HSNP C' and DBNP B are also observed, HSNP C' occurs in the early fractions and DBNP B in the later fractions.

The nucleic acid content of the fractions (Fig 19c) shows no signal in peak I. Peak II fractions show a smear ~ 10 kbp to ~2 kbp. A small amount of sample is also retained in the wells. The two components resolved by S-1000 chromatography may represent two forms of the nucleoid in *Sulfolobus acidocaldarius.* The first peak could be membrane associated nucleoid as the solution showed opalescence. The presence of membrane S-layer protein was tested in peak I.

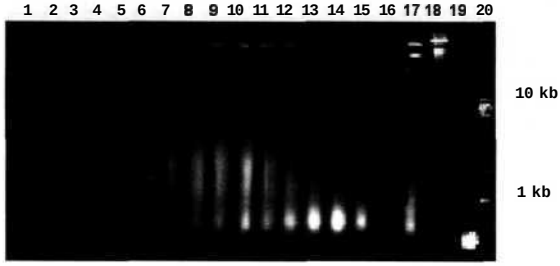


Fig 18b: Nucleic acid profile of nucleoid from stationary phase cells chromatographed on sephacryl S-1000 analyzed on a 1.4% agarose gel.
Lane 1-4: Peak I fractions 9-12.
Lane 5-16: Peak II fractions 13-24.
Lane 17: Nucleoid from stationary phase cells.
Lane 18: Nucleoid from exponential phase cells.
Lane \9: *E.coli* tRNA.
Lane 20: Molecular weight markers (1 kbp ladder).

Fig 19: Sephacryl S-1000 column chromatography of stationary phase nucleoid digested with RNase.

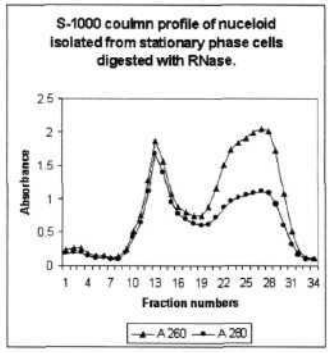


Fig 19a: Sephacryl S-1000 column elution profile of *Sulfolobus acidocaldarius* nucleoid at stationary phase digested with RNase.
Absorbance at 260 and 280 nm of fractions obtained from the sephacryl S-1000 column chromatography of nucleoid digested with RNase, isolated from cells harvested at stationary phase, was measured.

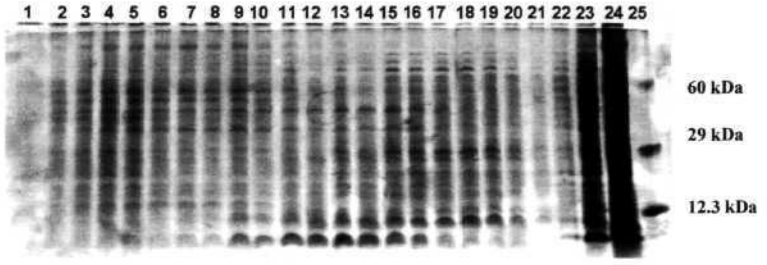


Fig 19b: SDS-PAGE analysis of peak fractions from sephacryl S-1000 column chromatography of nucleoid digested with RNase, from stationary phase cells.
Fractions (200 µl) were TCA precipitated and electrophoresed on an 18 % SDS-polyacrylamide gel followed by coomassie blue staining.
Lane 1-9: Peak I fractions 10-18 respectively.
Lane 10-22: Peak II fractions 19-31 respectively.
Lane 23: Nucleoid from stationary phase cells.
Lane 24: Nucleoid from exponential phase cells.
Lane 25: Molecular weight markers (BSA (60 kDa), carbonic anhydrase (29 kDa), cyt C (12.3 kDa).

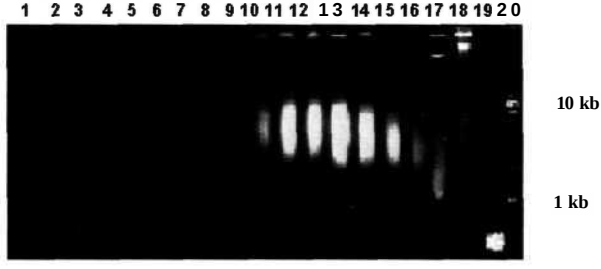


Fig 19d: Nucleic acid profile of sephacryl S-1000 column fractions of RNase digested nucleoid from stationary phase cells analyzed on a 1.4% agarose gel
Lane 1-9: Peak **I** fractions 10-18.
Lane 10-16: Peak II fractions 19- 25
Lane 17: Nucleoid.
Lane 18: *E. coli* tRNA.
Lane 19: Molecular weight markers (1 kbp ladder).

Nature of the nucleoid in regenerating (revived) *S. acidocaldarius* culture:

Nucleoid isolated from cells during the regeneration of culture from preserved stock shows a variant profile on sephacryl S-1000 column (Fig 20a). A small hump occurs at the elution volume corresponding to peak I and a distinct peak corresponding to peak II. The DNA (A 260) is considerably more than the protein (A 280) content of peak II.

SDS-PAGE analysis of column fractions (Fig 20b) showed the peak I fractions to contain high levels of HSNP A and HSNP C'and some proteins of ~50 kDa to 40 kDa molecular weight. Later fractions have very high levels of DBNP B and HSNP C along with HSNP A, ~ 30 to ~20 kDa proteins and ~ 60 to ~50 kDa proteins.

Occurrence of high levels of HSNP A in the high molecular weight aggregate along with HSNP C' hints at HSNP A playing an important role in the cell during stress conditions. Further studies were done on HSNP A to determine its properties and its ability to bind and stabilize DNA.

Effect of temperature on nucleoid fractions:

To check for the stability of the nucleoid of *Sulfolobus acidocaldarius* at temperatures ideal for growth of mesophilic organisms, the sephacryl S-1000 column peak fractions were incubated at 37°C (Fig 21a). Peak II fractions remained unaffected. Peak I fractions show low molecular weight proteins on incubation. Studies on the effect of temperature on peak I protein profile (Fig 21b) show incubation at 37°C to result in appearance of the histone like proteins, DBNP B and HSNP C' along with protein of molecular weights ranging from ~60 kDa to ~ 30 kDa. Incubation at 68°C apparently results in aggregation of these proteins.

Incubation at 37°C shows up the low molecular weight proteins either due to changes induced in the structure (disaggregation) of the high molecular weight aggregate at non physiological temperature or due to RNase or RNase like activity of some protein(s) in peak I not present in peak II.

Fig 20: Sephacryl S-1000 column chromatography of nucleoid isolated from *Sulfolobus acidocaldarius* cells during the regeneration of the culture.

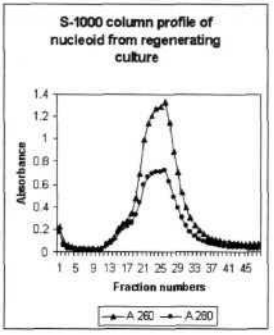


Fig 20a: Column elution profile of nucleoid from regenerating *S. acidocaldarius* cells, chromatographed on sephacryl S-1000.

Nucleoid isolated from culture being regenerated from cells stored at room temperature for 10 days was chromatographed on sephacryl S-1000 column. Fractions collected were tested for absorbance at 260 and 280 nm respectively.

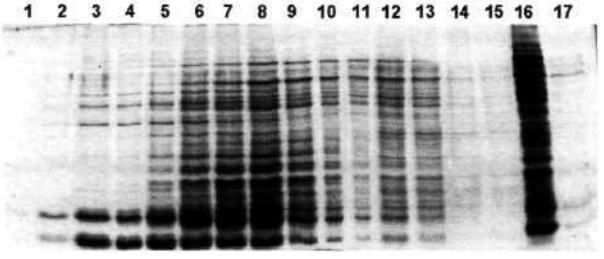


Fig 20b: SDS-PAGE analysis of peak fractions from sephacryl S-1000 column chromatography of nucleoid isolated from regenerating culture.

Nucleoid was isolated from regenerating culture and chromatographed on a sephacryl S-1000 column. Fractions (70 |il) were electrophoresed on an 18% SDS-polyacrylamide gel and visualized by coomassie blue staining.

Lane 1-15: Fractions 14-28 respectively.

Lane 16: Nucleoid.

Lane 17: Nucleoid acid extract.

Fig 21: Effect of temperature on sephacryl S-1000 peak fractions.

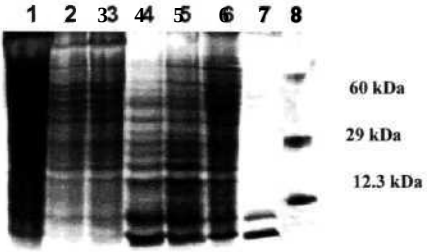


Fig 21a: SDS- PAGE analysis of Sephacryl S-1000 nucleoid peak fractions incubated at 37°C.

Sephacryl S-1000 peak fractions were incubated at 37 °C for 2 hrs, electrophoresed on an 18 % SDS- polyacrylamide gel and visualized by coomassie blue staining.

Lane 1-3: Peak I fractions.
Lane 3-6: Peak II fractions.
Lane 7: Nucleoid acid extract.
Lane 8: Molecular weight markers (BSA (60 kDa), carbonic anhydrase (29 kDa), cytochrome C (12.3 kDa).

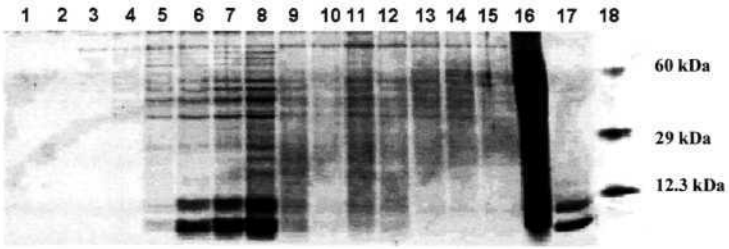


Fig 21b: SDS-PAGE analysis of effect of temperature on sephacryl S-1000 peak I nucleoid fractions.

Peak I was incubated at different temperatures, as described in methods section, TCA precipitated and analyzed by electrophoresis on an 18 % SDS polyacrylamide gel.

Lane 1-4: Peak I fractions (control).
Lane 5-8: Peak I fractions incubated at 37 °C for 1 hour.
Lane 9-12: Peak I fractions incubated at 68 °C for 15 mins.
Lane 13-15: Peak I fractions incubated at 68 °C for 1 hour.
Lane 16: Nucleoid.
Lane 17: Nucleoid acid extract.
Lane 18: Molecular weight markers (BSA (60 kDa), carbonic anhydrase (29 kDa), cyt C (12.3 kDa).

Purification and characterization of S-layer proteins:

S-layer of Sulfolobus acidocaldarius is a glycoprotein consisting of two sub units of 115 kDa and 60 kDa respectively. Peak I from the sephacryl S-1000 column chromatography of nucleoid has a turbid appearance suggesting the presence of membrane proteins. S- layer protein purification protocol, as described in the methods section was applied to the cells of *Sulfolobus acidocaldarius*, nucleoid and peak fractions of nucleoid chromatographed on sephacryl S-1000.

Purification of S-layer protein:

S-layer proteins were purified from the *Sulfolobus* cells, nucleoid and peak fractions of nucleoid chromatographed on sephacryl S-1000 (Fig 22a). Schiff s reagent staining of the gel was performed to confirm the proteins isolated to be glycoproteins (Fig 22b). Peak I has a higher content of the S-layer proteins with negligible amount of S-layer protein in peak II.

DNA cellulose chromatography of S-layer protein.

The S-layer proteins were analyzed for DNA binding activity since they were detected in the nucleoid preparations. Fig 23a shows the elution profile of S-layer protein on ds DNA cellulose column with one peak in the wash fractions and a second peak in the elute with 0.3 M NaCl. SDS -PAGE (Fig 23b) showed the unbound protein which eluted out in the 150 mM fractions to be the ~ 115 kDa sub unit and the bound fraction eluting at 300 mM is the 60 kDa sub unit of S-layer protein. These results show that 60 kDa subunit has DNA binding property.

S-layer proteins and effect of temperature on peak II:

S-layer proteins were predominantly isolated from peak I. Peak II was incubated with s-layer proteins to check for its role in temperature tolerance. As see in Fig 24, at 37 °C there is no difference between the control sample and peak II incubated with S-layer. However, at 70°C, there is a marked reduction in degradation among the samples incubated with S-layer proteins as compared to the control.

Fig 22: S-layer protein purification and characterization

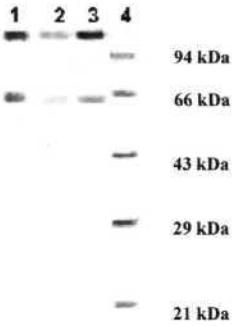


Fig 22a: Isolation of S-layer protein from peak fractions of nucleoid chromatographed on Sephacryl S-1000.
S-layer protein was isolated, as described in the methods section, from cells at exponential phase and from peak fractions of nucleoid chromatographed on sephacryl S-1000. The proteins were analyzed by electrophoresis on an 18 % SDS polyacrylamide gel followed by coomassie blue staining.
Lane 1: S-layer from peak I
Lane 2: S-layer from peak II
Lane 3: S-layer from *S.acidocaldarius* cells.
Lane 4: Molecular weight markers (94 kDa, 66 kDa, 43 kDa, 29 kDa and 21 kDa).

Fig 22b: Schiff's reagent staining of S-layer protein from peak fractions of nucleoid chromatographed on sephacryl S-1000.
S- layer proteins were electrophoresed on an 18% SDS polyacrylamide gel and visualized by Schiff's reagent staining, as described in the methods section, specific for detection of glycoproteins.
Lane 1: S-layer from peak I
Lane 2: S-layer from peak II
Lane 3: S-layer from *S.acidocaldarius* cells.

Fig 23: ds DNA cellulose chromatography of S-layer proteins

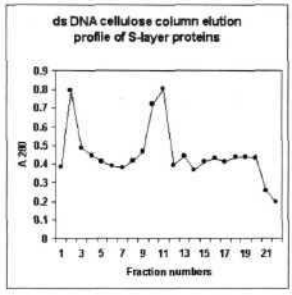


Fig 23a: ds DNA cellulose column elution profile of S-layer proteins.
S-layer proteins (200 µg) were chromatographed on a 3ml ds DNA cellulose column as described in materials and methods. The fractions collected were checked for absorbance at 280 nm.

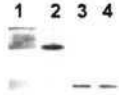


Fig 23b: SDS-Page analysis of fractions from ds DNA cellulose chromatography of S-layer proteins on an 18 % gel.
200 µg of S-layer protein was chromatographed on ds DNA cellulose. 0.5ml fractions were collected, peak fractions (70 µl) analyzed on 18% SDS polyacrylamide gels and visualized by coomassie blue staining.
Lane 1: S-layer protein from 48 hr cells
Lane 2: 150 mM wash fraction.
Lane 3-4: 300 mM wash fraction.

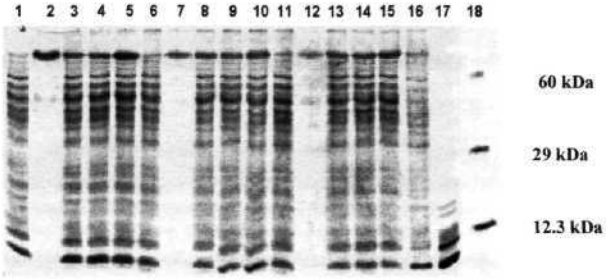


Fig 24: SDS-PAGE analysis of effect of S-layer proteins on thermal denaturation of sephacryl S-1000 peak II nucleoid fractions.

Peak II (20µg) pool of nucleoid chromatographed on sephacryl S-100 column was incubated with varying amounts of S- layer protein at increasing temperatures as described below. Samples were electrophoresed on an 18% SDS polyacrylamide gel and visualized by coomassie blue staining.

Lane 1: Peak II
Lane 2: S-layer protein (2µg).
Lane 3-5: Peak II (40 ug) and S-layer 2, 4 and 8 ug respectively incubated at 37 °C for 60 mins
Lane 6: Peak II incubated at 37 °C for 60 mins.
Lane 7: S-layer protein incubated at 70°C for 30 mins.
Lane 8-10: Peak II (40 µg) and S-layer 2, 4 and 8 µg respectively incubated at 70 °C for 30 mins
Lane 11: Peak II incubated at 70 °C for 30 mins.
Lane 12: S-layer protein incubated at 70°C for 60 mins
Lane 13-15: Peak II (40 ug) and S-layer 2, 4 and 8 ug respectively incubated at 70 °C for 60 mins
Lane 16: Peak II incubated at 70 °C for 60 mins.
Lane 17: Nucleoid acid extract.
Lane 18: Molecular weight markers (BSA (60 kDa), carbonic anhydrase (29 kDa), cyt C (12.3 kDa).

Purification of HSNP A and characterization of its DNA binding properties:

Reddy and Suryanarayana (1988, 1989) purified four acid soluble DNA binding proteins, HSNP A, HSNP C, HSNP C and DBNP B, from the nucleoid of *Sulfolobus acidocaldarius*. They occur in the order of abundance HSNP A<DBNP B<HSNP C'. HSNP A is a 12 kDa protein which binds DNA and protects it against thermal denaturation. It was found to be present localized exclusively on the nucleoid (Bohrmann et al., 1994). In the present study, a protocol was devised to purify HSNP A and its chemical and nucleic acid binding properties were studied.

Purification of HSNPA:

HSNP A was purified as described in the methods section. S-100 acid extract was chromatographed on CM-52 column. The column was eluted with 100 mM, 150 mM and 300 mM KCl containing buffers. Fig 25 shows that HSNP A elutes out in the 150 mM fractions along with HSNP C. The HSNP A containing fractions were pooled and chromatographed on ds DNA cellulose column as described in the methods section.

Chromatography of HSNP A containing fractions from CM-52 on ds DNA cellulose column (Fig 26a) in P-mercaptoethanol containing buffer results in HSNP A co eluting with HSNP C' at 300 mM.

In buffer devoid of P-mercaptoethanol, chromatography of HSNP A containing fractions from CM-52 on ds DNA cellulose column (Fig 26b) results in HSNP A eluting out in 300 mM fractions. HSNP C' elutes out in 150 mM fractions. The differential affinity of the protein to DNA in the absence and presence of mercaptoethanol resulted in the purification of HSNP A to homogeneity.

These results are indicative of two possible interpretations - either HSNP C" has weaker affinity to DNA in the absence of mercaptoethanol or HSNP C and HSNP A associate with each other in the presence of mercaptoethanol and the complex thus formed elutes -out in 300 mM salt buffer.

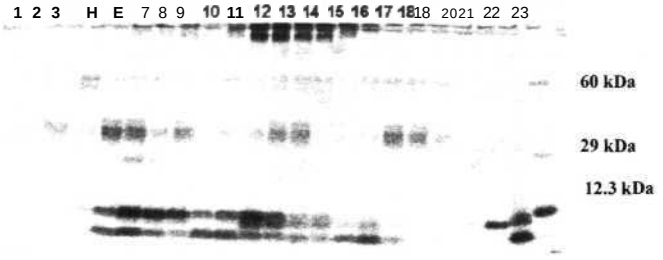


Fig 25: Purification of HSNP A
Elution pattern of acid treated post ribosomal supernatant of *S.acidocaldarius* on CM-cellulose analyzed by 18 % SDS-PAGE.
Lane 1-2: Flow through fractions 1 and 8 respectively
Lane 3-4: wash fractions 3 and 7 respectively
Lane 5-9: 150 mM wash fractions 5, 7, 9, 11 and 13 respectively.
Lane 10-14: 300 mM wash fractions 6, 8, 10, 12 and 14 respectively
Lane 15-20: 300 mM wash fractions 18, 20, 22, 24, 26 and 28 respectively
Lane 21: 600 mM wash fraction 9.
Lane 22: Acid extract
Lane 23: Molecular weight markers (Bovine serum albumin 66 kDa, carbonic anhydrase 29 kDa and cytochrome C 12.3 kDa).

Fig 26: dsDNA cellulose chromatography of HSNP A containing fractions of CM-52 column.

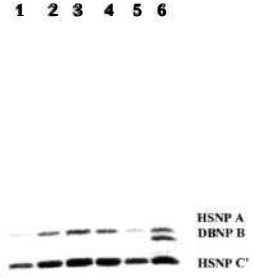


Fig 26a: DNA- cellulose chromatography of HSNP A containing CM cellulose column fractions in presence of β-mercaptoethanol.

Peak fractions from the 150mM and 300mM NaCl elute of CM cellulose column were pooled and dialyzed against column buffer in presence of β- mercaptoethanol and chromatographed on a ds DNA cellulose column as described in the methods section. Fractions (40µl) were electrophoresed on an 18% SDS polyacrylamide gel and visualized by coomassie blue staining.
Lane 1-5: 300 mM fractions 2, 4, 6, 8 and 10 respectively
Lane 6: Nucleoid acid extract.

Molecular weight determination of HSNP A:

Molecular weight determination of HSNP A was done by electrophoresing it on an 18 % polyacrylamide gel in the presence of SDS as described by Thomas and Kornberg (1974). As seen in Fig 27, comparison of the relative mobility of HSNP A with respect to the molecular weight markers indicated a molecular weight of 10.5 kDa.

Protein Crosslinking:

Cross linking studies were done to determine the solution state of HSNP A. The protein was cross linked using the bifunctional reagent Dimethyl suberimidate and also with zero level cross linker, HCHO at different temperatures.

The cross linking products were analyzed by SDS- PAGE. As seen in Fig 28a, Crosslinking with formaldehyde at 37°C and 55°C resulted in the formation of predominantly octamers (80 kDa). When the protein was incubated at 72°C in the absence of the crosslinking reagent octameric form could be seen along with monomer (lane 6). Dimer formation is observed when the protein is treated with formaldehyde at 72°C.

Crosslinking with Dimethyl suberimidate, (Fig 28b) shows the formation of octamers and hexamers on incubation of the protein for 24 hrs at both 37°C and 72°C with greater cross linked products at 72°C. Monomers are not observed in either lane.

Gel filtration of HSNP A also showed that the protein exists in an oligomeric form in solution as indicated by the elution of the protein at volume corresponding to octamer (80 kDa), hexamer (60 kDa) and dimer (24 kDa) (Fig 29). Negligible amount of protein eluted at volume corresponding to monomer

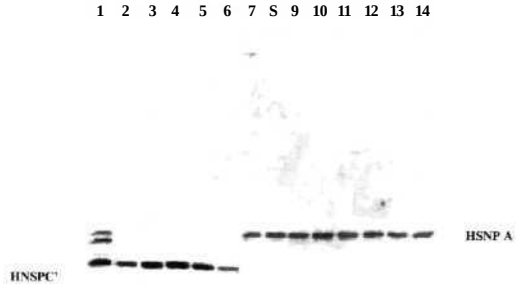


Fig 26b: DNA- cellulose chromatography of 150 and 300 mM HSNP A containing CM cellulose column fractions in absence of β mercaptoethanol.

Peak fractions from the 150mM and 300mM NaCl elute of CM cellulose column were pooled and dialyzed against column buffer in absence of β mercaptoethanol and chromatographed on a ds DNA cellulose column as described in the methods section. Fractions (40µl) were electrophoresed on an 18% SDS polyacrylamide gel and visualized by coomassie blue staining.
Lane 1: Nucleoid acid extract
Lane 2-6: 150 mM fractions 2, 3, 4, 5 and 6.
Lane 7-14: 300 mM wash fractions 2, 3, 4, 5, 6, 7, 8 and 9 respectively

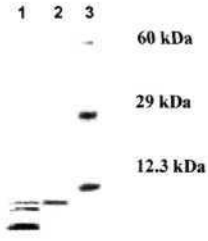


Fig 27: Determination of molecular weight by SDS-PAGE

SDS-PAGE analysis by Thomas and Kornberg method, using an 18 % acrylamide gel was used to determine the molecular weight of HSNP A. The gel was visualized by silver staining.
Lane 1: Nucleoid acid extract
Lane 2: HSNP A.
Lane 3: Molecular weight markers (BSA 60 kDa, carbonic anhydrase 29 kDa, cytochrome C 12.3 kDa)

Fig 28: Cross linking studies on HSNP A

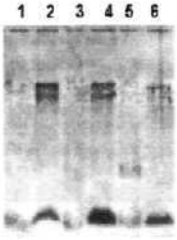


Fig 28a: SDS-PAGE analysis of HSNP A cross linked with formaldehyde in pH 5 buffer.

Formaldehyde cross linking of HSNP A was performed with 4 µg of HSNP A at 37°C, 55°C and 72°C for one hour in sodium acetate buffer (pH 5.0) and electrophoresed on an 18 % SDS-polyacrylamide gel followed by silver staining.
Lane 1: HSNP A (control) in sodium acetate buffer (pH 5.0).
Lane 2: Cross linking at 37°C for 1hour.
Lane 3: HSNP A incubated at 55°C (control).
Lane 4: Cross linking at 55°C for 1hour.
Lane 5: Cross linking at 72°C for 1hour.
Lane 6: HSNP A incubated at 72°C (control).

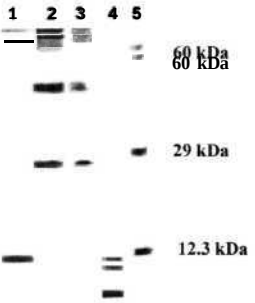


Fig 28b: SDS-PAGE analysis of HSNP A cross linked with DMS.

Dimethyl suberimidate cross linking was performed as described in the methods section with 4 µg of HSNP A at 37°C and 72°C for 24 hours and electrophoresed on an 18% SDS-polyacrylamide gel followed by silver staining.
Lane 1: HSNP A (control)
Lane 2: Cross linking at 72°C for 24 hrs.
Lane 4: Cross linking at 37°C for 24 hrs.
Lane 6: Nucleoid acid extract.
Lane 7: Molecular weight markers.

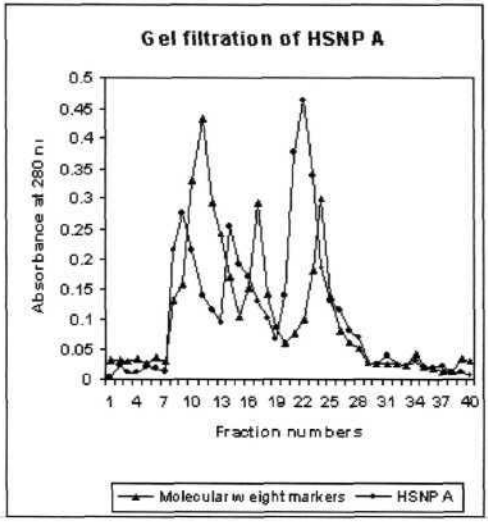


Fig 28c: Gel filtration of HSNP A.

HSNPA (100 µg) and molecular weight markers was chromatographed on sephadex G-50 as described in the methods section. Fractions were analysed at 280 nm.

Spectroscopic properties of HSNP A:

UV absorption spectrum (Fig 29a) and fluorescence emission spectrum (Fig 29b) were recorded. Absorption spectrum showed absorption maximum in the range 260 to 280nm. Fluorescence emission spectrum of the protein showed a broad spectrum from 295 nm to 330 nm with maxima at 305 nm with the excitation wave length of 285 nm. Fluorescence spectrum obtained with excitation wavelength of 272 nm showed separation of emission due to tyrosine and tryptophan indicating no fluorescence energy transfer from tyrosine to tryptophan.

Nucleic acid binding properties of HSNP A:

Fluorescence titrations:

Quenching of intrinsic fluorescence of proteins by nucleic acids can be used to understand interaction of proteins with nucleic acids. Reverse titrations were performed by adding nucleic acids to HSNPA and measuring the extent of fluorescence quenching. The quenched fluorescence spectra obtained by adding poly dA, poly U and calf thymus DNA are given in Fig 30. The data with amount of ligand added vs % quenching (Fig 30) showed that more quenching was observed with poly U which lacks ordered structure than double stranded DNA or poly dA (which has ordered helical structure).

Binding site size was calculated from the extent of quenching saturation. A binding site size of about 70 nt/monomer was obtained with native DNA.

Displacement of DNA bound ethidium bromide by HSNP A:

In order to understand the mode of interaction of the protein with DNA, HSNP A was added to ethidium bromide bound DNA

As seen in Fig 31a, addition of histones or HSNP A resulted in substantial decrease in the fluorescence of DNA-ethidium bromide complex with increasing amount of protein indicating release of ethidium bromide from DNA by HSNP A or histones. As control, Bovine serum albumin was added to DNA-ethidium bromide complex which did not show

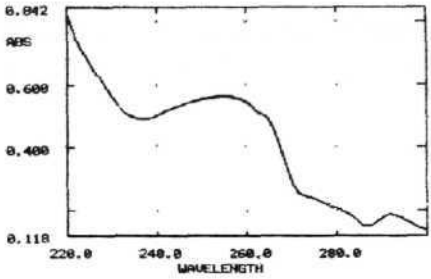


Fig 29a: UV absorption spectrum of HSNP A recorded between 220nm to 300nm in 1 **mM** Tris-Cl pH 7.6 and 25 mM NaCl.

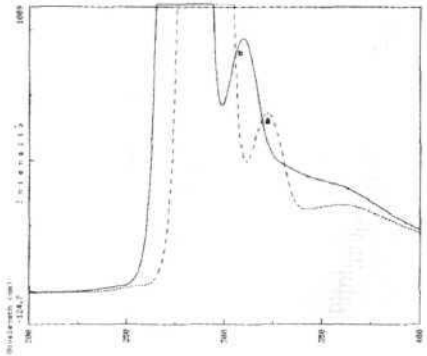
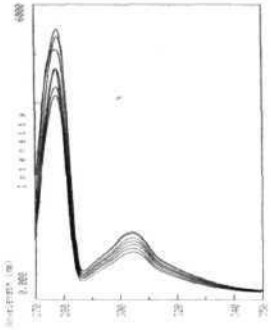
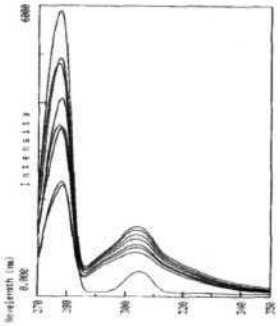


Fig 29b: Intrinsic fluorescence spectrum of HSNP A
a: 285 nm
b: 274 nm

Fig 30: Quenching of intrinsic fluorescence of HSNP A



a



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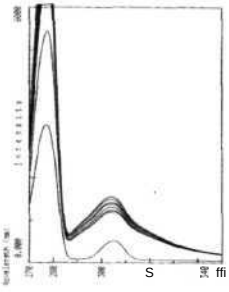
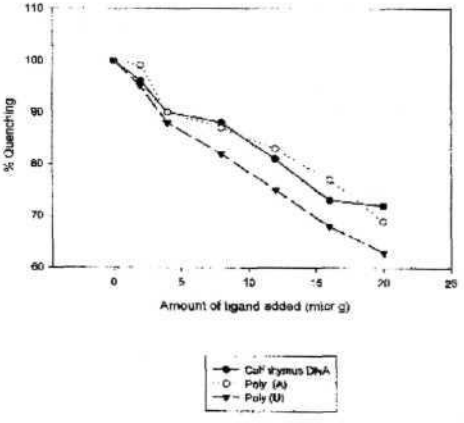


c



HSNP A (2 μM) was reverse titrated with poly A, poly U and calf thymus DNA.

a: Quenching of intrinsic fluorescence of HSNP A by Poly A
b: Quenching of intrinsic fluorescence of HSNP A by Poly U
c: Quenching of intrinsic fluorescence of HSNP A by calf thymus DNA

Fig 31: Ethidium bromide displacement assay

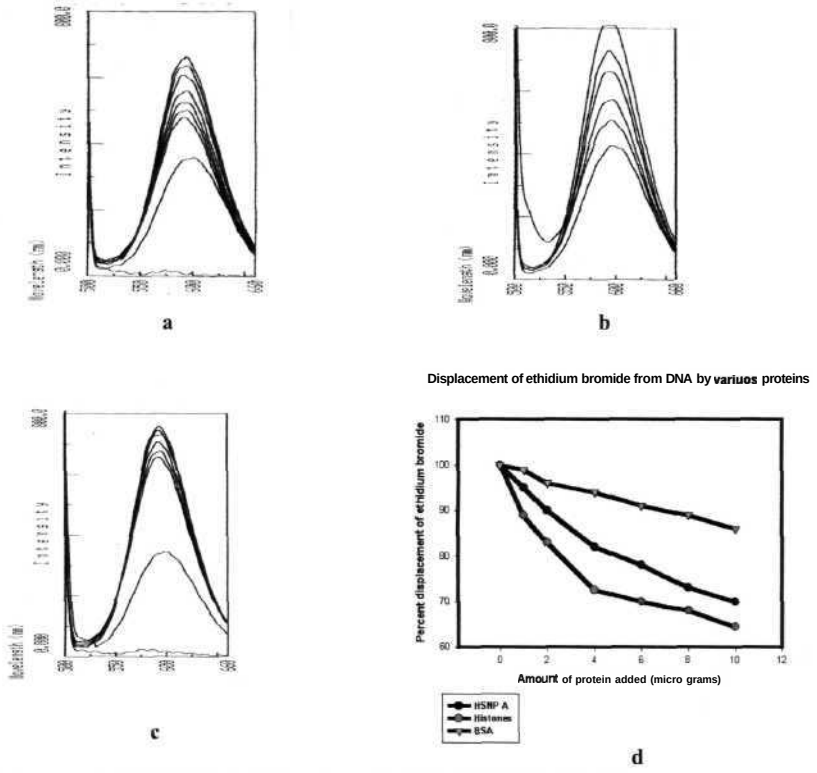


Fig 31a: Displacement of intercalated ethidium bromide from ds DNA by HSNP A.

DNA (2μg) was added to cuvette containing 500 ng ethidium bromide. Aliquots of **the** proteins were added, to final concentrations, as mentioned below and spectra were recorded after each addition.

a: Displacement of ethidium bromide from dsDNA (ct DNA) by bovine serum albumin (1, 2, 4, 6, 8 and 10 μg respectively).
b: Displacement of ethidium bromide from dsDNA (ct DNA) by histones (1, 2, 4, 6, 8 and 10 μg respectively).
c: Displacement of ethidium bromide from dsDNA (ct DNA) by HSNP A (1, 2, 4, 6, 8 and 10 μg respectively).
d: Displacement of ethidium bromide from ds DNA by bovine serum albumin, histones and HSNP A.
Data from Fig a, Fig b and Fig c was replotted as % Relative displacement of ethidium bromide vs protein concentration.

any significant release of bound ethidium. In the presence of magnesium the displacement of DNA bound ethidium was reduced (Fig 31b).

CT DNA melting curves:

Analysis of the thermal melting curves of calf thymus DNA in presence on HSNP A (Fig 32) shows that HSNP A increases the Tm of DNA by ~ 20°C at DNA to protein ratio of 60 nt/monomer of the protein.

Gel mobility shift assay:

Increasing amounts of HSNP A was added to pBS plasmid DNA and the protein- DNA complexes were electrophoresed in agarose gels. As seen in Fig 33a, addition of HSNP A resulted in progressively increasing amounts of DNA being retained in the wells of the agarose gel with no intermediate migrating DNA protein complexes.

Presence of β-mercaptoethanol was observed to affect the elution of HSNP A from ds DNA cellulose column, as seen in Fig 33b, it also affects the mobility of DNA. Greater retardation was observed in absence of P-mercaptoethanol indicating that HSNP A binds DNA more effectively under these conditions.

The protein showed similar binding affinity to DNA both at 37°C (Fig 33c) and 75°C (Fig 33d).

Binding of HSNP A to DNA was also studied by mobility shift assay in the presence of other nucleoid associated DNA binding protein HSNP C'(Sac7d). Sac7d is abundant non specific DNA binding protein. Electron microscopy has shown that it binds to DNA to form looped DNA structure and at higher concentration the Sac 7d forms highly condensed compact structure with DNA (Francina, 1995). Increasing amounts of Sac7d added to DNA progressively (Fig 34b (lanes 10-12)) show progressive increase in retardation of the DNA. However the presence of HSNP A, along with Sac 7d resulted in the decrease in retardation caused by Sac7d alone. This result indicates that either HSNP A interferes with the binding of Sac 7d to DNA or the DNA-protein complexes formed in the combined presence of the two proteins show decreased retardation (see EM picture below).

Effect of Magnesium on displacement of ethidium bromide from calf **thymus** DNA by HSNP A

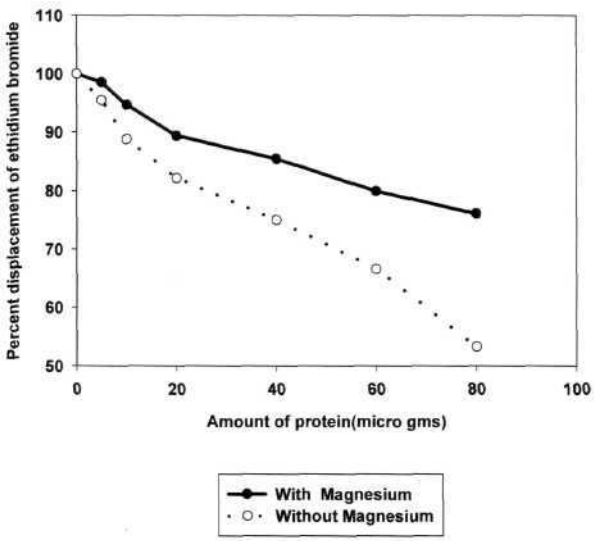


Fig 31b: Effect of magnesium on ethidium bromide displacement from ds DNA by **HSNP** A.

500 ng Ethidium bromide was added to CT DNA (2 µg) 20 mM Tris (pH 7.6) buffer. HSNP A was added to 5, 10, 20, 40, 60 and 80 µg final concentration respectively in absence of and in presence of magnesium (2 mM). Data was replotted as % Displacement of ethidium bromide vs Amount of protein (µg).

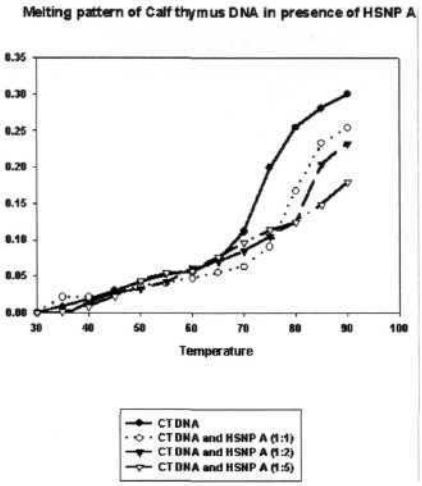


Fig 32: Effect of HSNP A on Tm of calf thymus DNA.
Calf thymus DNA was incubated with HSNP A at a DNA to protein ratio of 1:1, 1:2 and 1:5. The increase in A 260 was monitored between 30°C to 90°C and plotted.

Fig 33: Gel Mobility shift Assays:

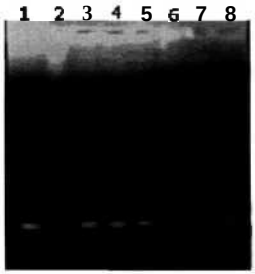


Fig 33a: Gel mobility shift analysis of HSNP A-DNA complexes.
pBS (1 µg) was incubated with increasing amounts of HSNP A, as described below and analyzed on 0.8% agarose gel.
Lane 1: pBS (control).
Lane 2: pBS incubated with histone (1 µg).
Lane 3: pBS incubated with BSA (5 µg).
Lane 4-8: pBS with HSNP A, 1 µg, 2 µg, 4 µg, 8 µg and 12 µg, respectively.

Fig 33b: Effect of p-mercaptoethanol on HSNP A- DNA complex formation.
pBS (1µg) was incubated with increasing amounts of HSNP A as described below, in presence and absence of β-mercaptoethanol. The products were analyzed on a 0.8% agarose gel.
Lane 1: pBS (control).
Lane 2-4: pBS incubated with 5, 10 and 20 µg of HSNP A in absence of P-mercaptoethanol
Lane 5-7: pBS incubated with 5, 10 and 20 ug of HSNP A in presence of 7 mM p-mercaptoethanol

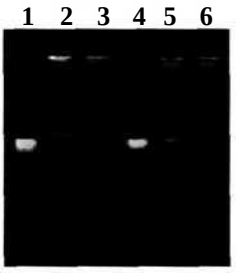


Fig 33c: Effect of HSNPA on the mobility of pBS (1 µg) containing supercoiled relaxed and linear forms at 37°C.
pBS (1 µg) was incubated with HSNP A at 37°C for different time periods as described below, and analyzed on a 0.8% agarose gel.
Lane 1: pBS DNA incubated at 37°C for 10 minutes in the absence of HSNP A (control).
Lane 2-3: pBS DNA incubated at 37°C for 10 minutes with 4 ug, 8 ug of HSNP A.
Lane 4-5: pBS DNA incubated at 37°C for 30 minutes with 4 ug, 8 ug of HSNP A.
Lane 6: pBS DNA incubated with 8 ug HSNP A at room temperature (control).

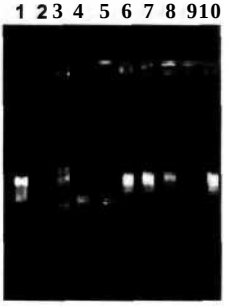


Fig 33d: Effect of HSNPA on the mobility shift of pBS (1 µg) containing supercoiled relaxed and linear forms at 75°C.
pBS (1µg) was incubated with HSNP A at 75°C for different time periods as described below, and analyzed on 0.8% agarose gel.
Lane 1: DNA incubated at 75°C for 10 minutes in the absence of HSNP A.
Lane 2-5: DNA incubated at 75°C for 10 minutes with 2 µg, 4 µg, 6 µg, 8 µg of HSNP A.
Lane 6-9: DNA incubated at 75°C for 30 minutes with 2 µg, 4 µg, 6 µg, 8 µg of HSNP A.
Lane 10: DNA incubated with HSNP A at room temperature (control).

Fig 34a: Mobility shift of pBS by HSNP A, C'and DBNP B.
pBS (2µg) was incubated with HSNP A, HSNP C'and DBNP A at ratios of 1:1, 1:2 and 1:4 and analyzed on a 0.8 % agarose gel.
Lane 1: pBS (control).
Lane 2-4: pBS (2 µg) with 2 µg, 4 µg and 8 µg HSNP A
Lane 5: pBS (2 µg) with HSNP A (2 µg) and HSNP C' (4 µg).
Lane 6: pBS (2 µg) with HSNP A (4 µg) and HSNP C'(8 µg).
Lane 7: pBS (2 µg) with HSNP C' (4 µg).
Lane 8: pBS (2 µg) with HSNP A (2 µg), HSNP C'(4 µg) and DBNP B (2 µg).

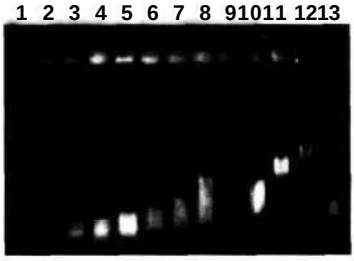


Fig 34b: Mobility shift of pBS by HSNP A, C and DBNP B.
pBS was incubated with HSNP A, HSNP C'and DBNP B at a DNA to protein ratio of 1:4, 1:8, 1:16, 1:32, 1:48 respectively and analyzed on a 0.8% agarose gel.
Lane 1: pBS (control).
Lane 2-6: pBS (1 ng) with 4 ug, 8 ug, 16 ug, 32 ug and 48 ug HSNP A respectively.
Lane 7: pBS (1 µg) with HSNP A (4 ug) and HSNP C (8 ng).
Lane 8: pBS (1 ug) with HSNP A (8 ng) and HSNP C (16 µg).
Lane 9: pBS (1 ug) with HSNP A (16 ug) and HSNP C (32 ug).
Lane 10-12: pBS (1 ug) with HSNP C', 8 µg, 16 ug and 32 ug respectively.
Lane 8: pBS (1 ug) with HSNP A (4 ug), HSNP C'(8 ug) and DBNP B (8 ug).

Studies on DNA binding properties of chemically modified HSNP A

Chemical modification of HSNP A:

Tryptophan and lysine residues in HSNP A were modified by BNPS-Skatole and pyridoxal phosphate and formaldehyde. The absorption spectra (Fig 35) and fluorescence spectra (Fig 36) of the modified proteins were recorded. Pyridoxal modified spectra clearly showed presence of pyridoxal moiety covalently linked to the protein. Modification did not result in any significant change in the spectra in the region from 250 to 300 nm both in the concentration of UV as well as fluorescence emission.

Mobility shift assays were carried out with the modified protein. There was no significant change in the binding of the protein by these modifications (fig 37).

Effect of chemical modification on the helix stabilizing property of HSNP A:

Fluorescence reverse titrations were also performed with modified proteins. Quenching of intrinsic fluorescence of both modified and unmodified proteins was obtained to the same extent by poly dG-dC (Fig 38a). However poly dA showed slightly decreased quenching of lysine modified HSNP A (Fig 38b).

Addition of HSNP A to DNA resulted in increase in Tm of DNA by about 10ºC (at 1:1 DNA: protein (w/w) ratio). There was increased helix stabilization with HSNP A modified at both tryptophan and lysine residues (indicating introduction of hydrophobic groups results in increased protection of DNA against thermal denaturation (Fig 39).

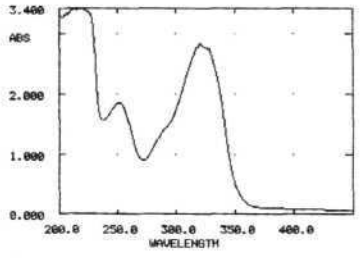
Electron microscopy of complexes of HSNP A with DNA:

Electron microscopy was performed to visualize complexes of HSNP A and HSNP C" with linearized pUC 18 DNA (Fig 40). The protein bound to the DNA uniformly all along the DNA with slightly greater amount of protein bound at the ends of DNA (Fig 40b).

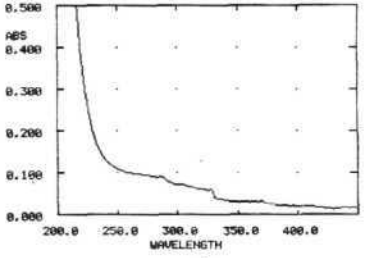
Chemical modification of HSNP A:

HSNP A protein was modified as described in the methods section at the lysine and tryptophan residues.

Fig 35: Absorbance spectra of modified HSNP A between 200nm to 450 nm recorded in 20 mM Tris pH 7.6 buffer



a



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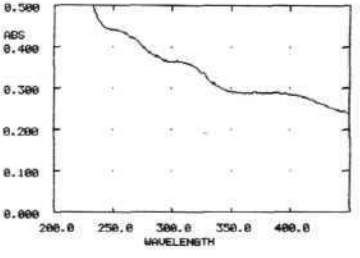

c

a: HSNP A modified with PLP
b: HNSP A modified with formaldehyde
c: HSNP A modified with BNPS-skatole.

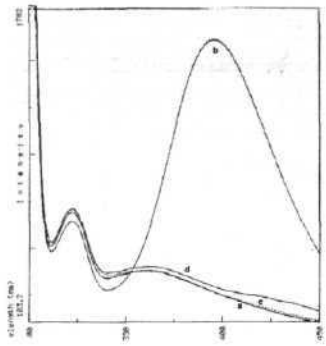

Fig 36: Intrinsic fluorescence spectrum of HSNP A and modified HSNP A.
a: unmodified HSNP A
b: plp modified HSNP A
c: tryptophan modified HSNP A
d: HCHO modified HSNP A.

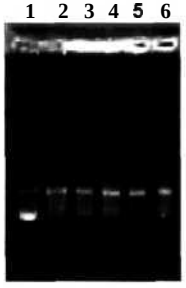

Fig 37: Mobility shift assay with modified HSNP A.

HSNP A protein was modified as described in the methods section at the lysine and tryptophan residues. To 2 µg pBS 10 µg protein was added and incubated for 10 mins at room temperature and electrophoresed on 0.8% agarose gel.

Lane 1: pBS (control)
Lane 2: pBS with unmodified HSNP A
Lane 3: pBS with HSNP A modified at lysine residues with pyridoxal phosphate.
Lane 4: pBS with HSNP A modified at lysine residues with formaldehyde.
Lane 5: pBS with HSNP A modified at tryptophan residues.
Lane 6: pBS with BSA.

Reverse titration of modified HSNP A with Poly d(G)-d(C)

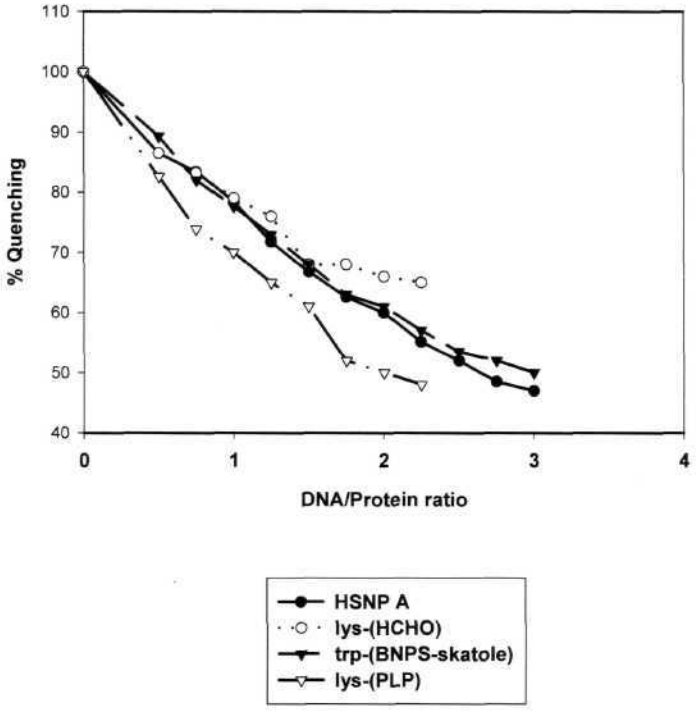

Fig 38a: Effect of modification on affinity of HSNP A to Poly dG-dC

Poly dG-dC was titrated as described in methods section with modified and unmodified HSNPA. The recorded spectral changes were replotted as % quenching vs DNA/protein ratio.

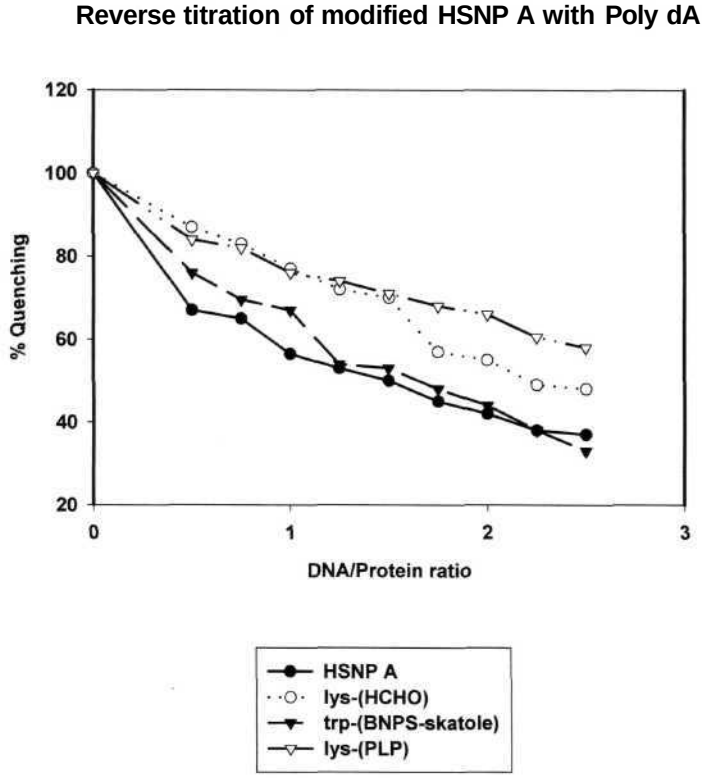

Fig 38b: Effect of modification on affinity of HSNP A to poly dA

Poly dA was titrated as described in methods section with modified and unmodified HSNPA. The recorded spectral changes were replotted as % quenching vs DNA/protein ratio.

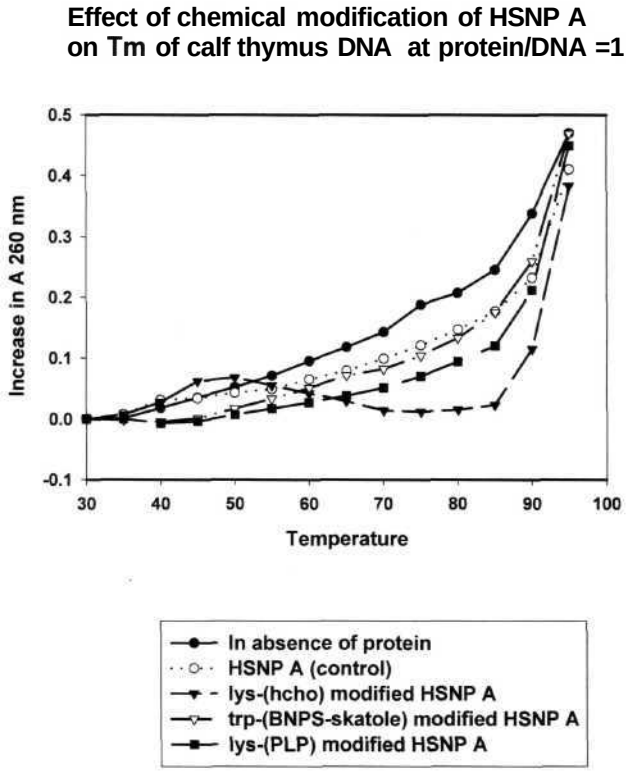

Fig 39: Thermal melting profile of Calf thymus DNA in presence of modified and unmodified HSNP A at a 1:1 DNA to protein ratio.

Fig 40: Electron microscopy of HSNP A: ds DNA complexes:

The experiment was performed by Dr. Rudi Lurz, Max Plank Institute for Molecular Genetics, Berlin, Germany.

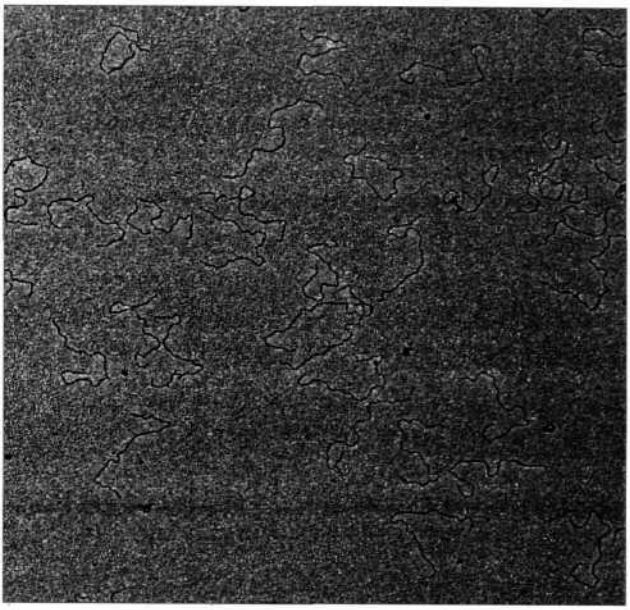

Fig 40a: Control panel of SmaI digested pUC18 plasmid DNA.

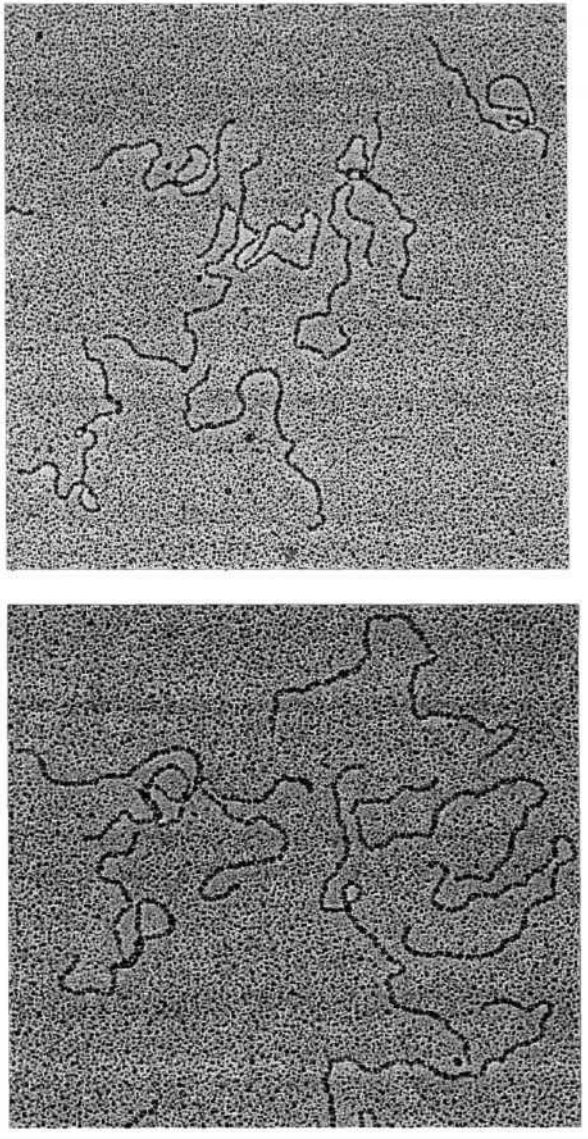

Fig 40b: HSNP A-ds DNA complexes formed at 1:10, DNA/protein (w/w) ratio.

HSNP C (Sac 7d) has been known to bind double stranded circular DNA forming looped DNA structure with central core of aggregated protein DNA complex (Fig 40c). Effect of HSNP A on the interaction of HSNP *C* (Sac 7d) was also studied. As shown in Fig 40d, when DNA was first incubated with Sac 7d for 5 minutes followed by incubation with HSNP A, the characteristic HSNP C core interacted with several molecules of ds DNA forming condensed structure. The looping here was not as pronounced as that observed in Fig 40c.

Incubating the DNA first with HSNP A for 5 minutes and then with HSNP C (Fig 40e) or incubation of DNA with HSNP A and Sac 7d together (Fig 40f) inhibited the formation of condensed structure by HSNP C. Implying that HSNP A coats the DNA uniformly and competes for the binding sites with HSNP C

Close examination reveals that HSNP C condenses the DNA binding to one of the ends of DNA molecule and brings rest of the DNA molecules together to the central core. In this complex HSNP A can be seen to bind at the other end of the DNA with progressively decreasing density of bound HSNP A towards the centre of the core.

These results suggest that HSNP A binds initially at one end of the DNA followed by binding of other HSNP A molecules.

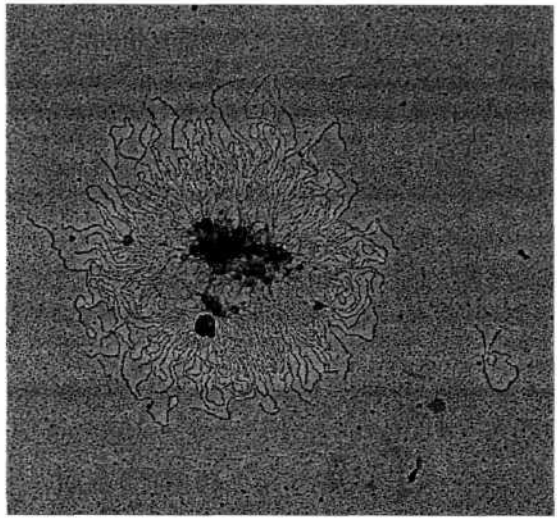

Fig 40c: HSNP C'-ds DNA complex.

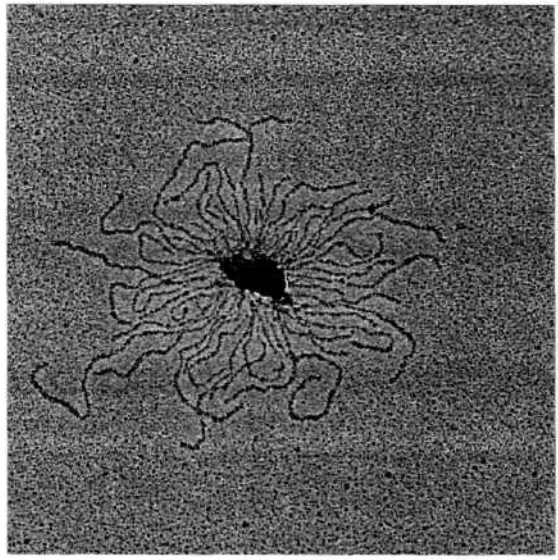

Fig 40d: Complex formed after incubating HSNP C and ds DNA for 5 minutes followed by addition of HSNP A.

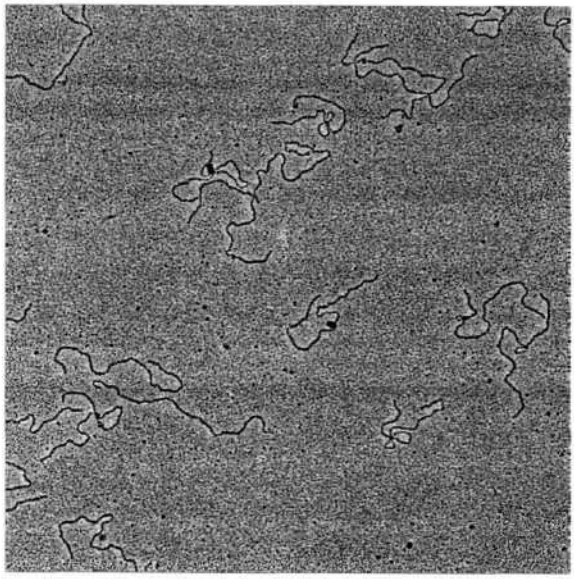

Fig 40e: ds DNA incubated with HSNP A for 5 minutes followed by HSNP C'

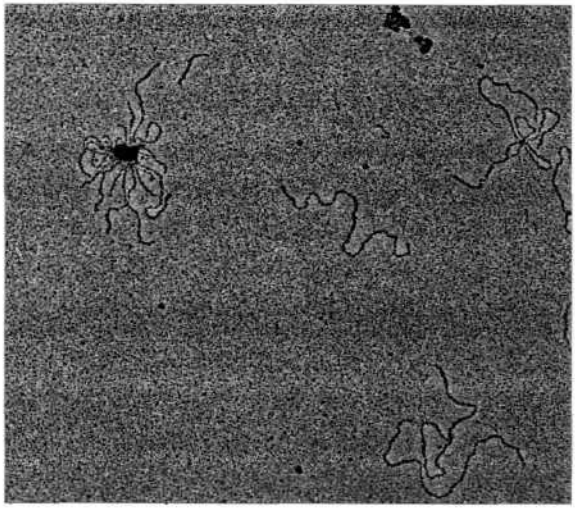

Fig 40f: ds DNA incubated with HSNP A and HSNP C' simultaneously.

Discussion

DISCUSSION

The focus of this study was the characterization of the nucleoprotein complexes present in the nucleoid of *Sulfolobus acidocaldarius*. The protein and the nucleic acids i.e., DNA and RNA components of the nucleoid, their roles in stabilization of the nucleoid and their variations during the midlogarithmic and stationary phases of the cell cycle were studied. Helix stabilizing nucleoid protein A (HSNP A) was purified and its properties were determined.

The histone like proteins of *Sulfolobus*, HSNP A, C and C have been shown to localize dominantly within the nucleoid (Bohrmann et al., 1994). The initial high molecular weight fraction from the sephacryl S-200 chromatography of nucleoid digested with micrococcal nuclease is free of HSNP A and HSNP C' whereas the latter fractions contain both these proteins along with some ~6- to 50 kDa and some ~25 to 15 kDa proteins. Previously, electron microscopy of HSNP C binding to DNA indicated that the protein condenses large regions of DNA to form a central DNA-protein core with loops of free DNA, invitro. HSNP C has been suggested to act as a scaffold protein for creating folded domains of intracellular DNA (Francina and Suryanarayana, 1995). During purification of HSNP A, it was found to constantly co elute with HSNP C, showing a high degree of affinity between the two proteins. The elution of HSNP C', a protein predominantly localized within distinct nucleoid subdomains (Bohrmann et al., 1994), separately, along with HSNP A and indicates its action, in vivo, as being similar to that of a scaffold protein.

The nucleoid of *S.acidocaldarius* fractionates into two distinct peaks on the sephacryl S-1000 column, peak I and peak II during the exponential phase. S-1000 elution profile of formaldehyde fixed nucleoid showing a single elution peak indicates that peak I and peak II are both components of the nucleoid interacting with each other.

Previously described histone like proteins, HSNP A, HSNP C, HSNP C' and DBNP B are present in both peak I and peak II. Peak II has a higher content of protein, DNA and RNA. In peak I, the protein to DNA ratio is higher than in peak I. S-layer membrane proteins were present in nucleoid which can be isolated. S-layer protein

subunit of 60 kDa were found to be mostly present in peak I. These results suggest that peak I may correspond to the nucleoid region associated with the membrane.

Small RNA molecules were observed in abundance in peak II, in exponential phase and to a lesser extent in stationary phase. Reduction of the level of these RNA molecules in the nucleoid, either upon addition of RNase or during stationary phase, corresponds to the appearance of low molecular weight SDS- PAGE protein pattern in peak I and a shift in the elution volume of peak II. Presence of abundant RNA molecules in peak II and lower abundance in peak II isolated from stationary phase cells indicate that the peak II may correspond to nucleoid region actively involved in transcription.

S-layer membrane proteins were detected in the nuceloid and peak I. S-layer proteins bind DNA and protected the peak II proteins against thermal denturation.The anomalous behaviour of peak I at different temperature conditions can also be attributed to these proteins. Disaggregation of peak I fractions at 37°C could be due to inability of S-layer protein to form complexes with DNA at lower than physiological temperatures.

Studies on the biochemistry of the nucleoid at different stages of growth correspond with the previous data collected from electron micrographs. Exponential phase cells which have a high transcription and translation rate show an abundance of RNA and S-layer proteins in the nucleoid. The decrease in the RNA and S-layer content in the stationary phase cells corresponds to a lowered rate of RNA synthesis. The nucleoid has been observed to have an organized structure at exponential phase. The sephacryl S-1000 profile of gives a distinct pattern of two markedly different peaks. Nucleoid organization at stationary phase is unstructured correspondingly the S-1000 elution pattern shows no distinct demarcation of peak 1 and peak II.

The variations in the sephacryl S-1000 elution profile of the nucleoid at exponential and stationary phases could be attributed to the difference in RNA and S-layer protein levels. If the coupled transcription, translation and insertion of membrane peptides occur in *Sulfolobus*, the above observations could account for the structured organization of the nucleoid at exponential phase and the unstructured organization at

stationary phase. The membrane peptide transcription complexes could anchor the genomic DNA to the membrane giving it an extended, structured appearance at exponential phase. The reduced transcriptional activity at stationary phase would leave the genomic DNA free of any anchor resulting in its relaxed unstructured appearance.

Micrococcal nuclease and DNase digestion studies indicated that the nucleoid in *Sulfolobus acidocaldarius* is poorly defined condensed structure with no repetitive nucleosome-like structure. DNA fragments in the size class of 500 kb are obtained when digested with nuclease. The DNA fragments could be looped regions of DNA with less protein bound at these regions. This is in contrast to the chromatin structure in euryarchaeota.

As already mentioned, *M.fervidus* and other methanogens contain histones which organize intracellular DNA into nucleosome-like structures. Chromosomal structure in halophilic euryarchaeon, *Halobacterium salinarium* was also shown to contain two components one being protein free DNA and the other DNA associated with nucleosomal structures as shown by electron microscopic studies (Takayangi et al., 1992).

The DNA helix stabilizing protein HSNP A was biochemically characterized and its interactions with DNA have been studied in HSNP A exists in oligomeric state in solution with predominantly tetrameric aggregation as indicated by gel filteration and cross linking experiments. The protein identity has to be estabilished through sequence analysis. This protein belongs to the Sac 10 group of proteins which are associated with intracellular DNA.

During purification the protein showed that it has strong interaction with Sac 7d protein. DNA cellulose chromatography in the absence and presence of mercaptoethanol showed that the protein has decreased affinity to HSNP C or that HSNP A binds weakly to DNA in presence of β-mercaptoethanol.

Fluorescence titration experiments showed that HSNP A binds to ss DNA and ds DNA with comparable affinity. The HSNP A could be binding at the minor groove as indicated by displacement of DNA bound ethidium by HSNP A.
Mobility shift experiments suggested the protein binding cooperatively to DNA as indicated by the presence of only free DNA and protein bound DNA species (in the wells) with no intermediately migrating DNA protein complexes.

Electron microscopy and mobility shift experiments with DNA protein complexes formed with HSNP A and HSNP AHSNP C showed that HSNP A interferes with the binding of Sac 7d to DNA. Furthermore, the electron micrographs indicate that HSNP A binds to DNA from the ends progressively binding to the internal region in the DNA.

Chemical modification experiments were performed to understand the nature of binding of the protein to DNA. Modification of lysine residues with formaldehyde or pyridoxal phosphate or modification of tryptophan with BNPS-skatole did not result in any significant change in the binding of the protein to DNA as shown by fluorescence titration and mobility shift assays. However modified proteins show increased protection of DNA against thermal denaturation. This could be due to increased hydrophobicity in the protein after modification, i.e., hydrophobic interactions of the protein to DNA could be responsible for the increased protection of DNA against thermal denaturation.

Finally, the protein could be binding to the looped domains of the nucleoid with condensed central core of the DNA bound by Sac 7d. Further experiments are needed to understand the mechanism of interaction of these proteins along with other proteins of the nucleoid.

Summary

Summary

Chapter 1 (Introduction):

A review of the cell and molecular biology of the archaea, their classification and characteristics followed by a review of the histone and histone like proteins present in the prokaryotes is covered in this chapter. This is followed by objectives and scope of the present investigation.

Chapter 2 (Materials and methods):

This chapter lists the materials used in the study and a brief description of the methods used during the course of the investigation. Purification protocol for nucleoid, S-layer and HSNP A are presented. The nucleoid was investigated and its components were determined and analyzed by micrococcal nuclease digestion, RNase digestion and formaldehyde fixation. The changes in the nucleoid composition during the growth phases were determined. Effect of temperature on the nucleoid components was monitored.

The solution state of HSNP A was studied by formaldehyde and dimethylsuberimidate cross linking. The nucleic acid binding properties of HSNP A were studied by flourimetry, gel mobility shift assay and thermal denaturation experiments. Effect of chemical modification of lysine and tryptophan residues of HSNP A on its DNA binding properties was also studied.

Chapter 3 (Results):

This chapter deals with the results that were obtained in the present investigation and contain the following sections.

3.1: Biochemical characterization of thermoacidophilic archaeal nucleoid of *Sulfolobus acidocaldarius.*

Sephacryl S-200 chromatography of the micrococcal nuclease digested nucleoid shows HSNP C and HSNP A coeluting in the later fractions. Of the two peaks resolved during the Sephacryl S-1000 column chromatography of the nucleoid peak I was found to have a higher protein to DNA ratio whereas the protein, DNA and RNA content was higher in peak II. The two peaks are different forms of the nucleoid. RNA was found to be a major component of the nucleoid and play an important role in maintaining the structural integrity of the nucleoid.

3.2: Studies on growth phase dependent variations in nucleoid

The elution profile of sephacryl S-1000 was found to vary during exponential and stationary growth phases. Peak I from nucleoid of stationary phase cells shows a profile similar to that of RNase digested exponential phase nucleoid. DBNP B protein is observed to be relatively more abundant whereas HSNP C levels are relatively reduced. Digestion of nucleoid with RNase does not have any effect on the peak I profile and only slightly shifts the peak II elution volume.

3.3: Effect of temperature on nucleoid fractions

The high molecular aggregate form of peak I was found to be maintained at the optimum growth temperature conditions of *Sulfolobus acidocaldarius* i.e., 70 C. The aggregate form is lost upon incubation at 37°C and regained on incubation at 70°C. There is no temperature induced change in the peak II profile.

3.4: Purification and characterization of S-layer proteins

The S-layer protein was purified as described by Grogan et al. The purified S-layer consists of two proteins, a 130 kDa protein and a 65 kDa protein. The 65 kDa protein bound to ds DNA cellulose column and eluted out at 300 mM. Nucleoid preparations and both peak I and peak II show a prominent band at ~ 65 kDa which could be this

protein which binds to the genomic DNA and is collected along with it during nucleoid purification.

3.5: Purification of HSNP A and characterization of its DNA binding properties

Helix stabilizing nucleoid protein A (HSNP A) is a nucleoid associated protein which protects DNA against thermal denaturation. The protein was purified and characterized for its biochemical and nucleic acid binding properties. It is a 10 kDa protein which was observed to occur as a tetramer or octamer in solution. Spectroscopic properties of the protein were also studied. The protein binds both ss and ds DNA, with greater affinity to ds DNA. The nucleic acid properties were studied by fluorescence titration, gel mobility shift assays and electron microscopy.

3.6: Studies on DNA binding properties of chemically modified HSNP A

HSNP A was treated with modification agents to modify the tryptophan and lysine residues. There was no inactivation of the DNA binding ability on modification. The modified protein showed an increased ability to protect the DNA against thermal denaturation.

Chapter 4: (Discussion):

This chapter deals with the conclusions derived from and the implications of the results observed during the course of the investigation.

Micrococcal nuclease digestion studies along with the electron microscopy studies of HSNP C' implicates HSNP C to act like a scaffold protein. The co elution of HSNP A along with HSNP C' during DNA cellulose chromatography and also during the chromatography of micrococcal nuclease digestion products shows a close interaction between the two proteins. The above observations and the occurrence of relatively high levels of HSNP A, DBNP B and HSNP C' during the regeneration of the *Sulfolobus* culture shows the need for further investigation into the roles of these proteins.

The two peaks resolved by the Sephacryl S-1000 chromatography of the nucleoid are both confirmed to be nucleoid components. The peak II has most of the nucleic acid

and the associated proteins of the nucleoid. Peak I has a higher protein to DNA ratio and is rich in S-layer proteins. The strong affinity to ds DNA observed in case of the small 65 kDa subunit of the S-layer protein along with the protein profile of peak I indicates peak I to comprise S-layer bound fraction of genomic DNA formed either during the purification of the nucleoid or comprising the genomic DNA and RNA involved in the coupled transcription and translation of the S-layer proteins.

RNA was observed to form a major component of the nucleoid. RNase digestion studies and the analysis of it effect on Sephacryl S-1000 column profile indicate a major role to RNA in maintaining the structural integrity of the nucleoid.

Temperature dependent variations are observed in peak I protein profile. Further investigation into the component(s) responsible for these changes is required.

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