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INTERACTION OF THE E. COLI SINGLE-STRANDED DNA-BINDING PROTEIN WITH NUCLEIC ACIDS AND ITS COMPARISON WITH PROTAMINE

A Thesis

Presented to

The Faculty of the Department of Chemistry

San Jose State University

In partial Fulfillment

of the Requirements for the Degree

Master of Arts

by

Shirin W. Hasan

August, 1995.

UMI Number: 1375698

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ABSTRACT

INTERACTION OF THE E. COLI SINGLE-STRANDED DNA-BINDING PROTEIN WITH NUCLEIC ACIDS AND ITS COMPARISON WITH PROTAMINE

by Shirin W. Hasan

Single-stranded DNA binding proteins or SSBs are nonspecific, single-stranded DNA binding proteins. They play a role in DNA replication, recombination, and repair. They are also referred to as unwinding, melting or helix-destabilizing proteins. SSBs are able to protect single-stranded DNA from nuclease digestion. Protamines are a class of low-molecular-weight proteins that protect the chromosomal DNA in the sperm cells of eukaryotic organisms. Protamines package the sperm DNA into a highly compact state and render it genetically inactive.

The binding properties of Eco SSB to single-stranded DNA have been integrated, correlated, and evaluated. The influence of different factors such as salt concentration, effect of ions, and temperature on the interaction of Eco SSB to DNA have been discussed. Finally, a comparison of the DNA-SSB binding with DNA-protamine binding has been presented.

ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Stacks, for her advice and understanding, for our innumerable discussions, and for all the time she gave me in completing this project. I am thankful to my committee members Dr. Biringer and Dr. Okuda, for a review of this manuscript and for all their helpful comments.

I would like to thank my husband, Waqar, for his moral support, help, inspiration and faith in me, without which, I would not have been able to complete this project. A very special thanks to my parents, for their love and encouragement all through, across the miles.

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INTRODUCTION

Proteins interacting with DNA can be divided into two classes characterized as "specific" and "nonspecific" respectively. Specific binding proteins bind with very high affinity to certain base sequences on DNA molecules. Examples of specific binding proteins are lac and lambda repressors. The lac repressor prevents the transcription of the lac operon, which encodes for the enzymes responsible for lactose metabolism. The lambda repressor prevents the release of the integrated lambda genome from its host's DNA (1).

The nonspecific binding proteins are those that have very little sequence specificity and may bind preferentially to either double or single-stranded DNA molecules. Examples of nonspecific binding proteins are protamines and histones, which bind to double-stranded DNA, and single-stranded DNA binding proteins such as *E. coli* single-stranded binding proteins (SSBs) and T4 gene 32 protein.

Protamines form a class of "low molecular weight" proteins that protect the chromosomal DNA in the sperm cells of eukaryotic organisms. They package the sperm DNA into a highly compact state, and the DNA-protamine complex coalesces into a highly condensed, biochemically and genetically inert state (2). Histones package the genomic DNA in somatic cells in a relatively less condensed state. Thus, DNA packaged by histones is available for DNA replication, transcription and repair processes (1).

Single-stranded DNA-binding proteins bind with high affinity to single-stranded DNA. The sequence of the single-stranded DNA-binding protein has been determined by Sancar et al. (3) These proteins play a role in DNA replication, recombination and repair. Because of their preferential binding to single-stranded DNA, these proteins have the ability to destabilize native, double-stranded DNA. For this reason they have been referred to as unwinding, melting or helix-destabilizing proteins. They are also able to protect single-stranded DNA from nuclease digestion and prevent replication and digestion, although the mechanisms by which these proteins function remain somewhat obscure (4, 5).

SSBs are known to be essential for DNA replication in phage T4, phage T7, *Escherichia coli* and yeast. They have the ability to convert double-stranded DNA into the single-stranded form at a temperature 40 ^{o}C below the melting temperature. The SSBs bind nonspecifically to single-stranded DNA at the

replication fork and stimulate the DNA polymerase. It is suggested that the polymerase and the SSB form a specific complex that interacts with DNA and other proteins during replication (6). The first example of this class of proteins, bacteriophage T4 gene 32, was discovered and characterized by Alberts and Frey in 1970 (6). *E. coli* SSB protein (Eco SSB) was first identified and purified by Sigal et al. in 1972 (7). These are the two SSBs that have been extensively studied. A basic difference between the two are that the T4 gene 32 protein functions as a monomer, whereas the Eco SSB functions as a homotetramer. The T4 gene 32 protein binds to single-stranded DNA with positive cooperativity, which allows continuous protein cluster formation and thus saturates the single-stranded DNA. The T4 gene 32 protein smoothes out the single-stranded DNA into a linear structure and protects it from nucleases. The role of SSBs *in vitro* may be to bring about denaturation of DNA, but their role *in vivo* may be to stabilize single-stranded DNA.

This paper is a review of the biochemical and biophysical studies carried out on the Eco SSB protein. It mainly deals with the single-stranded DNA binding properties of the Eco SSB protein in order to gain insight into the molecular basis of this phenomenon. The DNA binding properties of Eco SSB protein are compared with those of protamine binding to double-stranded DNA. The differences in the properties and functions of the Eco SSB and protamines lead to a clearer understanding of nonsequence-specific interactions of the DNA-binding proteins.

E. COLI SSB PROTEIN: STRUCTURE AND FUNCTION

Structure of Eco SSB

The *E. coli* SSB protein is a tetramer that is very stable at 30 nM NaCl, *in vitro* at 25 °C, and is most likely the active species *in vivo*. The monomer contains 177 amino acids (8, 9). Single crystals of tetramers have been obtained by Ollis and coworkers, but limited information was obtained due to proteolytic degradation of two of the four subunits (10). Preliminary X-ray crystallographic analysis of these partially proteolysed crystals indicates an asymmetric tetramer with D_2 symmetry. It was not possible to obtain high-resolution structural information with these crystals (10). The lack of high-resolution data has limited our complete understanding of the three-dimensional structure of the Eco SSB tetramer.

The physical properties of the Eco SSB tetramer can be summarized as follows (8):

frictional coefficient	1.42
sedimentation coefficient $S_{20,W}$	4.6S
isoelectric point	6.0

The value of the frictional coefficient (1.42) suggests that the tetramer is asymmetric in shape, which is also consistent with the X-ray crystallography data (9). Partial proteolysis studies have allowed a better definition of the structural and functional domains of Eco SSB protein (8).

The single-stranded DNA binding site of the Eco SSB protein lies within the N-terminal region (residues 1-105). This was evaluated by fluorescence quenching studies (8). A proteolytic fragment, containing the first 115 residues, bound with approximately the same affinity as native SSB did to single-stranded DNA, proving that the DNA binding site lies within the first 115 residues in SSB. A high content of basic amino acids lie in this region, which also suggests that the N-terminal region of the SSB is important in binding. All of the major proteolytic cleavage sites occurred in the region of SSB (residues 106-165). The amino acid sequence of the protein was determined by Sancar et al. (3). Based on the amino acid sequence, SSB can be divide into three structural domains. The amino-terminal two-thirds of the protein (residues 1

to 105) contains a large number of charged amino acids. Hence, it has been predicted to be highly ordered consisting almost entirely of α-helix and β-pleated sheets, although the model for this prediction was not cited (3). No cleavage sites were found between residues 5 and 105, indicating a compact and protease resistant structure in this region (8).

The function of the carboxy terminal domain in SSB can be summarized as follows: The carboxy terminal tail (residues 166 to 177) of the SSB protein is highly acidic and it can be removed by partial proteolysis (8). Upon binding to DNA, there is an increase in the susceptibility of the C terminus to proteolysis, which suggests that the carboxy terminus of SSB undergoes a conformational change. The increased exposure of these regions upon binding to single-stranded DNA has led to the suggestion that the carboxy terminal region becomes available for interaction with other proteins, such as proteins involved in DNA replication or repair, once the SSB is bound to the single-stranded DNA.

A finer dissection of domains was accomplished by tetramer formation and single-stranded DNA binding by different core polypeptides produced by partial proteolysis. SSB^*_T and SSB^*_C are the core polypeptides formed after trypsin cleavage at Arg115 or after chymotrypsin cleavage at Trp135, respectively. The ratio of the native tetrameric molecular weight to the subunit molecular weight for both SSB^*_T and SSB^*_C was found to be less than 4. This means that four subunits of SSB^*_T and SSB^*_C are not able to form the native core polypeptide tetramer. Both SSB^*_T and SSB^*_C lack the carboxy terminal domain. This suggests that the carboxy terminal domain may be required for the stabilization of tetrameric structure. Additionally, these core polypeptides show altered kinetics for DNA binding and enhanced helix-destabilizing activity. A marked decrease in the melting temperature, T_m, for poly[d(A-T)] was found if either SSB or the core polypeptides were present (Figure 1) (8). The T_m of poly[d(A-T)] in the presence of SSBs were in the order: native SSB > SSB^*_T > SSB^*_C (i.e., SSB^*_C was found to be the best helix destabilizer in this assay). These results led to the suggestion that the region on the SSB protein between residues 115 and 135 might be involved in stabilizing single-stranded DNA (8).

Eco SSB contains four tyrosine and four tryptophan residues per monomer (3). The tryptophan residues are important in the binding of SSB to

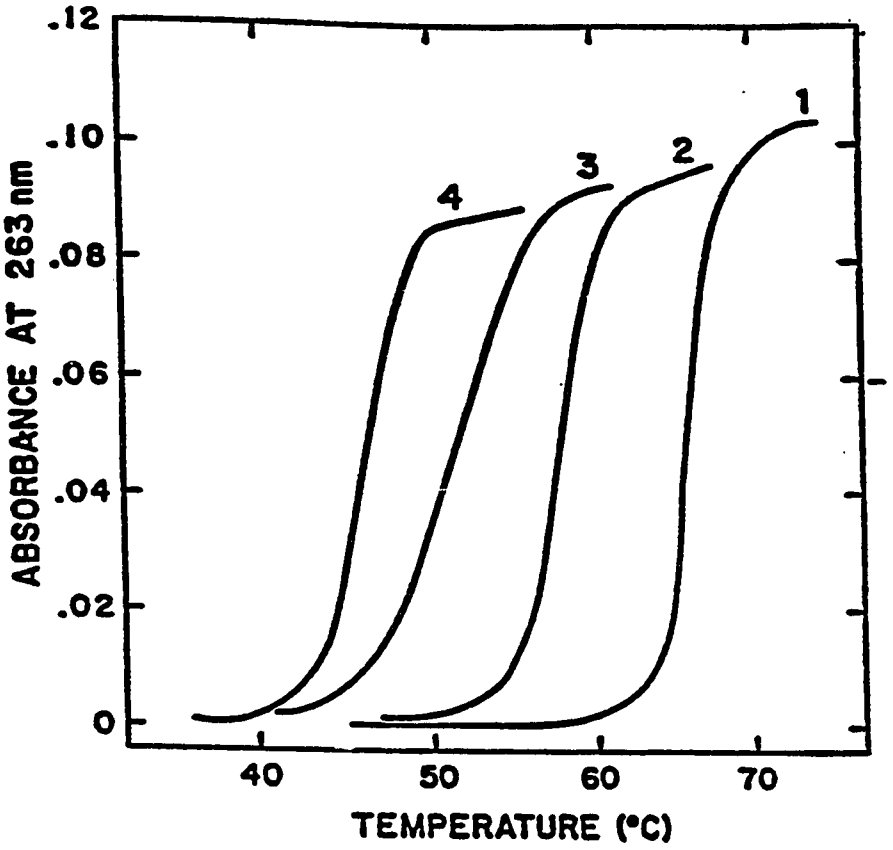

Fig. 1. Thermal denaturation of poly[d(A-T)] in the presence of SSB (8). "Poly[d(A-T)] at a concentration of 33.5 μM (phosphate) was melted in the absence of protein (Curve 1), in the presence of 2.84 μM (monomer) SSB (Curve 2), 4.52 μM SSB^*_T (Curve 3) or 3.92 μM SSB*C (Curve 4)". Absorbance at 263 nm was plotted against temperature. Reproduced with permission from Ref. 8. Copyright 1983 Dr. K.R. Williams.

DNA (11). The role of tryptophan in SSB-DNA binding will be elaborated upon later in the section "Binding Modes of *E. coli* SSB".

Many authors have worked on elucidating the native structure of the SSB-DNA complex. Sigal and coworkers (7) as well as Chrysogelos and Griffith (12) have used electron microscopy (EM). Both groups have used phage DNA. The results, however, are difficult to compare directly because sample preparation as well as fixing and staining procedures differed considerably.

Sigal et al. (7) visualized a complex of Eco SSB and circular single-stranded DNA of about 6600 nucleotides in length with electron microscopy using negative staining. The fully saturated complex had a circumference of 1.2 μm, which was 35% smaller than the circumference measured for the free circular DNA. The length increment was about 0.18 nm per nucleotide. Since the estimated internucleotide spacing in the complex is too small for a linear single-stranded DNA chain, these authors were the first to note that the DNA in the complex must be in a regularly folded state, or in a broad helix. The complex appeared as a "beaded necklace" of about 200 beads. The diameter of the beads was about 4.5-5 nm. The authors state a stoichiometry of about 33 nucleotides per bead (7). However, this holds true only if all the 6600 nucleotides were occupied by the 200 beads, since the beads were spaced at about 6 nm intervals along the DNA strand. Hence, this suggests that all the nucleotides are not occupied by beads.

Chrysogelos and Griffith (12) have given a somewhat different description of the structure of the Eco SSB-DNA complexes. They report an organization of circular single-stranded fd-DNA in a nucleosome-like structure. They have observed 38 to 40 beads per DNA circle using negative staining with electron microscopy. This gives a stoichiometry of 160 nucleotides per bead. The circumference of the DNA-SSB complex was found to be 0.54 μm, which was 30% of the length of the protein-free DNA (1.9 μm). The length increment was 0.8 nm per base and the diameter of each bead was found to be about 12 nm.

Chrysogelos and Griffith (12) have confirmed their results by nuclease digestion of native unfixed complexes as well as with equilibrium density banding of formaldehyde and glutaraldehyde fixed complexes. In contrast, Sigal's group (7) used electron microscopy and centrifugation to quantitate the weight ratio of SSB associated with DNA. In techniques such as EM, which use fixation methods, the structural features seen may reflect the preparative

procedure. Hence, to confirm such results, a strong accord needs to be established between the results obtained by EM and by methods which do not use fixation. The observed differences between groups may be due to the differences in sample preparation which could lead to different binding modes observed in these studies. These experiments will be discussed at greater length in the section, "Binding Modes of *E. coli* SSB".

On the basis of optical rotatory dispersion (ORD) and circular dichroism (CD) experiments, Kuil and coworkers (13) have reported a reduction in internucleotide distance from 3.0 Å to 1.8 Å, and a substantial tilt of the bases upon binding of single-stranded DNA to SSB, due to the coiling of the DNA around the SSB tetramer. They also used the electric-field-induced birefringence experiments to show that the projected base-base distance of the complex is about 0.23 nm, in agreement with electron microscopy results (0.18 nm) by Sigal and coworkers (7).

Function of Eco SSB

Genetic studies indicate that the Eco SSB protein is essential for replication, recombination and repair processes (4). The presumed role of the SSB protein in replication is that it binds cooperatively to the exposed single strands to prevent them from reannealing, and to also protect them from nuclease attack. The SSB protein has a large influence on the functions of many other proteins that interact with DNA. For example, Sigal et al. (7) have shown that the DNA polymerase II activity on gapped duplexes increases in the presence of SSB. The enzyme DNA polymerase II catalyses the synthesis of DNA from its deoxyribonucleotide precursors (1). The authors produced long single-stranded regions on intact double-stranded DNA by exonuclease III degradation. This was found to be a poor template for DNA polymerase II, but when Eco SSB was added to the solution, the rate of synthesis was stimulated at least ten-fold under the most optimum conditions. Thus, SSB was seen to enhance the activity of polymerase II on single-stranded DNA (7).

Polymerase II is not necessarily required in replication. Hence, *in vitro* studies do not prove the involvement of Eco SSB in replication, recombination and repair of single-stranded DNA. Its step-by-step involvement in these processes needs to be elucidated.

BINDING MODES OF E. COLI SSB

The tetrameric SSB protein binds to the single-stranded nucleic acids in a number of different binding modes *in vitro*. These modes differ in the number of nucleotides bound by the SSB tetramer. It is not yet known which of these modes function *in vivo*, but due to the highly different properties of the SSB tetramer in these different single-stranded DNA binding modes, it has been suggested that these different modes may function selectively in replication, recombination and repair (14).

Site size for SSBs

One of the primary physical quantities needed to characterize a protein-DNA interaction is its site size. "Site size" is the average number of nucleotides bound by a protein, and is characteristic for a particular protein-nucleic acid complex. It provides structural information and is also required for quantitative analysis of the nonspecific binding of a protein to linear DNA (15, 4). Different binding modes observed with single-stranded polynucleotides are designated by $(SSB)_n$, where n is the average site size per tetramer (15). In the Eco SSB system, the three main binding modes are the $(SSB)_{35}$, the $(SSB)_{56}$, and the $(SSB)_{65}$ modes with site sizes of n = 35($\pm$ 2), 56($\pm$ 3) and 65($\pm$ 3) nucleotides respectively (4). The solution conditions, particularly the salt concentration and type, lead to the formation of a particular binding mode.

The different binding modes differ in the following ways: in the case of $(SSB)_{35}$, single-stranded DNA is proposed to interact with only two subunits of the tetramer, whereas with the $(SSB)_{56}$ and the $(SSB)_{65}$ binding modes, single-stranded DNA is proposed to interact with all four subunits as shown in Figure 2 (4). Hence, the $(SSB)_{56}$ and the $(SSB)_{65}$ complexes provide greater compaction than does the $(SSB)_{35}$ complex. At 37 ^{o}C, a fourth binding mode with a site size of 42 ($\pm$ 2) nucleotides per tetramer is also seen for the SSB-poly(dT) interaction (16). A subset of the SSB tetramers can form octamers after binding to single-stranded DNA, as shown in Figure 2 (4). The exact details of this structure have not been determined.

The two morphologies of the SSB-DNA complex

Chrysogelos and Griffith have shown two distinct morphologies for the SSB-DNA complex by electron microscopy (12). A "beaded" complex was observed at high salt and low protein to DNA ratios, and a "smooth-contoured" complex was observed at low salt and high protein to DNA ratios. EM

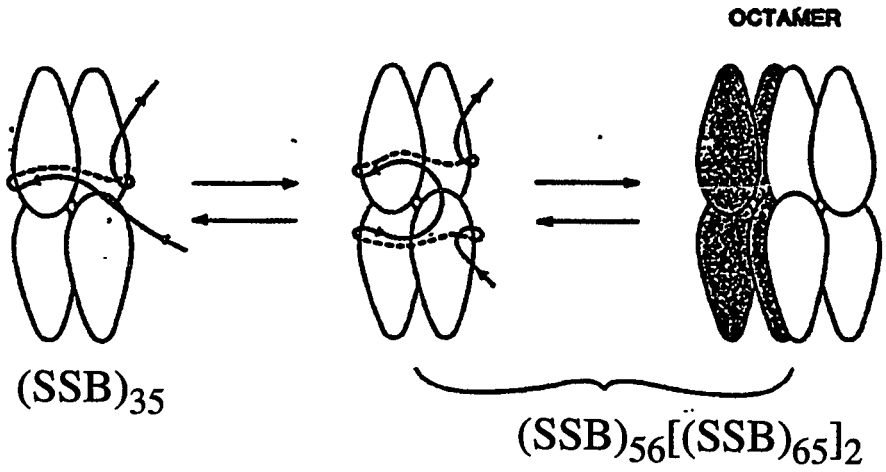

Fig. 2. Schematic depicting the different SSB-ssDNA binding modes. The interaction of SSB with DNA in the $(SSB)_{35}$ and $(SSB)_{65}$ modes is shown (4). Reproduced with permission from Ref. 4. Copyright 1994 Annual Reviews Inc.

measurements and stoichiometries were consistent with the beaded complex corresponding to the $(SSB)_{65}$ or the $(SSB)_{56}$ mode, and the smooth-contoured complex corresponding to the $(SSB)_{35}$ mode.

Extensive digestion of the native, unfixed SSB-fd DNA complexes (where fd DNA is the single-stranded DNA from phage fd), with micrococcal nuclease revealed a protected DNA fragment of 145 bases. DNase I digestion of native, unfixed complexes produced a repeating pattern of bands, thus confirming the presence of a "nucleosome-like repeating structure" or "beads" suggesting that the DNA is wrapped around a protein core. Based on the amount of DNA and SSB added to form the complexes, and the molecular weights of the fd DNA and Eco SSB, it was calculated that each bead is composed of two tetramers with about 73 nucleotides per tetramer, or 145 nucleotides per octamer (12). The reported binding site size for the SSB tetramer single-stranded DNA complex varies a great deal in the literature. The reasons for this may be due to the differences in SSB preparation and purification, or the different molar absorptivities that have been used for determining concentrations (4).

Between each "nucleosomal-like bead" is a stretch of 30 bases of protein-free single-stranded DNA. This could be available for interaction with other proteins like RecA, which is involved in DNA recombination (12). Figure 3 (17) shows the electron micrographs of the beaded and the smooth-contoured complexes. The procedure used for electron microscopy was as follows: Purified SSB was mixed with M13 single-stranded DNA in a buffer of 0.01 M tris (tris(hydroxymethyl) aminomethane) and 1 mM ethylenediaminetetraacetic acid (EDTA) at pH 7.5. Samples were incubated for 5 minutes in different concentrations of NaCl, chilled on ice and fixed with the sequential addition of formaldehyde and glutaraldehyde. The samples at different concentrations were prepared for EM by direct mounting onto thin carbon films and shadow casted with tungsten. In Figure 3, the first panel is the lowest magnification required to obtain a "field view". The magnification increases in the consecutive panels, from left to right, with the last panel being the highest magnification. Panel A (right) clearly shows the beaded form, whereas in panels C and D smooth-contoured forms are obvious. A drawback in this technique is that tungsten shadowing has a tendency to make objects look more rounded and hence, it is not so easy to distinguish between the two forms. Griffith and

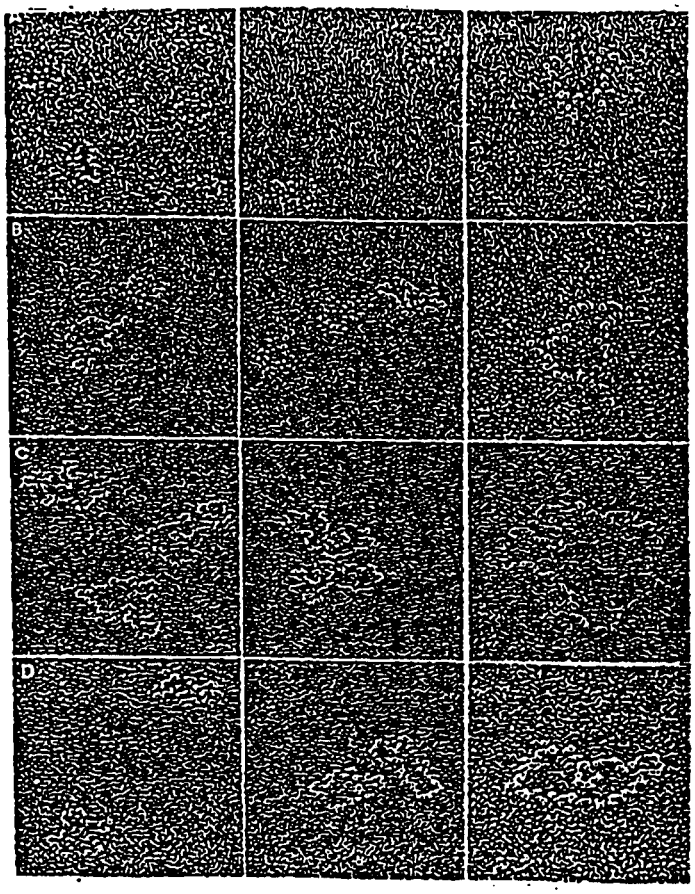

Fig. 3. Visualization of SSB-ssDNA interaction by electron microscopy (17). Purified SSB was mixed with M13 ssDNA in a buffer of 0.01 M Tris, 1 mM EDTA (pH 7.5) (Panels A-C), or 0.02 M HEPES (pH 7.5), 30 mM NaCl, 12 mM $MgCl_2$, 3 mM ATP (Panel D) and incubated for 5 min at 37 °C. Input SSB to DNA weight ratios in Panel A were 1.5 : 1, and 3 : 1 from left to right. In Panel B, all input weight ratios were 3 : 1, and for Panels C and D, the input weight ratios were 12 : 1. Reproduced with permission from Ref. 17. Copyright 1984 Cold Spring HarborLab. Symposia on Quant. Biology.

coworkers (17) have shown that the apparent contour length of the smooth-contoured complex (0.85 mm) is about twice as long as that of the "beaded" form (0.5 mm). Since DNA interacts with only two subunits of the SSB tetramer in the smooth-contoured complex, and it interacts with all four subunits of the tetramer in the beaded complex, the DNA is more compact in the beaded complex. In their work, a stoichiometry of the protein to DNA ratio of the beaded SSB-single-stranded DNA was reported to be 3 : 1, based on the amount of each material added to form the complexes.

In contrast, Sigal et al. (7) have shown a ratio of 8 : 1 SSB to DNA, based on the amount of SSB and DNA added in the test mixture. A constant amount of single-stranded DNA was mixed with different amounts of protein, and the instantaneous drop in absorbance at 260 nm upon Mg^{++} addition was measured and plotted for each DNA to protein ratio tested. At ratios equal to or exceeding saturation of the complex, no change in absorbance should be observed. The results obtained indicated that the DNA should be saturated with the protein at a protein to DNA ratio of 8 : 1. In another set of experiments, they ran the free DNA and the DNA-protein complexes on a sucrose gradient and the material was separated on the basis of size and molecular weight. By both these experiments, they found a weight ratio of 8 : 1 of protein to DNA (7). Since it was not recognized in 1972, that SSB can bind to single-stranded nucleic acids in multiple binding modes, these measurements were performed under conditions that favor a mixture of binding modes. A model to explain the basis of site size has been proposed by Lohman and coworkers (4). It is known that smooth-contoured complexes form at high protein to DNA ratios, which favors the mode with smaller site-size. As shown in Figure 4 A, only two subunits of the tetramer are shown to interact with DNA in the $(SSB)_{35}$ binding mode. Figure 4 B shows the proposed beaded forms of the larger site size mode. The single-stranded DNA is in an extended form in Figure 4 A, whereas it is more compact in Figure 4 B, with all four subunits interacting with DNA. This clustering is limited to the formation of octamers, or dimers of the tetramers (4, 10). The model does not allow more tetramers to cluster to an octamer after its formation.

Role of tryptophan in SSB-single-stranded DNA binding

Bandyopadhyay and Wu (11) have studied the role of tryptophan residues in the SSB-DNA interaction. Each Eco SSB monomer contains four

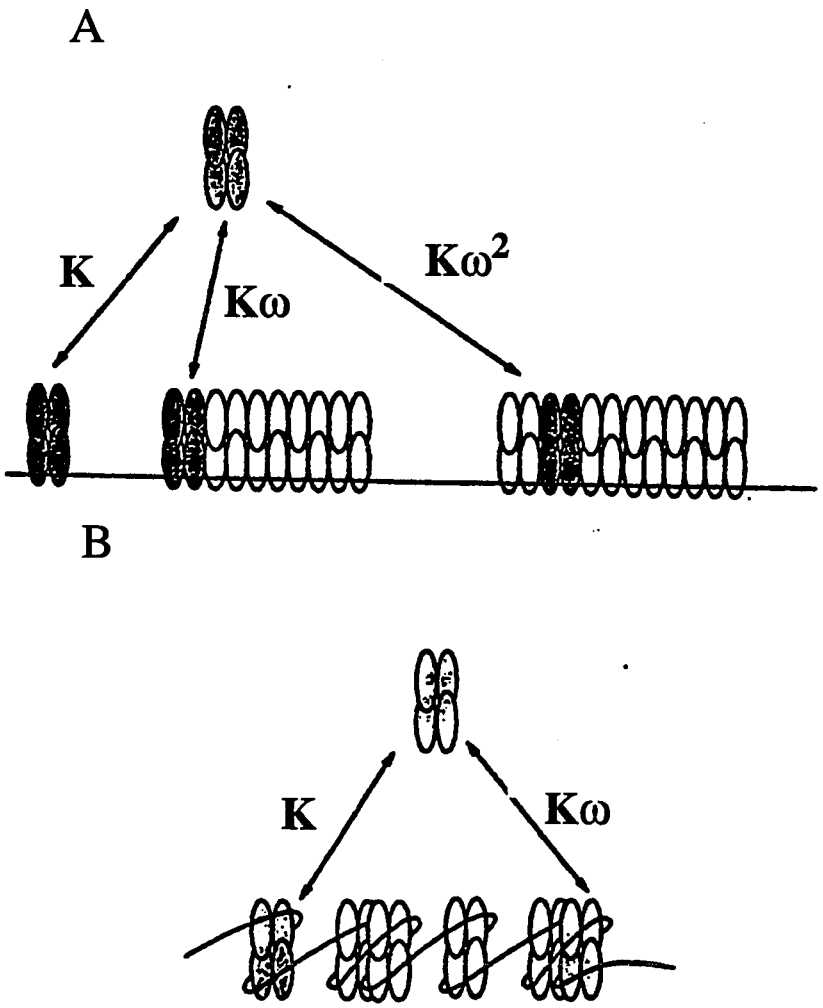

Fig. 4. Schematic view of two types of cooperative binding models (4). The three shaded SSB tetramers represent binding in an isolated manner (with equilibrium constant K), a singly contiguous manner (with equilibrium constant ($K\omega$), and a doubly contiguous manner (with equilibrium constant ($K\omega^2$), where ω is the cooperativity parameter. Reproduced with permission from Ref. 4. Copyright 1994 Annual Reviews Inc.

tyrosine and four tryptophan residues (3). For most proteins that contain tyrosine as well as tryptophan residues, tyrosine emission is found to be negligible due to the high efficiency of energy transfer from tyrosine to tryptophan. However, tyrosines located far from the tryptophan residues can contribute to the protein emission spectrum. To check the role of tyrosine and tryptophan in the emission spectrum of SSB, emission spectra at two excitation wavelengths were compared. The results showed that tyrosine emission is negligible in SSB. In addition, they found the emission maximum for native SSB protein was 345 nm, whereas for the denatured protein it was 350 nm. In the SSB-DNA complex it was 345 nm, suggesting that there was no structural change taking place in the SSB protein upon binding to DNA, as the tryptophan residues were neither buried nor exposed further. Furthermore, the quantum yield of the tryptophan emission of the native SSB protein was found to be 0.13, whereas the quantum yield of the tryptophan emission of the SSB bound to single-stranded DNA was reduced to 0.025. Hence, fluorescence quenching takes place upon SSB binding to DNA, suggesting that the tryptophan residues are involved in the interaction. The authors attempted to probe the accessibility of the tryptophan residues upon the binding of SSB to DNA via fluorescence quenching. They found that in the presence of DNA, the tryptophan residues became less accessible to ionic quenchers such as iodide. Stern-Volmer plots (fluorescence quenching plotted against the concentration of the quencher) indicated that the tryptophan residues were heterogeneous in their accessibility to quenchers.

These fluorescence experiments indicate that tryptophan residues on the SSB protein are involved in the interaction with single-stranded DNA, and are probably located in the hydrophobic pockets of the protein, which makes them even less accessible to the quenchers. These studies indicate that the tryptophan residues are near or at the DNA binding site.

Bujalowski and Lohman (18) have also used fluorescence quenching studies to determine the role of tryptophan in SSB-DNA binding. The tryptophan fluorescence was partially quenched upon binding single-stranded nucleic acids, with nearly complete quenching of Trp54 and Trp88 (18). In complexes with poly(dT), SSB tryptophan fluorescence was quenched 89 ± 2% in the $(SSB)_{65}$ and $(SSB)_{56}$ modes, and 53 ± 3% in the $(SSB)_{35}$ mode (18). These results are consistent with the proposal that tryptophan residues are involved

in the binding of Eco SSB to DNA, and also that only two subunits of the SSB tetramer interact with DNA in the $(SSB)_{35}$ mode and with all four subunits in the $(SSB)_{65}$ and $(SSB)_{56}$ modes.

Salt-dependent transitions between the binding modes

The binding modes are affected by a variety of factors such as the concentration of the monovalent salt, divalent cations, multivalent cations such as polyamines as well as by protein binding density, pH and temperature (18).

Researchers have shown that more than one binding mode exists for the SSB-DNA complex. The following section explains the salt-dependent transitions between the different modes. Different researchers have used varying concentrations of different salts to demonstrate these transitions. Lohman and coworkers (18-20) have used different monovalent and divalent cation salts, and polyamines. Poly(dT) was the model single-stranded DNA used for complex formation.

Lohman and Overman (20) performed fluorescence quenching experiments to examine the transitions in different binding modes with added salt. They titrated free SSB and SSB bound to a two-fold excess of poly(dT), while monitoring the SSB fluorescence. The extent of fluorescence quenching went through a transition as a function of NaCl concentration. They found that the quenching at low NaCl concentration was 51 ± 3%, the complex formed at this low NaCl concentration was referred to as the $(SSB)_{35}$ complex. As the NaCl concentration reached about 0.1 M, the quenching reached a plateau of 83 ± 3%. The complex formed at this high NaCl concentration was referred to as the $(SSB)_{65}$ complex. Thus, the fluorescence change can be described by a two-state model, which can be identified by different site-sizes and different extents of tryptophan fluorescence quenching. This effect seemed to be due to the cation alone, since NaCl, NaF and $NaC_2H_3O_2$ had the same effect on the transition. On the basis of these experiments, Lohman and Overman concluded that SSB binds to DNA in two different binding modes, the $(SSB)_{35}$ and the $(SSB)_{65}$ modes, respectively (20). Bujalowski and Lohman (18) followed up this study using both NaCl and $MgCl_2$. Figure 5 shows the salt-dependent transitions among the $(SSB)_{35}$, $(SSB)_{56}$ and $(SSB)_{65}$ binding modes of the tetramer-poly(dT) complex (18). The $(SSB)_{35}$ mode was found to be stable when the NaCl concentration was less than or equal to 10 mM, but increasing the

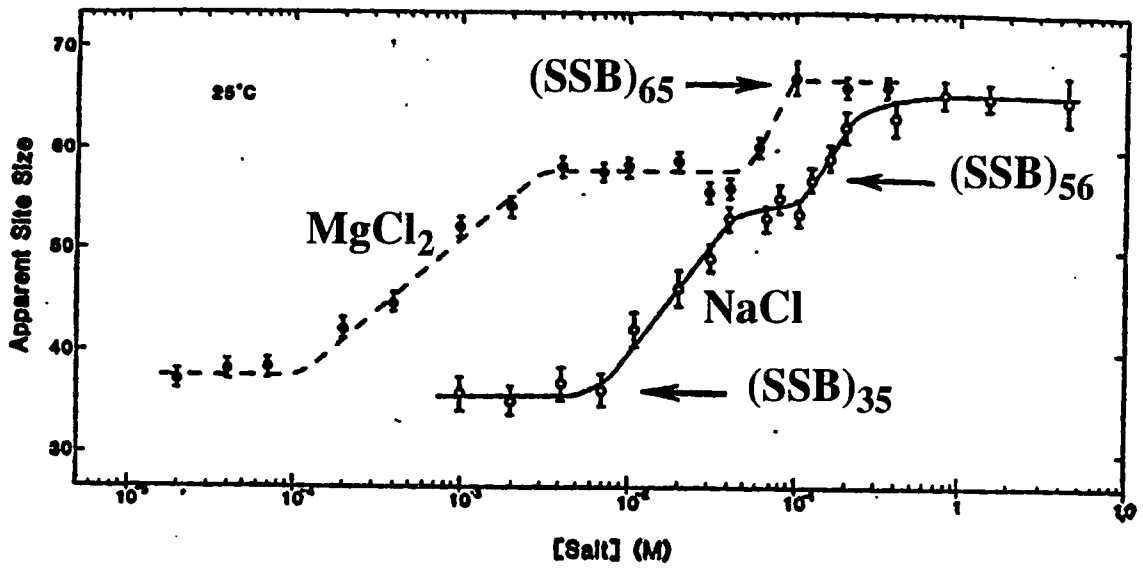

Fig. 5. Dependence of site size on salt concentrations as measured by monitoring the quenching of the SSB tryptophan fluorescence upon addition of poly(dT) (18). Reproduced with permission from Ref. 18. Copyright 1986 American Chemical Society.

NaCl concentration induced transitions from the $(SSB)_{35}$ mode to the $(SSB)_{56}$ mode, and finally to the $(SSB)_{65}$ mode, which was stable at NaCl concentration greater than or equal to 0.2 M. The $(SSB)_{56}$ mode was stable at intermediate concentrations of NaCl (18). $MgCl_2$ showed the same effects, although much lower concentrations were required. Wei, Bujalowski and Lohman (19) have shown that multivalent cations like the polyamines, $spermine^{4+}$ and $spermidine^{3+}$ are able to achieve the same effect with poly(dT) at micromolar concentrations (6 μM). These experiments reflect the fact that cation binding induces $(SSB)_{35}$ to $(SSB)_{65}$ transitions, and the more highly charged cations have a higher affinity for the complex, as they bind cooperatively (19), resulting in the uptake of a large number of cations. This additional cation binding may be required to partially neutralize single-stranded spacer DNA leading to its binding to the third and fourth subunits of the SSB tetramer in order to form the $(SSB)_{65}$ or the $(SSB)_{56}$ mode (19). Double-stranded DNA is stabilized by base pair stacking and a particular arrangement of the phosphate groups to lower charge-charge repulsion. On single-stranded DNA, the negative charges repel each other and there is less stabilization by base stacking. This results in a longer contour length compared to double-stranded DNA. With the $(SSB)_{35}$ complex formation (i.e. at low salt conditions), the positive charges of the protein lie on the DNA. This partially neutralizes the negative charges and lowers charge repulsion. Hence, the DNA becomes more stable, resulting in a shorter contour length. With the $(SSB)_{65}$ complex formation, (i.e. at high salt conditions), charge neutralization of the spacer DNA is more pronounced, which further enables the DNA to bind to the third and fourth subunits of the SSB tetramer and to wrap around it. Hence, the total compaction is much greater (4).

Mechanism of binding of single-stranded DNA to SSB tetramer

McGhee and von Hippel (15) have put forth a general model to explain the protein-DNA binding. There are three distinguishable types of ligand binding sites when only nearest neighbor ligand-ligand and direct ligand-lattice interactions are considered. These binding sites are schematically shown in Figure 6 (15). A ligand is assumed to bind to the lattice, (in this case single-stranded nucleic acid) and to cover 'n' consecutive lattice residues. Ligand-ligand interactions are only allowed between nearest neighbors bound without intervening free lattice residues. The three types of binding sites that result are:

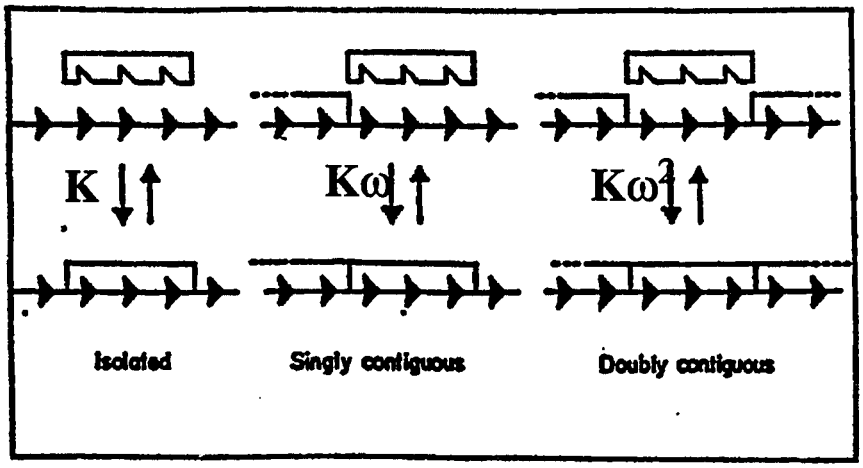

Fig. 6. The three types of ligand binding sites proposed by McGhee and von Hippel (15). K is the association constant, and ω is the cooperativity parameter. Reproduced with permission from Ref. 15. Copyright 1974 Academic Press Ltd.

(i) an 'isolated' site to which the ligand binds with association constant K, (ii) a 'singly contiguous' site to which the ligand binds with association constant Kω, and (iii) a 'doubly contiguous' site to which the ligand binds with association constant $K\omega^2$, where ω is the cooperativity parameter. When ω is greater than 1, there is attraction between the ligands and the binding is positively cooperative. When ω is less than 1, there is repulsion between the ligands and the binding is negatively cooperative. When ω is equal to 1, binding is noncooperative.

McGhee and von Hippel have also shown why the traditional Scatchard analysis fails even for noncooperative binding. The Scatchard equation was originally derived for the interaction of small ligands with multiple, but discrete and isolated binding sites on a lattice (15). This derivation neglects the important consideration that on a homogeneous lattice with no bound ligands, nearly every lattice residue is able to start a ligand binding site. As shown by McGhee and von Hippel in Figure 7 (a), a number of different binding sites can be the initial binding site. Thus, by binding the ligand on a particular lattice, other potential ligand binding sites are eliminated (15). Figure 7 (b) shows that when two ligands are bound on a homogeneous lattice with a gap of 'g' residues between them, the number of binding sites between them is g-n+1 if g is greater than or equal to 1, where n is the binding site size, or it is 0 if g is less than 1. These facts make lattice saturation difficult to achieve. Hence, the Scatchard equation cannot be used universally to analyze data in its original form. McGhee and von Hippel have derived an equation relating the binding parameters (K, n, ω) to free ligand activity and number of ligands bound. The model put forth by McGhee and von Hippel has been used by a number of authors, as will be evident later, to study the interaction between single-stranded DNA and SSB as well as protamine and double-stranded DNA.

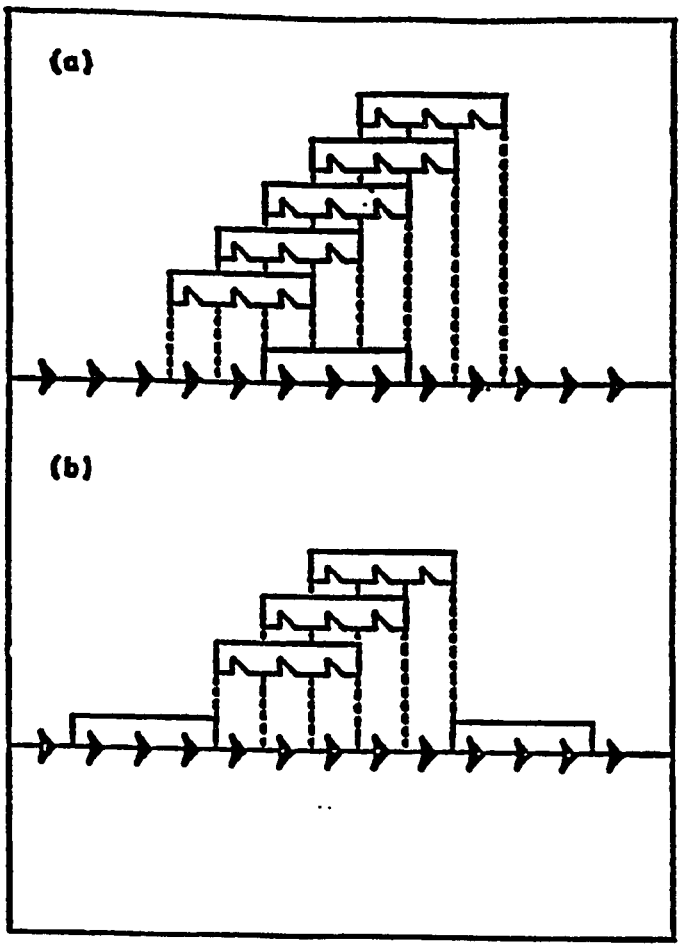

Fig. 7. "Potential ligand binding sites eliminated on a naked lattice by the binding of one ligand" (15). Reproduced with permission from Ref. 15. Copyright 1974 Academic Press Ltd.

THERMODYNAMICS AND EQUILIBRIUM BINDING OF SSB TO SINGLE-STRANDED DNA

A quantitative study of the equilibrium binding of a protein to a nucleic acid is done to obtain the following information: (i) the stoichiometry of interaction, (ii) the existence and the degree of positive or negative cooperativity, (iii) the binding constants, (iv) the dependence of the equilibrium binding parameters on solution variables such as pH, temperature and salt concentration, and (v) the thermodynamic quantities such as change in free energy (ΔG), change in enthalpy (ΔH) and change in entropy (ΔS). All of these parameters can give a great deal of information about the molecular forces involved in the interaction. In order to achieve these goals, equilibrium binding isotherms must be measured over a wide range of binding densities. An equilibrium binding isotherm is the relationship between the ligand (protein) bound per macromolecule (nucleic acid), and the free ligand concentration at a constant temperature (21, 22).

An understanding of macromolecular interactions such as protein-nucleic acid interactions require information about the thermodynamics and kinetics involved in the interactions, and their structures. The basis for the functions and control of the protein-DNA complexes requires an understanding of the stability, specificity and mechanisms of the interaction. Thermodynamic studies provide the information required to determine the forces that stabilize the protein-DNA interactions and structural changes that result upon binding (23).

A number of laboratories have studied the equilibrium binding properties of the SSB tetramer with single-stranded oligonucleotides to reduce the complexities present in the interaction of the SSB tetramer with single-stranded polynucleotides. Early work with SSB was difficult to interpret quantitatively, as SSB binding to single-stranded nucleic acids resulted in a mixture of binding modes. In fact, conditions that populate a single binding mode must be used. This ensures that all equilibrium isotherms are performed under conditions in which a single equilibrium species such as the $(SSB)_{65}$ mode is present, in order to provide a simple analysis of the thermodynamic parameters. The $(SSB)_{65}$ binding mode is the only one that exists under high salt conditions, hence it has been studied in isolation on single-stranded nucleic acids. The limited cooperativity model of McGhee and von Hippel (15) has been

used to analyze equilibrium binding in the $(SSB)_{65}$ mode and to obtain values for the association constant, K^{65}, and the limited cooperativity parameter $\omega_{65,lim}$, for $(SSB)_{65}$.

Fluorescence quenching assay for SSB binding to DNA

Quantitative studies of the effects of salt concentration on protein-nucleic acid interactions can provide useful information about the number of electrostatic interactions in the complex as well as the mechanism of the interaction. The interactions between proteins and nucleic acids are usually very sensitive to changes in the salt concentration in solution. Fluorescence quenching studies can be used to determine the maximum fluorescence quenching, Q_{max}, for the interaction of the SSB protein with DNA. Overman et al. (22) have used fluorescence quenching studies to determine the fraction of bound SSB protein in the $(SSB)_{65}$ binding mode as a function of the degree of fluorescence quenching.

A method of analysis was developed by these authors (22), which allows one to determine the binding density, υ, and the free protein concentration from titrations, which monitor a change in signal from the ligand upon binding to the macromolecule. Titrations of SSB with single-stranded homopolynucleotides were performed while monitoring the quenching of the intrinsic tryptophan fluorescence of SSB. If the total ligand concentration, L_T, is plotted against the total nucleic acid concentration, D_T, then the average binding density, υ, is equal to the slope of the plot, and the y-intercept is the free protein concentration, L_F. They have shown for this system that $L_B/L_T = Q_{obs} / Q_{max}$, where L_B is the bound ligand concentration, and Q_{obs} is the observed fluorescence quenching. Fluorescence quenching of tryptophan was monitored for SSB binding to poly(U) in 10 mM Tris, 0.1 mM EDTA at pH 8.1, and 0.25 M NaCl. The results were plotted against the fraction of bound SSB protein, L_B/L_T, as shown in Figure 8 (22). From Figure 8, it is evident that the degree of fluorescence quenching is directly proportional to the fraction of protein bound. By an extrapolation to $L_B/L_T = 1$, one finds that $Q_{max} = 0.59$ for the SSB-poly(U) interaction under the given conditions. The maximum fluorescence quenching, Q_{max}, was related to binding affinities, pH and temperature (22). The value of Q_{max} was found to be correlated to the affinity of SSB for different homopolynucleotides, ranging from 0.39 for poly(A) to 0.9 for poly(dT) (0.25 M NaCl, 10 mM Tris and 0.1 mM EDTA) (22). Overman (24) has shown that for

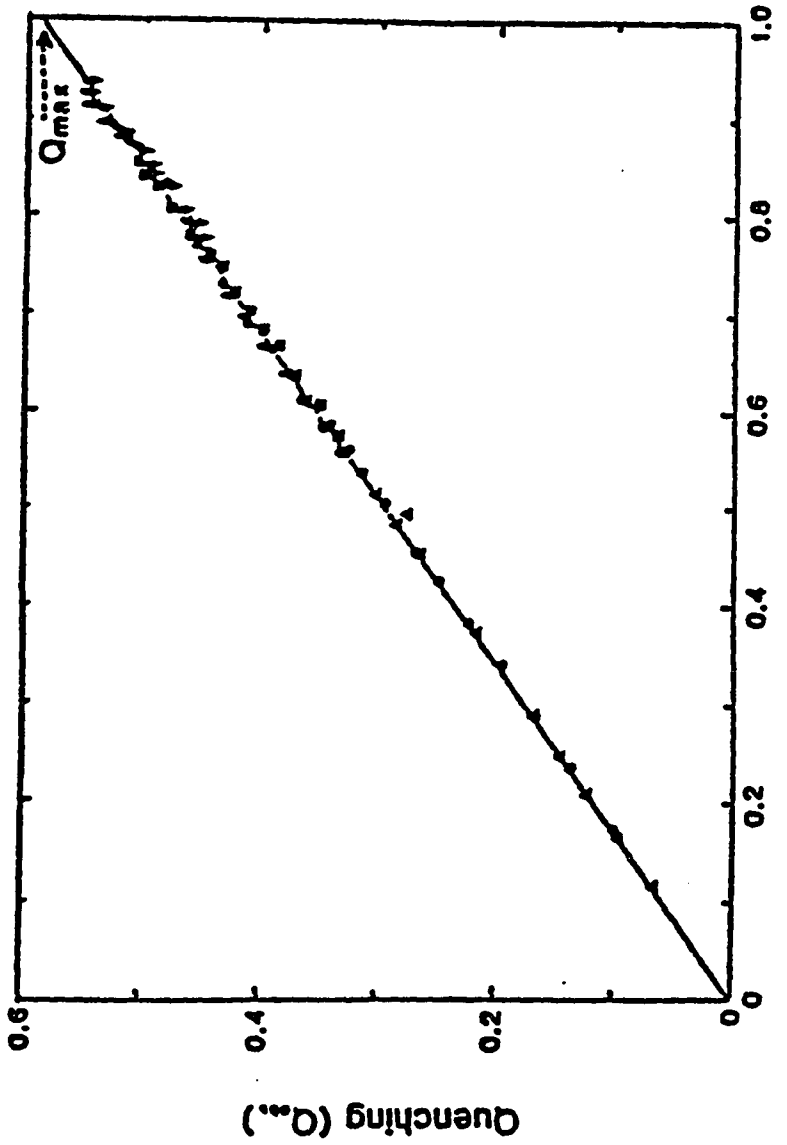

Fig. 8. "Determination of Q_{max}, and demonstration that $Q_{obs}/Q_{max} = L_B/L_T$" (22). L_B/L_T is the fraction of the total ligand bound. L_B is the amount of protein bound, and L_T is the total input protein. Reproduced with permission from Ref. 22. Copyright 1988 Dr. T.M. Lohman.

the SSB-poly(U) interaction, Q_{max} ranges from 0.5 at pH 9.0 to 0.72 at pH 5.5. Similarly, Q_{max} for the SSB-poly(U) interaction has been shown to range from 0.51 at 37 °C to 0.72 at 10 °C. The author states that the reason for this variation of Q_{max} is unknown (24).

Electrostatic interactions during SSB-DNA binding

Changes in salt concentration in solution play an important role in equilibrium binding and kinetics of protein-nucleic acid interactions. The major effect arises from the fact that linear nucleic acids are negatively charged, hence they attract cations which partially neutralize the high charge density on the nucleic acid.

In the equilibrium binding of a protein P, with nucleic acid D, to form a complex PD, the intrinsic equilibrium association constant, K_{obs}, is defined in terms of P and D (22).

$$P + D \rightleftharpoons PD \qquad (1)$$

$$K_{obs} = [PD] / [P][D] \qquad (2)$$

The intrinsic equilibrium association constant, K_{obs}, can be used to used to calculate the true thermodynamic quantities, ΔG^o, ΔH^o, and ΔS^o. The relationship between these quantities is given by the following equation:

$$\Delta G^o = -RT \ln K_{obs} = \Delta H^o - T\Delta S^o \qquad (3)$$

where ΔG^o is the Gibb's standard free energy change, ΔH^o is the change in intrinsic binding enthalpy, ΔS^o is the change in binding entropy, R is the gas constant and T is the absolute temperature. The temperature dependence of the intrinsic binding constant K_{16}, for $dT(pT)_{15}$ was shown in the form of van't Hoff plots (log K versus 1/T). From the temperature dependence of K_{16}, the intrinsic binding enthalpy, ΔH^o, was calculated to be -26 (± 3) kcal/mol and the binding entropy, ΔS^o, was found to be -60 (± 5) cal/mol K, at pH 7.4 and 0.2 M KCl. This indicates that the intrinsic binding interaction between $dT(pT)_{15}$ and the SSB tetramer is enthalpy driven (21).

An additional consideration in DNA-protein binding, is the role of electrostatics. The following derivation shows the relationship between K_{obs} and z, the number of ionic interactions made upon complex formation (22). Say a protein with a positively charged binding site or an oligocation of charge z^+ binds to a nucleic acid site in the presence of excess univalent salt (MX). Some

counterions $z\psi M^+$ will be released, where ψ is the number of counterions thermodynamically associated per nucleic acid phosphate. These counterions are released, since all cations are not needed to partially neutralize the negative charge on the phosphates involved in the protein-DNA interaction. If the protein also binds cations or anions and this ion binding is disturbed by its interaction with the nucleic acid, then a release or uptake of cations (M^+) or anions (X^-) from the protein can take place. Overman et al. (22) demonstrated that there is a net release of both cations (ΔC) and anions (ΔA) during the formation of the $(SSB)_{65}$ complex. A net release or uptake of water molecules (Δw) or preferential hydration can also accompany the formation of the $(SSB)_{65}$ complex. Equation (1) can be rewritten for the presence of excess monovalent salt as,

$$P + D \rightleftharpoons PD + \Delta CM^+ + \Delta AX^- + \Delta w H_2O \qquad (4)$$

Since ΔC has contributions from the "release of $z\psi$ cations from the nucleic acid as well as the uptake of b cations" (22), then

$$\frac{-\partial \log K_{obs}}{\partial \log [MX]} = (z\psi - b) + \Delta A - 2[MX]\frac{\Delta w}{[H_2O]} \qquad (5)$$

Some assumptions were made to simplify equation (5). For the binding of simple oligocations which do not bind anions, ΔA and b equal zero, and the preferential hydration is negligible at high salt concentrations (> 0.5 M). These equations allow equation (5) to be rewritten as

$$\frac{\partial \log K_{obs}}{\partial \log [M^+]} = -(z\psi) \qquad (6)$$

Thus, the number of ionic interactions formed in the complex, z, can be calculated if ψ is known (22).

As stated above, the value of the intrinsic equilibrium constant is highly dependent on the changes in salt concentration in solution. Overman et al. (22) have measured the K^{65}, equilibrium constants for the SSB tetramer using various salts, and over a range of monovalent salt concentrations with

poly(dA), poly(dT), poly(A) and poly(U). The conditions used favored only the $(SSB)_{65}$ binding mode (i.e. high salt concentrations such as $\geq$ 0.2 M NaCl). A high SSB concentration was used (5.2×10^{-6} M tetramer) to ensure stoichiometric binding. Titrations were performed which monitor the fluorescence quenching of the SSB tryptophan residues upon complex formation. It was seen that an increase in salt concentration led to a decrease in the equilibrium constant for the interaction of SSB in the $(SSB)_{65}$ mode with homopolynucleotides. This change in K^{65} with increasing salt concentration is consistent with a net release of cations from the nucleic acid upon formation of the complex (22).

In studies of protein-DNA interactions, two basic types of titrations can be performed to examine the binding equilibrium. In one case, the protein is added to a constant amount of DNA and this is referred to as a "normal" titration. The binding density, $\sqrt{}$, the average moles of DNA bound per mole of protein molecule, increases as the titration progresses. In the second case, the DNA is titrated with the protein. This is referred to as "reverse" titration, since the binding density decreases as the titration progresses (25). A variation of the "normal titration" is a salt-back titration. The salt-back titration involves the formation of an SSB-polynucleotide complex by first titrating the SSB protein with the nucleic acid. This is then followed by the addition of high concentrations of salt to dissociate the complexes. The increase in fluorescence due to the dissociation of the complex is monitored.

As shown in Figure 9, Overman (22, 24) has compared the binding constants obtained for $(SSB)_{65}$ complexes with poly(U) using reverse titrations in NaCl. The SSB protein was titrated with poly(U) at different NaCl concentrations (0.2 to 0.275 M). The fraction of protein bound to the nucleic acid was determined and plotted against the mole ratio of total nucleotides per total SSB tetramer. Additionally, Overman et al. (22) performed a reverse titration (Figure 10). From a quantitative analysis of the data, the authors were able to show that the affinity of SSB for poly(U) decreased as the salt concentration increased. The salt-back and reverse titrations were in good agreement with one another. This agreement verified that the system is at equilibrium, binding is reversible, and that either method yields valid binding constants.

The salt concentration affects the stability of the SSB-DNA complex (Figure 11) (22). Figure 11 is a plot of the increase in salt concentration versus

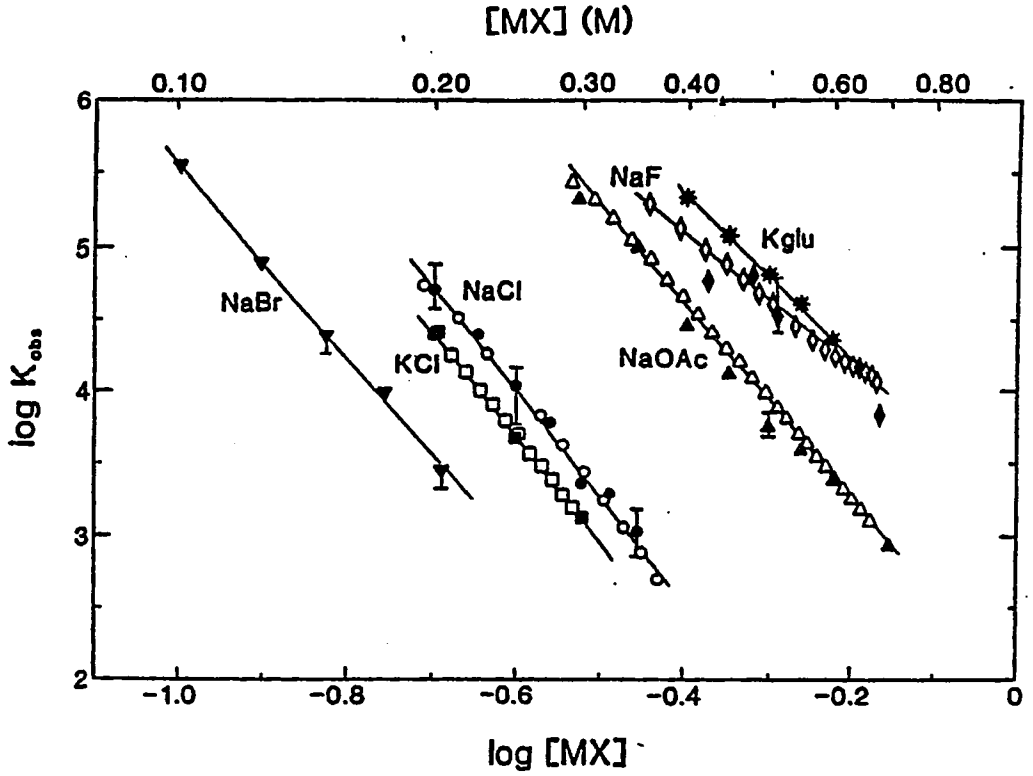

Fig. 9. Dependence of the binding constant on salt concentration for the interaction of the SSB tetramer with poly(U) in different salts (22). Open symbols represented data from salt-back titrations, and closed symbols represent data from reverse titrations performed at constant salt concentrations. Reproduced with permission from Ref. 22. Copyright 1988 Dr. T.M. Lohman.

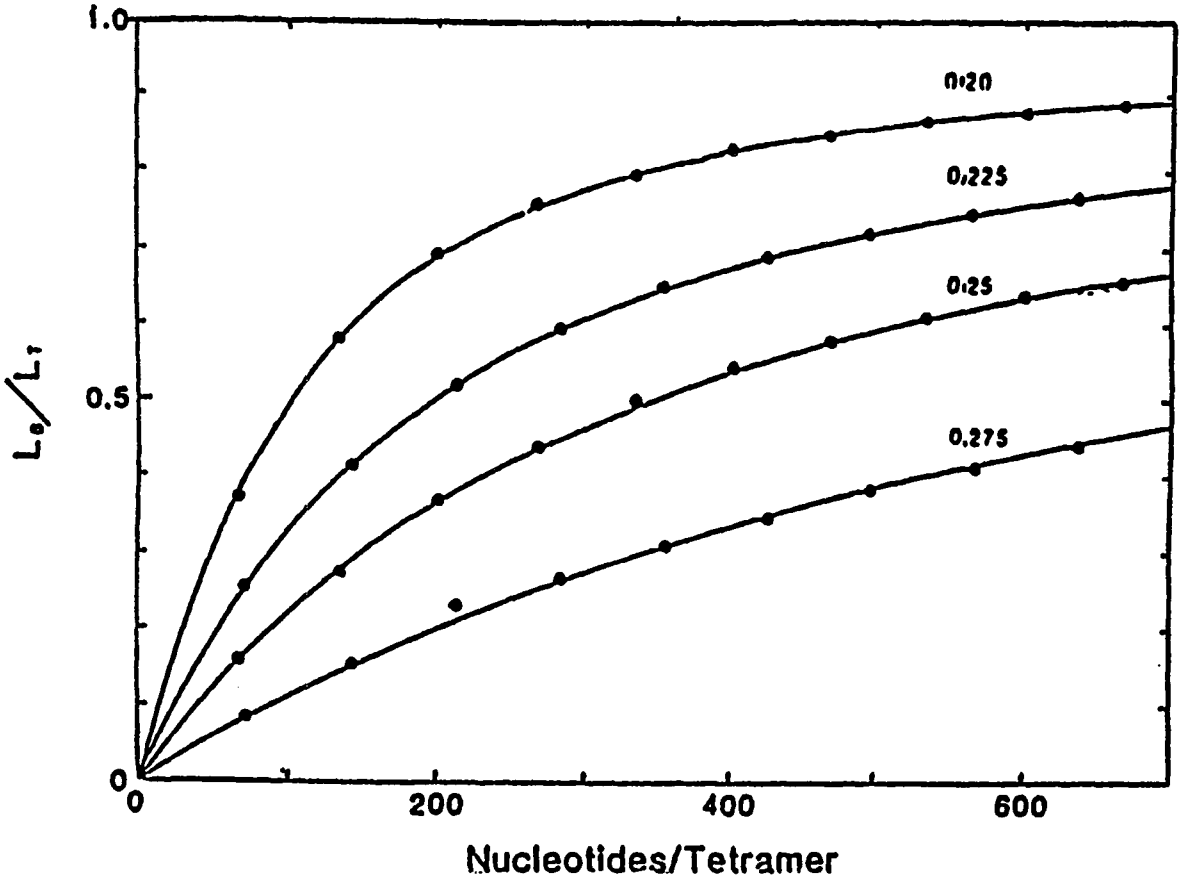

Fig. 10. "Reverse" titration of the SSB-poly(U) complex to show the dependence of the binding constant on salt concentration (22). The SSB (0.15μM) was allowed to titrate with poly(U) (117 μM nucleotide) at different NaCl concentrations (0.2 to 0.275 M) represented by the different curves. Reproduced with permission from Ref. 22. Copyright 1988 Dr. T.M. Lohman.

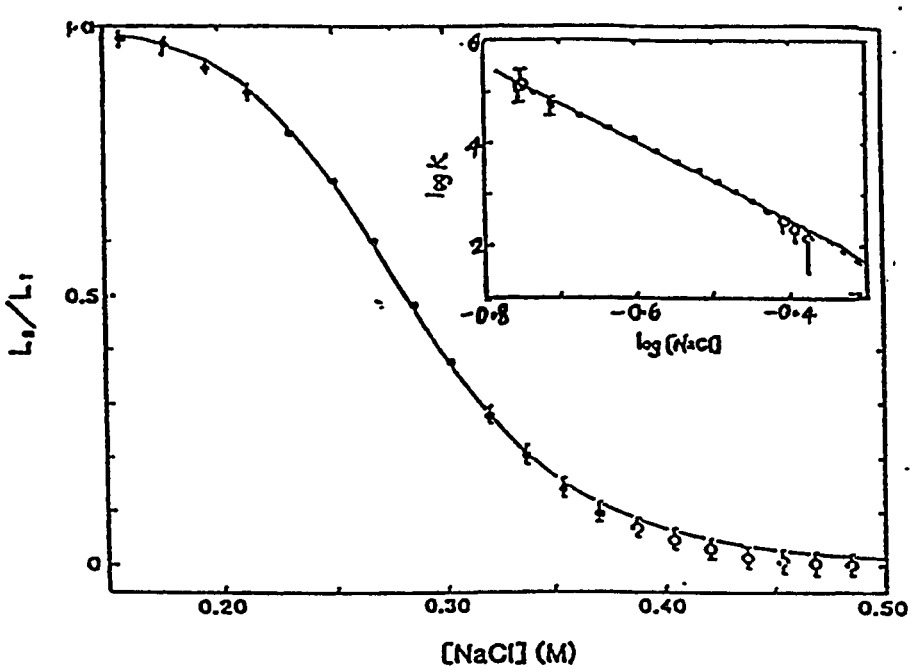

Fig. 11. Dependence of the binding constant on salt concentration (22). Closed symbols indicate the region in which $0.1 \leq LB/LT \leq 0.9$. The smooth curve is the theoretical prediction generated by using the tetramer/octamer model, with $\omega_{T/O} = 420$, the inset shows the log-log plot of the binding constant and the NaCl concentration. Reproduced with permission from Ref. 22. Copyright 1988 Dr. T.M. Lohman.

the fraction of bound SSB protein (L_B/L_T). The inset shows the log-log plot of the binding constant calculated from the data in the salt-back titration. The figure shows that dissociation occurs over the range of 0.2 M to 0.4 M NaCl. An increase in the salt concentration led to the dissociation of the SSB-poly(U) complex, which suggests that the complex is not stable at high salt concentrations. Since DNA is negatively charged, it attracts cations in solution which partially neutralize the high charge density on the DNA. This interaction leads to cation as well as anion release and uptake. It can thus be concluded that the intrinsic equilibrium constant for the interaction of SSB with single-stranded nucleic acids is highly salt-dependent, or that electrostatic interactions play a major role in SSB-DNA interactions.

The cooperativity parameter:

Cooperative binding of ligands or proteins to nucleic acids is possible when multiple proteins or ligands can bind to the same nucleic acid molecule. Cooperativity is a thermodynamic quantity which reflects the influence of one bound ligand on the binding affinity of the second.

Cooperativity can be either positive or negative. The following section explains positive as well as negative cooperativity that exists during SSB-DNA binding. Positive cooperativity is examined first. Two different models have been used to analyze the equilibrium binding data, the "tetramer/octamer" model proposed by Bujalowski and Lohman (23), and the formation of unlimited clusters of tetramers using the model of McGhee and von Hippel (15). The models and the associated data will be presented. The next section will deal with negative cooperativity and the associated data.

The tetramer/octamer model:

Although SSB exists as a free tetramer in solution, it can also adopt an octameric structure when bound to single-stranded DNA (12). In order to account for these observations, Bujalowski and Lohman (23) have presented a statistical thermodynamic model, the "tetramer/octamer" model. The tetramer/octamer model describes the equilibrium binding of SSB to single-stranded DNA in its beaded mode or the $(SSB)_{65}$ mode. The model assumes that the SSB tetramers bind to the single-stranded nucleic acids in a single orientation with respect to the nucleic acid polarity. SSB binding induces two configurations of the complex, both being equally populated. This model assumes nearest-neighbor cooperativity as explained by McGhee and von

Hippel (15), however, cooperativity is limited to the formation of octamers only. This means that the binding of SSB to single-stranded DNA induces positive cooperativity in the formation of an octamer of SSB by an additional tetramer binding. The binding continues only until the formation of an octamer, after which the binding again becomes noncooperative, and results in the binding of the single-stranded DNA to a new SSB tetramer. It also assumes that the octamers do not interact with adjacent tetramers or octamers, and it accounts for the fact that each nucleotide represents the start of a potential protein binding site.

The tetramer/octamer model is shown in Figure 12 (23). Figure 12 (a) shows the two configurations of the SSB tetramer-single-stranded nucleic acid complex. Figure 12 (b) shows the four possible states for two adjacent SSB tetramers. In the tetramer/octamer model, $\omega_1 = \omega_3 = \omega_4 = 1$ and $\omega_2 > 1$, where ω is the cooperativity parameter. As explained earlier, the model put forth by McGhee and von Hippel, states that when $\omega = 1$, there is binding noncooperativity, when $\omega > 1$, there is positive cooperativity, and when $\omega < 1$, there is negative cooperativity. In Figure 12 (b), only ω_2 shows positive cooperativity, whereas the other three states show no cooperativity in binding. The parameter ω is also referred to as $\omega_{T/O}$, a cooperativity constant for formation of octamers from two nearest-neighbor tetramers on the nucleic acid. The tetramer/octamer model suggests that SSB initially binds to single-stranded DNA in the form of tetramers. After binding to DNA, positive cooperativity induces a subset of the tetramers to form octamers.

A value of 410 ± 120 for the cooperativity parameter was obtained from analyses of equilibrium binding isotherms derived from the tetramer/octamer model (23). A range of protein concentrations was investigated, and it was confirmed that this value of $\omega_{T/O}$ can be used to fit the data irrespective of the NaCl concentrations. Thus, although the intrinsic binding constant was found to be dependent on the salt concentration, the cooperativity parameter was not (22). $\omega_{T/O}$ represents the cooperativity constant for formation of nucleic acid bound SSB octamer from two nucleic acid bound SSB tetramers. According to Bujalowski and Lohman (22), this value of 410 ± 120 suggests that cooperativity is not high enough to completely form octamers. The equilibrium distribution of nucleic acid bound SSB tetramers and octamers was predicted from the tetramer/octamer model, using $\omega_{T/O} = 410 \pm 120$. It was found that a binding

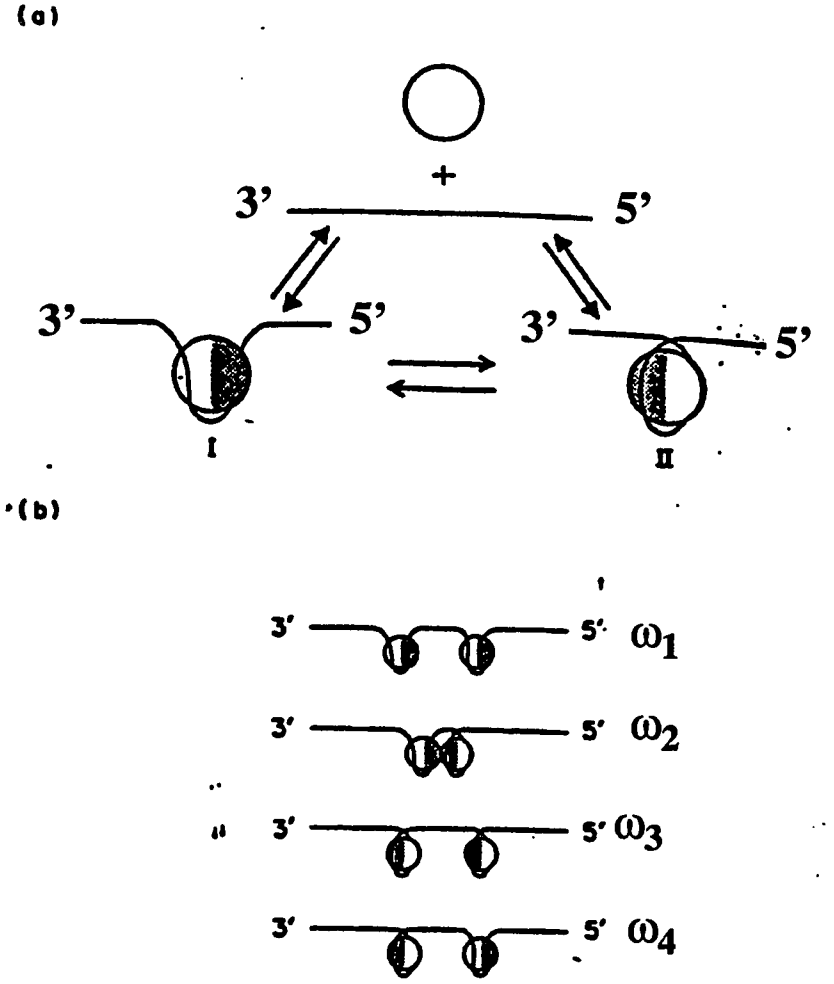

Fig. 12. Model for the cooperative interaction of SSB with ssDNA in the beaded $(SSB)_{65}$ binding mode, or the "tetramer/octamer" model (23). Each circle represents an SSB tetramer. Schematic (a) shows the two configurations of the SSB tetramer-single-stranded nucleic acid complex. Schematic (b) shows the four possible states for two adjacent SSB tetramers. Reproduced with permission from Ref. 23. Copyright 1987 Academic Press Ltd.

density-dependent mixture of tetramers and octamers should form on the nucleic acid (22). Bujalowski and Lohman (23) suggest that electron microscopy of SSB-DNA complexes may be used to detect the existence of both tetramers and octamers on the DNA. Such measurements have been made by Chrysogelos and Griffith (12) and Griffith et al. (17).

The tetramer/octamer model explains the binding of SSB to DNA in the $(SSB)_{65}$ binding mode. Another general model which has been used to explain the interaction of SSB to DNA in the $(SSB)_{65}$ binding mode has been proposed by McGhee and von Hippel (15). It is called the "limited" cooperativity model. The tetramer/octamer model and the "limited" cooperativity parameter do not explain the binding of SSB to DNA in the $(SSB)_{35}$ mode. McGhee and von Hippel (15) also have put forth the "unlimited cooperativity" model that can be used to explain the interaction of SSB with single-stranded DNA in the $(SSB)_{35}$ mode. These two statistical thermodynamic models have been presented for nonspecific, cooperative binding of SSB to single-stranded polynucleotides.

"Unlimited" cooperativity is shown in Figure 4 A (4), where nearest-neighbor interactions can occur on both sides of a protein bound to the DNA. Thus, depending on the binding density, $\sqrt{}$, long clusters of protein can form. This type of cooperativity is associated with the $(SSB)_{35}$ mode. When SSB tetramers bind in an isolated form, the binding is described by the equilibrium constant, K. When the tetramers bind in a singly contiguous manner, the equilibrium constant is $K\omega$, and when the tetramers bind in a doubly contiguous manner, the equilibrium constant is $K\omega^2$.

Figure 4 B (4) shows "limited cooperativity", which is associated with the $(SSB)_{65}$ mode where the DNA is more compacted. In this case, single-stranded DNA interacts with all four subunits of the Eco SSB tetramer. This "limited cooperativity" is limited to the formation of octamers or dimers of tetramers only, hence it does not allow doubly contiguously binding tetramers. Even when ω_{lim} is very large, complete saturation of the DNA is not reached (15).

The "limited cooperativity" model by McGhee and von Hippel (15) is essentially the same as the "tetramer/octamer" model by Bujalowski and Lohman (23). Lohman and coworkers (14) have used the "unlimited cooperativity" model to examine their data.

Lohman et al. (14) have determined that different degrees of binding cooperativity were associated with the two binding modes. They examined the

SSB binding to single-stranded nucleic acids over a wide range of salt concentrations (NaCl and $MgCl_2$). They attempted to obtain accurate measurements of the binding constant, K, and the cooperativity parameter, ω, for the two binding modes, $(SSB)_{35}$ and $(SSB)_{65}$. They obtained equilibrium binding isotherms at high salt concentrations ($\geq$ 0.2 M), where only the high site size SSB binding mode, $(SSB)_{65}$, exists. Under these conditions, using the unlimited cooperativity model, they found moderate cooperativity for SSB binding to nucleic acids ($\omega = 50 \pm 10$), independent of the salt concentrations. At low salt concentrations, the intrinsic binding constant, K, is very high, leading to stoichiometric binding. Therefore, there is not enough free protein to allow an accurate determination of the binding constant, K, and the cooperativity parameter, ω, from binding isotherms. In such cases, the extent of cooperative binding can be investigated by techniques that allow visual observation, like gel electrophoresis. At lower salt concentrations, where only the low site size SSB binding mode $(SSB)_{35}$ exists, the cooperativity, as observed by agarose gel electrophoresis, was found to increase dramatically. High cooperativity was observed only when SSB and single-stranded DNA were directly mixed at low NaCl concentrations. At equilibrium, the stable, low cooperativity form ($\omega = 50 \pm 10$) was seen to be present. When the complexes were formed at high concentrations of NaCl ($\geq$ 0.2 M), and subsequently dialyzed to lower concentrations, no highly cooperative binding was observed. This also shows that at low salt concentrations, the complexes were not at equilibrium, but were formed only transiently (14).

The model of "unlimited" cooperative binding, put forth by McGhee and von Hippel (15), was used for data analysis. This model assumes the formation of unlimited clusters of SSB tetramers on the nucleic acid. Chrysogelos and Griffith (12), and Bujalowski and Lohman (23) have shown that cooperativity is limited to the formation of octamers, i.e., binding continues only until the formation of an octamer, after which the binding again becomes noncooperative, hence, an indefinite clustering of tetramers is not formed. Hence, this value of $\omega = 50 \pm 10$ does not have much physical significance (22),

It has generally been assumed that helix-destabilizing proteins, such as SSB, must bind to the single-stranded DNA with a high degree of cooperativity at the replication fork *in vivo* to help in efficient replication (6). However, results reported by Lohman and coworkers (14) show that high cooperativity

may not be necessary for the helix-destabilizing function of SSB. Hence, it is not clear which of the binding modes function in replication. Eco SSB does not bind single-stranded nucleic acids with very high cooperativity under certain conditions. So, it is possible that the $(SSB)_{65}$ mode occurs in the binding to the nucleic acid during replication. One conjecture by Lohman and coworkers (14) is that equilibrium conditions presumably do not occur during replication. Hence, the $(SSB)_{35}$ binding mode might be involved in binding to the single-stranded DNA during replication. It is uncertain whether the $(SSB)_{65}$ mode or the $(SSB)_{35}$ mode functions during replication *in vivo*. Weiner and coworkers (5) have proposed that Eco SSB binds exclusively to single-stranded templates after which it can translocate rapidly on its template. During replication, the Eco SSB has to follow the replication fork. This can be achieved by dislocating the SSB protein in front of the polymerase, and reassociating at the site next to the helicase. Equilibrium conditions might exist during replication, but the value of the dissociation rate constant obtained (5) is too low to be compatible with such a mechanism. The answer to this question awaits a more detailed understanding of replication.

The square model for negative cooperativity in SSB-DNA binding

Bujalowski and Lohman (16) have performed experiments which show that negative cooperativity exists among DNA binding sites within the SSB tetramers, upon the binding of Eco SSB to single-stranded DNA. Their work shows that there was a dramatic decrease in the affinity for the second molecule of $dT(pT)_{34}$ with increasing salt concentrations, as compared to the first. This was measured by fluorescence quenching of the SSB tetramer upon titrating with $dT(pT)_{34}$ in 10 mM Tris and 0.1 mM EDTA at pH 8.1, and the desired NaCl concentration. Bujalowski and Lohman (16) have proposed an explanation for the existence of negative cooperativity within the binding sites of the SSB tetramer. They suggest that an uptake of cations may be essential to partially neutralize the negative charges on the single-stranded DNA, which would allow the reduction of repulsion if the binding sites are in close proximity. This would indicate a large electrostatic component to the free energy of interaction. The negative cooperativity within the binding sites of the SSB tetramer can be explained by the square model put forth by Bujalowski and Lohman (16).

The equilibrium binding constant decreases with an increase in the length of the DNA, indicating that negative cooperativity also exists. Figure 13

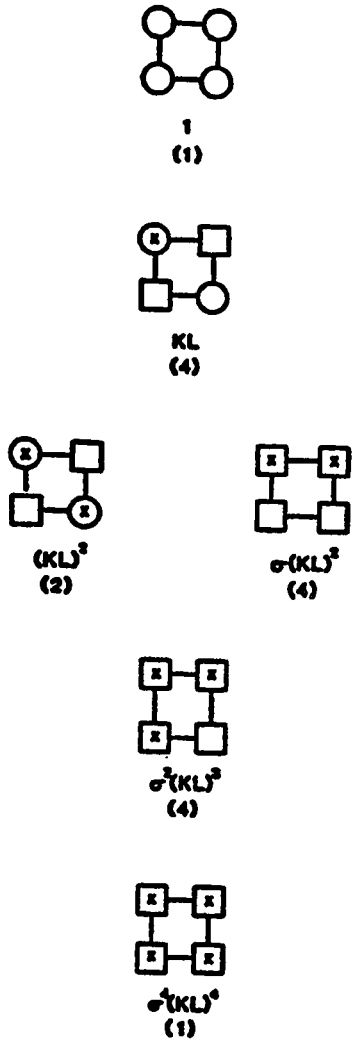

Fig. 13. "A schematic depicting the square model and the possible configurations of $dT(pT)_{15}$-SSB tetramer complexes with different number of oligonucleotides bound" (16). The oligonucleotides are represented by X. The statistical weight of each configuration relative to the free tetramer, is given below each configuration.The numbers in parentheses indicate the number of ways that each different configuration can be formed. The squares represent subunits that interact with negative cooperativity. Reproduced with permission from Ref. 16. Copyright 1989 Academic Press Ltd.

shows a schematic of the square model (16). The square model is described by two constants, the negative cooperativity constant, σ_N, where N is the length of the oligodeoxynucleotide, and the intrinsic equilibrium binding constant K_N. Negative cooperativity exists when $\sigma_N < 1$, and binding non-cooperativity exists when $\sigma = 1$. In the square model, the ligand, $dT(pT)_{15}$ can initially bind to any of the four equivalent subunits with an intrinsic association constant, K_{16}. The statistical weight for each configuration, relative to each tetramer is given by $K \times L$, where L is the concentration of free ligand (oligonucleotide). When $dT(pT)_{15}$ binds to a subunit, it exerts negative cooperativity on two of its neighboring subunits ($\sigma_{16} < 1$). The subunit that is diagonally opposite can bind DNA identically, and with no cooperative interactions. The binding affinity of the ligand to any of the neighboring subunits is characterized by the product $\sigma_{16} \times K_{16}$. The method of analysing fluorescence titrations to determine the equilibrium binding constant and the cooperativity constant has been explained in detail by Bujalowski and Lohman (16) using theoretical isotherms. Since $\Delta G = -RT \ln K$, the free energy of interaction between subunits is given by $-RT \ln \sigma_{16}$. The resulting isotherms are biphasic in which two ligands can bind to the SSB tetramer in each step. Bujalowski and Lohman have also found that σ_{16} was not temperature-dependent, whereas K_{16} was. Also, σ_{16} was not dependent on the type of anion, whereas K_{16} was slightly dependent (21).

Longer oligonucleotides such as $dT(pT)_{27}$ and $dT(pT)_{34}$ have only two binding sites per tetrameric protein. The different configurations of the two-site oligonucleotide-SSB tetramer complexes are shown in Figure 14 (16). The σ term comes into effect when the oligonucleotide occupies two adjacent units on the square. When the two diagonal units are occupied there is non-cooperative binding, and the σ parameter does not appear. The authors state that the square model is a statistical thermodynamic construct and does not explain the structure of the SSB tetramer.

Additional factors influencing the intrinsic binding constant

The above section explained the intrinsic binding constant and the cooperativity constants. Factors such as the effect of anions, temperature, length of the nucleotide binding site, and base specificity of the protein affects the intrinsic binding constant, although the positive cooperativity constant is not affected by these factors. These factors will be expanded upon in this section.

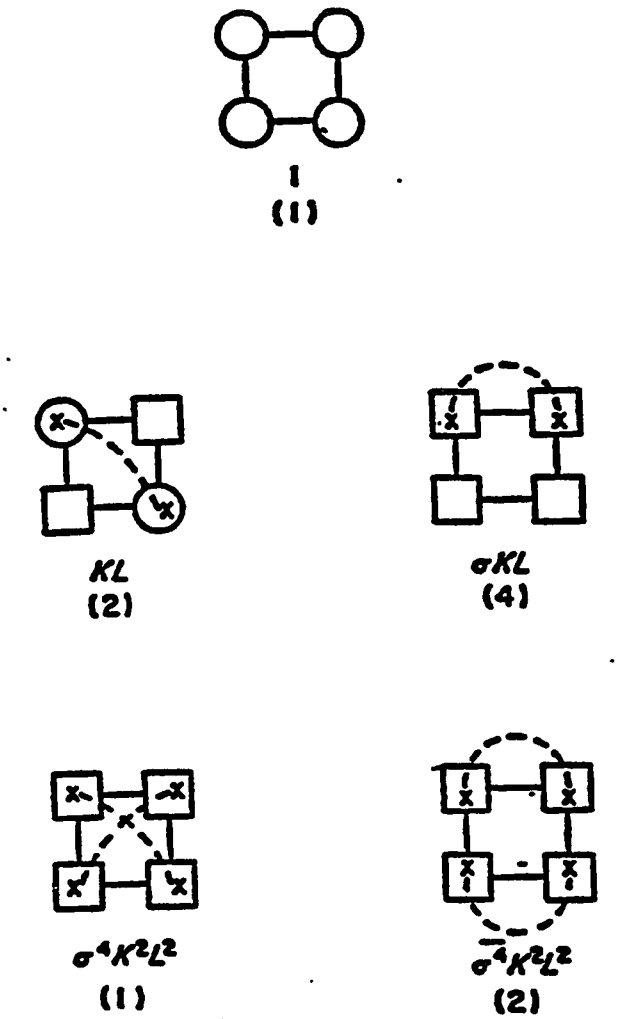

Fig. 14. The square model for the cooperative binding of longer oligonucleotides (16). Each oligonucleotide is represented by X---X. The statistical weight of each configuration relative to the free tetramer, is given below each configuration.The numbers in parentheses indicate the number of ways that each different configuration can be formed. The squares represent subunits that interact with negative cooperativity. Reproduced with permission from Ref. 16. Copyright 1989 Academic Press Ltd. Fig. 15. Dependence of the binding constant on type of anion (21). The open circles represent NaBr, and the closed circles represent NaCl. Reproduced with permission from Ref. 21. Copyright 1989 Academic Press Ltd.

Effect of anions: A dramatic effect of the type of anion on salt dependence and value of the intrinsic binding constant for poly(U) and SSB has been observed (22). The intrinsic binding constant value was highest in glutamate, followed by fluoride, acetate, chloride and finally, bromide. The anions lowered the binding constant for the SSB-poly(U) interaction (22). Glutamate is one of the free anions found in *E. coli* and its concentration increases to ~ 0.2 M when *E. coli* is grown in high osmotic media. This makes the effect of glutamate on the $(SSB)_{65}$ equilibria relevant, and for this reason, it was examined. It was seen that the K_{obs} value for the formation of $(SSB)_{65}$ complex was 1000 fold higher in the presence of sodium glutamate than in NaCl (22). One reason for the difference in affinities of anions may be that the anions compete with DNA for the same binding sites on the protein and exhibit different affinities for these sites (24). The effects of anions become larger as the oligonucleotide length increases. The anion type (Br^- versus Cl^-) had an effect on the intrinsic binding constant, K_{16} for $dT(pT)_{15}$, as shown in Figure 15 (21). K_{16} was shown to decrease as the concentrations of NaCl or NaBr were increased. This suggests that the intrinsic binding process leads to a net release of ions into solution. At low salt concentrations, the net cation release decreases, whereas at high salt concentrations it increases. However, the effect of the salt was higher in the presence of Cl^- as compared Br^-, suggesting that Br^- binds more strongly to the SSB than does the Cl^-. K^{35} was shown to be highly dependent on the salt concentration, indicating that some anion uptake occurs upon the binding of longer oligonucleotides, or oligonucleotides that span two or more SSB subunits (21).

Base specificity in DNA-SSB binding: Although Eco SSB is a nonspecific DNA-binding protein, it does show some base specificity. Lohman and coworkers (14, 22) studied this by monitoring the fluorescence quenching of tryptophan upon SSB binding to a variety of single-stranded nucleic acids in 0.2 M NaCl at 25 °C, pH 8.1. The K^{65}, equilibrium constants for the SSB tetramer in the $(SSB)_{65}$ binding mode were measured with the following nucleotides. The values of K^{65} with the different nucleotides were as follows: poly(dT) > (dC) >> (ssM13 DNA) > (rI) > (rU) > (dA) > (rA) >> (rC). The affinity of Eco SSB for poly (dT) was so high that it could not be measured accurately by the fluorescence quenching technique in NaCl. Thus, it was necessary to use solutions containing NaBr to lower the affinity of the SSB for poly(dT) into a range which

could be measured using fluorescence quenching measurements. The affinity for poly(dA) and poly(U) were moderately low.

Bobst et al. also determined the relative nucleic acid binding affinity of SSB by using electron spin resonance (ESR) techniques (26). They discovered that the intrinsic binding constant for the SSB tetramer in the $(SSB)_{65}$ binding mode exhibited greatest affinity for poly(dT), followed by ssM13 DNA, poly(dA), and finally, poly(A). Thus, the authors obtained the same ordering of affinities by the ESR technique as had been found with the fluorescence quenching technique.

Effect of oligonucleotide length: The intrinsic binding constant also depends on the length of the oligonucleotide. The intrinsic binding constant decreases as the length of the oligonucleotide increases (16, 21). As introduced earlier, Bujalowski and Lohman (16) have put forth the square model (figure 13) that explains the cooperative binding of long oligonucleotides. They have also determined stoichiometries of different lengths of oligonucleotide binding to SSB. The stoichiometries were determined under both high salt conditions ($\geq$ 0.2 M NaCl), in which the SSB exists in the $(SSB)_{65}$ mode, and under low salt conditions, in which the SSB exists in the $(SSB)_{35}$ mode. Under high salt conditions, the SSB tetramer binds four molecules of $dT(pT)_{15}$, two molecules of $dT(pT)_{34}$, or one molecule of $dT(pT)_{69}$. The degree of fluorescence quenching was about 90% in all cases. Under low salt conditions, in which the SSB exists in the $(SSB)_{35}$ mode, the tetramer binds only one $dT(pT)_{34}$ molecule, or two molecules of $dT(pT)_{15}$ with high affinity. This suggests that in this mode only two of the SSB subunits interact with the single-stranded DNA. This experimental finding led to the model depicted in Figures 2 and 4. In each case, a stretch of about 70 nucleotides is required to achieve a maximum quenching of 90% of the SSB tryptophan residues.

It was reported that the intrinsic binding free energy for an oligonucleotide of length N is not twice that for an oligonucleotide of length N/2. This was shown by fully saturating the SSB tetramer with the oligonucleotides of different lengths and then comparing the binding constants. It was calculated that the free energy change upon saturating the SSB tetramer was more favorable for shorter oligonucleotides and for noncontiguous DNA chains. The intrinsic binding constant, K_N, decreased as the length of the oligonucleotide increased (16, 21, 27). Increasing the length of the

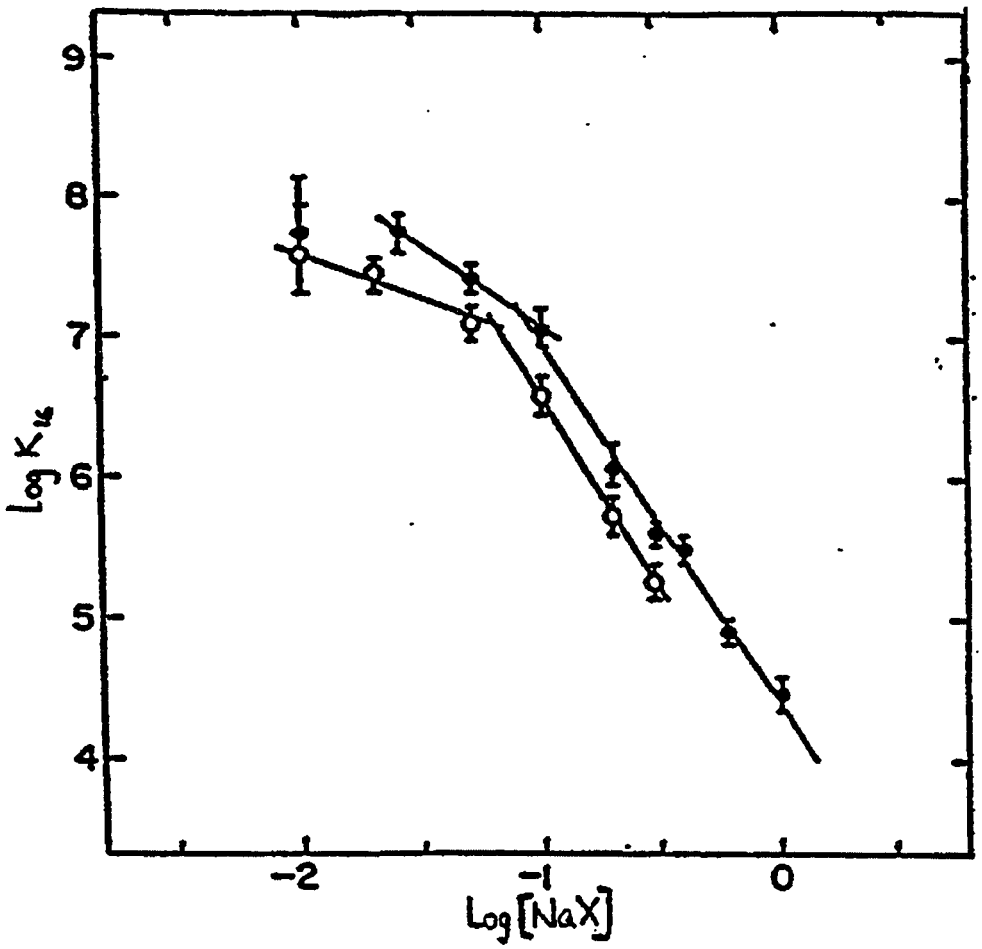

Fig. 15. Dependence of the binding constant on type of anion (21). The open circles represent NaBr, and the closed circles represent NaCl. Reproduced with permission from Ref. 21. Copyright 1989 Academic Press Ltd.

oligonucleotide by a factor of 2 from 16 to 35 or from 35 to 70 did not double the free energy of interaction, indicating that the interactions are not additive (21). As mentioned above, the intrinsic binding constant, K_N, was found to decrease with an increase in salt concentration, suggesting a net release of ions due to the protein-DNA interaction. The net ion release upon saturation of the SSB tetramer with four molecules of $dT(pT)_{15}$, two molecules of $dT(pT)_{34}$, or one molecule of $dT(pT)_{69}$ was compared by Bujalowski and Lohman (16). The extent of ion release decreased as the oligonucleotide length increased. Hence, it can be concluded that charge-charge interactions play a significant role in the formation of SSB-DNA complexes.

Effect of temperature: It was shown that the intrinsic binding constant, K_{16}, decreases with increasing temperatures. It is more difficult to saturate the four subunits of the SSB tetramer at higher temperatures (21), and hence, formation of the $(SSB)_{65}$ binding mode from the $(SSB)_{35}$ binding mode is more difficult to achieve at higher temperatures.

It can be concluded that the intrinsic binding constant is dependent upon factors such as the type of anions, temperature, length of the oligonucleotide, and base specificity of the protein. Hence, all these factors must be taken into consideration in the determination of the intrinsic binding constant in SSB-DNA interaction studies.

Kinetics of SSB binding

Along with the binding constant, it is important to know the rate of association and the rate of dissociation of the protein and DNA. This gives a measure of the kinetics of the reaction. Weiner et al. (5) have measured the rate of SSB binding to polynucleotides by two methods: (i) separation of a labeled protein-polynucleotide complex from free protein by gel filtration, and (ii) association of labeled polynucleotide with a protein-retaining nitrocellulose millipore filter. They mixed 1 to 5 nmol of the polynucleotide and 10 μg of the labeled SSB protein in buffer containing 20 mM Tris-HCl, 20 mM NaCl and 1 mM dithiothreitol. The mixture was layered on a gel filtration column. They found that binding of Eco SSB to DNA was complete in less than 10 seconds at 4 ^{o}C. The rate of release of the SSB protein from single-stranded DNA was measured from the rate of transfer from labeled to unlabeled single-stranded DNA. The rate of release was relatively slow even at 37 ^{o}C. The half-life was found to be 120 minutes under these conditions, with a dissociation rate

constant of 3×10^{-11} to 7×10^{-11} M per second at 37 °C (5). The low salt concentration (20 mM NaCl) leads to the formation of the $(SSB)_{35}$ mode, which has been shown to exist in nonequilibrium conditions (5).

Eco SSB binding site length

The binding site length of Eco SSB binding has been determined by different methods. Sigal et al. (7) have reported a stoichiometry of 7 nucleotides per SSB monomer by cosedimentation experiments. Chrysogelos and Griffith (12) have reported a binding site length of 20 nucleotides per monomer using nuclease digestion and electron microscopy experiments. Bobst et al. (26) have reported a stoichiometry of 6 to 25 nucleotides per monomer using electron spin resonance measurements. The inconsistencies in the stoichiometry values may either be due to the large differences in the methods used, but may also reflect the salt dependence of the binding stoichiometry, since these studies were conducted at a variety of salt concentrations.

Lohman and Overman (20) used fluorimetric titrations of Eco SSB with poly(dT) to demonstrate that there is a salt dependence of the binding stoichiometry. At low salt concentration (0.01 M NaCl, 25 °C, pH 8.1), the binding stoichiometry was 33 ± 3 nucleotides per tetramer, whereas at high salt concentration (0.2 M NaCl, 25 °C, pH 8.1), the binding stoichiometry was 65 ± 5 nucleotides per tetramer. As mentioned earlier, data obtained by fluorescence quenching experiments are more accurate than nuclease digestion or electron microscopy experiments. The values suggested by Lohman and Overman (20) are widely accepted for this reason.

All the above data suggest that significant cooperativity and salt effects occur in the SSB complexes formed with single-stranded nucleic acids. The intrinsic binding constant, K_{obs}, is highly dependent on the salt concentration. K_{obs} decreases as the salt concentration increases. These studies help to clarify the complexities in the interaction of the SSB tetramer with single-stranded nucleic acids.

PROTAMINES: STRUCTURE AND FUNCTION

Protamines fall in the class of nonspecific DNA binding proteins, and may bind to either double-stranded or single-stranded DNA molecules, but show very little base-sequence specificity.

A fully extended mammalian DNA molecule is about one meter long. It has to be compacted to a great extent for it to fit in a cell nucleus which is only a few micrometers in diameter. DNA in somatic cells is packaged in subunits called nucleosomes, which contain about 200 base pairs of DNA wound around a core of eight histone molecules (28). During spermiogenesis in mammalian and most of the vertebrate sperm cells, the spermatid chromatin undergoes reorganization and the histones are replaced by protamines. As a result, the nucleosomal-based packaging of DNA characteristic of somatic chromatin is transformed into a highly condensed form of chromatin referred to as nucleoprotamine (29, 30). The protamines are phosphorylated immediately after their synthesis. The phosphorylated protamines bind to chromatin to cause its initial condensation. Condensation is followed by a sequential release of histones. The final transformation into the nucleoprotamine complex is linked to dephosphorylation (29).

Protamines form a class of low-molecular-weight proteins that protect and package the chromosomal DNA in the sperm cells of eukaryotic organisms. Protamines are small, arginine-rich and hence, extremely basic proteins. The mode and location of protamine binding to DNA has not been established. Due to the large number of positive charges associated with protamines at physiological pH values, the main driving force for DNA-protamine binding may be due to electrostatic interactions. Interactions between the positively charged protamine and the negatively charged DNA leads to neutralization of the phosphodiester backbone of DNA. The neutralization allows DNA-protamine complex to coalesce into a highly condensed, biochemically and genetically inert state (31).

The fowl protamine, galline, and the herring sperm protamine, clupeine Z, have been the most extensively studied protamines. Mammalian protamines have not been studied extensively. The sperm of bull, ram, boar, rabbit and guinea pig contain only a single type of protamine, P1. Mouse, hamster and stallion sperm contain two types of protamine, P1 and P2, and sperm of humans have been found to contain four types of protamine,

although three of the protamines appear to be processed from the same precursor polypeptide (32, 33).

Galline is 68 amino acid residues long and has 38 arginine residues (34). Clupeine Z is 31 amino acid residues long and has 21 arginine residues (35). Protamine P1 is 50 amino acids long and has 26 arginine residues. Unlike mammalian protamine P1, clupeine and galline do not contain any cysteine residues, but they do contain proline residues. Cysteine residues help in cross-linking the protamine molecules by forming intermolecular and intramolecular disulfide bonds, thus leading to compaction of the DNA molecule. Galline and clupeine Z lack cysteine residues. It has been proposed that compaction is achieved by the hydrophobic proline residues, which help to fold the protein molecule around the DNA (35). Fish and fowl protamine sequences are less conserved, compared to mammalian protamines. Figure 16 shows the primary sequences of clupeine Z and galline.

Some of the common structural features in P1 molecules are as follows: each protamine P1 molecule begins with the hexapeptide sequence Ala-Arg-Tyr-Arg-Cys-Cys, and each P1 molecule has 50 amino acids. Protamine P1 can be divided into three structural domains, the highly positively charged, central polyarginine-rich or the DNA binding domain, which is flanked by two relatively arginine-deficient peptide domains containing serine, threonine and tyrosine residues located at specific positions. These residues are the probable sites of phosphorylation. The bull protamine P1 molecule has seven cysteine residues which are responsible for its intermolecular and intramolecular disulfide linkages (Figure 17) (32). Experiments by Balhorn and coworkers (2) have indicated that each protamine P1 in bull sperm likely has two intramolecular and three intermolecular disulfide linkages due to the interaction of cysteine residues (2). The disulfide bonds lock the neighboring protamines together around the DNA helix to stabilize the final structure of the chromatin (32).

Many model systems employ the commercially available clupeine Z or other fish protamines to study DNA-protamine interactions. These systems can be used as model systems to study the complex mammalian system. Although the fish, fowl and mammalian systems share similar DNA-binding domains, their intermolecular interactions are likely to be quite different. They differ from each other in their N and C-terminal ends, the presence of cysteine

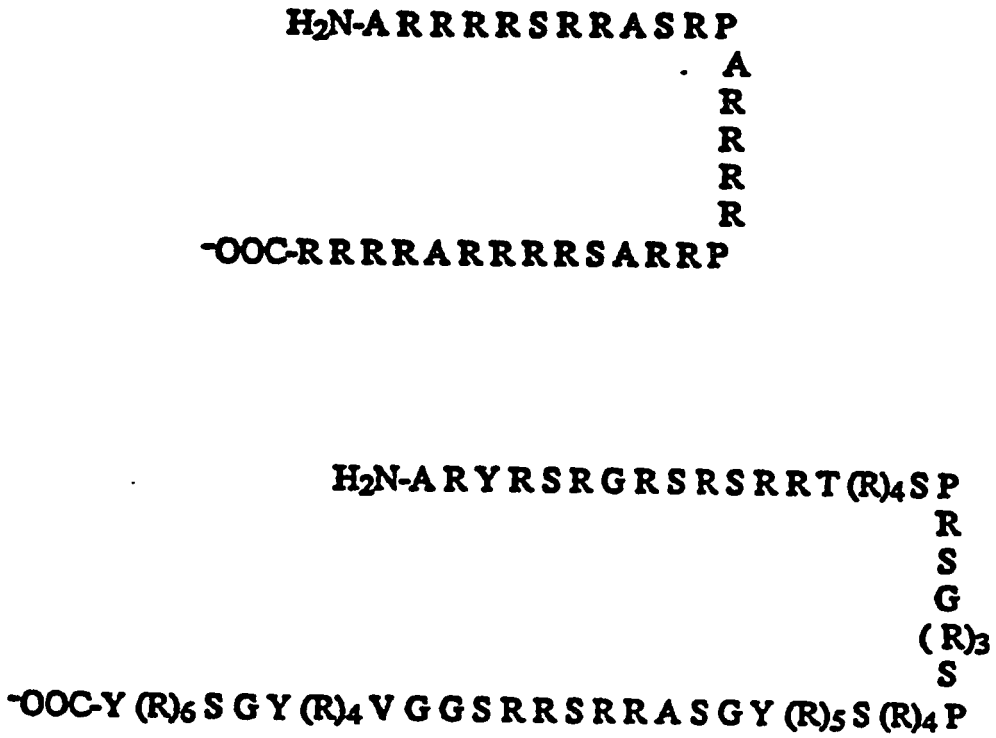

Fig. 16. Primary sequence of clupeine Z (42) and galline (34). Clupeine Z shown on top, galline on bottom.

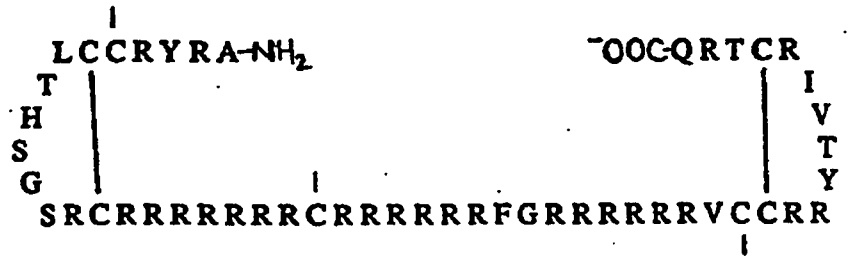

Fig. 17. Primary sequence of bull protamine P1 (2).

residues in protamine P1, and the presence of proline residues in galline and clupeine Z molecules.

PROTAMINE-DNA INTERACTIONS

The mode of protamine binding to DNA has not been established. To fully understand the mechanisms of reproduction, one must first understand the mode of DNA-protamine binding.

During late spermiogenesis or the transformation of spermatozoa into spermatids, the DNA in sperm cells becomes tightly packaged by protamines. The DNA in mature spermatozoa is condensed into a highly compact and transcriptionally inactive structure. The DNA molecule is a double helix. The B-form DNA has a major or a deep groove and a minor or a broad groove. It is thought that protamine binds to the DNA in the grooves, although it is not clear whether it binds in the major or in the minor groove. Balhorn (2) has proposed that protamine binds in the minor groove. This was supported by X-ray diffraction studies by Suau and Subirana (36). The portion of the protamine protruding from the minor groove, including the C and N-terminal tails of consecutive protamines, is positioned in the major groove of the adjacent DNA molecule, as shown in Figure 18 (Arrow A). This positioning allows the formation of disulfide bridges (arrow B), thus cross-linking each protamine molecule to the next. Thus, further compaction takes place (32).

Balhorn's model states that if the entire mammalian protamine P1 molecule were in an extended conformation, and were to bind in the minor groove of the DNA, the total length of the minor groove required to accommodate the protamine molecule in the sperm nucleus would be 2 to 3 times as much as that actually present. However, if only the central arginine rich region were to bind in the minor groove, then the length of the minor groove in mouse (2.3 m) would be just appropriate to fit the central arginine rich region, as measured by the mouse sperm DNA content (32). The C and N-terminals would then be free for inter and intraprotamine interactions. Thus, the model proposed by Balhorn states that the central polyarginine region of mammalian protamine binds in the minor groove of DNA, cross-linking and neutralizing the phosphodiester backbone of DNA. The carboxy and amino terminal ends are involved in inter and intraprotamine hydrogen, hydrophobic and disulfide bonds (31). It must be pointed out that most of the biophysical work reported has been on fish protamines, which only have structures comparable, and not identical to, the DNA-binding domain of

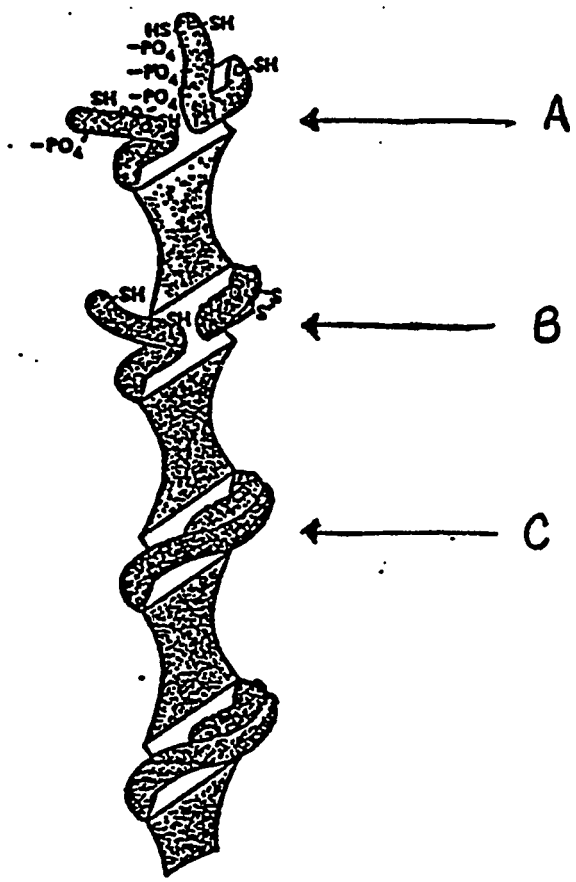

Fig. 18. Positioning of the protamine molecules in the minor grooves of DNA (32). The C and N-terminal tails of consecutive protamines are positioned in the major groove of the adjacent DNA molecule (Arrow A), leading to the formation of disulfide bridges (Arrow B), thus, cross-linking each protamine molecule to the next (Arrow C). Reproduced with permission from Ref. 32. Copyright 1982 The Rockefeller University Press.

mammalian protamines. Hence, this work cannot be extended directly to mammalian protamines.

Several studies have shown that protamine synthesis is initiated late in spermiogenesis (37, 38). Binding of protamines to DNA displaces the bulk of the histones and transition proteins, and the nucleosomal organization that is characteristic of somatic chromatin is lost. Protamine P1 is then post-synthetically modified by phosphorylation. Disulfide linkages are formed between the cysteine residues which help to stabilize the protamine molecule and further compact the DNA-protamine complex, as explained above (32, 39).

X-ray diffraction studies by Suau and Subirana (36) have shown that fish protamines stabilize DNA in the B form. They found that, in spite of the differences in the amino acid composition, all protamines that they studied (squid, fish and cephalopoda) seemed to stabilize DNA in the B form. That is, they had similar X-ray diffraction patterns. It was proposed by Balhorn (31) that adjacent arginine residues bind electrostatically to the opposite DNA strands, and thus, the phosphodiester chains are cross-linked along the entire length of the DNA molecule. This cross-linking would stabilize the DNA in the B form and prevent it from changing to the A or C form.

Since the basic region of protamine is proposed to be the interaction domain with DNA, studies on the interaction of basic oligo-L-amino acids (chain lengths between 2 and 18 residues) with DNA have increased our understanding of the system. Kawashima et al. (40) have studied the interaction of oligo-L-ornithines with native DNA. They found that addition of the oligomers to the DNA resulted in the stabilization of the double-stranded DNA against thermal denaturation. Kawashima and Ando (41) also have used oligo-L-arginines. DNA-oligo-L-arginine complexes were prepared by mixing. Precipitation of the complexes reflect strong and irreversible binding of oligo-L-arginine to DNA. The authors showed that as the chain length increased, the precipitate forming ability of the oligo-L-amino acids increased, hence, complex formation increased. With arginine, and with n = 5, more than 90% of the DNA was found in the precipitate. Figure 19 shows the relationships between chain lengths and salt concentration required for the gradient elution of oligo-L-arginine, poly-L-ornithine and poly-L-lysine in carboxymethyl cellulose column chromatography. Oligo-L-arginines had the strongest interaction with CM-cellulose, followed by oligo-L-lysines and finally oligo-L-ornithines.

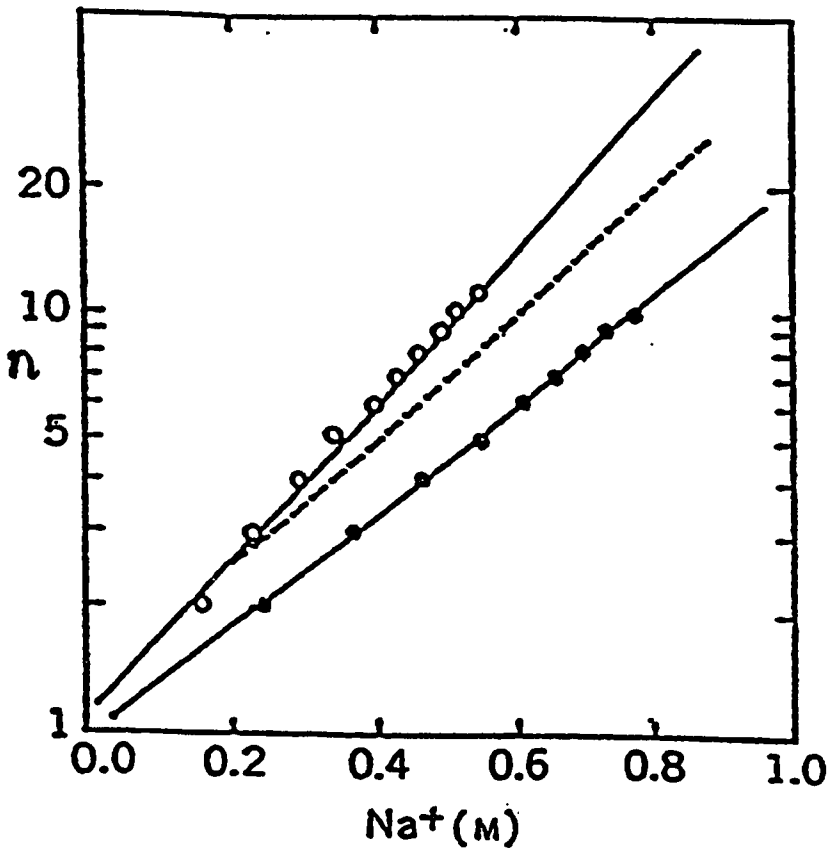

Fig. 19. Relationships between chain lengths and salt concentration necessary for the gradient elution of oligo-L-arginine, poly-L-ornithine, and poly-L-lysine (41). The number of residues n, was plotted against the Na^+ concentration. Closed circles represent (Arg)n data, open circles represent (Orn)n data, and dashed lines represent poly-L-lysine data. Reproduced with permission from Ref. 41. Copyright 1978 Dr. S. Kawashima.

THERMODYNAMICS AND EQUILIBRIUM BINDING OF PROTAMINE

Thermodynamics and kinetics of reactions such as protein-nucleic acid interactions help to increase the understanding of the stability, specificity and mechanisms of interaction.

DNA-protamine interactions can be used to study nonsequence specific binding of proteins to linear polymers such as nucleic acids. Binding of ligands to macromolecules may involve individual binding sites, that are independent of each other. This situation may not always occur. Most of the time, attractive forces between bound ligands at adjacent positions may be involved in the interactions (42).

Different researchers have shown that binding of protamine to DNA occurs with significant positive cooperativity. Positive cooperativity occurs when the binding of a ligand at a site on the polymer leads to further binding of another molecule at an adjacent site (35). The major techniques which have been employed to measure the extent of DNA-protamine binding are light scattering and fluorescence measurements. The different authors have proposed different models to explain the reason for positive cooperativity in DNA-protamine interactions.

Positive cooperativity in DNA-protamine binding

Willmitzer and Wagner (35) have studied the thermodynamics of protamine-DNA interactions *in vitro* using fluorescein-labeled clupeine Z, the protamine from herring sperm. As shown in Figure 20, upon addition of fluorescein-labeled clupeine to excess DNA (to ensure quantitative binding), fluorescence quenching was observed (35). The fluorescence quenching was substantial. The authors suggest that the quenching may be due to radiationless loss of energy caused by the contact of the fluorophor with the DNA surface. The McGhee and von Hippel model (15) states that a ligand covering more than one lattice residue has the choice to select any segment fitting to its size, and it includes the possibility of cooperative interactions. The protamine-DNA interaction is very well described by this concept, and Willmitzer and Wagner (35) used this model to analyze their results, and obtained values for the binding constant K, and the cooperativity parameter, ω. Willmitzer and Wagner (35) found that the high cooperativity can satisfactorily be explained by McGhee and von Hippel's model (15).

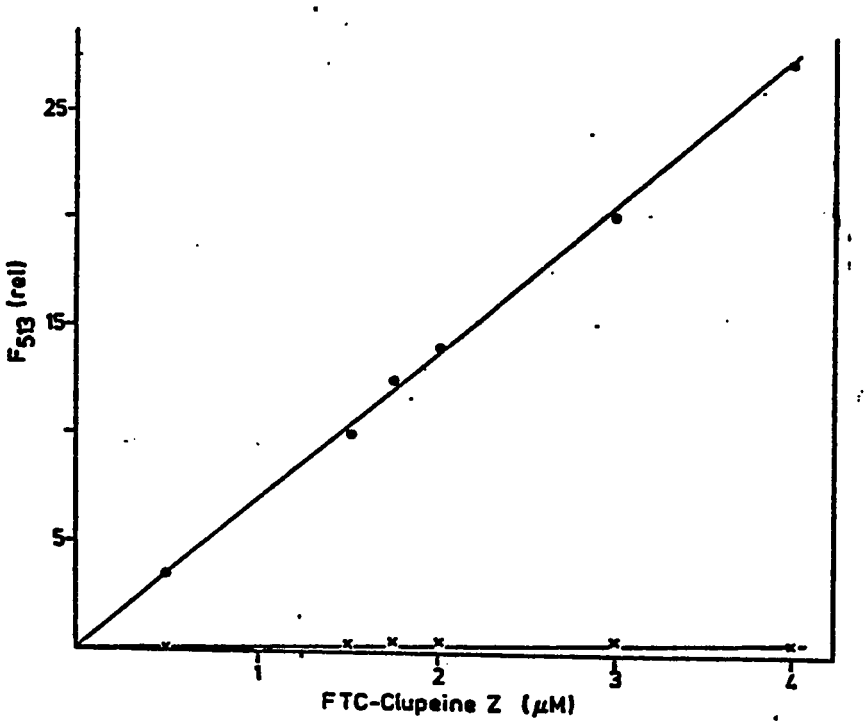

Fig. 20. Quenching of the fluorescence of protamine by the addition of DNA (35). Closed circles represent fluorescence of clupeine Z devoid of DNA, and crosses represent fluorescence in presence of 92 μM DNA. Reproduced with permission from Ref. 35. Copyright 1980 Springer-Verlag.

The results of Willmitzer and Wagner (35) were consistent with highly cooperative binding. Their model states that cooperativity, in general, can be due to two mechanisms: protein-protein interaction, or DNA-mediated cooperativity. They ruled out protein-protein interaction, as the cooperativity parameter also increased upon the addition of 2 M urea. Significant changes of the cooperativity parameter were seen by changing the ionic strength from 0.3 M to 0.5 M. As the NaCl concentration increased, the cooperativity parameter, ω, increased, but the binding constant decreased. The dependence of the affinity constant on the ionic strength or the salt concentration was used to find the number of ionic bonds participating in the interaction. This was found to be 15. This value also correlates with the stoichiometric value of 15 DNA nucleotides per clupeine molecule, as determined by performing fluorescence measurements on the binding radioactively labeled protamine with DNA (35). It was found that with increasing NaCl concentrations, the number of ionic interactions decreased.

Willmitzer and Wagner (35) also discovered that protamine binds with a higher affinity to denatured DNA than native DNA at a salt concentration of 0.3 M. The authors have explained this effect in the following way: The length of denatured DNA decreases more than twofold at high salt concentrations, whereas the length of native DNA remains unaffected. Hence, at high salt concentrations, the higher charge density of denatured DNA, which occurs due to compaction of the structure, may be responsible for the greater affinity in binding. There was a significant decrease in cooperativity upon binding protamine to denatured DNA. The lower cooperativity seen with binding to denatured DNA, as compared to native DNA, can be explained by assuming that the cooperative behavior arises from cross-linking of DNA strands with protamine molecules. The cross-linking would increase the charge density which would further increase the binding affinity of the next protamine molecule. Denatured DNA has a higher charge density, and the relative increase in charge density for denatured DNA would be less than that for native DNA. Hence, cross-linking of denatured DNA should result in a smaller binding cooperativity than for native DNA (35). The authors suggest that the ability of protamines to cross-link is better adapted for binding to native DNA, rather than to denatured DNA. The cross-linking, and hence cooperativity is

favored through the good fitting of the protamine molecules on adjacent DNA strands, as found in native DNA.

Watanabe and Schwarz (42) also have used fluorescein-labeled clupeine Z bound to calf thymus DNA to study the nonsequence specific binding of protamine to DNA. The equilibrium binding experiments were carried out by adding constant amounts of DNA and NaCl to varying amounts of the protamine solution. Fluorescence measurements were performed after the equilibrium had been reached. Equilibrium was attained by adding an excess of DNA to the protamine solution. A disturbance of the equilibrium state would lead to a relaxation process that could be monitored by the fluorescence signal. The authors discovered that the fluorescence intensity, F, decreased upon the addition of sufficient amounts of DNA. They evaluated their results by using the following equations:

$$F = f_A c_A + f_a c_a \tag{7}$$

$$c_A + c_a = c^o_A \tag{8}$$

where f_A and f_a are the molar fluorescence intensities of free and bound protamine, respectively, and c_A, c_a and c^o_A are the concentrations of free, bound and total protamine. They found that the magnitude of fluorescence intensity is proportional to the concentration of free ligand. As shown in Figure 21, the two broken lines labeled f_A and f_a, respectively, would be followed if the protamine was totally free or completely bound respectively (42). The distances (y, y_∞, x_A, x_a) in the figure can be used to calculate the degree of binding θ, or the concentration of free protamine c_A, by using the following equations:

$$\theta = y/y_\infty \tag{9}$$

$$c_A = [x_a/(x_A + x_a)]\, c^o_A \tag{10}$$

If the perpendicular to f_a is connected to the line labeled f_A, then the distance between the perpendicular line and the line labeled f_A, would give the distance y. The length of the perpendicular above the plateau (y), gives the distance x_A, and the length of the perpendicular below the plateau gives the distance x_a. The dependence of θ on c_A is shown in Figure 22 (42). The experiment was carried out at different fixed DNA concentrations, with increasing amounts of total protamine, c^o_A, and the fluorescence intensity in

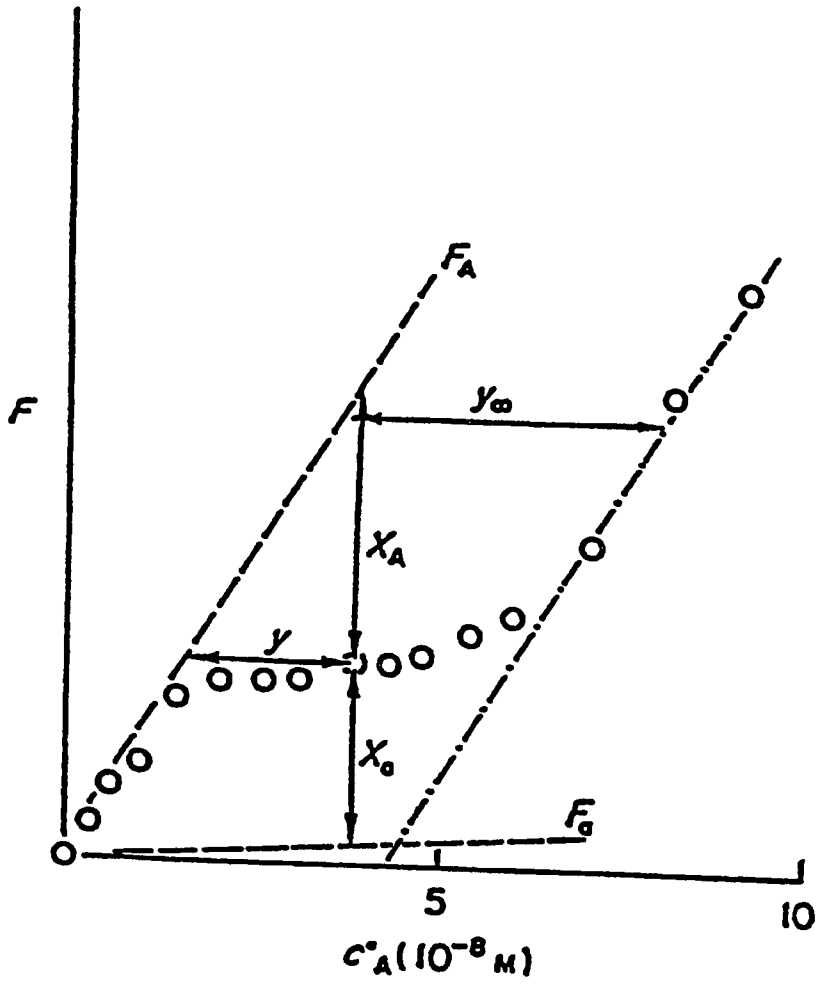

Fig. 21. Determination of the degree of binding of protamine to DNA at a fixed DNA concentration of 8.18×10^{-7} M (42). The degree of binding, θ, and free ligand concentration, c_A, can be measured directly by means of the y and x distances. Reproduced with permission from Ref. 42. Copyright 1983 Academic Press Ltd.

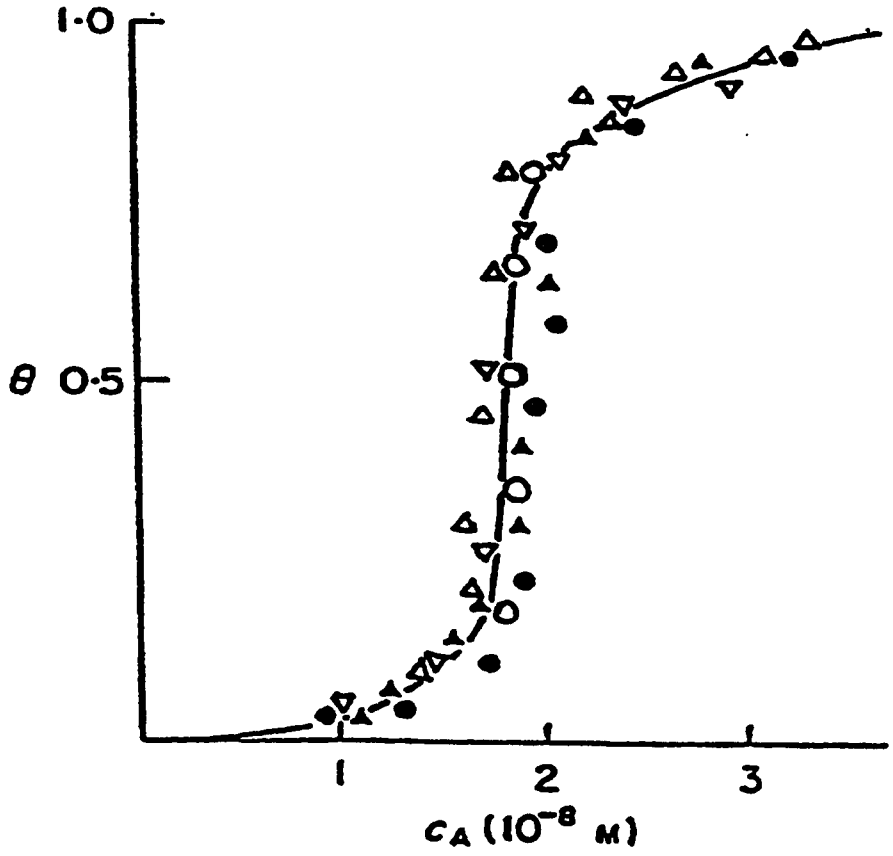

Fig. 22. The dependence of degree of binding of protamine to DNA on free protamine concentration (42). The various symbols represent the various concentrations of DNA at pH 7.5, 20 °C and in 0.4 M NaCl. Open circles represent a DNA concentration of 1.72×10^{-6} M, closed circles represent a DNA concentration of 8.18×10^{-7} M, closed triangles represent a DNA concentration of 6.1×10^{-7} M, open triangles represent a DNA concentration of 4.1×10^{-7} M, inverted triangles represent a DNA concentration of 3×10^{-7} M. Reproduced with permission from Ref. 42. Copyright 1983 Academic Press Ltd.

each case was measured. The range of DNA concentrations used were: 1.72×10^{-6} M to 3×10^{-7} M. Figure 22 shows that a change in DNA concentration did not significantly change the degree of binding when excess DNA was used.

Watanabe and Schwarz have also shown that the binding affinity between protamine and DNA became weaker, and the positive cooperativity became higher with increasing concentrations of NaCl. They proposed that the high number of arginine residues on protamine lead to electrostatic interactions with the DNA. Using titration curves, the authors were able to measure stoichiometries of the reaction. Keeping the total protamine concentration fixed, and adding increasing amounts of DNA, they measured the fluorescence intensities at 0.4 M NaCl. They found that F/F_o (F_o is the original fluorescence intensity) is related to n (number of nucleotides bound by a protamine molecule) by the following equation,

$$F/F_o = 1 - Z(\theta/n)p, \qquad (11)$$

where Z is the quenching efficiency and p is the DNA to protamine ratio, given in terms of nucleotides per protamine molecule. The value of n, or the number of nucleotides bound by a clupeine molecule, was found to be 20.

Watanabe and Schwarz (42) have developed a model similar to that of McGhee and von Hippel (15). It implicates two types of binding steps. The first one is the binding of a ligand (protamine molecule) on the lattice (DNA molecule) at a site where the neighboring lattice residues are empty. The binding constant in this case is referred to as K'. In the second case, the ligand binds to a site whose adjacent site is already occupied. The binding constant in this case is referred to as K. Binding of the ligand in this case leads to clusters of ligands bound consecutively to adjacent sites due to positive cooperative binding. Cooperativity between adjacent bound ligands can be measured by the parameter $q = K/K'$. All equilibrium parameters can be calculated using q, n and Kc_A, the binding affinity of free protamine to the DNA (42).

Thus, experiments performed by Willmitzer and Wagner (35) as well as Watanabe and Schwarz (42) suggest that the equilibrium binding constant for DNA-protamine binding decreases, and the cooperativity parameter increases with an increase in salt concentration. Both groups have used similar systems and models for the analysis of their data. They differ in their explanations of cooperativity.

Watanabe and Schwarz (42) propose the following hypothesis to explain the observed positive cooperativity. Free DNA is strongly charged, hence it is surrounded by an ionic cloud, the size of which is dependent on the salt concentration. At DNA sites occupied by protamine, there is no net charge and no ionic atmosphere. The negative charges on an unoccupied site adjacent to a bound ligand should be screened electrostatically to a lesser extent than one which is farther away, hence, an enhancement of the binding constant is expected at sites adjacent to cluster ends. This effect, which depends on the structure of the polymer (DNA) and of the ligand (protamine), explains the results in a qualitative way.

Willmitzer and Wagner (35) have explained the mechanism by stating that higher ionic strengths leads to cross-linking of the DNA strands with protamine molecules. The cross-linking creates a zone of higher charge density leading to further binding of protamine molecules, as an increase in charge density leads to an increase in the binding affinity.

Porschke (43) has used light scattering techniques to show positive cooperativity in DNA-protamine binding. He demonstrated that the positive cooperativity in DNA-protamine binding is protamine driven, which means that protamine binding induces the association of intermolecular DNA strands. Clupeine Z from herring sperm was used for the experiments, and was quantitated by absorbance measurements at 200 nm. The DNA was obtained from bacteriophage lambda. Protamine was added to DNA, the solution was incubated, and then centrifuged to pellet the bound DNA. Free protamine left in the supernatant was measured by absorbance at 200 nm. He also performed light scattering measurements. As protamine was added to DNA, the light scattering intensity increased as shown in Figure 23, indicating an increase in aggregation and thus, protamine-DNA binding. After a certain concentration of added protamine, the intensity increased dramatically, suggesting positive cooperativity in binding. The degree of protamine binding to DNA, was plotted against the total protamine concentration (Figure 23) (43). Porschke has interpreted the figure as showing that there is very little binding of protamine to DNA before the onset of cooperative transition, whereas the degree of binding increased to almost 70% beyond the transition range.

At ionic concentrations below 200 mM, and DNA concentration at 2 μM of nucleotide residues, the increase in light scattering took place at a protamine

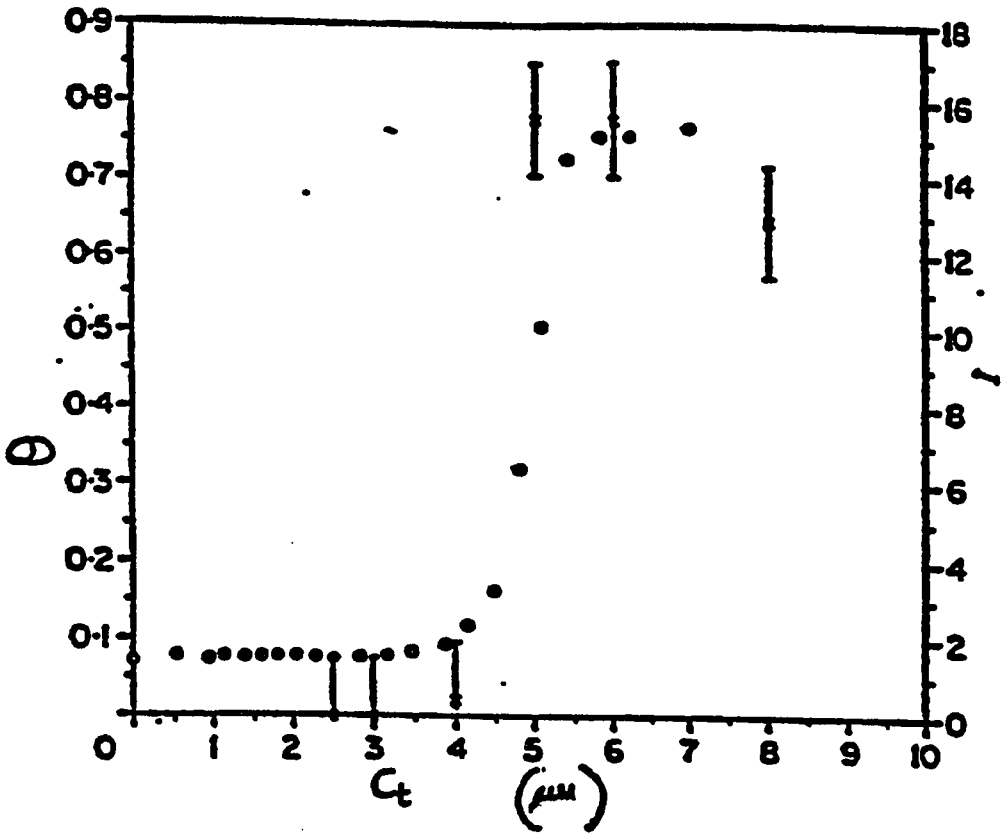

Fig. 23. The dependence of the degree of protamine (θ) binding to DNA as a function of total protamine concentration (c_t) (43). θ is obtained from a least squares fit of the obtained from light scattering intensity measurements. Open circles represent data from light-scattering experiments, and crosses represent data from centrifugation experiments. UV absorbance of the supernatant gave the free protamine concentration, and the data was converted into degrees of binding using the stoichiometric coefficient of 1 protamine molecule per 20 phosphate residues. Reproduced with permission from Ref. 43. Copyright 1991 Academic Press Ltd.

concentration of 0.1 μM. This corresponds to a stoichiometry of 20 nucleotide residues per protamine molecule, and it also agrees with the fact that there are 21 arginine residues in the sequence of clupeine Z. This calculation assumes complete binding. From the results obtained by centrifugation and light scattering, he was able to confirm that little or no binding took place before the onset of cooperativity. As the onset of cooperative transition took place, the degree of binding increased dramatically. Hence, very low protamine concentration was able to lead to this degree of binding as shown in Figure 23 (43).

Porschke (43) has also developed a model to explain cooperativity in DNA-protamine binding. He states that ligand binding itself is noncooperative. However, the ligands are assumed to induce a transition of the polymer to a different, more compact state at a defined degree of ligand binding. This transition implies an altered affinity of the ligand for the polymer. Hence, in this case, there are two equilibrium constants, one for the noncooperative binding of the ligand, K_b, and the other for the intramolecular polymer transition K_t. The minimum degree of binding required to induce this transition is defined by θ_x. Since protamines, like most ligands of biological interest, occupy more than one lattice residue, the binding of protamine to DNA can be described by McGhee and von Hippel's (15) excluded site model. Although, he has not presented this, Porschke's model can be combined with McGhee and von Hippel's (15) excluded site model to fully understand the mechanism of cooperativity.

Protamine-DNA binding measurements by intrinsic fluorescence

Willmitzer and Wagner (35), and Watanabe and Schwarz (42) have used fluorescein-labeled clupeine to study binding using fluorescence intensity measurements, but the effect of the fluorescent probe on the binding behavior cannot be evaluated. Nakano and coworkers (34) bypassed the use of fluorescein by using the fowl protamine, galline, which contains tyrosine, for quantitating total and free protamine concentrations by fluorescence. They also employed light scattering measurements to determine the actual binding parameters.

The equilibrium parameters of the binding of galline to DNA were determined as follows: The DNA and galline were directly mixed in ST buffer (100 mM NaCl in 50 mM Tricine-HCl, pH 7.4), ET buffer (0.25 mM EDTA in 10 mM Tricine-HCl, pH 7.4) or 20% solution of standard saline citrate or 0.2 × SSC

(150 mM NaCl in 15 mM sodium citrate). The scattered light intensity of the complex was measured at an angle of 90° with respect to the incident light, using a fluorimeter. The DNA-galline complexes were incubated for 1 hour at 37 °C, the intensity at 300 nm was monitored with excitation at 300 nm. The relative scattered light intensity for these complexes compared to free DNA was measured as function of R, which is defined as the arginine to nucleotide phosphate ratio. Figure 24 shows that as the arginine to nucleotide phosphate ratio (R) increased, the intensity increased initially, and then decreased. This result was thought to be due to the formation of a soluble DNA-protamine complex. The loss of light scattering was due to precipitation of the complex in ET buffer. There was very little binding of galline to DNA in SSC buffer, as judged by the low scattered light intensity of the DNA-galline complex in 0.2 × SSC.

Nakano et al. (34) have shown, using a Scatchard plot, the existence of positive cooperativity for DNA-protamine interactions (Figure 24). The DNA and galline samples were incubated for 1 hour at 37 °C. The samples were then centrifuged, so that the fluorescence from the supernatant could be measured without a contribution from light scattering. The concentrations of total, bound and free galline are denoted as C_t, C_b and C_f respectively. Unbound galline remained in the supernatant and its concentration, C_f, was determined by the measurement of the fluorescence of the tyrosine molecules in galline. Since C_t was known, C_b could be calculated by $C_b = C_t - C_f$. The DNA concentration in terms of the nucleotide phosphate is denoted by C_p. Figure 25 (34) shows a plot of C_f versus R. The DNA binding sites were saturated at R = 1.34. Above this value, galline was not able to bind to DNA, and remained free in solution. The number of nucleotides occluded by one molecule of galline, n, was calculated to be 28. The value for n was calculated as follows: Galline has 38 arginine residues. The arginine to nucleotide phosphate ratio for galline is 1.34, hence, n = 38/1.34 = 28. The data obtained was plotted according to Scatchard, i.e. r / C_f versus r, where $r = C_b/C_p$ (34), as shown in Figure 26. A strong positive cooperativity was observed (34). The authors derived an equation relating the degree of binding θ, concentration of free protamine C_f, and the cooperative binding constant K_c. The relationship between the degree of saturation θ, and C_f can be represented by,

$$(2\theta - 1)/ \text{sqrt}[\theta(1 - \theta)] = \text{sqrt}[(q/n) (K_c C_f - 1) \qquad (11)$$

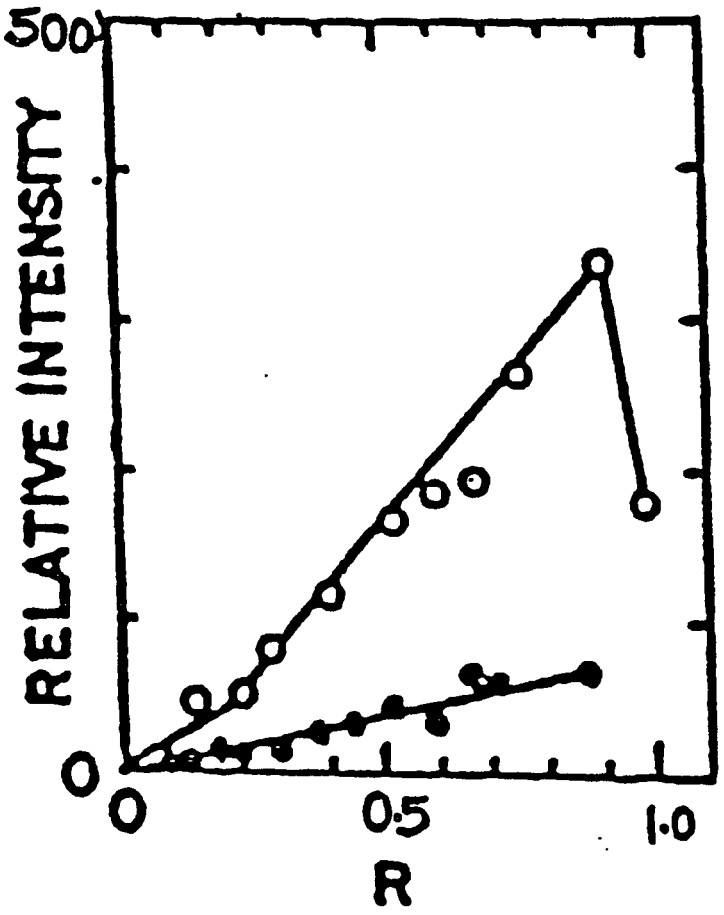

Fig. 24. The relative scattered light intensity of the DNA-protamine complex as a function of arginine to nucleotide ratio (34). Open circles represent the scattered light intensity of the complex in ET buffer. Closed circles represent the scattered light intensity of the complex in 0.2 x SSC buffer. Reproduced with permission from Ref. 34. Copyright 1989 Dr. N. Nakano.

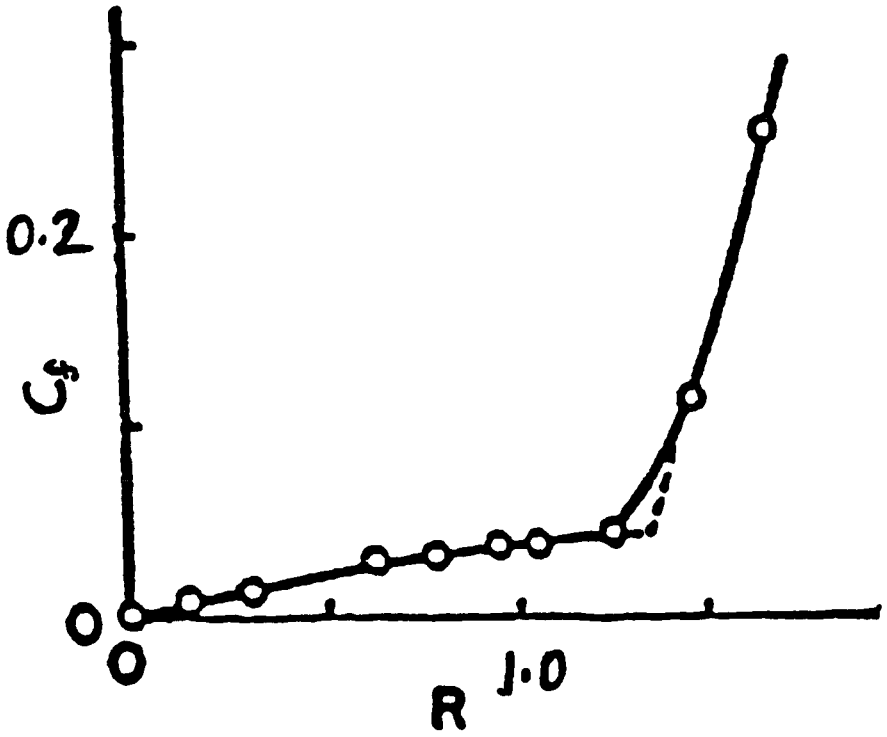

Fig. 25. Concentration of unbound protamine as a function of the arginine to nucleotide ratio in 100 mM NaCl/50 mM Tricine-HCl, pH 7.4 (34). The value of R, i.e. 1.34 is represented by the intersection of the dashed lines. Reproduced with permission from Ref. 34. Copyright 1989 Dr. N. Nakano.

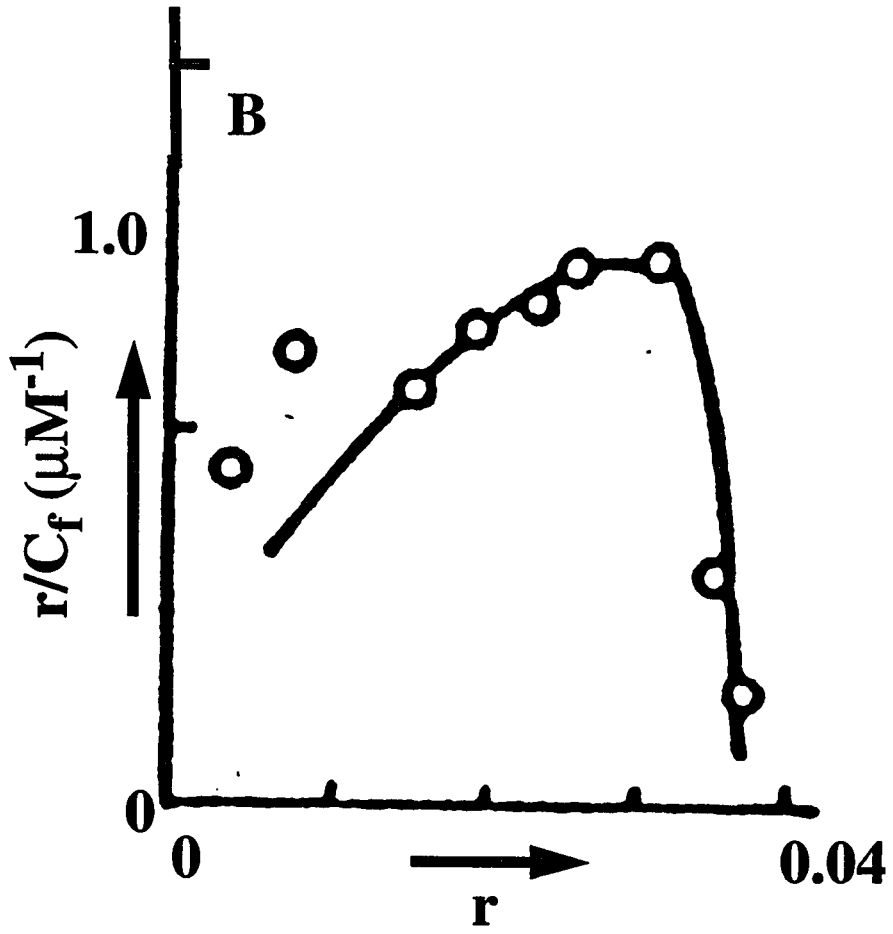

Fig. 26. Scatchard plot for the binding of protamine to DNA, where $r = C_b/C_p$ (34). Reproduced with permission from Ref. 34. Copyright 1989 Dr. N. Nakano.

A plot of $(2\theta - 1)/ \text{sqrt}[\theta(1 - \theta)]$ versus C_f gave a straight line and its X-intercept was $1/K_c$. Using this data, K_c for galline was determined to be 3.3×10^7 M^{-1}.

McGhee and von Hippel (15) have proposed that Scatchard analysis cannot be used in all cases of binding of a ligand to a polymer. As stated earlier, the Scatchard equation was originally derived for the interaction of small ligands with multiple but discrete and isolated binding sites on a lattice (15). It does not take into account the fact that nearly every lattice residue on a polymer is able to start a ligand binding site. Thus, by binding, other potential ligand binding sites are eliminated and lattice saturation is difficult to achieve. Nakano et al. (34) as well as Watanabe and Schwarz (42) have used the Scatchard analysis to analyze their data. This analysis is probably not appropriate, because the galline molecule does not have discrete and isolated binding sites on the DNA.

In all reports, it was found that protamine binds to DNA with positive cooperativity. Nakano's group (34) as well as Porschke (43) used methods which do not require the use of a covalently-attached fluorescent probe. All groups have put forth their own models to explain cooperativity in DNA-protamine binding, as explained earlier. Watanabe and Schwarz (42) and Willmitzer and Wagner (35) have proposed that the higher charge density around the DNA is responsible for the positive cooperativity seen in DNA-protamine interactions. Porschke has attributed the positive cooperativity to ligand-induced DNA association.

The effect of cations on DNA-protamine binding

Olins et al. (44) and Latt and Sober (45) examined the effect that cations would have on the binding of DNA to protamine. Since the positively charged protamine molecules bind to negatively charged phosphate groups on the DNA, it would be interesting to know if the cations present *in vivo* would alter the binding properties of protamine to DNA.

Olins et al. (44) formed soluble complexes of DNA and salmon protamine by direct mixing followed by low speed centrifugation. They observed that the solution showed increasing turbidity with increasing amounts of protamine added to the DNA. They saw that protamines acted like the cationic compounds such as spermine, spermidine and diamines, and

stabilized the native DNA against thermal denaturation as measured by changes in absorption at 260 nm.

Latt and Sober (45) have shown the influence of cations on the strength and specificity of the interaction between DNA and poly-L-lysine. They showed that in the interaction of basic proteins with nucleic acids, the monovalent and divalent cations compete with the basic groups of the proteins for nucleic acid phosphates. The authors (45) propose that cations have different specificities for the different bases. Hence, depending on the cation present, the basic protein may be displaced to another base-rich region. For example, Na^+ has a high specificity for A-T rich regions, hence, in this case, a basic protein such as protamine would be predicted to be displaced to a G-C rich region.

From the experiments carried out by Latt and Sober (45), it can be concluded that cations do influence the interactions between DNA and basic proteins. The base composition of polynucleotides somehow modulates the cation-phosphate interaction. Cations may compete with the positively charged groups on the protamine for the negatively charged phosphates on the DNA, and this may alter the DNA-protamine binding mechanism. However, it must be noted that protamines do not contain lysine, hence these results cannot be directly extended to the protamine system.

To summarize the work done on protamine, most of the research involving protamine has used fowl protamine or fish protamine. Most of the authors have evaluated their binding data on the basis of the model put forth by McGhee and von Hippel (15). However, McGhee and von Hippel's model does not explain aggregation of lattices in their approach. Nakano et al. (34) have used the Scatchard plot to analyze their data, which McGhee and von Hippel (15) suggest is inappropriate for proteins binding to DNA.

Protamines from different species have different chain lengths, hence, the number of arginine residues in each case varies. One has to be careful in extrapolating the results obtained from one protamine species to another. For example, galline has 38 arginine residues, and its arginine to phosphate ratio is 1.34 (34). Therefore, its binding stoichiometry n, or the number of nucleotides occupied by one galline molecule would be $38/1.34 = 28$ nucleotides, or 14 base pairs. Similarly the fish protamine, clupeine Z, contains 21 arginine residues, and its arginine to phosphate ratio is 0.95, hence its binding stoichiometry would be 22 nucleotides, or 11 base pairs (34).

The studies presented here show that the DNA-protamine binding is dependent on the surrounding salt concentration. The equilibrium binding constant decreases, and the cooperativity parameter increases, with an increase in salt concentration. The binding of DNA to protamine needs to be examined at physiological salt concentrations. However, these studies are hampered due to aggregation and precipitation of the complexes under these conditions. The work by these investigators explains the equilibrium of DNA-protamine interactions to some extent, although more work needs to be done to understand this process fully.

COMPARISON OF THE ECO SSB AND PROTAMINE SYSTEMS

Functions

Single-stranded DNA-binding proteins bind with high affinity to single-stranded DNA, and play a role in DNA replication, recombination, and repair. Because of their preferential binding to single-stranded DNA, these proteins have the ability to destabilize native, double-stranded DNA. Hence, they are also known as unwinding, melting or helix-destabilizing proteins. The function of protamine is very different from that of Eco SSB *in vivo*. Protamines bind to double-stranded DNA in the sperm nucleus, and condense it, rendering the DNA genetically inactive.

Both SSB and protamine are nonsequence specific DNA binding proteins. SSB binds to single-stranded DNA and destabilizes double-stranded DNA, as compared to protamine, which stabilizes double-stranded DNA to thermal denaturation. Protamines bind to double-stranded DNA, compacts it, and leaves the DNA in this stable form until fertilization occurs.

Structure and binding properties

The SSB protein is an asymmetric homotetramer containing 18,843 dalton subunits. The monomer contains 177 amino acids, as compared to the mammalian protamine, P1 molecule, which contains 50 amino acids. SSB is divided into two domains, a basic N-terminus and an acidic C-terminus. Protamine P1, on the other hand, contains a central domain which is highly basic due to its arginine-rich DNA binding domain. In fish and fowl protamines, the size varies from 31 to 68 amino acids, but apparently only contains sequences comparable to the central mammalian P1 DNA binding domain. Eco SSB binds preferentially to single-stranded DNA at a ratio of one monomer to every 32 nucleotides, whereas protamine binds to double-stranded DNA at a ratio of about 20 nucleotides or 10 base pairs per protamine molecule. The SSB protein monomer is about three times larger than the protamine molecule. Thus, the two proteins differ to a great extent in their structures, but the regions which bind DNA are basic in both proteins.

Proteolysis studies show that the single-stranded DNA binding site of the Eco SSB protein lies within the N-terminal region of the SSB (8). Since this region is highly basic, this suggests that it is involved in the binding process, as compared to the protamine P1, whose central arginine rich domain has been proposed to be the DNA-binding domain (32, 46).

The role of the different domains of the two proteins differs in the mode of binding. In the case of SSB, the amino terminal region includes most of the residues important for monomer-monomer interactions within the tetramer, and for cooperative SSB binding to the single-stranded DNA (8). The carboxy terminal region is important for tetramerization, and for interaction with other proteins involved in DNA replication or repair, once the single-stranded DNA is bound to the SSB. In the mammalian P1 protamine molecule, the carboxy and amino terminals are involved in the inter and intramolecular cross-linking via disulfide linkages. SSB does not form any inter or intramolecular linkages as in protamine, rather the tetramer stabilization is due to noncovalent forces.

Fluorescence spectra, experiments on accessibility of the SSB tryptophan residues to quenchers, and quantum yield measurements for SSB bound to single-stranded DNA revealed that the tryptophan residues on each monomer were responsible mainly for DNA-SSB binding (11). Bandyopadhyay and Wu (11) suggest that the tryptophan residues are present in the hydrophobic pockets of the protein. Thus, the tryptophan residues become even less accessible to quenchers upon binding to DNA. It has been suggested that tryptophan residues of the SSB protein can interact with nucleic acids by stacking of the indole ring with the purine or pyrimidine bases of DNA (11), in contrast to the electrostatic interactions involved in DNA-protamine binding, which involve the positively charged arginine residues binding to the negatively charged phosphate groups on the DNA (31). Balhorn's model (31) proposes that protamine binds to the DNA in the minor groove, but this has not been established experimentally.

Cooperative binding

Researchers have shown that DNA-SSB binding, and DNA-protamine binding are positively cooperative. For SSB-DNA interactions, it has been proposed that the binding of an SSB tetramer to single-stranded DNA induces the formation of an octamer of SSB by an additional tetramer binding (23). The binding continues only until the formation of an octamer, and it does not lead to indefinite protein clusters. It also assumes that the octamers do not interact with adjacent tetramers or octamers. However, when a protamine molecule binds to DNA, it induces further binding of another molecule, thus leading to an indefinite protamine cluster formation (43).

Two statistical thermodynamic models have been presented for nonspecific, positively cooperative binding of SSB to single-stranded polynucleotides, the "unlimited" cooperativity model applicable to the SSB binding in the $(SSB)_{35}$ mode, and the "limited" cooperativity model applicable to the SSB binding in the $(SSB)_{65}$ mode. The tetramer/octamer model proposed by Bujalowski and Lohman (23) also explains positive cooperativity for the interaction of DNA with SSB in the $(SSB)_{65}$ mode. A number of models have been proposed to explain the observed cooperativity in DNA-protamine binding. One of the models proposed by Willmitzer and Wagner (35) explains the mechanism by stating that higher ionic strengths leads to cross-linking of the DNA strands with protamine molecules. The cross-linking creates a zone of higher charge density leading to further binding of protamine molecules, as an increase in charge density leads to an increase in the binding affinity.

Porschke (43) also has developed a model to explain cooperativity in DNA-protamine binding. He states that ligand binding itself is noncooperative. However, the ligands are assumed to induce a transition of the polymer (DNA) to a different state at a defined degree of ligand binding. This transition implies an altered affinity of the ligand to the polymer. Since protamines like most ligands of biological interest occupy more than one lattice residue, the binding of protamine to DNA can be described by McGhee and von Hippel's (15) excluded site model. Porschke's model can be combined by McGhee and von Hippel's (15) excluded site model to fully understand the mechanism of cooperativity.

Bujalowski and Lohman (16) have shown that negative cooperativity also exists within the different subunits of the SSB tetramer upon the binding of SSB to single-stranded DNA. In the case of DNA-protamine binding, no negative cooperativity has been reported between the different protamine molecules upon binding to DNA. The square model proposed by Bujalowski and Lohman (16) explains negative cooperativity within the SSB tetramer.

Thus, different models have been proposed to explain the cooperativity associated with DNA-SSB binding and with DNA-protamine binding.

Effect of salt concentration

In both SSB-DNA binding and protamine-DNA binding, a decrease in the intrinsic binding constant with an increase in salt concentration was reported. The major difference observed for salt effects, was in the cooperativity

parameter. With SSB-DNA binding, the cooperativity parameter does not change with a change in salt concentration. Research on DNA-protamine interactions has shown that binding is highly cooperative. As the NaCl concentration increased, the cooperativity parameter increased, but the binding constant decreased (35, 42). Watanabe and Schwarz (42) have also shown that the binding affinity between protamine and DNA becomes weaker with increasing concentrations of NaCl. This is because of the competition between the high number of positively charged arginine residues on protamine and the cations present in solution for the negatively charged DNA.

Bujalowski and Lohman (16) have shown that there was a dramatic increase in the affinity for the second molecule of $dT(pT)_{34}$ interacting with SSB with increasing salt concentrations. An uptake of cations may be essential to partially neutralize the negative charges on the single-stranded DNA, and hence, reduce the repulsion if the binding sites are in close proximity. Changes in salt concentration in solution also play an important role in equilibrium binding and kinetics of protamine-nucleic acid interactions. The major effect arises from the fact that linear nucleic acids are negatively charged, hence, they attract cations which partially neutralize the high charge density on the nucleic acid. Thus, the binding of the SSB to DNA as well as that of protamine to DNA is highly salt-dependent, suggesting the electrostatic component in the cooperative and direct interactions.

Both, Eco SSB and protamine molecules differ in their structures. The SSB-DNA complex is soluble *in vitro*, whereas the protamine-DNA complex is not. Understanding the interactions between Eco SSB and DNA can extend our understanding of nonsequence specific DNA binding proteins. It may also help elucidate DNA-protamine binding mechanisms.

CONCLUSION AND FUTURE WORK

The nonspecific interactions of the Eco SSB protein with single-stranded DNA are necessary for various aspects of DNA metabolism and survival of the cell. Due to the multiple binding modes present in SSB it is not clear which of these modes is present *in vivo*. Eco SSB-single-stranded DNA binding is influenced by factors such as salt concentration, pH, and DNA to protein ratio. The studies presented here have provided a foundation on which the molecular basis of nonspecific DNA-protein interactions can be understood. The SSB protein exists in different binding modes in its interaction with single-stranded DNA.

The mechanism of action of the Eco SSB protein is not fully understood, hence, systematic quantitative studies of the effect of SSB on the individual stages of replication and recombination is required. Additionally, little is known about the three-dimensional structure of Eco SSB. A more accurate determination of the site size of Eco SSB binding is required to completely characterize SSB-DNA interactions. The general assumption that all DNA unwinding proteins mainly function by forming long protein clusters along the length of the single-stranded DNA is questionable, since the binding of Eco SSB *in vitro* differs considerably from that of the T4 gene 32 protein. Building upon the foundation of the work in prokaryotes, there should be progress in defining higher eukaryotic SSBs, and their relationship to the prokaryotic SSBs.

Interactions between DNA and protamine play a significant role in spermatogenesis and DNA condensation. This system can be used as a model to study nonspecific interactions between proteins and nucleic acids, or as mentioned by Watanabe and Schwarz, "a model for the nonspecific binding of large ligands to linear polymers" (42).

Although many studies have been done to elucidate the role of protamines in protecting the genetic material after meiosis has occurred, no detailed information on the specificity of its interaction with DNA is available yet. Further experimental work on its binding properties is required. Most of the work performed has been limited to fish protamines or to model homopolypeptides. It is clear that these model homopolypeptides cannot completely represent the naturally occurring protamines due to the difference in their structures, such as the absence of other amino acid residues in homopolypeptides, which may influence the DNA-protamine interaction.

Additionally, the folding of protamine molecule due to disulfide linkages is absent in homopolypeptides. Very little work on mammalian protamine has been reported and almost none on human protamines. This is due to the fact that fish and fowl protamines are more widely commercially available. Studies with mammalian protamines when undertaken, will help to clarify the biological significance of protamines in the sperm nucleus.

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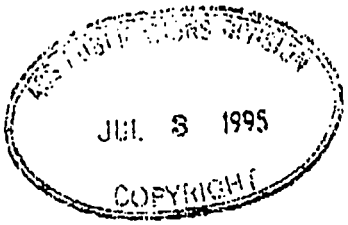

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