

Nonhistone Chromosomal Protein HMG 1 Interactions with DNA

FLUORESCENCE AND THERMAL DENATURATION STUDIES*

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The interaction of high mobility group protein 1 (HMG 1) isolated from chicken erythrocytes with DNA has been characterized using the intrinsic tryptophan fluorescence of the protein as a probe. It was found that the fluorescence is quenched approximately 30% upon binding to either single- or double-stranded DNA. Fluorescent titrations indicate that the physical site size for HMG 1 binding on native DNA is approximately 14 base pairs (or 14 bases for binding to single-stranded DNA). Binding to single-stranded poly(dA) is only slightly dependent on ionic strength, although the affinity for double-stranded DNA is strongly ionic strength-dependent and has an optimum at approximately 100–120 mM Na⁺. Above this range, binding to native DNA is virtually all electrostatic in nature. Although the affinity of HMG 1 for single-stranded DNA is higher than that for double-stranded DNA at the extremes of the ionic range studied, no clear evidence for a helix-destabilizing activity was obtained. At low ionic strength, the protein actually stabilized DNA against thermal denaturation, while at high ionic strength, HMG 1 appears to undergo denaturation below the T_m of the DNA. Studies of the environment of the tryptophan fluorophores using collisional quenchers iodide, cesium, and acrylamide suggest that the predominant fluorophore is relatively exposed but constrained in a rigid, positively charged environment.

Although eukaryotic chromatin is composed primarily of DNA and histones organized in a repeating array of nucleosomes (reviewed by McGhee and Felsenfeld (11), a wide variety of nonhistone proteins are also present. Among the most abundant of these nonhistones are the so-called high mobility group proteins, which are present at levels of at least 10⁶ molecules/nucleus (2, 3). HMG¹ proteins are defined

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¹ The abbreviations used are: HMG, high mobility group; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; l, liters; bp, base pairs; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

largely on an operational basis as chromatin-associated proteins which can be extracted from chromatin by 0.35 M NaCl, are soluble in 2% (w/v) trichloroacetic acid, and are relatively high (approximately 25 mol % of each) in both basic and acidic amino acids (4). The HMG proteins have been found throughout the animal, plant, and fungal kingdoms (5–7). Based on their molecular weights and biochemical properties, the HMG proteins are grouped into two classes: the low molecular weight HMG proteins (HMG 14 and 17 in mammals, H6 in trout testis), and the higher molecular weight HMG proteins, HMG 1 and 2 in mammals, and HMG 1, 2, and E in avian erythrocytes.

The low molecular weight HMG proteins are selectively released from chromatin by treatment of nuclei with DNase I under conditions which also preferentially degrade active genes (8, 9). The presence of these proteins apparently is required for the observed DNase I sensitivity of chromatin containing DNA sequences complementary to messenger RNA. Furthermore, this DNase I sensitivity can be restored by reconstituting chromatin washed with 0.35 M NaCl with HMG 14 or HMG 17 (10–12). These and other observations have led to the hypothesis that the low molecular weight HMG proteins are structural proteins in active chromatin. Reconstitution experiments also demonstrate that either HMG 14 or 17 can bind cooperatively to two sites on the 146 bp-nucleosome core particle (13, 14). By contrast, the high molecular weight HMG proteins are released from chromatin during the early stages of digestion of nuclei with micrococcal nuclease, suggesting a possible localization of HMG 1 and HMG 2 with the linker DNA, similar to the linker-specific histones H1 and H5 (15). The function of the high molecular weight HMG proteins has not been established. However, Stoute and Marzluff (16) reported that either HMG 1 or HMG 2 can stimulate transcription by an endogenous RNA polymerase in mouse myeloma nuclei, while Bonne-Andrea *et al.* (17) found that HMG 1 can mediate nucleosome assembly *in vitro*. Other laboratories have reported that HMG 1 and HMG 2 can alter the superhelicity of closed circular DNA (18, 19); in addition, HMG 1 and HMG 2 are selectively retained on single-stranded DNA columns and apparently destabilize native DNA towards thermal denaturation (19, 20). This helix-destabilizing activity may depend on the conditions of the experiment, however, since other studies concluded that HMG 1 and HMG 2 stabilize DNA against thermal denaturation (21).

HMG 1 and HMG 2 from calf thymus have been largely sequenced (22). These proteins share approximately 85% homology at the sequence level. Serological studies suggest that these two proteins differ by only 6–8% in overall structure and are highly conserved in different species (23). The distri-

bution of charged amino acids in these proteins is highly asymmetric, with the basic residues, lysine and arginine, distributed throughout the N-terminal two-thirds of the polypeptide, while many of the acidic residues, aspartate and glutamate, occur in a continuous run near the C terminus (residues 191 to 232 in calf thymus HMG 1). The native structures of HMG 1 and HMG 2 are approximately 40–50% α -helical (24, 25) and appear to fold into a domain structure (26, 27). According to this model, domain A includes amino acids at positions 1 to ~92, domain B includes positions ~98 to 176, and domain C (positions 177–259) includes the highly acidic region of the protein. Because of the high charge density of domain C and its paucity of hydrophobic residues, this region is not expected to form a compact globular structure. Domains A and B, on the other hand, are predicted to contain substantial amounts of α -helix and to form folded structures. These predictions are supported by the results of proteolytic digestion by both trypsin (26) and V8 protease (28).

In order to further characterize the interaction between HMG 1 and DNA, we have exploited the intrinsic fluorescence of the protein. Previous work has shown that HMG 1 contains two tryptophan residues whose fluorescence is sensitive to protein conformation (24). We now show that the tryptophan fluorescence of chicken erythrocyte HMG 1 is also partially quenched by polynucleotide binding. This fluorescence change was used to obtain quantitative DNA-protein binding data. We also report studies of the microenvironment of the fluorescent residues. In addition, we have reinvestigated the thermal denaturation characteristics of the HMG 1·DNA complex.

EXPERIMENTAL PROCEDURES

Preparation of HMG 1—Chicken erythrocyte nuclei were prepared from 35 l of fresh chicken blood and washed once with 155 l of RSB buffer (10 mM Tris (pH 7.5), 10 mM NaCl, 3 mM MgCl₂) containing 0.1 mM PMSF, which was used throughout the isolation to inhibit proteolysis. The suspension was centrifuged and the erythrocyte pellet was resuspended in 190 l of RSB plus 0.5% Nonidet P-40 and centrifuged again. The nuclear pellet (~3.5 l) was resuspended in 10 l of 0.35 M NaCl, 0.1 mM PMSF (pH 7) and stirred in the cold for 30 min to extract the HMG proteins. The nuclei were removed by centrifugation and the supernatant was made 2% in trichloroacetic acid to precipitate the "low mobility group" proteins (2). The solution was filtered and then centrifuged at 20,000 $\times g$ for 15 min. The clear supernatant was brought to 10% trichloroacetic acid and incubated overnight at 4 °C. The precipitate was collected by centrifugation, washed once with acetone:HCl (200:1, v/v) and then three times with cold acetone. The pellet, containing crude HMG 1, 2, and E, was dried under vacuum and stored desiccated at -20 °C. Portions of this mixture were subsequently fractionated by Cm-Sephadex C25 chromatography as previously described (29). The protein was first dissolved in 0.01 N HCl, 5 M urea, 50 mM DTT, 0.1 mM PMSF and incubated 1–2 h at 4 °C to reduce the sulfhydryl groups. The solution was dialyzed against 7.5 mM borate (pH 8.8), 1 mM DTT, 0.1 M NaCl, 0.1 mM PMSF, clarified by centrifugation, and loaded on a 2.5 \times 30-cm column of CM-Sephadex equilibrated in the same buffer. HMG 1 was eluted with about 200 ml of the starting buffer; the eluate was monitored at 230 nm. HMG 2 and HMG E were subsequently eluted with 7.5 mM borate (pH 8.8) containing 0.6 M NaCl. Fractions across the column peaks were analyzed by sodium dodecyl sulfate-gel electrophoresis in 15% polyacrylamide minislabs as previously described (30, 31). The gels were stained with Coomassie Brilliant Blue and those fractions containing pure HMG 1 were pooled and precipitated with acetone (Fig. 1). The purified HMG 1 was stored at -20 °C.

Fluorescence Procedures—HMG 1 stock solutions in 0.1 M NaCl, 10 mM Tris, 0.1 mM DTT, 0.1 mM PMSF were diluted to a concentration of 1–2 μ M just before use. UV spectra were recorded (Cary 15 or Cary 118) to verify protein concentration, using the published extinction coefficient of 20,900 at 280 nm (24). Fluorescence spectra were recorded using a 2-nm slit in a Perkin-Elmer MPF 44A spectro-

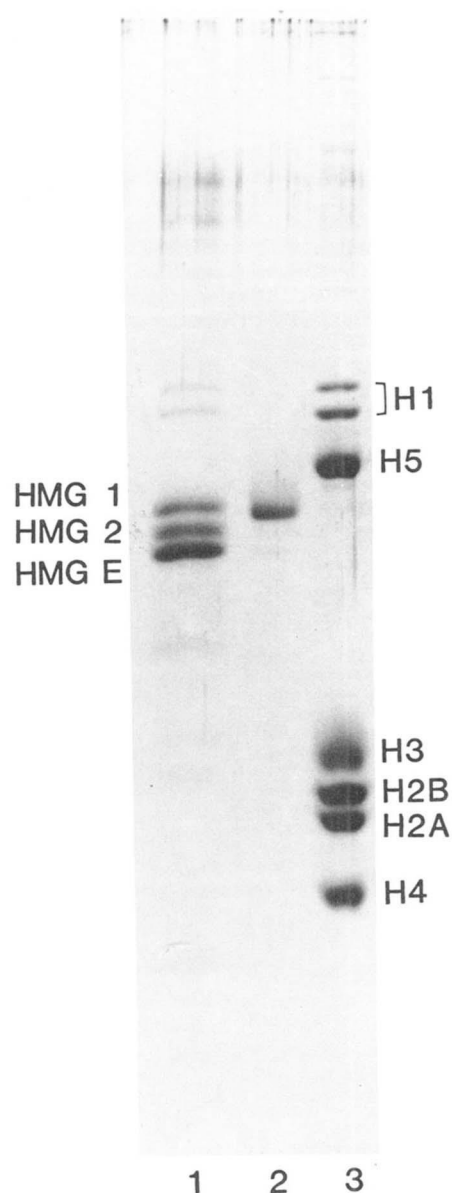


FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of chicken erythrocyte HMG proteins. Lane 1, chromatin proteins soluble in 2–10% trichloroacetic acid (predominantly HMG 1, 2, and E); this sample was further fractionated by Cm-Sephadex chromatography. Lane 2, HMG 1 purified by Cm-Sephadex chromatography. Lane 3, erythrocyte histones.

fluorimeter. For titrations, aliquots of DNA or quenchers were added directly to the protein solution and the fluorescence change at 330 nm was recorded. Unless otherwise indicated, excitation was at 295 nm. The data was corrected for dilution (less than 10% of the initial volume); parallel titrations of a solution of *N*-acetyltryptophanamide at the same absorbance were conducted to rule out artifacts due to inner filter effects.

Other Procedures—Analytical electrophoresis in sodium dodecyl sulfate-polyacrylamide gels followed the method of Laemmli (30); the separating gel was 15%. Thermal denaturation experiments were conducted with a Gilford 2000 spectrophotometer interfaced to a PDP 11-20 computer, as described (32). The buffers used for all thermal denaturation experiments contained 0.2 mM Na₂EDTA, 0.1 mM DTT, plus either 1 or 5 mM HEPES (pH 8.0) and NaCl to adjust the ionic strength. Cation (Na⁺) concentrations reported take into account the contribution of 0.58 mM Na⁺ added to 1 mM HEPES to adjust the pH. Samples were degassed under vacuum and the cuvettes were sealed tightly with Teflon stoppers prior to thermal denaturation. CD spectra were recorded on a JASCO J-40A; the α -helix

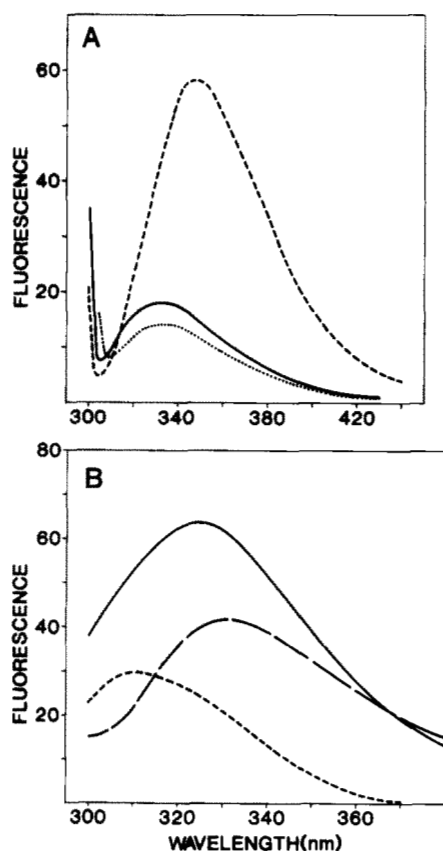


FIG. 2. Fluorescence emission spectra of HMG 1. A, identical concentrations of free HMG 1 (—), DNA-bound HMG 1 (.....), and the free protein denatured in 9 M urea (-----). B, fluorescence emission of HMG 1 after excitation at 295 nm (—) and 275 nm (---), normalized to equal intensity at 370 nm; the difference (.....) represents the tyrosine contribution to the emission spectra. The buffer for these experiments contained 0.1 M NaCl, 10 mM Tris-HCl (pH 8.0) and 0.1 mM DTT. Protein concentration was approximately 1.5 μ M and the emission monochromator slit was 2 nm.

content was calculated to be 40–45%, in agreement with published values for calf thymus HMG 1 (24).

RESULTS

HMG 1 Fluorescence—The fluorescence emission properties of HMG 1 are shown in Fig. 2. When tryptophan is selectively excited at 295 nm, the protein fluorescence peak is at 328–330 nm (Fig. 2A). Model tryptophan compounds, such as *N*-acetyltryptophanamide, fluoresce at 347–350 nm in the same buffer. In both cases, these values are uncorrected for the response characteristics of the instrument. The observed blue shift of \sim 20 nm indicates that one or both of the two tryptophan residues in each HMG 1 molecule are in an environment much different than that of liquid water. In addition to the blue shift, the relative intensity of HMG 1 fluorescence is substantially lower than of an equivalent concentration of *N*-acetyltryptophanamide. However, both the quantum yield and emission maximum are returned to the expected values when the protein is denatured in 9 M urea (Fig. 2A). These fluorescence properties are thus highly sensitive to the native state of the protein.

Addition of DNA to HMG 1 causes an additional quenching of protein fluorescence, with no observable shift in emission wavelength (Fig. 2A). Addition of the same concentration of DNA to *N*-acetyltryptophanamide produces no change in fluorescence intensity, demonstrating the absence of inner filter effects at the concentrations used (see “Experimental

Procedures”). Both single- and double-stranded DNA and the synthetic polynucleotides poly(dA) and poly[d(A·T)]-[d(A·T)] produce qualitatively similar fluorescence quenching. This suggests that DNA binds HMG 1 at a site near one or both tryptophan residues or that DNA binding induces a protein conformational change which affects the environment of the fluorophore. These fluorescence changes can be used to monitor DNA binding (see below).

We conducted a number of experiments to obtain additional information about the environment of the tryptophan residues and the effect of bound DNA on the protein conformation. Emission spectra were scanned as a function of excitation wavelength, at 5-nm intervals from 275 to 305 nm. At excitation wavelengths below \sim 285 nm, the emission maximum is about 325 nm. At longer excitation wavelengths (290–305 nm), the emission peak shifts to \sim 330 nm. This heterogeneity could reflect a contribution due to tyrosine fluorescence, since this amino acid can be excited at wavelengths below 290 nm. To confirm this, emission spectra derived from excitation at 295 nm (excitation of only tryptophan) and 280 nm (excitation of both tryptophan and tyrosine) were normalized to equal spectral intensities and a difference spectrum calculated as previously described (33). This difference emission spectrum (Fig. 2B) has a peak intensity of 309–310 nm, as expected for tyrosine fluorescence. Thus, it appears that at least some of the eight tyrosines (22) in HMG 1 fluoresce independently and are not quenched by nonradiative energy transfer to tryptophan, as is frequently the case in proteins (34).

The solvent accessibility of HMG 1 tryptophans was determined by the solute quenching method (35). The results of KI quenching studies are summarized in Table I. Experiments conducted at low iodide concentrations (<0.25 M) show the behavior expected for a collisional quenching mechanism (35, 36). Plots of F_0/F_i are linear, within experimental precision, indicating approximately equal accessibility for the majority of the protein fluorophores. The quenching constant (K_q) determined from the slope of these plots (Table I) is approximately 5.6 M $^{-1}$, as compared to 11.6 M $^{-1}$ for *N*-acetyltryptophanamide under the same experimental conditions. This represents considerable exposure of the fluorophore and is contrary to the expectation, based on the strongly blue-shifted emission spectrum (Fig. 2A) of a buried tryptophan fluorophore (see “Discussion”). Emission spectra of HMG 1 samples dissolved in 1 M KI are red-shifted 5–7 nm, as well as being strongly quenched, relative to a sample of HMG 1 in 1 M NaCl as control. This suggests that the most accessible tryptophan residues are those with fluorescence at the shortest wavelength. We note that previous studies have shown that, above a concentration of \sim 0.1 M NaCl, increasing ionic strength has little or no additional effect on the conformation of HMG 1 (24).

Because of the apparent contradiction between the dra-

TABLE I

Solute quenching of HMG 1 fluorescence

Buffer: 0.01 M Tris (pH 8.0), 0.1 M NaCl, 0.1 mM DTT. The protein concentration was between 1 and 2 μ M for each experiment shown.

Quencher	K_q		
	HMG M $^{-1}$	NATA ^a M $^{-1}$	HMG + DNA M $^{-1}$
Iodide	5.6	11.6	ND ^b
Acrylamide	4.5	30.5	3.7
Cesium	<0.2	1.5	ND

^a NATA, *N*-acetyltryptophanamide.

^b ND, not determined.

matic blue shift of the spectrum of native HMG 1 and the moderate exposure indicated by iodide quenching, we undertook quenching studies with an uncharged collisional quenching agent, acrylamide (37), and a positive quencher, cesium (38). The results are given in Table I. The quenching constant for acrylamide is $K_q = 4.5$, as determined from linear Stern-Volmer plots. Since acrylamide is an extremely effective quencher of *N*-acetyltryptophanamide ($K_q = 30$), the relative accessibility of HMG 1 tryptophans to acrylamide is somewhat less than the accessibility to iodide. This may be due to the larger size of acrylamide relative to iodide, or to a lack of favorable electrostatic interactions which may be present between the protein and iodide. Using the positively charged quencher Cs^+ , virtually no quenching of HMG 1 fluorescence could be detected ($K_q < 0.2$, Table I). Since the ionic radius of Cs^+ (1.69 Å) is actually slightly smaller than that of I^- (2.16 Å), we conclude that the relatively greater quenching by iodide is due to the presence of positively charged amino acid side chains near the fluorophore which enhance the effective local iodide concentration (and diminish the effective local Cesium concentration), rather than exposure of the fluorophore *per se*. An example of similar behavior has been reported in the case of human serum albumin (38). Acrylamide quenching experiments conducted with HMG 1 bound to double-stranded DNA (Table I) or poly(dA) suggest that the fluorophores may be slightly less accessible in the protein-DNA complex. However, the small differences observed indicate no major burial of tryptophan residues when the protein is bound to DNA.

Interaction of HMG 1 with DNA—The fluorescence quenching of HMG 1 by added DNA provides a convenient method for monitoring equilibrium titrations of the protein by polynucleotides. Fig. 3 illustrates a titration curve generated by the addition of chicken erythrocyte DNA to a solution of HMG 1. The maximal quenching is approximately 30% of the initial fluorescence and is similar for both double-stranded and single-stranded DNA. These titrations have been interpreted by means of the generalized Scatchard (39) binding equation developed by McGhee and von Hippel (40). This analysis takes into account the fact that the start of each potential nonspecific binding site overlaps other binding sites on the DNA lattice. From this approach, each titration curve can be described in terms of an association constant K_a and the site size n , the number of base pairs physically occluded

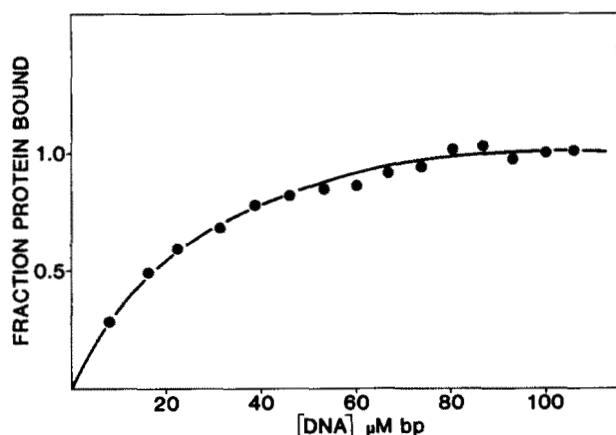


FIG. 3. Titration of HMG 1 with double-stranded DNA. HMG 1 concentration was 1.49 μM . Fluorescence intensity was measured at 330 nm after each addition of native chicken DNA (see "Experimental Procedures"). The data from three independent titrations were averaged. The final quenching was $30.1 \pm 1.2\%$ of the initial in intensity. Buffer composition was as in Fig. 2.

by the binding of one protein molecule. A Scatchard plot of fluorescence titrations of HMG 1 with native chicken erythrocyte DNA in 0.1 M NaCl is shown in Fig. 4. The discrete symbols are individual data points, while the continuous curves are calculated binding isotherms presented to show the effect of varying the assumed site size from $n = 12$ to $n = 16$ bp, with $K_a = 2.5 \times 10^5$. We conclude that the site size is 14 ± 3 bp. Titrations performed at 0.2 M NaCl, also shown in Fig. 4, were fit to a site size of $n = 14$ and $K_a = 2.5 \times 10^4 \text{ M}^{-1}$. Due to inner filter effects at high DNA and protein concentration, we estimate that the practical lower limit for obtaining K_a from these titrations is about 10^4 M^{-1} . Results from other titrations with both native chicken DNA and poly(dA) performed over a range of ionic strengths are summarized in Table II. Each value is the mean of at least three independent titrations, and all of the experimental data agree with a site size of approximately 14 bp (for native DNA) or 14 bases (for denatured DNA or poly(dA)). This site size is consistent with

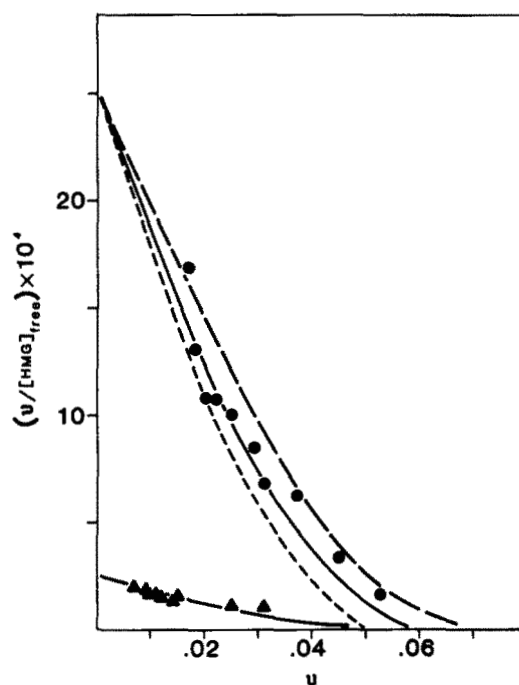


FIG. 4. Scatchard analysis of fluorescence binding data. Results from the experiment in Fig. 3 are indicated by \bullet ; a similar titration in 0.2 M NaCl is also presented (\blacktriangle). Theoretical binding curves were calculated according to Ref. 40 using association constants of $2.5 \times 10^5 \text{ M}^{-1}$ for the 0.1 M data and $2.5 \times 10^4 \text{ M}^{-1}$ for 0.2 M NaCl. The site sizes used for the calculations were 12 (----), 14 (—), and 16 (-·-·-).

TABLE II
Association of HMG 1 with DNA

[Na ⁺] M	Equilibrium constant (K_a) ^a M ⁻¹	
	Native chicken DNA	Poly(dA)
0.025	5×10^4	6×10^5
0.05	1.5×10^5	6×10^5
0.10	3×10^5	5×10^5
0.14	3×10^5	4×10^5
0.19	5×10^4	ND ^b
0.20	3×10^4	2×10^5

^a Equilibrium constants determined from Scatchard plots of the fluorescence data, assuming a site size of 14 base pairs. Buffer conditions: 10 mM Tris-HCl (pH 8.0), 0.1 mM PMSF, 0.1 mM DTT, and NaCl to give the indicated sodium concentration.

^b ND, not determined.

previous estimates of the site size for native DNA of 13–18 bp, based on other techniques (41).

The data in Table II show that binding of HMG 1 to double-stranded DNA has a substantial dependence on ionic strength. Binding to single-stranded poly(dA), however, appears to be much less sensitive to ionic strength. The ionic dependence of protein-DNA association constants can be analyzed according to the model of Manning (42) as elaborated by Record and co-workers (43, 44). These workers have shown that, for electrostatic interaction of a ligand with DNA, the dependence of the binding constant on monovalent cation concentration is expected to follow the relationship given below.

$$-\frac{\partial \log K_a}{\partial \log [M^+]} = m' \psi + k \quad (1)$$

In this equation, K_a is the observed association constant, $[M^+]$ is the monovalent cation molarity, m' is the number of ion pairs formed between protein and DNA (or, equivalently, the number of sodium ions displaced from DNA when the complex is formed), and ψ represents the fractional counterion thermodynamically bound per phosphate. This parameter is dependent on the geometry of the DNA *i.e.* for native B-form DNA, $\psi = 0.88$, while for single-stranded DNA, $\psi = 0.71$. These theoretical values have been verified experimentally (45). The term k represents the number of anions (if any) displaced by formation of the nucleic acid-protein complex. For the present discussion, we assume this term to be zero, although we have not experimentally determined if anion displacement is involved in the interaction.

According to this model, a plot of $\log K_a$ versus $\log [M^+]$ should have a slope proportional to m' , the number of ion pairs formed in the protein-DNA complex. When the titration data is plotted in this fashion, striking differences are observed between the ionic dependence of binding to single- and double-stranded polynucleotides (Fig. 5). Binding of HMG 1 to poly(dA) conforms approximately to the equation over the range $25 \text{ mM} < [Na^+] < 200 \text{ mM}$, although there may be a small decrease in slope below $\sim 100 \text{ mM NaCl}$. The slope at

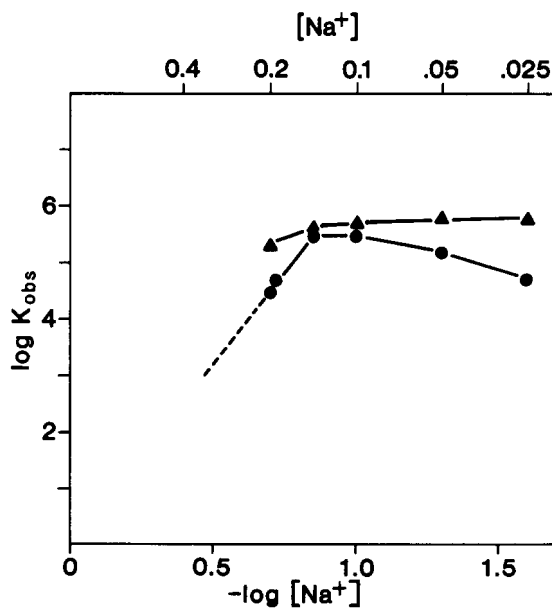


FIG. 5. Dependence of the observed association constants on $[Na^+]$. Binding constants (Table II) were plotted according to Equation 1. ● correspond to native chicken DNA; ▲ represent titrations with poly(dA).

higher ionic strength indicates that a maximum of 2–3 ion pairs are formed in the complex with single-stranded DNA and that much of the binding energy appears to be nonelectrostatic. The interaction of HMG 1 with native DNA shows a more complex ionic dependence. Above $\sim 130 \text{ mM NaCl}$, $\log K_a$ is a strong linear function of $\log [Na^+]$, as predicted by Equation 1. The slope of this line corresponds to the formation of 7 or 8 ion pairs in the DNA-protein complex. The extrapolation of this line to 1 M NaCl gives an intercept of $\log K_a \sim 0$. This implies that under these conditions, the binding of double-stranded DNA is virtually all electrostatic in nature. Below $\sim 130 \text{ mM Na}^+$, the value of K_a reaches a plateau and then decreases at the lowest ionic strengths studied. In the plateau region ($\sim 75\text{--}120 \text{ mM Na}^+$), the affinity of HMG 1 is similar for both single- and double-stranded DNA, while at the extremes of the ionic range studied there is a distinct preference for single-stranded DNA. The unusual ionic dependence of the binding to double-stranded DNA suggests that a conformational change may occur in the protein at ionic strengths below $\sim 100 \text{ mM Na}^+$ (see "Discussion").

Thermal Denaturation Studies—Although early studies of the interaction of calf thymus HMG 1 with DNA concluded that HMG 1 stabilizes native DNA against thermal denaturation (21), more recent experiments suggest that HMG 1 acts as a helix-destabilizing protein at moderate ionic strength (19). In an attempt to resolve this discrepancy, we undertook additional experiments to determine the effect of chicken HMG 1 on the thermal denaturation of DNA.

At ionic conditions of less than $25\text{--}30 \text{ mM Na}^+$, HMG 1 does stabilize DNA against thermal denaturation, as shown in derivative melting profiles (Fig. 6A). Although the observed

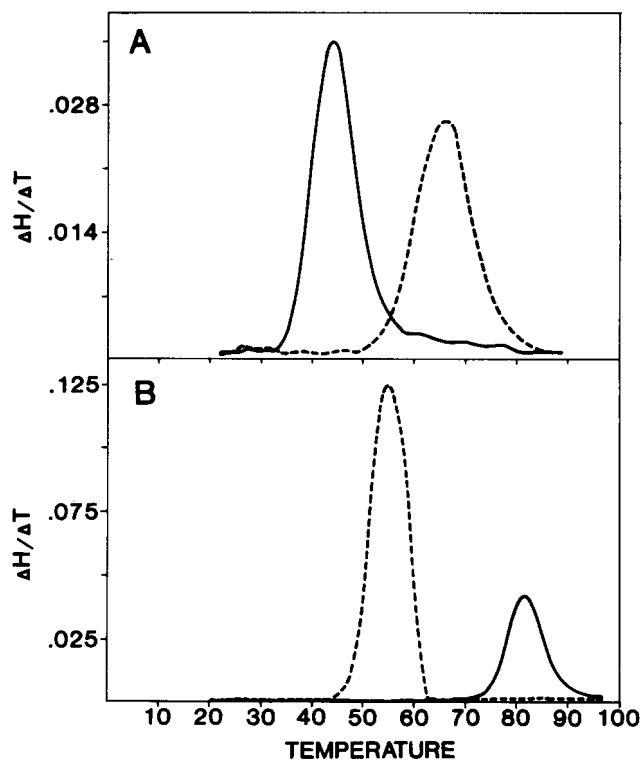


FIG. 6. Thermal denaturation of HMG 1-DNA complexes. Derivative melting profiles are shown under conditions of low ionic strength (A) and high ionic strength (B). When HMG 1 was present, the DNA-to-protein ratio was 15 bp/polypeptide; initial DNA absorbance was 1.0 at 260 nm. —, DNA alone; ----, DNA + HMG 1. The buffers were composed of 1 mM HEPES (pH 8.0), 0.2 mM Na_2EDTA ; in panel B, 100 mM NaCl was also present.

hyperchromicity transition is broadened relative to free DNA under these conditions, the melts are monophasic, suggesting a lack of binding cooperativity. The total hyperchromicity of the complexes under these conditions is 35–40%, similar to that of free DNA. Above 30 mM Na⁺, on the other hand, the HMG 1·DNA complex undergoes a transition below the temperature at which DNA alone melts (Fig. 6B). This result is comparable to that reported by Javaherian and co-workers (18, 19) and suggests that HMG 1 is a helix-destabilizing protein at physiological ionic strength. However, at these ionic strengths, interpretation of the denaturation profiles is difficult due to turbidity above the transition temperature, apparently caused by protein denaturation and aggregation. This leads to an apparent hyperchromicity at 260 nm, caused by light-scattering artifacts, substantially greater than that of free DNA (typically 3-fold greater), and in several cases the transitions also appear to be multiphasic. Furthermore, at all NaCl concentrations above 40 mM, the HMG 1-DNA thermal transitions appear to occur at 54–62 °C.

In order to distinguish the contribution of protein denaturation and aggregation to the overall thermal transition, we heated HMG 1 alone. At low ionic strength, a transition which resulted in significant light scattering (as monitored by absorbance at 340 nm) occurred near 56 °C (in 15 mM Na⁺) or 59 °C (in 48 mM Na⁺). In 180 mM Na⁺, no turbidity was observed (at 340 nm) between 20 and 100 °C, although a gradual increase in protein absorbance (at 260–280 nm) did occur between 50–70 °C. The final value of the protein absorbance at 90–100 °C was ~23% greater than at room temperature. We interpret these results to indicate that HMG 1 undergoes thermal denaturation between 55 and 65 °C, in the absence of DNA, over the range of ionic strengths studied. We note that, at low ionic strengths, this denaturation leads to aggregation, while at high ionic strengths the aggregation appears to be suppressed. This is in contrast to the behavior of the protein·DNA complexes, which aggregate at high ionic strength, but not at low ionic strength. This difference suggests that the protein remains bound to DNA above the transition point and may be protected against denaturation.

The stoichiometry of the protein·DNA complex was investigated by analyzing the melting profiles of poly[d(A·T)]-poly[d(A·T)] or native chicken DNA in the presence of increasing amounts of HMG 1, in 50 mM NaCl (Fig. 7A). Under these conditions, melts were biphasic at a DNA/protein ratio of 30 bp/HMG 1 molecule; about half the hyperchromicity was at the temperature expected for free DNA. However, at a 15 bp/HMG 1 or 10 bp/HMG 1 ratio, essentially all of the hyperchromicity occurred at the “destabilized” temperature, consistent with a site size of ~14 bp, as determined fluorimetrically.

In order to ensure that the thermal denaturation experiments were conducted under equilibrium conditions, we conducted a “transfer” experiment (46) in which HMG 1 was dialyzed with native chicken DNA at a ratio of 15 bp/polypeptide. Part of this DNA·protein complex was then mixed with enough additional DNA to produce a 2-fold excess of DNA (30 bp/polypeptide) and immediately melted. The denaturation profile was superimposable on that of a 30 bp/polypeptide complex prepared by prior dialysis (Fig. 7B), demonstrating that HMG 1 bound to DNA is free to equilibrate with other DNA molecules. This experiment also shows that the half-life of the DNA·protein complex must be less than ~10 min under the conditions used (5 mM HEPES (pH 8), 4.3 mM Na⁺). Because of the problems associated with aggregation at high temperatures in denaturation experiments at higher ionic strength, it has not been possible to perform

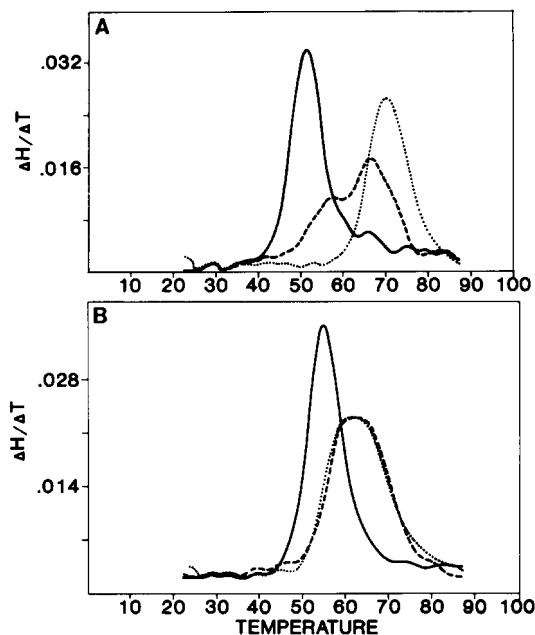


FIG. 7. A, derivative melting profiles as a function of DNA-to-HMG ratio. —, DNA alone; ----, DNA/HMG 1 at 30 bp/polypeptide; ····, DNA/HMG 1 at 15 bp/polypeptide. B, equilibration of bound HMG 1 with added DNA. Derivative thermal denaturation profiles are shown for DNA alone (—); a protein·DNA complex prepared by dialysis at a ratio of 30-bp DNA/HMG 1 (----) and a complex at 30 bp/HMG 1 prepared by adding free DNA to a dialyzed 15 bp/polypeptide complex immediately before the experiment. The buffer in both A and B was 5 mM HEPES (pH 8.0), 0.2 mM Na₂EDTA.

these transfer experiments under conditions of physiological ionic strength.

Other Experiments—We have confirmed previous findings of an 8–10% decrease in the intensity of the circular dichroism of DNA at 275 nm when bound to HMG 1 (21). CD titrations of either native DNA or poly[d(A·T)]-poly[d(A·T)] with increasing amounts of HMG 1 show a break point at approximately 12–16 bp/protein molecule (data not shown). However, the CD changes are sufficiently small that they have not proven reliable for quantitative determinations of HMG 1-DNA interactions.

Titration of poly(dA) with HMG 1 were also monitored by UV absorbance spectroscopy. We have not found any measurable changes in the poly(dA) spectrum, between 240–320 nm, after corrections for protein absorbance. This suggests that, unlike T4 gene 32 protein (47), HMG 1 does not distort the polynucleotide backbone. This difference may explain, in part, the lack of cooperativity of HMG 1 binding.

DISCUSSION

Our fluorescence studies reveal the following features of the interaction between HMG 1 and DNA. 1) The physical site size for the protein on DNA is approximately 14 bp. 2) The binding to double-stranded DNA is largely electrostatic in nature and varies strongly with ionic strength. 3) Binding to single-stranded DNA is somewhat less dependent on ionic interactions and may include a contribution from nonelectrostatic forces. 4) Above 0.1 M NaCl, HMG 1 displays preferential binding to single-stranded polynucleotides.

These results are comparable to earlier work in several respects. A site size of 13–18 bp was reported for the binding of calf thymus HMG 1 to phage T7 DNA based on the results of a sedimentation method (41). Association constants reported in that study for binding to double-stranded DNA

show virtually the same ionic dependence as our results; however, the binding constants which we have determined are approximately 5-fold weaker than those previously reported (41). More recently, the binding of HMG proteins to nucleosome particles was studied using both sedimentation velocity and gel electrophoresis (48). This study indicated that while both HMG 1 and HMG 2 bind to 180-base pair nucleosomes, HMG 1 interacts much more weakly. Furthermore, 140-bp nucleosome core particles bound only a trace of either protein. Consistent with the idea that HMG 1 or HMG 2 bind preferentially to linker DNA, free 180-bp DNA bound ~9-fold more HMG 1 or HMG 2 than the 180-bp nucleosome, a difference approximately proportional to the increased number of accessible binding sites on free DNA. Although binding constants to DNA were not reported, an affinity of $1.7 \times 10^7 \text{ M}^{-1}$ (in 0.15 M NaCl) was determined for the interaction of HMG 2 with 180-bp nucleosomes. Based on the relative affinities reported by Schröter and Bode (48), HMG 1 would be expected to bind 5–10-fold less tightly than HMG 2 to either DNA or 180-bp nucleosomes. This would also be approximately 5-fold greater than our results at the same ionic strength. The differences in the binding constants previously reported (41, 48) and those determined by us may reflect differences in the sources of protein or DNA as well as the possibility of a higher affinity for short DNA fragments due to binding of the protein at free ends, which could be easily denatured. Given the differences in experimental procedures, and other sources of variation, the agreement between the previous studies and our results is good. Our results do not agree as well with those of Shepelev *et al.* (49), who reported association constants for native DNA of 10- to 30-fold weaker than we find. The affinity which we measure for poly(dA) is also ~40-fold greater than reported in Ref. 49 at 0.075 M NaCl. These differences may be due to the chromatographic technique used in Ref. 49. Binding constants of HMG 1 to single-stranded mixed sequence DNA have not previously been reported. However, qualitative studies have shown that HMG 1 will bind to single-stranded DNA-cellulose columns in 0.2 M NaCl, while no retention is observed on a double-stranded DNA column at this ionic strength (11). At 0.2 M NaCl, HMG 1 displays ~10-fold preference for single-stranded DNA binding (Table II). This difference could be sufficient to lead to the observed separation on DNA-cellulose (50).

The dependence of the association constants on ionic strength do not conform to the log-log relationship predicted by Equation 1. Although the relationship is approximately valid for single-stranded poly(dA) binding, there may be a change in slope between 50 and 100 mM Na⁺. In any case, the ionic dependence for single-stranded DNA binding indicates that not more than two or three net ion pairs are formed under any of the conditions studied. Binding to double-stranded DNA has a much greater dependence on ionic strength, however. Above 100 mM Na⁺, the binding agrees with the relationship predicted if the interaction is essentially all electrostatic in nature. The slope of the line suggests that approximately 7 or 8 ion pairs may be involved. However, below 100 mM Na⁺, the affinity plateaus at approximately $3 \times 10^5 \text{ M}^{-1}$ and then decreases at low ionic strength. Among the possible explanations for this behavior is a protein conformational change at low ionic strength resulting in a form with lowered affinity for native DNA. We note that Baker *et al.* (24) reported small changes in fluorescence anisotropy and circular dichroism over this low ionic strength range which may be manifestations of a conformational change. The highly asymmetric charge distribution of HMG 1 suggests a possible

mechanism for reduction in affinity at low ionic strength. It is likely that at high ionic strength, the C-terminal, aspartate- and glutamate-rich region of the protein exists as a "random coil," extended domain. This prediction is based both on the fact that synthetic poly(L)-glutamic acid is in the random coil conformation at neutral pH and on NMR studies which indicate that the majority of HMG 1 aspartate and glutamate side chains are free and mobile (25). At low ionic strength, the C-terminal domain may partially collapse onto the structured, DNA binding domain, which has a net positive charge. This could occlude a portion of the DNA binding site; in a sense, the C-terminal domain may act as a competitive inhibitor of DNA binding at low ionic strength. Studies of the stability of proteolytic cleavage products of HMG 1 also suggest possible interactions between the C-terminal domain and the positively-charged central domain (amino acid residues 96–169) (27). In chromatin, the C-terminal domain may be involved in electrostatic interactions with the basic regions of histones, which would render it unlikely to interfere with ionic protein-DNA interactions.

The preferential affinity shown by HMG 1 for single-stranded DNA, at ionic strengths greater than 0.1 M NaCl, suggests that the protein should be able to destabilize double-stranded DNA at equilibrium, under physiological conditions of ionic strength. However, previously published thermal denaturation experiments reported contradictory results (19, 21). Our results strongly suggest that the apparent destabilization of DNA by HMG 1 is largely an artifact caused by aggregation of the protein and light scattering as the temperature is raised above ~56 °C. Marekov *et al.* (51) recently published results leading to the same conclusion. This artifact is present, although not commented on, in earlier studies (20).

Recent studies suggest that HMG 1 may differ somewhat in conformation when it is isolated by salt extraction and chromatography at normal pH, rather than by acid extraction (52). However, the DNA binding properties of the protein are apparently not substantially affected by the isolation procedure (52), and the salt-extracted protein also fails to lower the T_m of DNA (51). While it has been reported that HMG 1 isolated from regenerating rat liver, but not from normal rat liver, is capable of reducing the T_m of poly[d(A·T)] (17, 53), additional studies will be required to determine the cell-cycle dependence of HMG 1-DNA interactions. If these cell-cycle dependent changes in helix-destabilizing activity are confirmed, they could have an important role in modulating the functions of HMG 1, such as its proposed involvement in chromatin assembly (17).

The apparent inability of HMG 1 to denature DNA, despite preferential affinity for single-stranded DNA, implies that there may be a kinetic block which prevents DNA destabilization. This behavior is reminiscent of that displayed by phage T4 gene 32 protein (g32p) which can destabilize poly[d(A·T)]-poly[d(A·T)] but not native DNA, even though its preferential affinity for single-stranded DNA is substantial (47). Removal of a C-terminal peptide (residues 254–301), which has a net charge of -10, eliminates the g32p kinetic block (54). In this regard, it would be of interest to conduct DNA binding studies with a fragment of HMG 1 lacking the acidic C-terminal region (residues ~177–259). Although several laboratories have reported discrete proteolytic domains from HMG 1, we are not aware of any report of isolation of an intact peptide containing the N-terminal two-thirds of the protein.

In addition to its use as a probe of DNA binding, the intrinsic fluorescence of HMG 1 also reveals features of the microenvironment near the fluorophore. HMG 1 has strongly

blue-shifted fluorescence in the native state (Fig. 2A). Yet the fluorescent tryptophyl residues are apparently exposed to collisional quenchers such as iodide. The lack of quenching by Cs⁺ suggests a region of positive charge near the fluorophore. Taken together, these findings suggest that the dominant tryptophan is exposed but may be constrained in a rigid, positively charged environment. Analysis of the protein sequence (derived from calf thymus HMG 1) shows that Trp 56 is flanked by an arginine and a lysine while Trp 140 is flanked by a methionine and an asparagine (22). We tentatively conclude that Trp 56 is the "blue" fluorophore and that this residue may be at or near the DNA binding site. More detailed studies of the interaction of independent proteolytic domains of HMG 1 with free DNA, as well as with defined chromatin fragments, will be required to fully define the mode of HMG 1 binding.

The tryptophan fluorescence of HMG 1, 2, and E may prove useful as a probe of interactions with chromatin as well as DNA. Since the histones contain only weakly fluorescent tyrosine residues, they should not interfere appreciably. Such a selective probe of HMG binding promises to provide insight into the thermodynamics of the interaction with chromatin as well as knowledge of the conformation of the protein.

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