Thiol Reactivity of Histone H3 in Soluble and DNA-Associated Histone Complexes: Evidence for Allosteric and Torsional Regulation†

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ABSTRACT: The reactivity of chick erythrocyte and calf thymus histone H3 thiol groups toward 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) has been investigated both in the soluble, DNA-free state and in various nucleohistone complexes. We have found that the thiol reactivity of both tetramers and octamers decreases continuously as the ionic strength of the assay is increased, up to and beyond 2.0 M NaCl. Upon association of dimers with tetramers, there is loss of labeling by DTNB at one site, suggesting the existence of allosteric regulation [see also Godfrey, J. E., Eickbush, T. H., & Moudrianakis, E. N. (1980) *Biochemistry 19*, 1339–1346] of dimer–tetramer interfaces emanating from within the tetramer complex. Comparison of the thiol reactivities of chick and calf tetramers indicates that the thiol groups at amino acid positions 96 and 110 are not chemically equivalent. When the histones are associated with DNA, in either reconstituted complexes, core particles, or long soluble chromatin, the thiol reactivity is greatly diminished, and this "DNA effect" overwhelms any influence of dimers. However, if single-strand nicks are introduced into the DNA backbone of core particles and other chromatin-like complexes by the action of DNase I, the influence of the DNA double helix upon thiol reactivity is reduced, and the effect of dimers can be detected once again. We can therefore conclude that the DNA effect derives from *intra*nucleosomal torsional strain of the continuum of the double helix in equilibrium with coupled protein conformational changes. These observations support the concept that the octamer complex is a *dynamic tripartite* structure whose properties can be modulated through its interactions with DNA and by changes occurring in the dimer–tetramer interfaces.

The elementary subunit of chromatin structure, the nucleosome (Thomas & Kornberg, 1974), consists of a histone octamer containing two molecules each of histones H2A, H2B, H3, and H4 around which approximately 165 base pairs of nuclear DNA are wound [cf. McGhee & Felsenfeld (1980) for a review]. Each octamer is comprised of a central $(H3–H4)_2$ tetramer and two symmetrically situated H2A–H2B dimers (Eickbush & Moudrianakis, 1978). In the absence of DNA and at low ionic strength, the octamer complex dissociates into these two species (Eickbush & Moudrianakis, 1978; Godfrey et al., 1980). Studies using the purified histone complexes have revealed that their assembly into octamers follows a reversible mixed-association reaction scheme involving cooperative interactions (Godfrey et al., 1980) and a net loss of protons (Benedict et al., 1984). These and other solution studies indicate that the histone octamer is a dynamic tripartite structure. Our recent 3-Å resolution X-ray crystallographic study (Burlingame et al., 1985) describes the octamer as having the overall shape of a rugby ball and an internal tripartite organization consistent with that originally proposed by Eickbush and Moudrianakis (1978).

In order to understand the changes that occur within chromatin fibers in the various stages of cellular physiology, it is necessary to explore the dynamics of the internal rearrangements available to the histone cores. Various probes have been used to look at the intermolecular interactions that take place between individual histones, between histones and DNA, and between neighboring nucleosomes along the chromatin fiber. Of particular value in studying histone conformation are molecular probes of sulfhydryl reactivity, since of the four core histones in chick and calf, only histone H3 contains any cysteine residues (Brandt & von Holt, 1974; Panyim et al., 1971). A number of studies of the accessibility of these thiol groups to various biochemical reagents have demonstrated differences in thiol activity between histones in the soluble, DNA-free state, and histones associated with DNA (Hyde & Walker, 1973; Zama et al., 1977; Wong & Candido, 1978; Bode & Standt, 1978; Palau & Daban, 1979; Manosa et al., 1984).

In this study, we have systematically explored the reactivity toward 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB)[1] of both chick and calf histone H3 thiol groups. Using isolation procedures that avoid acid denaturation and alcohol precipitation of the histones (Eickbush & Moudrianakis, 1978), we have been able to characterize some of the properties of H3 thiol reactivity in native histone complexes. We have found that thiol reactivity of soluble, DNA-free $(H3–H4)_2$ tetramers is dependent upon the ionic strength of the assay as well as the presence of the H2A–H2B dimers. Comparisons of thiol reactivity of calf and chick samples allow us to conclude that the thiol group of cysteine-110 is more reactive than the thiol at amino acid position 96. Upon association of histones with the DNA, thiol reactivity is greatly diminished, and any effects of dimers is overwhelmed by the influence of the DNA double helix. However, after treatment of the DNA in chromatin complexes with DNase I, an enzyme that introduces single-strand nicks into the DNA wrapped around the histones, the influence of the DNA upon thiol reactivity is decreased, and an effect of dimers upon thiol reactivity can again be detected. These observations support the concept that the octamer

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[1] Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); LSC, long soluble chromatin; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DTT, dithiothreitol; BIPM, *N*-[*p*-(2-benzimidazolyl)phenyl]maleimide; EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance.

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complex is a dynamic *tripartite* structure whose properties can be modulated through its interactions with DNA and by changes occurring in the dimer–tetramer interfaces.

Materials and Methods

Protein Samples. Chick nuclei were prepared from packed red blood cells by the method described by Burgoyne et al. (1970). Chick erythrocyte and calf thymus chromatins were isolated essentially as described earlier (Eickbush & Moudrianakis, 1978). Chromatin prepared by this procedure is extensively washed in 85 mM sodium pyrophosphate, which removes all non-histone proteins as well as histone H1. Homogeneous preparations of histone octamers and its subunits, i.e., the H2A–H2B dimer and the $(H3–H4)_2$ tetramer, were isolated from the corresponding chromatin preparation by a column chromatographic fractionation procedure as described (Benedict et al., 1984). The histone fractions off the anion-exchange column were pooled, concentrated either by vacuum dialysis or by dialysis against solid polyethylene glycol (average molecular weight of 20000), and dialyzed against the desired buffers. Phenylmethanesulfonyl fluoride was included throughout the purification procedures to minimize proteolytic degradation. The integrity of the histone molecules was verified during the course of these experiments by $NaDodSO_4$–urea–polyacrylamide gel electrophoresis (Eickbush & Moudrianakis, 1978) and revealed no detectable breakdown of histones during storage at 4 °C for up to 3 weeks. The amount of the specifically cleaved H2A molecule (Eickbush et al., 1976) was less than 5% of the total amount of H2A.

Preparation of Calf Thymus DNA. Calf thymus DNA was prepared from pellets taken from the histone preparations as described (Rubin & Moudrianakis, 1975). The extent of protein contamination was determined from concentrated solutions of the DNA by the Lowry procedure (Lowry et al., 1951) and amounted to less than 2% by weight. DNA solutions at approximately 4 mg/mL in 1 mM EDTA, pH 7.5, were sheared in a Virtis homogenizer to yield a size range of approximately 5000–50000 base pairs as determined by agarose gel electrophoresis.

Preparation of Nucleoproteins. Long soluble chromatin (LSC), H1-stripped LSC, and core particles were prepared from chick red blood cell nuclei or calf thymus chromatin essentially as described (Lutter, 1978). Dimer subunits were removed from samples of LSC by the addition of 5 M NaCl stock, to bring the final concentration to 1.1 M, and passage over a Bio-Gel A 50 M column equilibrated with 1.1 M NaCl, 5 mM Tris-HCl, pH 7.4, 0.2 mM EDTA, and 0.2 mM β-mercaptoethanol. Fractions were checked for DNA size by 0.7% agarose gel electrophoresis and for protein content by $NaDodSO_4$–urea–polyacrylamide gel electrophoresis. Fractions containing no detectable H2A or H2B, and ranging in DNA size from about 500 to 10000 base pairs, were pooled, concentrated, and dialyzed against 10 mM Tris-HCl, pH 7.4, and 1 mM EDTA. We refer to this material as "dimer-stripped LSC".

Reconstitution of Histones onto DNA. Calf or chick histones were reconstituted to sheared calf thymus DNA by salt gradient dialysis. The protein, either tetramer or octamer, and DNA were mixed at a weight/weight ratio of 0.8 in 2.0 M NaCl, 10 mM HEPES, and 1 mM EDTA, pH 7.5. The mixture was dialyzed stepwise against 2.0 M NaCl for 2 h, 1.0 M NaCl for 4–6 h, and finally 1 mM EDTA for 12–16 h, all at 4 °C and at pH 7.5.

DNase Treatment of Nucleoproteins. Samples of LSC or dimer-stripped LSC at a concentration of 1–2 mg/mL were incubated at 37 °C in the presence of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1.7 mM $MgCl_2$, and 20 units/mL bovine pancreatic DNase I (Sigma) for the desired time, after which the reaction was quenched by the addition of EDTA to a final concentration of 10 mM. DNA was precipitated by the addition of 0.33 volume of 800 mM NaCl and 800 mM H_3PO_4 to an aliquot of the sample, followed by centrifugation at 11000*g* for 3 min at 4 °C. The extent of reaction was determined by calculating the amount of nucleotides released into the resulting supernatant. Products of DNase digestion were examined by electrophoresis in denaturing polyacrylamide gels.

Protein Determination and Gel Electrophoresis. Protein determination was as described (Benedict et al., 1984), using the following extinction coefficients in 2.0 M NaCl, A(277 nm, 1 mg/mL): 0.464 for octamer; 0.444 for tetramer; and 0.484 for dimer. Corrections to account for scattering were always less than 5%. Molecular weights of 28000, 53000, and 109000 were assumed for dimer, tetramer, and octamer, respectively. Agarose gel electrophoresis was carried out by using Tris–borate–EDTA buffer (90 mM Tris–borate, pH 8.3, and 2.5 mM EDTA) (Peacock & Dingman, 1967) with 0.7% or 1.0% agarose, and using *Hind*III-cut λ DNA fragments as size markers. Denaturing polyacrylamide gel electrophoresis, used to examine the products of DNase digestion, was carried out as described (Lutter, 1978). DNA gels were stained in 20 μg/mL ethidium bromide and destained in distilled water.

Assay for Thiol Reactivity. Unless otherwise indicated, the pH of all assays was 7.5, and the buffer used was 10 mM HEPES against which all histone stocks were dialyzed. The reaction of various histone samples with DTNB (Aldrich) was carried out as follows: To a test tube containing the desired concentrations of NaCl and 1 mM EDTA at pH 7.5 was added an aliquot of the buffered histone or nucleohistone sample. The final concentration of histone thiol groups was kept at 30 μM or less. After the initial absorbance of this sample at 412 nm was recorded, an aliquot of buffered DTNB was added to a final concentration of 250 μM. After various times of incubation (room temperature for the soluble histone samples and 37 °C for the nucleohistone samples), the absorbance at 412 nm was recorded. Product yield was calculated by employing an extinction coefficient of 13.6 mM^{-1} for DTNB (Ellman, 1959), and taking all blanks into account. Each reaction was monitored for a length of time twice that required for the signal at 412 nm to reach a plateau value. Furthermore, a direct test of whether or not this value indeed represented the completion of the reaction was applied to each new set of conditions. For this, after a reaction had reached "apparent" saturation, the mixture was brought to a final concentration of 4 M urea (for histone) or 5 M urea–1 M NaCl (for nucleohistones) and incubated for 10 min at 37 °C, before its absorbance was measured again, multiplied by the appropriate dilution factor, and compared to the apparent saturation value. These final conditions always yielded full (100%) reactivity for all samples tested. Data are presented (except for Figure 8 and Table I) as "reaction %", where 100% corresponds to a molar ratio of 2.0 reactive thiols per octamer or tetramer for the chick histone samples, and 3.8 for the calf histone samples rather than 4.0. The deviation from an integer number for the saturation of the reactivity of H3 is due to the well-known polymorphism of histone populations in general and specifically to the fact that some of the calf H3 variants contain only one rather than two cysteines (Panyim et al., 1971). The relative abundance of these variants in different histone populations has not been quantitated yet, and methods for their isolation in homogeneous states are not available at

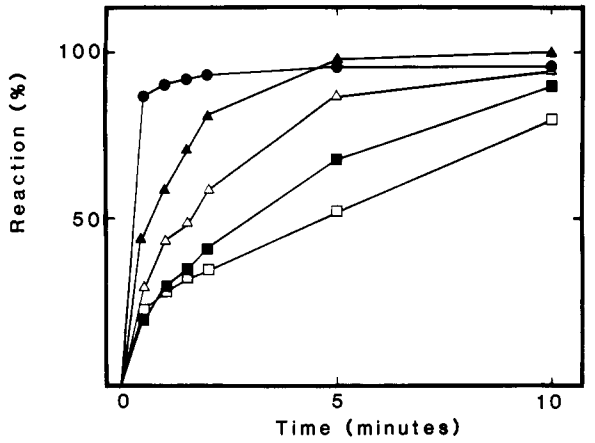

FIGURE 1: Time course of DTNB reaction with soluble chick tetramer as a function of NaCl concentration. Samples of chick tetramer were assayed for thiol reactivity at room temperature in the presence of 1 mM EDTA, pH 7.5, and (●) 16 mM NaCl, (▲) 400 mM NaCl, (△) 800 mM NaCl, (■) 1.56 M NaCl, or (□) 2.0 M NaCl for the indicated amounts of time.

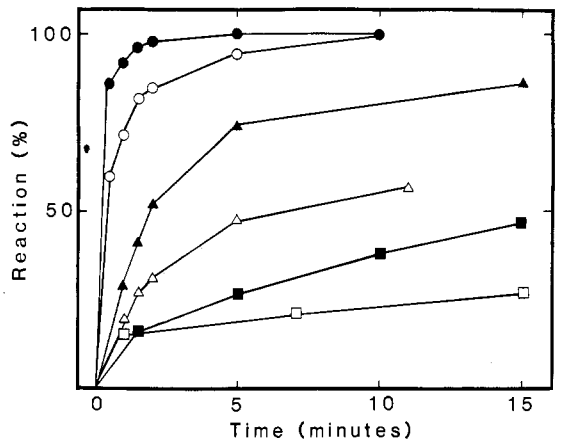

FIGURE 2: Time course of DTNB reaction with soluble chick octamer as a function of NaCl concentration. Samples of chick octamer were assayed for thiol reactivity at room temperature in the presence of 1 mM EDTA, pH 7.5, and (●) 16 mM NaCl, (○) 200 mM NaCl, (▲) 400 mM NaCl, (△) 800 mM NaCl, (■) 1.50 M NaCl, or (□) 2.0 M NaCl for the indicated lengths of time.

this time. However, for ease of comparison, 100% reactivity of *calf* histones is referred to as a molar ratio of 4.0 throughout the text. A thorough kinetic analysis of the reactions described here as well as the possibility of differential thiol reactivity of H3 variants will be addressed in a future communication.

RESULTS

In the absence of DNA, the soluble octameric complex of the core histones dissociates at low ionic strengths into discrete dimer and tetramer subunits (Eickbush & Moudrianakis, 1978) which can subsequently be separated from each other. This property has allowed us to isolate large quantities of pure subunits which can be used for studies of their properties and interactions. In this study, we have examined the changes in thiol reactivity which occur as the complexity of the histone sample increases from isolated purified tetramers and dimers, to soluble octamer, to tetramer associated with DNA, and finally to chromatin-like systems containing all four histones complexed to DNA.

Reactivity of Soluble Chick Histone Complexes. In the isolated chick $(H3–H4)_2$ tetramer complex, there are a total of two cysteine residues, one located at amino acid position 110 of each of the two H3 molecules. Figure 1 shows the time course of reaction of DTNB with this complex as a function of the ionic strength of the assay. At the lowest ionic strength used, the reaction was complete within 2 min of incubation, and two –SH groups reacted per tetramer. As the ionic strength was increased, the rate of the reaction decreased, such that at 2.0 M NaCl between 10 and 20 min of incubation was required for full reactivity to occur. However, at all ionic strengths tested, sufficient incubation time resulted in the same extent of reaction (2.0 thiols per tetramer), indicating that accessibility to the thiol groups is kinetically hindered, but not completely blocked, in high salt conditions.

Having characterized the reaction of DTNB with one of the simplest physiologically relevant histone complexes, the tetramer, we next looked at the same reaction when the H2A–H2B dimer subunits also were present, by using the purified octamer. The results of this experiment are shown in Figure 2. The thiol reactivity at low salt concentration was again very rapid and reached a value of two –SH groups per octamer, consistent with the fact that at low ionic strengths the octamer is fully dissociated into dimers and tetramers (Eickbush & Moudrianakis, 1978). Increasing the ionic strength of the assay resulted in a decrease in the rate of the reaction (even to a greater degree than that seen with the tetramer alone) and reduced the extent of labeling (at 2.0 M NaCl) by 1.0 thiol per octamer. Prolonged incubations at 2.0 M NaCl did not lead to any significant increase in labeling, but the addition of urea to a final concentration of 4 M led rapidly to the complete (100%) labeling of the octamer. Similar results were obtained when the purified dimer was added to a sample of the purified tetramer, indicating that, to the extent detectable by this assay, the properties of the histone subunits are not altered by our purification procedures. We conclude that the binding of the H2A–H2B dimers to the tetramer causes one of the two sulfhydryl groups to become inaccessible to DTNB modification. Since the two thiol groups of the $(H3–H4)_2$ tetramer are initially in the same conformation, this result indicates that in the octamer complex the binding of DTNB to one of the two thiol groups, or the binding of the first dimer to the tetramer, alters in some way the accessibility of DTNB to the second thiol within the same octameric structure. The mechanism of this "half-of-sites reactivity" is currently under investigation.

The relative effectiveness of ionic strength upon the thiol reactivity of tetramers compared to octamers can be evaluated by comparing the corresponding lines in Figures 1 and 2. It can be seen that an increase in salt concentration causes a decrease in the rate of labeling of both tetramers and octamers, that the reaction of the octamer is more drastically affected (ca. 6-fold), and that this differential effect first detectable between 200 and 400 mM NaCl continues to increase with increasing salt concentration. It should be noted that the rate of reaction of DTNB with free cysteine is not affected by NaCl concentration (Hyde & Walker, 1973). The differential effect in inhibition of octamer–H3 labeling is most likely due to the increased association between the dimer and tetramer subunits observable in higher ionic strengths (Eickbush & Moudrianakis, 1978), which, in turn, should be expected to limit the accessibility of the "solvent channels" delineating the dimer–tetramer interfaces (Burlingame et al., 1985).

Reactivity of Soluble Calf Histone Complexes. While each chicken H3 molecule contains only a single cysteine residue at position 110, the H3 molecules from higher eukaryotes such as mouse, rat, and calf contain a second cysteine at position 96 (Panyim et al., 1971). We have therefore used calf thymus histones to evaluate thiol reactivity at position 96. The rate of reaction of the purified calf thymus tetramer with DTNB was very similar to that found with the chick tetramer. At

Table I: Summary of Thiol Labeling Obtained with Tetramers and Octamers in High and Low NaCl Concentrations[a]

sample	-SH groups reacted per complex	
	16 mM NaCl	2.0 M NaCl
chick tetramer	2	2
chick octamer	2	1
calf tetramer	4	3
calf octamer	4	2

[a] Samples of chick or calf histones were incubated with DTNB at room temperature in the presence of 1 mM EDTA, pH 7.5, and the indicated concentration of NaCl. The extent of labeling was determined after 30 min for the low salt samples (at which point complete labeling was obtained) or after 3 h for the high salt samples (after which time no further reaction was detected). The ratios presented for the calf samples have been normalized to 4.0 for ease of comparison.

the lowest ionic strength used, the reaction was rapid, and full modification of all four thiols was attained in a few minutes. As the ionic strength was increased, a decrease in the rate of reaction was observed. However, when the assay was carried out at 2.0 M NaCl, the maximum ratio attained with the calf tetramer was 3.0, indicating that at this salt concentration one of the four -SH groups became inaccessible to modification by DTNB. Since the thiol groups of both cysteine-110 residues were accessible when the chick tetramer was used, we have assigned the "unreactive" thiol in the calf tetramer to one of the cysteine-96 residues.

The influence of the dimers upon the reactivity of the calf tetramer thiol goups was next examined by assaying the purified calf octamer. At low ionic strength, where the octamer is fully dissociated into its subunits, the reaction quickly reached an -SH to octamer ratio of 4.0. At 2.0 M NaCl, where the octamer is fully assembled, the reaction proceeded very slowly, and two of the four -SH groups remained unreactive. If we assume that in 2.0 M NaCl the unreactive cysteine-96 thiol observed with the calf tetramer remains unreactive when in the octameric complex, then the second unreactive thiol is most likely one of the cysteine-110 residues, as was observed with the chick octamer. A prolonged time course of labeling in 2.0 M NaCl showed that even after 3 h of incubation at room temperature, calf tetramer reaches a ratio of 3.0 while the octamer plateaus at 2.0. These results are summarized in Table I.

Thiol Reactivity in High Ionic Strength. Earlier studies on soluble histones have demonstrated that in the presence of 2.0 M NaCl, the histones have many properties similar to those they exhibit when associated with DNA in low salt (Thomas et al., 1977; Bidney et al., 1977; Cotter & Lilley, 1977). Additionally, measurements of salt-induced changes in DNA-free histone preparations indicate that by 2.0 M NaCl, the majority of conformational and structural changes have taken place (Bradbury, 1965). We have examined the effects of high ionic strength conditions upon the thiol reactivity of the calf octamer to determine if any further changes take place under salt concentrations up to 4.0 M NaCl or 60% saturation with ammonium sulfate (Figure 3). The latter reagent was chosen to probe the effect(s) of our crystallization conditions (Burlingame et al., 1985) upon the state of the octamer. For both reagents tested, there was a continual decrease in thiol reactivity as the salt concentration was increased. At 55–60% saturation with ammonium sulfate, the reactivity of the octamer was comparable to its reactivity in 2.0 M NaCl. Similar decreases in thiol reactivity were also found when the purified calf tetramer was assayed, indicating an ionic strength induced tightening of tetramer structure in the absence of dimers. The low level of DTNB reactivity with the calf octamer or tetamer

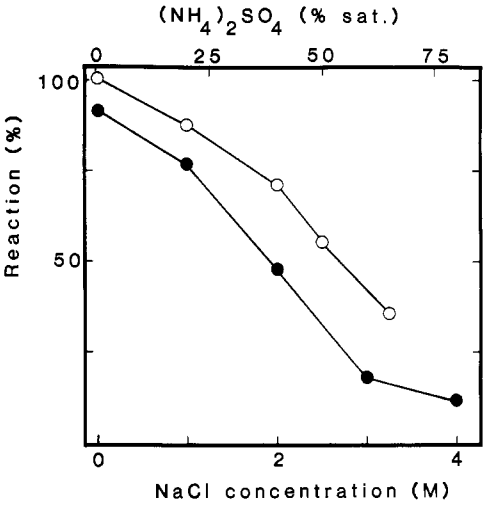

FIGURE 3: Time course of DTNB reaction with soluble calf octamer as a function of ionic strength. Samples of calf octamer were incubated with DTNB for 2 h at room temperature in the presence of 1 mM EDTA, pH 7.5, and the indicated concentrations of NaCl (○) or for 1.5 h at room temperature in the presence of 10 mM sodium pyrophosphate, 10 mM HEPES, pH 6.8, and the indicated amounts of ammonium sulfate (●), after which time the amount of DTNB reacted was determined.

in high ionic strengths is comparable to the slight labeling of H3 molecules obtained when either chromatin or nucleosomes are assayed (Wong & Candido, 1978; Olins et al., 1977).

Effect of Urea Concentration on Thiol Reactivity. To further characterize the nature of the forces responsible for the modulation of histone thiol reactivity toward DTNB, calf and chick octamers were assayed in the presence of 2.0 M NaCl and varying amounts of urea (Figure 4). In studies of nucleohistone complexes, it has been shown that the thiol groups of the DNA-bound histones are not susceptible to DTNB modification in urea concentrations of up to 5–6 M (Wong & Candido, 1978; Olins et al., 1977). In contrast, the reactivity of the thiol groups of soluble, DNA-free histones was greatly increased by the presence of relatively low amounts of urea. In the case of chick octamer, a 1 M urea concentration was sufficient to allow full modification of both thiol groups to occur (Figure 4A). On the other hand, incubation of calf octamer in 1 M urea for 2 h resulted in approximately 2.5 mol of -SH reacting per mole of octamer, suggesting that one of the thiol groups is still essentially unreactive at this urea concentration (Figure 4B). All four -SH groups of the calf octamer were modified at urea concentrations of 2.0 M or greater. These results support the idea that in the calf octamer, the thiol group of cysteine-96 is less reactive, since a higher concentration of urea was needed for its exposure than for exposure of the cysteine-110 thiol group. Experiments carried out with octamers in higher ionic strengths (4.0 M NaCl) revealed that urea concentrations of greater than 3 M were necessary to stimulate thiol reactivity, suggesting that at even higher ionic strengths the thiol reactivity of soluble histones may start to resemble that of core particles and long soluble chromatin, where 6–8 M urea is necessary to induce thiol exposure.

The thiol reactivity of purified calf tetramer in 3.0 M NaCl also was increased in the presence of urea (Figure 5), at concentrations somewhat lower than those which stimulated the reaction of the octamer thiol groups, indicating a direct effect of urea upon the unfolding of the tetrameric complex.

Thiol Reactivity of Reconstituted Nucleohistone Complexes. Since the presence of DNA in nucleoprotein complexes may induce conformational and structural changes in the

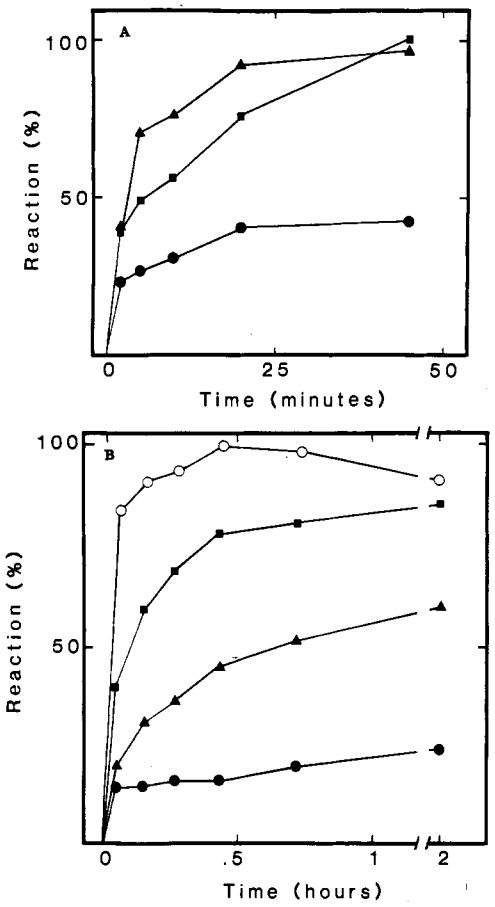

FIGURE 4: Time course of DTNB reaction with soluble octamer as a function of urea concentration. (A) Samples of chick octamer were assayed for thiol reactivity at room temperature for the indicated times in the presence of 2 M NaCl, 1 mM EDTA, pH 7.5, and (●) no urea, (■) 1 M urea, or (▲) 3 M urea. (B) Samples of calf octamer were assayed for thiol reactivity at room temperature for the indicated times in the presence of 2 M NaCl, 1 mM EDTA, pH 7.5, and (●) no urea, (▲) 1 M urea, (■) 2 M urea, or (○) 3.8 M urea.

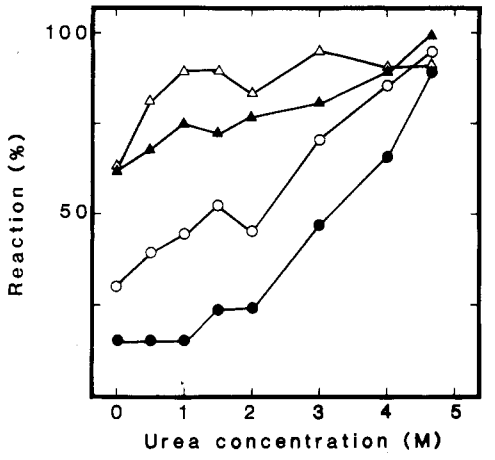

FIGURE 5: Effect of urea concentration on the reaction of DTNB with the soluble calf tetramer. Samples of calf tetramer were assayed for thiol reactivity at room temperature in the presence of 3 M NaCl, 1 mM EDTA, pH 7.5, and the indicated concentration of urea for (●) 10 min, (○) 30 min, (▲) 1 h, or (△) 2.5 h.

histones that are absent in the soluble, DNA-free state, we decided to next examine the thiol reactivity of tetramers and octamers after reconstituting them onto calf thymus DNA. Preliminary experiments under the conditions used to study the soluble histone complexes indicated that negligible labeling of the thiols occurred at room temperature; therefore, these experiments were carried out at 37 °C. Figure 6 shows the

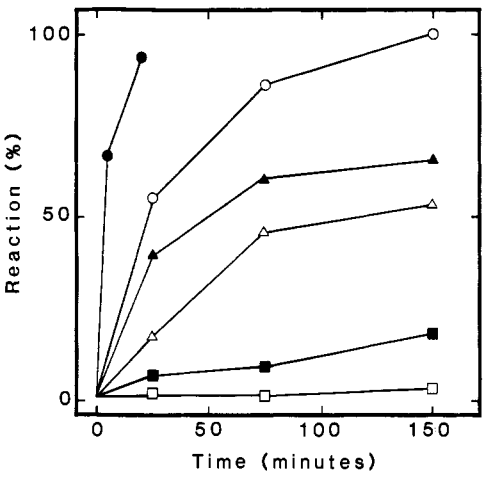

FIGURE 6: Time course of DTNB reaction with reconstituted chick tetramer–DNA as a function of NaCl concentration. Isolated chick tetramer was reconstituted onto sheared calf thymus DNA via salt gradient dialysis as described under Materials and Methods. After a final dialysis to 1 mM EDTA, pH 7.5, samples were made (□) 400 mM NaCl, (■) 800 mM NaCl, (△) 1.0 M NaCl, (▲) 1.5 M NaCl, or (○) 2.0 M NaCl and assayed for thiol reactivity at 37 °C for the indicated amounts of time. (●) Time course of DTNB reaction with DNA-free soluble chick tetramer at 37 °C in the presence of 2.0 M NaCl and 1 mM EDTA, pH 7.5.

time course of the purified chick tetramer reacting with DTNB after reconstitution onto calf thymus DNA. In contrast to the results obtained when the soluble, DNA-free tetramer was assayed, in this case increasing the ionic strength of the assay led to an increase in thiol reactivity. In 2.0 M NaCl, modification of both thiol groups was complete after 2.5 h of incubation. Essentially identical results were obtained when calf tetramer alone was reconstituted onto DNA, with the exception that in 2.0 M NaCl three thiol groups reacted per tetramer molecule, the same ratio as was obtained with DNA-free calf tetramer.

Figure 6 indicates that in 2.0 M NaCl the rate of the reaction of the "reconstitute" was approximately 8-fold slower than the rate obtained when the DNA-free tetramer was assayed at the same concentration of NaCl (closed circles). Since at this ionic strength histones are mostly dissociated from DNA, this result points to either an influence of DNA upon the conformational state of the histones in the absence of any strong association between these macromolecules or an effect on the histones due to the additional ionic strength contributed by the free DNA in solution.

The influence of dimers upon thiol reactivity in nucleohistone reconstitutes was evaluated by carrying out the reconstitution procedure with the purified octamer complex (data not shown). With either type of histone octamer (chick or calf) on DNA, the increase in thiol reactivity as a function of NaCl concentration was found to be identical with the response obtained when only tetramer was bound to DNA. Thus, while the addition of dimers to tetramers in the soluble, DNA-free environment results in a marked decrease in the thiol reactivity of the tetramer, it appears that the binding of these subunits onto DNA makes any dimer-induced effects undetectable under these conditions.

DTNB Modification of Long Soluble Chromatin. While the results of the above experiments with reconstituted nucleoprotein complexes demonstrated an effect of DNA upon tetramer thiol reactivity, it was important to determine if the properties of the histones were further modified when they were associated with DNA in a more physiologically relevant form, i.e., long soluble chromatin (LSC) or core particles. The

procedures used to prepare these samples removed all detectable amounts of histone H1, and thus the influence of this histone on thiol reactivity was not evaluated. However, limited studies carried out using chromatin or LSC samples containing H1 generated results identical with those obtained with the fully H1-stripped chromatin samples (data not shown), indicating that H1 does not measurably affect the thiol reactivity of DNA-bound tetramer.

The effect of NaCl on the thiol reactivity of calf LSC was investigated. Calf LSC was unreactive toward DTNB up to a NaCl concentration of approximately 0.8 M, in agreement with the findings of others (Bode & Standt, 1978; Wong & Candido, 1978; Zama et al., 1977). Further increase in ionic strength resulted in a sigmoidal increase of thiol reactivity, reaching a midpoint at about 1.1 M NaCl and a maximum level of about 3 mol of thiol per mole of octamer at 2.0 M NaCl. Comparison of this value with that obtained when soluble, DNA-free calf octamer is assayed in 2.0 M NaCl at room temperature (2 mol of thiol per mole of octamer) suggests that the higher temperature allows modification of additional thiol groups. Similar curves obtained for dimer-stripped LSC (not shown) demonstrate that the sigmoidal increase in reactivity is not dependent upon the salt-induced dissociation of dimer subunits from the chromatin, but rather upon a salt-dependent loosening of either tetramer–DNA contacts or the tetramer structure itself. This conclusion is also supported by the results we obtained using the reconstituted calf tetramer–DNA system (see above). In all cases examined, we were unable to discern any difference in the labeling patterns of the thiol groups of cysteine-96 and cysteine-110, indicating a simultaneous unmasking of these two groups of sites (Bode & Standt, 1978) in this type of preparation.

Effect of DNase Treatment on Thiol Reactivity. The results of the above experiments in which the thiol reactivity of nucleohistone complexes containing all four histones was compared to the reactivity of complexes lacking the dimer subunits indicate that both of these macromolecular complexes exhibited a similar response to changes in the ionic strength of the environment. In fact, no reactivity was observed until the NaCl concentration was approximately 1 M, at which point most of the dimer subunits would have dissociated from the DNA. However, the results using soluble histones as substrates for DTNB modification demonstrate that some protection of thiol groups occurs in the presence of dimers and 2.0 M NaCl. A possible explanation for the apparent lack of dimer influence in chromatin-like structures is that the effect of the DNA double helix upon thiol group reactivity overwhelms any possible effect the dimers may have. If it were possible to reduce this double-helix effect in DNA–histone complexes, it might then be possible to observe changes in tetramer reactivity due to the presence of dimers.

To approach this problem, we made use of the endonuclease DNase I (Noll, 1974) to test the possibility that the presence of single-strand nicks in the DNA wrapped around the core histones would relieve some of the torsional energies stored within the DNA supercoil and thereby minimize the double-helix effect on the histones while they remain bound to the nicked DNA. Table II summarizes the effect of increasing DNase treatment upon the thiol reactivity of core particles when assayed in 1 M NaCl. It can be seen that even a low level of endonucleolytic cleavage resulted in a dramatic increase in the susceptibility of the -SH groups to DTNB modification. Denaturing polyacrylamide gel electrophoresis of the core particles revealed that after the longest time of digestion (26% acid-soluble nucleotides) most of the DNA existed as discrete fragments between 10 and 80 base pairs in length, which is consistent with earlier results by others (Noll, 1974; Lutter, 1978). To determine if any histones had dissociated from the DNA as a consequence of DNase treatment during the time period of the labeling reaction (2 h at 37 °C in 1 M NaCl), we carried out a similar experiment in which the DTNB was not added until after a 2-h preincubation under the same conditions. At that time, the sample was put at room temperature and reacted with DTNB for 5 min. During this brief reaction, histones complexed to DNA will not react significantly, while the reaction of DTNB with any tetramers which might have dissociated from the DNA will react to at least 75% completion [Figure 1 and Reactivity of Soluble Calf Histone Complexes (Results)]. Whereas 2–3% of the total thiols were reactive when control particles were assayed (i.e., not treated with DNase), approximately 10% of total thiols were reactive when DNase-treated particles were used. We conclude that although DNase treatment caused a measurable increase in histone dissociation to occur during the 2-h preincubation, this increase (10%) cannot account for the levels of reaction we observe (up to 100%) when DTNB is present throughout the 2-h incubation. It has also been reported that upon extensive digestion of core particles with micrococcal nuclease (Rill et al., 1975) or with exonuclease III (Ariel, 1983) the histones remain associated with the DNA in solutions of low ionic strengh.

Table II: Effect of DNase Treatment on Thiol Reactivity of Calf Thymus Core Particles[a]

incubation time (s)	% acid-soluble nucleotides	% reaction
0	0	4
15	3	37
30	15	60
60	26	100

[a] Samples of calf thymus core particles were treated with 20 units of DNase I at 37 °C for the indicated amounts of time. The percentage of acid-soluble nucleotides released by DNase treatment was determined as described under Materials and Methods. After DNase treatment, samples were incubated with DTNB for 2 h at 37 °C in the presence of 1 mM EDTA, pH 7.5, and 1.0 M NaCl, after which time the extent of thiol reactivity was determined. 100% reactivity corresponds to complete labeling of all the calf H3 thiol groups.

The dependence of thiol reactivity of calf core particles on NaCl concentration was next reexamined, this time following DNase treatment (Figure 7). A comparison of the curves obtained with and without DNase treatment revealed two distinct changes associated with the nucleolysis. First, the thiol groups in the DNase-treated samples reacted with DTNB at a lower salt concentration than that at which native core particles reacted. Second, the cooperative response in thiol modification normally seen with chromatin and core particles between 1.0 and 1.3 M NaCl was not observed with the DNase-treated samples. Instead, a linear increase in reactivity as a function of the salt concentration was found. We conclude that the presence of numerous single-strand nicks in the DNA duplex bound to the histones results in a reduction in the influence of the DNA upon the histone thiol reactivity and thus demonstrates for the first time that the torsional energy of the double helix can modulate the chemical properties of the histones and contributes greatly to the cooperative effects manifested within the tetramer.

We next compared the effects of DNase treatment upon dimer-stripped samples vs. samples containing all four core histones (Figure 8). In this experiment, dimer-stripped LSC was treated with DNase to produce approximately 65%

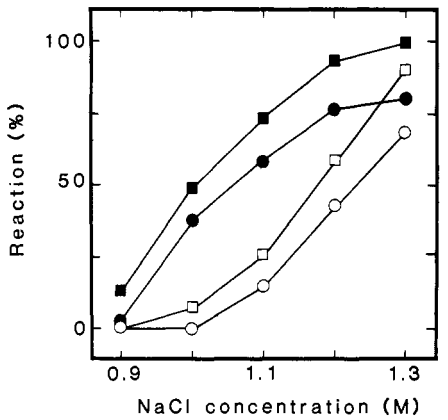

FIGURE 7: Effect of DNase treatment on the reaction of DTNB with calf thymus core particles. A sample of calf thymus core particles was treated with DNase I to yield approximately 25% acid-soluble nucleotides. Following DNase treatment, aliquots were assayed for thiol reactivity at 37 °C in the presence of 1 mM EDTA, pH 7.5, and the indicated concentration of NaCl. Untreated particles incubated for 15 min (○) or 30 min (□). DNase-treated particles incubated for 15 min (●) or 30 min (■).

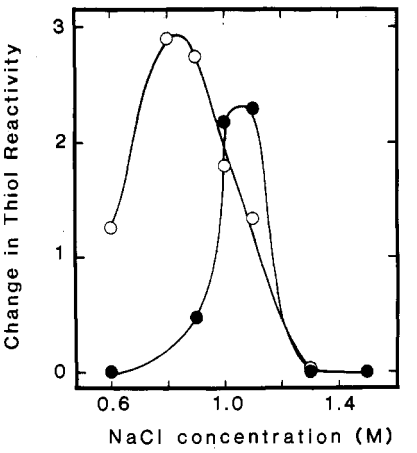

FIGURE 8: Effect of DNase treatment on the reaction of DTNB with native and dimer-stripped nucleohistone samples. A sample of calf thymus long soluble chromatin was stripped of dimer subunits as described under Materials and Methods and then treated with DNase I to the extent of 65% acid-soluble nucleotides. The samples (before and after DNase treatment) were then incubated with DTNB for 2 h at 37 °C in the presence of 1 mM EDTA, pH 7.5, and the indicated concentration of NaCl. For each NaCl concentration used, the difference obtained between the degree of thiol modification for the two samples was determined ("change in thiol reactivity" equals ratio obtained with DNase-treated samples minus ratio obtained with untreated samples) and is plotted against the NaCl concentration (○). A similar graph is shown for core particles which contain all four histones (●). Note that for core particles, there was no detectable reaction with DTNB at NaCl concentrations of 0.8 M or less, either for untreated or for DNase-treated samples.

acid-soluble nucleotides and then was incubated with DTNB at various salt concentrations. The molar ratio of thiol groups that reacted after 2 h of incubation was determined, and the difference obtained between control, dimer-stripped LSC and the DNase-treated samples was calculated. A similar plot was also constructed for core particles (which contained all four histones) before and after DNase treatment. LSC containing all four histones was found to precipitate following DNase treatment and DTNB modification and thus could not be used for a direct comparison. It can be seen that for dimer-stripped LSC, the thiol groups of the DNase-treated samples began to react at a NaCl concentration of between 0.6 and 0.7 M, while the untreated sample became reactive to DTNB at approximately 1.0 M NaCl, consistent with a loosening of histone–DNA interactions due to the DNase treatment. The influence of dimers upon thiol reactivity can be seen by comparing the curves for DNase-treated dimer-stripped LSC with those for DNase-treated core particles. While the nuclease-treated core particles did not react at salt concentrations of less than 0.8 M, removal of the dimers (the DNase-treated dimer-stripped LSC) allowed the thiols to react at a salt concentration of only 0.6 M. We therefore conclude that when the influence of DNA on tetramer conformation is reduced by nicking with DNase, an influence of dimers upon the DNA-bound tetramer structure can be readily detected, measurable as an increase in the salt concentration necessary to allow the thiol groups to react.

Discussion

We have used a relatively simple assay, that of following the reaction of DTNB with the thiol groups of histone H3, to examine the dynamics of both histone–histone and histone–DNA interactions, interpretable as changes occurring in the structure of the $(H3–H4)_2$ tetramer. Since the tetramer is the central subunit of the tripartite histone octamer (Eickbush & Moudrianakis, 1978; Burlingame et al., 1985), its chemical properties could be modulated by (as well as cause the modulation of) the properties of the flanking dimer subunits. Understanding the overall regulation of these interactions is the ultimate goal of our studies. In the experiments presented here, we have been careful to use only salt-extracted histone complexes in order to avoid possible artifacts due to improper and/or inefficient reannealing of acid-extracted histones. Additionally, we are aware of the possibility that covalent modification of proteins (i.e., prelabeling) can lead to structural rearrangements within the protein and subsequent loss or change of its normal response to various perturbants (Wingender et al., 1981). In light of this problem, we feel that earlier studies of thiol reactivity in which measurements were carried out under conditions different from the conditions of the labeling reaction itself may be open to alternate interpretations.

The results of our present study allow us to conclude the following: (1) thiol reactivity of both tetramers and octamers is dependent upon ionic strength; (2) the thiol groups at amino acid positions 96 and 110 are not chemically equivalent; (3) the thiol reactivity of the soluble, DNA-free tetramer is influenced by the presence of the dimer subunits; (4) upon association of the histones with DNA, thiol reactivity is greatly diminished, and this DNA effect overwhelms any influence of dimers; (5) with the introduction of single-strand nicks into the DNA backbone of core particles and other chromatin-like complexes, the influence of the DNA double helix upon thiol reactivity is reduced, and the effect of dimers can be detected once again; (6) thus, the DNA effect derives from *intra*nucleosomal torsional strain of the continuum of the double helix in equilibrium with coupled protein conformational changes. We will now consider these findings separately.

Effect of Ionic Strength on Thiol Reactivity. We have found that the thiol reactivity of isolated tetramers and octamers decreases as the ionic strength of the assay conditions is increased, in general agreement with findings from other laboratories (Palau & Padros, 1972; Puigdomenech et al., 1977; Palau & Daban, 1979; Manosa et al., 1984). In the present study, we have used "native" histone subunits and have observed a continual decrease in the thiol reactivity of the isolated tetramer in concentrations of up to 3.0 M NaCl, in accord with previous spectroscopic data of others (Manosa et al., 1984). Additionally, we have found that the decrease in thiol reactivity of octamers is much more pronounced than that

seen with tetramers (see below). The difference in response of these two types of complexes is most likely due to the formation of hexamers and octamers from the dimer and tetramer subunits and indicates that some type of dimer–tetramer interactions occur at ionic strengths as low as 200–400 mM NaCl, the lowest salt concentration at which a difference between tetramer and octamer reactivity was observed. Previously, we have been able to demonstrate dimer–tetramer interactions occurring at a salt concentration as low as 800 mM, as monitored by small-zone column chromatography (Eickbush & Moudrianakis, 1978). While the chromatographic method of analysis resolves relatively stable complexes, it appears that the thiol reactivity of the tetramer is a sensitive indicator of much weaker dimer–tetramer interactions.

Relative Reactivities of Cysteines-96 and -110 toward DTNB. While some studies using native calf chromatin and core particles have concluded that there is a simultaneous unmasking of reactivity of both cysteine-96 and cysteine-110 thiol groups (Bode & Standt, 1978; Wong & Candido, 1978; Zama et al., 1977), other studies have indicated that the reactivity of these two sites is not equal (Palau & Padros, 1972; Hyde & Walker, 1973; Bode & Standt, 1978). We have documented (Figure 1 and Table I) that in 2.0 M NaCl, at neutral pH, complete labeling of chick tetramer was obtained if sufficient incubation time was used, whereas only three of the four thiols in calf tetramer were modified even after extensive incubation. If we assume that the tertiary structure and chemical environment of the cysteine-110 residues are identical in calf and chick tetramers, we can conclude that in 2.0 M NaCl at pH 7.5, the cysteine-96 thiol is less reactive than that of the cysteine-110 residue. Further evidence supporting this conclusion comes from Figures 4 and 5, in which 1 M urea was adequate to promote labeling of both cysteine-110 residues in chick octamer but was insufficient to allow complete labeling of the four calf thiol groups. Thus, the tertiary structure surrounding the cysteine-110 residue appears to be more susceptible to protein denaturants than that surrounding the cysteine-96 residue, in agreement with the conclusions of others (Palau & Padros, 1972). Although a difference in the reactivities of the two thiol groups was easily seen when soluble, DNA-free histones were used, we have not been able to discern any difference in reactivities when DNA-bound histones are assayed.

Nature of Dimer Interactions with the Tetramer. It has been well established that upon interaction of isolated histone H3 with histone H4, easily detectable changes occur in the thiol reactivity and microenvironment of H3 (Padros et al., 1977; Puigdomenech et al., 1977; Bode & Standt, 1978). However, studies on the influence of dimers upon tetramer thiol reactivity have yielded varying conclusions (Palau & Daban, 1976; Manosa et al., 1984; Bode & Standt, 1978). Previous work from our laboratory has demonstrated that upon the interaction of dimers with tetramers there is a net loss of protons from the complex (Benedict et al., 1984), cooperativity in the binding of a second dimer to the hexameric $(H3–H4)_2$–H2A–H2B complex (Eickbush & Moudrianakis, 1978), and a change in the kinetics of hydrogen exchange of tetramers (McCarthy et al., 1984). These phenomena indicate that changes within the tetramer occur upon the binding of dimers and therefore predict that the thiol reactivity of the tetramer may be modulated by association of these subunits. Comparison of the data in Figures 1 and 2 shows that in the presence of dimers there is a more pronounced decrease in the rate of reaction as the NaCl concentration is increased. Additionally, while modification of the chick tetramer in 2 M NaCl after prolonged incubation reached a molar ratio of 2.0 (Figure 1), the maximum labeling of chick octamer under the same conditions was never much greater than 1.0 (Figure 2). Similar assays using calf histones also revealed a loss in the labeling of one thiol group when dimers were present. Thus, dimers not only cause a decrease in the overall rate of thiol modification but also fully prevent labeling at one of the cysteine-110 sites. Ion-exchange chromatography of octamers labeled in 2 M NaCl indicated that 100% of the tetramers were singly labeled (data not shown), ruling out the possibility that in 2 M NaCl a molar ratio of 1.0 reflects complete labeling of 50% of the molecules and no labeling of the remaining 50% of the molecules. The ability of very low concentrations of urea to stimulate modification of the chick and calf H3 thiol groups (Figures 4 and 5) is an expected result, since in solution low amounts of urea have been shown to promote dissociation of dimers from tetramers (Eickbush & Moudrianakis, 1978).

These results are consistent with a model for $(H3–H4)_2$ tetramer thiol reactivity in which the binding of DTNB to a thiol group on one of the two H3 molecules prevents the modification of the homologous thiol group on the other H3 molecule in the same complex. We propose the term "trans-regulation" to describe this phenomenon. Our results indicate that while trans-regulation of cysteine-96 can occur in the absence of dimer, as observed with the calf tetramer, trans-regulation of the cysteine-110 residue requires a tight association of dimers with tetramers and therefore is detectable with chick octamer but not with chick tetramer. Inspection of the 3.3-Å crystallographic structure of the octamer (Burlingame et al., 1985) suggests that the decrease in the rate of modification of cysteine-110 upon association of dimers with tetramers could be due to steric hindrance, by dimers, of solvent channels that exist within the tetramer. However, the overall decrease in extent of modification brought about by dimers cannot be due to steric hindrance, since the dynamic equilibrium that exists between dimers, tetramers, hexamers, and octamers dictates that over a finite period of time dimers reversibly dissociating from tetramers will allow DTNB access to the cysteine residue. Thus, to account for the dimer-dependent decrease in thiol reactivity, we further propose that upon binding of a DTNB molecule to the tetramer the association of a dimer subunit with the modified tetramer is dramatically strengthened. The resulting complex, a tetramer labeled with one molecule of DTNB and having one tightly bound dimer, is therefore resistant to further modification by DTNB. Our previous studies, which demonstrated a 4-fold increase in the association constant for binding of the *second* dimer subunit to the tetramer, support this type of modulation. Further support comes from the data of Figures 1 and 2, where it can be seen that while the reaction of DTNB with tetramers appears primarily monophasic, reflecting the existence of two equivalent reactive sites, the time courses obtained with octamer samples (at 0.8 M NaCl and above) are more complex, suggestive of unequal reactive sites and/or allosteric effects. Integration of the above arguments allows us to conclude that the coupling of dimer–tetramer interfaces is sequential and can be allosterically controlled by subtle modifications emanating from the centrally located tetramer subunit.

DNA Torsional Effects on Thiol Reactivity. The results of our experiments using LSC, dimer-stripped LSC (Figure 8), and reconstituted tetramer–DNA (Figure 6) indicate that the salt dependence of the thiol reactivity of these complexes is very similar and that no detectable reaction occurs until a concentration of approximately 1 M is reached, at which point

most of the dimers would be dissociated from the LSC. It therefore appears that while the dimers can influence the thiol reactivity of tetramers in the soluble, DNA-free state, they do not have a readily detectable role in modulating thiol exposure in nucleoprotein complexes. Upon treatment with DNase (Figure 8), this apparent difference in behavior was eliminated, indicating that the effects of the DNA double helix upon thiol group exposure overwhelm any effect the dimers may have and thus render their contribution undetectable.

It has previously been suggested that the unwinding of a left-handed DNA supercoil can lead to formation of a lexosome, a transcriptionally active region of chromation structure that is also susceptible to labeling of the H3 thiol groups by iodoacetimidofluorescein (Prior et al., 1983). Our findings regarding the effects of DNase treatment upon H3 thiol reactivity are in agreement with this suggestion, since the introduction of nicks into the DNA backbone may be reducing the torsional energies of the complex in a manner similar to that achieved by the unwinding of a supercoil. Furthermore, our experiments using dimer-stripped LSC (Figure 8), in which there was no detectable thiol reactivity in the absence of DNase treatment, indicate that when the histones are bound to DNA, the H2A–H2B dimers do not play a major role in modulating H3 thiol reactivity, and again emphasize the role of DNA–tetramer interactions. Our results indicate that neither histone dimer subunits nor any other non-histone proteins are required for the formation of a thiol-reactive chromatin structure and thus eliminate the need to hypothesize the formation of heterotypic (i.e., H2A–H2B–H3–H4) tetramers in order to explain the increased thiol reactivity of active chromatin.

The control exerted by DNA on the thiol reactivity of histones can be broken down into two components, one ionic and the other torsional. Due to the highly localized concentration of phosphate groups in the DNA backbone, the effective local "ionic strength" experienced by the histones when bound to DNA is possibly as great as 7 M. Our studies with DNA-free histones have documented a decrease in thiol reactivity with increasing ionic strength up to 4 M NaCl (Figure 3). If this effect could be extended upward to 7 M ionic strength (experimentally unattainable conditions), we would predict that the thiol reactivity of the soluble histones would be negligible, as indeed has been observed with chromatin at physiological (bulk phase) ionic strengths. The precise value of the local ionic strength activity at the DNA–histone interfaces will depend upon the balance of all interactions between the DNA phosphates and small ions such as NaCl and any other competitively binding peptides.

The second possible component of DNA influence upon histone conformation is the torsional strain exerted by the DNA double-helix continuum. Our finding that the DNA effect upon thiol reactivity of core particles can be relieved by limited treatment with DNase demonstrates the existence of a double-helix-derived *intra*nucleosomal torsional strain, in addition to any internucleosomal torsional stain that may exist in chromatin structures (Germond et al., 1975). The torsional energy of the DNA double helix can be finely modulated by a number of mechanisms, including the intercalation and/or binding of various ligands to the DNA backbone; the interaction of various protein molecules, such as the single-strand DNA binding proteins, with the DNA; changes occurring in local DNA tertiary structure, by hairpin loop, cruciform, or Z-DNA formation; the action of various topoisomerases on the number of DNA supercoils; and possibly the covalent modification of the histone molecules themselves (Bode et al., 1980). The demonstration that changes in the superhelicity of DNA can influence histone conformation raises the intriguing possibility that changes in histone conformation can have an effect upon the degree of DNA supercoiling. Such studies, utilizing histones reconstituted onto supercoiled plasmids, are now in progress.

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Partial Amino Acid Sequence of Human Thrombospondin As Determined by Analysis of cDNA Clones: Homology to Malarial Circumsporozoite Proteins†

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ABSTRACT: A λgt 11 library prepared from human umbilical vein endothelial cell RNA was screened for cDNAs encoding thrombospondin. Reagents included a monospecific antibody to human thrombospondin and a mixture of four synthetic oligodeoxyribonucleotides derived from an amino acid sequence near the NH_2 terminus of mature human thrombospondin. Two series of cDNA clones coding for sequences at the 5′ and 3′ ends of thrombospondin mRNA, respectively, were isolated. The nucleotide sequence of a 1.3-kilobase (kb) 5′ clone (λTS-33) coded for 99 bases of 5′ untranslated RNA, a signal peptide of 18 amino acids, and the first 379 amino acids of thrombospondin. Northern blot analysis with λTS-33 detected a single mRNA species of ~6.0 kb in rat aortic smooth muscle cell RNA. Thrombospondin mRNA levels increased rapidly, but transiently, in quiescent smooth muscle cells treated with platelet-derived growth factor. The kinetics of this response were very similar to those of the thrombospondin protein to this growth factor. There was significant homology in amino acid sequence between thrombospondin and a conserved region in the circumsporozoite protein of two malarial sporozoites. This region of thrombospondin may therefore represent a potential recognition site for a cell surface thrombospondin receptor.

Thrombospondin (TS)[1] is a high molecular weight glycoprotein (M_r 450 000) that is both stored in α granules of platelets and secreted by a wide variety of mesenchymal cells [see Silverstein et al. (1986) and Majack and Bornstein (1986) for recent reviews]. There is evidence that TS functions in the secondary, irreversible phase of platelet aggregation (Leung, 1984; Dixit et al., 1985; Silverstein et al., 1986). TS also forms complexes with histidine-rich glycoprotein and plasminogen (Silverstein et al., 1985a) and may regulate the release of plasmin from complexes containing plasminogen and tissue plasminogen activator (Silverstein et al., 1985b).

The role of TS in the extracellular matrix is less well understood. TS binds to cell surfaces (McKeown-Longo et al., 1984; Roberts et al., 1985) and to a variety of matrix macromolecules including collagen, fibronectin, and heparin (heparan sulfate) Dixit et al., 1984; Majack & Bornstein, 1986), but the functional consequences of these interactions are not known. Recently, this laboratory has provided evidence that PDGF and heparin-like glycosaminoglycans act antagonistically in regulating the production of a TS-rich extracellular matrix by rat aortic SMC (Majack et al., 1985). Asch et al. (1986) have also shown that PDGF stimulates the production of TS by cultured human glial cells. Furthermore, TS can act synergistically in conjunction with epidermal growth factor to facilitate SMC growth (Majack et al., 1986). Thus, evidence exists for an autocrine, growth-promoting function for matrix TS.

The regions of the TS protein chain responsible for binding to cell surfaces and to extracellular macromolecules have been identified in a preliminary fashion by a combination of limited protease fragmentation and the use of monoclonal antibodies (Galvin et al., 1985). Limited amino acid sequence information is also available (Coligan & Slayter, 1984; Dixit et al., 1984, 1985; Raugi et al., 1984; Galvin et al., 1985). These findings suggest that TS, like fibronectin, is a member of a class of proteins termed "modular glycoproteins" (Hynes & Yamada, 1982). In all likelihood, the TS molecule consists of three identical chains of M_r 140 000–150 000, but variations in structure resulting from alternative splicing of the TS mRNA, as has been shown for fibronectin (Tamkun et al., 1984), may exist.

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[1] Abbreviations: TS, thrombospondin; PDGF, platelet-derived growth factor; SMC, smooth muscle cells; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; SSC, standard saline citrate.

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