Journal of Cellular Biochemistry 47:11–17 (1991)

Probing Nucleosome Core Secondary Structure Before and After α-Chymotrypsin Treatment By Raman Spectroscopy and Thermal Denaturation

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Abstract Nucleosome cores were digested with α-chymotrypsin until histone H3 was degraded to a partial histone, CP1. As we reported previously, cleavage occurred at leucine 20 to H3 and resulted in an increase in circular dichroism between 265 to 285 nm. Some modest core unfolding was also observed as determined by a small decrease in the sedimentation coefficient. Studies reported here deal with the analysis of core secondary structure and subsequent perturbation caused by treatment with α-chymotrypsin. Raman spectroscopy indicated that chymotryptic treatment promoted a change in the conformational environment of a population of core histone tyrosines. In addition, a shift from B-form to an intermediate B- or A-form was observed for core DNA. High-resolution thermal denaturation was used to determine alterations in the stabilization of core DNA related to perturbation of the core histones. Brief chymotryptic treatment indicated changes in both pre-melt and irreversible transitions.

Key words: nucleosome structure, chromatin, histone, Raman spectroscopy, thermal denaturation

Eukaryotic chromatin may be described as a dispersed network of nucleohistone fibers composed of equal weights of DNA and basic histone proteins and a variety of other less abundant protein species [1]. Chromatin consists of repeat structural building blocks, nucleosomes, composed of a core and spacer region. The core, a roughly cylindrical unit 11 nm wide and 5.5 nm high contains an octamer of four histones, H3, H4, H2A, and H2B, and it is associated with approximately 146 base pairs of supercoiled DNA about its periphery [2–4]. The spacer or linker region that connects successive cores possesses a variable length of DNA (depending on the species) and one molecule of histone H1 or H5. Although the nucleosome's general structure is well defined, relatively little is known about the fine secondary structure associated with specific histone-histone and histone-DNA interactions.

Molecular recognition of such key cellular processes such as transcription is most likely to be affected by chromatic native structure. Theoretically, therefore, the structural integrity of the nucleosome core and other chromatin structural domains related to the nucleosome could affect the modulation of such processes. Changes in nucleoprotein folding and compaction were shown to be essential for the transcription efficiency of ribosomal DNA [5–7].

The basic results described in this report indicated that if the nucleosome core is perturbed at a critical histone site by α-chymotrypsin, a site at the juncture between the N-termini and central globular domains, some changes in secondary structure occur. Using Raman spectroscopy, we found that a population of nucleosome histone tyrosines underwent a change in conformational environment after chymotryptic treatment. Also observed was a shift of the core DNA from B form to an intermediate B- or A-form. High-resolution thermal denaturation studies indicated that brief treatment of cores with α-chymotrypsin resulted in destabilizing of the core DNA.

MATERIALS AND METHODS

Preparation of Nucleosome Cores

Chicken erythrocytes were chosen as the source of chromatin because of low endogenous protease activities. Erythrocyte nuclei were isolated and lysed according to the method of Libertini and Small [8]. A modified version of this

Received March 26, 1991; accepted March 29, 1991.

method was then used to obtain nucleosome cores from chromatin. Digestion of nuclei was performed using 125 units of micrococcal nuclease (Worthington, 14,925 units/mg) per 6.25 mg of chromatin in a digestion buffer containing 0.01 M Tris/HCl and 1 mM $CaCl_2$, pH 7.8. After digestion time of 30 min at room temperature, the reaction was terminated by adding Na_2 EDTA to a final concentration of 10 mM. The chromatin was then centrifuged for 30 min using a Sorvall Rotor at 12,000 rpm, after which the supernatant was removed and cooled on ice. The digestion and ensuing steps were repeated twice, after which the pooled supernatants were clarified by centrifugation at 4°C for 30 min, using an SB 283 rotor at 28,000 rpm. After clarification, the chromatin was stripped of H1 by adding, for each 6.25 mg of chromatin, 80 mg of dry CM-Sephadex (Sigma Chemical), followed by slow addition of 0.01 volume of 5 M NaCl at 0°C and stirring for 1 h. The stripped chromatin was then dialyzed overnight against 0.02 M Tris/HCl, 0.2 mM Na_2EDTA, pH 7.8. Then the stripped chromatin was digested for 3 min at 37°C (using 0.25 units of micrococcal nuclease per 50 μg of chromatin DNA) and layered onto sucrose gradients (5–30%). Nucleosome cores were then isolated after centrifugation (4°C) for 24 h at 26,000 rpm, using a Beckman SW 28 rotor.

Digestion With α-Chymotrypsin

Nucleosome cores were dialyzed into 50 mM Tris/HCl, pH 7.8, and diluted to a concentration of 1 mg/ml. The cores were treated with α-chromatrypsin (Worthington, 50 units/mg), 0.25 unit enzyme/100 μg of total core histone, and analyzed after increasing periods of digestion at 37°C. Digestion was terminated by the addition of phenylmethylsulfonyl fluoride to a concentration of 0.1 mM.

Protein Electrophoresis

High-resolution polyacrylamide electrophoresis was performed on a 15 cm × 0.3 cm slab gel of 18% acrylamide containing SDS, using a modification of the Laemmli procedure [9] as described by Thomas and Kornberg [10]. Gels were then stained with napthol blue black (Sigma, 1% napthol blue black in 50% methanol, 40% glass-distilled H_2O, 10% glacial acetic acid).

Raman Spectroscopic Analysis

Samples of undigested and chymotryptic-treated cores were concentrated, to 1 mg/50 μl, respectively, in the digestion buffer described using a millipore immersible CX-10. An internal standard, sodium sulfate, was added to a concentration of 0.14 mM before spectroscopic analysis. Raman spectra were obtained with a SPEX model 403 spectrophotometer and photon counting system. The 514.5 non-excitation line of 140 mW power was tuned from a Spectra-Physics Model 165 AR^+ laser. Solution samples of both control and α-chymotrypsin-treated nucleosome cores were placed in capillary tubes 2 mm in diameter with both ends sealed. Signals were averaged by a SPEC Datamate, and DM1 and peak frequencies were calibrated using the CCl_4 454 cm^{-1} band. Spectra over a region of 200–2000 cm^{-1} were scanned with a speed of 1 sec/cm^{-1} and a spectrum band result of 4 cm^{-1}. Accuracy of the measurement is believed to be ± 1 cm^{-1}.

High-Resolution Thermal Denaturation

Samples were dialyzed against 1 liter of 2.5×10^{-4} Na_2EDTA adjusted to pH 8 by the addition of a small amount of NaOH. After dialysis, samples were diluted to have optical absorbance ranging from 1 to 1.5 at 260 nm. Samples and a portion of the medium were bubbled for about 5 min at room temperature with helum gas to displace dissolved O_2 and N_2 (to prevent bubble formation in cuvettes during heating), and then were centrifuged at 3500*g* for 5 min in stoppered microtubes to remove particulates. About 0.3 ml of clarified sample was placed in a minicuvette, put into a 4-place Beckman-style holder along with about 0.8 ml of medium in the "blank" position, and overlaid with several drops of silicone oil. The three samples were tightly stoppered with teflon caps, and the blank cuvette was closed with a thermistor probe assembly.

All melting experiments were conducted at 270 nm so that AT and GC base pairs were weighted about equally. Sample temperature was elevated at the rate of 30° per h. A computer-controlled single-beam spectrophotometer was used to record data and monitor hyperchromicities during the experiment. The initial disk records were subsequently processed by a microcomputer program (in BASIC). The program corrects all absorbances for thermal expansion

and then fits successive groups of 13 hyperchromicity-temperature pairs to a cubic polynomial to obtain best-fit values at the center point for smoothed hyperchromicity, first derivative with respect to temperature, and estimated error of the derivative. This fitting was continued repeatedly after advancing one data pair at a time was advanced until the data set was exhausted. Readings made at 320 nm before and after experiments, and at several points during the first experiments, indicated that changes in light scattering were small in all samples.

RESULTS

α-Chymotryptic Treatment of Nucleosome Cores

Brief treatment of nucleosome cores with α-chymotrypsin (0.25 μg of enzyme/100 μg of core histone for 5 min) yielded a major partial H3-derived product, CP1 [11] (Fig. 1). A detailed description of the relative rates of histone degradation resulting from α-chymotrypsin treatment was described elsewhere [11]. Using a combination of tryptic digest and end-group analyses, we identified the primary site of cleavage as ^{20}Leu in H3 [11]. Changes in core histone and DNA secondary structure corresponding to the initial chymotrypsin induced degradation of H3 to a partial product (lanes 8 and 9) were further analyzed by both Raman spectroscopy and high-resolution thermal denaturation.

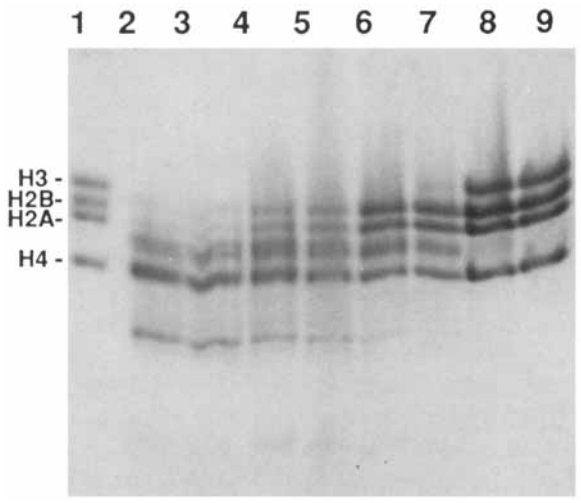

Fig. 1. The action of α-chymotrypsin on nucleosome cores monitored by polyacrylamide gel electrophoresis in the present of SDS. Chicken erythrocyte nucleosome cores were digested with 0.25 units of enzyme/100 μg of core histone for various digestion periods. **Lane 1,** control histones (Sigma); **lane 2,** nucleosome cores digested for 45 min; **lane 3,** 30 min; **lane 4,** 20 min; **lane 5,** 15 min; **lane 6,** 10 min; **lane 7,** 5 min; **lane 8,** 1.5 min; **lane 9,** control nucleosome cores.

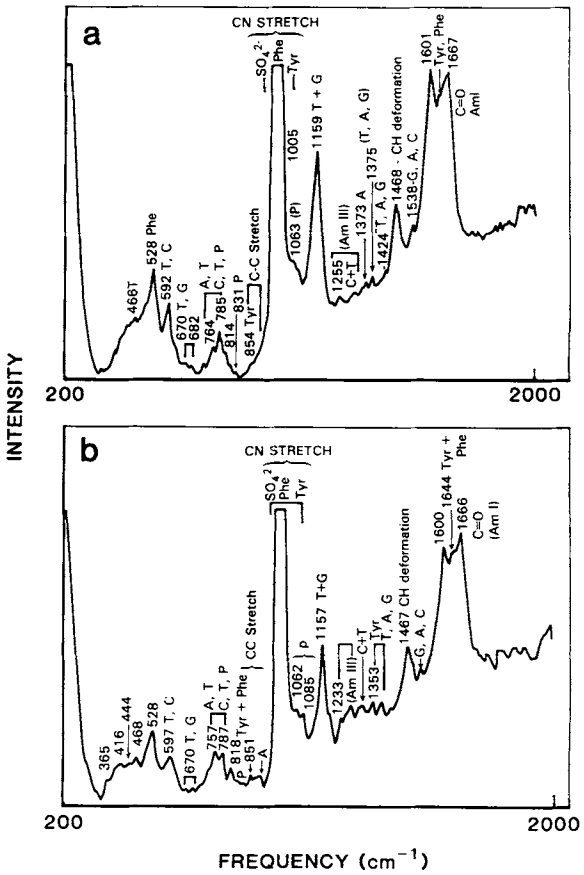

Fig. 2. Raman spectra of (**a**) control nucleosome cores and (**b**) cores digested for 5 min with α-chymotrypsin (0.25 units of enzyme/100 μg of core histone). Chymotryptic treatment induces change in a population of core histone tyrosines as indicated by a line near 1353 cm^{-1}. Perturbation of core DNA thymine (T), cytosine (C), and guanine (G) rings are indicated by changes at frequencies near 597 cm^{-1} and 670 cm^{-1}. A change in the adenine (A) ring environment is indicated by the appearance of a peak at 1373 cm^{-1}. P, PO_3^{2-}.

Raman Spectra of Untreated Cores Compared With Cores Treated With α-Chymotrypsin

Raman spectra performed on untreated cores corresponded well with results obtained by Thomas et al. [12]. The characteristic presence of a PO_4^{2-} group frequency near 1065 cm^{-1} was noted in this analysis, as well as the DNA backbone group frequency at 831 cm^{-1} indicative of phosphodiester stretching (Fig. 2). Also present were Raman lines at 670 cm^{-1} and 682 cm^{-1} typical of base interactions of B-form DNA, the predominant type of DNA believed to be associated with nucleosome cores. In this particular control sample, however, there was the presence of some A-form DNA confirmed by a minor line at 814 cm^{-1}. Distinguishable Raman lines associated with the nucleosome core octamer were ob-

served as well. Lines near 1667 cm^{-1} caused by amide I, and near 1468 cm^{-1} caused by CH deformations, were observed. Amide III was represented by several weak components near 1255 cm^{-1}. Also notable were lines representative of histone aromatic side chains at 1005 cm^{-1} and 854 cm^{-1}. All correlated well with the findings of Thomas et al. [12] and showed again that because of the relative positions and intensities of amide I and amide III Raman lines, core protein is primarily in the α-helical conformation with some, theoretically contributing random coil. Because of the absence of an intense Amide III line below 1240 cm^{-1}, these data reaffirmed that no significant β-sheet structure is associated with the core histones.

After digestion with α-chymotrypsin to the point at which H3 was degraded to a partial histone CP1, some clear, albeit subtle, secondary structural changes were apparent in both DNA and protein portions of the Raman spectra. The most significant change in the protein spectra was associated with the aromatic tyrosine residues. Although the initial site of chymotryptic cleavage was shown to be ^{20}Leu in H3, tyrosine is the preferred substrate of α-chymotrypsin. Therefore, the perturbation of aromatic tyrosine residues by chymotrypsin after initial cleavage of ^{20}Leu is reasonable. The tyrosine doublet near 851 cm^{-1} generally obscured by overlapping DNA lines in the control, was more defined in the digested cores. A tyrosine line near 1353 cm^{-1}, also became more delineated upon digestion. This finding suggested that upon digestion, a population of core histone tyrosines undergoes a distinctive change in conformational environment. Apparaently α-chymotrypsin induces a definitive change associated with amide III frequency. A distinctive line was present below 1240 cm^{-1}, implying that a small percentage of the core histone protein chains were shifted to a potential β-sheet conformation when the core is subjected to α-chymotrypsin treatment.

There were also some structural changes associated with the core DNA after digestion with α-chymotrypsin. Most notable was a relative shift to A-form DNA or intermediate B-form as indicated by frequencies near 597 cm^{-1} and 670 cm^{-1}, which suggested some perturbation of thymine, guanine, and cytosine rings and perhaps affected base-pair tilt. Also indicative of base ring perturbation was the appearance of a defined peak for adenine near 851 cm^{-1} in the digested cores compared with the presence of an adenine at 1373 cm^{-1} in the control cores. A small frequency shift from 764 cm^{-1} to 757 cm^{-1} is apparent for adenine and thymine when comparing control and digested cores. The PO_4^{2-} group frequency near 831 cm^{-1} was shifted to 851 cm^{-1} and considerably more pronounced in the treated cores as compared with the control. This observation suggests that chymotryptic treatment promotes breathing for the core DNA phosphodiester backbone. Corraborating this is the presence of a more defined peak at 1085 cm^{-1} in the treated cores. Arnott et al. [13] have defined a series of A-structures—DNA, RNA, and RNA/DNA hybrids—as differing in base-pair tilt, and Thomas and Kornberg [10] suggested that the A structure is implicated in transcription. An increase in the circular dichroism in the near UV region, suggesting a small relaxation of the core DNA upon treatment with α-chymotrypsin, has been described by my research group [11]. It is, therefore, not surprising that the enzyme action would promote change in the DNA bases' environment, but it is difficult to say precisely what these changes are.

High-Resolution Thermal Denaturation Analyses of Cores Treated With α-Chymotrypsin Compared With Untreated Cores

Thermal denaturation of DNA in nucleoprotein complexes provides information about the extent of stabilization of the DNA double helix by associated chromosomal proteins. First derivative thermal denaturation profiles of nucleosome cores typically are characterized by a reversible, pre-melt transition occuring from 40° to 60°C. It is believed that the pre-melting transition behavior of nucleosome cores corresponds to the disruption of 20 base pairs at each end of the particle segments from the (14) N-termini. A larger irreversible transition is characterized by a T_m of about 75°C. First derivative thermal denaturation profiles were obtained for aliquots of total digests corresponding to increasing digestion time (Fig. 3). The first derivative melting profile obtained for control cores in these experiments agreed well with those reported in the literature. The results in Figure 3 indicate that melting corresponding to early digestion was biphasic in nature. The derivative profiles showed that both transitions appeared to undergo an overall decrease in T_m, with a maxi-

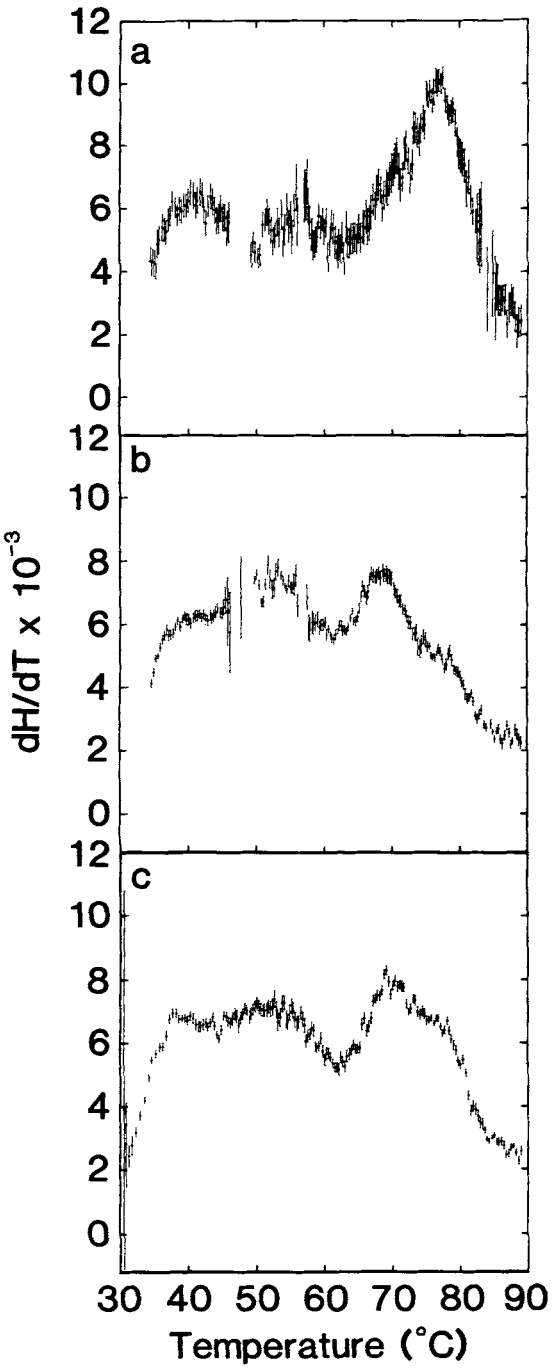

Fig. 3. High-resolution first derivative thermal denaturation profiles of (a) untreated nucleosome cores, or nucleosome cores treated with α-chymotrypsin for (b) 5 min or (c) 10 min. The control profile agrees with previous reports and is biphasic in nature. After minimal chymotryptic treatment (5 min of 0.25 units of enzyme/100 μg of core histone) an overall decrease in T_m occurs at about 68°C and 35°C. There is also a decrease in the apparent magnitude of both T_m transitions. A curious "break" at 45–49°C is noted in the profile of the control and the nucleosome cores after 5 min treatment (a,b) that is not seen after 10 min of treatment with α-chymotrypsin (c).

mum of about 68°C and 55°C, respectively. Also noted was a generalized decrease in the magnitude of the transitions. A curious "break" at 45–49°C is noted in both the control core and 5-min chymotryptic-treated core profile. This apparent "break" is absent in the 10-min condition. The break is reproducible, but it is difficult to speculate on the implication of this observation. Since the pre-melting behavior of nucleosome cores appears to correspond to the disruption of 20 base pairs at each end of the particle segment from the core [14], perhaps the high-resolution thermal denaturation profile "break" corresponds to end melting that is not simultaneous. As digestion progressed for increasing time intervals, there appeared to be no further decrease in the overll T_m of the irreversible and pre-melt transitions (data not shown). By the 40-min interval, the first derivative denaturation profile was no longer typically biphasic (unpublished observations). In fact, extensive digestion indicated that the pre-melting phase was nearly contiguous with the irreversible transition, with only a slight break occurring at about 60°C to 65°C. By this point in the digestion, it was clear that critical histone-DNA contacts had been disrupted so as to alter the core DNA's apparent stability. After prolonged digestion, some of the core DNA is not stabilized.

Apparently, chymotryptic treatment of cores affected the stabilization of the DNA to some extent even early in the digestion. This observation, and the observed perturbations in the Raman spectra, further supports that chymotrypsin affects the core DNA's disposition. Circular dichroic spectra in the near μv indicated that chymotryptic treatment of cores caused the relaxation of about one DNA turn on each DNA terminus [11]. It is likely that alterations in the Raman spectra and high-resolution thermal denaturation patterns of the chymotryptic-treated cores reflected this change in core DNA relaxation.

DISCUSSION

Previous studies indicated that treatment of nucleosome cores with specific proteases did not have profound effects on the generalized structural integrity of the nucleosome core [11,15,16]. These studies showed that sedimentation changes corresponded to changes in particle mass rather than to any major unfolding. But the unfolding change was subtle and corresponded

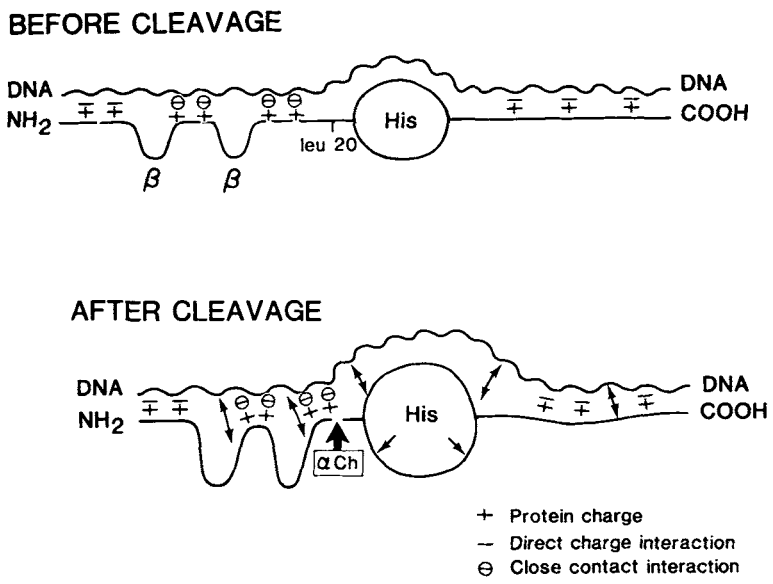

Fig. 4. Schematic representation of nucleosome core histone-histone, histone-DNA, or both types of interactions that might be affected by perturbation with α-chymotrypsin. Charge interactions vs. close contact and direct contact are depicted. Theoretical central domain core DNA contraction juxtaposed to relaxation of DNA at the N-termini has been indicated as a possible consequence of α-chymotrypsin treatment of nucleosome cores.

to a relaxation of about 1–2 turns of DNA on each DNA terminus [11]. The Raman spectra presented here suggest that some of the core DNA undergoes a conformational change to an intermediate B- or A-form DNA. In addition, the thermal denaturation studies showed that chymotrypsin promoted a destabilization of the core DNA. Although both circular dichroic and sedimentation data suggested that protease treatment induced some modes of unfolding and partial relaxation of at least the ends of the core DNA, this conclusion could be a theoretical oversimplification of the structural changes when considering the secondary protein and DNA structure.

The Raman spectra presented here indicated that α-chymotrypsin promoted a change in the environment of a population of the core histone tyrosine residues. The majority of tryosine residues found in the nucleosome core are found in the central histone sequences, with the exceptions of Tyr^{121} in H2b and Tyr^{88} and Tyr^{98} in H4. Since it was shown that the ^{20}Leu in H3 was the initial site of chymotryptic cleavage of the core, even through the other aromatic residues are preferred substrates over leucine, it is unlikely that the aromatic residues are topographically exposed in the core's native state. The Raman spectra clearly showed, however, that a population of core tyrosines underwent a change in conformational environment. Therefore, it is possible that chymotryptic treatment promotes a partial exposure of some of the tyrosine aromatic residues.

When considering the potential effects of chymotryptic treatment on the core, a hypothetical equilibrium between histone-histone and/or histone-DNA interactions, or both, must be considered (Fig. 4). It is theoretically possible that a histone-histone interaction, such as a change in exposure of the tyrosine aromatic residues, could alter histone-histone charge-charge interactions that indirectly affect histone-DNA contacts. A potential tyrosine conformational transition is unlikely to occur through a direct interaction with the core DNA, since treatment of nucleosome cores with 2 M NaCl has been shown to not cause any major disruption of the core DNA-tyrosine disposition, both Raman spectra and tyrosine iodination patterns remaining unchanged [12,17]. In the case of chymotryptic action on the core, however, cleavage at a site close to the internal globular domain and located near (only 20 residues away from) the N-termini could, in fact, induce relaxation at the ends, but promote potential change from a B- to A-form in the core central DNA domain. Poten-

tial DNA contraction in the central domain in possible (Fig. 4).

A thorough understanding of secondary structural changes in core nucleoprotein compaction and DNA superhelicity should eventually yield insights into such cellular processes as transcription. We have isolated large nucleoprotein structures with transcription capability that contain histones and a semi-nucleosomal arrangement [6,7]. We observed that transcriptional efficiency is modulated by the state of the nucleoprotein compaction or constituent DNA superhelicity or both [6]. Further analyses of the secondary fine structure and potential transitions of the nucleosome core, which is a substructure of the large transcription-capable nucleoprotein particles, should enhance our knowledge of transcriptional fine-tuning.

ACKNOWLEDGMENTS

I thank Garth L. Nicolson for critical discussion and help in preparing the manuscript. Special thanks are due to Dr. Spencer Linn, Dr. Allen Ansevin, and Dr. Randolph Rill. This work was supported in part by NIH grant R35-CA44352 from the National Cancer Institute to Professor Garth L. Nicolson.

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