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Crosslinked histone octamer as a model of the nucleosome core

(chromatin/superhelical DNA/DNA flexibility)

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ABSTRACT **When histones in chromatin core particles were crosslinked with dimethylsuberimidate, the resulting particles had properties closely similar to those of native core particles. A crosslinked octameric histone complex was isolated from these particles under nondenaturing conditions. Upon reaction with DNA, this octameric protein folded the DNA into a structure closely resembling that of native core particles as verified by various techniques; protein denaturants were necessary for reassociation. The histone octamer is useful as a model of the nucleosome protein core and for studying histone–DNA interactions that occur in nucleosomes.**

A major step toward understanding the structure of eukaryotic chromatin has been the realization that DNA is folded in a periodic fashion by complexing with histones H2A, H2B, H3, and H4, a concept initially suggested by Olins and Olins (1) and Kornberg (2). The first-order compaction of chromosomal DNA is thought to arise from wrapping of 140 to 200 base-pair lengths of DNA around a protein core composed of two each of these histones to form the chromatin core particle (nucleosome, nu-body) (2–6). In this manner the octameric histone complex serves as the primary building block in construction of a chromosome. Certain features characteristic of the DNA structure in nucleosomes have been found including characteristic circular dichroism spectra, melting profile, DNase I digestion pattern, torsional constraints, and degree of compaction (7–11).

The details of the condensation of DNA by histones into nucleosomes are of considerable interest and seem a necessary prelude to understanding the functional properties of chromatin. From the viewpoint of solution physical chemistry, sorting out the relevant interactions among the components of this system is a formidable problem. The equilibria governing histone associations in solution are apparently quite complex and depend upon solution conditions (12–15). In addition, each of the four histones interacts individually with DNA (16).

It would seem profitable to investigate the interaction of DNA with the whole octameric histone core in an attempt to study histone–DNA interactions that occur in the nucleosome while omitting from the analysis both protein–protein interactions and histone–DNA interactions not leading to nucleosome formation. Although histones extracted from chromatin in 2 M NaCl at pH 9.0 can be crosslinked into an octameric complex by using dimethylsuberimidate (14), hydrodynamic and other studies of histones dissociated from DNA under these conditions indicate that an octamer may not be the predominant species present (12, 15). Also, for protein and NaCl concentrations that are convenient for studying histone–DNA interactions, the octameric histone complex is unstable (A. Stein, unpublished data).

In this paper, we describe a model system for studying histone–DNA interactions that occur in the nucleosome. We have prepared a histone octamer by treating purified core particles with dimethylsuberimidate. This crosslinked octamer interacts with DNA to form a structure closely resembling the native nucleosome and can therefore serve as a useful model of the histone core of the nucleosome.

Abbreviation: SV40, simian virus 40.

MATERIALS AND METHODS

Preparation of Core Particles. Chicken erythrocyte nuclei were prepared as described by Hymer and Kuff (17). Nuclei at 100 A_{260} units/ml in 0.25 M sucrose/10 mM Tris·HCl, pH 8.0/1 mM $CaCl_2$ were digested with micrococcal nuclease (Worthington) at 200 units/ml to an extent maximizing the production of monomer chromatin particles. The digest was fractionated by sucrose gradient centrifugation using the Ti 15 zonal rotor as described by Olins *et al.* (18); the gradient contained 0.025 M Tris·HCl, pH 8.0, 1 mM Na_2EDTA, and 5 mM $NaHSO_3$. The monomer fraction was then refractionated on a sucrose gradient containing, in addition, 0.6 M NaCl. Core particles thus purified contain the four smaller histones in correct proportion, approximately 140 base pairs of DNA, no H1 or H5 histone, and a protein/DNA ratio of 1.2 as determined by methods previously described (8).

Preparation of DNA. The 140 base-pair DNA was extracted from core particles with 3 M NaCl/0.05 M sodium phosphate buffer, pH 7.0, and purified with hydroxylapatite in a batch procedure. Simian virus 40 (SV40) DNA I (strain 776) was isolated from mature virions as previously described (19).

Octamer Preparation. Purified core particles were crosslinked at 2 A_{260} units/ml in 0.01 M sodium borate, pH 10.0, with dimethylsuberimidate (Pierce), 10 mg/ml, at 25° for 40 min. After dialysis and refractionation, the modified particles were extracted with 3 M NaCl/10 mM sodium phosphate, pH 7.0, several times to remove DNA, yielding a highly purified octameric protein. The crosslinked histone octamer is insoluble in 3 M NaCl, pH 7.0.

Octamer–DNA Reconstitution. Octamer (dissolved in 0.05 M sodium phosphate buffer, pH 7.0) and DNA were mixed at 0° in 1.0 M NaCl/0.25 mM Na_2 EDTA/10 mM Tris·HCl, pH 8.0/bovine serum albumin (2.0 mg/ml). The mixture was dialyzed overnight at room temperature against 0.6 M NaCl in the same buffer and then fractionated on a sucrose gradient.

Gel Electrophorersis. The 5% (vol/vol) sodium dodecyl sulfate tube gels were run according to Weber and Osborn (20). DNase I digestions and denaturing DNA gels were as previously described (21). Agarose gel electrophoresis of SV40 DNA was performed as previously described (19, 22).

Electron Microscopy. Micrographs were made with a Philips 300 electron microscope at 40 kV. DNA length measurements were made with a Numonics, Inc., X-Y recording stage (23). Grids were prepared as previously described (24). Samples were fixed with 1% formaldehyde, stained with 1% uranyl formate, and rotary-shadowed with Pt–Pd.

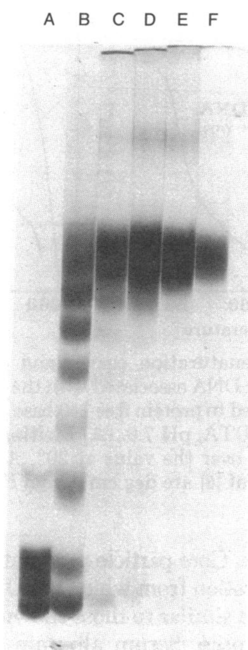

FIG. 1. Five percent sodium dodecyl sulfate gel of histones from purified core particles crosslinked for increasing times of reaction with dimethylsuberimidate as described in the *text*. The reaction was stopped after 0, 5, 10, 15, 40 min (*A–E*) by adjusting the reaction mixture to pH 5 with sodium acetate buffer. Tube F shows the histone from fractionated, crosslinked core particles.

Other Measurements. Thermal denaturation measurements were made by using a Beckman Acta III spectrophotometer with the platinum probe temperature sensor in a dummy cell. The platinum probe was calibrated against a Fisher Hg-glass thermometer. Absorbance values were corrected for solvent expansion. Circular dichroism measurements were performed on a Cary model 61 at room temperature. Sedimentation velocities were measured at 20° and 44,000 rpm with a Beckman model E ultracentrifuge equipped with UV scanner. The extent of lysine modification was assessed by using 2,4,6-trinitrobenzenesulfonic acid (25).

RESULTS

The approach used for preparation of a crosslinked core protein was as follows. Purified core particles were treated with a protein-crosslinking reagent to covalently link the histones together under conditions that minimally perturb the structure of the particle. DNA was then removed from the crosslinked protein core under nondenaturing conditions.

Core Particles Can Be Extensively Crosslinked without Substantial Structural Pertubations. By using a slight modification of the method used by Thomas and Kornberg (14), it is possible to crosslink all of the histones of isolated core particles into an octameric complex. Fig. 1 shows the time course of the reaction. At early times, eight bands, corresponding to monomer through octamer histone complexes, were visible (14). As the reaction proceeded, the lower molecular weight bands were driven into the octamer band and some material appeared in higher order bands, presumably deriving from interparticle crosslinking.

The extensively crosslinked core particles were fractionated by sucrose gradient centrifugation to remove these higher order products and any nonspecific aggregates. Approximately 40% of the starting material appeared as a symmetrical band sedimenting at about 11 S. Over 95% of the histone in the reisolated crosslinked core particle migrated as a single band on electro-

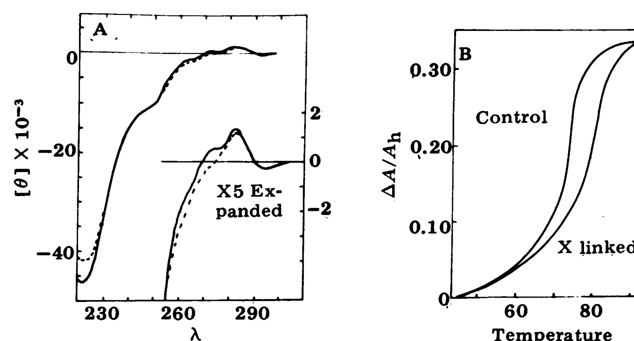

FIG. 2. Circular dichroism spectra and thermal denaturation curves for crosslinked and noncrosslinked core particles. All samples were in 0.25 mM Na_2EDTA, pH 7.0. (*A*) Circular dichroism spectra. Units of $[\theta]$ are deg cm^2/dmol PO_4; wavelength, λ, in nm. The dashed curve is the crosslinked sample; the right-hand scale corresponds to the ×5 greater sensitivity scan through the region of the DNA maximum. (*B*) Melting curves. ΔA is the increase in absorbance over the value at 20°, A_h.

phoresis with a mobility corresponding to that of the octameric histone species (Fig. 1*F*). About 80% of the lysyl residues of the histones were modified.

Because core particles lacking H1 and H5 are soluble at moderate salt concentrations, a solvent that suppresses electrostatic effects, 0.1 M NaCl/1 mM Tris·HCl, pH 8.0, could be used for evaluation of shape changes by analytical centrifugation. $s^0_{20,w}$ fo the crosslinked core particle was 10.8 ± 0.2 compared to 10.4 ± 0.2 for native monomeric core particles; this increase is consistent with the increase in molecular weight of the particle due to the crosslinking reagent. Both samples sedimented as symmetrical boundaries.

The crosslinking reaction does not alter the UV absorption spectrum of core particles, allowing examination of the circular dichroism spectrum of the modified particle. The greatly decreased ellipticity of crosslinked core particles at 280 nm, <2000 deg cm^2/dmol PO_4 compared to that for protein-free DNA, 8300 deg cm^2/dmol PO_4 (Fig. 2*A*), is characteristic of the DNA conformation in native core particles (7, 8). This finding provides a sensitive indication that the DNA remained folded in the modified particle. The near-identity of the circular dichroism spectra of modified and native core particles in the region of absorption of the peptide bond suggests that few alterations in secondary structure of the histones resulted from crosslinking.

Compared to native core particles, the DNA in the crosslinked core particle was more resistant to thermal denaturation (Fig. 2*B*). The midpoint of the predominant transition was 6° higher whereas the early melting was unchanged. This enhanced thermal stability apparently is a result of linking histones together rather than simply the monofunctional modification of the lysyl residues, because the high temperature transition of chromatin modified to the same extent with ethyl acetimidate is only 1° higher than that of unmodified chromatin (25).

The characteristic spacing and intensity distribution of single-strand DNA fragments resulting from DNase I digestion of native chromatin core particles (9) was also observed for crosslinked core particles (Fig. 3). Nuclease succeptibility is expected to be a highly sensitive structural probe for chromatin (26, 27).

Lastly, no morphological differences were seen in electron micrographs of the native and crosslinked core particles; both appeared as compact spherical particles, approximately 80 Å in diameter (data not shown).

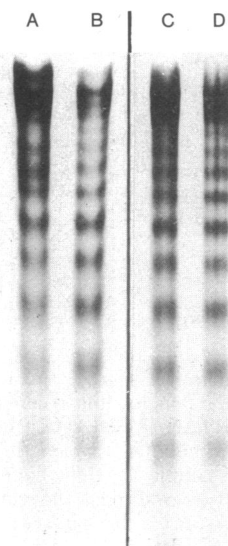

FIG. 3. Twelve percent polyacrylamide gels (containing 7 M urea) of single-stranded DNA fragments from DNase I digestion. (*A* and *B*) Uncrosslinked and crosslinked core particles, respectively, at equivalent amounts of digestion. (*C* and *D*) Crosslinked histone octamer associated with 140 base-pair DNA and crosslinked core particles, respectively, at equivalent amounts of digestion. Sample loads were unequal.

DNA Can Be Separated from the Crosslinked Octamer by Using Nondenaturing Conditions. DNA was completely removed from the octameric protein core of crosslinked core particles by extraction with 3 M NaCl/10 mM sodium phosphate, pH 7.0. The protein was recovered in highly purified form with a yield of about 85%. Thus, the chemical modification did not lead to protein–nucleic acid crosslinks or to entrapment of the DNA under crosslinked lattices. The purified octamer was homogeneous on analytical centrifugation in 0.1 M NaCl/5 mM sodium phosphate, pH 7.0, at a concentration of 0.5 mg/ml. $s^0_{20,w}$ in this solvent was approximately 5.3. A more detailed characterization of the crosslinked octamer will be published elsewhere.

The Crosslinked Octameric Core Protein Folds DNA into Nucleosome-Like Structures. It was of interest to determine whether the crosslinked octamer could fold DNA into a conformation like that present in native core particles. Of particular interest was whether or not denaturants such as urea are required to disrupt protein–protein interactions to allow reassociation. Purified DNA extracted from nucleosomes was mixed with a stoichiometric amount of octamer as described in *Materials and Methods*. The annealing mixture was then fractionated by sucrose gradient centrifugation to remove the serum albumin added to the mixture and aggregated material. Greater than 60% of the material sedimented as an 11S fraction which resembled closely the parent crosslinked core particles.

The same criteria as used above for comparison of the properties of native and crosslinked core particles indicated that the annealing generates a nucleosome-like structure. $s^0_{20,w}$ determined in 0.1 M NaCl/1 mM Tris·HCl, pH 8.0, was 10.9 ± 0.2. The melting curve and circular dichroism spectrum of the reconstituted particles resembled those of the crosslinked core particles closely and differed strikingly from those for protein-free DNA (Fig. 4). The slightly greater ellipticity at 280 nm for reconstituted crosslinked particles compared to native nucleosomes and the slightly increased lower melting transition likely result from more length heterogeneity in the DNA sample

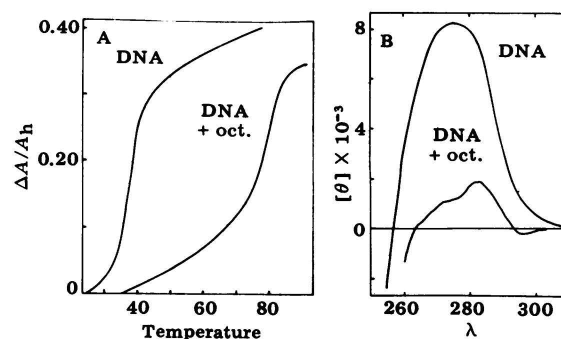

FIG. 4. Thermal denaturation curves and circular dichroism spectra for 140 base-pair DNA associated with the crosslinked histone octamer (OCT.) compared to protein free 140 base pair DNA. Samples were in 0.25 mM Na_2EDTA, pH 7.0. (*A*) Melting curves. ΔA is the increase in absorbance over the value at 20°, A_h. (*B*) Circular dichroism spectra. Units of $[\theta]$ are deg cm^2/dmol PO_4; wavelength, λ, in nm.

used for reconstitution. Core particle preparations vary to some extent, and the preparation from which the DNA was extracted had optical properties similar to those shown for the reconstituted crosslinked sample. Serum albumin in amounts contaminating the reconstituted samples did not affect the optical properties of DNA. Finally, at equivalent levels of digestion with DNase I, the single-strand DNA fragment size distribution for crosslinked core particles before dissociation was closely similar to that for the reassociated octamer (Fig. 3).

The Crosslinked Octameric Core Protein Core Imposes Torsional Constraints on Closed Circular DNA. It is known that nucleosome formation with closed circular DNA leads to the topological equivalent of unwinding the DNA by approximately one turn per nucleosome formed (10). Bina-Stein and Simpson (24) have shown that the H3, H4 tetramer alone introduces similar torsional constraints in closed circular DNA. We found that the nondenatured, crosslinked, octameric histone complex leads to the same torsional constraints when associated with SV40 DNA. Under nondenaturing conditions, crosslinked octamer was associated with superhelical SV40 DNA (DNA I) and relaxed closed circular SV40 DNA (DNA Ir). The resulting complexes were treated with relaxing extract from LA9 cells. This activity relaxes superhelical turns present in the regions of DNA not bound by proteins (10). After deproteinization of the complexes, the DNA was analyzed by agarose gel electrophoresis. In this system, each successive band represents a DNA species differing from its neighbor by one superhelical turn, the more superhelical species having the higher mobility (22). A distribution of bands, initially Gaussian, is generally observed due to thermal fluctuations in the DNA conformation at the time of closure (28, 29).

If torsional constraints are introduced into the closed circular DNA upon complex formation with the octameric histone, removal of these constraints by deproteinization *after relaxation* will result in DNA with negative superhelical turns for both DNA I and DNA Ir. Fig. 5 shows the results of such an experiment for association of the crosslinked octamer with DNA I and DNA Ir at protein/DNA mass ratios of 0.0, 0.4, 0.8, 1.0, and 1.2. In both cases the band distribution was shifted toward greater mobility, clearly demonstrating that the octamer imposes torsional constraints upon the DNA. A significant shift was observed for protein/DNA mass ratios less than 1.0 for DNA I, suggesting that most of the total added octamer is effective in influencing the torsional properties of the DNA. A greater shift in the band distribution for a given protein/DNA ratio was observed for DNA I than for DNA Ir because a decrease in the

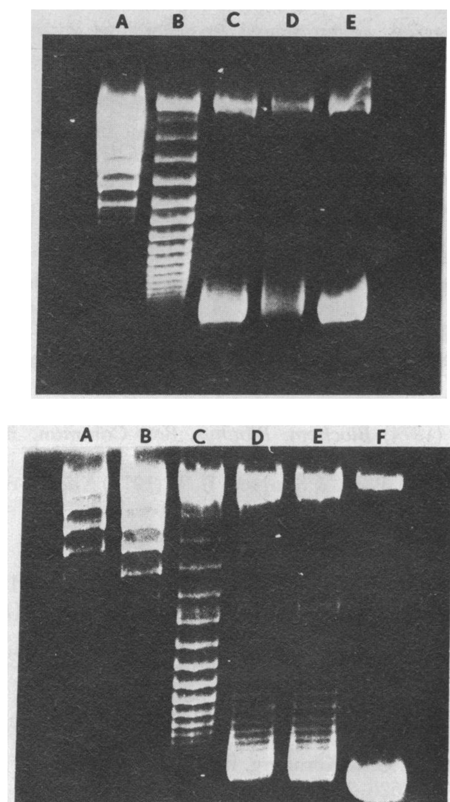

FIG. 5. Agarose gel electrophoresis of SV40 DNA associated with the crosslinked histone octamer and treated with relaxing extract. (*Upper*) SV40 DNA I. Channels A–E correspond to octamer/DNA I ratios of 0.0, 0.4, 0.8, 1.0, and 1.2 (wt/wt), respectively. Electrophoresis was from top to bottom. (*Lower*) SV40 DNA Ir. Channel A is the starting material, DNA Ir; channels B–D correspond to octamer/DNA Ir ratios of 0.0, 0.4, 0.8, and 1.0 (wt/wt), respectively. Channel F is a control DNA I marker. Electrophoresis was from top to bottom.

number of negative superhelical turns is energetically favored compared to the introduction of positive superhelical turns (10, 30).

The Crosslinked Octamer-SV40 DNA Complex Is Morphologically Similar to the SV40 Minichromosome. Fig. 6 compares the crosslinked histone octamer associated with SV40 DNA I to protein-free SV40 DNA Ir, protein-free SV40 DNA I, and uncrosslinked salt-extracted histones associated with SV40 DNA I. The morphological characteristics of the DNA–octamer complex were nearly identical to those of DNA I reconstituted with uncrosslinked histones or of the native SV40 minichromosome at the same ionic strength (10, 11). SV40 DNA I reconstituted with the crosslinked octamer at a protein/DNA ratio of 1.2 contained 21 ± 3 beads and appeared to be relaxed and contracted 2.75 ± 0.25 times in overall contour length (Fig. 6*A* and *D*). The size of the beads depends upon the extent of shadowing. The average diameter of the histone octamer associated with 140 base-pair length DNA and not shadowed is 85 ± 15 Å (data not shown). These measurements imply approximately 175 base pairs of DNA within the beads for both the crosslinked and uncrosslinked histone–DNA complexes (24).

The morphology of the histone–SV40 complexes has been studied by Germond *et al.* (10) and Bellard *et al.* (11). Bellard *et al.* (11) reported 190–200 base pairs of DNA within nucleosomes, 120 Å in diameter, for shadowed SV40 minichromosomes. The value obtained for the diameter of nucleosomes and of the octamer–140 base-pair DNA reconstituted complex, 85

FIG. 6. Electron micrographs. (*A*) SV40 DNA Ir, (*B*) SV40 DNA I. (*C*) SV40 DNA I associated with H2A, H2B, H3, and H4. (*D*) SV40 DNA I associated with the crosslinked histone octamer. (Bar indicates 0.2 μm.)

± 15 Å, is consistent with the value reported by Olins *et al.* (18) for native core particles.

DISCUSSION

A number of implications follow directly from the results showing that is possible (*i*) to crosslink the histones of isolated core particles into an octamer without significant structural pertubations, (*ii*) to remove the DNA from this protein under mild, nondenaturing conditions, and (*iii*) to reconstitute the nucleosome from isolated DNA and the crosslinked protein core, again using nondenaturing conditions.

1. The crosslinked core particle serves as a suitable control to eliminate the possibility that the characteristic chromatin DNase I digestion pattern might arise from histone rearrangement during digestion.

2. Although the model for nucleosome structure in which DNA is wound about the outside of a protein core is generally accepted, the evidence for it is rather circumstantial. Neutron scattering results indicate that the radius of gyration for the DNA is greater than that for the protein (4, 5). DNase I digests in which DNA is nicked at sites separated by 10 nucleotides (9, 21) may reflect the periodicity of the DNA backbone lying on a surface. Neither of these approaches can eliminate the possibility that a portion of the DNA is buried inside the protein core of the chromatin core particles. If the histones in the crosslinked core particle are rather tightly held together by a sufficient number of crosslinks, the observations reported here—that the DNA is extractable by simply increasing the ionic strength and that reconstitution occurs under nondenaturing conditions—would make it unlikely that there is any portion of the DNA buried inside the protein core of the nucleosome. How tightly the histones are fixed in crosslinked particles will be presented elsewhere.

3. The results imply that the mechanism of DNA compaction by histones does not necessarily arise from histone distribution along the whole length of DNA to be compacted, with subsequent folding driven by histone–histone interactions. Because the persistence length of DNA is about 660 Å (31), DNA 140 base pairs in length with a contour length of 476 Å is a stiff chain. The root mean square end-to-end distance in solution is calculated to be approximately 425 Å (32). The crosslinked histone octamer cannot span a very large fraction of this extended DNA length because the histones were fixed together while in a particle roughly 85 Å in diameter, in which the radius of gyration of the protein component is about 30 Å (5). Yet, this single protein can still effect a roughly 5- to 7-fold contraction in the length of the DNA.

An understanding of the mechanism of DNA folding around the preformed octamer should provide useful information on both histone–DNA interactions and the flexibility of DNA. Information necessary includes the activation energy of DNA folding, the number of DNA binding sites on the histone octamer, and the conformational change of very short lengths of double-stranded DNA upon interaction with the octamer. One can, however, imagine several possible mechanisms for DNA folding around a preformed histone core. The torsional constraints imposed on closed circular SV40 DNA by the preformed octamer (Fig. 5), together with the decrease of the DNA contour length visualized in Fig. 6, are most simply explained by attributing enough flexibility to the DNA backbone to allow a looping about the protein core one or more times. If this explanation is correct, it may be that: (*i*) DNA is more flexible than the putative worm-like chain model indicates (31); or (*ii*) upon contact with the octamer, regions of the DNA backbone are strongly perturbed so that it can collapse around the core; or (*iii*) a low-energy flexibility mode operative over short stretches of the DNA backbone may exist for DNA in solution, allowing the backbone to kink around the core as recently suggested by Crick and Klug (33) and by Sobell *et al.* (34); or (*iv*) loops in the DNA backbone resulting from thermal fluctuations tighten around the protein core in some fashion. This last possibility would apply to high molecular weight DNA or circular DNA for which the folding mechanism could differ from that of 140 base-pair DNA. The 140 base-pair–octamer complex formation could possibly be faciliated by rotational diffusion during the reconstitution process.

A number of nontrivial experimental difficulties in studying the solution physical chemistry of histone–DNA interactions occurring in the nucleosome can be circumvented by using this crosslinked octamer rather than purified histones as the protein component. The crosslinked octamer serves as a realistic model for the nucleosome protein core. Proper histone stoichiometry is ensured and the spatial relationships of the various histones are likely to be close to those that exist in the native nucleosome. In experiments designed to study the interactions between histones and DNA shorter than 140 base pairs, for example, aggregation and nonspecific complex formation are highly probable if individual histones are used. Addition of urea would be expected to minimize aggregation but increase nonspecific complex formation. Because the crosslinked octamer can be reconstituted with 140 base-pair DNA in the presence of 6 M urea (A. Stein, unpublished observations), it can be a highly useful reagent for experiments of this nature. Also, in kinetic studies designed to measure the energy required for folding DNA around the nucleosome protein core, the number of possible kinetic mechanisms that must be considered is substantially decreased when a preformed octamer of histones is used as the protein reagent. Finally, the structure and physical properties of the crosslinked histone core of the nucleosome can be studied as a single protein.

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