Nucleosome Core Particle Self-Assembly Kinetics and Stability at Physiological Ionic Strength†

Pilar Diaz and Joan-Ramon Daban*

Departament de Bioquimica i Biologia Molecular, Facultat de Ciències, Universitat Autònoma de Barcelona, Bellaterra (Barcelona), Spain

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ABSTRACT: Micrococcal nuclease, DNase I, and trypsin have been employed to study the kinetics of core particle self-assembly by salt jump from 2.0 to 0.2 M NaCl. A few seconds after the initiation of the reassociation reaction, the bulk of core particle DNA becomes protected from digestion by micrococcal nuclease, whereas free DNA, under the same conditions, is completely hydrolyzed. The central and C-terminal regions of core histones are also protected from trypsin digestion immediately after the 2.0–0.2 M NaCl salt jump. Moreover, the extent of degradation produced by trypsin is the same for samples digested a few seconds after the salt jump and for samples digested 20 min after the salt jump. With DNase I, minor structural differences have been detected between samples obtained at different times during the reaction. However, even in this case our results indicate that many of the characteristic histone–DNA contacts within the core particle are made a few seconds after the initiation of the self-assembly reaction. Furthermore, core particles have been labeled with the fluorescent reagent *N*-(1-pyrenyl)maleimide (NPM), which was previously used as a sensitive probe for nucleosome conformation. Extensive DNase I or trypsin digestion of NPM-labeled core particles in 0.2 M NaCl does not produce significant changes in excimer fluorescence. This allows us to conclude that the covalent continuity of DNA is not required for the maintenance of the folded conformation of the core particle and that the trypsin-resistant domains of core histones play a fundamental role in the stabilization of this structure.

The basic structural features of the nucleosome are well established (McGhee & Felsenfeld, 1980a). Recently, the results of X-ray diffraction studies of crystals of both nucleosome core particles (Richmond et al., 1984) and isolated histone octamers (Burlingame et al., 1985) have been reported. Although there are discrepancies between the structures proposed in these two reports (Klug et al., 1985; Moudrianakis et al., 1985), these results and those obtained in histone–DNA cross-linking studies (Shick et al., 1980) afford a detailed knowledge of the three-dimensional structure of the nucleosome core particle. Nevertheless, in addition, direct information about the forces that stabilize this structure and about its dynamics in solution, under physiological conditions, is necessary for an understanding of the physical basis of the cellular function of the nucleosome.

Core particles can be reconstituted in vitro from histones and DNA, at physiological ionic strength, in the absence of other factors (Ruiz-Carrillo et al., 1979; Stein et al., 1979). In principle, these results allow the investigation of the kinetics of this self-assembly process. Obviously, such an investigation can be useful in understanding the mechanism of nucleosome formation during the replication of eukaryotic chromosomes. However, the kinetics of the core particle self-assembly reaction have been difficult to study due to methodological problems (Daban & Cantor, 1982a). Direct kinetic data of this self-assembly process at physiological ionic strength have been obtained previously with the fluorescent label *N*-(1-pyrenyl)maleimide (NPM)[1] (Daban & Cantor, 1982a,b) and circular dichroism spectroscopy (Erard et al., 1982). Here we report the kinetic results obtained using various enzymes (micrococcal nuclease, DNase I, and trypsin) as structural probes for the core particle self-assembly reaction in 0.2 M NaCl. Moreover, taking advantage of the fluorescent properties of the NPM label, we have studied the role of histones and DNA in the stabilization of the nucleosome core particle.

Materials and Methods

Preparation of Nucleosome Core Particles, DNA, and Histones. Nucleosomes were parpared from chicken erythrocyte nuclei by digestion with micrococcal nuclease (Lacy & Axel, 1975). The digested nuclei were suspended in 0.2 mM Na_2EDTA and centrifuged for 20 min at 58 000 rpm in a Beckman SW60 rotor. The resulting supernatant was fractionated by sedimentation on a 5–20% linear sucrose gradient. The mononucleosome peak was further purified by treatment with 0.1 M KCl (Olins et al., 1976) for 3–4 h, followed by centrifugation at 17000*g* for 15 min. The soluble particles obtained by this method are completely free of histones H1 and H5 and contain the four core histones and DNA fragments from about 145 bp to about 170 bp. For DNase I digestion experiments carried out with ^{32}P-labeled core particles, a more homogeneous preparation of nucleosome cores was used. In this case, isolated chicken erythrocyte nuclei were lightly digested with micrococcal nuclease, and the resulting long chromatin was solubilized in 40 mM NaCl and depleted of very lysine-rich histones following the method of Ruiz-Carrillo et al. (1980). The H1–H5-depleted chromatin was further digested with micrococcal nuclease under conditions

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* Address correspondence to this author.

[1] Abbreviations: bp, base pair(s); Na_2EDTA, disodium ethylenediaminetetraacetate; $NaDodSO_4$, sodium dodecyl sulfate; NPM, *N*-(1-pyrenyl)maleimide; PMSF, phenylmethanesulfonyl fluoride; Tris, tris(hydroxymethyl)aminomethane; Tricine, *N*-[tris(hydroxymethyl)methyl]-glycine.

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similar to those of Lutter (1978). The digest was centrifuged for 20 min at 58 000 rpm in a Beckman SW60 rotor, and the supernatant was fractionated by sedimentation on a sucrose gradient and treatment with 0.1 M KCl as described above. The resulting core particles were finally dialyzed against 2 mM Na_2EDTA and 10 mM Tris-HCl, pH 7.0. The DNA concentration of nucleosome solutions was calculated by using $A_{260nm} = 20.0$ for a solution of 1.0 mg/mL and by assuming that the absorbance of histones at 260 nm is negligible.

Nucleosomal DNA was prepared from core particles as described previously (Daban & Cantor, 1982a). Calf thymus DNA (Sigma, type I) was purified by treatment with proteinase K followed by phenol–chloroform extraction. The resulting high molecular weight DNA was fragmentated by sonication. The DNA fragments obtained by this procedure show a wide range of sizes with a mean length of around 900 bp as estimated by gel electrophoresis analysis.

Total histones were prepared from chicken erythrocyte chromatin by extraction with 0.25 M HCl (Johns, 1964). Core histones were separated from very lysine-rich histones by precipitation with 0.5 M $HClO_4$ (Oliver et al., 1972). Histone solutions were prepared as described previously (Daban & Cantor, 1982b).

Kinetic Studies. (*1*) *Trypsin Digestions.* The appropriate conditions for the rapid digestion of core histones with trypsin (Sigma, type IX) were determined in experiments in which the concentration of enzyme and the time of digestion were varied widely. In order to compare the results obtained in trypsin digestions of reconstituted core particles performed in 0.2 M NaCl, 0.2 mM Na_2EDTA, and 10 mM Tris-HCl, pH 7.0, with those obtained in the same buffer but in the presence of 0.6 M NaCl, the activity of this enzyme in 0.2 or 0.6 M NaCl was determined with *N*-benzoyl-L-arginine ethyl ester as substrate (Schwert & Takenaka, 1955).

For kinetic studies, the reassociation of core histones and DNA was carried out by salt jump from 2.0 to 0.2 M NaCl at 20 °C. For each sample of a kinetic experiment, 0.9 mL of a buffer containing 2 mM Na_2EDTA and 10 mM Tris-HCl, pH 7.0, was added quickly to 0.1 mL of a solution of core histones (100 μg/mL) and DNA (~900 bp, 100 μg/mL) or core particles (200 μg of DNA/mL) in the same buffer but containing, in addition, 2 M NaCl. Mixing was by vortexing immediately after the addition of the dilution buffer. At the indicated times after the salt jump (see legend of Figure 1), 100 μL of a concentrated solution of trypsin (generally 55 μg/mL) was added, and the sample was mixed immediately by agitation in a vortex shaker. The time of digestion (5–30 s) was the same for all the samples of each kinetic experiment. Control samples in which DNA or trypsin was omitted were prepared following the same procedure, but with the addition of blank solutions so that the concentrations of all the other components were identical in all the samples. The digestion was stopped by the quick addition of trichloroacetic acid to a final concentration of 21% and cooling on ice. Finally, samples were washed with acetone, dried under vacuum, and analyzed on 15% polyacrylamide–$NaDodSO_4$ gels with a 6% polyacrylamide stacking gel (Laemmli, 1970). Slab gels were stained with Coomassie blue and scanned with a Beckman DU-8B densitometer.

(*2*) *Micrococcal Nuclease Digestions.* A procedure similar to that described for trypsin in the preceding section was followed in kinetic studies of core particle self-assembly performed at 20 °C using micrococcal nuclease as structural probe. The salt jump from 2.0 to 0.2 M NaCl was carried out by the rapid addition of 0.45 mL of 10 mM Tris-HCl, pH 7.0, to 50 μL of core particles (100 μg of DNA/mL) in 2.0 M NaCl, 2 mM Na_2EDTA, and 10 mM Tris-HCl, pH 7.0. At the specified times after the salt-jump (see legend of Figure 3), 80 μL of a concentrated solution of micrococcal nuclease (Sigma) in water containing 6 mM $CaCl_2$ was added to the core particle sample. After 1 min of digestion, the reaction was terminated by the quick addition of Na_2EDTA to 10 mM and 1 volume of phenol (saturated with 0.2 M NaCl, 11 mM Na_2 EDTA, and 55 mM Tris-HCl, pH 8.0), followed by vortexing and cooling on ice. Carrier DNA (25 μg of high molecular weight DNA from calf thymus) was added to each sample, and the DNA fragments were deproteinized 3 times with phenol, precipitated and washed with ethanol, and analyzed on 8% polyacrylamide gels (Maniatis et al., 1975). After being stained with ethidium bromide, gels were photographed under UV illumination. Negatives were scanned with a Beckman DU-8B densitometer. Control samples containing free core particle DNA were salt-jumped and digested as indicated above for reconstituted core particles. As a reference, native core particles in 0.2 M NaCl, 0.2 mM Na_2EDTA, and 10 mM Tris-HCl, pH 7.0, were digested following the same procedure.

(*3*) *DNase I Digestions.* In this case samples containing 50 μL of core particles (200 μg of DNA/mL) in 2.0 M NaCl were salt-jumped to 0.2 M NaCl by dilution as indicated in the preceding section. The digestion with DNase I (Sigma, type II) was initiated, at the specified times after the salt jump (see legend of Figure 4), by the quick addition of 100 μL of a concentrated solution of this enzyme in water containing 60 mM $MgCl_2$. All the samples were digested for 5 s, and finally, the reaction was stopped by the addition of Na_2EDTA to 40 mM and 1 volume of phenol and cooling on ice. Samples were deproteinized with phenol in the presence of carrier DNA, precipitated and washed with ethanol, denatured with deionized formamide, and analyzed on 12% polyacrylamide gels in the presence of 7 M urea (Maniatis & Efstratiadis, 1980).

In some experiments, core particle preparations obtained by digestion of soluble chromatin fragments depleted of histones H1 and H5 as described above were labeled with ^{32}P at the 5′ ends of the DNA (Noll, 1977; Lutter, 1978). Core particles were dialyzed extensively against 3.8 mM dithiothreitol and 50 mM Tris-HCl, pH 7.5, concentrated to approximately 2 mg of DNA/mL by ultrafiltration on a Centricon-10 microconcentrator (Amicon), and incubated for 3–4 h at 25 °C with [γ-^{32}P]ATP (0.9 Ci/mmol; 0.29 nmol/μg of DNA) and polynucleotide kinase (Cultek, 0.06 unit/μg of DNA) in the presence of 10 mM $MgCl_2$. Labeled core particles were separated from unreacted ATP by Sephadex G-50 column chromatography (0.8 × 25cm), eluted with 2 mM Na_2EDTA and 10 mM Tris-HCl, pH 7.0. Fractions corresponding to core particles were pooled, diluted with 0.1 volume of unlabeled core particles (2 mg of DNA/mL), and dialyzed extensively against 2 M NaCl, 2 mM Na_2EDTA, and 10 mM Tris-HCl, pH 7.0. After reassociation by salt jump from 2.0 to 0.2 M NaCl and digestion with DNase I under the same conditions as indicated above for unlabeled samples, DNA fragments were deproteinized and analyzed on 12% polyacrylamide slab gels (30 cm long) under denaturing conditions. Gels were dried and autoradiographed by using MAFE RPX1 X-ray film and an intensifying screen (3–4 days of exposure at −80 °C). Autoradiographs were scanned with a Beckman DU-8B densitometer. As a reference, ^{32}P-labeled native core particles in 0.2 M NaCl, 0.2 mM Na_2EDTA, and 10 mM Tris-HCl, pH 7.0, were digested and analyzed following the same procedure.

Fluorescence Studies. Fluorescence measurements were performed on a Perkin-Elmer 650-40 spectrofluorometer interfaced to a Perkin-Elmer 3600 data station. The temperature in the sample compartment was maintained at 20 °C with a Heto CB7 circulating water bath attached to the cell holder. The excitation wavelength was 340 nm. The excimer fluorescence was measured at 460 nm (Daban & Cantor, 1982a).

Fluorescent labeling of nucleosome core particles with the sulfhydryl-specific reagent NPM (Molecular Probes) was carried out essentially as described previously (Daban & Cantor, 1982a). After extensive dialysis against a buffer containing 2 M NaCl, 2 mM Na_2EDTA, and 10 mM Tricine, pH 7.2 (adjusted with NaOH), core particles (200 μg of DNA/mL) were reacted at room temperature for 2–3 h with a 2-fold molar excess of NPM (dissolved at a concentration of 0.54 mg/mL in dimethyl sulfoxide). The extent of labeling was estimated from the degree of conversion of monomer to excimer emission, assuming a random assortment of NPM-labeled and unlabeled H3 (Daban & Cantor, 1982a). This represents an underestimate of the extent of labeling because the yield of reconstitution is lower than 100%, and consequently, not all the core particles containing two NPM labels can produce excimers. In all of the experiments carried out as described below, samples were labeled and used the same day.

To study the effect of DNase I on nucleosome structure, NPM-labeled core particles in 2 M NaCl, 2 mM Na_2EDTA, and 10 mM Tricine, pH 7.2, were reconstituted by salt jump to 0.2 M NaCl as described above, except that Tricine was used instead of Tris in the dilution buffer. After incubation for 90 min at 20 °C, labeled core particles (20 μg of DNA/mL) were digested with different concentrations of DNase I in the presence of 10 mM $MgCl_2$. At the indicated times during the digestion (see Figure 5), aliquots were withdrawn, the fluorescence spectrum of each was obtained immediately, and finally, the reaction was stopped by the addition of Na_2EDTA to 40 mM and 1 volume of phenol and cooling on ice. Deproteinization and electrophoresis under denaturing conditions were carried out as described above.

In control experiments, core particles were radioactively labeled at the 5′ ends of the DNA as described in the preceding section. After extensive dialysis against 2 M NaCl, 2 mM Na_2EDTA, and 10 mM Tricine, pH 7.2, ^{32}P-labeled core particles were reacted with NPM under the conditions indicated above. The resulting double-labeled core particles were salt-jumped to 0.2 M NaCl, incubated for 90 min at 20 °C, and finally digested for 5 s with DNase I (60 units/μg of DNA). DNA fragments were analyzed on polyacrylamide gels. Autoradiography was carried out as described in the preceding section.

To study the effect of trypsin digestion on nucleosome structure, fluorescent-labeled core particles (20 μg of DNA/mL), salt jumped from 2.0 to 0.2 M NaCl, were incubated for 20 min at 20 °C and finally digested with 0.5 μg of trypsin/mL. For the fluorescence measurements, aliquots were removed at specified times (see Figure 6) during the digestion. In addition, 1-mL samples were removed at the indicated times and immediately precipitated with 20% trichloroacetic acid, washed with acetone, and analyzed by electrophoresis on a 15% polyacrylamide–$NaDodSO_4$ gel. The fluorescent bands containing NPM-labeled histone H3 or its tryptic peptides were photographed by using a 302-nm transilluminator (Ultra-Violet Products), Polaroid 665 film, and a Wratten 8 gelatin filter (Kodak).

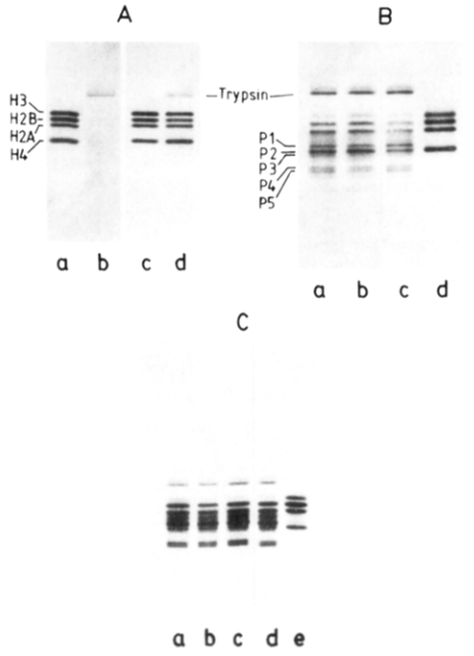

FIGURE 1: Rapid trypsin digestion of reconstituted core particles at different times after the initiation of the reassociation reaction. Tryptic products were analyzed on a 15% polyacrylamide–$NaDodSO_4$ gel. (A) Samples containing core histones and DNA (~900 bp) or histones alone in 2.0 M NaCl were salt-jumped to 0.2 M NaCl, incubated for 20 min at 20 °C, and further digested for 5 s with trypsin (3 μg/mL). Trypsin digestion of core histones (b) and DNA–core histone complex (d). Undigested core histones (a) and DNA–core histone complex (c) are shown as reference. (B) Samples containing core histones and DNA (~900 bp) in 2.0 M NaCl were salt-jumped to 0.2 M NaCl and further digested for 15 s with trypsin (5 μg/mL). Trypsin digestion was started 2 s (a), 5 s (b), or 20 min (c) after the salt jump. (d) Undigested DNA–core histone complex. P1, P2, P3, P4, and P5 denote the limit peptides obtained by trypsin digestion of chromatin. (C) Chicken erythrocyte core particles were salt-jumped from 2.0 to 0.2 M NaCl and further digested for 30 s with trypsin (5 μg/mL). Trypsin digestion was started 4 s (a), 5 s (b), 15 s (c), or 20 min (d) after the salt jump. (e) Undigested reconstituted core particles. The final concentration of core histones and DNA was 10 μg/mL (A and B); the final concentration of core particle DNA was 20 μg/mL (C).

RESULTS

Enzymes as Structural Probes for Studying Core Particle Self-Assembly Kinetics. (1) Trypsin. Digestion of chromatin or isolated nucleosomes with trypsin results in the gradual degradation of histones, accompanied by the simultaneous appearance of relatively stable limit peptides (Weintraub & van Lente, 1974; Böhm & Crane-Robinson, 1984). During the digestion, 11–26 amino acid residues are cleaved from the N-terminal ends of the four core histones, whereas only histones H2A and H3 lose 6–10 amino acid residues from the C-terminal sequences (Böhm et al., 1980, 1981, 1982). The digestion of free core histones in 0.2 M NaCl with relatively high concentrations of trypsin does not show the characteristic pattern of trypsin resistance (Figure 1A, lane b). After 5 s of digestion, core histones are completely degraded. In contrast, DNA–core histone complex treated with trypsin under the same conditions appears to be completely resistant to this enzyme (Figure 1A, lane d). Conditions were optimized so that a partial degradation of core histones complexed with DNA could be achieved with relatively short digestion times (15–30 s). These results allowed us to study the sensitivity of core histones to trypsin at different times after the initiation of the reassociation reaction of core histones with DNA. These studies were carried out with core histones and DNA (~900

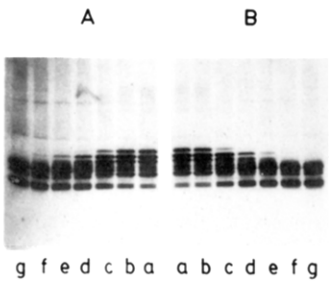

FIGURE 2: Effect of NaCl concentration on core particle sensitivity to trypsin. Time course of trypsin digestion of reconstituted core particles in 0.2 (A) or 0.6 M (B) NaCl. Core particles in 2.0 M NaCl were salt-jumped to 0.2 or 0.6 M NaCl, incubated for 20 min at 20 °C, and further digested with trypsin (0.5 μg/mL). At different times during the digestion, aliquots were withdrawn and analyzed on a 15% polyacrylamide–$NaDodSO_4$ gel. Times of digestion with trypsin were (a–g) 0.5, 1, 5, 15, 30, 60, and 90 min, respectively. The final concentration of core particle DNA was 20 μg/mL. Trypsin activity (measured as described under Materials and Methods) in 0.2 and 0.6 M NaCl was respectively 5.8 ± 0.7 and 6.0 ± 0.7 units/μg. These values correspond to the mean ± 1 standard deviation for five determinations.

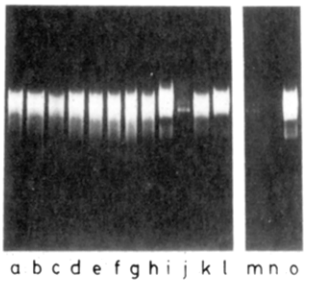

FIGURE 3: Rapid micrococcal nuclease digestion of reconstituted core particles at different times after the initiation of the reassociation reaction. Chicken erythrocyte core particles were salt-jumped from 2.0 to 0.2 M NaCl and further digested for 1 min with micrococcal nuclease (230 units/μg of DNA). Micrococcal nuclease digestion was started 3 s (a), 5 s (b), 15 s (c), 30 s (d), 1 min (e), 5 min (f), 10 min (g), or 20 min (h) after the salt jump. The DNA from the different samples was analyzed on a 8% polyacrylamide gel. (i) The same sample as that in (h) untreated with micrococcal nuclease. Native core particles in 0.2 M NaCl undigested (l) and digested (k) and purified core particle DNA in 0.2M NaCl undigested (o) or digested (n) with micrococcal nuclease as indicated above are shown as reference. The main band in lanes j and m corresponds to 146-bp DNA. The DNA concentration of all samples before micrococcal nuclease digestion was 10 μg/mL.

bp) (Figure 1B) or core particles (Figure 1C). In both cases, reassociation was by salt jump from 2.0 to 0.2 M NaCl.

The densitometric analysis of the gel shown in Figure 1C indicates that the extent of histone degradation produced by trypsin is roughly the same for samples digested a few seconds after the salt jump and for samples digested 20 min after the salt jump. Moreover, all the samples produce the typical trypsin-resistant histone digest (limit peptides P1–P5; Figure 1B, and C). These observations suggest that the interactions that originate the histone resistance to trypsin are produced within the first seconds of the reassociation reaction.

A detailed study of the results shown in Figure 1 indicates that histone H3 is the most sensitive to proteolytic degradation by trypsin. This observation is in agreement with previous reports (Böhm & Crane-Robinson, 1984). On the other hand, the relative trypsin resistance of histone H2A is higher than that observed by others with native core particles at low ionic strength (Lilley & Tatchell, 1977; Whitlock & Simpson, 1977). Nevertheless, according to the study of Harborne and Allan (1983) showing that the relative trypsin resistance of histone H2A increases dramatically when NaCl concentration increases from 5 to 80 mM, the discrepancies considered above could be due exclusively to the relatively high concentration of salt (0.2 M) present in our digestion solutions.

Previous fluorescence studies on the kinetics of core particle self-assembly (Daban & Cantor, 1982a) have shown that the binding of core histones to DNA is a very fast process that takes place before the intramolecular structural transition that yields the final folded structure. This structural transition is also observed when the NaCl concentration of reconstituted core particle solutions decreases from 0.6 to 0.2 M. Nucleosome conformational changes in the same ionic strength region have been demonstrated in other studies using different techniques (Dieterich et al., 1979; Wilhelm & Wilhelm, 1980; Harrington, 1982; Yager & van Holde, 1984). In order to examine whether these conformational changes can be detected using trypsin as structural probe, reconstituted core particles were digested with this enzyme in the presence of 0.2 or 0.6 M NaCl. The results of this experiment, presented in Figure 2, indicate that a change of NaCl concentration from 0.2 to 0.6 M does not produce any significant change in the time course of core particle digestion by trypsin. Taking into account that trypsin activity measured with an artificial substrate is roughly the same in 0.2 and 0.6 M NaCl (see legend of Figure 2), our results indicate that the accessibility of core histones to trypsin attack is not modified by this ionic strength change.

(*2*) *Micrococcal Nuclease.* At 20 °C, under physiological conditions (0.2 M NaCl, pH 7.2), micrococcal nuclease activity is about $^1/_{26}$th of that observed at 37 °C and pH 8.8 in the absence of salt (Diaz & Daban, 1986). However, as shown in Figure 3 (lanes n and o), with high concentrations of this enzyme it is possible to produce in 1 min the complete degradation of free core particle DNA under physiological conditions at 20 °C. This result allowed us to investigate the core particle susceptibility to micrococcal nuclease at different times during the self-assembly process.

The results of this study are presented in Figure 3 (lanes a–h) and indicate that a significant fraction of the initial amount of core particle DNA is protected against nuclease digestion even when micrococcal nuclease is added about 3 s after the initiation of the reassociation reaction. The quantitative estimation of the amount of protected core DNA at different times after the salt jump from 2.0 to 0.2 M NaCl (not shown) indicates that the amount of undigested DNA increases slightly with time (about 11% from 3 s to 20 min). Similar results were obtained in another experiment carried out under similar conditions. By comparison of the densitometry areas of lanes h and i (Figure 3), we have estimated that, 20 min after the salt jump, about 85% of the initial amount of core particle DNA is protected from nuclease digestion. A similar degree of protection is observed when native core particles in 0.2 M NaCl are digested under the same conditions (compare lanes k and l). All these results suggest that the contacts between core histones and DNA that cause the resistance to micrococcal nuclease are made a few seconds after the salt jump from 2.0 to 0.2 M NaCl.

(*3*) *DNase I.* The conformation of DNA in the nucleosome has been investigated in various laboratories with endonucleases such as DNase I (McGhee & Felsenfeld, 1980a). This enzyme produces single-strand nicks in the nucleosomal DNA at aproximately 10-nucleotide intervals (Simpson & Whitlock, 1976; Noll, 1977; Lutter, 1978). Native core particles in 0.2 M NaCl digested with DNase I have electrophoretic patterns in denaturing gels with the characteristic modulation in intensity of the bands observed at lower ionic strength. Under the conditions indicated in Figure 4A, di-

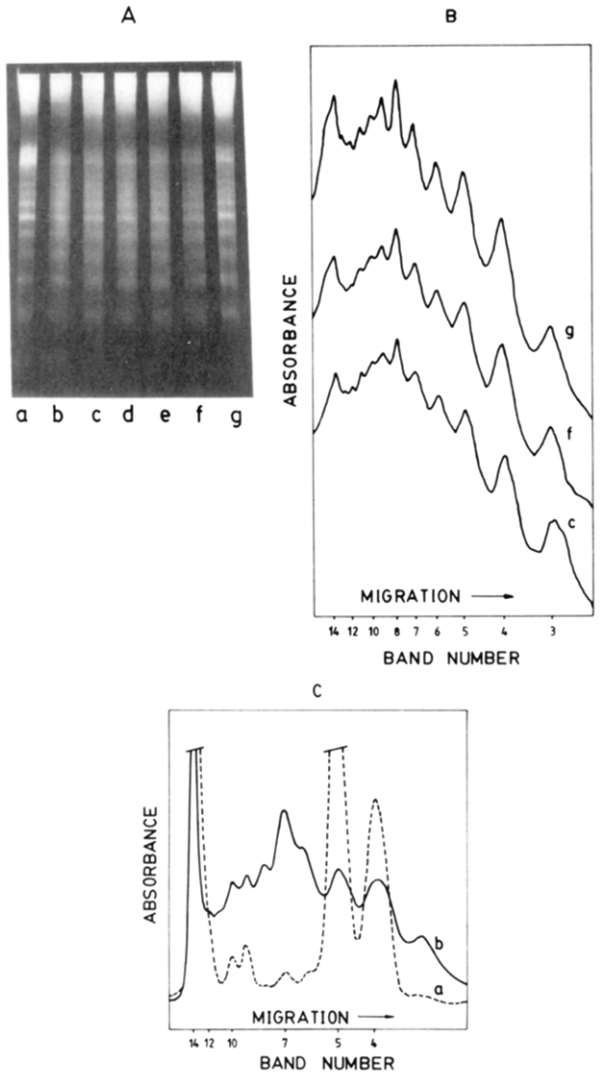

FIGURE 4: Rapid DNase I digestion of reconstituted core particles at different times after the initiation of the reassociation reaction. (A) Chicken erythrocyte core particles were salt-jumped from 2.0 to 0.2 M NaCl and further digested for 5 s with DNase I (40 units/μg of DNA). DNase I digestion was started 2 s (b and c), 5 s (d), 15 s (e), 1 min (f), or 30 min (g) after the salt jump. The DNA from the different samples was analyzed on a 12% polyacrylamide gel in the presence of 7 M urea. (a) Native core particles in 0.2 M NaCl digested with DNase I under the same conditions indicated for reconstituted samples are shown as reference. The broad band at the top of the gel corresponds to carrier DNA added to facilitate the recovery of DNA fragments. (B) Densitometer scans of lanes c, f, and g of the gel shown in (A) stained with ethidium bromide. The high intensity of the slowly migrating bands is partially due to the background produced by carrier DNA. (C) Densitometer scan of the autoradiograph of ^{32}P-labeled core particles digested for 5 s with DNase I (60 units/μg of DNA), 2 s after the salt jump from 2.0 to 0.2 M NaCl (—). The densitometer scan of native core particles in 0.2 M NaCl labeled with ^{32}P and digested with DNase I under the same conditions indicated for reconstituted samples is shown as reference (– –). The DNA concentration of all samples before DNase I digestion was 20 μg/mL.

gestion for 5 s is enough to produce a significant extent of nicking of the DNA of native core particles (lane a). This result made possible the use of DNase I to probe the structure of DNA at different times after the initiation of the reassociation reaction.

Typical kinetic results obtained from such experiments are shown in Figure 4A (lanes b–g). It can be seen that the characteristic banding patterns are obtained even when the brief (5 s) digestion is started about 2 s after the salt jump from 2.0 to 0.2 M NaCl. Densitometer scans (Figure 4B) show that the relative intensity of the 146-bp fragment (band 14) increases with increasing time of reassociation. Furthermore, all the bands corresponding to digests obtained 30 min after the salt jump are better defined than bands of samples digested 2 s after the salt jump. In some experiments, carried out under conditions that produce a higher extent of degradation, we have observed more pronounced differences between the digests obtained a few seconds and 20–30 min after the salt jump.

The above results suggest that during the self-assembly reaction core particles undergo structural transformations that change the susceptibility of DNA to DNase I digestion. However, these results also indicate that the basic structural features of core particles are formed a few seconds after the initiation of the reassociation reaction. In fact, core particles labeled at the 5′ ends of the DNA with ^{32}P and digested with DNase I about 2 s after the salt jump from 2.0 to 0.2 M NaCl produce a banding pattern in the autoradiograph (Figure 4C, curve b) similar to the pattern obtained with samples digested 30 min after the salt jump, but with lower intensity in band 14 (not shown). The autoradiographs of ^{32}P-labeled core particles reconstituted by salt jump and digested with DNase I show a higher background than native core particles (Figure 4C, curve a). As observed for native core particles (Simpson & Whitlock, 1976; Noll, 1977; Lutter, 1978), reconstituted core particles show a relatively low intensity in bands 3 and 11. However, the relative intensity of band 7 is higher than that produced in the case of native core particles. A similar observation was reported previously by Tatchell and van Holde (1979) in a study of core particles reconstituted by stepwise dialysis. Although it is possible that this observation could reflect structural differences between reconstituted and native core particles, the banding pattern in the autoradiograph indicates a nonrandom placement of DNA with respect to core histones.

Stability of Reconstituted Nucleosome Core Particle. Chicken erythrocyte nucleosome cores can be labeled with the fluorescent reagent NPM at position 110 of histone H3 (Zama et al., 1978a; Daban & Cantor, 1982a). This fluorescent label has been employed to investigate conformational changes of nucleosome produced by salt, pH, urea, and organic solvents (Zama et al., 1978a,b; Daban & Cantor, 1982a,b). These previous investigations allowed us to examine the effect of DNase I and trypsin on the conformation of core particles at physiological ionic strength.

Figure 5A shows the electrophoretic analysis of single-stranded DNA extracted from NPM-labeled core particles after digestion with DNase I for increasing times in the presence of 0.2 M NaCl at 20 °C. The banding pattern obtained with NPM-labeled core particles in gels stained with ethidium bromide is equivalent to that obtained with unlabeled core particles. Furthermore, the autoradiograph of ^{32}P-labeled core particles containing NPM-labeled H3 and digested with DNase I (Figure 5B) is similar to that obtained with reconstituted core particles unreacted with NPM (see above). The extent of fluorescent labeling of the sample used in Figure 5B is higher than 88% as estimated from fluorescence spectra (not shown). These results indicate that labeling with NPM does not change the relative exposures of the DNase I accessible sites of DNA in reconstituted core particles.

The fluorescence spectra of NPM-labeled core particles dissociated (in 2.0 M NaCl) or reassociated (in 0.2 M NaCl) are shown in Figure 5C. As described previously (Daban & Cantor, 1982a), NPM-labeled core particles in 0.2 M NaCl have an emission peak (centered at about 460 nm) due to the

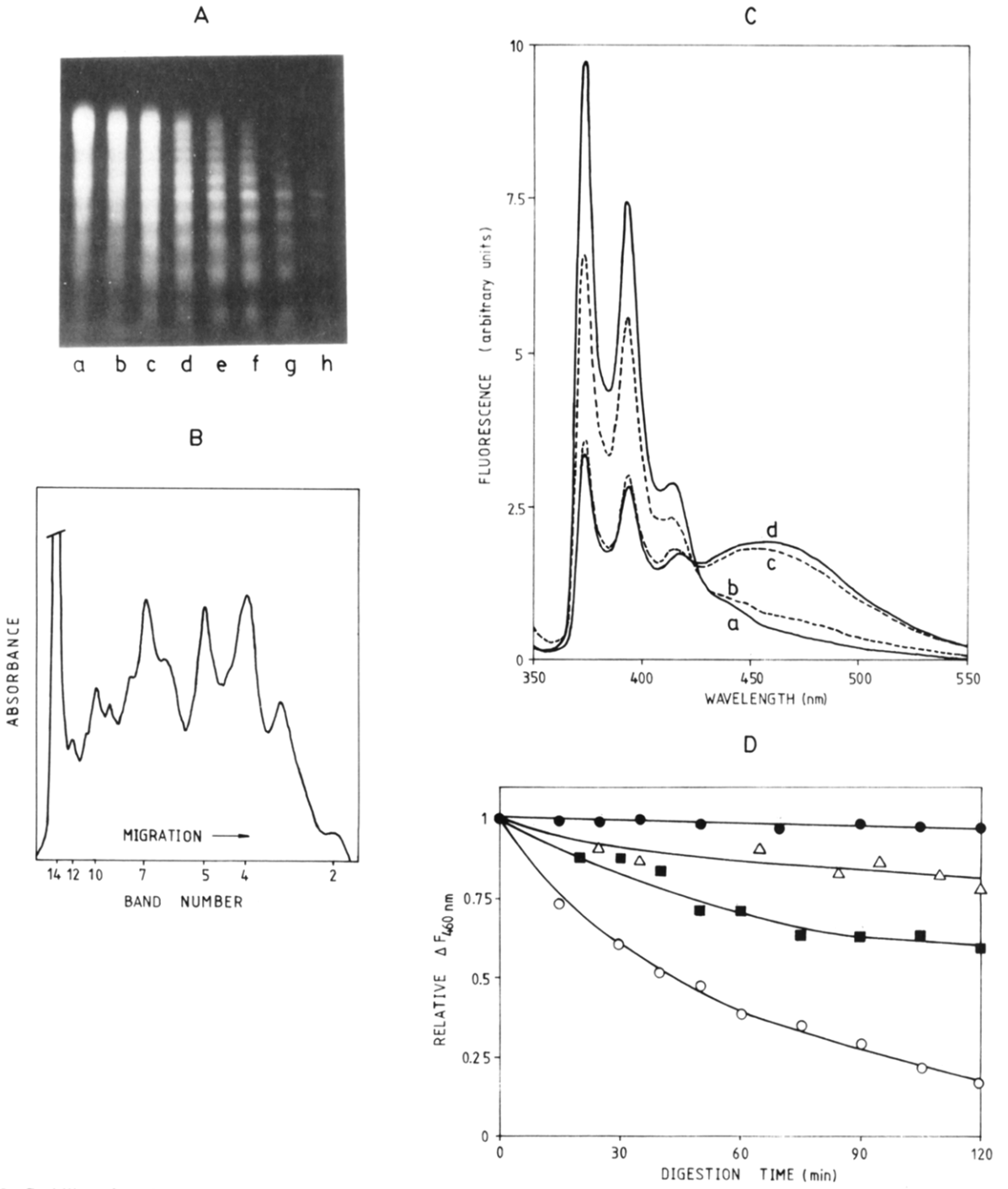

FIGURE 5: Stability of nucleosome core particle. Effect of DNase I digestion on the fluorescent properties of NPM-labeled core particles. Nucleosome cores labeled with NPM in 2.0 M NaCl were salt-jumped to 0.2 M NaCl, incubated for 90 min at 20 °C, and further digested with DNase I. (A) 12% polyacrylamide–7 M urea gel of the DNA fragments obtained at different times during the digestion of NPM-labeled core particles with DNase I (0.6 unit/μg of DNA): (a–h) 5, 10, 15, 30, 45, 60, 90, and 120 min, respectively. (B) Densitometer scan of the autoradiograph of ^{32}P-labeled core particles containing NPM-labeled H3 digested for 5 s with DNase I (60 units/μg of DNA) in 0.2 M NaCl. (C) Uncorrected fluorescence emission spectra of NPM-labeled core particles undigested with DNase I (—), before (a) and after (d) the salt jump from 2.0 M to 0.2 M NaCl. Fluorescence spectra of samples digested with DNase I (– –): the digestion was carried out for 90 min with 0.6 unit/μg of DNA (c) [sample analyzed in lane g of the (A) gel] and for 120 min with 6 units/μg of DNA (b). (D) Intensity of excimer emission of NPM-labeled core particles as a function of time of digestion with different amounts of DNase I in 0.2 M NaCl: 0 (●), 0.6 (△), 1.2 (■), and 6.0 (○) units/μg of DNA, respectively. The DNA concentration of all samples before DNase I digestion was 20 μg/mL.

formation of the excimer form of the pyrene label (spectrum d). The appearance of this peak is accompanied by a marked decrease of pyrene monomer fluorescence in the 370–400-nm region (see spectra d and a). Conparison of spectra c and d shows that NPM-labeled core particles extensively digested with DNase I (lane g of the gel shown in Figure 5A) have fluorescent properties similar to those observed for undigested particles. These results indicate that extensive nicking of DNA does not induce significant conformational changes in nucleosome cores. When higher concentrations of DNase I are used, a significant decrease of excimer fluorescence is observed (spectrum b). As expected, this decrease of fluorescence intensity of the excimer peak produces an increase in monomer fluorescence. A detailed kinetic study on the effect of different concentrations of DNase I on the fluorescence at 460 nm of NPM-labeled core particles is presented in Figure 5D. Rel-

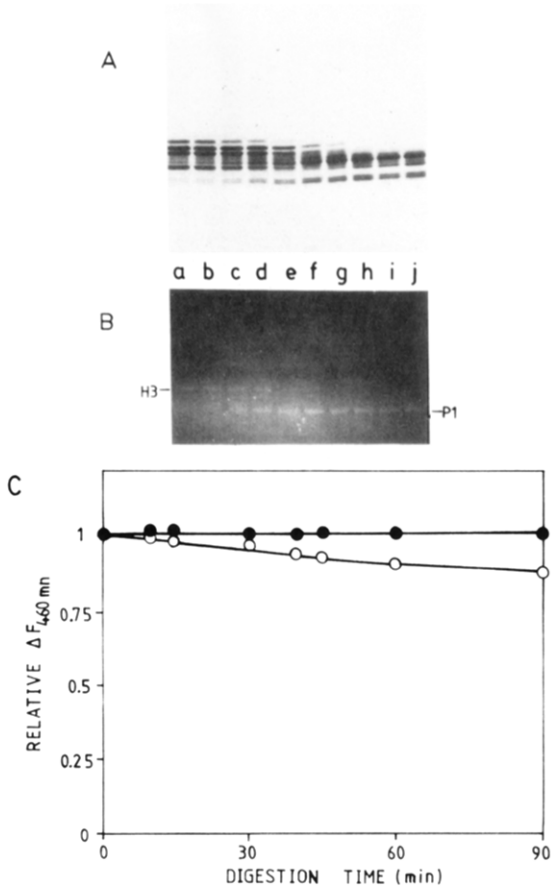

FIGURE 6: Stability of nucleosome core particle. Effect of trypsin digestion on the fluorescent properties of NPM-labeled core particles. Nucleosome cores containing NPM-labeled H3 were salt-jumped from 2.0 to 0.2 M NaCl, incubated for 20 min at 20 °C, and further digested with trypsin (0.5 μg/mL). Tryptic products were analyzed on a 15% polyacrylamide–$NaDodSO_4$ gel (A and B). Times of digestion with trypsin were (a–j) 0.5, 1, 2, 3, 5, 10, 15, 30, 40, and 60 min, respectively. The photograph shown in (B) was obtained by UV transillumination of the gel before staining with Coomassie blue (A). (C) Intensity of excimer emission of NPM-labeled core particles as a function of time of digestion with trypsin (0.5 μg/mL) (○). The fluorescence of undigested core particles is shown as reference (●). The final concentration of core particle DNA was 20 μg/mL.

atively high concentrations of DNase I and long digestion times are required to preclude the formation of excimers.

Figure 6A shows the time course of NPM-labeled core particle digestion by trypsin in 0.2 M NaCl at 20 °C. The histone limit peptides obtained are equivalent to those observed for unlabeled core particles under the same conditions (see above). As expected, taking into account the identity of the histone peptides obtained by trypsin digestion (Böhm et al., 1981), the limit peptide (P1) corresponding to histone H3 contains the NPM label and can be detected by UV transillumination of the gel (Figure 6B). Furthermore, the relative susceptibility observed for the different core histones is similar to that found for unlabeled particles. These results indicate that the covalently bound NPM label does not change the structural features that determine the specific accessibility of histones to trypsin.

As observed above for DNase I, trypsin digestion of NPM-labeled core particles does not produce significant changes in excimer emission intensity (Figure 6C). In fact, NPM-labeled core particles extensively digested with trypsin (lane h in panels A and B of Figure 6) show only about a 5% decrease of fluorescence at 460 nm. Taking into account that the degraded terminal regions produced by trypsin digestion dissociate from the DNA–limit peptide complex (Grigoryev & Krasheninnikov, 1982), our results indicate that the removal of the basic terminal regions of core histones does not induce significant conformational changes in nucleosome core particles.

Discussion

Our kinetic results indicate that a few seconds after the initiation of the reassociation reaction in 0.2 M NaCl the bulk of core particle DNA is bound to the histone core and becomes protected from hydrolysis by micrococcal nuclease. The relatively small fraction of DNA that is degraded in these experiments is similar to that found for native core particles in 0.2 M NaCl. The dissociation of DNA from nucleosomes caused by salt (Cotton & Hamkalo, 1981; Ausió et al., 1984; Yager & van Holde, 1984; A. M. Aragay and J.-R. Daban, unpublished results) could be responsible for this partial degradation.

The kinetic results obtained by using trypsin as a structural probe indicate that in the self-assembly reaction the interactions between DNA and the central and C-terminal regions of core histones are established rapidly. From the results obtained in DNase I digestions, we conclude that the specific histone–DNA contacts that originate the characteristic modulation of DNA fragment frequencies in gels stained with ethidium bromide are essentially made within the first seconds of the reassociation reaction. Furthermore, the relatively well-defined bands seen in the autoradiograph of reconstituted samples, labeled with ^{32}P and digested with DNase I immediately after the salt jump, suggest that the positioning of the DNA ends with respect to histones is also essentially produced a few seconds after the initiation of the reassociation reaction.

All of the above considerations indicate that in the self-assembly reaction many structural features of core particles are rapidly formed. These observations agree with previous findings indicating that the binding of core histones to DNA is a very fast process (Daban & Cantor, 1982a; Erard et al., 1982). In the case of DNase I, our results show minor structural differences between the digests obtained a few seconds and 30 min after the salt jump. The increase of intensity of band 14 observed in these kinetic experiments suggests that the interactions between the histone core and the DNA tails are strengthened during the reaction. With micrococcal nuclease and trypsin, no significant structural changes have been detected. This observation is in contrast with previous kinetic results obtained with the fluorescent label NPM (Daban & Cantor, 1982a,b), which indicate the existence of intermediate structures related to the folding of the core particle that takes place after the initial binding of core histones to DNA. This intramolecular structural change that occurs during the self-assembly reaction is the same as that which is found between 0.6 and 0.2 M NaCl with this fluorescent label (Daban & Cantor, 1982a) and is probably equivalent to the conformational change observed in this ionic strength region by other techniques (Wilhelm & Wilhelm, 1980; Harrington, 1982; Yager & van Holde, 1984). Therefore, the apparent discrepancies considered above suggest that the structural characteristics that determine the resistance to trypsin and micrococcal nuclease do not change during the folding reaction previously reported. In fact, our results showing that the accessibility of core histones to trypsin attack is not modified when NaCl concentration is changed from 0.6 to 0.2 M support this interpretation. These results are not surprising considering that histone–DNA cross-linking experiments (Zayetz et al., 1981) have shown that nucleosome cores can undergo significant conformational transitions and unfolding while the sequential arrangement of histones along

core DNA remains essentially unaffected.

Previous fluorescence quenching and lifetime studies (Daban & Cantor, 1982b) have shown that in 0.2 M NaCl the reconstituted NPM-nucleosome cores that can produce excimers are in a folded conformation in which the two pyrene rings are very close and buried inside the particle. Recently, it has been determined by X-ray diffraction of core particle crystals (Richmond et al., 1984) that the heavy atom cluster tetrakis(acetoxymercuri)methane, which is bound to cysteine-110 of histone H3, lies precisely on the dyad axis between the central turn of the DNA superhelix and the center of the particle. Therefore, the two pyrene rings that produce excimers in 0.2 M NaCl are probably located in this region of the nucleosome core. The dramatic changes of the excimer fluorescence of NPM-labeled core particles produced by different agents that induce structural transitions (Zama et al., 1978a,b; Daban & Cantor, 1982a,b) indicate that NPM is a sensitive probe for nucleosome conformation. Thus, from our results showing that extensive trypsin or DNase I digestion of NPM-labeled core particles does not produce significant changes in excimer fluorescence, we can conclude that neither the N-terminal regions of core histones nor the covalent continuity of DNA is required for the maintenance of the folded conformation of the nucleosome core particle.

The above conclusions suggest that, at physiological ionic strength, core particles adopt a stable conformation in which the folded DNA is entirely relaxed. Alternatively, folding could produce strain in DNA. In this case, however, DNA strain would not induce significant deformations of the core histone structure; otherwise, extensive nicking should have produced significant changes in the fluorescence of the NPM label. Furthermore, the N-terminal regions of histones are not necessary to stabilize this conformation. Thus, the trypsin-resistant domains of core histones appear to be responsible for the core particle folding. This is consistent with the observation that partially trypsinized histones are able to form nucleosome-like particles in reconstitution experiments (Whitlock & Stein, 1978). Asymmetric lateral neutralization of DNA phosphate groups produced by histones may be a significant force for core DNA folding (Mirzabekov & Rich, 1979). The folding process can be driven entropically by counterion release (Bina et al., 1980), even if the number of charge–charge interactions between core histones and DNA is relatively small (McGhee & Felsenfeld, 1980b). Moreover, histone–histone interactions can also provide part of the free energy of stabilization of the folded conformation (Benedict et al., 1984).

The nucleosome represents the first level of DNA packaging in chromatin (McGhee & Felsenfeld, 1980a). As discussed above, the central and C-terminal regions of core histones play a primary role in the stabilization of this level of chromatin condensation. Very lysine-rich histones (Thomas, 1984) and probably the basic N-terminal regions of core histones (Böhm & Crane-Robinson, 1984) are involved in higher order structures. Our findings, together with the observation that the integrity of the condensed chromatin fiber is maintained even when DNA is partially fragmentated (Ruiz-Carrillo et al., 1980; Pérez-Grau et al., 1982; Lasters et al., 1985), stress the fundamental role of histones in organizing the chromatin structure.

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Effects of Hydration on Purine Motion in Solid DNA†

Rolf Brandes, Regitze R. Vold, Robert L. Vold, and David R. Kearns*
Department of Chemistry, University of California at San Diego, La Jolla, California 92093
Received June 12, 1986

ABSTRACT: Deuterium quadrupole echo spectra and spin–lattice relaxation rates measured at 76.8 and 38.4 MHz as a function of relative humidity are reported for calf thymus DNA deuterated at positions A8 and G8. The amplitude of base pair motion is observed to increase slightly with increasing degree of hydration (up to ~20 mol of H_2O/nucleotide), and the onset of motion is associated with a more than 100-fold drop in T_1. This observed decrease in T_1 parallels that observed previously for the phosphate backbone and appears to be characteristic of collective modes of motion. Above ~20 mol of H_2O/nucleotide, the amplitude of the base motion increases substantially up to a point where slow components of motion lead to a complete loss of the quadrupole echo.

The conformational flexibility of polynucleotides in solution has been the subject of numerous studies (Cohen & Eisenberg, 1969; Allison & Schurr, 1979; Barkley & Zimm, 1979; Englander et al., 1980; Hogan & Jardetzky, 1980; Early et al., 1981; Keepers & James, 1982; Levy et al., 1983; Thomas & Schurr, 1983; Kearns, 1984) using several kinds of physicochemical techniques over the last few years, but significant questions about the nature of this internal mobility still remain to be answered. In particular, it remains to be established whether internal motions detected by NMR are "localized" (Bolton & James, 1980; Hogan & Jardetzky, 1980; Assa-Munt et al., 1984) or the result of longer range cooperative torsional and bending modes of the double helix (Allison & Schurr, 1979; Barkley & Zimm, 1979). The extent to which these motions are influenced by metal ions, hydration, or helix-binding agents is also not understood in detail. One difficulty associated with the interpretation of many measurements on DNA solutions is the presence of overall tumbling of the molecule, and accordingly, interest has developed in investigating solid DNA where the nature of internal motions may be more easily explored. Crystallographic measurements (Drew et al., 1980, 1982) have clearly shown that large thermal parameters are associated with atomic coordinates in fibrous and crystalline DNA. Recently, Holbrook and Kim (1984) used a "segmented rigid body" model to determine directions and magnitudes of translational and rotational motion of individual polynucleotide subgroups from single-crystal X-ray diffraction data. They find librational motions of the bases and ribose units with amplitudes up to ±20° and larger motions of the phosphate unit in solid samples of double- and single-stranded polynucleotides.

Solid-state NMR spectra and relaxation rates may be used to evaluate not just the amplitudes, but also the rates of internal motions in nucleic acids. During the last few years, several NMR studies of solid polynucleotides have been published. At present, it appears that ^{31}P (Shindo et al., 1980, 1983; DiVerdi & Opella, 1981; Nall et al., 1981; Opella et al., 1981; Mai et al., 1983; Fujiwara & Shindo, 1985) and ^{2}H (DiVerdi & Opella, 1981; Bendel et al., 1983; James et al., 1983; Vold et al., 1986) are the most readily accessible and informative nuclear spin probes. On the basis of the NMR reports available up to now, it appears that internal motion is quite limited in amplitude in dehydrated A-form DNA (Shindo et al., 1980; DiVerdi & Opella, 1981; Nall et al., 1981; Opella et al., 1981) but that the amplitudes of motion increase with increasing degree of hydration. Early studies (Shindo et al., 1980, 1983; DiVerdi & Opella, 1981; Nall et al., 1981; Opella et al., 1981) have shown that the phosphate backbone of hydrated B-form DNA undergoes rather large angular

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