Urea Denaturation of Chromatin Periodic Structure†

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ABSTRACT: Isolated chicken erythrocyte nuclei dispersed in urea solutions (0–5.0 *M*) have been examined in terms of their low-angle X-ray diffraction and electron microscopic properties. At high urea concentrations, the characteristic low-angle X-ray reflections of chromatin are absent, and the spheroid chromatin particles (ν bodies) are markedly perturbed. This lability of chromatin periodic structure to high concentrations of urea is consistent with previous hydrodynamic and spectroscopic studies.

The determination of the structure of chromatin continues to be a problem of considerable interest and difficulty. Electron microscopy has shown that chromatin is fibrous, but different methods have led to considerable disagreement over the nature of these fibers (Ris and Kubai, 1970; DuPraw, 1970). X-Ray diffraction studies of whole nuclei or isolated chromatin have shown a characteristic series of low-angle X-ray reflections suggesting that there is a periodic structure along chromatin fibers. These X-ray data have been interpreted primarily in terms of a model which has the chromatin fiber constrained into a supercoil as a result of histone–histone and histone–DNA interactions (Pardon and Wilkins, 1972; Pardon et al., 1967). Recently it has been observed that chromatin fibers visualized by electron microscopy appear to consist of globular subunits (denoted ν bodies) occurring along the fibers like beads on a string (Olins and Olins, 1973, 1974; Olins et al., 1975; Woodcock, 1973). Additional biochemical data have also suggested that there are periodic nucleoprotein units in chromatin (Hewish and Burgoyne, 1973; Rill and Van Holde, 1973; Sahasrabuddhe and Van Holde, 1974; Van Holde et al., 1974; Kornberg, 1974; Noll, 1974; Senior et al., 1975). Together these data argue for a new concept of the chromatin fiber; namely, repeating spheroid subunits (the ν bodies) composed of DNA condensed by association with histones.

One way to gain structural information about biological macromolecules is to study conformational perturbations of the native form. It is well known that in solutions of high urea concentration, proteins can reversibly unfold (Anfinsen, 1973). Similar, apparently reversible changes have been observed in the hydrodynamic properties of chromatin (Bartley and Chalkley, 1968; 1973), in its circular dichroic properties (Shih and Lake, 1972; Bartley and Chalkley, 1973; Chang and Li, 1974), and in its thermal melting behavior (Ansevin et al., 1971; Shih and Lake, 1972; Chang and Li, 1974). Electron microscopy has also shown that there are changes in fiber dimensions (Georgiev et al., 1970). We have utilized low-angle X-ray diffraction and electron microscopy to study these conformational changes, with emphasis upon the disruption of chromatin periodic structure.

Materials and Methods

(1) Nuclei. Chicken erythrocyte nuclei were isolated by a method previously described (Olins and Olins, 1972). A detailed analysis of nuclei prepared in this manner is presented elsewhere (Olins et al., 1975). After washing the nuclei in CKM buffer[1] (0.05 *M* sodium cacodylate–0.025 *M* KCl–0.005 *M* $MgCl_2$ (pH 7.5)) and in 0.2 *M* KCl, the nuclei were swollen by diluting them approximately 100-fold with glass-distilled H_2O. To fix samples, formaldehyde was added to give a final concentration of 1% HCHO (pH 7.0, 4°). After fixation, excess HCHO was removed by dialyzing against 0.002 *M* KCl.

(2) Chemicals. Formaldehyde (10%) was prepared fresh from a stock solution of ~37% analytical reagent grade formaldehyde. The pH was adjusted to 7.0 with KOH. Stock urea solutions (~8 *M*) were prepared from crystalline analytical reagent grade urea. Ionic impurities were removed by passing the urea solution through a column of Bio-Rad AG 501-X8 analytical grade mixed-bed resin. The conductivity of each solution was measured and was always as low as for solutions made with commercially available ultrapure urea. The concentrations of deionized urea stock solutions were determined by measuring the index of refraction of each solution (Fasman, 1963). All other chemicals used were analytical reagent grade.

(3) X-Ray. After the nuclei had swelled in distilled H_2O, one sample was fixed overnight in 1% HCHO. Each sample was then brought to the desired urea concentration by adding urea stock solution. To some of the samples containing urea an equal volume of 2XCKM buffer (double-concentration CKM) containing the same concentration of urea was added. An equal volume of 2XCKM buffer without urea was added to each sample in which no urea was originally present.

Each of the above samples was pelleted for X-ray diffraction experiments by centrifuging it at 58,000 rpm for 16–20 hr in a Beckman Type 65 rotor at 4°. A portion of each pellet was pushed into a separate thin-walled glass capillary tube 1 mm in diameter (Uni-Mex Company, Griffith, Ind.). A drop of solvent was added at the top of the capillary so the sample would not dry out, and the capillary was then sealed with sealing wax. After the X-ray experiment was completed, the cross-sectional diameter of the capillary and the length of the sample were measured in a light microscope, the volume was calculated, and the portion of the

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[1] Abbreviation used is: CKM buffer, 0.05 *M* sodium cacodylate–0.025 *M* KCl–0.005 *M* $MgCl_2$ (pH 7.5).

capillary containing the sample was crushed into 6–12 *M* nitric acid. The DNA concentration of the sample was determined by measuring the total phosphate content of this solution (Ames and Dubin, 1960).

Low-angle X-ray diffraction data were collected on film using a Searle X-ray camera with toroid optics (Baird and Tatlock Ltd., England). This camera was mounted on an Elliott GX6 rotating-anode microfocus X-ray generator operated at 40 kV and 40 mA, with a focal spot 2.0 × 0.2 mm. Data were collected on three sheets of Kodak Type T Industrial X-ray film. Exposures were for 2 hr in air at room temperature with a film-to-sample distance of 73 mm. The positions of the reflections were measured by eye using a Supper film-measuring instrument with a movable hairline. These measurements were used in calculating the equivalent Bragg spacings. In general it was necessary to use more than one film to measure the positions because of the intensity differences of the reflections.

(4) Electron Microscopy. Isolated chicken erythrocyte nuclei suspended in 0.2 *M* KCl were diluted 1:200 in 0.001 *M* EDTA (pH 7.0) (Figure 2a and c), or in 5 *M* urea (pH 7.0) (Figure 2b) and allowed to swell for 15 min; 10% HCHO (pH 7.0–7.3) was then added to these swollen nuclei for a final concentration of 1% HCHO. The samples shown in Figure 2a and b were fixed for 30 min; that shown in Figure 2c was fixed overnight at 4°. The nuclei were then centrifuged onto freshly glowed, carbon-coated grids through 10% HCHO (pH 7.0) (Figure 2a); 10% HCHO with 5 *M* urea (pH 7.3) (Figure 2b); and 5 *M* urea–0.001 *M* EDTA (pH 7.0) (Figure 2c). All samples were then washed in dilute Kodak Photo-Flo (pH 7), air-dried, and stained. The negative stain used was 1% sodium phosphotungstate (pH 7.1). Positive staining with ethanolic phosphotungstic acid was accomplished by diluting a stock solution of 4% aqueous phosphotungstic acid with 95% ethanol to yield 0.7% solution. Samples were examined on a Siemens I electron microscope.

Results

Low-angle X-ray diffraction experiments were performed on nuclei in urea–CKM solutions where the urea concentration was 1, 2, 3, 4, and 5 *M*. In urea concentrations of up to 3.0 *M*, the diffraction pattern of the nuclei is virtually indistinguishable from that of nuclei in CKM buffer (Figure 1a). In 4.0 *M* urea (Figure 1b), the reflections are still present and their positions are unchanged, but their intensities are less pronounced compared to the background scatter. Figure 1c shows that in 5.0 *M* urea there is no evidence of the characteristic reflections at ~110, 55, or 37 Å. They are replaced by a diffuse central scatter that gradually falls off in intensity over this angular range. Measurement of the phosphate content of these samples showed that the loss of the reflections was not a result of concentration differences. Similar X-ray data were obtained on nuclei in urea without CKM, although these samples did not pellet as well and the diffraction patterns were less intense. To establish that these data are not due to a submerging of the chromatin diffraction pattern by increased background scattering from urea rather than the result of a conformational change in the chromatin, the following experiment was performed. Swollen nuclei were fixed for 17 hr in 1% HCHO prior to exposure to 5.0 *M* urea. After fixation under these conditions, virtually none of the histones could be extracted by acid, high salt, guanidine hydrochloride, or sodium dodecyl sulfate (Senior and Olins, unpublished data; Brutlag et al., 1969). In addition, we have previously shown that fixation of nuclei with 1% HCHO for up to 24 hr does not alter the low-angle X-ray pattern (Olins et al., 1975). Figure 1d shows that nuclei fixed in H_2O, then adjusted to 5.0 *M* urea–CKM and pelleted, give the normal low-angle X-ray diffraction pattern even though the concentration of this pellet was the lowest measured. This experiment demonstrates two important points: (1) the periodic structure of chromatin fixed with HCHO is stable to the denaturing effects of urea; (2) the background scattering from 5.0 *M* urea is not sufficient to obscure the low-angle reflections from chromatin. We have also observed that the denaturing effects of urea are, to some extent, reversible. When 5.0 *M* urea was removed by dialysis vs. CKM (or vs. 0.002 *M* KCl, and then adjusted to CKM), the X-ray pattern of the pellet was normal.

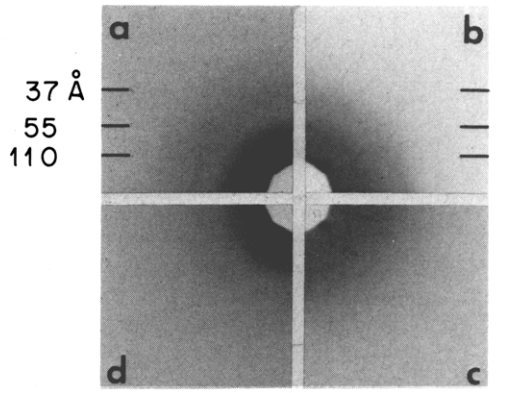

FIGURE 1: Low-angle X-ray diffraction data from nuclear pellets: (a) in CKM; (b) in 4 *M* urea–CKM; (c) in 5 *M* urea–CKM; (d) fixed for 17 hr in 1% HCHO, then made 5 *M* urea–CKM. Diffraction patterns are similar to those previously obtained for nuclear pellets (Olins et al., 1975). Reflections occur at 107 ± 10, 56 ± 3, and 37 ± 2 Å. The first two reflections are seen as edges, while the ~37-Å reflection is a distinct ring in a and b and an edge in c.

Figure 2 presents electron micrographs which correspond to three of the X-ray experiments. Figure 2a shows negatively stained chromatin fibers from nuclei which have not been exposed to urea. Chromatin particles (ν bodies) are observed along the lengths of the fibers. Figure 2b demonstrates the effect of 5.0 *M* urea on these chromatin fibers. In positively stained preparations we visualized fibers of varying diameters exhibiting irregularly shaped areas. After negative staining, these aggregated areas revealed the possible remains of chromatin particles, but with highly distorted size and shape. Figure 2c presents the control experiment, where the nuclei were fixed with HCHO before the solution was adjusted to a final concentration of 5.0 *M* urea. The ν bodies persisted and were virtually indistinguishable from nuclei that were not treated with urea.

Discussion

The results presented in this paper agree with data from other laboratories, which have shown that high concentrations of urea alter the native structure of chromatin. We demonstrate that this structural change involves a loss of periodic structure as measured by low-angle X-ray diffraction and by electron microscopic methods.

As the concentration of urea is increased from 0 to 5 *M* there is a two- to three-fold increase in the viscosity of chromatin solutions (Bartley and Chalkley, 1968; 1973). This increase occurs primarily in the range of 2–4 *M* urea for chromatins from several different sources, leveling off a

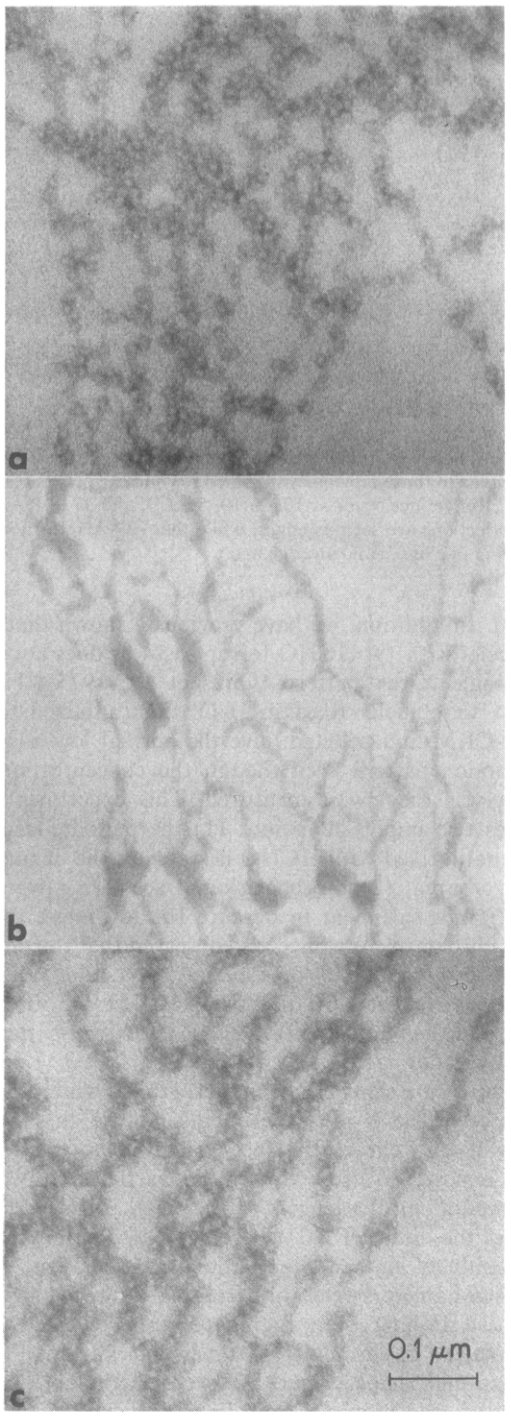

FIGURE 2: Chicken erythrocyte nuclei: (a) fixed in 1 m*M* EDTA, spun through 10% HCHO, and negatively stained; (b) fixed in urea, spun through 10% HCHO in urea, and positively stained; (c) fixed in 1 m*M* EDTA, spun through urea, and negatively stained.

concentration of 5 *M* urea. Data for chicken erythrocyte chromatin show that the major change in viscosity occurs at urea concentrations between 3 and 4 *M*. These authors suggest that the magnitude of the changes in viscosity rule out changes in hydration as a primary cause. Their additional observation that the sedimentation coefficient decreases with increased urea concentration rules out aggregation as the cause of the increased viscosity. These hydrodynamic data have been interpreted (Bartley and Chalkley, 1968; 1973) as indicating that chromatin unfolds to a less compact structure in high concentrations of urea.

Changes have also been observed in the circular dichroism (CD) and thermal denaturation properties of chromatin (Ansevin, 1971; Shih and Lake, 1972; Bartley and Chalkley, 1973; Chang and Li, 1974). Changes in regions of the CD spectrum attributable to protein conformation and to DNA conformation parallel the changes in viscosity, with increasing urea concentration (Bartley and Chalkley, 1973). These data suggest that there is a loss of histone secondary structure which is accompanied by a change to a DNA conformation more like that of free DNA. Thermal denaturation studies of chromatin in concentrated urea solutions have shown that there is a destabilization of the DNA complexed with histones (Chang and Li, 1974).

The conformational changes measured by these methods are all largely reversible. Furthermore, it is clear that they cannot be attributed to a decrease in the amount of bound histone. It has been shown that at these solvent conditions (low ionic strength, neutral pH, high urea concentration) the histones remain bound to the DNA (Bartley and Chalkley, 1968; Shih and Lake, 1972) although there may be some histone migration (Clark and Felsenfeld, 1971). The CD data of Chang and Li (1974) suggest that there is no increase in the fraction of histone-free DNA.

Conformational changes have also been observed by electron microscopy (Georgiev et al., 1970). Using the Kleinschmidt spreading procedure Georgiev and coworkers observed that chromatin dissolved in a solution containing 4 *M* urea appeared to consist of thin fibers with an average fiber diameter of approximately 40 Å.

Our data show that by a concentration of 5 *M* urea, the low-angle X-ray reflections are gone, and electron micrographs no longer reveal clear particulate structure along the fibers. These data are consistent with an unfolding of chromatin to a less compact conformation in high urea solutions. A possible model involves an unfolding of the ν bodies resulting from a disruption of the histone–histone interactions within these particles, probably between the more hydrophobic parts of the histones (Van Holde et al., 1974). As they unfold, the constraints on the folded DNA are lessened, allowing a relaxation to a DNA conformation more like that of free DNA. Weintraub and Van Lente (1974) recently observed that extensive digestion of chicken erythrocyte chromatin with trypsin yielded a mixture of high molecular weight peptides; whereas pretreatment of the chromatin with 6.0 *M* urea, followed by digestion with trypsin, drastically reduced the yield of the large trypsin-resistant peptide fragments. These data suggest that specific histone conformations and histone–histone interactions within chromatin are responsible for the resistance to digestion apparent in these fragments.

Because of the tendency of chromatin to aggregate unless the ionic strength is low, even in the presence of high concentrations of urea, studies of the solution properties of chromatin must be performed at low ionic strength. Likewise, our samples for electron microscopy were fixed at low ionic strength. As was previously mentioned, at these conditions there is no loss of histone from the DNA. For the X-ray experiments, however, samples were adjusted to urea–CKM to facilitate pelleting. While we cannot rule out the possibility that there is some loss of histone in urea–CKM, we believe such loss is not a significant factor contributing to our observations. We have made the same observations when no additional salt was added to the urea solution, although the chromatin did not pellet as well and the X-ray diffraction pattern was much less intense. Addition-

ally Georgiev et al. (1970) have observed that while there is some loss of histone when calf thymus chromatin in 4 *M* urea is precipitated with 0.15 *M* NaCl, there is no loss when it is repeatedly precipitated in 0.05 *M* NaCl. The ionic strength of CKM buffer is intermediate between these values.

The concomitant loss of low-angle X-ray reflections and of the *ν* body structure with increased urea concentration is consistent with the suggestion that these periodicities are related to each other (Kornberg, 1974; Van Holde et al., 1974; Senior et al., 1975; Olins et al., 1975; Carlson and Olins, 1975). In support of the possibility that an ordered array of *ν* bodies may be the source of the X-ray periodicity, we have performed theoretical calculations for different arrays of identical spherical particles of uniform electron density (Carlson and Olins, 1975). The size of these particles is consistent with that expected for hydrated *ν* bodies (Olins and Olins, 1974). These calculations show that certain linear and helical arrays of particles are capable of accounting for the spacings of the characteristic X-ray reflections. It is therefore tempting to speculate that the disappearance of the low-angle reflections, caused by high concentrations of urea, is due in large part to the destruction of an ordered array of *ν* bodies, which is due in turn to the disruption of their internal structure.

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