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H3 Cys-110 Is in Close Proximity to the C-Terminal Regions of H2B and H4 in a Nucleosome Core with an Altered Internal Arrangement of Histones†

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ABSTRACT: A particle obtained by nuclease digestion of nucleohistone complexes prepared by direct mixing of histones with DNA in 0.15 M NaCl was indistinguishable by composition and physical properties from nucleosome cores prepared under the same conditions from nucleohistone preannealed in 0.6 M NaCl. We show here that different photo-cross-links form when these particles are prepared from H3 labeled with photoaffinity reagents on the unique histone H3 cysteine. H3-H3 histone dimers were dominant when the particles were prepared by dilution of the nucleohistone from 0.6 M NaCl while H3-H2B and H3-H4 histone dimers were prominent if the nucleohistone complex was prepared directly in 0.15 M NaCl. Peptide mapping of the novel H3-H4 and H3-H2B dimers showed that Cys-110 of histone H3 is cross-linked to the 18 amino acid C-terminal end of H4 or to the 66 amino acid C-terminal half of H2B.

The nucleosome core is composed of 145 base pairs of DNA wrapped in a left-handed supercoil around a histone octamer containing two molecules each of H2A, H2B, H3, and H4 [for a review, see McGhee and Felsenfeld (1980) and van Holde (1989)]. Crystallographic studies have shown that the cysteine-110 residues of the H3 histones are close to each other across the dyad axis (Richmond et al., 1984; Uberbacher & Bunick, 1985). The histone pairs H2A-H2B and H3-H4 interact sufficiently strongly to form heterotypic dimeric structures in the absence of DNA. These can further associate to form $(H3-H4)_2$ tetramers and $2(H2A-H2B)/(H3-H4)_2$ octamers under conditions of high histone concentration or elevated ionic strength (D'Anna & Isenberg, 1974; Roark et al., 1974, 1976; Thomas & Kornberg, 1975; Eickbush & Moudrianakis, 1978; Ruiz-Carrillo & Jorcano, 1979; Godfrey et al., 1980; Benedict et al., 1984). A dyad axis within the

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central $(H3–H4)_2$ tetramer gives the completed octamer 2-fold symmetry (Burlingame et al., 1985).

Interactions among histones are more complex in the presence of DNA than indicated by studies of histones alone. The range of possible binding contacts among histones is limited since the population of nucleohistone particles does not consist of a mixture of randomly disordered species. Two discrete complexes that form in the presence of DNA require the histones to interact in ways that are not predicted by standard models of the nucleosome core. Octameric particles with the composition $(H3–H4)_4$/145 bp DNA are obtained when H3 and H4 are mixed with DNA in the absence of H2A and H2B (Simon et al., 1978; Stockley & Thomas, 1979; Thomas & Oudet, 1979; Read & Crane-Robinson, 1985). The existence of these particles suggests that $(H3–H4)_2$ tetramers may interact through the sites normally occupied by H2A–H2B pairs. The site on the H3–H4 dimer which is normally occupied by the second H3–H4 dimer at the dyad axis of the $(H3–H4)_2$ tetramer may also be occupied by an H2A–H2B dimer. This is seen in a hexameric particle (P2) found among the products of nuclease digestion of nucleohistone complexes prepared in 0.15 M NaCl (Ellison & Pulleyblank, 1983a–c). The formation of hexameric P2 particle is blocked by the preassociation of H3–H4 pairs in $(H3–H4)_2$ tetramers when nucleohistone complexes associate in the high ionic strength buffers usually used for nucleohistone assembly.

A nucleohistone particle (P3b) whose composition and physical properties are similar to those of the standard nucleosome core (P3a) is found among the products assembled at physiological ionic strength (Ellison & Pulleyblank, 1983a–c). Two observations have suggested that the internal arrangement of histones in P3b may differ from that found in the standard P3a particle. In P3b particles, unlike the standard (P3a) nucleosome core, the molecules of H2A and H2B can be cross-linked through lysine residues to form homodimers (Ellison & Pulleyblank, 1983b). P3b particles labeled with fluorescein on H3 also have different fluorescence properties from those of similarly derivatized annealed P3a nucleosome cores (Ellison & Pulleyblank, 1983b). We proposed that histones within the octameric P3b particle are stably arranged in a structure in which the H3 cysteines, instead of facing each other across the dyad axis, turn outward to face the H2A–H2B pairs (Ellison & Pulleyblank, 1983c). In order to test the proposed spatial orientation of histone H3 in P3b, we have mapped the environment of Cys-110 in these particles through the use of photoaffinity reagents.

Materials and Methods

Preparation of Nucleohistone Particles. Core histones were obtained from chicken erythrocyte nuclei as described in Ellison and Pulleyblank (1982). Individual histones, where needed, were isolated by reverse-phase HPLC on an RP-318 column (Bio-Rad, 250 × 4.6 mm) eluted at 1 mL/min with 0.05% trifluoroacetic acid in a linear 35–50% gradient of acetonitrile/water. For the preparation of hexameric P2 and rearranged (P3b) octameric particles, 1 mg of core histone in 100 μL of 10 mM HCl was diluted to 1 mL with 20 mM Tris-HCl buffer/0.15 M NaCl, pH 8.0, containing 1 mM phenylmethanesulfonyl fluoride (PMSF).[1] The histone solution was added slowly with rapid mixing to 1 mL of 2 mg/mL sonicated, phenol-extracted, Sigma grade X salmon sperm DNA in 20 mM Tris-HCl/0.15 M NaCl, pH 8.0. The mixture was centrifuged to remove precipitated material. The soluble supernatant was made 2 mM in $CaCl_2$ and digested with 2 units of micrococcal nuclease (Sigma) for 20 min at 23 °C. Digestions were terminated by addition of NaEDTA (5 mM final concentration). For annealed nucleosome cores (P3a), the nucleohistone complex was prepared at an initial salt concentration of 0.6 M NaCl instead of 0.15 M NaCl. The complex was annealed for 2 h at 23 °C, centrifuged to remove insoluble material, and then diluted to a final NaCl concentration of 0.15 M with 3 volumes of 20 mM Tris-HCl, (pH 8.0)/1 mM PMSF before digestion with micrococcal nuclease as described for P2 and P3b. Nucleohistone particles were concentrated by ultrafiltration and separated by sedimentation through 5–20% sucrose gradients (Ellison & Pulleyblank, 1983a).

Preparation of Labeled H3. Five microliters of *N*-(4-azidophenylthio)pthalimide (APTP, Pierce, 10 mg/mL dissolved in dimethylformamide) or *p*-azidophenacyl bromide (APAB, Pierce, 10 mg/mL dissolved in methanol) was added to 100 μL of a 10 mg/mL histone solution in 10 mM HCl. Where needed, illumination was by red safelight. The histones were incubated for 30 min to 1 h in the dark and then diluted to 1 mL with 20 mM Tris-HCl buffer/0.15 M NaCl, pH 8.0.

Photoactivation. Assembled complexes of DNA with APAB- or APTP-labeled histones as well as nonlabeled control samples in 20 mM Tris-HCl (pH 8.0)/0.15 M NaCl were placed in plastic petri dishes and irradiated from a distance of 5 cm with a long-wave UV light box for 10 min.

Electrophoretic Analysis of Histones. For peptide mapping where high histone concentrations were required, DNA was initially removed from nucleohistone samples by precipitation with 0.4 M HCl. Histones were precipitated from the supernatants with trichloroacetic acid (25% final concentration), collected by centrifugation, exchanged to the chloride salt with 0.1% HCl in acetone, and then rinsed with acetone. Samples were analyzed by electrophoresis on 0.75-mm 15% SDS–polyacrylamide slab gels (Laemmli, 1970). APTP-cross-linked histones were analyzed by two-dimensional gel electrophoresis. Lanes excised from the first-dimension 15% SDS–polyacrylamide gels were soaked for 30 min in 0.1 M β-mercaptoethanol in 0.125 M Tris-HCl, pH 6.8 (stacking gel buffer), to reverse the cross-links and then placed across the top of another 15% SDS–polyacrylamide gel for electrophoresis in the second dimension. Histones were visualized by silver staining as described in Ellison and Pulleyblank (1983a).

Cyanogen Bromide Cleavage Analysis. A standard set of H2B fragments was generated by incubating a 2-μL sample containing 20 μg of H2B with 20 μL of 50 mg/mL CNBr in 70% formic acid for 2 h at 23 °C. Reactions were terminated by lyophilization. Samples were relyophilized from 10 μL of H_2O. Complete separation of the cross-linked histone pairs from annealed nucleosome particles was achieved by electrophoresis on long 26 cm × 0.75 mm thick 15% SDS–polyacrylamide gels for 12 h at 250 V. Gels were stained with Coomassie Brilliant Blue R-250 (Pierce) in 50% methanol/10% acetic acid for 10 min and then rinsed for 15 min in water. Gel slices containing histones or cross-linked histones were excised and immersed in 50 mg/mL cyanogen bromide (CNBr, Pierce) in 70% formic acid for 2 h and then washed repeatedly with stacking gel buffer (lacking SDS) in the presence of bromophenol blue. Complete removal of formic acid was evident when this indicator remained blue. Stacking gel buffer containing 0.5% SDS was then added, and the tubes

[1] Abbreviations: APTP, *N*-(4-azidophenylthio)phthalimide; APAB, *p*-azidophenacyl bromide; bp, base pair(s); CD, circular dichroism; UV, ultraviolet; DNase, deoxyribonuclease; SDS, sodium dodecyl sulfate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; aa, amino acid(s); PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetate.

were incubated at 90 °C for 15 min. For two-dimensional electrophoresis of peptides, lanes containing the CNBr-treated cross-linked histones were excised, cross-links were reduced, and the peptides were analyzed as described above for cross-linked histones. Peptides were detected by silver staining.

Microsequencing of CNBr-Cleaved H2B. CNBr-treated histone H2B was electrophoresed in a 0.75-mm SDS–polyacrylamide gel. The fragments were then transferred onto an Immobilon-P membrane (Millipore) using a Tyler semi-dry electrophoretic transfer system (ET-20) with Laemmli (1970) electrophoresis buffer containing 10% methanol at 500 mA for 1 h. Transferred proteins were Coomassie blue stained and dried, the H2B peptide was cut out, and the N-terminal 15 aa were microsequenced as described by Matsudaira (1987).

DNase I Digestion of Particles. Particles assembled either in 0.15 M NaCl or in 0.6 M NaCl were isolated from 5–20% sucrose density gradients and dialyzed against 20 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, and 4 mM dithiothreitol (DTT). The DNA within the particles was end-labeled with [γ-^{32}P]-ATP and polynucleotide kinase. Nucleohistone particles were digested with DNase I as described by Lutter (1978). Briefly, an equal volume of 20 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, and 4 mM DTT was added, and 200-μL aliquots (OD_{260} = 0.8) were digested with 1.5 units of DNase I (Boehringer Mannheim) at 23 °C. At different times, 50-μL aliquots were removed and added to 5 μL of 0.1 M NaEDTA. Samples were heated to 100 °C for 2 min, cooled, and digested with proteinase K at 37 °C for 30 min. DNA fragments were analyzed by electrophoresis on an 8% acrylamide/0.4% bis(acrylamide) denaturing gel.

Results

Identification of Cross-Linked Products. The sucrose gradient profiles in Figure 1A show the particles P1, P2, and P3b [(---) and (···)] resolved after micrococcal nuclease treatment of nucleohistone samples obtained by direct assembly of nucleohistone in 0.15 M NaCl. Particles P1 and P3a (—) are obtained by assembly in 0.6 M NaCl followed by dilution to 0.15 M NaCl and subsequent nuclease digestion. P1 and P2 are respectively a dimer of histones H2A–H2B associated with about 40 bp of DNA and the 2(H2A–H2B)/H3–H4 hexamer particle which contains 92–102 bp of DNA (Ellison & Pulleyblank, 1983a). The yield of the hexameric P2 particle decreased when H3 was modified with either APAB or APTP shown in Figure 1A when using APTP [compare (---) and (···)]. APTP-photo-cross-linked histone pairs H3–H2B, H3–H4, and a small amount of H3–H2A arise in physiological ionic strength (0.15 M NaCl) assembled P3b particles which were irradiated in the same buffer (Figure 1C). The identity of these histones was determined by using two-dimensional gel analysis (see Figure 3). These cross-linked dimers do not form in P3a particles assembled in 0.6 M NaCl which were diluted to 0.15 M NaCl before irradiation (Figure 1B). Photo-cross-linking of histones within the particles caused no detectable change in their sedimentation properties. A non-reversible cross-linked species which forms when the H2A–H2B dimer particle P1 is irradiated (see Figure 1B,C) is a UV-cross-linked product of H2A with H2B previously reported by DeLange et al. (1979) and Callaway et al. (1985).

Figure 2 shows the photo-cross-linked products obtained from isolated P3 core particles in which H3 was modified on Cys-110 with the noncleavable reagent APAB. Although H3–H3 dimers formed by oxidation of Cys-110 can participate in the formation of nucleosome particles (Camerini-Otero & Felsenfeld, 1977; Lewis & Chiu, 1980; Ellison & Pulleyblank, 1983c), the dimers in lanes 1 and 3 (Figure 2) were resistant

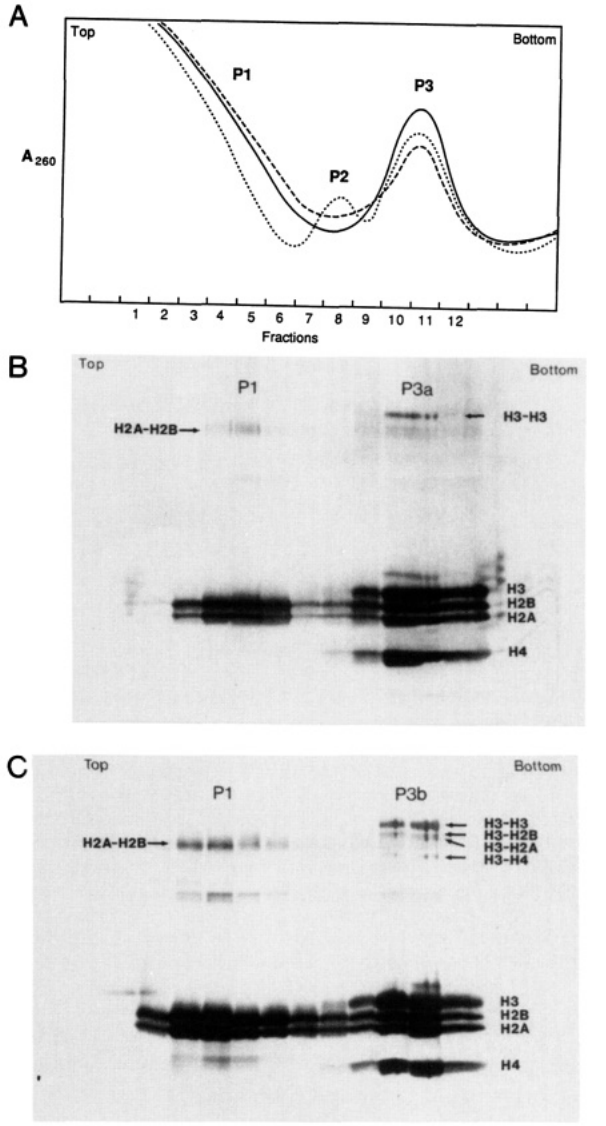

FIGURE 1: Assembled nucleohistone particles separated in 5–20% sucrose gradients. (A) Complexes formed between chicken erythrocyte core histones and salmon sperm DNA in 0.15 M NaCl [(---) and (···)] and in 0.6 M NaCl (—). P3 is P3a in the sample assembled in 0.6 M NaCl (—) and P3b in the sample assembled in 0.15 M NaCl [(---) and (···)]. Particles assembled in 0.15 M NaCl where H3 was not modified with APTP are indicated by (···). The particles P1 (H2A–H2B dimer associated with ~40 bp of DNA), P2 [2(H2A–H2B)/H3–H4 hexamers containing 92–102 bp of DNA], P3a [2-(H2A–H2B)/2(H3–H4) octamers containing 145 bp of DNA annealed at high ionic strength], and P3b [2(H2A–H2B)/2(H3–H4) octamers containing 145 bp of DNA annealed at low ionic strength] which can be resolved are indicated. (B and C) 15% SDS–polyacrylamide gel of fractions obtained from gradient separation of particles assembled in 0.6 M NaCl (—) and in 0.15 M NaCl [(---) Figure 1A] in which histone H3 was previously modified on Cys-110 with APTP and the particles were irradiated with UV light. The cross-linked products in P3a and P3b (arrows) were identified by subsequent 2-D gel analysis (Figure 3). Zero-length UV-cross-linked H2A–H2B pairs are indicated. These were identified by their absence in non-UV-irradiated samples and their comigration with P1 particles containing non-cross-linked histones H2A and H2B.

to reduction by β-mercaptoethanol and were therefore linked through the photoaffinity ligand. Cross-linked products were identified by two-dimensional electrophoresis using the reversible cross-linking reagent APTP as shown in Figure 3. The patterns of cross-linked species formed by APTP or APAB cross-linking reagents were similar (compare Figure 1B,C lanes corresponding to P3a and P3b with Figure 2, lanes 3 and 1, respectively). The major species that formed within nucleo-

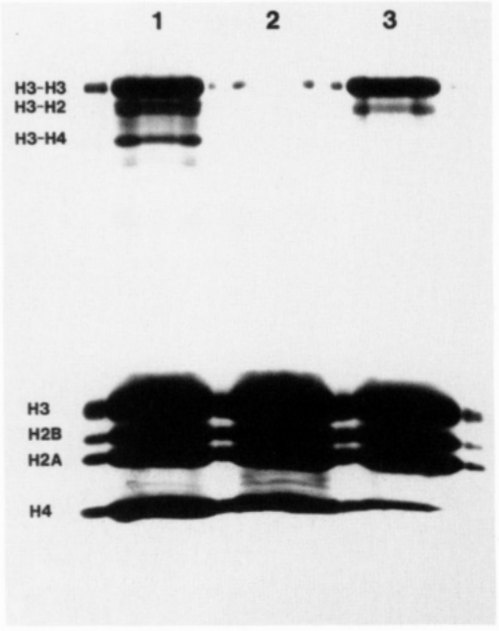

FIGURE 2: Analysis of APAB-cross-linked histones formed after nucleosomes were assembled in 0.15 and 0.6 M NaCl. Core histones and histone H3 modified with APAB were assembled into nucleohistones, treated with micrococcal nuclease, and separated by sedimentation through a 5–20% sucrose gradient. Fractions corresponding to P3a and P3b were treated with 20 mM β-mercaptoethanol to reduce H3–H3 oxidation products and electrophoresed on a 15% SDS–polyacrylamide gel. Lane 1, histones of UV-irradiated P3b particles assembled in 0.15 mM NaCl. Lane 2, histones from P3b particles prepared in 0.15 M NaCl which had not been pretreated with cross-linking agent or UV light. Lane 3, histones of UV-irradiated P3a particles assembled in 0.6 M NaCl. The cross-linked products evident in lanes 1 and 3 were identified by comparison of their mobilities with those of cross-linked histones.

some particles assembled in 0.15 M NaCl were identified as H3–H3, H3–H2B, H3–H4, and trace amounts of H3–H2A dimers (Figure 3A). When the nucleohistone complex was annealed in 0.6 M NaCl and then diluted to 0.15 M NaCl for micrococcal nuclease digestion, the only abundant cross-linked species formed was H3–H3 (lane 3, Figure 2). The small amount of an H3–H2A cross-linked product that was observed when APAB was used may be due to the greater reach (~9 Å) of APAB when compared to APTP. Cyanogen bromide peptide fragments of APAB- and APTP-cross-linked H3–H3, H3–H2B, and H3–H4 dimers were indistinguishable (not shown).

Cyanogen Bromide Cleaved Histones. The cyanogen bromide cleavage products of core histones and cross-linked dimers are shown in Figure 4A. Fragments containing greater than 45 amino acids from APAB-cross-linked, UV-cross-linked, and core histones were detected by SDS gel electrophoresis. Under the conditions used, we obtained partial CNBr cleavage products. The lowest mobility fragments are uncleaved histone. The methionine sites and potential cleavage products of histones H2B, H3, and H4 are illustrated in Figure 4B. Cross-linked fragments derived from P3b H3–H4 and H3–H2B dimers are also depicted in Figure 4B. The sites of APAB cross-linking of H3–H3 dimers in the P3a and P3b (Figure 4A, lanes 2 and 1, respectively) preparations are probably the same since treatment with CNBr yielded identical sets of cleavage products (see Discussion). Fragments of UV-cross-linked H2A–H2B pairs from P3a and P3b were also indistinguishable (Figure 4A, lanes 9 and 10). The cleavage patterns of H3–H2B and H3–H4 cross-linked pairs obtained from P3b are shown in lanes 4 and 7. CNBr fragments of H3 corresponding to the amino-terminal peptide H3(1–90) and

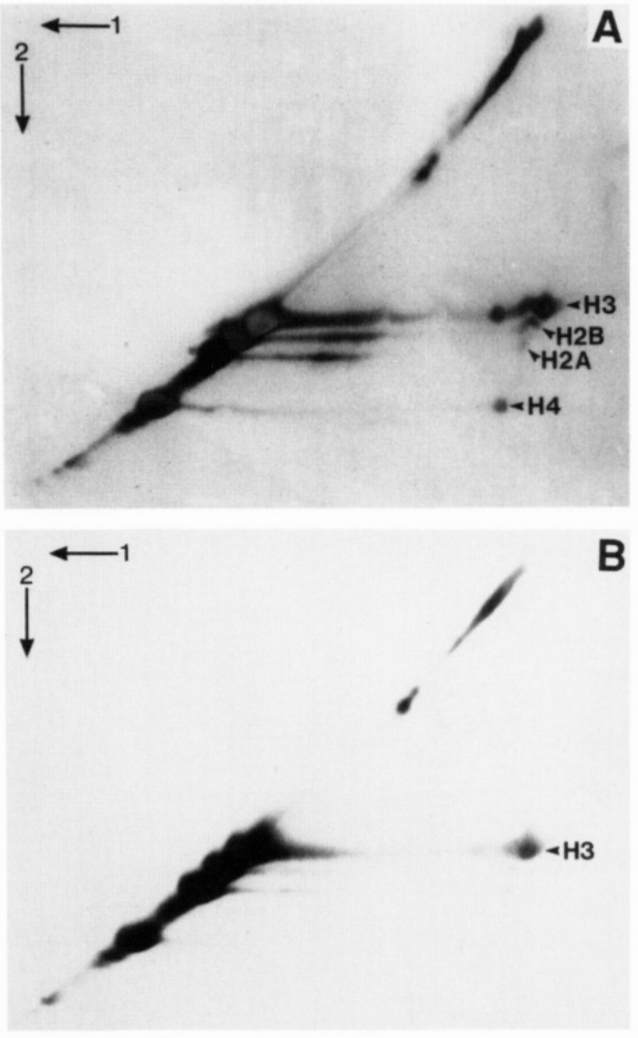

FIGURE 3: Identification of APTP-cross-linked histones by two-dimensional gel electrophoresis. Nucleohistone samples containing APTP-cross-linked histones were separated in a 15% SDS–PAGE in the first dimension. Channels were excised, soaked in 0.1 M β-mercaptoethanol, and placed across the top of another 15% SDS–PAGE. (A) Cross-linked histones assembled in 0.15 M NaCl are identified as H3–H3, H3–H2B, H3–H4, and a small amount of H3–H2A. (B) Nucleohistones assembled in 0.6 M NaCl contain only cross-linked H3–H3 dimers.

the partial cleavage product H3(1–120) were observed (lanes 3 and 6, Figure 4A). Uncleaved H4 and the peptide H4(1–84) are shown in lane 5 (Figure 4A). Histone H2A contains no methionine residues and was not cleaved (lane 11, Figure 4A). Lane 8 shows uncleaved histone H2B as well as two faster migrating bands comprised of the unresolved peptides H2B-(1–59) with H2B(1–62) and H2B(60–125) with H2B(63–125). Although these pairs of fragments, which correspond respectively to the N- and C-terminal ends of H2B, are approximately equal in molecular weight, their mobilities in SDS–PAGE are very different (Figure 4A, lane 8). The higher mobility of the C-terminal fragment was established by microsequencing the H2B fragment transferred to an Immobilon-P membrane (Millipore).

Sites of Cross-Links Formed between Cys-110 of H3 and H4 and H2B. Two-dimensional in situ CNBr peptide analysis of the APTP-cross-linked products H3–H4 is shown in Figure 5A. The peptides on the diagonal correspond to those seen in lane 4 of Figure 4A. Under these conditions which were chosen so as to minimize product diffusion, both cyanogen bromide cleavage and subsequent reduction with β-mercaptoethanol are incomplete. Band a was undegraded H3–H4

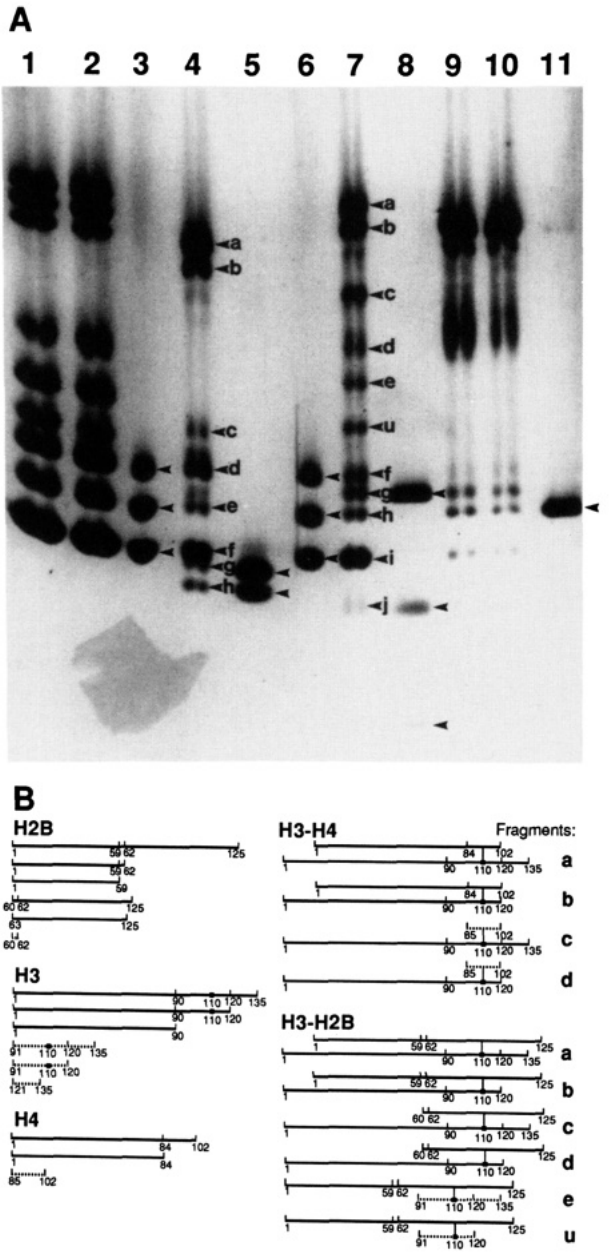

FIGURE 4: Cyanogen bromide cleavage of histones and cross-linked products. (A) Gel slices containing individual histones or cross-linked histone pairs were treated with cyanogen bromide and placed on top of another 15% SDS–PAGE. The least mobile bands are uncleaved histone. Lanes 1 and 2 contain APAB-cross-linked H3–H3 dimers isolated from nucleohistones assembled at low and high ionic strength, respectively. APAB-cross-linked H3–H4 and H3–H2B pairs assembled at 0.15 M NaCl are present in lanes 4 and 7, respectively. (The indicated peptides correlate with those shown in Figure 5.) CNBr-treated H3 (lanes 3 and 6), H4 (lane 5), H2B (lane 8), and H2A (lane 11) are shown. Lanes 9 and 10 are CNBr-cleaved UV-cross-linked H2A–H2B dimers assembled at low and high ionic strength, respectively. (B) CNBr cleavage sites in histones H2B, H3, and H4 and cross-linked dimers H3–H2B and H3–H4. Met-59 and -62 of H2B, Met-90 and -120 of H3, Met-84 of H4, and Cys-110 of H3 are shown. The resulting fragments which can be resolved (solid line) and those not detected (stippled line) in our gel system are indicated. A small peptide (stippled line) can be detected when cross-linked to a larger peptide. Cross-linked fragments a, b, c, and d from H3–H4 dimers seen in lane 4 (A) are depicted. Bands e, f, g, and h are not drawn but correspond to H3(1–120), H3(1–90), H4, and H4(1–84), respectively. Fragments a, b, c, d, and e, and u from cross-linked H3–H2B pairs seen in lane 7 (A) are illustrated. Only one of the two possible C-terminal halves of H2B are shown for c and d. Bands not drawn are f, g, h, i, and j corresponding to H3, H2B, H3(1–120), H3(1–90), and H2B(1–59,62), respectively. The identity of these fragments was determined as described in Figure 5.

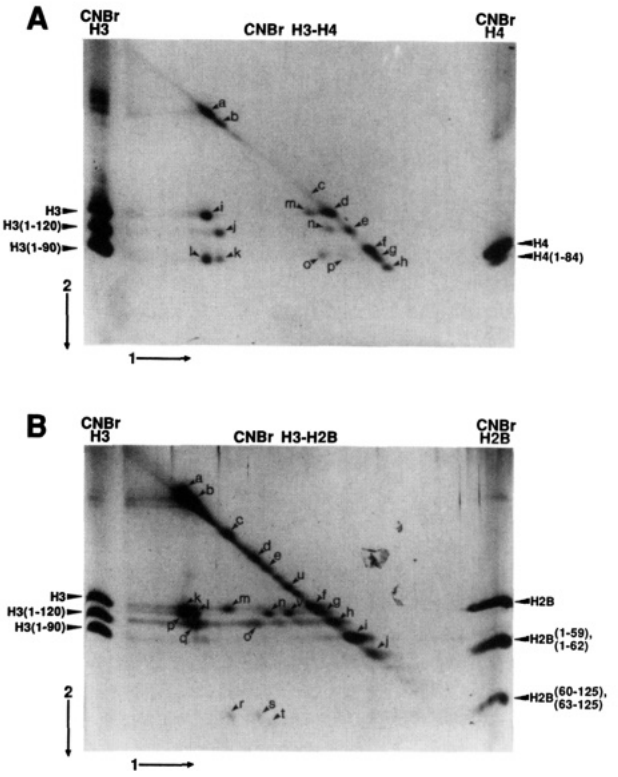

FIGURE 5: Two-dimensional analysis of cyanogen bromide treated cross-linked H3–H4 and H3–H2B pairs from P3b assembled at low ionic strength. Gel slices containing APTP-cross-linked histone pairs were treated with cyanogen bromide, placed on top of a 15% SDS–polyacrylamide gel, and electrophoresed. The lanes were cut out, immersed in stacking gel buffer containing 0.1 M β-mercaptoethanol, and subsequently placed on top of another 15% SDS–PAGE. Marker histones cleaved with CNBr were run alongside. The fragments of CNBr-treated cross-linked H3–H4 (A) and H3–H2B (B) dimers are shown. For identification of the indicated peptides, see Results.

since it yielded histone H3 (band i) and H4 (band l) upon reduction in the second dimension. Fragment b gave intact histone H4 (k) and the H3(1–120) peptide (j). Fragment c, which had a slightly lower mobility than whole histone H3 in the first dimension, yielded H3 upon reduction (m). This indicates that in c, H3 was cross-linked to the small 18 aa C-terminal peptide fragment of histone H4 which was too small to be detected. This assignment of the site of H3–H4 cross-linking to the C-terminal 18 amino acids of H4 is confirmed by the reduction product (n) of the peptide (d) corresponding to the H3(1–120) peptide in the second dimension. The change in mobility corresponds to that predicted for loss of this C-terminal fragment from H4. Bands e, f, g, and h are H3(1–120), H3(1–90), H4, and H4(1–84), respectively. The presence of H3(1–120) (e) and whole H4 (g) on the diagonal is probably due to partial cleavage of the disulfide linkages during isolation of the cross-linked species. For this reason, d contained intact histone H3 as well as the H3(1–120)–H4(85–102) fragment. o and p are histone H4. The first-dimension mobilities of the cross-linked parent species of o and p are consistent with their being H4 cross-linked to H3(91–135) and to H3(91–120). The absence of H4(1–84) off the diagonal of Figure 5A is also consistent with the site of the cross-link being within the 18 aa C-terminal peptide of H4.

An equivalent analysis of cross-linked H3–H2B dimers is illustrated in Figure 5B. Here the peptides on the diagonal correspond to those of lane 7 in Figure 4A. Band a yielded histones H3 (k) and H2B (p) upon reduction. Fragment b consisted of histone H2B (l) and peptide H3(1–120) (q).

Peptide c defined the carboxyl half of H2B as the site of cross-linking since it consisted of histone H3 (m) and the 63 or 66 amino acid C-terminal fragment of H2B (r). Peptide d is H3(1–120) (a) which before reduction was cross-linked to the 63 or 66 amino acid C-terminal fragment of H2B (s). Fragment e is resolved to histone H2B (n) and the very small peptide t which is probably H3(91–135). This fragment was detected here because of the increased protein loading relative to other gels shown. The H3(91–135) fragment is not seen in the CNBr H3 lane where it is likely to have been lost to diffusion during the CNBr treatment. Fragment u gave histone H2B (v) and a peptide, probably H3(91–120), which was too small to be detected. The fragments f, g, h, i, and j are H3, H2B, H3(1–120), H3(1–90), and H2B(1–59,62), respectively. Partial cleavage of disulfide cross-links prior to running the first dimension accounts for the presence of H3 (f), H2B(g), and H3(1–120) (h). These observations indicate that Cys-110 of H3 is cross-linked to the C-terminal half of H2B in P3b. The absence of off-diagonal peptides corresponding to H2B(1–59,62) supports this interpretation.

DNase I Analysis of DNA Folding within P3a and P3b. The folding of the DNA in P3a and P3b particles was analyzed by digesting the particles with DNase I under conditions of approximately one cut per nucleosome. Nucleosomes obtained from chromatin as well as those obtained by annealing histones to DNA in 0.6 M NaCl are relatively resistant to cleavage at sites 30, 60, 80, and 110 residues from the 5′ termini (Lutter, 1978). As shown in Figure 6, the P3b particle population shows a similar digestion pattern to that of P3a particles. Although it is probable that the two P3a and P3b particles have similar digestion profiles, contamination of the P3b sample by P3a particles could have obscured a distinctive P3b pattern if the particles are digested at markedly different rates. Some increase in background was observed in P3b lanes (compare lane 2, P3b, to lane 2, P3a).

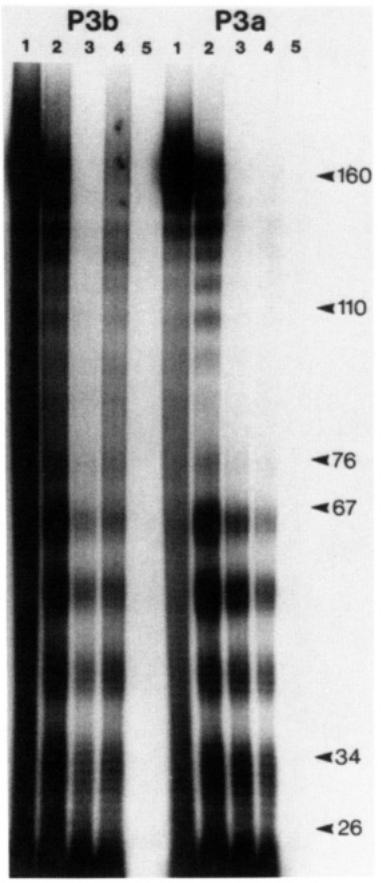

FIGURE 6: DNase I digestion of P3a and P3b particles. Nucleohistone particles assembled in 0.15 M NaCl (P3b) and in 0.6 M NaCl (P3a) were isolated from 5–20% sucrose density gradients and end-labeled with [γ-^{32}P]ATP. The nucleohistones were digested with DNase I at 22 °C for 10 s and 2, 5, 10, and 15 min (lanes 1–5, respectively). Samples were analyzed by electrophoresis on an 8% denaturing polyacrylamide gel. The size marks indicated on the side are from a parallel sequencing lane.

Discussion

In most protocols for reconstitution of nucleosome core particles in vitro, histones are mixed with DNA in a concentrated salt solution which is then reduced in ionic strength by dialysis or dilution (McGhee & Felsenfeld, 1980; Wu et al., 1986). This method minimizes nonspecific ionic interactions between the cationic histones and the anionic DNA and promotes the association of $(H3–H4)_2$ tetramers, thereby facilitating the formation of the most thermodynamically stable configuration of the nucleosome. These conditions of assembly prevent detection of the metastable population of particles found in P3b preparations. For assembly of P3b, it is necessary to avoid long periods of exposure of the histones to neutral low ionic strength conditions since they aggregate extensively. The even faster association of previously acid-solubilized histones with DNA under neutral conditions permits 20–40% of the histone to be recovered in soluble nucleohistone particles. Although P3b may represent an aberrant structure peculiar to the use of acid-dissociated histones, Isenberg (1979) showed that acid-extracted histones are capable of the specific associations involved in nucleosome formation while Greyling et al. (1983) established that acid-extracted histones and histone octamers prepared by extraction at high ionic strength under neutral conditions from identical helical structures. Previous work (Ellison & Pulleyblank, 1983b) has shown that the $t_{1/2}$ of the fluorescein-labeled P3b particle is greater than 24 h at 23 °C in 0.15 M NaCl. In the present work, P3b particles containing modified H3 were stable for a period of at least 6 h prior to photoactivation of the cross-linker. Detection of P3b is prevented by preincubation of the particles at high (0.6 M) ionic strength, and therefore conversion of P3b to P3a particles occurs rapidly under these conditions; however, it is not certain whether the rearrangement pathway requires the histone to dissociate from the DNA. Since the sulfhydryl modifying reagents used in these studies may alter the kinetics of rearrangement, these results do not directly address the question of the kinetic stability of unmodified P3b. Direct assessment of the stability of unmodified P3b is prevented by the lack of a quantitative assay for this particle; however, the related P2 particle is stable under assembly conditions for at least 48 h (Ellison, unpublished results).

Conformational perturbation of the normal P3a nucleosome core by changes in ionic strength, pH, or temperature (Uberbacher et al., 1983; Sibbet et al., 1983; Ausio et al., 1984; Walker, 1984; Yager & van Holde, 1984; Libertini & Small, 1984; Chung & Lewis, 1985; Greulich et al., 1985; Oohara & Wada; Hirai et al., 1988; Yager et al., 1989) differs from those reported here in that they are fully reversible. The H3–H3 cross-links generated in P3b samples appear to be at the same site as those found in P3a preparations (Figure 4A, lanes 1 and 2), and it is probable that normal P3a nucleosomes which contaminate P3b preparations are the precursors of this cross-linked species. Although the relative abundances of the cross-linked species may not reflect the relative abundances of their precursors, the yield of H3–H4 and H3–H2B cross-links in P3b preparations is approximately equal to that of H3–H3 cross-links, suggesting that in these preparations rearranged particles constitute about half the total.

Despite the internal differences between P3a and P3b particles, they cannot readily be distinguished by their physical properties. P3b particles are compact since their sedimentation properties are identical with those of P3a particles (Ellison & Pulleyblank, 1983a). The DNA folding around P3a and P3b nucleosome cores also appears to be similar as indicated by their indistinguishable CD spectra and thermal denaturation profiles (Ellison & Pulleyblank, 1983b). Although the DNase I digestion patterns of P3a and P3b preparations shown in Figure 6 are not significantly different, the possibility remains that subtle alterations in the digestion profile may have been obscured by contaminating P3a particles if the digestion rate of the two particles differ.

The four core histones are present in P3b samples in equimolar ratios (Ellison & Pulleyblank, 1983a). If the P3b population includes particles with the composition $(H3–H4)_3/H2A/H2B$, a compensating octameric H2A–H2B rich particle would also have to be present. The existence of the latter is unlikely since histones H2A and H2B do not form multimeric structures (van Holde, 1989) or compact nucleohistone particles (Aragay et al., 1988).

Cysteine-110 residues are buried in the hydrophobic interiors of both normal P3a cores and P3b core particles (Wong & Candido, 1978; Dieterich et al., 1979; Ellison & Pulleyblank, 1983b). In normal core (P3a) particles, the C-terminal end of H4 can be cross-linked to the C-terminal half of H2B (Martinson et al., 1979). Our ability to cross-link the 18 amino acid C-terminus of histone H4 to APTP-derivatized Cys-110 residues in P3b particles suggests that in these particles this portion of H4 is within 7 Å of H3 Cys-110. Our inability to photo-cross-link H3 to H4 in normal nucleosomes is consistent with the models proposed by both Richmond et al. (1984) and Burlingame et al. (1985) where this site is proposed to lie in the interaction face between H3 molecules. Other sites in these histones can be cross-linked with a carbodiimide (Bonner & Pollard, 1975). Our cross-linking data indicate that the C-terminal half of H2B must be in close proximity to the Cys-110 residue in some particles of the P3b population. Previously cross-linked H2A–H2B dimers can be assembled into both P3a and P3b particles (Ellison & Pulleyblank, 1983b,c), and UV-cross-linked sites within H2A–H2B dimers from these particles appear to be the same as those previously reported in intact chromatin (Callaway et al., 1985). Therefore, the H3–H2B cross-link found in the present study is likely to reflect an altered relationship of Cys-110 to an otherwise normal H2A–H2B pair rather than a dislocation of part of H2B within the H2A–H2B pair. The positions of the two H2A–H2B pairs with respect to the $(H3–H4)_2$ tetramer in P3b differ from those in P3a since cross-linked H2A–H2A and H2B–H2B homodimers can be formed in P3b but not in P3a core particles (Ellison & Pulleyblank, 1983b).

We cannot yet ascertain whether the P3b particles which yield cross-linked H3–H2B pairs have the same internal arrangement as those which yield cross-linked H3–H4 pairs. If for simplicity sake we assume that P3b represents a single altered particle, then among possible models there is an asymmetric case [Figure 7, P3b(ii)], where an interface of an H3–H4 pair containing cysteine interacts with a H2A–H2B pair. Here the cysteine of the second H3–H4 dimer faces the side of the first H3–H4 dimer which would normally interact with the H2A–H2B pair. In this model, an H3 of one H3–H4 pair may cross-link to the H4 of the second H3–H4 pair while the Cys-110 of that H3–H4 dimer could be cross-linked to H2B and some H2A. This model is related to a "pseudo-core" model for the octameric $(H3–H4)_4$ nucleohistone particle

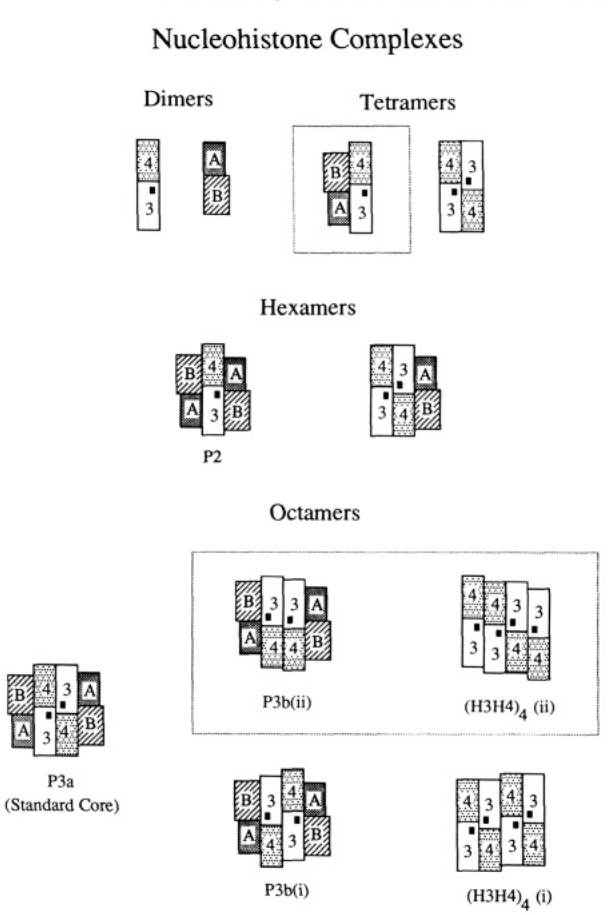

FIGURE 7: Family of nucleohistone particles. Histones H2A, H2B, H3, and H4 are shown as A, B, 3, and 4, respectively. The DNA component is not depicted for clarity. The cysteine-110 residue of H3 is drawn as a black square. Particles which have not been isolated but which may be intermediates in the assembly of known particles (the H2A–H2B–H3–H4 tetramer) or which are considered to be the less likely among alternative arrangements [$(H3H4)_4$(ii) and P3b(ii)] are boxed. The formation of P2 is cooperative since intermediate heterotypic tetramers (H2A–H2B–H3–H4, boxed) cannot be isolated. The normal P3a nucleosome assembled at elevated ionic strength is built out of an $(H3–H4)_2$ tetramer end-capped by two H2A–H2B pairs. In P3a, the Cys-110 residues interact at the dyad axis of the core particle (Richmond et al., 1984; Uberbacher & Bunick, 1985), and the orientation of H2A–H2B pairs is based on cross-linking studies (Martinson et al., 1979). The models of the P3b particle illustrated P3b(i–ii) correspond to different possible arrangements of the octameric H3/H4-rich particle $(H3–H4)_4$(i–ii).

$(H3–H4)_4$(ii) (Figure 7) where the octamer is composed of a central tetramer through the addition of H3–H4 pairs to the sites normally occupied by H2A–H2B pairs. Both cross-linking (Stockley & Thomas, 1979; Read & Crane-Robinson, 1985) and the nuclease accessibility at the center of the $(H3–H4)_4$ particle (Read & Crane-Robinson, 1985) indicate that the octamer forms from two tetrameric subunits $(H3–H4)_4$(i) and is not a pseudocore particle where H2A–H2B pairs are replaced by H3–H4 pairs. This latter method requires conformational adjustments within the $(H3–H4)_2$ tetramers to prevent the appearance of particles containing higher H3–H4 polymers or further addition of histones H2A and H2B (Daban & Cantor, 1982). These considerations suggest that interactions of H3–H4 pairs in P3b particles may resemble the contacts between the $(H3–H4)_2$ tetramers in octameric nucleohistones depicted in $(H3–H4)_4$(i) (Figure 7). The generation of H3–H2B cross-linked pairs in P3b is consistent with a model [P3b(i), Figure 1], in which the contacts along the dyad axis between the H3–H4 pairs in the central $(H3–H4)_2$ tetramer are exchanged with those between the

H3–H4 pairs and the H2A–H2B pairs. This produces particles where the H3 thiols are turned toward H2A–H2B pairs. Abundant H2B–H3 and relatively small amounts of H2A–H3 cross-linked products may form in the rearranged P3b(i) particle if the H3 sulfhydryl sits within 7 Å of the boundary between the H2 molecules. The cooperativity with which P2 and octameric P3b particles form indicates that their assembly is accompanied by conformational adjustments within H3–H4 pairs which could permit the observed cross-linking of H3 with H4. Conformational changes within the $(H3–H4)_2$ complex are believed to be responsible for the cooperative association of H2A–H2B pairs with $(H3–H4)_2$ tetramers (Godfrey et al., 1980; Benedict et al., 1984; Feinstein et al., 1986) and the biphasic release of H2A–H2B pairs from nucleosomes (Jordano et al., 1985; Nieto & Palacián, 1988). The interaction of H2A with H2B within H2A–H2B pairs of P2 and P3b probably does not differ significantly from those in annealed nucleosomes since previously cross-linked H2A–H2B dimers can be assembled into these particles (Ellison & Pulleyblank, 1983b).

Allfrey and co-workers (Sterner et al., 1987; Allegra et al., 1987; Chen & Allfrey, 1987) suggested that transcriptionally active chromatin may be enriched in nucleosome particles in which the cysteines of histone H3 are exposed to solvent. This interpretation has been disputed (Moudrianakis et al., 1989) although Chan et al. (1988) have shown that the H3 sulfhydryl in acetylated mono- and polynucleosomes is more accessible to modification than in unacetylated core particles. In the altered nucleosome described here, the sulfhydryl of H3 does not appear to be accessible to thiol reagents (Ellison & Pulleyblank, 1983b). Ridsdale et al. (1988) have shown that nucleosomes isolated from active chromatin contain histones bound to DNA in a less thermodynamically stable arrangement when compared to nucleosomes from transcriptionally inactive chromatin as assessed by their greater susceptibility to dissociation by ethidium bromide. The rearranged core particle described here provides a candidate for an active nucleosome structure which could not readily be distinguished from a standard nucleosome by commonly used physical criteria but which may nevertheless have sufficient kinetic stability to be retained for long periods of time in vivo.

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On the Nature of the Structural Change of the Colicin E1 Channel Peptide Necessary for Its Translocation-Competent State†

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ABSTRACT: Acidic pH conditions required in vitro for membrane binding and activity of the channel-forming colicin E1 resulted in an increased susceptibility to proteases of the 178-residue thermolytic channel peptide, an increased accessibility to acrylamide of a fluorescence probe linked to cysteine-505 of the peptide, and an increased partition into nonionic detergent. The structural change in the peptide sensed by the fluorescence probe caused by a transition from pH 6.0 to 3.5 occurred in <1 s. The presence of low concentrations of detergents (0.001% SDS or 0.44% octyl β-D-glucoside) or urea (0.2 M) at pH 6 or 4 also increased the susceptibility of the channel peptide to proteases. The increase in protease susceptibility and acrylamide accessibility at low pH, as well as partition of the peptide into nonionic detergent, suggested that acidic pH or the detergents might cause peptide unfolding. However, the hydrodynamic radius of the channel peptide at pH 6, 21–23 Å, was not changed at pH 3.5 or by detergents or urea under conditions that increased the susceptibility of the peptide to protease. The activity of the channel peptide at pH 6 measured with liposomes and planar bilayers, which was a factor of 10^3–10^4 smaller than that at pH 4, was increased by 2–4 orders of magnitude by 0.001% SDS or 0.44% octyl β-D-glucoside, with an additional small increment of activity on planar bilayers caused by 0.01% SDS. A small increase in Stokes radius of the peptide in the presence of SDS could be detected that was approximately correlated with increased activity.

Colicin E1 is a bactericidal protein whose cytotoxic action results from formation of a channel in the cytoplasmic membrane sufficiently conductive to depolarize and deenergize the cell (Gould & Cramer, 1977; Schein et al., 1978; Bullock et al., 1983; Cleveland et al., 1983). The prerequisites for channel formation are binding of the colicin molecule to a receptor in the outer membrane, translocation across this membrane, and binding, import, and channel formation in the cytoplasmic membrane. The molecular events associated with colicin channel formation in the cytoplasmic membrane can be mimicked in vitro by using artificial planar bilayer membranes [e.g., Bullock et al. (1983)] and membrane vesicles (Peterson & Cramer, 1987).

Translocation competence of proteins that are imported or secreted across the membranes of organelles and bacterial cells is believed to require an "unfolded" conformation. The definition of this state is mainly based on the following observations: (i) Mitochondrial import of a fusion protein of cytochrome oxidase subunit IV with dihydrofolate reductase is inhibited if the native folded structure of reductase is stabilized with respect to protease digestion by the compound methotrexate, which binds to its active site (Eilers & Schatz, 1986). (ii) Nascent pre maltose-binding protein that is competent for membrane assembly is also sensitive to protease digestion, whereas the protein loses translocation competence as it folds into a mature protease-resistant structure (Randall & Hardy, 1986). Although these protease data are consistent with an unfolded state, there is a paucity of physical–chemical information concerning the characterization of this state.

The in vitro activity of colicin E1 requires an acidic pH with an optimum ≤4.0 (Davidson et al., 1985). The requirement of an acidic pH for optimum activity is shared by the channel-forming colicin A (Lazdunski et al., 1988), several toxins

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