Proc. Nat. Acad. Sci. USA
Vol. 72, No. 7, pp. 2626–2630, July 1975
Biochemistry

An octamer of histones in chromatin and free in solution

(di-imidoester crosslinking/dimers)

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Communicated by F. H. C. Crick, April 30, 1975

ABSTRACT **Crosslinking with dimethyl suberimidate reveals a chain of histone octamers in chromatin. The octamer can be isolated free in solution at high ionic strength and pH. The identification of dimers formed by crosslinking reveals two or more contacts of each histone with others within the octamer. The molecular weight (110,000) and pattern of dissociation of the octamer are compatible with the composition $(F2A1)_2(F3)_2(F2A2)_2(F2B)_2$.**

The four main types of histone associate in pairs in solution: histones F2A1 and F3 constitute a tetramer (1–3) of composition $(F2A1)_2(F3)_2$, and histones F2A2 and F2B a mixture of oligomers, possibly dimers or short polymers (1, 4–6). The finding of a histone tetramer led to a model for chromatin structure (7): the composition of the tetramer suggested a repeating unit comprising eight histone molecules (two each of the four main types) and 200 base pairs of DNA; the globular nature of the tetramer suggested that the DNA is on the outside, surrounding a histone core. The spacing of tetramers, or length of the repeating unit in the chromatin fiber direction, was taken on the basis of x-ray evidence to be about 100 Å, which corresponds to a packing ratio (ratio of extended length of DNA to length of unit) of 6.8:1.

The main quantitative features of the model are the number of base pairs in a repeating unit, the number of histone molecules, and the packing ratio. Two of these features have been tested, and the results agree well with the model. Noll (8) has shown that endonucleases cut rat liver chromatin at sites occurring every 205 ± 15 base pairs. Griffith (9) has measured contour lengths of DNA of simian virus 40, naked and in its histone-associated form, and found a packing ratio of 7 ± 0.5:1. Oudet *et al.* (10) have obtained similar values for the number of base pairs per repeating unit and for the packing ratio in chromatin from other sources. Here we report evidence in support of the remaining quantitative feature of the model, the number of histone molecules in the repeating unit.

MATERIALS AND METHODS

Dimethyl suberimidate (Me_2Sub) was prepared according to Davies and Stark (11). Dithiobis(succinimidyl propionate) from Pierce was a gift of Dr. U. Laemmli to Dr. A. Klug. Native chromatin containing DNA of weight-average size 1600 base pairs was prepared from rat liver nuclei as described (12). Chromatin monomer (pure fraction containing 200 base pairs of DNA) was prepared and its sedimentation coefficient determined (to ±3%) in 5–28.8% isokinetic sucrose gradients (8) containing 0.2 mM NaEDTA, pH 7. F1 was removed by the tRNA exchange method (13) in 40 mM NaCl/3 mM NaEDTA, pH 8.

Procedure for Crosslinking. Buffers were 70 mM sodium phosphate for pH 8, ionic strength 0.2; 60 mM NaCl/100 mM sodium borate for pH 9, ionic strength 0.1; and 1.95 M NaCl/100 mM sodium borate for pH 9, ionic strength 2.0. Chromatin in buffer was treated at 23° with Me_2Sub, freshly dissolved at 20 mg/ml in buffer, or with dithiobis(succinimidyl propionate), freshly dissolved at 50 mg/ml in dimethylformamide. Solutions were dialyzed against 0.2 mM phenylmethylsulfonyl fluoride/0.1 mM NaEDTA, pH 7 and freeze-dried for analysis in gels.

Sodium Dodecyl Sulfate ($NaDodSO_4$)/Polyacrylamide Gel Electrophoresis. $NaDodSO_4$/5% polyacrylamide tube gels (14), 0.4 cm × 7 cm, were run at 6 mA per gel. $NaDodSO_4$/polyacrylamide slab gels contained 18% acrylamide and were run according to Laemmli (15) with three modifications: the concentration of Tris buffer in the separating gel was increased to 0.75 M; the ratio acrylamide: *N,N'*-methylene bis-acrylamide was lowered (16) to 30:0.15; and the electrode buffer contained 0.05 M Tris, 0.38 M glycine, and 0.1% $NaDodSO_4$. The slabs were 0.15 cm thick and either 15 cm or 30 cm long. The short gels were run at 30 mA for about 6 hr and the long ones at 4 W or less for about 24 hr, until the bromophenol blue tracking dye had reached the bottom. Tubes and slabs were fixed for at least 1 hr in methanol/acetic acid/water (5:1:5), stained in 0.1% (w/v) Coomassie brilliant blue in the same solvent, and destained by diffusion at 37° in 5% methanol/7.5% acetic acid. Migration was from top to bottom or left to right in all gels and densitometer traces shown.

RESULTS AND DISCUSSION

Native chromatin

Chromatin prepared by a method involving limited nuclease digestion is native, whereas chromatin prepared by conventional methods involving shear is not (12). In the experiments reported here we have either worked directly with the product of nuclease digestion (a mixture of chromatin fragments containing DNA of weight-average size 1600 base pairs, which we refer to as "1600 base-pair chromatin") or with pure chromatin fragments (monomer, dimer, trimer, etc. of the repeating unit) obtained from the mixture by fractionation on a sucrose gradient.

Most of the experiments described here involve di-imidoester crosslinking of the amino groups in histones. Since interactions between the negative charges of DNA phosphates and the positive charges of amino and guanidino

Abbreviations: Me_2Sub, dimethyl suberimidate; $NaDodSO_4$, sodium dodecyl sulfate.

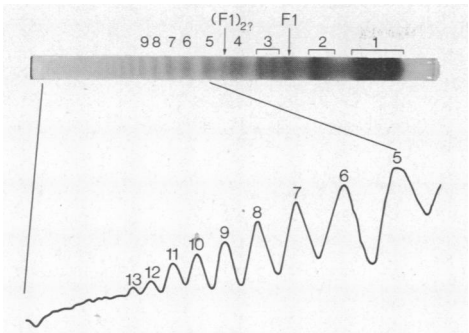

FIG. 1. Crosslinking of chromatin (0.1 mg/ml) with Me_2Sub (1 mg/ml) at pH 8, ionic strength 0.2, for 3 hr.

groups bind the DNA to the histones in chromatin, crosslinking of amino groups might perturb the structure and, in the extreme case, displace the DNA from the histone core. We have investigated this possibility by sedimentation analysis of the chromatin monomer (detected by the absorbance at 260 nm of its DNA) before and after crosslinking. The sedimentation coefficient of the untreated monomer was 11.2 S, and after crosslinking (at pH 9, ionic strength 0.1, for 3 hr) it was 11.3 S; for free monomer DNA a value of 5.3 S would be expected (17). Thus, the structure is not grossly perturbed by crosslinking, and in particular, there is no displacement of the DNA.

A chain of histone octamers in chromatin

We have investigated the associations of histones in chromatin by the crosslinking method of Davies and Stark (11), which we used previously to determine the associations of histones in free solution (1). In this method a dilute solution of a multimeric protein is treated with a di-imidoester, dimethyl suberimidate (Me_2Sub), and the products of crosslinking are analyzed in a $NaDodSO_4$/polyacrylamide gel. The results of treating 1600 base-pair chromatin with

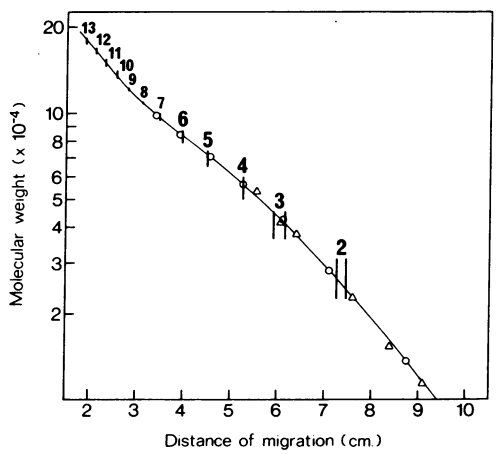

FIG. 2. Molecular weights of products formed by crosslinking of chromatin; comparison with molecular weights expected from a model (7) based on a repeating unit containing two each of F2A1, F3, F2A2, and F2B. A standard curve was constructed from the known molecular weights (1) and mobilities in a 5% gel of the products of crosslinking the $(F2A1)_2(F3)_2$ tetramer (△) and F2A2-F2B oligomers (○). The vertical bars mark the distances of migration of the bands in the gel in Fig. 1. The bars span the ranges of molecular weights of all possible dimers, trimers, etc. expected from the model. Since the bars intersect the standard curve, the molecular weights of the crosslinked products are compatible with the model.

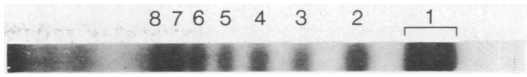

FIG. 3. Crosslinking of chromatin (0.1 mg/ml) with Me_2Sub (3 mg/ml) at pH 9, ionic strength 0.1, for 3 hr.

Me_2Sub at pH 8 and ionic strength 0.2 are shown in Fig. 1. The bands identified as F1 and its possible dimer were absent when the experiment was done on F1-depleted chromatin. The remaining bands, which must have arisen from the other four histones, form a regular progression in spacing and intensity. Since the four histones differ considerably in molecular weight (F2A1, 11,300; F2A2, 14,500; F2B, 13,800; and F3, 15,300), a mixture of all possible crosslinked products would give a complex pattern of bands or smear on the gel, and thus the regular spacing observed is evidence of a limited range of products, for example, the multiples (dimers, trimers, etc.) that would arise from a simple repeating arrangement of the histones. The molecular weights of the crosslinked products were compatible (Fig. 2) with the arrangement of histones proposed previously (7) based on a repeating unit containing two each of the four main types.

The pattern of bands was different in two respects when the pH was raised to 9 (Fig. 3). First, the bands due to F1 were absent; experiments with cleavable crosslinking reagents (J. O. Thomas and R. D. Kornberg, in preparation) showed that F1 was removed to the top of the gel. Second, the bands corresponding to species up to 8-mer were present (those due to 7-mer and 8-mer being relatively more intense), but the bands due to 9-mer and beyond were absent (except for two weak bands near the top of the gel whose mobilities were consistent with their being due to 16-mer and 24-mer). Crosslinking of purified chromatin monomer under these conditions also gave bands corresponding to species up to 8-mer and no bands beyond.

Another crosslinking reagent, dithiobis(succinimidyl propionate), Lomant's reagent, which reacts with amino groups and has roughly the same span as Me_2Sub, gave results at pH 8 identical with those obtained by using Me_2Sub. However at pH 9, crosslinking with Lomant's reagent (Fig. 4) was more extensive than with Me_2Sub: all the histone was converted to 8-mer and multiples (16-mer, 24-mer, and higher molecular weight product). (The product formed with Lomant's reagent was identified as 8-mer by comparison in a slab gel with the products of crosslinking with Me_2Sub at pH 8, ionic strength 0.2.)

These results suggest that there are strong associations of the histones in sets of eight and weaker associations between the sets that break down under the conditions of crosslinking at pH 9. The breakdown of associations could correspond to a transition from a compact state of chromatin to an open one in which the DNA has come partly unwound off the units of structure and extends between them. This would be similar to the effect of lowering the ionic strength observed by Griffith (9). The result obtained for chromatin monomer at pH 9 shows that the sets of eight histones correspond to the units of chromatin structure defined by cleavage with

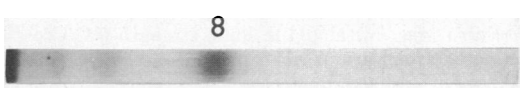

FIG. 4. Crosslinking of chromatin (0.1 mg/ml) with dithiobis-(succinimidyl propionate) (1 mg/ml) at pH 9, ionic strength 0.2, for 1 hr.

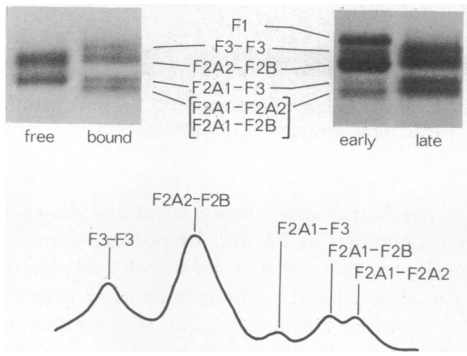

FIG. 5. Dimers formed by crosslinking with Me_2Sub (1 mg/ml). Insets show dimer regions of short slab gels. *Upper left:* comparison of products of crosslinking the histones "free" in solution [a mixture of the tetramer $(F2A1)_2(F3)_2$ and oligomers of F2A2 and F2B, at pH 8, ionic strength 0.2] and "bound" in chromatin (crosslinked for 2 hr as in Fig. 6). *Upper right:* chromatin crosslinked for 15 min ("early") and 2 hr ("late") as in Fig. 6. The densitometer trace shows the dimer region of a long slab gel containing chromatin crosslinked for 40 min as in Fig. 6.

micrococcal nuclease, or in other words, that a set of eight histones is associated with 200 base pairs of DNA.

Arrangement of histones within the octamer

Analysis of the dimers formed by crosslinking should reveal histone–histone contacts in chromatin. Four bands were resolved in the dimer region of a short $NaDodSO_4$/polyacrylamide slab gel (Fig. 5, "bound") after crosslinking 1600 base pair chromatin at pH 9 with Me_2Sub. The same pattern of bands was obtained from purified chromatin monomer as from 1600 base pair chromatin, showing that all the contacts revealed by the formation of dimers occur within a set of eight histones. Three of the bands had mobilities identical with those of the dimers $(F3)_2$, F2A2-F2B, and F2A1-F3 formed by crosslinking the histones free in solution (Fig. 5, "free") [bands corresponding to $(F3)_2$ and $(F2A1)_2$ formed in solution were too weak to be seen in the photograph]. The fourth band was intermediate in mobility between the bands due to the dimers F2A1-F3 and $(F2A1)_2$ formed in solution, so it could have arisen from either or both of the dimers F2A1-F2A2 and F2A1-F2B; on a longer gel the band was a

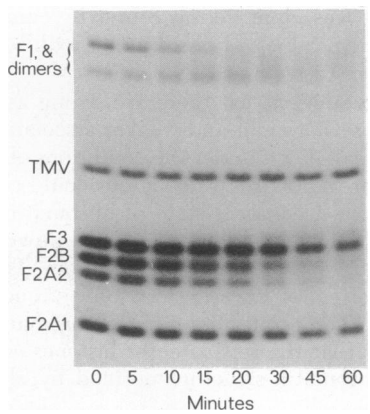

FIG. 6. Time course of crosslinking of chromatin (0.1 mg/ml) with Me_2Sub (1 mg/ml) at pH 9, ionic strength 0.1. Crosslinking was terminated after various times by adding 1 ml of reaction mixture to 0.2 ml of 2 M sodium acetate pH 5 on ice; 25 μg of tobacco mosaic virus (TMV), a gift of Dr. P. J. G. Butler, was also added as an internal standard for densitometry (see Fig. 7). The monomer and dimer regions of a long slab gel are shown.

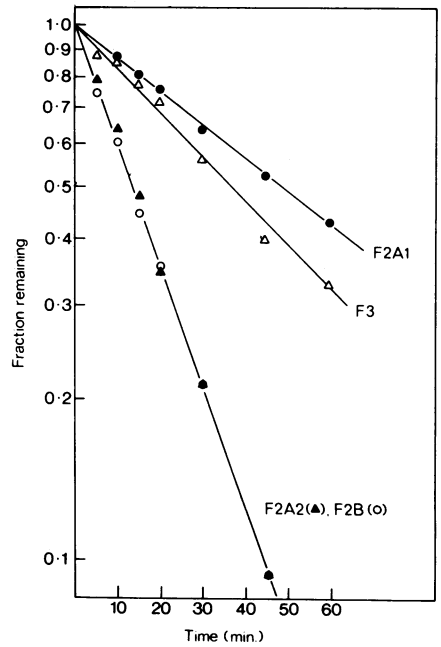

FIG. 7. Time course of crosslinking of chromatin (0.1 mg/ml) with Me_2Sub (1 mg/ml) at pH 9, ionic strength 0.1. The plots were constructed from densitometer traces of the gel in Fig. 6. The peaks in the traces were sufficiently well resolved and constant in width that peak heights could be used as a direct measure of the amounts of protein in the bands. Peak heights were corrected for slight losses during dialysis and handling by normalizing to the peak from tobacco mosaic virus.

doublet (Fig. 5, densitometer trace), so it must have arisen from both of the dimers.

The identification of the dimers described above was confirmed by experiments with cleavable crosslinking reagents (J. O. Thomas and R. D. Kornberg, in preparation). Chromatin crosslinked at pH 9 with these reagents gave the same four dimer bands on a short slab gel as described above, and when the crosslinks were cleaved, the bands (in order of increasing mobility) were found to contain: F3; F2A2 and F2B; F2A1 and F3; and F2A1, F2A2, and F2B. The identification of dimers from these compositions is unambiguous for all bands except the one containing F2A2 and F2B. This could have arisen from some combination of the dimers $(F2A2)_2$, $(F2B)_2$, and F2A2-F2B, which would have similar mobilities in a $NaDodSO_4$ gel since F2A2 and F2B have very similar molecular weights. However, analysis of the kinetics of crosslinking suggested that the band was due to the heterodimer F2A2-F2B. A time course of crosslinking was constructed by following the disappearance of histone bands from the monomer positions in a $NaDodSO_4$ gel (Fig. 6). Semi-logarithmic plots (Fig. 7) confirmed the visual impression that F2A2 and F2B were crosslinked at the same rate, which suggests that they were crosslinked to each other. Their disappearance from the monomer positions in the gel paralleled the appearance of the strong dimer band shown above to contain F2A2 and F2B (see Fig. 5, "early" and "late").

The semi-logarithmic plots showed that F2A1 and F3 were crosslinked more slowly than F2A2 and F2B; consistent with this, the dimer bands containing F2A1 and F3 appeared more slowly than the band due to F2A2-F2B. The plots also showed that although the rates of crosslinking of F2A1 and F3 were similar, they were not identical, suggest-

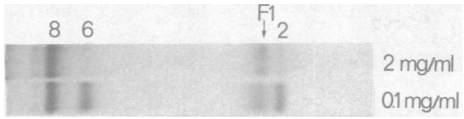

FIG. 8. Crosslinking of histone octamer free in solution. Chromatin was crosslinked at the concentrations indicated with Me_2Sub (1 mg/ml) at pH 9, ionic strength 2.0, for 1 hr and analyzed in short slab gel. (A tetramer band was visible in the gel at the lower chromatin concentration, but was too weak to be seen in the photograph.)

ing that F2A1 and F3 were not crosslinked entirely to each other; in accord with this, the bands due to $(F3)_2$, F2A1-F2A2, and F2A1-F2B appeared more rapidly than that due to F2A1-F3.

A histone octamer free in solution

The dimers F2A1-F2A2 and F2A1-F2B mentioned above are evidence of two interactions between the pairs F2A1,F3 and F2A2,F2B in chromatin. In contrast, no interaction could be demonstrated between the pairs in solution (ref. 1 and Fig. 5 above), possibly because the pairs were damaged by some step during isolation or because they were driven apart in the absence of DNA by repulsion between their positive charges. These difficulties could be circumvented by working with chromatin at ionic strength 2.0 and pH 9 as a means of obtaining the histones free in solution (18) with a minimum of manipulation, and of reducing repulsions. When chromatin was crosslinked at 2 mg/ml under these conditions, two strong bands were resolved in a $NaDodSO_4$ gel (Fig. 8). The band assigned to F1 was absent when the experiment was done on F1-depleted chromatin. The other band had a mobility identical (based on a comparison in a slab gel) with that of the 8-mer formed at pH 8, ionic strength 0.2 (Fig. 1). The same band was obtained when crosslinking was carried out after removal of DNA by gel filtration or sedimentation, showing that the octamer of histones observed did exist free in solution. The relative rates of crosslinking of the histones under these conditions (half-times of 2.5 min for both F2A2 and F2B and 5.3 min and 4.7 min for F2A1 and F3, respectively), and the compositions of the dimers formed at an early stage in the crosslinking, were the same as described above for histones in chromatin at low ionic strength, suggesting that the arrangement of histones in the octamer free in solution was the same as in chromatin.

The octamer began to dissociate when the chromatin concentration was reduced to 0.1 mg/ml (Fig. 8), giving rise to dimer and hexamer (identified by comparison in a slab gel with the products of crosslinking at pH 8, ionic strength 0.2). Experiments with cleavable crosslinking reagents (J. O. Thomas and R. D. Kornberg, in preparation) showed that the dimer contained F2A2 and F2B but virtually no F2A1 or F3, the hexamer contained all four histones but more of F2A1 and F3 than of F2A2 and F2B, and the octamer contained roughly equal amounts of all four histones.

Dissociation of the octamer by loss of a dimer of F2A2 and F2B is in keeping with evidence for pairwise association of the histones in solution (1–6). However, we were unable to reconstitute the octamer from pairs of histones [isolated by the method of Van der Westhuyzen and Von Holt (19)] by simply mixing the pairs in solution at ionic strength 2.0 and pH 9. The histones may have been damaged during isolation despite the mild methods used, and it would seem that future studies on histones in solution should start with the octamer isolated at high ionic strength.

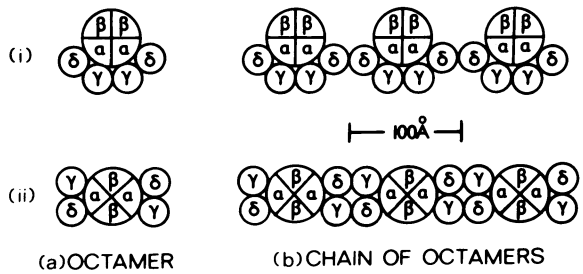

FIG. 9. Possible arrangements of histones (a) within the octamer and (b) in chains of octamers, as previously suggested (7). α, β, γ, and δ represent F2A1, F3, F2A2, and F2B, respectively.

Models

Neither the composition of the octamer nor the arrangement of histones within it is uniquely determined by the crosslinking results described above. However, dissociation of the octamer by loss of a dimer of F2A2 and F2B (see above) and the previous finding (1–3) of a homogeneous tetramer $(F2A1)_2(F3)_2$ do suggest an octamer of composition $(F2A1)_2(F3)_2(F2A2)_2(F2B)_2$. Two families of arrangement within the octamer can arise from dimers and a tetramer (Fig. 9a): in (*i*) the dimers of F2A2 and F2B are in contact; in (*ii*) they are not. The octamers can be joined in various ways to form chains. The two ways shown in Fig. 9b are those suggested previously (7), in which F2A2 and F2B occur (*i*) as a polymer or (*ii*) as pairs of dimers.

Five of the 10 possible types of histone–histone contact within an octamer are revealed by the formation of crosslinked dimers (Fig. 5). Additional contacts may occur but not lead to the formation of dimers. For example, F3 may touch on F2A2 and F2B but be crosslinked so slowly compared with the formation of the dimer F2A2-F2B that only the trimer F3-F2A2-F2B and higher multimers are observed. Although Fig. 9 was drawn with the present results on crosslinked dimers in mind, additional interactions are readily accommodated in both families of arrangement.

Formation of the crosslinked dimers F2A1-F2A2 and F2A1-F2B is direct evidence of contact between the pairs F2A1,F3 and F2A2,F2B. This is in keeping with our previous finding (1) that both pairs are required together with DNA to generate the x-ray diffraction pattern characteristic of chromatin. It is also in keeping with the work of D'Anna and Isenberg (20) and Clark *et al.* (21) but not with the suggestion of Hyde and Walker (22) that the pairs F2A1,F3 and F2A2,F2B do not interact.

The crosslinking results also give information about F1. There is little if any crosslinking of F1 to the other four histones, either in chromatin or in solution. However, F1 is crosslinked to itself in chromatin, giving a low molecular weight product, possibly dimer, at pH 8, and high molecular weight product at pH 9. Two observations suggest that this pH dependence reflects a change in reactivity of F1 (due to its high lysine content) rather than dissociation from chromatin and aggregation in solution: no high molecular weight product is formed in crosslinking of purified chromatin monomer (which does contain F1) at pH 9; and F1 exists as a monomer in solution over the pH range 7–9 (ref. 1 and results above). The results are all consistent with a picture in which one F1 molecule is bound to the DNA on the outside of every repeating unit of chromatin structure (7), and in which F1 molecules on adjacent units or neighboring fibers interact (23, 24).

We thank V. Furber for expert technical assistance and our colleagues for many helpful discussions. J.O.T. thanks the Science Research Council for a grant (B/RG/3508.3).

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