ULTRACENTRIFUGATION AS A PROBE OF CONFORMATION OF UNHYDROXYLATED PROCOLLAGEN AND COLLAGEN

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SUMMARY

Unhydroxylated and hydroxylated procollagen and collagen were compared by zone velocity and isopycnic centrifugation. The sedimentation coefficient of unhydroxylated procollagen(3.7 S) was slightly less than that of hydroxylated procollagen (3.9 S) and the sedimentation coefficient of unhydroxylated collagen (2.9 S) was slightly less than hydroxylated collagen (3.2 S). These differences could be accounted for largely by the slight increase in molecular weight and density of the hydroxylated molecules. The results indicate that the unhydroxylated molecules are in a triple helical conformation composed of three chains rather than analogous helical structures formed by the back-folding of individual chains. We conclude, therefore, that previous experiments demonstrating the decreased thermal stability of unhydroxylated collagen relative to hydroxylated collagen have measured the denaturation of true triple helices.

Under conditions in which hydroxylation was inhibited, chick tendon fibroblasts synthesized procollagen which was retained intracellularly at 37° as three disulfide-linked chains in a random coil conformation (1). When this unhydroxylated procollagen was extracted from the cells at 4° with 0.5 M acetic acid, it appeared to reform triple helical structures as measured by resistance to pepsin digestion at 15°, optical rotation, and by the ability of the molecules to form normal segment long spacing at 4° (2-4). However, the first two techniques may not unequivocally distinguish between true triple helices composed of three chains and analogous, though perhaps less stable conformations in which the individual chains fold back upon themselves (5, 6). In the third technique precipitates are examined in the electron microscope and it is difficult to estimate the fraction of molecules found in segment long spacing. The question of the exact conformation of these unhydroxylated molecules is important since they have been shown to have a denaturation temperature of 24° in comparison to a denaturation temperature of 39° for fully hydroxylated collagen;

this observation has been interpreted to mean that hydroxyproline contributes significantly to the thermal stability of the collagen triple helix (2, 3).

Since all back-folded, more compact conformations would sediment more rapidly than fully extended triple helical molecules (7), we have used zone sedimentation velocity to distinguish between these possible alternatives. We have found that the sedimentation coefficient of unhydroxylated procollagen was 3.7 S and the sedimentation coefficient of hydroxylated procollagen was 3.9 S. As expected, the unhydroxylated and hydroxylated collagens obtained by pepsin digestion of procollagen at 15° sedimented more slowly, with sedimentation coefficients of 2.9 S and 3.2 S respectively. Thus, pepsin-resistant, unhydroxylated procollagen sedimented as triple helices composed of three chains. The results indicate, therefore, that previous experiments performed on these unhydroxylated molecules have in fact measured the denaturation of true triple helices.

MATERIALS AND METHODS

Fibroblasts were isolated from 17-day old chick embryo tendons as previously described (2). Subsequently, 10^7 cells/ml were incubated for 3 hr at 37° in Krebs medium containing 2% fetal calf serum, 25 µg/ml ascorbic acid, [^{14}C]proline or [^{3}H]proline, and either no α,α'-dipyridyl or 5×10^{-4} M α,α'-dipyridyl. Secreted, hydroxylated procollagen was prepared from the medium and unhydroxylated procollagen was extracted from the cells with 0.5 M acetic acid and prepared as previously described (2). Collagen was prepared from the procollagen by digesting the sample in 0.5 M acetic acid with 100 µg/ml pepsin for 16 hr at 15° (2). After digestion the samples were dialyzed against 0.4 m NaCl, 1 mM disodium EDTA[1], 0.1 M Tris·HCl, pH 7.4 at 4°. The untreated procollagen samples were also dialyzed against the same buffer solution.

Aliquots of the hydroxylated and unhydroxylated procollagen and colla-

[1]Abbreviation: EDTA, ethylene diamine tetraacetic acid.

gen samples were analyzed by zone velocity sedimentation at 6° by the method of Martin and Ames (8) using a 5-20% sucrose gradient containing 0.4 M NaCl, 1 mM EDTA, 0.5 M urea and 0.1 M Tris·HCl, pH 7.4. A sedimentation coefficient of 3.2 $S^{\circ}_{20,w}$ was taken for pepsin digested collagen as determined by Beier and Engel (9) and all sedimentation coefficients were calculated relative to this standard. Isopycnic ultracentrifugation in a CsCl density gradient with 0.02 M sodium phosphate buffer, pH 7.4 and 1 mM EDTA was performed in the Spinco Ti50 fixed angle rotor at 6° (10). The mean density of the solution was 1.347 (2.75 M) measured at 20°.

RESULTS AND DISCUSSION

When [^{14}C]proline-labeled hydroxylated procollagen was sedimented with [^{3}H]proline-labeled unhydroxylated procollagen, the unhydroxylated molecules sedimented slightly more slowly (Fig. 1A). Approximately 55% of the [^{3}H]proline was found in a sharp single peak; the remainder was found in slower sedimenting, more heterogeneous molecules, and no significant amounts of more rapidly sedimenting molecules were observed. This result is consistent with previous polyacrylamide analyses of unhydroxylated procollagen preparations in which approximately 63% of the radioactivity was found in procollagen chains and the remainder in lower molecular weight material (2). The [^{14}C]proline-labeled hydroxylated procollagen sedimented as a single peak which contained 86% of the total radioactivity found in the sample. This observation is also consistent with polyacrylamide gel electrophoresis analyses of such preparations (2).

In order to determine whether pepsin-resistant unhydroxylated collagen molecules sedimented similarly to hydroxylated collagen, pepsin digested samples of the two were sedimented together (Fig. 1B). As observed for the procollagen, the hydroxylated collagen sedimented slightly more rapidly than the unhydroxylated. The fraction of unhydroxylated procollagen chains which resisted pepsin digestion and which were found in the single, sharp peak illustrated in Fig. 1B was approximately 72%. This value is consistent with pre-

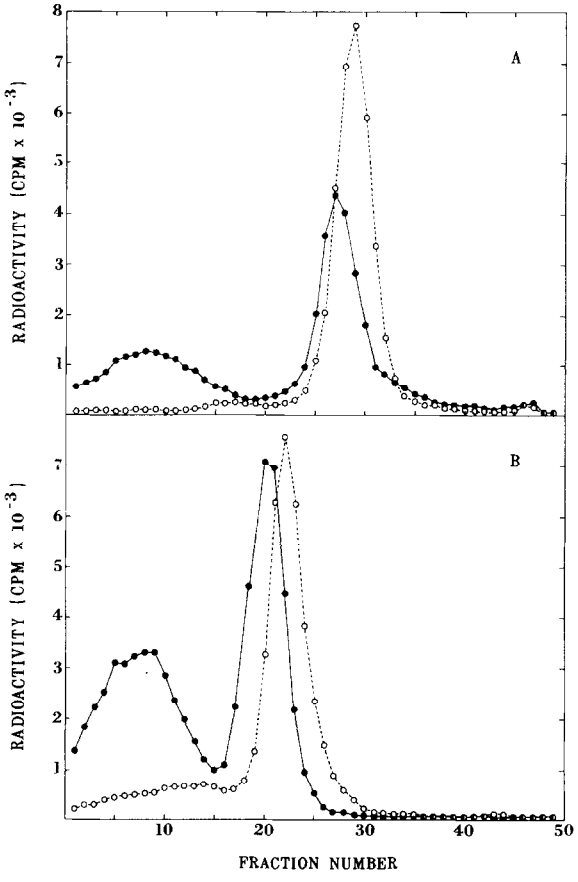

Fig. 1. Zone velocity sedimentation of [^{14}C]labeled hydroxylated (o-o-o) and [^{3}H]labeled unhydroxylated (●—●—●) procollagen (A) and collagen (B). Centrifugation was performed for 18 hr in the SW 50.1 Spinco rotor at 50,000 rpm at 6°. Sedimentation is from left to right. At the end of the run the gradient was displaced by 60% sucrose and 6-drop fractions collected directly into counting vials. A Triton-based counting solution was added and the fractions counted in a Packard scintillation counter (2).

vious estimates of pepsin resistance made by polyacrylamide electrophoresis (2). Sedimentation coefficients of the various samples are listed in Table I.

It is difficult to predict on theoretical grounds what net effect the hydroxylation of proline and lysine would have on the sedimentation behavior of collagen, since the partial specific volume, hydration and conformation may all be affected in addition to the slight molecular weight increase (7). In order to compare the relative densities of hydroxylated and unhydroxylated

TABLE I

Relative Sedimentation Coefficients and Buoyant Densities of Hydroxylated and Unhydroxylated Procollagen and Collagen

Sample	$S_{20,w}$ [a]	Buoyant density [b]
Hydroxylated procollagen	3.9	1.339
Unhydroxylated procollagen	3.7	1.319
Hydroxylated collagen	3.2	1.325
Unhydroxylated collagen	2.9	1.309

[a] Sedimentation coefficients were determined by the method of Martin and Ames (8) using pepsin digested collagen which had a $S^{\circ}_{20,w}$ of 3.2 as a standard (9).

[b] Densities were calculated from the refractive index of the fractions, measured at 20°, while the actual centrifugation was performed at 6° (Figure 2). Thus, the values reported are intended to illustrate the relative densities of the molecules rather than their absolute values.

molecules, procollagen and collagen were sedimented to equilibrium in a CsCl density gradient in which the average CsCl concentration was 2.75 M (Fig. 2). The observed buoyant densities are listed in Table I. Hydroxylated procollagen was more dense than unhydroxylated procollagen as was hydroxylated collagen relative to unhydroxylated collagen. Each type of procollagen was slightly more dense than its counterpart collagen. If we assume that such relative differences in density exist between unhydroxylated and hydroxylated molecules in the somewhat more dilute salt solutions in which the sedimentation velocity experiments were carried out, then such density differences as well as the increased molecular weight can account for the slight differences in sedimentation coefficient between the two types of molecules.

Previous experiments have shown that procollagen synthesized at 37° in the presence of α,α'-dipyridyl to inhibit hydroxylation is found intracellu-

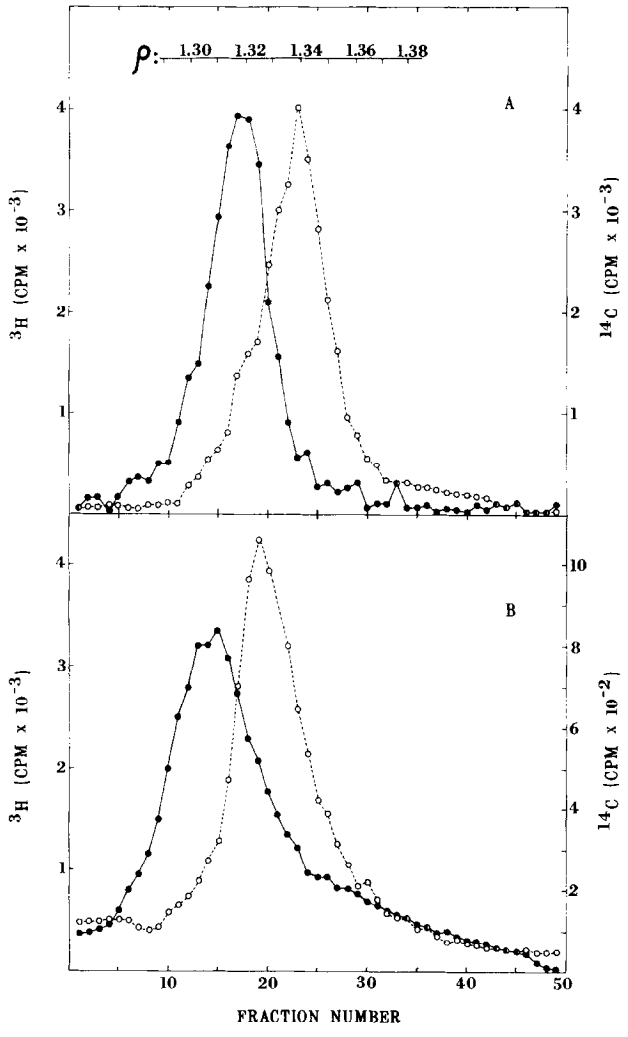

Fig. 2. Isopycnic centrifugation in a CsCl density gradient of [^{14}C]labeled hydroxylated (o-o-o) and [^{3}H]labeled unhydroxylated (●-●-●) procollagen (A) and collagen (B). Centrifugation was as described in the text at 6°. Centrifugal direction is to the right. The density throughout the gradient was determined by measuring the refractive index of the collected fractions at 20°. The relationship between density (ρ) and fraction number was linear so that a density scale has been superimposed on the horizontal axis.

larly as three chains linked by disulfide bonds (1). However, these chains are in a random coil conformation (1) while intracellular hydroxylated procollagen is found largely as triple helices (11). Sometime during the extraction and purification from the cells, the unhydroxylated procollagen forms structures

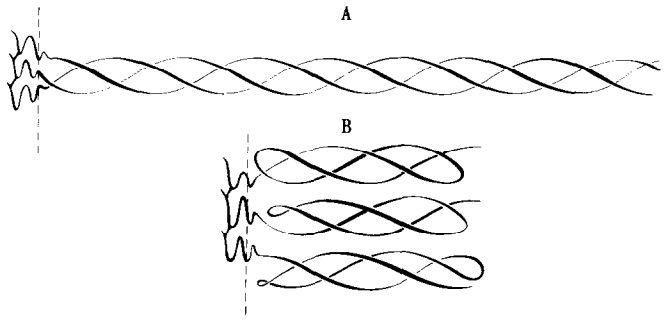

Fig. 3. Proposed models for the structures of refolded unhydroxylated procollagen. Structure A represents helices formed of three separate chains while structure B represents helices formed by the back-folding of individual chains. The dashed line indicates the position of cleavage by pepsin at low temperatures in which the product in both cases would be the size of single α chains upon examination by polyacrylamide gel electrophoresis in sodium dodecyl sulfate.

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which appear to be triple helices by the criteria of resistance to proteolytic enzymes, optical rotation, and characteristic precipitates observed in the electron microscope (2-4). All of the above techniques have pitfalls, and in addition denatured collagen is known to form back-folded triple helical structures when renaturation is allowed to take place at concentrations comparable to those usually employed with procollagen solutions (5, 6). Moreover, the back-folded triple helix appears to be the stable conformational state of the subunit chain in native Ascaris cuticle collagen (12, 13). The two possible helical structures considered here are illustrated in Fig. 3. Note that after pepsin digestion at low temperatures, both yield products which would be the size of single α chains upon exmaination by polyacrylamide gel electrophoresis in sodium dodecyl sulfate, a commonly used technique for estimating the molecular weight of these molecules. Presumably, since the chains in the procollagen studied here are joined at the amino terminus, true triple helix formation would be favored rather than back-folded structures. However, it is important to unequivocally eliminate the possible occurrence of such back-folded structures since they may be inherently less stable than true triple helices (5, 6)

and conclusions drawn from their denaturation properties concerning the stabilizing effect of hydroxyproline would be misleading. Zone velocity sedimentation is a good technique to substantiate the similarity of conformation of unhydroxylated and hydroxylated molecules at low temperatures since back-folded structures would have a markedly reduced axial ratio and hence a greatly increased sedimentation coefficient (7). We observed no such rapidly sedimenting class of unhydroxylated molecules, rather they sedimented slightly more slowly than the hydroxylated ones; this difference can probably be accounted for by the lower molecular weight and density of the unhydroxylated ones. We conclude, therefore, that the unhydroxylated molecules are in triple helices composed of three chains and that previous experiments have measured the denaturation of such true triple helices.

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