Journal of Supramolecular Structure 2:103–107 (1974)

RENATURATION OF DISULFIDE-LINKED PROCOLLAGEN*

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Disulfide-linked, triple-stranded procollagen (pro γ 1 1 2) was isolated from chick embryo skull bones. It was reversibly denatured and renatured as judged by sedimentation properties and susceptibility to digestion with pepsin. Refolding was an intramolecular process and aggregation between molecules did not occur.

Current schemes for collagen synthesis require the self-assembly of procollagen first (for reviews see Refs. 2–6). The original proposal for the self-assembly of procollagen (7) required first association of the N-terminal extension (or registration) peptides of each of the three procollagen chains, followed by folding of the collagen portions into a triple helix. Recently it was shown that association of the registration peptides is accompanied by disulfide linkage between them (8–14), and that this occurs between fully formed procollagen chains (1). The disulfide bridges between the registration peptides are even made when association and folding of the collagen portions of the strands is interfered with by preventing post-ribosomal hydroxylation of proline (15). The disulfide-linked, triple-stranded procollagen of chick skull bones (calvaria) contains two pro α 1 chains and one pro α 2 chain (11, 14, 15) and is called pro γ 112. We have studied the effects of temperature and urea on the denaturation of pro γ 112 (15). In 0.05 Tris, pH 7.5, buffer containing 1 mg Triton X-100/ml, the normal hydroxylated form denatured at approximately 41° and the nonhydroxylated form at approximately 25°. In 0.1 M NaCl, 0.04 M sodium acetate, pH 4.5, buffer containing 1 mg Triton-X100/ml at 20° the hydroxylated form denatured at 6–7 M urea, while the nonhydroxylated form denatured at 0.5–1.0 M urea. The denaturation temperatures of collagens, derived proteolytically from hydroxylated and nonhydroxylated procollagens, are approximately 38° and 25°, respectively (16, 17).

*This is part VI in a series on collagen synthesis. Part V is Ref. 1.

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The tenets of self-assembly suggest that the denatured collagen portions of pro γ 112 should refold upon restoring native solvent-temperature conditions and our report is to this effect. In contrast the reassociation of separated collagen strands is much slower and leads to many imperfect structures (for review of the problem see Ref. 18), indicating the important role of the registration peptides of procollagen in assembly. The translational frictional coefficient of pro γ 112 changes considerably with the state of folding (9), and this enabled us to follow structural changes by velocity sedimentation analysis of radioactive material. Susceptibility to digestion by pepsin was also used to investigate the state of folding, as the native collagen triple helix is resistant to this, while denatured or incompletely folded collagen chains are digested; (the registration peptides do not have the collagen helical form and are also digested).

EXPERIMENTAL PROCEDURE

Calvaria were incubated with [^{3}H] proline for 10 min, extracted with 0.5 M acetic acid containing inhibitors and dialyzed against 6 M urea-containing buffers at pH 4.5, as previously described (15). After dialysis the extract was denatured for 30 min at 40° and the pro γ fraction was isolated by ultracentrifugal velocity-band sedimentation on sucrose gradients containing 6 M urea, 40 mM sodium acetate, 100 mM NaCl and 0.1% Triton X-100 at pH 4.5. Sedimentation was in a Beckman SW56 rotor at 20° for 18 hr at 53 krpm.

Renaturation of isolated pro γ fraction was performed in a dialysis bag. All experiments were made in a standard buffer (100 mM KCl, 0.1% Triton X-100, 50 mM Tris, pH 7.2) to which urea was added, just before use, to various concentrations. First the sample was made 6 M with respect to urea by dialysis and complete denaturation was ensured by heating to 40° for 30 min. After gradually cooling to 22° during approximately 1 hr, the urea concentration was lowered over the next 12 hr to 1 M by dilution with standard buffer. Finally, the sample was cooled to 0° and exhaustively dialyzed against standard buffer for 24 hr.

Velocity sedimentation analysis was performed in standard buffer made 5 M with respect to urea for analysis of renatured materials, and 6 M urea for denatured samples, which were reheated to 40° for 30 min just before analysis. Other details were as previously described (15).

For pepsin digestion samples were dialyzed into 0.5 M acetic acid, 0.1% Triton X-100. Denatured samples were heated to 50° for 10 min. Incubation with 100 μg pepsin per ml was carried out for 18 hr at 0° and then 6 hr at 15°, and the reaction was stopped by adding pepstatin, 200 μg/ml. After addition of standard buffer containing 2 M urea, samples were analyzed by velocity sedimentation in 2 M urea-standard buffer. Collagen marker was prepared by incubating calvaria with [^{14}C]-proline for 3 hr as described (15). Electrophoretic analyses in 5% acrylamide-SDS gels were performed as described (11). Sedimentation coefficients were calculated as described (15).

RESULTS

When isolated, denatured pro γ 112 was renatured, better than 80% of the material became resistant to pepsin digestion, and cosedimented with native collagen marker, as is shown in Fig. 1A. If the denatured pro γ 112 was digested with pepsin it was totally altered and the mixture of products clearly sedimented much slower than native collagen marker, as is shown in Fig. 1B. Figure 1C shows that denatured collagen was digested by pepsin to products which similarly sedimented much slower than native collagen marker. We showed previously (15) that the 2 M urea buffer used in these sedimentation analyses maintains native hydroxylated collagens in that state.

The pepsin-resistant renatured procollagen was not aggregated in any way, i.e., existed as individual native procollagen molecules. This is shown in Fig. 2B, in which 85% of the material sedimented as a single peak of 3.2 S material, and 15% sedimented somewhat faster, probably representing material which had not quite renatured. No material had formed higher aggregates, as these would have been caught by the 60% sucrose pad at the bottom of the centrifuge tube (15). The renatured procollagen sedimented just faster than native collagen marker, as expected from its larger size (11). As a control, the renatured procollagen was again denatured and sedimented under denaturing conditions in

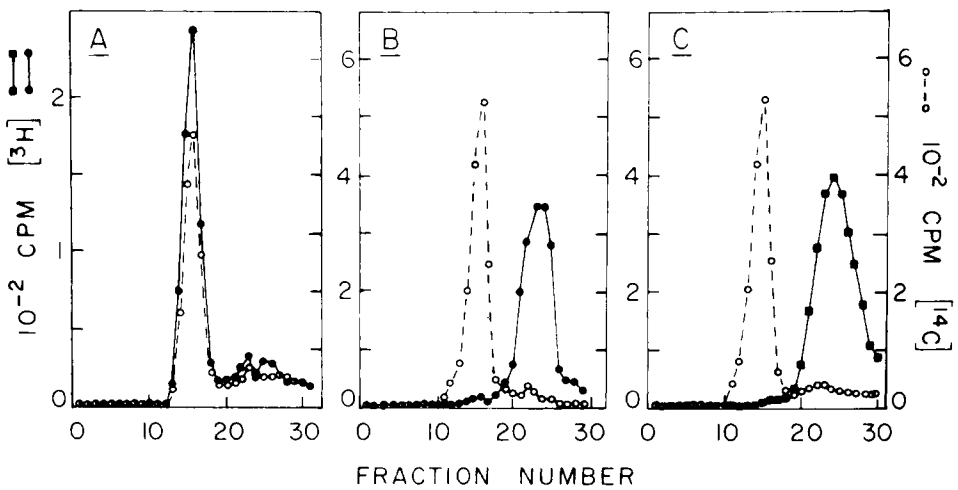

Fig. 1. Pepsin digestion of procollagen and collagen. (A) Renatured [^{3}H] pro γ (●) and [^{14}C] collagen (○) were treated separately with pepsin. The reaction was stopped with pepstatin, and then the samples were mixed and additions to a final concentration of 2 M urea, 40 mM sodium acetate, 100 mM NaCl, 0.1% Triton-X-100, at pH 4.5 were made. Sedimentation from right to left in 5–20% sucrose gradients containing the above was at 7°, for 19 hr at 53 krpm. (B) [^{3}H] pro γ (●) was denatured by warming to 50° for 10 min before digestion with pepsin. [^{14}C] collagen (○) was added as a marker for sedimentation under conditions as in A. (C) [^{3}H] collagen (■) was denatured by warming to 50° for 10 min before digestion with pepsin. [^{14}C] collagen (○) was added as a marker for sedimentation under conditions as in A.

6 M urea. It moved as a single peak with the sedimentation coefficient of 4.4 S, characteristic of denatured pro γ 112.

When renaturation was carried out for various shorter times, such as 2 and 6 hr at 22°, without or with subsequent cooling, sedimentation analyses showed progressive partial renaturation, the sedimentation diagrams being compounded forms of the patterns shown in Fig. 2A and B. Denatured pro γ 112 was also isolated by carboxymethyl cellulose chromatography (11), and this also renatured to better than 70% as shown by the above tests.

Nonhydroxylated pro γ 112, which has a much lower stability than the hydroxylated form (15), could also be substantially renatured at pH 7.2, 0° in the absence of urea. The resistance of the renatured product to pepsin was further checked by SDS-acrylamide gel electrophoresis which yielded collagen α chains comigrating with [^{14}C] marker α chains.

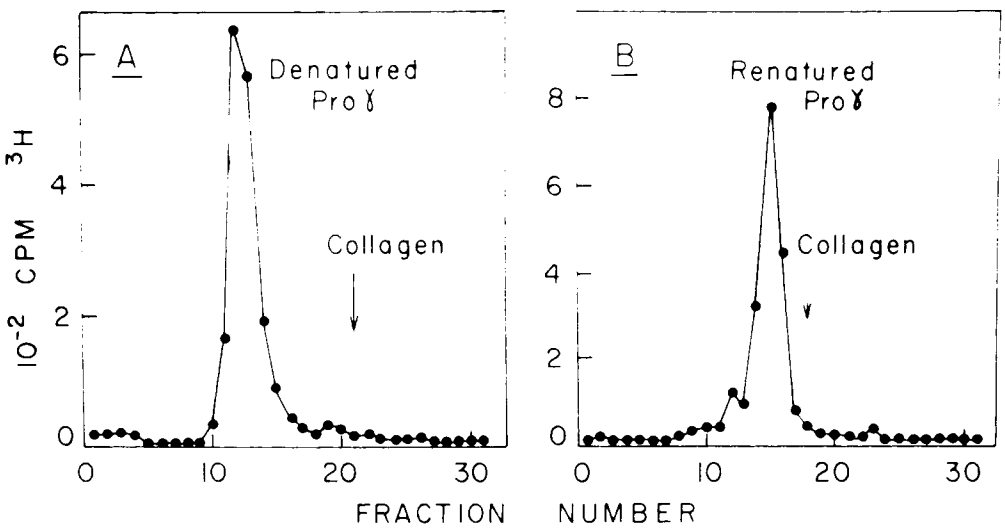

Fig. 2. Sedimentation analysis of denatured and renatured pro γ collagen. Sedimentation is from right to left at 53 krpm, 20° for 20.2 hr in standard buffer, pH 7.2, to which urea was added and a supporting gradient of 5–20% sucrose. A 0.25 ml cushion of 60% sucrose formed the bottom of each gradient. A) Sample denatured at 40° for 30 min before sedimented in 6 M urea. B) Renatured sample sedimented in 5 M urea. Arrows indicate sedimentation peak positions of collagen markers centrifuged as in A and B, in companion tubes.

DISCUSSION

Our evidence clearly shows that the disulfide-linked pro γ 112 isolated by two different procedures renatures readily and in good yield, and that the product is not stabilized by aggregation between chains belonging to different molecules, as tends to occur when collagen is renatured. The concentrations of these solutions are not known but are certainly very low. The refolding is therefore an intramolecular rearrangement.

The association of the registration peptides of procollagen and their disulfide linkage thus appears as the critical step in assembly of procollagen, as this work proves that subsequent folding occurs readily for both hydroxylated and nonhydroxylated states. Although the registration peptides may associate while they are still parts of nascent chains on ribosomes, our investigations indicate that disulfide bridge formation occurs between completed procollagen chains (1). It will therefore be of particular interest to see whether isolated separate procollagen chains can specifically reassociate.

ACKNOWLEDGMENTS

Financial support is gratefully acknowledged from National Institutes of Health Grants AM 13748 and AM 11546, and American Heart Association Research Grant 71-1031.

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