On the state of aggregation of newly secreted procollagen

(collagen/segment-long spacing/dilution-concentration effects/sedimentation analyses/electron microscopy)

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Contributed by Jerome Gross, October 18, 1982

ABSTRACT Procollagen and partially processed procollagen from cultures of primary chicken embryo tendon cells appeared as segment-long-spacing (SLS)-like aggregates when drops of medium were negatively stained and examined by electron microscopy. Similar aggregates were obtained after negative staining of medium partially purified by gel filtration and also after staining thin sections of fixed, dehydrated, and embedded pellets formed by prolonged ultracentrifugation of whole culture medium. In contrast to results from electron microscopy, analysis by velocity density gradient sedimentation or sedimentation equilibrium indicated the exclusive presence of procollagen or partially processed procollagen monomers in solution. These contradictory data can be reconciled if procollagen exists in monomeric form when greatly diluted (as in culture medium), and in specific aggregated form (SLS) at high concentration. We believe that cells *in vivo* secrete procollagen in high, local concentration packaged in the SLS form. We propose that such zero-*D* arrayed packages are the precursors of native collagen fibrils.

The presence of large numbers of procollagen aggregates in zero-*D* array, analogous to packing of adenosine triphosphoric acid (ATP)-produced segment-long-spacing crystallites (SLS) (1), in culture media of freshly released chicken embryo tendon cells and in homogenates of these cells has led us to propose that the procollagen aggregate and not the individual molecule is the kinetic unit for fibril formation *in vivo* (2). SLS-like aggregates found in culture fluids were generally 5–20 nm in diameter and about 300 nm long, bearing large terminal extensions at one end. These aggregates appeared in the culture medium within hours of cell plating and were abundant after 24 hr. They were observed directly by negative staining, after fixation and shadow casting, and also in close association with whole mounted fibroblasts cultured on Formvar films. Bundles of these structures in antiparallel, zero-*D* array, also were seen in large numbers associated with membranous material in embryonic chicken tendon and cartilage homogenates, similar to those seen in thin sections of fixed and embedded fibroblasts (2). It should be emphasized that no extraneous additives (e.g., ATP) were required to form these structures.

Ordered aggregates were identified as collagenous by their size and shape [quite similar to published pictures of purified procollagen SLS aggregates “produced” by ATP (3–5)], their disappearance on exposure to bacterial collagenase (2), and their reduction to three-quarter length by vertebrate collagenase.

These earlier observations left unanswered some important questions as to whether the procollagen SLS aggregates are formed as a result of preparation for electron microscopy or whether they preexist in the culture medium? If the latter is correct, then what proportion of the total secreted procollagen do the aggregates represent? Are they incorporated into striated

fibrils in the SLS form? How does further enzyme processing and their intermolecular rearrangement influence native fibril structure and formation?

This report describes our efforts to further characterize procollagen aggregates in terms of environmental conditions affecting their structure and formation as observed by electron microscopy and by hydrodynamic methods.

MATERIALS AND METHODS

Chicken embryo tendon cells were isolated according to the method of Dehm and Prockop (6). [³H]Proline-labeled procollagen, partially processed and intact, was obtained from the medium of 24-hr monolayer cultures and the medium of 4-hr suspension cultures, respectively. Molecular-sieve chromatography on Bio-Gel P-300 to remove low molecular weight ³H-labeled material, NaDodSO₄ gel electrophoresis, and amino acid analyses were carried out by standard methods.

Density Gradient Velocity Sedimentation. Linear density gradients were formed with sucrose (5–20%) or with deuterium oxide/water (20–95%) at 22°C in standard buffer (50 mM Tris·HCl, pH 7.5/0.15 M NaCl/2.5 mM EDTA/1 mM *N*-ethylmaleimide/1 mM phenylmethylsulfonyl fluoride). The sucrose gradients were formed on top of a 60% sucrose cushion. A 0.25-ml sample was layered on top of each gradient and centrifuged for 16 hr at 20°C in a Beckman SW 65 Ti swinging-bucket rotor operated at 50,000 rpm in a Beckman L8-70 preparative ultracentrifuge. Approximately 20 fractions of 0.25 ml each were collected from a hole in the bottom of the centrifuge tube. Aliquots of 50 μ l were used for the determination of [³H]proline/[³H]hydroxyproline radioactivity.

Sedimentation Equilibrium. The Airfuge technique of Pollet *et al.* (7) was modified for use in the L8-70 ultracentrifuge. Beckman Airfuge tubes (240 μ l) were used in a SW 65 Ti swinging-bucket rotor fitted with special Teflon adapters. The final sample (volume, 100 μ l; radial path length, 5 mm) was made up of an initial step gradient of a 50- μ l sample in buffer containing bovine serum albumin (10 mg/ml) overlaid with 50 μ l of buffer. For the sample with [¹⁴C]albumin, the buffer used was 0.02 M Na phosphate, pH 7.2/0.15 M NaCl/0.02% NaN₃; for the procollagen samples labeled with [³H]proline/[³H]hydroxyproline, standard buffer was used. Equilibrium experiments were performed at 20°C and at 7,500 or 10,000 rpm for 24–90 hr. High-speed runs (40,000 rpm for 18 hr at 10°C) were also carried out, beginning with a uniformly distributed 100- μ l sample containing bovine serum albumin (5 mg/ml) to check for the presence of any nonsedimenting radioactive material (7). The gradients were fractionated within 30 min from the end of the run with the Beckman microtube fractionator.

Abbreviation: SLS, segment-long spacing.

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DATA ANALYSIS

The following equations were used for velocity density gradient centrifugation. The sedimentation coefficient, S , is related to the particle weight, M , by the following equation:

$$S = M(1 - \bar{v}_2 \rho) / Nf$$

where ρ is the solvent density; $\bar{v}_2$, the solute partial specific volume; N , Avogadro's number; and f , the frictional coefficient (8). For rod-like particles of length L and diameter d where $L/d > 60$,

$$f \approx k\eta(L/d)^{0.48} R_o,$$

in which k is a constant; η , solvent viscosity; and R_o , radius of the sphere with the same hydrated volume as the particle,

$$R_o = (\bar{v}_2/16)^{1/3} (Ld^2)^{1/3}, \quad f \approx k\eta(\bar{v}_2/16)^{1/3} L^{0.81} d^{0.19}.$$

The normalized sedimentation coefficient ($S_{w,20}$) for collagen monomers, of length 300 nm and diameter 1.4 nm, is 3×10^{-13} sec (9). Consider a thin SLS aggregate composed of seven collagen molecules—i.e., a central molecule surrounded by a hexagonal shell of six nearest neighbors. The diameter of such an aggregate is 4.2 nm; thus, the ratio of the frictional coefficient of the SLS aggregate (f_{SLS}) to that of the monomer (f_{mon}) is

$$f_{SLS}/f_{mon} = 3^{0.19} = 1.2.$$

The ratio of the sedimentation coefficients is

$$S_{SLS}/S_{mon} = 5.7.$$

For an SLS aggregate of diameter 7.0 nm composed of 19 molecules (i.e., a central molecule surrounded by concentric hexagonal shells containing 6 and 12 molecules, respectively), the ratio is

$$S_{SLS}/S_{mon} = 19/5^{0.19} = 14.1.$$

Electron microscopy has indicated (2) that the average diameter of the procollagen SLS aggregates is 5–10 nm. Thus, the above analysis shows that, if the SLS aggregates do exist in solution with the same diameters as determined by electron microscopy, then sedimentation coefficients should be an order of magnitude greater than that of monomer.

In sedimentation equilibrium experiments, molecular weights were determined from the slope of the $\ln(\text{cpm})$ vs. r^2 plot—i.e.,

$$\ln(\text{cpm}) = \frac{M\omega^2(1 - \bar{v}_2\rho)}{2RT} r^2 + \text{constant},$$

where ω is the angular velocity, R the gas constant, and T the absolute temperature. Partial specific volumes, $\bar{v}_2$, were taken as 0.72 ml/g for [^{14}C]albumin (7) and 0.75 ml/g for procollagen. Solvent density, ρ , was measured to be 1.007 g/ml (7).

RESULTS

The apparent stability of the SLS aggregates, as judged from the electron microscopic results (2), suggested that it would be possible to quantify by using hydrodynamic techniques the proportion of newly synthesized procollagen present in aggregate form. It was desired to determine the extent of aggregation in conditions as close as possible to those pertaining to the original

FIG. 1. SLS crystallites in the negatively stained void-volume peak from Bio-Gel P-300 chromatography of 24-hr chicken embryo tendon fibroblast culture medium. Negative staining was with 4% aqueous sodium silicotungstate. ($\times 82,000$.)

FIG. 2. Fluorogram showing the chain composition of procollagen (pro $\alpha 1$ and $\alpha 2$) in 4-hr chicken embryo tendon fibroblast suspension culture medium (lane a) and partially processed procollagen (pC $\alpha 1$ and $\alpha 2$) in 24-hr monolayer culture medium (lane b), as well as collagen chains $\alpha 1$ and $\alpha 2$.

culture medium—i.e., in the presence of noncollagenous material that might be involved in stabilizing the SLS aggregates. Thus, purification was restricted to nonprecipitating procedures such as gel filtration, after which SLS aggregates were observed in the radiolabeled, eluted peaks and in samples where total $[^3\text{H}]$ proline/ $[^3\text{H}]$ hydroxyproline radioactivity could be used to monitor the state of aggregation of procollagen molecules in subsequent centrifugation experiments (see below).

Total recovery of nondialyzable ^3H cpm after Bio-Gel P-300 chromatography of the 24-hr monolayer medium was at least 90%, and 76% of these cpm was incorporated into proteins that were eluted in the void volume. The void-volume fractions were pooled and analyzed by electron microscopy, NaDodSO₄/polyacrylamide gel electrophoresis, and fluorography. Large numbers of well-formed procollagen SLS were present in the void-volume peak (Fig. 1). Radioactivity in this peak was distributed among four distinct bands on electrophoresis (Fig. 2, lane b). The hydroxyproline/proline ratio was 0.94:1, and pepsin digestion of this material resulted in all of the radioactivity appearing in the lower two bands. By comparison with collagen standards, the lower bands were assigned to collagen chains $\alpha 1(\text{I})$ and $\alpha 2$. From the well-documented (10, 11) order of procollagen processing in this system, the upper two bands in Fig. 2, lane b were assigned to the partially processed procollagen chains designated pC $\alpha 1(\text{I})$ and pC $\alpha 2$ —i.e., procollagen chains with NH₂ propeptides not visible.

FIG. 3. Velocity sedimentation analysis of 24-hr fibroblast culture medium (i.e., partially processed procollagen). The medium was partially purified by Bio-Gel P-300 chromatography, and the void-volume peak was centrifuged in a SW 65 Ti rotor for 16 hr at 50,000 rpm in a 5–20% linear sucrose gradient. The position of the radioactive peak indicates the expected sedimentation coefficient for monomers. An identical experiment with 4-hr suspension culture medium (i.e., with procollagen) also gave the same S value for monomers. The arrow shows the direction of sedimentation. Hyp, hydroxyproline.

FIG. 4. Velocity sedimentation analysis of 24-hr (partially processed procollagen) culture medium chromatographed on Bio-Gel P-300 and centrifuged for 4 hr at 4°C in a SW 65 Ti rotor at 50,000 rpm in a 20–95% $^2\text{H}_2\text{O}$ linear gradient. The sedimentation coefficient indicates the presence of monomers, but SLS were seen in negatively stained drops of the peak fraction. The arrow shows the direction of sedimentation. Hyp, hydroxyproline.

Gel filtration of the 4-hr suspension culture medium showed a recovery of nondialyzable ^3H cpm of at least 95% (84% was found in the void-volume peak). SLS aggregates were seen in void-volume fractions, and electrophoretic analysis of this material (Fig. 2, lane a) showed that the ^3H radioactivity corre-

FIG. 5. Sedimentation equilibrium centrifugation of ^{14}C -labeled bovine serum albumin ($\blacksquare$), procollagen ($\bullet$), and partially processed procollagen ($\circ$) indicates particle weights of $65,900 \pm 5,400$ ($N = 5$); $372,000 \pm 19,000$ ($N = 6$); and $326,000 \pm 28,000$ ($N = 6$), respectively. The sedimentation buffer contained 5 mg of bovine serum albumin per ml for stabilization. The ^{14}C albumin analysis, used as an internal control, confirmed the validity of the technique. SLS were seen by electron microscopy in the samples containing albumin immediately prior to centrifugation.

FIG. 6. Thin section of a pellet formed by high-speed centrifugation (40,000 rpm for 48 hr) of 48-hr chicken embryo tendon fibroblast culture medium. The pellet was fixed with Karnovsky's fixative, post-fixed with OsO_4 , stained *en bloc* with uranyl acetate, dehydrated, and embedded in Epon. Sections were stained with uranyl acetate and lead citrate. Note the wide SLS forms. ($\times 90,000$.)

sponds to two bands which, by analogy with other observations on this system (4, 12), were assigned to procollagen chains pro $\alpha 1(I)$ and pro $\alpha 2$. Thus, total $[^3\text{H}]$ proline/ $[^3\text{H}]$ hydroxyproline cpm in the void-volume peak fractions after chromatography on Bio-Gel P-300 could be used as a specific label for procollagen or partially processed procollagen, under conditions where SLS aggregates were observed by electron microscopy.

From amino acid analyses, maximum protein concentration in the pooled void-volume fractions for both procollagen and partially processed procollagen samples was about $10 \mu\text{g}/\text{ml}$.

Sedimentation coefficients ($S_{w,20}$) were determined for the Bio-Gel P-300-purified procollagen (4-hr suspension culture) and partially processed procollagen (24-hr primary monolayer culture) by velocity sedimentation in sucrose and $^2\text{H}_2\text{O}$ gradients. Sucrose gradients show only a single peak at $S = 3 \times 10^{-13}$ sec for both the unprocessed (4 hr) and partially processed (24 hr) (Fig. 3) procollagen. This is the expected sedimentation coefficient for monomers. Total recovery of ^3H throughout all fractions of the sucrose gradient was approximately 40%.

Because of the difficulties in making electron microscopic observations of gradient fractions in the presence of sucrose, and because sucrose may have caused the dissociation of SLS aggregates into monomers, sedimentation coefficients in an $\text{H}_2\text{O}/^2\text{H}_2\text{O}$ density gradient were determined. Recoveries of $[^3\text{H}]$ proline-labeled material for the entire gradient were about 80% of that in starting solutions. Again the results (Fig. 4) showed a single peak sedimenting at $S = 3 \times 10^{-13}$ sec, with no evidence of any more rapidly sedimenting species. However, examination of the peak fractions by electron microscopy re-

vealed typical SLS aggregates (not shown). Thus, whereas the sedimentation coefficients indicated the exclusive presence of procollagen or partially processed procollagen monomers, electron microscopy of the peak fractions showed SLS aggregates.

In analysis by sedimentation equilibrium with an initial step gradient, equilibrium was attained for both Bio-Gel P-300-purified procollagen and -purified partially processed procollagen after 65 hr at 7,500 rpm and 20°C .

No change in the shape of the gradient was observed after centrifugation up to 90 hr. For $[^{14}\text{C}]$ albumin samples, equilibrium was attained in 41 hr (again from an initial step gradient). The presence of nonsedimenting ^3H - or ^{14}C -labeled material (9) was checked by centrifugation at 40,000 rpm for 18 hr at 20°C . No such material was found for the procollagen or partially processed procollagen samples. Total ^3H or ^{14}C cpm recoveries in experiments were $\approx 90\%$. The results (Fig. 5) demonstrated excellent linearity and permitted accurate estimates of particle weights. Thus, sedimentation equilibrium analysis indicated the sole presence of monomers of procollagen or partially processed procollagen. No deviations from linearity of the $\ln(\text{cpm})$ vs. r^2 plot were observed near the base of the centrifuge tube, which if present, would have been indicative of aggregation.

High-speed, prolonged velocity sedimentation in standard buffer of labeled crude medium from monolayer cultures resulted in complete depletion of radioactive material from the supernatant fluid and the formation of a small, compact pellet. Electron microscopic examination of the sectioned pellets revealed large areas of procollagen SLS similar to, although usu-

ally broader than, those deposited from drops of culture medium (Fig. 6).

DISCUSSION

The presence of intracellular cigar-shaped, membrane-bound structures about 300 nm in length were interpreted by Trelstad (13) to be packages of newly synthesized procollagen awaiting secretion. Subsequently, others (14–18) reported more readily identifiable SLS-type aggregates with collagen-like band patterns both within and just outside cells in tissue sections. The broad question addressed here is whether procollagen normally might be secreted in local domains of high concentration that are ordered in the form of SLS (or zero-*D*) staggered aggregates or whether the aggregates seen in tissue sections are a rare event—a transient form that rapidly disperses to monomers before fibril formation.

The problem arises with the reproducible appearance of SLS in large numbers from a drop of culture medium and in cell homogenates in the absence of ATP or other added aggregating agent. There is no way of evaluating from the electron micrographs what proportion of the total procollagen is in the aggregate form, if any, prior to drying and staining of the drop on the grid. The experiments in which the conditions of the medium were broadly altered prior to grid preparation—high and low pH or ionic strength; the presence of detergents such as NaDodSO₄, Triton X-100, and Brij; and various methods of grid drying, including the “drop-wash” technique of Schwartz and Veis (19) and the presence of SLS aggregates lying on top of each other—indicate that the procollagen aggregates are not the result of drying patterns reflecting the effect of surface tension.

We anticipated that the aggregates would be present, preformed, in the culture medium but, as mentioned, could not estimate what proportion of the total procollagen they might represent. However, density gradient velocity sedimentation analysis in both sucrose and ²H₂O confirmed previously obtained sedimentation coefficients (20), which corresponded to monomer molecular weight. Direct measurements of particle weight by sedimentation equilibrium yielded values of ≈350,000, again consistent with monomer weight, with no indication of a rapidly sedimenting component.

However, in experiments in which similarly prepared media were sedimented in high centrifugal fields, pellets containing all of the radioactivity and large arrays of SLS-like aggregates were found.

We are faced with two clearly conflicting sets of data. There are a number of explanations for this discordance arrived at by two very different approaches. One we favor at this time is based on the fact that the material revealed by the electron microscope was deposited on the grid at a high concentration at the last moments before drying. Strong support is furnished by those experiments in which all of the procollagen in solution was highly concentrated in the hydrated pellet by prolonged sedimentation in a strong centrifugal field and in which, after fixation and staining of the pellet, both procollagen SLS and collagen fibrils in large numbers were observed in thin sections of the embedded material. How much of the total procollagen was in the ordered aggregate form cannot be determined; however, from the dimensions of the pellet compared with the volume and procollagen concentration of the starting solution (about 10 μg/ml), we estimate a minimum of 16 mg/ml.

Procollagen under analysis in the ultracentrifuge was in solution at low concentration. We propose that at high concen-

tration, procollagen monomers associate in a preferred zero-*D*-arrayed aggregate, which readily dissociates on dilution. There is a good precedent for this phenomenon recently described for spectrin, a major fibrillar structural protein of the erythrocyte. Morrow and Marchesi (21) conclude that the fibrillar state of spectrin in the cell is a consequence of its high degree of dependence on concentration, as duplicated in their *in vitro* experiments with the isolated purified protein.

We propose here that procollagen is secreted in high local concentrations in the tissue, under which condition its preferred state is the zero-*D*-array, or SLS, form. In cell culture, however, under the artificial conditions of great dilution in the liquid environment (the culture medium), the aggregates readily dissociate to monomers. Thus, it would appear to us that the conditions of preparation for electron microscopy may, in this particular case, more nearly approximate conditions *in vivo*.

We have suggested previously that the SLS procollagen aggregates, further described here, may be immediate precursors of the fibril *in situ* and that they may overlap one another in polarized, *D*-staggered array, giving rise to the characteristic axial period. Molecular rearrangements within the fibril, in association with enzymatic processing, may modify the structure. Recent x-ray diffraction and electron microscopic studies of fixed and embedded tendon fibers reported by Hulmes *et al.* (22) and the occurrence of “polyα1CB6” (23, 24) provide increased support for this concept.

This work was supported by the U.S. Department of Health and Human Services Grants AM3564 and EY02252. This is publication no. 906 of the Robert W. Lovett Memorial Group for the Study of Diseases Causing Deformities.

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