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Biochemistry

Triple-helix formation on ribosome-bound nascent chains of procollagen: Deuterium-hydrogen exchange studies

(collagen synthesis/post-translational modifications/chain registration)

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ABSTRACT **Polyribosomes containing nascent [^{3}H]proline-labeled collagen chains were isolated from chick embryo fibroblasts in culture. These nascent chains were nearly completely hydroxylated, as indicated by the presence of [^{3}H]hydroxyproline and high hydroxyproline/proline ratios. The polyribosomes were suspended in D_2O at 15° and the infrared spectrum was determined using a reference cell containing collagen-depleted polyribosomes in D_2O, matched to equal RNA content. The amide I and amide II bands were observed. When the polyribosomes were heated in D_2O at 44° in the infrared cells, the N–D amide II absorbance at 1480 cm^{-1} increased markedly, indicating that H → D exchange had occurred. Collagen-depleted polyribosomes showed no such changes in absorbance at 1450–1480 cm^{-1} upon heating. Polyribosomes recovered from the infrared cells after treatment at 44° and cooling still contained collagen, as indicated by their [^{3}H]hydroxyproline content. These data indicate that nascent collagen bound to the polyribosomes can assume a hydrogen-bonded structure. Taken with prior data showing that the nascent collagen was also resistant to pepsin digestion, it is suggested that the collagen examined is in triple-helix conformation. Because the nascent polyribosome-bound collagen is nearly fully hydroxylated, it must be considered that triple-helix formation can occur between nascent chains while they are attached to the endoplasmic reticulum surface and that chain association and triple-helix formation *in vivo* may well occur before rather than after release.**

Recent studies have demonstrated that the thermal stability of the collagen triple-helix is directly related to the degree of hydroxylation of the constituent peptide chains (1). Because it has also been found that hydroxylation of proline ceases when the collagen peptide chains are in triple-helix conformation (2), triple-helix formation has clearly occurred after hydroxylation. It has been hypothesized that hydroxylation takes place in the cisternal space of the endoplasmic reticulum after release of the nascent chains from their ribosomes, and hence, that triple-helix formation is a late event in which separated chains must find each other and register properly (3). This postulate was reached on the basis of the susceptibility of nascent polyribosome-bound or newly released protein to pepsin digestion.

Isolated polyribosomes from chick embryo fibroblasts do commit a major part of their synthesis machinery to procollagen production, but we have demonstrated (4, *) that only about 30% of the newly synthesized protein of the polyribosome fraction is in peptides with the typical collagen triplet -Gly-X-Y- repeat sequences. We have also shown, in support of other studies (5–7), that both proline (4, *) and lysine (8) in sequences of nascent polyribosome-bound collagen are hydroxylated. Treatment of [^{3}H]proline-labeled polyribosomes containing hydroxylated nascent collagen with pepsin releases about 67% of the radioactivity but only about 28% of the hydroxyproline. The remaining hydroxyproline was released only after treatment of the polyribosomes with purified bacterial collagenase (4, *). Moreover, the pepsin-resistant collagen was nearly fully hydroxylated. These data were taken to signify that pepsin-resistant, and hence presumably triple-helical, collagen could be formed while nascent collagen chains were still bound to polyribosomes. This conclusion has many implications in considering the *in vivo* processes of chain registration and triple-helix formation.

The enzyme probe techniques, while widely used, are nevertheless indirect measures of the triple-helix conformation. Another property of collagen chains in triple-helix formation is the presence of hydrogen bonds of interchain character. Native collagens, or collagen-fold triple-helix units, placed in D_2O retain the characteristic infrared N–H deformation amide II band at 1565 cm^{-1}. This is replaced by an absorbance of about 1450 cm^{-1} upon denaturation and substitution of N–D for N–H hydrogen bonds. This transformation is readily measured, even for low levels of collagen-fold formation (9). We therefore set out to examine by infrared absorbance measurements the D → H exchange process in the polyribosome-bound nascent collagen chains. Estimates of the amount of collagen on the ribosomes suggested that their exchange spectra might be detectable if suitable blanks could be used.

These studies show directly that triple-helix formation can occur among nascent collagen chains while they are still attached to the polyribosome, because N–H hydrogen bonds persist until denaturing temperatures are reached, at which time the N–D amide II absorbance is increased.

EXPERIMENTAL PROCEDURES

Polyribosomes with Nascent Collagen. The procedures for isolation of chick embro fibroblasts, their culture, labeling with [^{3}H]proline, disruption, and subcellular fractionation, and sucrose density gradient fractionation of the polyribosomes have all been described in detail (4, 8,*). It is important to emphasize that the temperature was dropped to 4° immediately after protein synthesis was stopped by the addition of cycloheximide to the culture, and the cells were dislodged by trypsinization. Gradients were centrifuged at 2–4°, but the gradient fractionation was at room temperature (about 25°). Polyribosomes were isolated on a preparative scale using large 38.5-ml gradients in an SW27 rotor. Still, to obtain sufficient material, the polysomes from several gradients had to be combined for each study. The combined polysome fraction was diluted in buffer without sucrose and pelleted by low-speed centrifugation.

In order to be sure that the polysomes utilized contained bound nascent collagen even after thermal denaturation, [^{3}H]proline-labeled polysomes were used. The polysomes were collected from the infrared cells after the conclusion of the

* A. G. Brownell and A Veis, unpublished data.

exchange experiments and hydrolyzed in 6 M HCl for 20 hr. The hydrolysates were examined in an amino acid analyzer utilizing a stream splitting device. Radioactivity attributable to hydroxyproline, and hence the presence of collagen, was thus determined directly.

Polyribosomes Cleared of Collagen. Polyribosomes were digested with bacterial collagenase (CLSPA grade, Worthington) further purified by the method of Peterkofsky and Diegelmann (10). The digestions were done in a 0.01 M Tris (pH 7.4), 0.24 M KCl, 0.01 M $CaCl_2$, and 0.01 M $Mg(CH_3COO)_2 \cdot 4H_2O$ buffer, made 2.5 mM in *N*-ethylmaleimide to inhibit other nonspecific proteases. At least 90% of the hydroxyproline is released from the ribosomes by this treatment*. Moreover, the polysomes remain undegraded by the collagenase, in contrast to exposure to either ribonuclease or a protease such as trypsin (4).

Collagen and Gelatin for Reference Spectra. Acid-soluble collagen was extracted from rat-tail tendon with 0.5 M acetic acid and purified by repeated precipitation with 10% NaCl. Gelatin was prepared by thermally denaturing the acid-soluble collagen.

Infrared Spectra. An aliquot of pelleted polysomes was suspended in 2 ml of D_2O, of which 0.2 ml was injected into the infrared cells. Spectra were obtained using a Beckman IR-12 spectrophotometer. One hundred-fold vertical scale expansion was used for selected regions. Transmission spectra were obtained with the photometer in the double beam mode with D_2O or collagenase-treated polysomes in the reference cell. Water jacketed FH-01 cells (Research and Industrial Instruments Co., London) were used with Irtran-2 windows (International Crystal Laboratories, Irvington, N.J.).

The water flow was arranged so that the temperature could be alternated between two levels during the course of an analysis. Spectra were taken at 15°; the photometer was then set at the absorption maximum of interest, 1480 or 1565 cm^{-1}. The temperature was raised to 44° in both sample and reference cells to denature the collagen or other proteins and all contents were held at that temperature for 10–30 min. After exchange was complete, the temperature was returned to 15° and, after thermal equilibration, the spectrum was recorded. The final cycling to the original measurement temperature was required for quantitation because small dimensional changes in the cell and mounting were noted on going from 15 to 44°.

RNA Content of Polyribosomes. A modification of the orcinol procedure (11) was used to determine the ribonucleic acid content of the polyribosomes.

RESULTS

The sucrose density gradient distribution of the post-mitochondrial supernatant fraction of the fibroblasts is illustrated in Fig. 1. Gradient fractions 1 through 22 were pooled in order to obtain sufficient polyribosomes, although this pool obviously contains a heterogeneous range of polyribosome sizes. These polyribosomes did contain [^{3}H]hydroxyproline and, hence, collagen triplet sequences that had been hydroxylated. The average [^{3}H]hydroxyproline/[^{3}H]proline ratio, *R*, was 0.11. After collagenase treatment *R* decreased to 0.03, indicating that most of the collagen was removed. The collagenase digestions were carried out in dialysis bags* and, although most of the hydroxyproline was removed as dialyzable peptides, the supernatant obtained after centrifugation of the retentate contained larger peptides with an *R* of 0.22, showing preferential release of the collagen from the polyribosomes.

Control Experiments. Measurement of the RNA content of the polyribosomes showed them to have about 0.4 μg of RNA per μg dry weight. Thus, the first concern was to determine if the constitutive ribosomal proteins would obscure the spectra in the amide I-amide II range and if these proteins themselves would show H–D exchange under the conditions of collagen denaturation. The spectrum of collagenase-treated polyribosomes in D_2O at 15° compared to D_2O in the reference beam shows strong absorbance due to protein in the 1700–1400 cm^{-1} region (Fig. 2, spectrum A). Nevertheless, when the reference cell is filled with an equivalent amount of collagen-depleted polyribosomes on the basis of RNA content, the spectrum can be blanked out quite effectively (Fig. 2, spectrum B) without loss in total transmission. The spectrum of the collagen-depleted polysomes in D_2O is unaffected by temperature cycling from 15° to 44° to 15°, indicating that either all the hydrogens are readily exchangeable or that those that are not are not made exchangeable by heating to 44°.

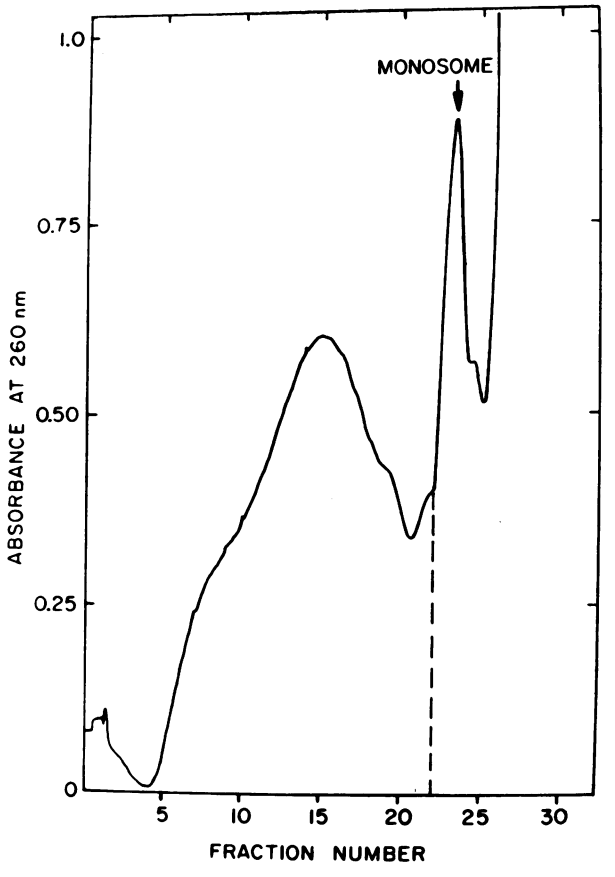

FIG. 1. Density gradient distribution of post-mitochondrial supernatant containing polyribosomes. Fractions were collected manually and the absorbance of each was read at 260 nm. This plot represents the average of five similar gradients. The fractions from 5 through 21, up to the dashed line, were taken for infrared analysis. All fractions in this region contained [^{3}H]hydroxyproline, but *R* was greater for the lower number fractions. All of the material in fractions 5–22 could be moved under the monosome peak by the action of ribonuclease.

The most serious question is that of sensitivity. Is there enough collagen on the polysomes to be detected? A standard amount of about 690 μg of polysomes was suspended in 2 ml of D_2O, corresponding to about 175×10^{-6} μmol of ribosomes, assuming that the ribosome assembly has a weight of about 4×10^6 μg/mol. As indicated earlier, about 30% of the nascent protein produced is in -Gly-X-Y- triplet sequences. Because the procollagen contains substantial quantities of nontriplet sequences, we can estimate that approximately 50% of the protein

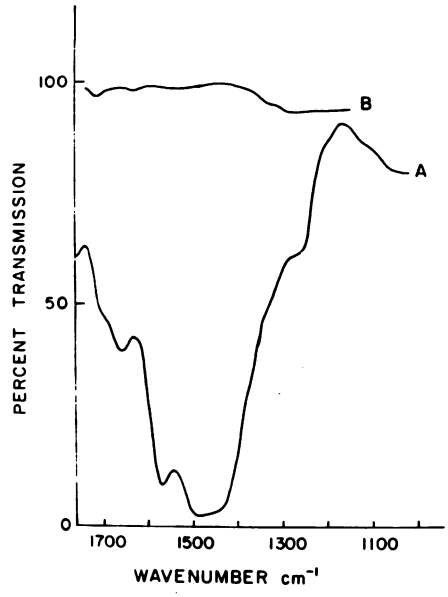

FIG. 2. Infrared spectrum of collagenase-treated polyribosomes in D_2O. *A*. Spectrum obtained at 15°, or at 15° after heating to 44° for 15–30 min in the infrared cell, using D_2O alone in the reference cell. *B*. Spectrum of polyribosomes at 15° when the sample and reference cells contain collagenase-treated polyribosomes of equal RNA content.

being produced is in procollagen chains, or about 50% of the ribosomes are producing collagen. Nascent chains in all stages of elongation are present at any instant; on average, each ribosome would thus contain the equivalent of a half-completed chain or 50×10^3 μg of collagen triplet sequence per mol of ribosome, yielding a very approximate 4.5 μg of collagen per 690 μg of polysomes, less than 1%. Fig. 3 shows the spectrum of 225 μg of rat-tail tendon collagen mixed with 500 μg of collagen-depleted ribosomes and blanked with an equivalent concentration of collagen-depleted polysomes. The collagen

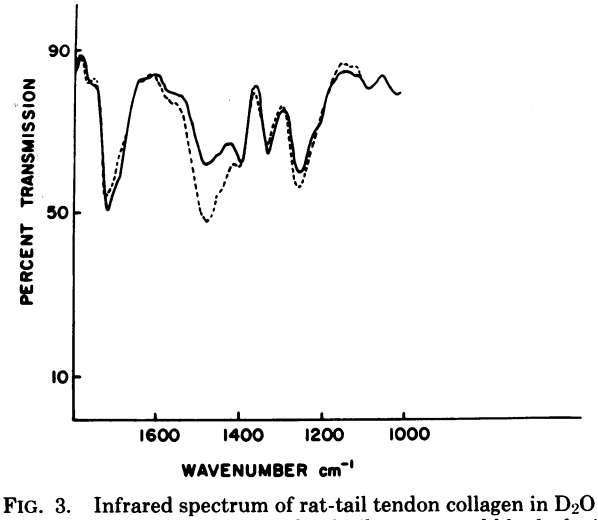

FIG. 3. Infrared spectrum of rat-tail tendon collagen in D_2O in the presence of collagen-depleted polyribosomes and blanked with collagen-depleted polyribosomes. Collagen (225 μg) in 500 μg of polyribosomes. (—) Spectrum at 15°, native collagen. (- - - -) Spectrum at 15° after conversion to gelatin by heating to 44° in the cell. Without scale expansion and in the presence of the polyribosomes the amide II N–H band is obscured compared to spectra obtained in systems without polysome material present (9). However, the increase in amide II N–D is large and unmistakable upon conversion of the collagen to gelatin.

Table 1. Relative changes in absorption at the 1480 cm^{-1} N–D deformation absorbance upon heating at 44°

Sample*	$\Delta A_{1480\ cm^{-1}}/A_{1675\ cm^{-1}}$†
Rat-tail tendon collagen	1.09
Polysome-bound collagen	1.00

* Collagen-containing polysomes and rat-tail tendon collagen plus collagen-depleted polysomes.

† $\Delta A_{1480 cm^{-1}} = (A_{denatured,\ 1480\ cm^{-1}} - A_{baseline,\ heated,\ 15°}) - (A_{native,\ 1480\ cm^{-1}} - A_{baseline,\ 15°})$. $A_{1675\ cm^{-1}} = (A_{1675\ cm^{-1}} - A_{baseline})$.

spectrum is readily visible at this high collagen concentration although in the presence of the polyribosomes the 1565 cm^{-1} difference spectrum absorbance of the N–H amide I was not very prominent. A small amount of H_2O associated with the rat-tail tendon preparation gave a background H–O–D absorbance at 1480 cm^{-1}. The minimum amount of collagen whose spectrum could be seen without scale expansion was at a concentration of 25 μg in 690 μg of polyribosome, or about 3% of the total polyribosome weight. However, using the 100-fold scale expansion available on the IR-12, the spectrum of a suspension of 5 μg of rat-tail tendon collagen in 690 μg of polyribosomes was readily observable, indicating that the projected experiment was feasible although we were working near the limit of our instrumentation. No difference spectrum was detectable at maximum sensitivity at a level of 1 μg of collagen per 690 μg of polyribosomes.

Denaturation of the collagen by heating to 44° and then recooling produced a spectrum (Fig. 3) in which, as expected (9), the carbonyl, amide I absorbance was nearly unchanged while a large increase in absorbance at the N–D amide II region at 1480 cm^{-1} was very prominent. Clearly, exchange had occurred on heating.

Two technical difficulties made quantitation difficult at these low concentrations. First, small changes in cell dimensions and alignment on temperature cycling caused shifts in the spectrum baseline that were not identical from run to run. Second, the small but variable amounts of H_2O present in the control rat-tail tendon collagen dried simply by lyophilization altered the absorbance ratios ($A_{1480\ cm^{-1}}$, native)/($A_{1660\ m^{-1}}$, native) from control run to control run, but this did not affect the increase in $A_{1480\ cm^{-1}}$. We therefore chose to quantitate the extent of denaturation in terms of $\Delta A_{1480\ cm^{-1}}/A_{1660\ cm^{-1}}$, implicitly assuming that the absorbance at 1660 cm^{-1} is proportional to the collagen concentration (9). This calculation avoids the problem of differences in H_2O content of different preparations because the measurements in the native and denatured states are made on the same collagen preparation without removal from the infrared cell. This calculation also minimizes a further difficulty in the case of the polysome-bound collagen. Pepsin digestion experiments (4, *) indicated that only about 70% of the collagen was pepsin resistant, that is, 30% was presumably in a nontriple-helical form with amide hydrogens available for rapid exchange as soon as the polysomes were exposed to D_2O. Thus, in this situation a "native state" absorbance at 1480 cm^{-1} might be expected. The term $\Delta A_{1480\ cm^{-1}}/A_{1660\ cm^{-1}}$ would, however, still be a measure of the additional exchange of amide N–H to N–D after heating to 44° in the collagen-containing polyribosomes as compared with collagenase-cleared polyribosome reference sample, otherwise treated identically.

Exchange of Polysomes with Nascent Collagen. The spectra of collagen-containing polyribosomes, blanked with collagen-depleted polysomes containing the same absolute

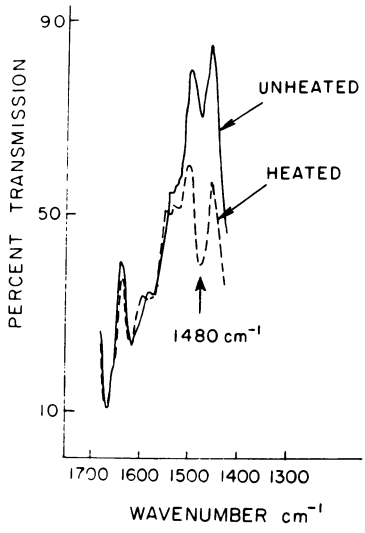

FIG. 4. Infrared spectra of collagen-containing polyribosomes in D_2O using collagen-depleted polyribosomes in the reference cell. (—) Spectrum at 15°, before heating. (- - - -) Spectrum at 15° after heating to 44° and then cooling to 15°. The only marked change is in amide II absorbance. Expanded scales measurement ×55. The % transmission figures are relative only, not absolute.

amounts of RNA, are compared at maximum scale expansion in Fig. 4 before and after the polysomes were heated in the infrared cell. In Fig. 4 the two spectra are adjusted to make up for baseline shift so that the 1660 cm^{-1} (C = 0) absorbances are matched for the initial and 44° denatured states. As in the case of native rat-tail tendon collagen in the presence of the polyribosomes, the 1550 cm^{-1} absorbances are obscured, but the increase in N–D amide II absorbance at 1480 cm^{-1} is large and unmistakable, leaving no doubt that additional exchange has occurred upon heating.

The changes in absorbance at 1480 cm^{-1} are summarized in Table 1. The relative increase in the N–D amide II absorbance upon denaturation is nearly equivalent in native rat-tail tendon collagen and nascent polysome-bound collagen.

CONCLUSION AND SUMMARY

The D → H exchange data are clear in demonstrating that nascent polysome-bound collagen does not exchange D for H prior to heating above denaturing temperature. In conjunction with the companion studies (4, *), which show that the nascent polysome-bound collagen is also resistant to digestion by pepsin, these data imply that the polysome-bound collagen is in a triple-helical state under the conditions of isolation of the polyribosomes. Nascent chains must therefore be sufficiently close in the polyribosome assembly system to recognize each other and form a triple helix while still attached to the ribosome. These data, of course, apply to isolated polyribosomes that have been cooled immediately after their isolation, and hence, triple-helix formation may have been an *in vitro* processing event. However, because the nascent collagen is also nearly fully hydroxylated and glycosylated while on the polyribosome (4, 8, *), it must be considered that triple-helix formation also occurs while the nascent chains are attached to the endoplasmic reticulum surface and does not occur after release.

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