Biosynthesis of Cartilage Procollagen. Influence of Chain Association and Hydroxylation of Prolyl Residues on the Folding of the Polypeptides into the Triple-Helical Conformation†

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ABSTRACT: Cells isolated by enzymic digestion of embryonic cartilage were used to study the biosynthesis of type II procollagen. The results demonstrated that interchain disulfide bonds among the three pro-α chains were not synthesized until well after assembly of amino acids into the polypeptide chains was completed. Also, the collagen portion of the molecule did not fold into a stable triple-helical structure which resisted pepsin digestion until well after assembly of the polypeptide chains was completed. In contrast, prolyl residues in the pro-α chains were maximally hydroxylated before the synthesis of interchain disulfide bonds and helix formation. The results indicated therefore that the rate of helix formation during biosynthesis under the incubation conditions employed was not limited by the synthesis of hydroxyproline but appeared to be limited by chain association. For reasons which were not apparent, chain association and helix formation occurred at a later stage in cartilage cells synthesizing type II procollagen than in tendon cells synthesizing type I procollagen. In further experiments cartilage cells were incubated under anaerobic conditions so that they synthesized and retained protocollagen which was comprised of nonhydroxylated pro-α chains largely linked by interchain disulfide bonds. When the cells were subsequently exposed to atmospheric O_2, the rate of helix formation closely paralleled the hydroxylation of prolyl residues, indicating that after temporary anoxia synthesis of hydroxyprolyl residues limited helix formation.

One of the critical steps in the biosynthesis of collagen is folding of three precursor polypeptide chains known as pro-α chains into the triple-helical procollagen molecule (for recent reviews, see Schofield and Prockop, 1973; Miller and Matukas, 1974; Martin *et al.*, 1974). Recent observations have demonstrated that one of the necessary requirements for the formation of the triple helix at 37° is the hydroxylation of appropriate number of prolyl residues in the peptide chains to hydroxyproline (Berg and Prockop, 1973a,b; Jimenez *et al.*, 1973; Rosenbloom *et al.*, 1973; Sakakibara *et al.*, 1973; Uitto and Prockop, 1973a,b, 1974a). Other recent observations have emphasized that a second requirement for folding of the chains into triple-helical conformation is the association of the three polypeptide chains, a process which is apparently closely associated with the formation of interchain disulfide bonds (Dehm *et al.*, 1972; Fessler *et al.*, 1973; Grant *et al.*, 1973; Harwood *et al.*, 1973; Monson and Bornstein, 1973; Scherr *et al.*, 1973; Uitto and Prockop, 1973b; Schofield *et al.*, 1974a,b).

We previously demonstrated that there was a close relationship in time between the synthesis of interchain disulfide bonds and the formation of triple-helical structure during the biosynthesis of procollagen in cells isolated from embryonic tendons (Schofield *et al.*, 1974a,b). In the present report, we have carried out similar studies on cells which are isolated by enzymic digestion of embryonic cartilage (Dehm and Prockop, 1973) and which synthesize and secrete a procollagen (type II) (Miller, 1971) structurally distinct from the procollagen (type I) synthesized by tendon cells. Because folding of the procollagen molecule into the triple-helical conformation occurs at a somewhat later stage during its biosynthesis in cartilage cells than in tendon cells, it was possible to examine the question of whether the rate of triple-helix formation is limited by hydroxylation of prolyl residues or by association of the three pro-α chains.

Experimental Section

Materials. Unless otherwise indicated, these were purchased from the same suppliers as previously (Dehm and Prockop, 1972; Schofield *et al.*, 1974a).

Isolation and Incubation of Matrix-Free Cells from Cartilage. Cells were isolated by enzymic digestion of the sterna from 17-day old chick embryos using trypsin and collagenase as described previously (Dehm and Prockop, 1973). The cells were filtered through lens paper and then washed with modified Krebs medium containing 10% fetal calf serum. In most experiments 8–15 $\times$ 10^6 cells were obtained from each sternum. The cells were incubated in modified Krebs medium containing 20% fetal calf serum and the incubations were carried out with moderate shaking at 37°.

Pulse-chase experiments were carried out as described previously in experiments with tendon cells (Schofield *et al.*, 1974a). In a typical experiment, 7.8 $\times$ 10^8 cells were incubated with 100 μCi of [^{14}C]proline in 24 ml of modified Krebs medium containing 20% fetal calf serum. After a pulse-labeling period of 4 min, the label was chased by adding 1 ml of Krebs medium containing 2.5 mg of [^{12}C]proline. After the chase periods indicated, one 2.0-ml aliquot of the incubation system (cells plus medium) was quickly pi-

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petted into a test tube containing 10% SDS[1] and 0.25 M iodoacetamide in 0.5 ml of 0.1 M sodium phosphate buffer (pH 7.4) (Schofield *et al.*, 1974a). The sample was immediately heated to 100° for 3 min, incubated for an additional 120 min at 37° in order to fully denature the protein, and then dialyzed against 0.1 M sodium phosphate buffer (pH 7.4) containing 0.1% SDS prior to gel filtration as described below. A comparable aliquot of 2.0 ml was removed from the incubation system after the same chase period and rapidly pipetted into a homogenizer vessel containing 0.5 mg of pepsin and 0.25 M iodoacetamide in 0.5 ml of 5.5 M acetic acid cooled to 4° (Uitto and Prockop, 1973b). The sample was immediately homogenized and the digestion with pepsin was allowed to proceed at 15° for 6 hr (Layman *et al.*, 1971; Müller *et al.*, 1971; Uitto and Prockop, 1974a,b). To inactivate pepsin, the digest was dialyzed against 0.4 M NaCl and 0.1 M Tris-HCl buffer (pH 7.5), and the sample was then treated with SDS as described above prior to gel filtration in SDS.

To synthesize intracellular [^{14}C]protocollagen, cartilage cells were incubated under nitrogen with [^{14}C]proline as described previously (Uitto and Prockop, 1973b, 1974a).

Gel Filtration and Polyacrylamide Gel Electrophoresis in Sodium Dodecyl Sulfate. Gel filtration in SDS of the ^{14}C protein was performed on a 6% agarose column as described previously (Jimenez *et al.*, 1971; Uitto and Prockop, 1974a,b). Polyacrylamide gel electrophoresis in SDS was carried out on 5% polyacrylamide gels with minor modifications (Uitto *et al.*, 1972) of the method of Weber and Osborn (1969). Protein in gels was visualized by staining with Coomassie Brilliant Blue as described elsewhere (Uitto and Prockop, 1974b).

Assays for [^{14}C]hydroxyproline in column fractions and other samples were carried out with a specific radiochemical procedure (Juva and Prockop, 1966).

Results

Demonstration that the Procollagen Synthesized and Secreted by the Cartilage Cells Contains Interchain Disulfide Bonds. As reported previously (Dehm and Prockop, 1973), the cells isolated from cartilage by digestion with collagenase and trypsin synthesize and secrete procollagen comprised of a single type of pro-α chain. Digestion with pepsin demonstrated that the collagen portion of the procollagen molecule recovered from the medium was in a triple-helical conformation. Also, the amino-terminal extensions of procollagen polypeptides were shown to contain cystine and tryptophan, amino acids not found in type I or type II collagen (Dehm and Prockop, 1973).

To test whether the secreted procollagen contained interchain disulfide bonds among the three pro-α chains, cartilage cells were incubated for 4 hr and the protein in the medium was examined by polyacrylamide gel electrophoresis in SDS either without reduction or after the protein was reduced with 2-mercaptoethanol (Figure 1). When the sample was not reduced, the protein was recovered as a somewhat diffuse band which had a lower mobility than γ chains of collagen. When the protein was reduced prior to electrophoresis, most of it was recovered in a relatively sharp band of about the same mobility as the pro-α1 chains of tendon procollagen (Figure 1). Further evidence that this band corresponds to pro-α chains of type II procollagen was provided by studies on the content of free and glycosylated hydroxylysine in the chains (Dehm and Prockop, 1973). Also, carboxymethylcellulose chromatography of the peptides obtained by cyanogen bromide cleavage demonstrated that essentially all [^{14}C]procollagen synthesized by freshly isolated cartilage cells was of type II (J. Uitto, R. Timpl, and D. J. Prockop, in preparation).

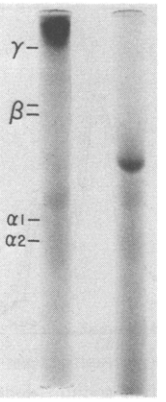

FIGURE 1: Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of procollagen secreted by the cartilage cells. Cells, 1×10^8, were incubated in modified Krebs medium containing 20% fetal calf serum for 4 hr. ^{14}C protein in the medium was precipitated by addition of 176 mg of ammonium sulfate/ml. To partially purify procollagen from precipitated serum proteins, the precipitate was extracted with 0.5 M acetic acid for 3 hr at 4°, and the extracted procollagen was reprecipitated with ammonium sulfate. The precipitate was dispersed in 1% SDS in 0.1 M sodium phosphate buffer (pH 7.4), heated at 100° for 3 min, and then incubated at 37° for 3 hr (Uitto and Prockop, 1974c). Polyacrylamide gel electrophoresis in SDS was carried out as described in the Experimental Section. The electrophoretic mobilities of γ, β, α_1, and α_2 chains of acid soluble calf skin collagen in the same electrophoretic run were as indicated. Left-hand gel: One-half of the sample which was electrophoresed without reduction. Right-hand gel: The other half of the sample which was reduced with 1% 2-mercaptoethanol at 37° for 30 min prior to electrophoresis.

To determine whether the cells also contained pro-α chains linked by interchain disulfide bonds, cells were incubated with [^{14}C]proline for 2 hr so that the intracellular polypeptides were labeled under steady-state conditions (Dehm and Prockop, 1973). The intracellular ^{14}C protein was then examined by gel filtration in SDS with or without prior reduction with 2-mercaptoethanol, and column fractions were assayed for [^{14}C]hydroxyproline in order to follow the elution of collagenous polypeptides. As indicated (Figure 2), most of the intracellular [^{14}C]hydroxyproline was in pro-α chains when the samples were reduced prior to gel filtration. With samples which were not reduced, less than 10% of the intracellular [^{14}C]hydroxyproline eluted ahead of the elution position of pro-α chains, indicating that less than 10% of the pro-α chains were disulfide linked (see Schofield *et al.*, 1974a,b). Also, digestion of the intracellular ^{14}C protein with pepsin under standardized conditions (see Uitto and Prockop, 1974a,b) demonstrated that less than 10% of the [^{14}C]hydroxyproline was in polypeptides which were in a stable triple-helical conformation at 15°. Since a large fraction of the [^{14}C]hydroxyproline was in polypeptides of the size of pro-α chains, the results indicated that under steady-state conditions the cells contain a large fraction of pro-α chains which are neither linked by disulfide bonds nor in a stable triple helix.

Synthesis of Interchain Disulfide Bonds and Formation of the Triple-Helical Conformation. To study the formation of interchain disulfide bonds during the biosynthesis of cartilage procollagen, cells were pulse-labeled with

[1] Abbreviations used are: SDS, sodium dodecyl sulfate; V_0, void volume of gel filtration column; V_t, total volume of gel filtration column.

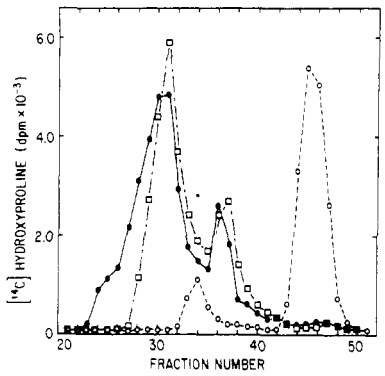

FIGURE 2: Gel filtration in sodium dodecyl sulfate of intracellular [^{14}C]hydroxyproline containing polypeptides labeled under steady-state conditions. Cartilage cells, 5 × 10^7, were incubated for 2 hr with 5 μCi of [^{14}C]proline in 5 ml of modified Krebs medium containing 20% fetal calf serum. The cells were recovered by centrifugation and part of the cells was homogenized in 50 mM iodoacetamide, 2% sodium dodecyl sulfate, and 0.1 M sodium phosphate (pH 7.4). Half of this sample was subsequently treated with 1% 2-mercaptoethanol. Another part of the cells was homogenized in 0.5 M acetic acid containing 0.1 mg of pepsin/ml at 4°; proteolytic digestion was allowed to proceed for 6 hr at 15°. The samples with and without proteolytic digestion were prepared for gel filtration as described in Methods. Symbols: Elution pattern of [^{14}C]hydroxyproline in sample chromatographed without reduction or proteolytic digestion (●——●); elution pattern of [^{14}C]hydroxyproline in sample reduced with 2-mercaptoethanol (□ - · · · · □); elution pattern of [^{14}C]hydroxyproline in sample digested by pepsin (O - - - - O). The V_0 was 48 ml (fraction 24) and the V_t was 132 ml (fraction 66). α Chains of acid soluble calf skin collagen eluted in fractions 33-34.

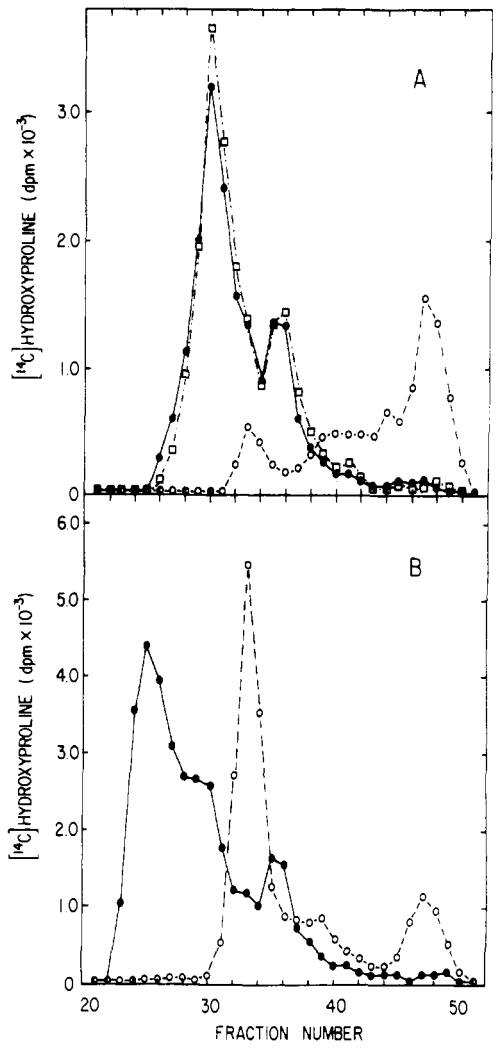

FIGURE 3: Pulse-chase experiments with cartilage cells. Cartilage cells were pulse-labeled with [^{14}C]proline for 4 min, and the label was then chased with [^{12}C]proline for 3 min (frame A) or 36 min (frame B). Symbols: Elution pattern of [^{14}C]hydroxyproline in sample chromatogaphed without reduction or proteolytic digestion (●——●); elution pattern of [^{14}C]hydroxyproline in sample reduced with 2-mercaptoethanol (□ - · · - □); elution pattern of [^{14}C]hydroxyproline in samples digested by pepsin (O - - - - O). The chromatographic conditions were as in Figure 2.

[^{14}C]proline for 4 min and then the label was chased with carrier [^{12}C]proline for 0 to 36 min. In order to terminate the chase period rapidly and to begin processing of the samples immediately, no attempt was made to separate cells from medium, and aliquots of the total system (cells plus medium) were examined after the chase times indicated. After a chase period of 3 min, most of the peptide-bound [^{14}C]hydroxyproline was in polypeptides of about the same size as pro-α chains (Figure 3A). The elution pattern was essentially the same with and without reduction, indicating that little of the [^{14}C]hydroxyproline-containing polypeptides were disulfide linked. Digestion of the protein with pepsin demonstrated that in the same sample less than 10% of the [^{14}C]hydroxyproline was in polypeptides which were in a stable triple-helical conformation (Figure 3A). It should be noted that about half of the [^{14}C]hydroxyproline-containing polypeptides were digested to dialyzable peptides and therefore were not recovered in the chromatogram.

If the chase period with [^{12}C]proline was increased to 36 min or about the time required for procollagen to be secreted by the same cells (Dehm and Prockop, 1973), a large fraction of the [^{14}C]hydroxyproline was recovered in polypeptides which eluted before pro-α chains in the gel filtration chromatogram if the protein was not reduced prior to chromatography (Figure 3B). However, if the protein was reduced with 2-mercaptoethanol prior to gel filtration, essentially all of this [^{14}C]hydroxyproline was recovered in polypeptides with the elution position of pro-α chains (not shown). In the same sample, digestion of the protein with pepsin indicated that a large fraction of the [^{14}C]hydroxyproline was in pro-α chains in which the collagen portion of the molecule was in a stable triple-helical conformation (Figure 3B).

Comparison of the data on the synthesis of interchain disulfide bonds and helix formation indicated that these processes occurred at about the same time (Figure 4) but more slowly than in tendon cells (see Schofield *et al.*, 1974a,b).

In the same pulse-chase experiments, the time required for maximal hydroxylation of prolyl residues in newly synthesized polypeptides was examined by determining the ratio of [^{14}C]hydroxyproline to total nondialyzable ^{14}C protein (Figure 4). The results indicated that the maximal value for the degree of hydroxylation was reached after a chase period of about 9 min or before maximal values were obtained for interchain bonding and helical structure.

Hydroxylation of Intracellular [^{14}C]Protocollagen after Temporary Anoxia. Cartilage cells were incubated with [^{14}C]proline under N_2 so that they synthesized [^{14}C]protocollagen (Uitto and Prockop, 1974a). Gel filtration demonstrated that a large fraction of the ^{14}C protein which was recovered in the cells at the end of the incubation under N_2 consisted of pro-α chains of protocollagen which were linked by interchain disulfide bonds (Figure 5). Essentially none of the ^{14}C protein was triple helical as tested by diges-

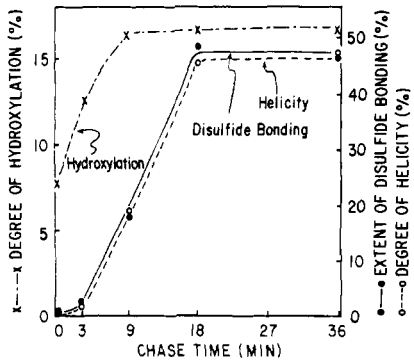

FIGURE 4: Interchain disulfide bonding, helicity, and hydroxylation of proline in a pulse-chase experiment similar to the one shown in Figure 3. The extent of disulfide bonding was calculated as the ratio of [^{14}C]hydroxyproline which eluted in the V_0 of the gel filtration column to total [^{14}C]hydroxyproline in the chromatogram (see Schofield *et al.*, 1974a,b). Degree of helicity was calculated as the ratio of [^{14}C]hydroxyproline recovered in the α-chain peak after digestion by pepsin to total nondialyzable [^{14}C]hydroxyproline in the sample before the proteolytic digestion (Uitto and Prockop, 1974a). Degree of hydroxylation was calculated as the ratio of [^{14}C]hydroxyproline to total ^{14}C protein.

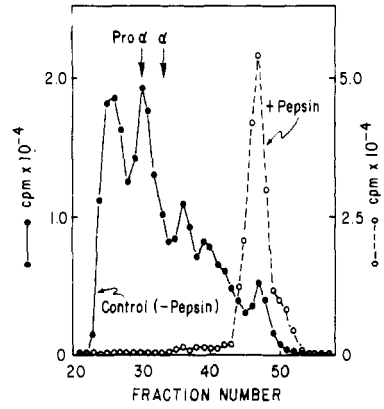

FIGURE 5: Gel filtration in SDS of unreduced ^{14}C protein which accumulated in cells incubated under anaerobic conditions. Cartilage cells, 1.3 × 10^9, were incubated under N_2 for 90 min with 100 μCi of [^{14}C]proline in 27 ml of modified Krebs medium containing 20% fetal calf serum (Uitto and Prockop, 1973b, 1974a). After 90 min of incubation under N_2, 2.8 ml of cycloheximide in 1 ml of Krebs medium was added to the incubation, and a 3.0-ml aliquot of the sample was then pipetted into 0.5 ml of the 0.1 M phosphate buffer (pH 7.4) containing 10% SDS, 25 mM iodoacetamide, and 11 mM α,α'-dipyridyl. The sample was immediately heated to 100° for 3 min and then incubated at 37° for 2 hr prior to chromatography in SDS. Another 3.0-ml aliquot was pipetted into 0.5 ml of 5.5 M acetic acid containing 25 mM iodoacetamide and 0.35 mg of pepsin for digestion at 15° as described in the Experimental Section. Symbols: ^{14}C protein in sample chromatographed without reduction and without proteolytic digestion (●——●); ^{14}C protein in a similar aliquot digested by pepsin (o - - - - o).

tion with pepsin. After incubation under N_2 for 90 min, further protein synthesis was stopped by addition of cycloheximide, and the cells were then exposed to atmospheric O_2 so that the intracellular [^{14}C]protocollagen was hydroxylated (see Uitto and Prockop, 1974a). As indicated (Figure 6), a large fraction of the ^{14}C protein became triple helical about 5 min after the cells were exposed to O_2 and the maximal value for triple-helical polypeptides was reached in about 10 min. Assays of [^{14}C]hydroxyproline indicated that hydroxylation of the ^{14}C protein occurred approximately at the same rate as folding of the protein into the triple-helical conformation (Figure 5). As indicated, the fraction of ^{14}C polypeptides linked by interchain disulfide bonds remained about the same throughout the incubation under atmospheric O_2 (Figure 5).

Discussion

In considering the folding of procollagen during its biosynthesis, it is apparently necessary to make a distinction between the two parts of the molecule, the collagen portion and the amino-terminal extensions on the pro-α chains. Because the unique triple helix of collagen depends on hydrogen bonds which are interchain, the individual polypeptides in the collagen portion of the molecule cannot assume a defined structure without chain association (Berg *et al.*, 1973; for review, see Traub and Piez, 1971). In contrast, the amino-terminal extensions appear to be globular in nature (see Schofield and Prockop, 1973). Therefore, the individual chains in this portion of the molecule probably fold into a defined structure prior to chain association, and probably the folded structure of the individual chains then directs association of the three subunits into quaternary structure in much the same manner as occurs with globular proteins in general (Anfinsen, 1972; Wetlaufer and Ristow, 1973).

The recent demonstrations (Berg and Prockop, 1973a,b; Jimenez *et al.*, 1973; Rosenbloom *et al.*, 1973; Sakakibara *et al.*, 1973; Uitto and Prockop, 1973a,b, 1974a) that hydroxyprolyl residues play an essential role in allowing collagen polypeptides to form a stable triple helix at 37° has focused attention on hydroxylation of prolyl residues as a critical step in determining the conformation of newly synthesized procollagen. The results presented here, however, demonstrated that in cartilage cells in which prolyl hydroxylase activity is uninhibited and the cells have adequate access to O_2, synthesis of hydroxyprolyl residues does not limit the rate of helix formation. Under the control conditions for the pulse-chase experiments, it was possible to demonstrate that maximum levels of prolyl hydroxylation were achieved well before the collagen portion of the molecule assumed a stable triple-helical conformation. These results are consistent with previous studies demonstrating that in several cell systems hydroxylation of prolyl residues be-

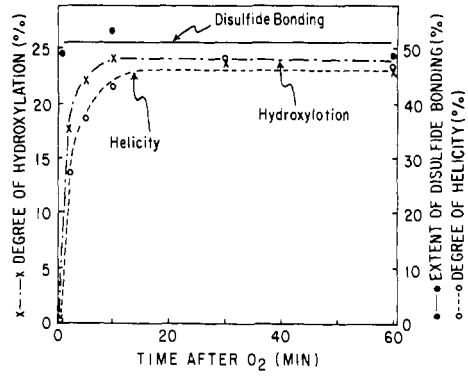

FIGURE 6: Rate of helix formation, hydroxylation of proline, and interchain disulfide bonding in cartilage cells subjected to temporary anoxia. To synthesize intracellular [^{14}C]protocollagen, cartilage cells were incubated under N_2 (see Figure 5), cycloheximide was added, and 10 min later the cells were exposed to atmospheric O_2. Disulfide bonding, helicity, and hydroxylation were then measured as in Figures 3–5, and in the Experimental Section. Because the accumulated [^{14}C]protocollagen did not contain [^{14}C]hydroxyproline, the values for extent of disulfide bonding were calculated as the ratio of ^{14}C protein eluting in the V_0 to total ^{14}C protein in the V_0 peak plus the pro-α chain peak (see Figure 5). Also, the degree of helicity was calculated as the ratio of ^{14}C protein eluting as α chains after pepsin digestion to the total ^{14}C protein recovered in the V_0 peak plus the pro-α chain peak in undigested samples. Degree of hydroxylation was calculated as in Figure 3.

gins on nascent, ribosomal peptides and is apparently maximal at about the same time or shortly after assembly of amino acids into the polypeptide chains is completed (Miller and Udenfriend, 1970; Lazarides *et al.*, 1971; Uitto and Prockop, 1974c; Cardinale and Udenfriend, 1974).

The process by which the newly synthesized pro-α chains associate has not been defined, but association of the extensions seems to be stabilized by interchain disulfide bonds. For example, experiments with disulfide-linked amino-terminal extensions isolated by collagenase digestion of procollagen demonstrated that the three extensions dissociated after mild reduction under nondenaturing conditions (Dehm *et al.*, 1974). Because denaturing conditions are required for adequate extraction of procollagen polypeptides from cells, it was not possible to follow chain association *per se* in the pulse-chase experiments carried out here. Instead biosynthesis was followed by monitoring the formation of the interchain disulfide bonds, which must be synthesized either at about the same time or, as is more likely, shortly after chain association. With this measure it was found that both in tendon cells (Schofield *et al.*, 1974a,b) and in cartilage cells there was a close correlation between the time at which the interchain bonds formed among the amino-terminal extensions and the time at which the collagen portion of the molecule folded into a stable triple helix. The results strongly suggested therefore that under the conditions employed here chain association is a limiting step in the folding of procollagen into a triple-helical conformation.

Assembly of the triple-helical, type II procollagen molecule in the cartilage cells required almost twice as long as the assembly of a comparable type I molecule in tendon cells studied previously (Schofield *et al.*, 1974a,b). The slower assembly is not explained by a slower rate of incorporation of amino acids into the polypeptide chains, since the synthesis time for the polypeptide chains is about 7 min for both type I and type II collagens (Vuust and Piez, 1972; Miller *et al.*, 1973). From the results presented here it would appear that folding of the chains into the triple helix occurs more slowly in the cartilage cells because, for some yet unexplained reason, association of the pro-α chains occurs more slowly than in tendon cells. Since under steady-state conditions only a small fraction of the intracellular pro-α chains were disulfide linked and triple helical, it appears that both of these processes occur shortly before the protein is secreted and well after the message for procollagen is translated. Similar observations were previously made in cells synthesizing basement membrane collagen (Grant *et al.*, 1973).

Recent evidence has demonstrated that lysyl hydroxylase (Ryhänen and Kivirikko, 1974; Uitto and Prockop, 1974e), like prolyl hydroxylase (Berg and Prockop, 1973b; Murphy and Rosenbloom, 1973), cannot hydroxylate protocollagen in a triple-helical conformation and it was therefore suggested that the stage at which the triple helix forms may determine the hydroxylysine content of the molecule (Kivirikko *et al.*, 1973; Ryhänen and Kivirikko, 1974). On this basis, the late stage at which procollagen becomes helical in cartilage and lens cells may help to explain the relatively high content of hydroxylysine in these collagens (Miller, 1971; Kefalides, 1971; Grant *et al.*, 1973). However, the factors which limit the hydroxylysine content of collagen are probably complex and include the relative activities of prolyl and lysyl hydroxylases and the relative concentrations of substrates and cofactors in the same compartments of cells.

As shown by the results presented here, the factors limiting helix formation change if the cells are subjected to temporary anoxia so that they synthesize and accumulate protocollagen. After anoxia, a large fraction of the intracellular protocollagen was already associated and disulfide linked. When the cells were subsequently exposed to O_2, hydroxylation of prolyl residues was the limiting step in helix formation. As discussed elsewhere (Uitto and Prockop, 1974d,e), temporary anoxia may well occur in a number of connective tissues under normal and pathological conditions, and hydroxylation of prolyl residues may well be a limiting reaction under such circumstances *in vivo*.

Acknowledgments

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References

Anfinsen, C. B. (1972), *Biochem. J. 128,* 737.

Berg, R. A., and Prockop, D. J. (1973a), *Biochem. Biophys. Res. Commun. 52,* 115.

Berg, R. A., and Prockop, D. J. (1973b), *Biochemistry 12,* 3395.

Berg, R. A., Kishida, Y., Kobayashi, Y., Inouye, K., Tonelli, A. E., Sakakibara, S., and Prockop, D. J. (1973), *Biochim. Biophys. Acta 328,* 553.

Cardinale, G., and Udenfriend, S. (1974), *Advan. Enzymol.* (in press).

Dehm, P., Jimenez, S. A., Olsen, B. R., and Prockop, D. J. (1972), *Proc. Nat. Acad. Sci. U. S. 69,* 60.

Dehm, P., Olsen, B. R., and Prockop, D. J. (1974), *Eur. J. Biochem. 46,* 107.

Dehm, P., and Prockop, D. J. (1972), *Biochim. Biophys. Acta 264,* 375.

Dehm, P., and Prockop, D. J. (1973), *Eur. J. Biochem. 35,* 159.

Fessler, L. I., Burgeson, R. E., Morris, N. P., and Fessler, J. H. (1973), *Proc. Nat. Acad. Sci. U. S. 70,* 2993.

Grant, M. E., Schofield, J. D., Kefalides, N. A., and Prockop, D. J. (1973), *J. Biol. Chem. 248,* 7432.

Harwood, R., Grant, M. E., and Jackson, D. S. (1973), *Biochem. Biophys. Res. Commun. 55,* 1188.

Jimenez, S. A., Dehm, P., and Prockop, D. J. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 17,* 245.

Jimenez, S. A., Harsch, M., and Rosenbloom, J. (1973), *Biochem. Biophys. Res. Commun. 52,* 106.

Juva, K., and Prockop, D. J. (1966), *Anal. Biochem. 15,* 77.

Kefalides, N. A. (1971), *Biochem. Biophys. Res. Commun. 45,* 226.

Kivirikko, K. I., Ryhänen, L., Anttinen, H., Bornstein, P., and Prockop, D. J. (1973), *Biochemistry 12,* 4966.

Layman, D. L., McGoodwin, E. B., and Martin, G. R. (1971) *Proc. Nat. Acad. Sci. U. S. 68,* 454.

Lazarides, E. L., Lukens, L. N., and Infante, A. A. (1971), *J. Mol. Biol. 38,* 831.

Martin, G. R., Byers, P. H., and Piez, K. A. (1974), *Advan. Enzymol.* (in press).

Miller, E. J. (1971), *Biochemistry 10,* 1652.

Miller, E. J., and Matukas, V. J. (1974), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 33,* 1197.

Miller, E. J., Woodall, D. L., and Vail, M. S. (1973), *J. Biol. Chem. 248,* 1666.

Miller, R. E., and Udenfriend, S. (1970), *Arch. Biochem.*

Biophys. 139, 104.
Monson, J. M., and Bornstein, P. (1973), *Proc. Nat. Acad. Sci. U. S. 70,* 3521.
Müller, P. K., McGoodwin, E., and Martin, G. R. (1971), *Biochem. Biophys. Res. Commun. 44,* 110.
Murphy, L., and Rosenbloom, J. (1973), *Biochem. J. 135,* 249.
Rosenbloom, J., Harsch, M., and Jimenez, S. A. (1973), *Arch. Biochem. Biophys. 158,* 478.
Ryhänen, L., and Kivirikko, K. I. (1974), *Biochim. Biophys. Acta 343,* 129.
Sakakibara, S., Katsuhiko, I., Shudo, K., Kishida, Y., Kobayashi, Y., and Prockop, D. J. (1973), *Biochim. Biophys. Acta 303,* 198.
Scherr, C. J., Taubman, M. B., and Goldberg, B. (1973), *J. Biol. Chem. 248,* 7033.
Schofield, J. D., and Prockop, D. J. (1973), *Clin. Orth. Rel. Res. 97,* 175.
Schofield, J. D., Uitto, J., and Prockop, D. J. (1974a), *Biochemistry 13,* 1801.
Schofield, J. D., Uitto, J., and Prockop, D. J. (1974b), *Biochem. Soc. Trans. 2,* 90.
Traub, W., and Piez, K. A. (1971), *Advan. Protein Chem. 25,* 243.
Uitto, J., Jimenez, S. A., Dehm, P., and Prockop, D. J. (1972), *Biochim. Biophys. Acta 278,* 198.
Uitto, J., and Prockop, D. J. (1973a), *Proc. Int. Congr. Biochem., 9th,* 425.
Uitto, J., and Prockop, D. J. (1973b), *Biochem. Biophys. Res. Commun. 55,* 904.
Uitto, J., and Prockop, D. J. (1974a), *Eur. J. Biochem. 43,* 221.
Uitto, J., and Prockop, D. J. (1974b), *Biochim. Biophys. Acta 336,* 234.
Uitto, J., and Prockop, D. J. (1974c), *Arch. Biochem. Biophys. 164,* 210.
Uitto, J., and Prockop, D. J. (1974d), *in* Molecular Pathology, Good, R. A., Day, S. B., Jorge, Y., Ed., Springfield, Ill., Charles C Thomas, (in press).
Uitto, J., and Prockop, D. J. (1974e), *Biochem. Biophys. Res. Commun.* (in press).
Uitto, J., Schofield, J. D., and Prockop, D. J. (1974), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 33,* 2300.
Vuust, J., and Piez, K. A. (1972), *J. Biol. Chem. 247,* 856.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.
Wetlaufer, D. B., and Ristow, S. (1973), *Annu. Rev. Biochem. 42,* 135.

Ferrous Porphyrins in Organic Solvents. I. Preparation and Coordinating Properties†

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ABSTRACT: Preparation and coordinating properties of ferrous deuteroporphyrin dimethyl ester and ferrous mesotetraphenylporphin in various organic solvents are investigated. Some methods of reduction are presented and discussed. In benzene, the hemes are proved to be free of ligands. Deuteroheme binds two pyridine molecules (K = (1.3 ± 0.2)10^8 M^{-2}) leading to the well-known hemochrome. The large negative changes of free energy, enthalpy, and entropy which accompany this reaction further support the conclusion that deuteroheme in benzene is a ligand-free monomer. In the case of ferrous mesotetraphenylporphin, successive binding of two pyridine molecules is suggested and the two affinity constants are estimated ($K_1 \simeq 1.5 \times 10^3$ M^{-1}; $K_2 \simeq 1.9 \times 10^4$ M^{-1}). Solvents such as alcohols, ethers, and substituted formamides are slightly coordinating. A thermodynamic study demonstrates that only one molecule of these solvents binds to the heme, with a low affinity (K = 1-6 M^{-1}). In these solvents, the heme can simultaneously bind two pyridine molecules with an affinity constant weaker than that obtained in benzene but similar to that reported for studies performed in water.

The prosthetic group (an iron porphyrin) of hemoproteins is located in a hydrophobic crevice of the protein (Kendrew, 1963; Perutz *et al.,* 1968; Dickerson *et al.,* 1971) and bound to one or two amino acid residues. The ability of iron porphyrins to complex with ligands has long been recognized (for a review see Falk, 1964) but the knowledge of bonds in terms of electronic or thermodynamical parameters requires further work. The hydrophobic environment of iron porphyrin in many hemoproteins has led us and others (see, for instance, Kassner, 1972, 1973; Caughey *et al.,* 1965) to think that a nonaqueous solution may be a more adequate medium for the iron porphyrin studies.

Thermodynamical studies of the binding of ligand by iron(II) porphyrins (hemes) require the preparation of bare hemes (bare heme is heme without any axial ligand). Unfortunately, these compounds are readily oxidized by air. On the other hand, dipyridinate complexes of hemes (hemochromes) are easily prepared and characterized (Alben *et al.,* 1968). They are relatively stable against oxidation. Alben *et al.* (1968) pointed out that hemochromes can lose their pyridine ligands under vacuum and prepared bare heme in solid state. In the same way, Kobayashi *et al.* (1970) and Kobayashi and Yanagawa (1972) prepared

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