Denaturation–Renaturation Properties of Two Molecular Forms of Short-Chain Cartilage Collagen†

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ABSTRACT: Certain physical properties of two molecular forms of short-chain (SC) cartilage collagen [Schmid, T. M., & Linsenmayer, T. F. (1983) *J. Biol. Chem. 258*, 9504–9509] have been determined. The 59K form has both a collagenous and a noncollagenous domain, and the 45K form has only the collagenous one. By circular dichroic spectropolarimetry, both forms show the characteristic spectrum of a collagen triple helix with a maximum ellipticity at 222 nm and a minimum at 197 nm. The denaturation temperature (T_m) of the helical structure of both forms, as monitored at 222 nm, is approximately 47 °C. Thus, the presence of the nonhelical domain does not greatly affect this property. After thermal denaturation, however, the renaturation of the 59K form is much more rapid than that of the 45K form, regaining >60% of its helical structure within 40 min. The 45K form regains at most 15%, even after 24 h. Gel filtration on Sephacryl S-500, run under nondenaturation conditions, showed that the molecules renatured from the 59K form had regained a structure indistinguishable from native ones, while the 45K had not. The noncollagenous domain of the 59K form could be obtained by digestion with bacterial collagenase. This domain, as previously reported, contains no disulfide bonds. But, it is very stable, requiring both detergent and heating to separate its component chains. We hypothesize that the chains within this domain are tightly held together by strong, noncovalent forces, such as hydrophobic bonds, which are refractory to thermal denaturation. These maintain the chains in proper registry, thus facilitating rapid renaturation of the helical domain.

We have recently reported that chondrocyte cultures derived from specific zones in the epiphyseal growth region of 12-day embryonic chick tibiotarsi produce a small collagen molecule that we have termed short-chain (SC)[1] collagen (Schmid & Conrad, 1982a,b; Schmid & Linsenmayer, 1983). On the basis of its cyanogen bromide peptides, amino acid composition, and other characteristics, SC collagen appears to be distinct from any previously described collagen including the HMW and LMW cartilage collagens isolated by Reese & Mayne (1981). This molecule may be of developmental significance since its synthesis appears to correlate with maturity of the chondrocytes (Schmid & Conrad, 1982b). In short-term organ cultures of the growth regions, it is synthesized only in zones containing the older, hypertrophying chondrocytes; it is not detectable in the region of younger, rapidly proliferating cells. Likewise, long-term mass cell cultures of passaged chondrocytes progressively increase synthesis of this molecule relative to that of type II collagen. Eventually, it becomes the predominant collagen type, constituting more than 90% of the collagen present in the culture medium (Schmid & Linsenmayer, 1983).

SC collagen can be isolated and purified from culture medium of mass cell cultures in quantities sufficient for biochemical and biophysical analyses. Thus, proteolytic isolation procedures are completely avoided. The molecule contains both a collagenase-sensitive, triple-helical domain and a pepsin-sensitive nonhelical one. The complete molecule, with both domains intact, is referred to as the 59K form since its constituent chains have an M_r of 59 000, as determined by SDS–PAGE. Limited pepsin digestion of the native 59K form removes the nonhelical domain, generating a molecule with M_r 45 000 chains, termed the 45K form.

The molecule is unique since its triple helix is approximately half the length of the helical domain in a typical interstitial collagen molecule. In fact, the helical domain of SC collagen is less than the size of the larger fragment (TC^A) produced by animal collagenase digestion of interstitial collagens (Gross & Nagai, 1965; Sakai & Gross, 1967). The TC^A fragment of type I collagen denatures about 4 °C lower than the intact molecule (Sakai & Gross, 1967), which has a denaturation temperature of 42 °C (Hayashi et al., 1979). From these data, one might expect that a collagen molecule the size of SC collagen would have a relatively low denaturation temperature, possibly even being unstable under physiological conditions.

In the present study we have examined the denaturation and renaturation properties of the 59K and 45K forms of SC collagen to determine (1) if the small size of SC collagen is indicative of a low thermal stability within the helical domain, (2) whether the nonhelical domain in the 59K form influence the thermal stability of the helical domain, and (3) if the presence of the nonhelical domain will influence the renaturation of the helical one.

Materials and Methods

Collagen Isolation. SC collagen was isolated from the culture medium of chick embryo chondrocytes obtained from 12-day tibiotarsi and cultured in medium supplemented with ascorbic acid and 2-aminopropionitrile as previously described (Kim & Conrad, 1977; Stocum et al., 1979; Schmid & Linsenmayer, 1983). Twenty 150-mm tissue culture dishes were harvested daily for 6 weeks of culture, yielding 50 mg of SC collagen. Protease inhibitors were added to the pooled harvest each day (5 mM EDTA, 1 mM *N*-ethylmaleimide, 1 mM phenylmethanesulfonyl fluoride), and the SC collagen was precipitated by the addition of solid ammonium sulfate to 30% of saturation. The precipitate was resolubilized, and the same precipitation was repeated a second time. The SC collagen was separated from type II collagen in the preparation by

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[1] Abbreviations: SC, short chain; 59K, 45K, etc., 59 000, 45 000, etc.; HMW, high molecular weight, LMW, low molecular weight; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; KP_i, potassium phosphate; CD, circular dichroism; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; T_m, midpoint denaturation temperature.

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fractional salt precipitation at acid pH (0.9 M NaCl, 0.5 M acetic acid). When dialyzed into this solution, the SC collagen remained soluble, whereas the type II precipitated. The SC collagen was subsequently precipitated from the solution by raising the NaCl concentration to 2 M. All precipitates were recovered by centrifugation at 10000*g* for 30 min. They were resolubilized in KP_i buffer (0.13 M K_2HPO_4 and 15 mM KH_2PO_4), pH 7.6, and stored at −20 °C. This procedure yielded the 59K form of SC collagen.

The 45K form of SC collagen was generated by limited pepsin digestion of the 59K form. For this, the 59K form was dialyzed into 0.5 M acetic acid and incubated with pepsin (100 μg/mL) for 18 h at 4 °C. The enzyme reaction was stopped by neutralizing the solution with 2 M NaOH, and the sample was dialyzed against the KP_i buffer. The 45K form thus produced was recovered by a 30% ammonium sulfate precipitation.

As a final purification step, each of the two forms of native SC collagen was chomatographed on a water-jacketed 2 × 90 cm column of Sephacryl S-500 gel-filtration resin (Pharmacia) run under nondenaturing conditions at 8 °C (Schmid & Linsenmayer, 1983). The column was equilibrated with the pH 7.6 KP_i buffer containing 25% ethylene glycol to minimize hydrophobic interactions (Hofstee, 1973; Schmid & Linsenmayer, 1983) and 0.5% 1-butanol as a bacteriostatic agent. Samples of 3 mL were loaded and chromatographed at a flow rate of 15 mL/h. The column effluent was continuously monitored at 230 nm, and 5-mL fractions were collected. The fractions containing the native SC collagen molecules were pooled, concentrated by ammonium sulfate precipitation, and redissolved in KP_i buffer.

CD Measurements. CD spectra were obtained with a Cary 61 spectropolarimeter. The collagen samples (20–100 μg/mL) were dialyzed into 0.2 M NaF and 5 mM NaP_i buffer, pH 7.5, and the measurements made in a water-jacketed cuvette with a 1-cm path length.

After each experiment, precise measurement of the amino acid concentration of the collagen sample was determined by a ninhydrin reaction performed according to the method of Hennessey & Johnson (1981). An aliquot of each sample was hydrolyzed in constant-boiling HCl at 108 °C for 24 h. The hydrolysate was dried and redissolved in 2 mL of water, and 1 mL of a ninhydrin solution (Piez & Morris, 1960) was added. The sample was heated for 20 min at 100 °C and cooled to 25 °C and the absorbance recorded at 570 nm. The amino acid concentration of each sample was determined from a calibration curve produced with a standard amino acid mixture (Calbiochem). The molar color yield ratios of the standard mixture (0.83), the 59K form of SC collagen (0.79), and the 45K form (0.74) were calculated from their known amino acid compositions (Schmid & Linsenmayer, 1983). The amino acid concentrations of the samples were corrected with these calculated molar color yield ratios.

Thermal-denaturation studies of SC collagen were performed by increasing the temperature of the water-jacketed cuvette while continuously monitoring the helical structure of the samples as determined by their circular dichroism at 222 nm. The temperature of the sample within the cuvette was measured with an internal thermistor probe coupled to a digital thermometer (Omega Engineering). The temperature was raised at a rate of 30 °C/h with a circulating water bath (Haake) controlled by a temperature programmer (Neslab).

To examine renaturation, samples that had been denatured by heating to 55 °C were rapidly cooled to 25 °C. The extent of renaturation was determined at several time points by scanning the CD spectrum from 250 to 210 nm. The samples were removed from the light path between measurements to minimize UV damage (Hayashi et al., 1979).

Measurement of Native Structure by Gel-Filtration Chromatography. The two forms of SC collagen were denatured at 60 °C for 30 min, cooled to 25 °C for 1 h, and chromatographed on a 0.9 × 98 cm column of Sephacryl S-500 under the nondenaturing conditions described above (Schmid & Linsenmayer, 1983). The column was run at a flow rate of 3 mL/h, the effluent was continuously monitored at 230 nm, and 1-mL fractions were collected. The void volume of the column was measured with $^{35}SO_4$-labeled chondroitin sulfate proteoglycan aggregate and the total column volume with tritiated water.

Bacterial Collagenase Digestion. Bacterial collagenase (Worthington CLSIII) was further purified by gel-filtration and ion-exchange chromatography on a DE-52 column (Peterkofsky, 1982). The enzyme showed no proteolytic activity toward a high specific activity casein substrate that had been radiolabeled with tritium by reductive methylation (Kumarasamy & Symons, 1979). The digestion of the 59K form of SC collagen was performed at 37 °C for 18 h in a pH 7.5 buffer containing 0.4 M NaCl, 10 mM *N*-ethylmaleimide, 10 mM $CaCl_2$, and 50 mM Tris. The reaction was terminated by the addition of EDTA to 15 mM.

SDS–PAGE. Samples were analyzed on a 12% polyacrylamide gel. The samples were mixed with equal volumes of sample buffer (Laemmli, 1970) containing 20% glycerol, 2% SDS, 10% 2-mercaptoethanol, and 0.125 M Tris at pH 6.8. Some samples were heated to 100 °C for 5 min; others were not heated prior to electrophoresis (see results). Electrophoresis was performed at 45 V for 18 h to minimize heating of the gel. The molecular weight determinations by SDS–PAGE was based on the mobility of the globular proteins standards: bovine serum albumin (M_r 67 000), ovalbumin (M_r 45 000), soybean trypsin inhibitor (M_r 21 500), and myoglobin (M_r 17 000).

Results

CD Spectra of SC Collagens. The circular dichroic spectrum of the collagen triple helix of type I collagen is characterized by a positive ellipiticity at 221 nm and a strong negative ellipticity at 197 nm (Hayashi et al., 1979). As can be seen in Figure 1, both the 59K and 45K forms of SC collagen exhibit these characteristics with the positive peak being shifted slightly to 222 nm. Both forms of SC collagen contain the same triple-helical domain, but the 59K form contains, in addition, a nonhelical one. The diminished molar ellipticity of the 59K form when compared to the 45K form presumably reflects the additional amino acid residues in the nonhelical domain that do not contribute to the positive and negative ellipticity maxima of the triple helix.

Thermal Denaturation. Thermal denaturation of collagen molecules results in a loss of their helical structure and the generation of individual α chains in a random-coil conformation. This conformational change is accompanied by an equivalent loss of the positive (222 nm) and negative (197 nm) ellipticity in their CD spectra. As shown in Figure 1, this is true for both forms of SC collagen. If the CD spectra of SC collagens are measured at 55 °C, the positive ellipticity at 222 nm is no longer present, and the strong negative ellipticity at 197 nm is greatly diminished.

For most collagen molecules in a neutral pH solution, the midpoint temperature of the helix to random-coil transition (T_m) occurs at or below 44 °C (see Discussion). As can be seen in Figure 2, both forms of SC collagen exhibit similar,

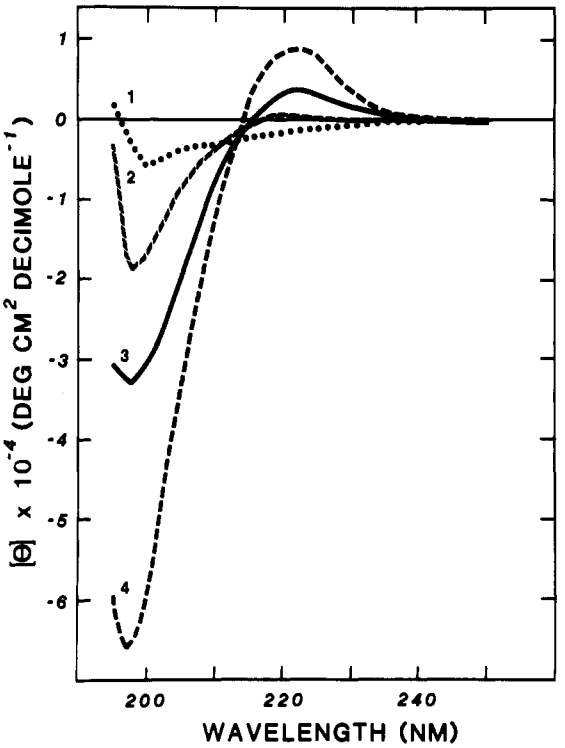

FIGURE 1: CD spectra of the forms of SC collagen. Spectra of 59K (curve 3) and 45K (curve 4) forms obtained at 25 °C. Spectra of 59K (curve 1) and 45K (curve 2) forms obtained at 55 °C.

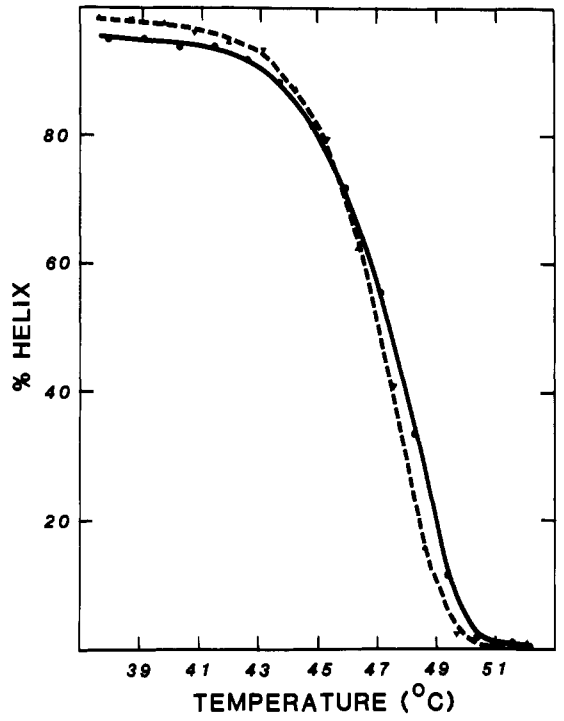

FIGURE 2: Thermal denaturation curve obtained by monitoring samples of the 59K (—) and 45K (--) forms of SC collagen at 222 nm. The temperature was raised at a rate of 30 °C/h.

rather high denaturation temperatures. The T_m of the 59K form of SC collagen was 47.4 ± 0.7 °C and that of the 45K from was 47.0 ± 1.0 °C. These data indicate the size, per se, does not determine melting temperature of a collagen molecule nor does the nonhelical domain of the 59K form greatly affect the melting temperature of the helical domain.

Denaturation–Renaturation. Certain preliminary observations suggested that even when the helix of the 59K form was completely denatured, the component chains still remained

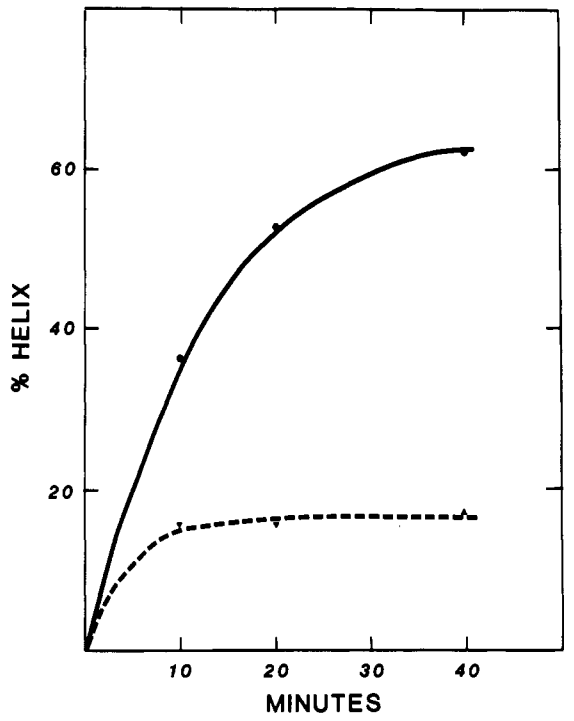

FIGURE 3: Renaturation of helical structure of the 59K (—) and 45K (--) forms at SC collagen at 25 °C following denaturation at 55 °C. The helical structure was measured at the time points shown by scanning the CD spectra from 250 to 210 nm and determining the areas under the curves.

associated, most probably through interactions in the nonhelical domain. If so, then such associations might keep the chains in "register", facilitating rapid renaturation of the helical domain upon a lowering of the temperature. We tested this possibility by examining the rates of renaturation of the two forms of SC collagen. After denaturation of the collagen samples at 60 °C, as verified by the complete loss of the positive ellipticity at 222 nm, the samples were rapidly cooled to 25 °C, and the degree of renaturation was determined at the time points shown in Figure 3. Within 10 min at 25 °C, about 36% of the positive ellipticity of the native 59K form returned, whereas only 16% of the ellipticity of the 45K form had returned. In the next 30 min, the 59K form of SC collagen recovered another 30% of its helical structure, while the 45K form of SC collagen recovered little if any additional helix. No additional changes in either form of SC collagen were observed when the samples were monitored for longer periods, up to 24 h.

These results showed that the 59K form of SC collagen regained greater than 60% of its original helical content within 40 min. Such measurements, however, did not provide information as to whether a subpopulation of the molecules had undergone complete renaturation or all of the molecules had partially renatured. To determine this, we compared the elution positions of renatured samples and undenatured ones from a calibrated Sephacryl S-500 column run under nondenaturing conditions.

Each of the SC collagen forms was denatured and allowed to renature at 25 °C for 1 h. They were then applied to the column. In frame A of Figure 4, the solid line shows the elution profile of the native 45K form of SC collagen; the dashed line shows the profile of a denatured 45K sample after a 1-h renaturation period. The native 45K form eluted at fraction 45. The denatured–renatured 45K material eluted as a single symetrical peak at fraction 56, which is much later than the native molecules and even slightly later than the

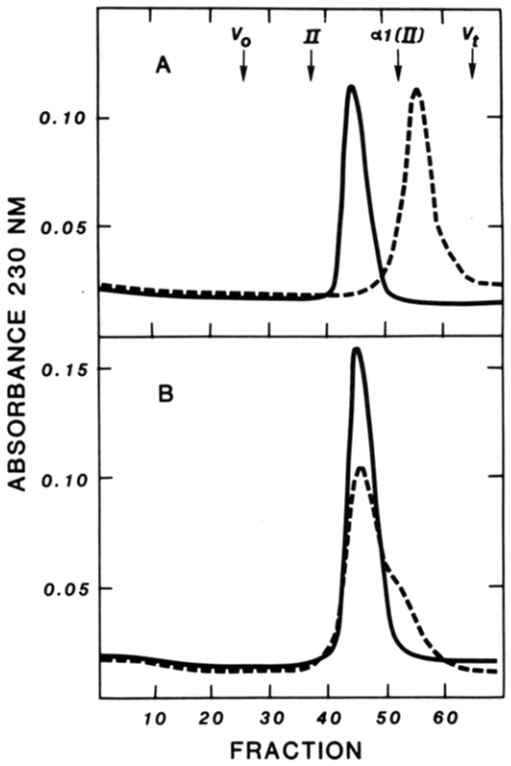

FIGURE 4: Profiles obtained by gel-filtration chromatography on Sephacryl S-500 columns run under nondenaturing conditions: (frame A) 45K form; (frame B) 59K form. (—) Undenatured samples; (--) samples denatured at 60 °C and then allowed to renature at 25 °C for 1 h before being loaded into the column. The elution positions of standards of native type II collagen molecules (II) and denatured α1(II) chains are also shown.

α1(II) chain standard that eluted at fraction 53 (arrow). Thus, none of the denatured 45K chains re-formed molecules with a structure resembling native 45K molecules.

Conversely, when denatured–renatured samples of the 59K form were chromatographed (frame B, dashed line), a major peak containing about 70% of the material coeluted with native 59K molecules (solid line; fraction 45). This is the same position where the native 59K and 45K forms eluted. The remainder of the material eluted as a shoulder at about fraction 52; only a very small portion was found in the region of fraction 56, the position of denatured 45K chains. These data indicate that approximately two-thirds of the sample had re-formed a molecular structure that is indistinguishable from the native molecules. The remainder of the sample was also larger than denatured 45K chains, suggesting that the chains had undergone partial renaturation of their helical structure or at least had remained strongly associated within their nonhelical domain.

Isolation of the Nonhelical Domain. What type of chain interactions are responsible for stabilizing the nonhelical domain and promoting helical renaturation? Disulfide bridges cannot be involved since amino acid analysis shows the molecule to be devoid of cysteine (Schmid & Linsenmayer, 1983). It is also unlikely that covalent cross-links are responsible for the large extent of renaturation observed, since collagens were isolated from cultures incubated with 2-aminopropionitrile and only a small proportion of the molecules contain covalent cross-links (see below). Instead, we thought that a likely possibility might be the involvement of strong, noncovalent bonding, possibly of a hydrophobic nature.

To examine this, we isolated the nonhelical domain of the 59K form by bacterial collagenase digestion and examined its sensitivity to heating in SDS. The results were analyzed by

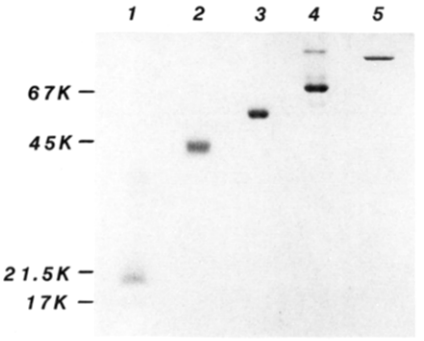

FIGURE 5: SDS–PAGE (12% gel): (lane 1) bacterial collagenase digest of 59K form of SC collagen heated at 100 °C in starting buffer before loading; (lane 2) same as lane 1 but not heated before loading; (lane 3) chains of 45K form of SC collagen; (lane 4) chains of 59K form of SC collagen; (lane 5) chains of type II collagen.

SDS–PAGE as shown in Figure 5. In this figure, lane 1 contains the bacterial collagenase digestion products that were mixed with SDS-containing sample buffer and heated to 100 °C before application on the gel. There is a major diffuse band with an approximate M_r 20000. There is also a small amount of material of about M_r 40000 that probably represents cross-linked dimers. If the sample was treated in an identical fashion but was not heated to 100 °C before being applied to the gel (lane 2), a single band with an apparent molecular weight of 45000 was observed. That 45000 is an accurate estimate for the size of the globular domain was verified by gel-filtration chromatography. When the bacterial collagenase digest was run on a Bio-Gel P-60 column in KP_i buffer without SDS present, the included volume of the column contained a single component that comigrated with an M_r 45000 ovalbumin standard (data not shown).

On the SDS gel shown in Figure 5, we have included the 45K and 59K forms of SC collagen used in the CD studies (lanes 3 and 4, respectively) plus a type II collagen standard (lane 5). Although the chondrocyte cell cultures included 2-aminopropionitrile, the 59K preparation (lane 4) contains a faint band at 120K, representing less than 10% of the material. This is always present in our preparations and most likely represents a small amount of covalently cross-linked dimers of the 59K chains. No equivalent dimers are ever present in the 45K form of SC collagen generated by pepsin treatment (lane 3). Thus, covalent cross-links, when present, are probably in the nonhelical domain.

Discussion

The 59K form of SC collagen is analogous to a procollagen or a procollagen-like molecule, in that it contains a nonhelical domain. We have demonstrated that this domain can be isolated by digestion of the 59K form with bacterial collagenase. The molecular weight of this intact globular domain, presumably containing the nonhelical portions of three chains, is 45000 as determined by SDS–PAGE run on unheated samples and by gel-filteration on a P-60 column. This size is consistent with the pepsin digestion of a molecule comprised of three 59K chains (total M_r of 177000) being converted to an intact triple-helical domain composed of three 45K chains (total M_r of 135000). The data suggest that a single domain accounts for all the pepsin-sensitive region on the 59K form of SC collagen. In agreement with this, preliminary data for rotary-shadowed preparations show the presence of a single "knob" at one end of the 59K form of SC collagen that is not present in the 45K form (R. Mayne, unpublished observation). There is, however, some discrepancy as to the molecular weight of the individual chains that comprise this domain. When the

isolated nonhelical domain is heated in the presence of SDS, the chains dissociate and exhibit a molecular weight of approximately 20 000 by SDS–PAGE. This disparity may be due to differential binding of SDS in the heated vs. the unheated samples, the difference in migration of collagenous vs. globular proteins in SDS–PAGE (Furthmayr & Timpl, 1973), or a combination of both. At present then, our best estimate of the size of the individual chains in this domain is somewhere between 15K and 20K.

The denaturation temperatures of both SC collagen forms were about 47 °C, a temperature several degrees higher than other interstitial collagens. For comparison, we obtained a T_m of 44 °C for chicken type II collagen, about 2 °C higher than has been reported for type I collagen in neutral buffer (Hayashi et al., 1979); for type V collagen, we obtained a T_m of 41 °C (Linsenmayer et al, 1983). A likely possibility for the elevated thermal stability of SC collagen is its rather high content of imino acids. In the helical domain of this molecule, proline and hydroxyproline constitute 25% of the amino acids (Schmid & Linsenmayer, 1983) as compared to about 21% in chicken collagen types I and II (Trelstad et al., 1972).

These results show the small size of a collagen molecule, per se, need not result in a low thermal stability. This is intriguing since the major vertebrate collagenase cleavage product of type I collagen (TC^A), which is even larger than SC collagen, has a much lower denaturation temperature than either native type I collagen or SC collagen. Since the SC molecule contains at least one cleavage site for vertebrate collagenase (Schmid & Conrad, 1982a), it will be informative to determine the thermal stability of the products generated by this enzyme digestion.

We have observed that the presence of the noncollagenous domain does not greatly influence the denaturation temperature of the adjacent, helical one. Similar results have been obtained for type I collagen in which the procollagen and collagen forms exhibit identical denaturation temperatures (Hayashi et al., 1979).

The nonhelical domain, however, does dramatically increase both the rate of helical renaturation, as determined by CD measurements, and the percentage of molecules that regain their native size, as determined by gel filtration. The kinetics of renaturation of the 59K form of SC collagen (>60% reformation of helix in 40 min), again, are similar to those that have been described for type I procollagen (Bruckner & Prockop, 1981) in which >60% of the ellipticity at 221 nm returned within 60 min. In both molecules, it seems likely that the ability of the chains to remain associated and maintain proper chain registration greatly facilitates the kinetics of triple-helix formation. In type I procollagen, the interchain disulfide bonds are thought to perform this function; in SC collagen, such bonds are clearly not involved.

In these studies, we consistently observed in all denatured collagen samples that about 10–15% of the ellipticity at 222 nm returned almost immediately (<10 min) upon cooling the samples to 25 °C. We have no explanation for this. Type II collagen (not shown) also displayed this characteristic. We think it unlikely that this reflects the renaturation of a small portion of highly cross-linked molecules within the population; the 45K form also exhibits this behavior, yet it shows no cross-linked chains by SDS–PAGE analysis and no renaturation to native-size molecules as detected by gel filtration. Instead, this possibly represents the rapid formation of left-handed helix within individual chains. Such structures may be stabilized by interchain hydrogen bonds as suggested by studies on synthetic polyproline peptides (Brahmachari & Ananthanarayanan, 1979; Chopka & Ananthanarayanan, 1982).

Experiments presented here and elsewhere (Schmid & Linsenmayer, 1983) show that within the nonhelical domain, the chains of SC collagen are not held together or stabilized by disulfide bonds. Also, the chains remain associated even after being heated to 100 °C in 1 M $CaCl_2$ for 20 min (unpublished observation). They are, however, dissociated into constituent chains by a combination of the detergent SDS plus heating, although neither by itself is sufficient. These experiments indicate strong noncovalent interactions between the chains, possibly by hydrophobic bonding. The hydrophobic nature of the nonhelical domain has previously been suggested by the observation that the 59K form of SC collagen is retained on a Sephacryl S-500 column unless ethylene glycol is added to disrupt such interactions (Schmid & Linsenmayer, 1983). The relatively high concentration of aromatic amino acids present within this domain may contribute to such forces (Schmid & Linsenmayer, 1983).

What is the role of the nonhelical domains in assembly of procollagen molecules? One model of procollagen assembly (Bachinger et al., 1981) postulates that interchain association within the carboxyl-terminal propeptide is an early and requisite event in the formation of triple-helical procollagen. In procollagen types I, II, and III, interchain disulfide bonding occurs in this domain. It seems reasonable to assume that before disulfide bonds form, information contained in the primary structure of the chains or in the biosynthetic machinery of the cell must bring the three chains within the distances necessary to form such bonds. The nonhelical domain of the 59K form of SC collagen appears to be a site of noncovalent chain association, which is very resistant to thermal denaturation and facilitates a rapid refolding of the adjacent helical domain. Therefore, at least in SC collagen, a noncovalent association of individual chains is sufficient to maintain proper chain registration and allow rapid renaturation of the molecule's triple helix. Whether during the synthesis of the molecule this domain is responsible for the proper chain association and molecular assembly is an intriguing possibility. Experiments with antibodies sensitive to the native conformation of each domain [see Fitch et al. (1982) and Mayne et al. (1983)], now in production, will be helpful in elucidating such a role for this domain.

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Determination of the Metabolic Origins of the Sulfur and 3′-Nitrogen Atoms in Biotin of *Escherichia coli* by Mass Spectrometry†

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ABSTRACT: Two steps in the biosynthesis of biotin in *Escherichia coli*, incorporation of the nitrogen atom of methionine into 7-keto-8-aminopelargonic acid and of the sulfur atom into dethiobiotin, were examined. Sulfur and nitrogen metabolism were monitored by gas chromatography–mass spectrometry of volatile derivatives of internal (protein-bound) amino acids and excreted biotin. We were able to show that internal cysteine and excreted biotin were labeled to the same extent with ^{34}S from either of two exogenous sulfur sources, $^{34}SO_4^{2-}$ or L-[*sulfane*-^{34}S]thiocystine. Internal methionine was eliminated from consideration, while cysteine, or possibly a closely related intermediate, was implicated as providing the sulfur atom for biotin biosynthesis. Also, in experiments designed to follow the metabolism of the nitrogen atom of methionine, it was found that biotin excreted into the culture medium by this organism grown with 95 atom % [^{15}N]methionine contained greater than 70 atom % excess ^{15}N in one of the nitrogens over that obtained from cultures grown with methionine of natural abundance ^{15}N. These results provide evidence for the direct transfer of the methionine nitrogen as the role of *S*-adenosylmethionine in the conversion of 7-keto-8-aminopelargonic acid to 7,8-diaminopelargonic acid.

As shown previously (DeMoll & Shive, 1982, 1983), *Escherichia coli* will employ exogenous cystine, to the exclusion of methionine, as a sulfur source for biotin biosynthesis. Also, it was determined that all of the sulfur atoms of thiocystine [bis(2-amino-2-carboxyethyl) trisulfide] contribute approximately equally toward biotin biosynthesis. White (1982) has shown that both types of the sulfur atoms of thiocystine are eventually cycled through cysteine when the former is used as the sole sulfur source for the growth of the organism. Evidence has been presented that once transported into the organism, cystine is metabolized by both direct reduction to cysteine and by β-elimination to yield thiocysteine, pyruvate, and ammonia (White, 1983). Consequently, it was necessary to extend our investigation to the internal cysteine pool of *E. coli* to ensure that the ^{35}S incorporation into biotin we had previously observed was due to reduction of cystine to cysteine followed by (perhaps several steps later) transfer of sulfur to biotin.

Methionine has been shown by Pai (1971) to be 7–20 times as effective as any other amino acid in the production of 7,8-diaminopelargonic acid from 7-keto-8-aminopelargonic acid by crude extracts from several strains of *E. coli* K-12. A requirement for pyridoxal phosphate or pyridoxamine or its phosphate indicated the possibility of transamination. Eisenberg & Stoner (1971) have since shown that the ability of methionine to stimulate the in vitro synthesis of 7,8-diaminopelargonic acid is dependent upon ATP and Mg^{2+} and that *S*-adenosylmethionine is about 10 times as effective as methionine, ATP, and Mg^{2+}. In a purified system, methionine, ATP, and Mg^{2+} were shown to be ineffective in replacing *S*-adenosylmethionine. The direct participation of *S*-adenosylmethionine in a transamination reaction was proposed. However, attempts to detect the keto acid derived of *S*-adenosylmethionine were unsuccessful, and the possibility that *S*-adenosylmethionine might have an essential role in the reaction without transfer of nitrogen must be considered.

In this investigation, procedures for determining the atom percent excess ^{34}S and ^{15}N in excreted biotin, using gas chromatography–mass spectrometry techniques, were devel-

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