Characterization of a Unique Collagenous Fraction from Limited Pepsin Digests of Human Placental Tissue: Molecular Organization of the Native Aggregate†

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ABSTRACT: A high molecular weight, disulfide-bonded, collagenous aggregate obtained from limited pepsin digests of human placenta has been chromatographed under nondenaturing conditions on carboxymethylcellulose to achieve a high state of purity. The native aggregate exhibits a specific negative optical rotation approaching that for native triple-helical collagen molecules and a melting profile extending over the range of 31–70 °C. When disulfide bonds in the aggregate are reduced under nondenaturing conditions, the protein exhibits a sharp helix to random coil transition over the range of 27–37 °C suggesting that disulfide bonds are responsible for the increased thermal stability of the unreduced aggregate. When rechromatographed on carboxymethylcellulose under nondenaturing conditions, the reduced aggregate separates into two fractions: an unretained, acidic polypeptide and a retained fraction comprised of two relatively basic polypeptides. Thermal denaturation studies revealed that the fraction containing the basic polypeptides is responsible for the helical structure of the native aggregate and that no helical structure can be attributed to the fraction containing the acidic polypeptide. Additional data concerning the structural features of the native aggregate were obtained in experiments utilizing enzyme probes. Incubation of the unreduced aggregate with bacterial collagenase at 37 °C had no measurable effect on the constituent polypeptide chains, whereas incubation of the reduced aggregate with bacterial collagenase at 37 °C resulted in the cleavage of all components to small molecular weight fragments indicating that the presence of intact disulfide bonds in the aggregate rendered potential collagenase cleavage sites inaccessible to the enzyme. Incubation of the unreduced aggregate with pepsin at 37 °C results in selective destruction of the acidic polypeptide, supporting the thermal denaturation studies indicating that this chain does not participate in helix formation. However, incubation of the reduced aggregate with pepsin at 37 °C resulted in the cleavage of all component peptides to small molecular weight fragments, confirming that under these conditions the constituent chains contain little, if any, helical structure.

Previous studies have demonstrated that a unique, high molecular weight, disulfide-bonded, collagenous aggregate could be isolated from limited pepsin digests of vascular tissues (Chung et al., 1976) and placenta (Furuto & Miller, 1980). When reduced and carboxymethylated, the aggregate is dissociated to yield collagenous peptides with an apparent M_r of 40 000 and a heterogeneous mixture of low molecular weight, noncollagenous peptides (Furuto & Miller, 1980). It was further shown that the collagenous peptides could be accounted for in terms of a single relatively acidic peptide plus two more basic peptides (Furuto & Miller, 1980). The acidic peptide was further purified by DEAE[1]-cellulose chromatography, and its major CNBr cleavage products have been isolated and characterized (Furuto & Miller, 1980). The present report describes the isolation of the native aggregate by chromatography on CM-cellulose under nondenaturing conditions and details investigations on its unique molecular organization as evaluated by studies on its thermal stability and susceptibility to proteolysis.

Materials and Methods

Preparation of Collagen. The high molecular weight, disulfide-bonded, collagenous aggregate was isolated from limited pepsin digests of human placenta as detailed in an earlier report (Furuto & Miller, 1980).

CM-cellulose Chromatography (Nondenaturing Conditions). CM-cellulose chromatography under nondenaturing conditions was performed essentially as described previously (Kresina & Miller, 1979). Lyophilized protein was dissolved in 0.5 M acetic acid and then dialyzed into starting buffer, 40 mM (Na^+) sodium acetate (pH 4.8) containing 2.0 M urea at 4 °C. The dialyzed solution was centrifuged at 48000*g* for 15 min to clarify the solution. The supernatant containing the collagenous aggregate was applied to a jacketed 2.5 × 10 cm column of CM-cellulose (Whatman, microgranular CM 32), equilibrated with the starting buffer, and maintained at 8 °C by a circulating water bath. Chromatography was performed at a flow rate of 100 mL/h by employing a linear gradient formed by a two-chamber constant-leveling device containing 500 mL of starting buffer in the mixing chamber and 500 mL of limit buffer (starting buffer plus 0.4 M NaCl) in the second chamber. The column effluent during this procedure and all subsequent chromatographic separations were monitored, recorded, and collected as described (Rhodes & Miller, 1978). The fractions containing the native collagenous aggregate were pooled, desalted by dialysis against 0.5 M acetic acid at 4 °C, and lyophilized.

Polarimetry. Optical rotation was determined in the native aggregate isolated from CM-cellulose chromatography under nondenaturing conditions by using a Perkin-Elmer 241 polarimeter as previously described (Rhodes & Miller, 1978). Thermal denaturation curves were generated by stepwise temperature increases beginning at 20 °C. The readings were taken at 1 °C increments allowing 20-min equilibration times at each temperature.

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[1] Abbreviations used: CM, carboxymethyl; DEAE, diethylaminoethyl; DTT, dithiothreitol; M_r, molecular weight; $NaDodSO_4$, sodium dodecyl sulfate; T_m, melting temperature; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

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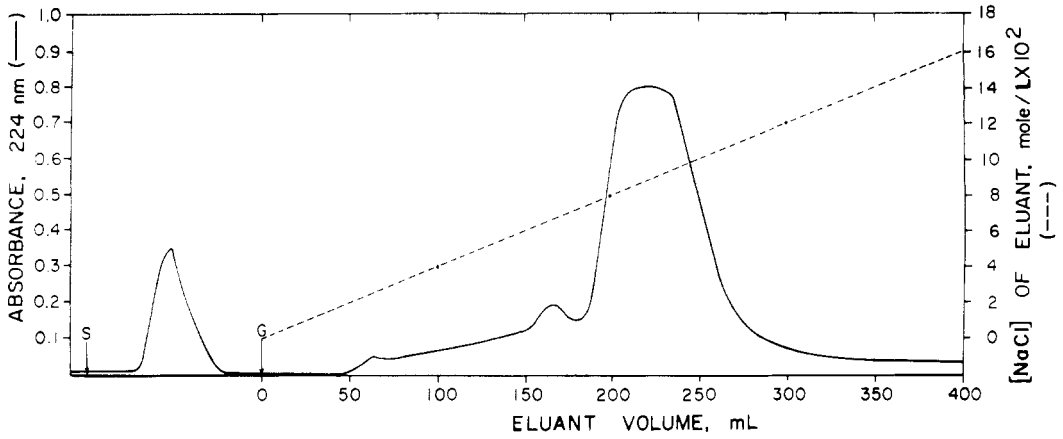

FIGURE 1: Chromatography of the native, disulfide-bonded, collagenous aggregate on CM-cellulose under nondenaturing conditions. The chromatogram represents the elution of a 30-mg sample dissolved initially in 30 mL of starting buffer. The conditions for chromatography are described in the text. S represents the point of sample application, and G is the point of gradient initiation.

Reduction–Alkylation (Nondenaturing Conditions). The protein recovered following CM-cellulose chromatography under nondenaturing conditions was dissolved in 0.5 M acetic acid at 4 °C (3 mg/mL) and dialyzed extensively against 2.0 M urea and 50 mM Tris, pH 8.0. After dialysis, DTT was added to the protein solution at a concentration of 20 mM, and reduction was allowed to proceed at 12 °C for 24 h. Following reduction, alkylation of the protein was performed by adding a 4-fold molar excess of iodoacetamide (relative to DTT) dissolved in 2.0 M urea and 50 mM Tris, pH 8.0. Alkylation was conducted in the dark at 12 °C for 8 h, after which time the reaction was terminated by dialysis against 0.5 M acetic acid. The dialyzed solution was subsequently lyophilized.

Portions of the reduced and alkylated native aggregate were then redissolved in 0.5 M acetic acid at 4 °C for polarimetry or subsequently dialyzed into starting buffer for CM-cellulose chromatography under nondenaturing conditions and rechromatographed as described above. On retrieval from the CM-cellulose column, fractions separated by this procedure were pooled, dialyzed vs. 0.5 M acetic acid at 4 °C, and lyophilized. These fractions were then examined for optical rotation as described above.

Polyacrylamide Gel Electrophoresis. Identification of the peptides contained in fractions eluted from CM-cellulose under nondenaturing conditions was performed by polyacrylamide disc gel electrophoresis utilizing 5% acrylamide gels in the presence of 0.1% $NaDodSO_4$ as previously described (Furthmayr & Timpl, 1971). Gels were scanned on a Gilford spectrophotometer, Model 240, equipped with a linear transport between 625 and 650 nm.

Use of Enzymatic Probes. Duplicate 2-mg samples of the native, disulfide-bonded, collagenous aggregate as isolated by chromatography on CM-cellulose under nondenaturing conditions were incubated with bacterial collagenase (Advance Biofactures, form III) or pepsin (Worthington Biochemicals, A, 3× recrystallized) at 37 °C.

In preparation for incubation with bacterial collagenase, the samples were redissolved in 0.5 M acetic acid (1 mg/mL) and then dialyzed vs. collagenase incubation buffer (2.5 mM Tris, pH 7.4, containing 300 mM $CaCl_2$ and 8 mM *N*-ethylmaleimide). The samples were preincubated at 37 °C for 1 h in this buffer, after which time bacterial collagenase (100:1, substrate to enzyme weight ratio) was added, and the samples were allowed to incubate with the enzyme for 18 h. Proteolysis was terminated by heating the digestion mixture at 90 °C for 15 min. The reaction mixture was subsequently desalted on Bio-Gel P-2 (Bio-Rad Laboratories, 100–200 mesh). Reduction and alkylation of control and collagenase-digested samples were performed under denaturing conditions as previously described (Furuto & Miller, 1980), except that DTT was substituted for 2-mercaptoethanol. The reduced and alkylated samples were desalted on Bio-Gel P-2, lyophilized, and subsequently examined by disc gel electrophoresis as described above.

Incubation with pepsin was performed by redissolving 2-mg samples in 0.5 M acetic acid (1 mg/mL) followed by a preincubation period of 1 h at 37 °C. Pepsin was added (100:1, substrate to enzyme weight ratio) to the experimental samples, and the mixture was incubated for an additional 18 h at 37 °C. At the termination of the reaction, the pH of the incubation mixture was adjusted with 4 M Tris base to pH 7.5 to inactivate the pepsin. The mixture was then desalted on a Bio-Gel P-2 column, lyophilized, and subsequently examined by disc gel electrophoresis. The effects of reduction of the disulfide bonds on enzyme susceptibility were examined in the same fashion as described above with the exception that the protein used for incubation was reduced and alkylated under nondenaturing conditions prior to incubation with the enzymes.

Results

CM-cellulose Chromatography of Native Aggregate (Nondenaturing Conditions). Collagen isolated from the 1.8 M NaCl precipitate as described (Furuto & Miller, 1980) was chromatographed on CM-cellulose under nondenaturing conditions to isolate the native aggregate. A representative chromatogram obtained during this procedure is shown in Figure 1. Approximately 90% of the protein applied to the column is eluted in a single peak over a gradient volume of 185–265 mL. This elution position closely corresponds to that observed for native type I, II, and III collagens as well as collagens containing the $\alpha 1(V)$[2] and $\alpha 2(V)$ chains, while collagens containing the $\alpha 1(IV)$ and $\alpha 2(IV)$ chains are eluted much earlier under the chromatographic conditions employed here (Kresina & Miller, 1979). The material which eluted during the application of the sample and prior to initiation of the gradient was shown by amino acid analysis to represent

[2] $\alpha 1(V)$ chain has been previously designated as B chain (Chung et al., 1976; Rhodes & Miller, 1978), and $\alpha 2(V)$ has been previously termed A chain (Chung et al., 1976; Rhodes & Miller, 1978). The $\alpha 1(IV)$ chain has been called C chain (Kresina & Miller, 1979), and the $\alpha 2(IV)$ chain has also been called D chain (Kresina & Miller, 1979).

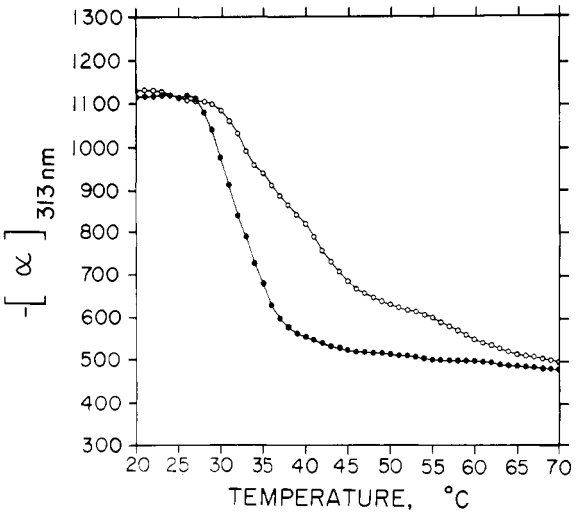

FIGURE 2: Thermal denaturation curves for native, disulfide-bonded, collagenous aggregate isolated from CM-cellulose chromatography (Figure 1). Native sample (O, upper curve) and native sample reduced and alkylated at 12 °C (see Materials and Methods) (●, lower curve) were dissolved (1 mg/mL) in 0.5 M acetic acid at 4 °C and subjected to thermal denaturation between 20 and 70 °C.

noncollagenous contaminants in the collagen preparation. Variable amounts of protein eluted between 50 and 180 mL of the gradient, and this material represented an additional portion of noncollagenous protein not removed during purification. The major fraction eluted from CM-cellulose chromatography was found upon reduction and alkylation and chromatography on Bio-Gel A-5m to correspond to the high molecular weight collagenous aggregate as described previously (Furuto & Miller, 1980).

Thermal Denaturation. The thermal stability of the collagenous aggregate isolated from CM-cellulose chromatography under nondenaturing conditions (Figure 1) was evaluated by optical rotation measurements as a function of temperatures. Figure 2 depicts the thermal denaturation pattern for the native, disulfide-bonded, collagenous aggregate. At 20 °C, the native aggregate exhibits a negative specific optical rotation of ~1130, which is approximately two-thirds that observed for native helical collagens (Rhodes & Miller, 1978). During stepwise increases in temperatures, the specific optical rotation gradually diminishes over the range from 31 to approximately 65–70 °C. Several deflections apparent in the melting profile of the native aggregate reveal that the aggregate probably contains several different regions of structure which exhibit various degrees of thermal stability. The apparent high resistance of the aggregate to melting with increasing temperature suggests a certain degree of structural stability derived from the presence of disulfide bonds.

This was confirmed in similar experiments on the reduced and alkylated native aggregate. As also shown in Figure 2, the reduced and alkylated native aggregate likewise exhibits an initial specific optical rotation of about −1110 at 20 °C but exhibits a relatively sharp helix to random coil transition over the range of 27–37 °C with a T_m of ~32 °C. This T_m is somewhat lower than that commonly observed for native collagen molecules but is consistent with the presence of relatively short helical domains within the native collagenous aggregate.

CM-cellulose Chromatography (Nondenaturing Conditions) of Reduced and Alkylated Native Aggregate. When the native, collagenous aggregate eluted from CM-cellulose under nondenaturing conditions (Figure 1) was reduced and alkylated under conditions expected to preserve native structure and rechromatographed on CM-cellulose under nondenaturing conditions, a much different elution profile is obtained. As shown in Figure 3, rechromatography of the reduced aggregate revealed that approximately one-fifth of the protein applied to the column was no longer retained and was eluted during sample application, whereas the remainder of the protein was more strongly retained from the unreduced aggregate and eluted as a single peak over a gradient volume of 260–340 mL.

Thermal Denaturation of Dissociated Components. Since reduction and alkylation of the aggregate resulted in the dissociation of at least two distinct molecular species from the original high molecular weight aggregate (Figure 3), these two fractions were evaluated for the presence of helicity and stability of the helical structure by polarimetry. As shown in Figure 4, the components present in the fraction retained on CM-cellulose exhibit an initial negative specific optical rotation and a melting profile remarkably similar to that observed for the reduced and alkylated aggregate prior to resolution of the components by CM-cellulose chromatography (Figure 2, solid circles). On the other hand, the material contained in the fraction which is unretained on CM-cellulose exhibits only a small initial negative optical rotation which is essentially unaltered at increasing temperatures, suggesting the presence of a random coil polypeptide. These data are interpreted as indicating that the components in the retained fraction contain all of the helical structure of the native aggregate as isolated here.

Polyacrylamide Gel Electrophoresis. The reduced and alkylated material in the fractions eluted from CM-cellulose under nondenaturing conditions (Figure 3) was denatured and identified by electrophoresis on 5% polyacrylamide disc gels

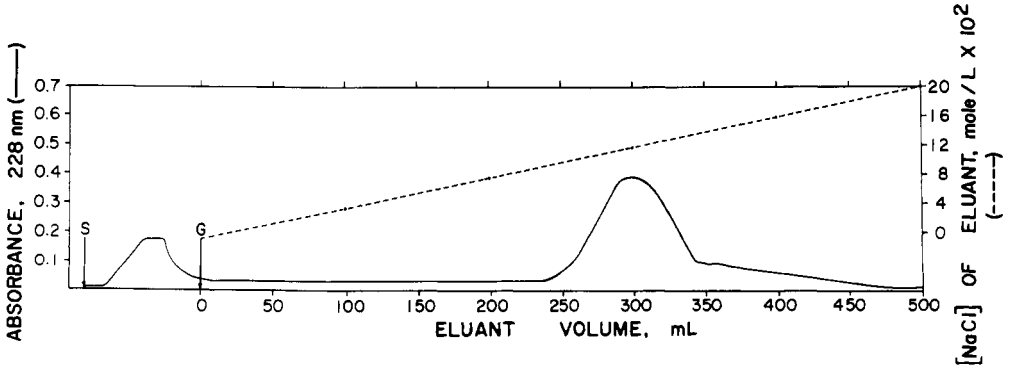

FIGURE 3: Chromatography of reduced and alkylated native aggregate on CM-cellulose under nondenaturing conditions. The sample (27 mg) containing disulfide-bonded, collagenous aggregates recovered from CM-cellulose under nondenaturing conditions (Figure 1) was reduced and alkylated at 12 °C (see Materials and Methods) and rechromatographed on the same column. S denotes the point of sample application, and G represents the point at which gradient elution was initiated.

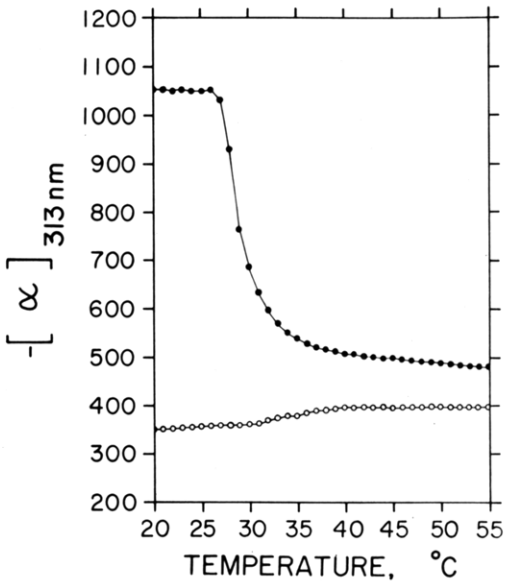

FIGURE 4: Thermal denaturation curves for components resolved on reduction and alkylation of native collagenous aggregate: material retained on CM-cellulose (●, upper curve); material unretained on CM-cellulose (○, lower curve).

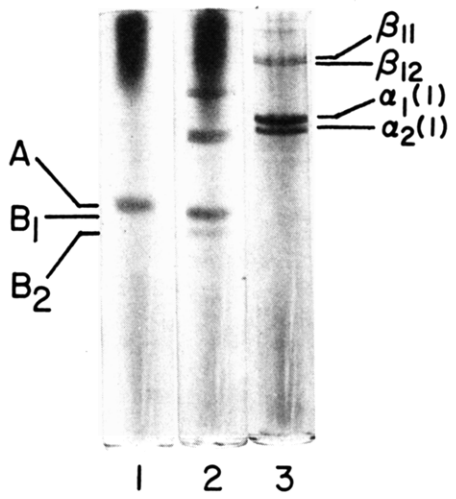

FIGURE 5: Polyacrylamide gel electrophoresis of reduced and alkylated components recovered from CM-cellulose under nondenaturing conditions (Figure 3). (Gel 1) Unretained fraction; (gel 2) retained fraction; (gel 3) α1(I) and α2(I) chains as well as β components from type I collagen. 16, 28, and 20 μg of protein were applied to gels 1, 2, and 3, respectively.

containing 0.1% $NaDodSO_4$ as shown in Figure 5. The unretained peak (gel 1) contained principally one peptide designated here as 40A, corresponding to the previously characterized 43 000-dalton, acidic polypeptide (Furuto & Miller, 1980). The retained fraction recovered from CM-cellulose contained two basic peptide bands (gel 2). The faster migrating band is designated $40B_2$, while the slower migrating band is designated $40B_1$. These two basic peptide bands correspond to the two ~40 000-dalton peptide bands previously recovered from CM-cellulose chromatography under denaturing conditions (Furuto & Miller, 1980). Gel 2 also reveals the presence of additional bands at molecular weights of approximately 80 000–90 000 and 130 000–140 000, corresponding to dimers and trimers of the individual basic peptides as judged by relative mobility compared with the α chains and β components of type I collagen shown in gel 3. The apparent ratio of $40B_1$ and $40B_2$ peptide bands varies between 3.2:1 and 4.2:1 in different preparations as judged by scans of the gels

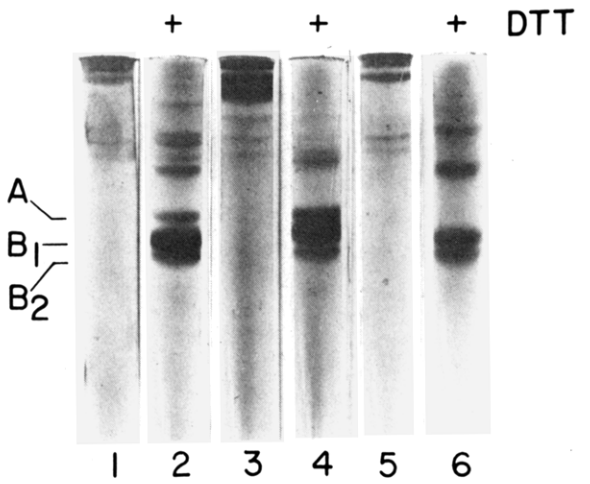

FIGURE 6: Polyacrylamide gel electrophoresis of aggregate control without enzyme treatment (lanes 1 and 2), after incubation with bacterial collagenase (lanes 3 and 4), and after incubation with pepsin (lanes 5 and 6). The protein on gels 2, 4, and 6 has been reduced under denaturing conditions prior to electrophoresis. A total of 40 μg of protein was loaded on each gel.

between 625 and 650 nm. Therefore, the $40B_1$ peptide band comprises 76–81% the basic peptide fraction. Preliminary experiments in this laboratory suggest that the $40B_1$ fraction on polyacrylamide gels contains two distinct polypeptide chains and that the $40B_2$ fraction contains these same two chains which have been further modified by pepsin.[3]

Incubation of the Aggregate with Collagenase and Pepsin. Figure 6 presents the results obtained on incubation of the aggregate with bacterial collagenase at 37 °C for 18 h (gels 3 and 4) and with pepsin at 37 °C for 18 h (gels 5 and 6). Only a small proportion of the undigested control sample (gel 1) penetrates into the upper portion of the gel. On reduction and electrophoresis of the control sample, the polypeptide bands (40A, $40B_1$, and $40B_2$) corresponding to the major reduction products of the aggregates are observed (gel 2). On incubation of the aggregate with bacterial collagenase, a somewhat greater proportion of the aggregate is able to penetrate the upper region of the gel (gel 3). However, on reduction of the collagenase-treated protein, the three major polypeptide bands are clearly still present (gel 4). These results, then, strongly suggest that proteolysis of the constituent chains in the aggregate on the part of bacterial collagenase is greatly restricted in the presence of intact disulfide bonds.

Similar results were observed on incubation of the aggregate with pepsin at 37 °C. Exposure to pepsin under these conditions did not greatly alter the general properties of the aggregate (Figure 6, gels 1 and 5). However, on reduction of the pepsin-treated aggregate, the band corresponding to the acidic 40A polypeptide is no longer present among the reduction products, whereas the basic polypeptide bands ($40B_1$ and $40B_2$) apparently remain intact (Figure 6, gels 2 and 6). In conjunction with results of the thermal denaturation studies, these results strongly suggest that resistance to proteolysis with pepsin is conferred largely through the participation of the $40B_1$ and $40B_2$ polypeptides in the formation of triple-helical segments. These results further support the conclusion that disulfide bonding considerably stabilizes the helical segments to thermal denaturation, since on reduction and alkylation under nondenaturing conditions the helical segments are fully denatured at temperatures above 27 °C (Figure 4, closed circles).

[3] D. K. Furuto, and E. J. Miller, unpublished experiments.

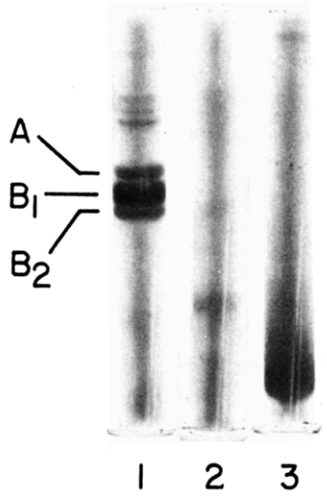

FIGURE 7: Polyacrylamide gel electrophoresis of reduced and alkylated aggregate digested with pepsin and bacterial collagenase. (Gel 1) Control sample; (gel 2) sample incubated with pepsin; (gel 3) sample incubated with bacterial collagenase. A total of 40 μg of aggregate was used in each experimental procedure.

Incubation of Reduced and Alkylated Aggregate with Collagenase and Pepsin. The conclusions reached above concerning the role of intact disulfide bonds in resistance to proteolysis were substantiated in additional experiments in which the enzymes were incubated with reduced and alkylated samples of the aggregate. In these experiments, samples of the native aggregate were reduced and alkylated under nondenaturing conditions prior to incubation with collagenase or pepsin at 37 °C for 18 h and then electrophoresed. All three major polypeptide bands are clearly present along with products of incomplete reduction in the control gel (Figure 7, gel 1). However, incubation of the reduced and alkylated aggregate with pepsin (gel 2) or bacterial collagenase (gel 3) results in degradation of all major components, remnants of which are observed to migrate near the bottom of the gels. These results not only verify the presence of collagenous regions in each of the major polypeptides but clearly indicate that each polypeptide is susceptible to degradation with collagenase in the absence of disulfide linkages. Moreover, these results clearly establish that each polypeptide is susceptible to degradation with pepsin, provided disulfide bonds are reduced and the samples are incubated with the enzyme above the denaturation temperature of the helices in which the $40B_1$ and $40B_2$ polypeptides participate.

Discussion

The studies presented here were initiated in order to elucidate the molecular organization of the major, 40 000-dalton collagenous polypeptides within a unique, high molecular weight, disulfide-bonded, collagenous aggregate isolated from limited pepsin digests of human placenta (Furuto & Miller, 1980). In preparation for these studies, we have isolated the native aggregate in a high state of purity by CM-cellulose chromatography under nondenaturing conditions. The native aggregate displays a rather high specific negative optical rotation indicative of the presence of regions of helicity. Dissociation of the aggregate and rechromatography under nondenaturing conditions allowed the recovery of the collagenous components in two fractions. The more acidic fraction was comprised of a single collagenous polypeptide, 40A, which has been shown to contain alternating regions of collagenous and noncollagenous sequences (Furuto & Miller, 1980). The more basic fraction contained polypeptide bands, $40B_1$ and $40B_2$, which in native conformation accounted for most, if not all, of the helicity of the native aggregate. Thermal denaturation studies revealed that regions of helicity within molecules formed by these chains are considerably stabilized by disulfide linkages. The presence of intact disulfide linkages renders the aggregate nonsusceptible to proteolysis with bacterial collagenase and greatly limits its susceptibility to degradation with pepsin. Only the acidic polypeptide which apparently does not participate in helical structures was susceptible to degradation with pepsin on reexposure of the disulfide-bonded aggregate to pepsin at 37 °C. At 4 °C, however, even the acidic polypeptide is somewhat stable to proteolysis with pepsin, since the starting aggregate is isolated from limited pepsin digests of whole placenta under such conditions.

The data presented here agree quite well with previous observations indicating that interchain disulfide bonds located in or near relatively short regions of collagen helicity increase the thermal stability of these helices. In this regard, the collagenous domain in the NH_2-terminal precursor-specific region of type III procollagen contains interchain disulfide bonds at its COOH terminus, and reduction and alkylation of this domain under nondenaturing conditions reduces its T_m by ~20 °C (Bruckner et al., 1978).

Furthermore, the collagenous domain within the NH_2-terminal precursor-specific region of type III procollagen is not readily susceptible to digestion with bacterial collagenase, except when the precursor-specific region is reduced and alkylated (Nowack et al., 1976) or when it is treated with collagenase at elevated temperatures (Bruckner et al., 1978). Although the homologous domain of type I procollagen does not contain interchain disulfide bridges, it likewise exhibits relative resistance to digestion with bacterial collagenase (Morris et al., 1979). Resistance to collagenase was not observed, however, after denaturation of the NH_2-terminal precursor-specific region at 60 °C, leading to the proposal that the globular domain preceding the collagenous domain in the NH_2-terminal precursor-specific region was folded back over the collagenous domain thereby restricting the activity of bacterial collagenase on the native protein (Morris et al., 1979). This proposal is compatible with the data of Bruckner et al. (1978) as well as the data of Nowack et al. (1976), since reduction and alkylation of intrachain disulfide bonds in the NH_2-terminal globular region might very well alter the configuration of this domain. A similar mechanism might also be proposed to explain the relative resistance to bacterial collagenase on the part of the unreduced aggregate observed in the present studies, since the native aggregate contains low molecular weight noncollagenous fragments which are dissociated from the complex on reduction (Furuto & Miller, 1980).

It is of interest to note that a collagenous aggregate has been isolated from the collagens extracted from a murine basement membrane tumor as the result of its resistance to proteolysis when exposed to pepsin and bacterial collagenase (Timpl et al., 1979). This aggregate consisted of three chains of different molecular weight and exhibited a T_m of ~70 °C with a thermal transition between 55 and 80 °C. The high thermal stability of the triple-helical portions of the aggregate as well as its resistance to proteolysis during isolation was attributed to extensive interchain disulfide bonding. In addition, bacterial collagenase and Pronase digestion of bovine glomerular basement membrane yields a high molecular weight fraction which can be further digested with these enzymes following performic acid oxidation of disulfide linkages (Levine & Spiro, 1979). In this latter study, oxidation of the aggregate followed by reexposure to the enzymes allowed the isolation of a rela-

tively short sequence of 15 amino acids which was apparently comprised of collagenous and noncollagenous sequences. The relationship of the aggregate isolated in our studies to those isolated in the above studies remains to be determined.

In summary, the data on the high molecular weight, disulfide-bonded, collagenous aggregate suggest that the $40B_1$ and $40B_2$ components form separate helical segments with the majority of the helicity derived from segments formed by the $40B_1$ chains. In contrast, the 40A subunit does not participate in the formation of helical structures. Further, each helical segment contains disulfide bridges between chains participating in the helix, and all major elements of the structure are linked through intermolecular disulfide bonds, including the attachment of relatively short noncollagenous chains to each of the major polypeptides. This latter conclusion is supported by the observation that on reduction and alkylation under denaturing conditions, the aggregate is completely dissociated to yield the three major polypeptides plus a number of small molecular weight, noncollagenous peptides (Furuto & Miller, 1980). Furthermore, as discussed above, disulfide linkages between the chains involved in helix formation would contribute to the thermal stability of the native, unreduced aggregate. This in turn would render the helices relatively resistant to proteolysis with pepsin even at relatively high temperatures provided disulfide linkages were present. Resistance to proteolysis on exposure to collagenase, on the other hand, is likely to be conferred by the presence of disulfide-linked, noncollagenous components in the unreduced protein.

Although numerous roles of the native aggregate might be suggested at this time, the fact that the aggregate can be reproducibly isolated following limited pepsin proteolysis of placenta and other tissues (Chung et al., 1976) certainly suggests that is represents a portion of a unique macromolecule or a highly specialized junction region connecting different segments of several distinct macromolecules which have been fragmented by limited pepsin digestion. The apparent complexity of the aggregate favors the latter possibility.

Acknowledgments

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Procedure for Purification of *Escherichia coli* Ribonucleic Acid Synthesis Termination Protein ρ†

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ABSTRACT: An improved purification procedure is described for the ρ transcription termination factor of *Escherichia coli*. The method involves lysozyme–sodium deoxycholate lysis, Polymin P fractionation, and chromatography on phosphocellulose, poly(uridylic acid)–Sepharose, and AMP–agarose. The method yields up to 9 mg of electrophoretically pure protein from 200 g of *E. coli* MRE 600. From quantitative amino acid analysis ρ is calculated to have an $E_{280nm}^{1\%}$ of 3.7 ± 0.3. The purified ρ has an ATPase specific activity of 32 nmol of P_i released min^{-1} μg^{-1} when poly(cytidylic acid) is used as a cofactor, and it functions effectively in termination of T7 DNA transcription. A subunit molecular weight of 48 000 for ρ was determined by phosphate-buffered sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The amino acid composition and circular dichroism spectrum in the far-ultraviolet for ρ are presented.

The activity of the DNA[1]-dependent RNA polymerase isolated from *Escherichia coli* can be modulated by several protein factors (Losick, 1972). One of these, called ρ, causes termination of RNA synthesis at specific sites on the DNA template and release of the termination RNA molecules (Roberts, 1969, 1976; Adhya & Gottesman, 1978). The highly purified ρ is also an RNA-dependent nucleoside triphosphate phosphohydrolase (NTPase). Its NTPase activity is required for termination; however, ρ can catalyze the NTPase reaction in the absence of termination (Lowery-Goldhammer & Richardson, 1974).

For elucidation of the mechanism of action and physical properties of ρ, large amounts of the enzyme will be required.

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[1] Abbreviations used: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; poly(C), poly(cytidylic acid); poly(U), poly(uridylic acid); DNase, deoxyribonuclease; DEAE, diethylaminoethyl; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; EDTA, ethylenediaminetetraacetic acid; ATPase, adenosine 5′-triphosphatase; ATP, adenosine 5′-triphosphate; GTP, guanosine, 5′-triphosphate; UTP, uridine 5′-triphosphate; CTP, cytidine 5′-triphosphate.

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