ULTRAVIOLET ABSORBANCE CHANGES ACCOMPANYING THE DENATURATION OF SOLUBLE COLLAGEN AND ATELOCOLLAGEN

R. J. Doyle and Jake Bello

Roswell Park Memorial Institute, Buffalo, N. Y. 14203

Received May 10, 1968

Recent studies on the structure of collagen have shown that small extrahelical appendages protrude from its polypeptide core. These protease-labile appendages, termed telopeptides (Drake et al., 1966; Rubin et al., 1965), contain most, if not all, of the tyrosyl residues found in collagen. In addition, tyrosyl groups have been implicated in the formation of native fibers from neutral collagen solutions (Bensusan, 1966). We now present evidence that the near ultraviolet absorption spectrum of soluble collagen is conformation dependent.

EXPERIMENTAL

Rat tail tendon acid soluble collagen was obtained as previously described (Bello and Bello, 1967). Treatment of acid-soluble collagen with pepsin and pronase (Calbiochem., Los Angeles, California) was carried out according to Drake et al., (1966) and Rubin et al., (1965) using a protein: enzyme ratio of 75 to 1 and an incubation temperature of 18°. Incubation times were 16 hrs.

Difference spectra were obtained with a Cary Model 15 spectrophotometer. Collagen solutions were solubilized overnight at 5° in 1.0 M acetic acid, centrifuged 30 minutes at 30,000 xg and denatured by heating 15 minutes at 60°. Following denaturation, the collagen solutions were cooled to room temperature (25°) in a water bath and the difference spectra obtained. Collagen solutions without heat treatment

were placed in the sample compartment while the denatured solutions were in the reference beam. Concentrations were 3 mg/ml except for calfskin soluble collagen which was 2.5 mg/ml. Paired 1-cm silica cuvettes were used as sample holders.

Thermal transitions representing the helix-coil transformation of soluble collagen were obtained by use of two 1-cm cells in each compartment. The use of four cells (each containing 3 mg/ml collagen in 1.0 M acetic acid) provided a longer path length, hence larger absorbance changes. The base line was adjusted to zero while each compartment was thermostatted at 25°. The temperature of one compartment was raised by 1° increments and the difference spectra obtained after 25 minute equilibrations. Control experiments established that 25 minute equilibrations were adequate to obtain maximum absorbance changes. The use of 1.0 M acetic acid as a solvent largely eliminated turbidities of denatured collagen solutions. Viscosities were obtained by use of a capillary Ubbelohde viscometer. In these measurements the collagen concentration was 1 mg/ml in 1.0 M acetic acid. Melting temperatures, T_m, are taken as the midpoint of the slope of difference spectra- or viscosity-temperature profile.

RESULTS AND DISCUSSION

The ultraviolet absorption spectrum of rat tail acid soluble collagen, shown in Fig. 1, is characterized by maxima at 257, 263, 267 and 276 mμ and shoulders at 251 and 283 mμ. The absorptions at 283 and 276 mμ are due to tyrosine while the other maxima are attributed to phenylalanine (see Fujimora, 1966). Following protease treatment, soluble collagen exhibits reduced absorption in the near ultraviolet region (Fig. 1). Analysis for tyrosine in denatured native collagen by the tetranitromethane reagent of Sokolovsky *et al.* (1966) gave a value of 13.8 tyrosines/mole, while pepsin-treated and pronase-treated collagens gave values of 4.1 and 2.9 or 70 and 79% loss, respectively, of tyrosines. Lyophilized tetranitromethane-treated collagens were solubilized by hydrolysis in 6 N HCℓ under nitrogen. Following solubilization the absorbance was obtained at 381 mμ and the nitrotyrosyl content calculated according to Sokolovsky *et al.* (1966). Tetranitromethane promotes a crosslinking reaction in collagen (Doyle, Bello

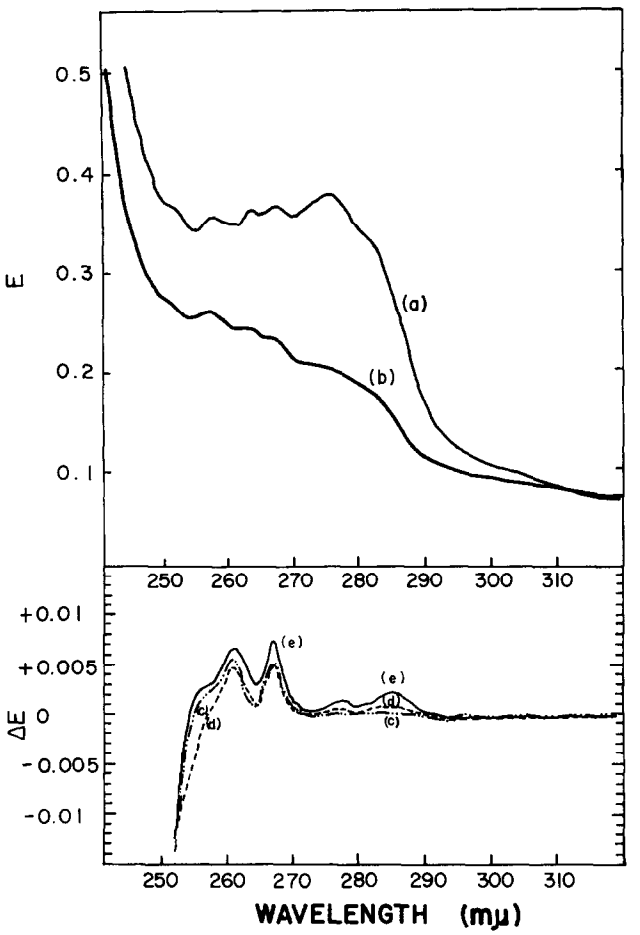

Fig. 1 Difference spectra of heat denatured soluble collagens. (a) and (b), UV absorption spectra for 3 mg/ml solutions of rat tail tendon soluble collagen and atelocollagen (pronase), respectively; (c), atelocollagen (pronase) vs heat denatured atelocollagen; (d) calfskin soluble collagen vs denatured calfskin collagen; (e) rat tail tendon soluble collagen vs denatured rat tail collagen.

---

and Roholt, 1968) thereby necessitating hydrolysis prior to nitrotyrosine analysis. Upon denaturation of soluble collagen an ultraviolet difference spectrum is generated characterized by rather wide shoulders at 284 and 276 mμ, maxima at 267 and 261 mμ and a shoulder at approximately 257 mμ. Below 255 mμ the absorption of the denatured collagen is much greater than

that of the native collagen.

Previously Wood (1963), Gratzer et al. (1963) and Katz et al. (1964) reported large differences in absorption between native and denatured calfskin soluble collagen below 250 mμ. These changes largely reflect modifications of the peptide bond environment (see review by von Hippel, (1967)). We have performed similar experiments using dilute (0.1 mg/ml) collagen solutions, and confirmed their findings. However, much higher collagen concentrations are needed to detect UV changes in the region 250-300 mμ.

Enzyme-treated soluble collagen also gave rise to difference spectra above 250 mμ. Major peaks appeared at 261 and 267 mμ, similar to untreated collagen, while maxima at 276 and 284 mμ were absent (Fig. 1, for pronase-treated collagen). Similar results were obtained following pepsin treatment. Apparently the enzyme removed the tyrosyl-laden telopeptides while leaving intact most of the phenylalanine residues which are found in the main structure (Drake et al., 1966). In consistency with the naming of various collagens (e.g. eucollagen, procollagen, etc.), we propose the name "atelocollagen" for protease-treated soluble collagens.

It is unlikely that the difference spectra for native and denatured soluble collagen reported here arise from contaminants. A sample of calfskin acid soluble collagen prepared in another laboratory gave rise to identical difference spectra (Fig. 1). Thus, it is probable that these spectra are due to changes in the environment of phenylalanine and tyrosine in the native and denatured states of soluble collagen.

That the difference spectrum originates from the helix-coil transition is apparent from the data in Fig. 2 and Table 1.

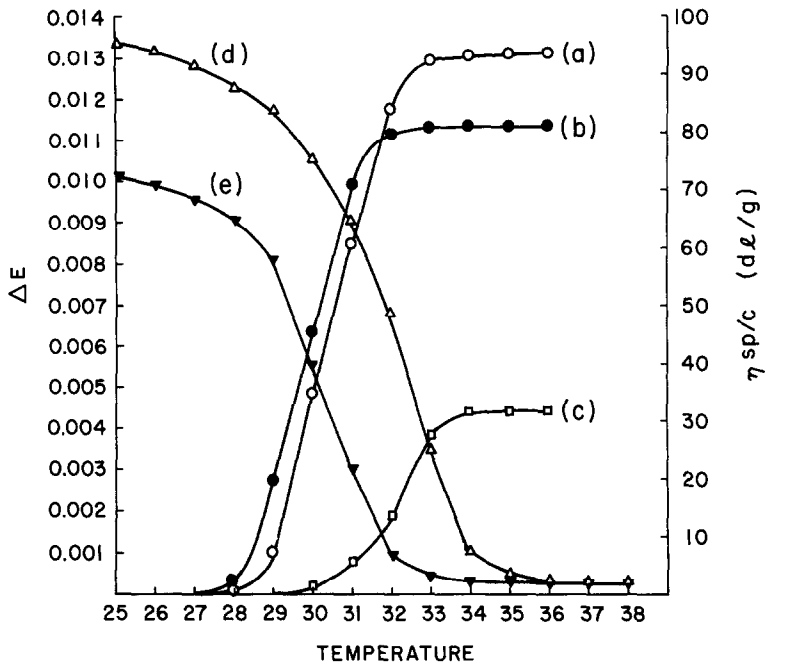

Fig. 2 The helix-coil transformation of rat tail tendon collagen. (a), ΔE at 267 mμ as a function of temperature for rat tail tendon collagens; (b), ΔE at 267 mμ as a function of temperature for atelocollagen (pepsin); (c) ΔE at 284 mμ as a function of temperature for rat tail tendon collagen; (d), viscosity-temperature for rat tail tendon soluble collagen; (e), viscosity-temperature curve for atelocollagen (pepsin). Curves similar to (a) and (b) can be obtained from 261 mμ measurements. Melting temperatures are summarized in Table 1.

Table I. Melting temperatures of rat tail soluble collagen and atelocollagen.[a]

	Rat tail tendon	Atelocollagen[b]
267 mμ	30.9 ± 0.7	29.7 ± 0.6
261 mμ	31.0 ± 0.6	29.5 ± 0.7
284 mμ	32.2 ± 0.9	
Viscosity	32.0	30.1

[a]Values given are the average of 4-5 separate experiments with their standard error. Transition temperatures based on viscosities are the average of two determinations.

[b]Obtained by treating rat tail soluble collagen with pepsin.

Thus, native rat tail tendon soluble collagen in 1.0 M acetic acid exhibits a T_m of approximately 31° when measured by difference spectra at 261 or 267 mμ. In contrast, atelocollagen (pepsin) has a T_m of approximately 29.7° based on spectral measurements.

The T_m difference between native collagen and atelocollagen may be due to a cleavage of intramolecular crosslinks in the native collagen resulting in a molecule which has less resistance to heat. Drake et al. (1966) have shown that in addition to removing telopeptides proteolytic enzymes convert a large percentage of β strands into their α components. However, in our hands, carboxymethyl-cellulose elution profiles of denatured atelocollagen indicate that the action of proteases on soluble collagens may be more complex than the simple conversion of β strands into their α components. It is possible that telopeptides may impart some structural rigidity upon the stranded core of tropocollagen. Support for such an explanation comes from a study by Fujimora (1966) who showed that ultraviolet-irradiated soluble collagen had a lower T_m than controls. In his experiments it was shown that the helical nature of soluble collagen was unchanged following UV irradiation and that the telopeptides were the major loci of UV action.

The higher T_m as measured at 284 mμ as compared to 261 or 267 mμ suggests that the telopeptides of collagen may be less susceptible to denaturation than phenylalanine regions. It is possible that telopeptide conformations may be important in the formation of native fibrils. The transition profile at 284 mμ shows a fairly steep slope at 30-31.5°. This suggests the possibility of a transition occurring earlier than the main transition, but the data are not precise enough to decide. The T_m of

the 284 mμ profile was taken as the mid-point of the steepest slope. An alternative method taking the point halfway along ΔE gives a T_m 0.2° lower, which is less than the experimental uncertainty.

The close correspondence between T_m (284 mμ) and T_m (viscosity) suggests that the telopeptide is related to the basic conformation of collagen, while the lower T_m (261, 267 mμ) suggests that some of the phenylalanine regions are more easily denatured. The somewhat lower values of T_m (261, 267 mμ) for atelocollagen are close to T_m (viscosity) for atelocollagen; this suggests that after removal of the telopeptides the remaining phenylalanine residues are dependent on the gross conformation. Since atelocollagen has fewer phenylalanines than native collagen, the difference between T_m (261, 267 mμ) for native and atelocollagen may be due to the loss of phenylalanine from the more thermally resistant telopeptides. This cannot be decided because of the low T_m (viscosity) which reflects a decrease in overall conformational stability.

Taking a value of 14 tyrosyl residues/mole of collagen and a molecular weight of 300,000 (Piez et al., 1963), it can be shown that ΔE/tyr/mole collagen is approximately 17 (Fig. 1, 284 mμ). This value contrasts with that for a fairly typical globular protein, ribonuclease, where ΔE/tyr/mole RNA'se is approximately 500 for acid denaturation (Bigelow and Krenitsky, 1064). Probably the 1.0 M acetic acid used in these studies elicited a partial exposure of the tyrosyl groups. The small ΔE for collagen suggests that the tyrosines are not buried in native collagen, but that only small conformational changes take place around the phenolic groups. This is supported by the observation that all of the tyrosines of collagen are

accessible to iodination (Bensusan, 1966).

ACKNOWLEDGEMENTS

The authors express gratitude to Professor H. B. Bensusan who supplied a sample of calfskin acid soluble collagen. This investigation was supported in part by Grant GM-14004-01 from the National Institutes of Health.

REFERENCES

Bello, J. and Bello, H. (1967) Biochim. Biophys. Acta, 147, 272.

Bensusan, H. B. (1966) Arch. Biochem. Biophys., 155, 77.

Bigelow, C. C. and Krenitsky, T. A. (1964) Biochim. Biophys. Acta, 88, 130.

Doyle, R. J., Bello, J. and Roholt, O. A. (1968) Biochim. Biophys. Acta (in press).

Drake, M. P., Davison, P. F., Bump, S. and Schmitt, F. O. (1966) Biochem., 5, 301.

Fujimora, E. (1966) Biochem. 5, 1034.

Gratzer, W. B., Rhodes, W. and Fasman, G. (1963) Biopolymers. 1, 319.

Katz, E. P., Nishigai, M. and Bonar, L. C. (1964) Collagen Currents, 4, 32.

Piez, K., Eigner, E. A. and Lewis, M. S. (1963) Biochem., 2, 58.

Rubin, A. L., Drake, M. P., Davision, P. F., Pfahl, D., Speakman, P. and Schmitt, F. O. (1965) Biochem., 4, 181.

Sokolovsky, M., Riordan, J. F. and Vallee, B. (1966) Biochem. 5, 3582.

Von Hippel, P. in G. Ramachandran, "Treatise on Collagen", Vol. I, Academic Press, New York, 1967, p. 270.

Wood, G. C. (1963) Biochem. Biophys. Res. Comm., 13, 95.