Thermal Stability of the Triple Helix of Type I Procollagen and Collagen. Precautions for Minimizing Ultraviolet Damage to Proteins during Circular Dichroism Studies†

Toshihiko Hayashi,‡ Samantha Curran-Patel, and Darwin J. Prockop*

ABSTRACT: The helical stability of type I procollagen and type I collagen was examined by circular dichroism (CD) under a variety of conditions. The temperature at midpoint of the helix-to-coil transition (T_m) of both collagen and procollagen was reduced by prolonged exposure of the proteins to the ultraviolet beam of the CD instrument. The reduction in T_m was apparently explained by random scission of the polypeptide chains, since peptide fragments of varying size were generated by the ultraviolet irradiation. Although the phenomenon was not explored in detail, the observations raised the possibility that ultraviolet damage may be encountered in CD studies on other proteins. With collagen and procollagen irradiation damage was minimized by interrupting the ultraviolet beam or carrying out the thermal transition rapidly. The same T_m values were obtained by increasing the temperature at rates varying from 0.8 to 65 °C/h. When the effects of ultraviolet irradiation were minimized, it was possible to examine the thermal transitions of procollagen and collagen in neutral buffer and at concentrations low enough to avoid precipitation. Under these conditions the T_m of type I collagen from chick embryo tendons was 42 °C. Type I procollagen from the same source had the same T_m. The results demonstrated therefore that the propeptides found at both the N and C terminals of type I procollagen have no effect on the thermal stability of a triple-helical domain of the molecule. Partially purified procollagen from chick embryo fibroblasts had the same T_m as purified procollagen from the same source, an observation which does not support the hypothesis that the protein is secreted as an aggregate in which lateral association of monomers increases their thermal stability.

Collagen fibers from homeothermic organisms are stable up to temperatures of 58–60 °C [see Traub & Piez (1971), Rigby & Robinson (1975), and Bornstein & Traub (1978)]. The same collagens in solution, however, have been observed to undergo a helix-to-coil transition whose T_m[1] is either at the normal body temperature of the organism or 2–5 °C higher. Therefore, one of the unanswered questions about collagen biosynthesis is: how do cells synthesize a protein which in solution is at the limit of its thermodynamic stability at body temperature?

Several suggestions have been made to explain the apparent dilemma posed by this question. One suggestion has been that the T_m of collagen in solution, as usually measured in vitro, does not reflect the T_m in vivo because collagen spontaneously forms fibrils at body temperature and neutral pH. Therefore, it is difficult to measure accurately the T_m under physiological conditions. Gross (1964) used 0.1–0.4 M arginine to prevent fibril formation and found a T_m of 42 °C from rat tail in neutral phosphate buffer. Hayashi & Nagai (1973) used 1 M glucose to prevent fibril formation. Under the neutral conditions they employed, the T_m of calf skin collagen was 39–40 °C. A second suggestion has been that collagen in vivo may be associated with other molecules, such as proteoglycans, and that this association increases its thermal stability. Gelman & Blackwell (1973, 1974) added mucopolysaccharides to solutions of calf skin collagen and found that the T_m increased from 38 to 46 °C. They suggested, therefore, that such interactions may be important in vivo. A third suggestion has been based on the discovery that collagen is first synthesized as the larger protein known as procollagen. The additional *N*- and *C*-propeptides on procollagen may increase the thermal stability of the collagen domain, and cleavage of procollagen to collagen may take place under circumstances in which collagen itself is not in solution for any considerable time [see Prockop et al. (1979)].

We report here on the thermal stability in solution of type I procollagen and collagen from chick embryos.

Materials and Methods

Materials. Bacterial collagenase was purchased from Worthington Biochemical Corp. and purified by chromatography on Sephadex G-200 (Peterkofsky & Diegelmann, 1971). Other materials were obtained from sources previously specified (Dehm & Prockop, 1972; Hoffmann et al., 1976).

Preparation of Type I Procollagen. Fibroblasts were isolated from the tendons of 17-day-old chick embryos by controlled enzymic digestion with the procedures employed previously (Dehm & Prockop, 1972; Hoffmann et al., 1976). From (1 to 2) × 10^9 cells were incubated in a concentration of (10 or 25) × 10^6 cells/mL at 37 °C in Krebs modified medium without fetal calf serum for 4 h. [^{14}C]Proline, 240 μCi/μmol (New England Nuclear), was added to the incubation medium to a concentration of 1–2 μCi/mL in order to label the procollagen synthesized by the cells. After the incubation, the cells were removed by centrifugation at 12000*g* for 5 min at room temperature, and a mixture of protease inhibitors was added to the medium [see Hoffmann et al. (1976)]. The medium was processed as described below.

Partial Purification of Type I Procollagen by Gel Filtration. The medium from fibroblasts incubated at a concentration of 25 × 10^6 cells/mL was removed and immediately passed through a 2.5 × 60 cm column of polyacrylamide (Bio-Rad P-200; 200–400 mesh) which was equilibrated and eluted at room temperature with the same Krebs medium used to incubate the cells. The sample volume was 8 mL, the flow rate was 18 mL/h, and fractions of 2 mL were collected. Fractions containing ^{14}C-labeled procollagen were taken immediately for CD studies, since preliminary experiments indicated that

† From the Department of Biochemistry, College of Medicine and Dentistry of New Jersey—Rutgers Medical School, Piscataway, New Jersey 08854. *Received January 23, 1979.* This work was supported in part by Research Grant AM-16516 from the National Institutes of Health.

‡ Present address: Department of Tissue Physiology, Medical Research Institute, Tokyo Medical and Dental University, Chiyoda-ku, Tokyo, Japan.

[1] Abbreviations used: T_m, temperature at midpoint of the helix-to-coil transition; CD, circular dichroism; $NaDodSO_4$, sodium dodecyl sulfate.

0006-2960/79/0418-4182$01.00/0

the partially purified procollagen precipitated if stored at 4 °C overnight.

Purification of Type I Procollagen by DEAE-cellulose Chromatography. The medium from the fibroblasts was precipitated by addition of 176 mg/mL ammonium sulfate and then chromatographed on a DEAE-cellulose column in the presence of 2 M urea as described previously [see Hoffmann et al. (1976)]. Fractions from the column containing the ^{14}C-labeled procollagen were dialyzed against 0.4 M NaCl in 0.1 M Tris-HCl buffer, pH 7.9 at 4 °C, and concentrated by precipitation with 176 mg/mL ammonium sulfate. The sample was suspended in the 0.4 M NaCl in 0.1 M Tris-HCl buffer (pH 7.4 at 25 °C), the "standard neutral buffer" employed here, and stored frozen at −20 °C. The protein was homogeneous when examined by polyacrylamide gel electrophoresis in $NaDodSO_4$ (Hoffmann et al., 1976).

Preparation of Type I Collagen from Chick Embryos. One milligram of β-aminopropionitrile fumarate in 0.2 mL of distilled water was placed on the chorioallantoic membrane of 14-day-old embryos, and the treatment was repeated on days 15 and 16. On day 17, leg tendons were removed and collagen was extracted from the tendons at 4 °C for 24 h with 10 volumes of 0.1 M Tris-HCl (pH 7.4), 0.4 M NaCl, and 1 M glucose. The samples were centrifuged at 20000*g* for 30 min, and collagen in the supernate was precipitated by adding NaCl to a final concentration of 2.1 M. The collagen was recovered by centrifuging at 20000*g* for 30 min. The protein was then further processed by one of two procedures. In one procedure the protein was dissolved at a concentration of ~1 mg/mL in 0.4 M NaCl and 0.1 M Tris-HCl buffer, pH 7.4. The sample was dialyzed against the same buffer, and the precipitation with 2.1 M NaCl was repeated 2 times. In the alternate procedure, the pellet from the first 2.1 M NaCl precipitate was dissolved in 0.5 M acetic acid, centrifuged, and lyophilized. Polyacrylamide gel electrophoresis (see below) with and without reduction indicated that there was essentially no type III collagen present and that the ratio of α1 to α2 chains was ~2:1.

CD Studies. CD was examined in a Model 61 Varian spectropolarimeter and in water-jacketed cells. The temperature was controlled by a water bath with an automatic programmer (Neslab Instrument, Inc.; Model TP-2), and the temperature was recorded with a thermistor. In initial experiments a cell with a 1-mm light path was employed, and the thermistor (YSI, Thermilinear Yellow Springs Instruments, Yellow Springs, OH) was inserted into the jacket of the cell. In most of the experiments reported here, however, 2- and 5-mm cells were employed and the thermistor was inserted into the top of the sample chamber. To ensure that the procollagen did not precipitate during measurements of thermal transition, we removed 10-μL aliquots of the sample from the top of the cuvette before and after each experiment. The aliquots were then assayed for ^{14}C in a liquid scintillation counter. In all the experiments reported here there was no apparent precipitation of ^{14}C-labeled procollagen during examination of the CD spectrum or the thermal transition.

Gel Electrophoresis. Polyacrylamide gel electrophoresis was performed with the standard procedures as described previously (Hoffmann et al., 1976). Slabs of 2-mm thickness were used, the stacking gel was 4.5% polyacrylamide, and the separating gel was 6% polyacrylamide. Samples in 0.1 M Tris-HCl buffer, pH 7.4, containing 0.4 M NaCl were prepared for electrophoresis by adding a one-fifth volume of 10% $NaDodSO_4$ and 2-mercaptoethanol to a final concentration of ~5%. The samples were heated at 100 °C for 3 min.

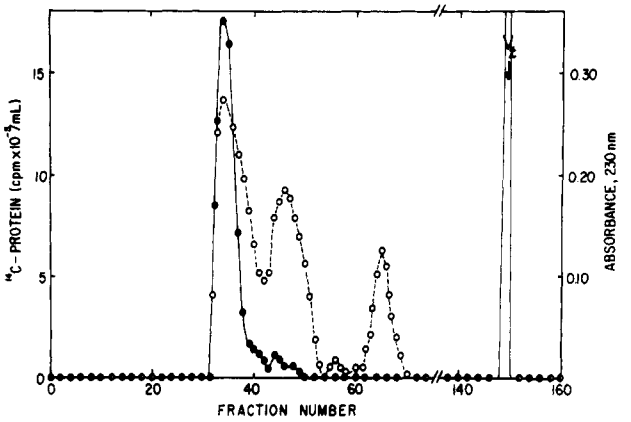

FIGURE 1: Gel filtration of medium from matrix-free tendon fibroblasts. Medium from fibroblasts was passed through a 2.5 × 60 cm column of polyacrylamide (Bio-Rad P-200) which was equilibrated and eluted at room temperature with culture medium. The void volume appeared in about fraction 35 and the total volume in about fraction 149. Absorbance at 230 nm (○); [^{14}C]protein (●); and [^{3}H]H_2O (—).

Glycerol was added to a final concentration of 10% and bromophenol blue to a concentration of 0.001%. For fluorography, the gels were impregnated with 2,5-diphenyloxazole (Eastman), dried, and exposed to preflashed RP Royal X-OMAT X-ray film at −70 °C for 24 h (Bonner & Laskey, 1974; Laskey & Mills, 1975).

Assays of Procollagen Concentrations. The concentration of purified procollagen was assayed by hydrolyzing the protein in 6 M HCl under N_2 and carrying out an amino acid analysis on a Model JLC-6AH amino acid analyzer. The ^{14}C in the same samples was assayed by liquid scintillation counting so that assays of ^{14}C could be used to estimate the procollagen concentration.

Results

CD Studies on Partially Purified Procollagen. Preliminary experiments demonstrated that although procollagen was the major constituent of the medium of the fibroblasts employed here [see Hoffmann et al. (1976)], it could not be used directly for CD studies because the spectrum of the procollagen was obscured by other components. Therefore, the procollagen was partially purified by passing the medium through a column of P-200 polyacrylamide which was equilibrated and eluted at room temperature with the modified Krebs medium used to incubate the fibroblasts (Dehm & Prockop, 1972). To obtain adequate protein concentrations, we incubated the cells at concentrations of ~25 × 10^6 cells/mL, or about threefold higher than used in previous experiments (Dehm & Prockop, 1972; Hoffmann et al., 1976). Most of the ^{14}C-labeled protein eluted in or near the void volume of the column, and the recovery of nondialyzable ^{14}C was ~78% (Figure 1). Adequate CD spectra were obtained from fractions in the leading edge of the protein peak and, in occasional experiments, from the peak fractions. The other fractions contained ultraviolet-absorbing materials which obscured the spectrum. The CD spectrum of fractions from the leading edge or the peak indicated a broad negative deflection below 250 nm with a small peak at ~223 nm (not shown). The difference spectrum obtained by comparing the protein before and after heat denaturation indicated a positive peak at ~221 nm similar to the positive peak at this wavelength seen with solutions of collagen [see Brown et al. (1969) and Brodsky-Doyle et al. (1976)]. The signal at 221 nm was therefore used to examine the helix-to-coil transition. As indicated in Figure 2, the

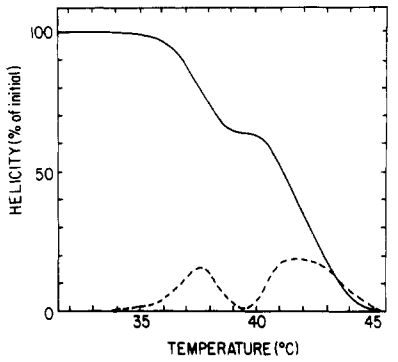

FIGURE 2: Thermal transition of partially purified procollagen from chick embryo fibroblasts. The sample used for the experiment shown in Figure 1 was examined at 221 nm in a 5-mm cell. The temperature was increased at a rate of 15 °C/h. Change in ellipticity at 221 nm expressed as $100(\theta^{20°C} - \theta^{t})/(\theta^{20°C} - \theta^{50°C})$ (—); first derivative of the transition curve (---).

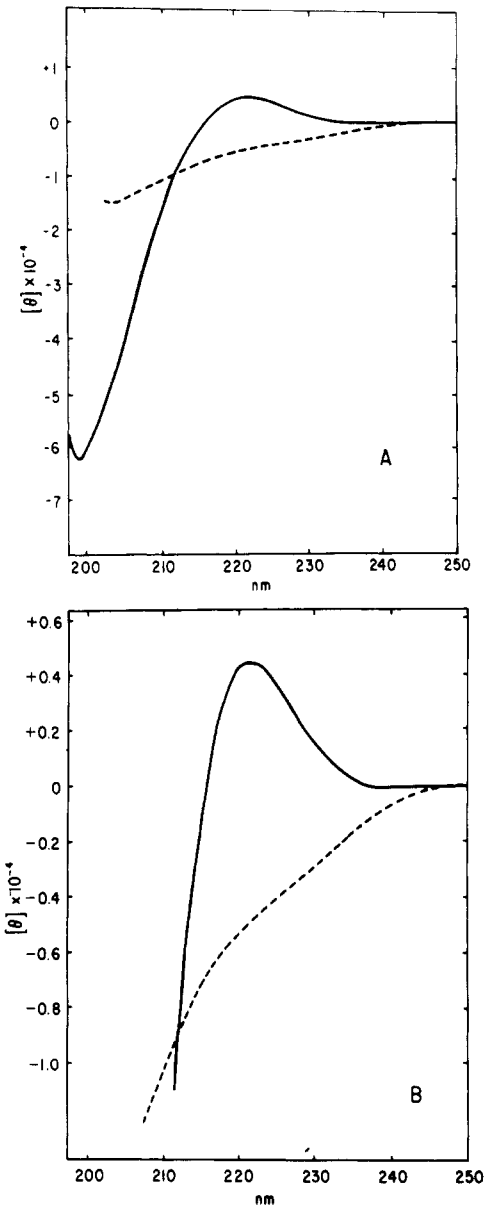

FIGURE 3: CD spectrum of purified type I procollagen from chick embryo fibroblasts. Procollagen purified by chromatography on DEAE-cellulose was dialyzed against the standard neutral buffer and examined in a 2-mm cell. (A) CD spectrum between 200 and 250 nm. (B) CD spectrum between 210 and 250 nm. Spectrum of a native protein (—); spectrum of protein after melting under conditions similar to those used in Figure 2 (---).

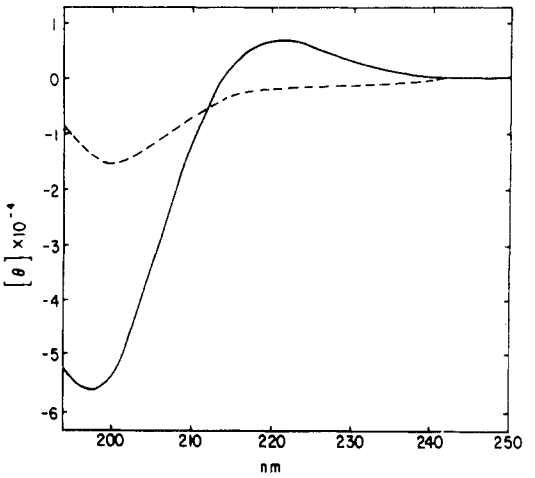

FIGURE 4: CD spectrum of type I collagen from lathyritic chick embryo tendon. The protein was examined in a 2-mm cell. The collagen was dissolved in 0.5 M acetic acid and then dialyzed extensively against water to a final pH of 4.3. CD spectrum of a native protein (—); CD spectrum after melting protein under conditions similar to those used in Figure 2 (---).

thermal transition appeared to occur in two phases: one with an apparent T_m of ~37 °C and another with an apparent T_m of ~42 °C. There was no change in the ^{14}C concentration of the sample during measurement of the thermal transition, suggesting that the biphasic transition was not explained by the fibril formation. Experiments with purified bacterial collagenase supported the conclusion that the thermal transition (Figure 2) reflected a conformational change in the triple helix of procollagen. Five microliters of a collagenase solution containing 300 μg/mL enzyme, 5 mM $CaCl_2$, and 50 mM Tris-HCl buffer, pH 7.6, was added to 600 μL of the same sample of partially purified procollagen used for the thermal transition shown in Figure 2. The sample was placed in the spectropolarimeter and incubated at 20 °C for 120 min. The temperature was then increased at a rate of 2.5 °C/h. All the transition in the signal at 221 nm occurred before 31 °C in the collagenase-treated sample, whereas a biphasic melt with T_m values of 37 and 42 °C was seen with the control sample.

CD Spectrum of Native and Denatured Type I Procollagen. More satisfactory spectra were obtained after the procollagen was purified by ammonium sulfate precipitation and chromatography on DEAE-cellulose in 2 M urea [see Hoffmann et al. (1976)]. When the protein was examined in 0.4 M NaCl and 0.1 M Tris-HCl, pH 7.4, the spectrum of native protein (Figure 3) was similar to the spectrum of collagen (Brown et al., 1969, 1972; Brodsky-Doyle et al., 1976) with a positive peak at ~221 nm and a negative peak at ~198 nm. In the neutral buffer employed here the mean residue ellipticity at 221 nm was ~5000 deg cm^2 $dmol^{-1}$ and the mean residue ellipticity at ~198 nm was −60 000 deg cm^2 $dmol^{-1}$. These values are similar to the values of 5000 to 10 000 deg cm^2 $dmol^{-1}$ at 221 nm and −50 000 to −60 000 deg cm^2 $dmol^{-1}$ at 198 nm for collagen from guinea pig skin (Brown et al., 1969, 1972) or lathyritic rat skin (Brodsky-Doyle et al., 1976). For purposes of comparison with chick embryo procollagen, type I collagen from lathyritic chick embryos was also examined here (Figure 4). In aqueous solution at pH 4.3, the mean residue ellipticity of the collagen was ~7000 deg cm^2 $dmol^{-1}$ at 221 nm and ~−55 000 deg cm^2 $dmol^{-1}$ at 198 nm. The ratio of the 221-nm peak to the 198-nm peak was less for the procollagen than for the collagen, apparently because of the contribution of the procollagen propeptides which are globular [see Becker et al. (1976) and Olsen et al. (1977)].

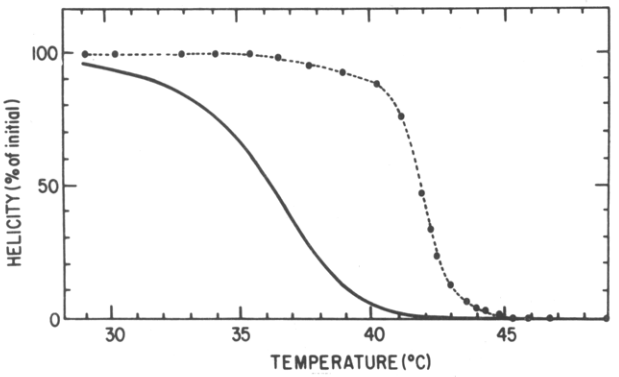

FIGURE 5: Effect of ultraviolet irradiation on the thermal transtition of purified procollagen from chick embryo fibroblasts. The thermal transition of purified procollagen was examined either with the slit open throughout the measurement or by closing the slit on the instrument and opening it for 30–60 s at the times indicated. The samples were examined in a 2-mm cell at a concentration of ~50 μg/mL in the standard neutral buffer. The temperature was increased at a rate of 15 °C/h and the transition monitored at 221 nm. Sample irradiated for 15 h at 221 nm at 5 °C prior to measuring the transition with the slit open (—); sample in which irradiation was minimized (●).

After denaturation of the procollagen, the positive peak at 221 nm disappeared (Figure 3), much as was seen with denaturation of collagen (Figure 4). The spectrum of the denatured procollagen between 245 and 220 nm, however, was more negative than the spectrum of denatured collagen. The negative peak of procollagen also decreased after heat denaturation, much as was observed with collagen (Figure 4). However, a full spectrum below 200 nm could not be obtained for the denatured procollagen because of increase in absorbance.

Thermal Transition of Purified Type I Procollagen. The helix-to-coil transition of purified procollagen was examined as a function of temperature. In initial experiments the protein was dissolved in 0.4 M NaCl and 0.1 M Tris-HCl buffer, pH 7.4, and it was examined in a 1-mm cell and with a protein concentration of ~100 μg/mL. In these initial experiments a two-phase thermal transition was observed, much as was seen with the partially purified protein (Figure 2). However, the relative fraction melting in each of these phases varied substantially in different experiments. Subsequently, it was observed that the relative fraction melting in the first phase was dependent on the time that the sample was exposed in the ultraviolet beam of the instrument before initiating the melting curve. As indicated in Figure 5, the transition occurred with a T_m of 36 °C when the protein was left in the spectropolarimeter and exposed to the beam at 221 nm for 15 h prior to measuring the thermal transition. In contrast, the transition occurred in a single phase and with an apparent T_m of ~42 °C when ultraviolet irradiation of the sample was minimized by intermittently closing the slit on the instrument during the thermal transition. Also, most of the procollagen underwent a thermal transition with an apparent T_m of ~42 °C when irradiation was minimized by increasing the rate of temperature change from 0.8 to 65 °C/h. As indicated in Figure 6, increasing the rate of temperature change did not have a major effect on the apparent T_m of samples. Therefore, it was possible to minimize the effects of irradiation on the thermal transition and obtain estimates of the T_m independent of the effects of irradiation by melting the samples rapidly and completing the thermal transition within 1 h or less.

Effects of Ultraviolet Irradiation and Salt Concentration on the Thermal Transition of Procollagen and Collagen. Although irradiation of the procollagen in the instrument under the conditions described in Figures 2 and 5 decreased the apparent T_m of the protein, the irradiation did not in itself change either the CD spectrum or the initial mean residue ellipticity at 221 nm. Irradiation under these conditions, however, produced scission of the pro-α chains. As indicated in Figure 7, essentially all the procollagen was present as intact pro-α1 and pro-α2 chains in samples which were not irradiated. After irradiation, a large fraction of the pro-α chains was degraded. The degraded peptides were in part recovered as small peptides which migrated at the dye front of the 6% acrylamide gels (Figure 7), but a broad smear of peptides of sizes intermediate between pro-α chains and peptides migrating at the dye front were detected after longer exposure of the films (not shown).

Similar effects of irradiation on thermal transition were observed with type I collagen. As indicated in Table I, the T_m of the lathyritic collagen was ~4 °C lower when the sample was irradiated for 15 h at 5 °C prior to measuring the thermal transition.

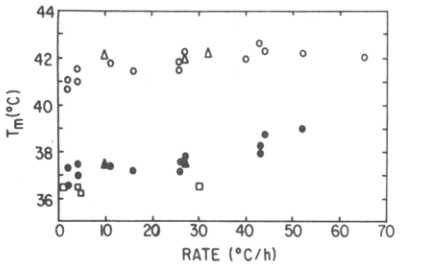

FIGURE 6: T_m of collagen and procollagen from chick embryos as a function of the rate at which the temperature is increased. The proteins were dissolved in the standard neutral buffer and examined in a 2-mm cell. T_m of collagen (○) and procollagen (△) in experiments in which irradiation was minimized by intermittently closing the slit as described in Figure 5; T_m for the first phase of the thermal transition (see Figure 2) with samples of collagen (●) and procollagen (▲) which were irradiated by exposure to the ultraviolet beam during the thermal transition and procollagen (□) which was irradiated for 15 h at 221 nm at 5 °C under the conditions described in Figure 5 prior to initiating the thermal transition curve.

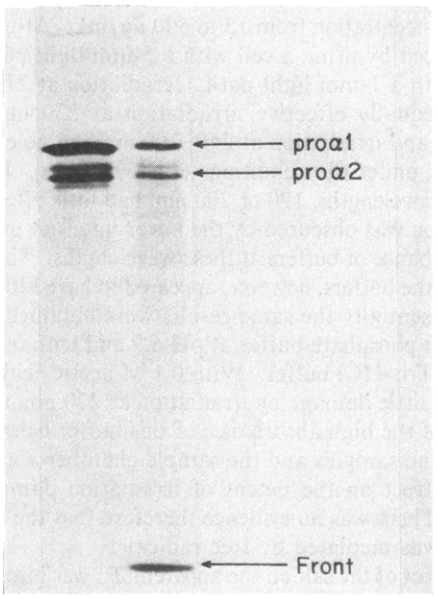

FIGURE 7: Polyacrylamide gel electrophoresis of purified procollagen from chick embryo fibroblasts before and after ultraviolet irradiation. Purified type I procollagen was electrophoresed either with or without prior irradiation at 221 nm at 5 °C for 15 h. The protein was dissolved in standard neutral buffer at a concentration of ~50 μg/mL and irradiated in the Cary spectropolarimeter in a 2-mm cell. Left: sample without ultraviolet irradiation. Right: sample with irradiation.

Table I: T_m of Type I Procollagen and Collagen from from Chick Embryos

sample	T_m (°C)[a] irradiated	nonirradiated
procollagen in standard neutral buffer	37.4 ± 0.6[b]	42.4 ± 0.7
collagen in neutral buffer	37.9 ± 0.8	42.1 ± 0.4
collagen in 0.5 M acetic acid		39.1 ± 0.5
collagen in 0.5 M acetic acid + 0.2 M NaCl	32.6 ± 0.6	36.3 ± 0.4

[a] For most experiments the temperature was increased at ~30 °C/h. [b] Standard error. Number of values varied from 5 to 16.

Several qualitative observations were made as to how the effects of irradiation were influenced by the protein concentration, the wavelength used to irradiate the sample, and the buffer employed. In each case the extent of irradiation damage was assayed by the relative fraction of collagen or procollagen which melted with a T_m of 37 °C. By this criterion, irradiation damage was reduced by increasing the protein concentration from 15 to 500 μg/mL. Also, the effect was reduced by using a cell with a 5-mm light path instead of one with a 1-mm light path. Irradiation at 215 and 220 nm was equally effective, irradiation at 230 nm was less effective, and irradiation at 240–260 nm had no effect in 15 h at 4 °C under the conditions employed here. Irradiation at lower wavelengths, 190 or 200 nm, had little effect, but this observation was obscured by the lower intensity of the beam and absorbance of buffers at these wavelengths. The chemical nature of the buffers, however, appeared to have little influence in that essentially the same results were obtained with 0.15 M sodium phosphate buffer at pH 6.9 and with 0.4 M NaCl in 0.1 M Tris-HCl buffer. With 0.1 M acetic acid there was relatively little damage by irradiation at 220 nm, apparently because of the high absorbance of this buffer below 230 nm. Purging the samples and the sample chamber with nitrogen had no effect on the extent of irradiation damage to the protein. There was no evidence therefore that the irradiation damage was mediated by free radicals.

The effect of the salt on the apparent T_m was also examined. When collagen was melted in either 0.45 or 0.01 M acetic acid, low concentrations of NaCl reduced the apparent T_m of type I collagen and the effect was nonlinear (Figure 8). The effect of salt appeared to be less in neutral buffer in that essentially the same T_m was seen with 0.2–0.4 M NaCl. Lower concentrations of NaCl could not be examined at neutral pH because the protein precipitated. Essentially the same T_m was found when the neutral buffer containing 0.2 or 0.4 M NaCl was replaced with the modified Krebs medium used to incubate the chick embryo fibroblasts (Dehm & Prockop, 1972).

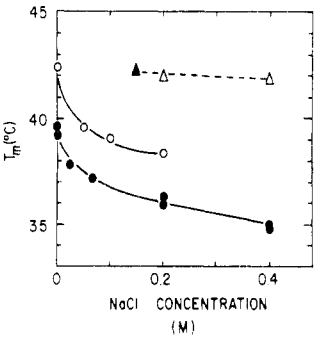

FIGURE 8: Effect of NaCl on the thermal transition of type I collagen from lathyritic chick embryos. The protein was examined in a 2-mm cell at a concentration of ~100 μg/mL. The temperature was increased at a rate of about 30 °C/h. Collagen dissolved in 0.45 M acetic acid (○); collagen dissolved in 0.01 M acetic acid (●); collagen dissolved in 100 mM Tris-HCl buffer, pH 7.4 (△); and collagen dissolved in incubation medium containing 250 mM glucose to prevent fibril formation (▲) (Hayashi & Nagai, 1972).

Discussion

The thermal transitions of collagens in solution have been extensively investigated in the past [see Traub & Piez (1971), Rigby & Robinson (1975), and Bornstein & Traub (1978)]. The results presented here, however, emphasize a new potential source of error in such studies through ultraviolet damage to the protein.

Exposure of either collagen or procollagen in dilute solution to the ultraviolet beam of the CD spectropolarimeter reduced the apparent T_m of the proteins by as much as 5 °C. Ultraviolet irradiation has previously been shown to cleave the polypeptide chains of collagen. Miyata et al. (1971) extensively irradiated collagen solutions at 253.7 nm and showed both that the polypeptide chains were cleaved and that the T_m of the protein was decreased. Sudo & Noda (1973) demonstrated that the action spectrum for cleavage of collagen by ultraviolet light was similar to the absorption spectrum for peptide bonds, and they therefore suggested that the irradiation produced random cleavage of the polypeptide backbone. The extent of irradiation damage observed in these previous studies, however, was apparently greater than that observed here in that there was no evidence of damaged protein with discrete thermodynamic properties. Although the phenomenon was not explored in detail, it seems likely that the irradiated forms of procollagen and collagen encountered here largely consisted of molecules which remained intact but in which there was scission of one of the three pro-α chains at a single locus. The biphasic thermal transition curves observed (Figure 2) demonstrated a discrete change in the thermodynamic properties which is unlikely to reflect multiple sites of damage. Also, even after irradiation extensive enough to produce a monophasic transition with a T_m of 37 °C (Figure 5), the mean residue ellipticity was unchanged and a large part of the protein consisted of intact pro-α chains (Figure 7). Although other proteins were not examined here, it is unlikely that the effects of irradiation on collagen are unique and therefore irradiation damage may well be encountered in CD studies with other proteins.

Part of the data presented here also emphasize earlier observations indicating that the presence of salt can be a potential source of error in estimating the T_m of collagen or procollagen. von Hippel & Wong (1963) reported that salt altered the thermal transition of collagen according to the formula $T_m = T_m^0 + K_m$, in which T_m is the midpoint temperature of the phase transition at a given salt concentration m, T_m^0 is the T_m at $m = 0$, and K is a constant characteristic of the particular salt–gelatin system. The results presented here illustrate that the effect of salts is markedly dependent on pH. When collagen was examined in neutral buffer, the effect of NaCl was small and consistent with the K value of −3.0 determined by von Hippel & Wong (1963) with ichthyol collagen. In acetic acid, however, the effect of NaCl at the low concentrations employed here was much larger and not a linear function of salt concentration. The effects of salt as well as the effects of irradiation in CD studies may well explain reports indicating that some collagens from homeothermic animals have T_m values as low as 35 °C [see Timpl et al. (1978) and Fujii & Kühn (1975)].

Of special interest was the observation that the same T_m values were obtained when the temperature for the thermal transition was increased at a rate of 0.8 or 65 °C/h. It is

apparent therefore that measurements of the thermal transitions of collagens or procollagens are not limited by the kinetics of unfolding.

Type I collagen from chick embryos was found here to have a T_m of 42.1 ± 0.4 °C when examined in a neutral buffer. Type I procollagen from the same source had the same T_m. Therefore, it is apparent that the propeptides of type I procollagen have no effect on the thermal stability of the triple-helical domain of the molecule. As discussed elsewhere (Becker et al., 1976), the collagen-like domains found in the *N*-propeptides of both pro-α chains of type I procollagen may play a role in initiating formation of the triple helix in that they may serve as a nucleus for folding. They may therefore influence the kinetics of folding. Based on the results obtained here, however, it is apparent that neither the *N*-propeptides nor the *C*-propeptides of type I procollagen play an important role in the thermodynamic stability of the triple helix.

The partially purified procollagen from the medium of fibroblasts had the same thermal stability as the purified protein. Since the partial purification consisted of gel filtration at room temperature, the partially purified procollagen was probably in the same form in which it was secreted by the fibroblasts. Previous experiments in which the helicity of the procollagen was examined by limited proteolytic digestion suggested that newly secreted procollagen had a higher thermal stability than collagen (Uitto & Prockop, 1974). These observations initially suggested to us that procollagen may be secreted as an aggregate in which lateral association of the protein, similar to the lateral association seen in a collagen fibril, increases the thermal stability of the triple helix. The data obtained here, however, do not support the hypothesis that procollagen is secreted as an aggregate with a greater thermal stability than the monomer. We have recently observed (T. Hayashi, C. Nagy, and D. J. Prockop, unpublished experiments) that the sedimentation coefficient of procollagen in the medium of tendon fibroblasts is 4.3 S, a value which is identical with that of purified procollagen and only slightly greater than the value of 3 S for collagen. Our results suggest therefore that the procollagen recovered from the medium of fibroblasts may well be in a monomeric form. This conclusion may or may not be consistent with electron microscopic observations suggesting that procollagen within cells and associated with cells is in an aggregated form (Weinstock & Leblond, 1974; Trelstad et al., 1976; Bruns et al., 1979).

Acknowledgments

We thank Nancy Kedersha for amino acid analysis. We are grateful for helpful discussions with Drs. Angelo A. Lamola, Gary Patterson, and Dinshaw Patel of Bell Laboratories, Murray Hill, N.J. Several of the preliminary experiments which provided the basis for this work were carried out in collaboration with Drs. Jürgen Engel, Hans-Peter Bächinger, and Peter Bruckner, Biozentrum, Basel, Switzerland.

References

Becker, U., Timpl, R., Helle, O., & Prockop, D. J. (1976) *Biochemistry 15*, 2853.

Bonner, W. M., & Laskey, R. A. (1974) *Eur. J. Biochem. 46*, 83.

Bornstein, P., & Traub, W. (1978) in *The Proteins* (Neurath, H., Ed.) Academic Press, New York and London (in press).

Brodsky-Doyle, B., Leonard, K. R., & Reid, K. R. M. (1976) *Biochem. J. 159*, 279.

Brown, F. R., III, Carver, J. P., & Blout, E. R. (1969) *J. Mol. Biol. 39*, 307.

Brown, F. R., III, Hopfinger, A. J., & Blout, E. R. (1972) *J. Mol. Biol. 63*, 85.

Bruns, R. M., Hulmes, D. J. S., Therrien, S. F., & Gross, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 313.

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

Fujii, T., & Kühn, K. (1975) *Hoppe-Seyler's Z. Physiol. Chem. 356*, 1793.

Gelman, R. A., & Blackwell, J. (1973) *Connect. Tissue Res. 2*, 31.

Gelman, R. A., & Blackwell, J. (1974) *Biochim. Biophys. Acta 342*, 254.

Gross, J. (1964) *Science 143*, 960.

Hayashi, T., & Nagai, Y. (1972) *J. Biochem. (Tokyo) 72*, 749.

Hayashi, T., & Nagai, Y. (1973) *J. Biochem. (Tokyo) 73*, 999.

Hoffmann, H-P., Olsen, B. R., Chen, H-T., & Prockop, D. J. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4304.

Laskey, R. A., & Mills, A. D. (1975) *Eur. J. Biochem. 56*, 335.

Miyata, T., Shoda, T., Rubin, A. L., & Stenzel, K. H. (1971) *Biochim. Biophys. Acta 229*, 672.

Olsen, B. R., Guzman, N. A., Engel, J., Condit, C., & Prockop, D. J. (1977) *Biochemistry 16*, 3030.

Peterkofsky, B., & Diegelmann, R. (1971) *Biochemistry 10*, 988.

Prockop, D. J., Kivirikko, K. I., Tuderman, L., & Guzman, N. A. (1979) *N. Engl. J. Med. 301*, 13, 77.

Rigby, B. J., & Robinson, M. S. (1975) *Nature (London) 253*, 277.

Sudo, K., & Noda, H. (1973) Abstracts of the 10th Annual Collagen Symposium, Tokyo, Japan, Collagen Society of Japan, p 1.

Timpl, R., Martin, G. R., Bruckner, P., Wick, G., & Wiedemann, H. (1978) *Eur. J. Biochem. 82*, 43.

Traub, W., & Piez, K. A. (1971) *Adv. Protein Chem. 27*, 243.

Trelstad, R. L., Hayashi, K., & Gross, J. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4027.

Uitto, J., & Prockop, D. J. (1974) *Eur. J. Biochem. 43*, 221.

von Hippel, P. H., & Wong, K-Y. (1963) *Biochemistry 2*, 1387.

Weinstock, M., & Leblond, C. P. (1974) *J. Cell Biol. 60*, 92.