FTIRS in H_2O Demonstrates That Collagen Monomers Undergo a Conformational Transition Prior to Thermal Self-Assembly in Vitro†

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ABSTRACT: The assembly of type I collagen molecules into native fibrils can be accomplished in vitro in solutions at physiological ionic strength and pH by raising the temperature above 30 °C. The thermal self-assembly reaction exhibits a distinct lag phase. This lag phase has been proposed to be evidence for a conformational transition in the monomer. Fourier transform infrared spectroscopy (FTIRS) is a very sensitive probe of the H-bonded states within the triple helix. The carbonyl group spectrum (amide I, 1700–1600 cm^{-1}) has been investigated in collagen/H_2O solutions at 1 mg/mL under self-assembly conditions from 4 to 34 °C and, in the same range, at a higher ionic strength where self-assembly does not occur. The deconvoluted spectra show three very clear bands at ≈1660, 1644, and 1630 cm^{-1}. These bands vary in both frequency maxima and relative intensity over the temperature range examined. Spectra were also obtained in the amide II and III regions. Spectral changes were evident in the 22–26 °C range, under fibril-forming conditions, which lead to the hypothesis that the triple helix of the semiflexible collagen molecule is actually perfected during the lag phase, facilitating nucleation and intermolecular interaction. Further spectral changes after fibrils do form show that the molecules are once again distorted as they are bent to fit within the fibrils.

The thermally driven in vitro self-assembly of type I collagen monomers into native type D-periodic fibrils is a complex reaction (Veis & Payne, 1988). It is characterized by a high degree of specificity of the intermolecular interactions (Helseth et al., 1979) which establish the axial registration of molecules to generate the D-periodic arrangement. The kinetics of the self-assembly reaction are particularly interesting, dominated by a lag period during which the hydrodynamic parameters of the monomers do not appear to change, and followed by a very rapid fibril growth phase (Comper & Veis, 1977). In a clean system, homogeneous nucleation theory would predict that the lag phase is related to the probability of forming a nucleating aggregate of sufficient size to support fibril growth. However, an alternate explanation that a thermally driven conformational transition is required in the collagen molecule, either in the telopeptide region or in the triple-helical region or both, has also been proposed (Helseth & Veis 1981; Veis, 1988). A number of recent studies of the collagen monomer in solution have suggested that the helical domain is semiflexible, with several subdomains of enhanced flexibility (Nestler et al., 1983; Bernengo et al., 1983; Amis et al., 1985; Hoffman et al., 1984). From this perspective, the proposed conformational change might involve transitions within these flexible subdomains. Analysis of such transitions in solution, within limited sequence domains, requires methods for very high resolution. We have selected Fourier transform infrared spectroscopy (FTIRS) for this study.

The amide groups of polypeptides and proteins possess a number of characteristic vibrational modes or group frequencies (Susi, 1972; Miyazawa et al., 1956). The amide I, II, and III band regions of the spectrum are directly related to the polypeptide conformation (Susi, 1969, 1972; Elliot & Ambrose, 1950; Parker, 1983). The amide I band, with characteristic frequencies in the range from 1600 to 1700 cm^{-1}, is associated with the stretching vibrations of the peptide carbonyl groups. The exact frequencies of these stretching vibrations depend both upon the strength of the hydrogen bond to the carbonyl oxygen and upon the environment dictated by the local peptide conformation. In a complex polypeptide, the set of carbonyl vibrations produces a fairly broad band in the infrared spectrum, but recently developed computational procedures, coupled with Fourier transform methods of obtaining the spectrum, permit resolution enhancement leading to the deconvolution of the broad band into its underlying components.

The amide I region of the spectrum of native collagen is particularly interesting because the type I collagen triple-helix conformation does not fit the standard α-helix, β-sheet, and β-turn conformations. Payne and Veis (1988) have shown that the broad amide I band of the native type I collagen molecule in aqueous solution can be deconvolved into three distinct bands with maxima at 1660, 1643, and 1633 cm^{-1}. Upon denaturation, the bands do not shift appreciably in position, but the relative intensities of the 1660 and 1633 cm^{-1} bands shift from >1 to <1. In a preliminary study several years ago, Jakobsen et al. (1983) had shown that the structure of the amide I band of collagen changed during in vitro fibrillogenesis, suggesting a possible conformational transition at some point during fibril assembly. The present work was undertaken to explore more fully the structure of the amide I band of monomeric type I collagen as a function of temperature under fibril-forming conditions to see if more specific evidence for a conformational transition could be obtained.

Materials and Methods

Monomeric Collagen. Lathyritic rat skin collagen was prepared as previously described (Payne & Veis, 1988). The stock solution in 0.01 M acetic acid was aliquoted into a number of identical portions and stored frozen.

SDS–polyacrylamide gel electrophoresis in a gradient gel system (Payne & Veis, 1988) showed the lathyritic collagen to be essentially non-cross-linked type I with a trace of type V collagen. Amino acid analysis data indicated that any

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noncollagenous contaminants were at trace levels.

Fibrillogenesis. (*A*) *Tyndallograms.* Collagen fibril assembly was monitored by spectrophotometric turbidity measurements, at 313 nm, as a function of time at 26 and 34 °C. The cold-start method was used to initiate fibrillogenesis (Holmes et al., 1986). At 4 °C, 1.0 mL of a solution containing 1.00 mg/mL collagen in 0.01 M acetic acid was mixed with 1.0 mL of double-strength phosphate buffer (final concentration: 9.2×10^{-3} M Na_2HPO_4, 5.83×10^{-3} M KH_2PO_4, and 0.15 M NaCl, pH 7.04) by repeated inversion in precooled cuvettes.

(*B*) *FTIRS.* For the infrared studies, the collagen aliquots in acetic acid were thawed at 4 °C and then dialyzed into the final buffer containing as above 9.2×10^{-3} M Na_2HPO_4, 5.83×10^{-3} M KH_2PO_4, and 0.15 M NaCl at pH 7.04 (ionic strength 0.20). These solutions were clarified by centrifugation at 40000*g* for 60 min at 4 °C. Final collagen concentrations were determined with a differential refractometer (Brice & Halwer, 1951). Sample concentrations were adjusted to 1 mg/mL for all analyses. In some experiments where fibrillogenesis was inhibited, the collagen was dialyzed into a buffer containing 6.2×10^{-2} M Na_2HPO_4 and 1.4×10^{-2} M KH_2PO_4, pH 7.04 (ionic strength 0.266).

The principal hardware consists of an Analect (Laser Precision Corp., Irvine, CA) FX6260 FTIR spectrometer, a MAP 67 data processor, and a RAM 56 color display unit. The system normally carries out one scan per second at a 4 cm^{-1} resolution with the signal gain set at 2. The sample cell is a demountable, thermally jacketted, nine-pass internal reflectance cell with a ZnSe crystal designed by Harrick Scientific Co. (Ossning, NY). Circulating water from either of two water baths is continuously pumped through the thermal jacket. Temperature can be controlled to ±0.1 °C within the sample cell, and the temperature within the cell can be changed and reequilibrated within 2 min. Four milliliters of solution is sufficient to completely fill the cell.

For any series of analyses, the spectrometer was left continuously powered up to avoid warm-up instabilities. The sample compartment was purged with nitrogen gas before any spectra were collected. Purging was continued until the CO_2 asymmetric stretch absorption peak at 2350 cm^{-1} disappeared.

All sample spectra were normalized against a dry cell background of 128 scans collected at the desired working temperature. The spectrum of the background and each protein and buffer blank solution were collected at 4 cm^{-1} resolution at a series of temperatures over the range from 4 to 34 °C. The buffer and collagen solution spectra averaged 384 scans per collection. The Happ–Genzel apodization function was used to improve the signal to noise ratio, and the Analect "Badscan" program minimized the incorporation of spurious signals. The data presented for the fibrillogenesis conditions represent the average from 10 or more independent sets of data collection. Different temperature change programs were used in some cases where 2 °C intervals were followed. Standard deviations are indicated in the figures and figure legends. Four independent data sets were collected under non-fibril-forming conditions.

The collagen spectrum was obtained by subtracting the buffer spectrum from the total spectrum of collagen in buffer at the same temperature, with both spectra in the absorbance mode. It is crucial to note that the buffer spectrum is highly temperature dependent; thus, the buffer spectrum must be recorded at the same temperature as the collagen solution.

The solvent H_2O has a very strong OH-bending absorption at 1640 cm^{-1}. The comparatively weak amide I band (carbonyl stretching) characteristic of peptide bonds is normally superimposed on or within a few wavenumbers of this strong water absorption peak. Therefore, excellent quality spectra and precise subtraction are necessary in order to correctly record the amide I bands. In this work, the "Autosubtract" option on our computer was used. This algorithm scales the reference spectrum so that the absorption of both spectra will be identical at some specified point. The choice of the specified point of equivalence is crucial.

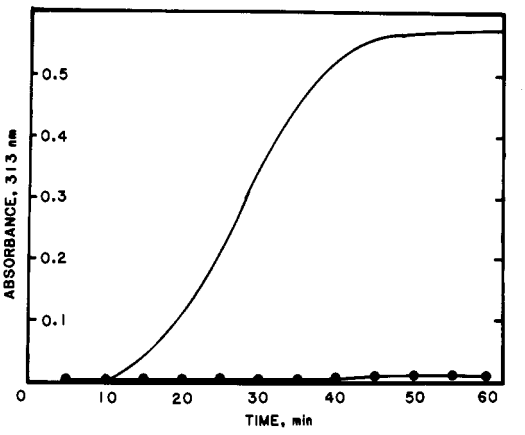

FIGURE 1: Effect of temperature on thermally driven collagen self-assembly. Tyndallograms of native monomeric rat skin collagen at 1 mg/mL, pH 7.04, in phosphate buffer permissive for fibrillogenesis, as a function of time at 26 and 34 °C. Solid line, 34 °C; (●) 26 °C.

The spectra were deconvolved by an Analect algorithm based on a program developed by Kauppinnen et al. (1981, 1982). The deconvolution was optimized by adjusting the apodization function: the mean value of the half-width at half-height was set at $\sigma = 7$ cm^{-1}; the resolution factor was $K = 1$. Two absorption bands can be resolved if their maxima are separated by 4 cm^{-1}; otherwise, a composite peak is observed. The position of a peak maximum can be determined to 0.1 cm^{-1}.

RESULTS

Fibrillogenesis. Figure 1 shows the Tyndallograms (turbidity vs time plots) describing the thermally induced fibrillogenesis at 26 and 34 °C in the phosphate buffer system. There is no evidence for fibril formation at 26 °C within an hour of initiating heating. The lag phase extends for only 10 min at 34 °C, and the turbidity plateau is reached within 40 min. Previous studies have shown that the fibrils produced in this manner are in the native D-periodic form.

FTIRS—Native Collagen in Solution at 4 °C. The structure of the native, monomeric collagen under nonaggregating conditions at 4 °C and physiological pH and ionic strength is taken as the reference point for all of the subsequent structural arguments. The 4 °C spectrum under these conditions is shown in Figure 2. The three principal amide group related absorption bands, at 1660, 1560, and 1244.5 cm^{-1}, are the prominent features of the spectrum. The amide I with its sharp maximum at 1660.1 cm^{-1} clearly is complex with a distinctive shoulder on the lower frequency side. The deconvolved amide I (Figure 3) consists of three sharply defined peaks with maxima at 1660.1 ± 0.29, 1644.7, and 1630.5 cm^{-1}, in good accord with the earlier measurements of Payne and Veis (1988) obtained under less sensitive measurement and deconvolution programs. These data are very reproducible in regard to the peak maxima positions and the relative peak intensities. Note that the three bands of the amide I region are separated by ≈15 cm^{-1}, well beyond any limitations posed by the instrument resolution.

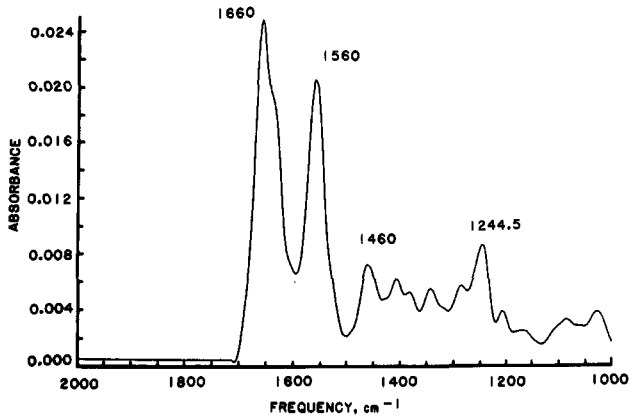

FIGURE 2: FTIR absorption spectrum of native monomeric lathyritic rat skin collagen at 1 mg/mL, pH 7.04, 4 °C, in H_2O. The numerals represent the frequency in cm^{-1} of each band at maximum intensity.

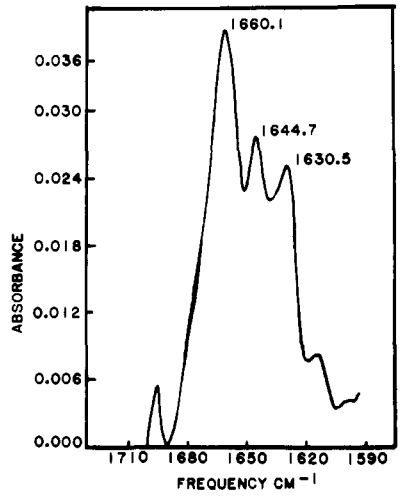

FIGURE 3: Deconvolution of the amide I absorption band of native monomeric lathyritic rat skin collagen at 1 mg/mL in phosphate buffer, pH 7.04, 4 °C (Figure 2), from 1710 to 1590 cm^{-1}. Deconvolution parameters are described in the text. Peaks can be resolved if they are separated by ≥4 cm^{-1}; the frequency at the peak absorbance maxima can be measured to 0.1 cm^{-1}.

FTIRS—Native Collagen in Fibril Form at 34 °C. The spectrum of the collagen in fibrillar form, at the plateau region of fibrillogenesis at 34 °C in Figure 1, is very similar to the native solution spectrum at 4 °C, as shown in Figure 4A. However, there are subtle changes in the spectrum which are important. In the deconvolved spectrum at 34 °C, the highest frequency band at 1660.1 cm^{-1} is sharpened substantially, and the 1660 cm^{-1}/1630 cm^{-1} absorbance ratio at the maxima is increased relative to the 4 °C spectrum. There is virtually no shift in the positions of the three principal deconvoluted peaks within the broad amide I spectrum comparing these two temperatures, although there is an overall enhancement of the absorbance at the higher temperature.

FTIRS—Thermally Driven Fibrillogenesis Reaction. The spectra of the amide I, II, and III regions were monitored as a function of temperature in the buffer system leading to fibrillogenesis. The amide II band shifted from 1560 cm^{-1} at 4 °C to 1558 cm^{-1} at 34 °C. There was a similar but more pronounced decrease in the amide III band frequency from 1244.8 to 1241.0 cm^{-1} over the same temperature range. A plot of these frequency–temperature data in the fibril-forming system (Figure 5) shows that in each case significant shifts in band frequency (before deconvolution) can be seen. The amide I band shifts gradually to higher frequency from 4 to 15 °C but drops back to the original value sharply over the

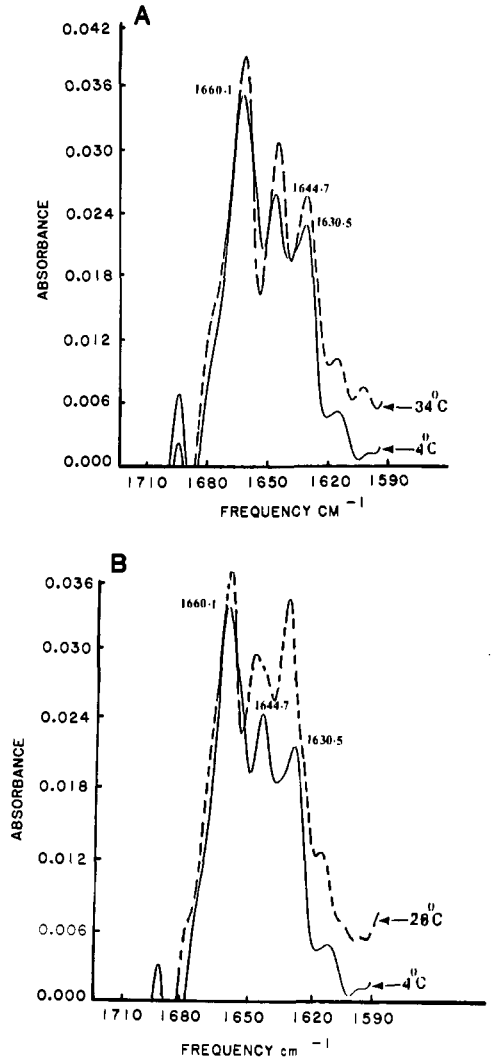

FIGURE 4: Comparison of the deconvoluted amide I spectra of collagen under fibrillogenesis conditions at 4, 26, and 34 °C in the region 1710–1590 cm^{-1}. (A) Comparison of 4 °C monomer solution and 34 °C data, after fibril formation. (B) Comparison of 4 °C monomer solution and 26 °C data; no fibrillogenesis.

interval from 24 to 28 °C. The amide II and III bands both show a decrease in frequency beginning at 22–24 °C and becoming essentially complete at 28 °C. These data suggest that one or more local conformational transitions affecting the states of the amide bond take place within this temperature range.

The three bands of the deconvolved amide I were analyzed in the same way and found to be even more complex. The high-frequency, 4 °C band at 1660.1 cm^{-1} increased to 1661.4 cm^{-1} at 15 °C and remained at that level until 26 °C, when it decreased to 1660.1 cm^{-1} and remained constant through 34 °C. The 1630 cm^{-1} band shifts in a related fashion over the same temperature range to 1633 cm^{-1} and back. There are also very significant changes in the peak extinction coefficients, as seen in the comparison of 4 and 26 °C deconvoluted spectra in Figure 4B. The absorbances of the three deconvoluted amide I bands increase during the transition range and decrease as fibrils form at higher temperature. Figure 6 shows that the deconvolved 1660 and 1630 cm^{-1} bands increase in intensity over the range from 4 to 22–24 °C but decrease more sharply from 26 to 34 °C.

Comparable measurements have been made under conditions of higher ionic strength where fibrillogenesis does not occur and continued heating of the collagen solutions leads

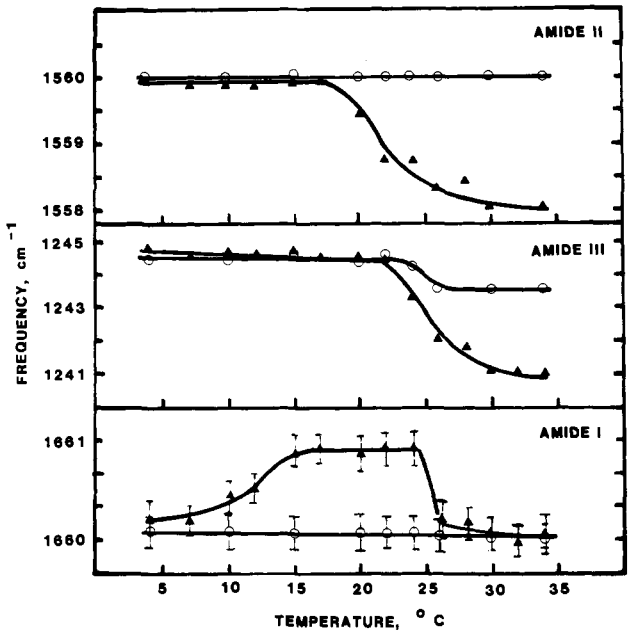

FIGURE 5: Plots of raw, nondeconvoluted peak maxima frequencies in the spectra versus temperature for the amide I, II, and III bands during fibrillogenesis and under non-fibril-forming conditions. (▲) Monomeric lathyritic rat skin collagen at 1 mg/mL in phosphate buffer of ionic strength 0.20 during fibrillogenesis. (○) Monomeric collagen, as above except at an ionic strength of 0.266, under non-fibril-forming conditions. Standard deviations are shown for the data points determined under fibrillogenesis conditions, determined from 10 independents sets of data obtained from fresh collagen solutions. Where the error bars are not seen, the deviations are less than the size of points used. This was true for every measurement under nonfibrillogenesis conditions.

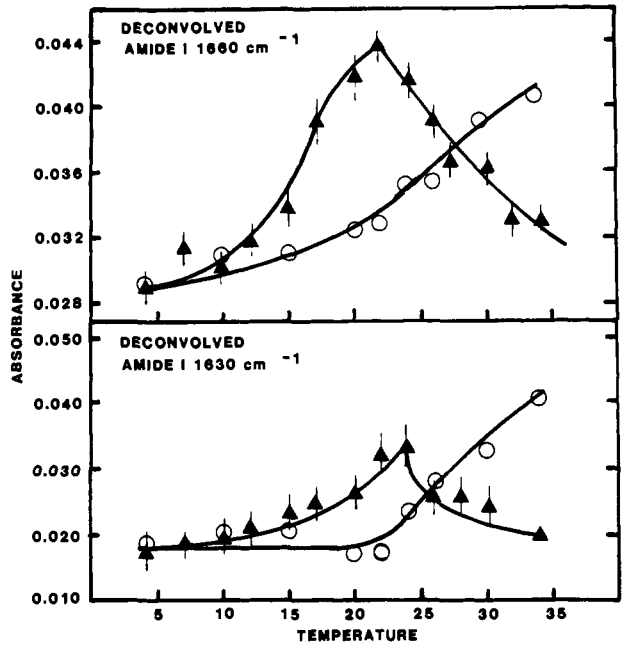

FIGURE 6: Plots of the absorbances of the 1660 and 1630 cm^{-1} deconvoluted amide I bands as a function of temperature. (▲) Monomeric lathyritic rat skin collagen at 1 mg/mL in phosphate buffer of ionic strength 0.20 during fibrillogenesis. (○) Monomeric collagen, as above except at an ionic strength of 0.266 under non-fibril-forming conditions. Standard deviations are shown for the amide I data as described in the legend of Figure 5.

to melting of the triple helix. Under these conditions (Figure 6), the extinction coefficients of the "1660" and "1630" bands increase gradually and continuously up to 22–24 °C. In that temperature range, the absorbance begins a more marked rate of increase with temperature. The deconvolved amide I 26

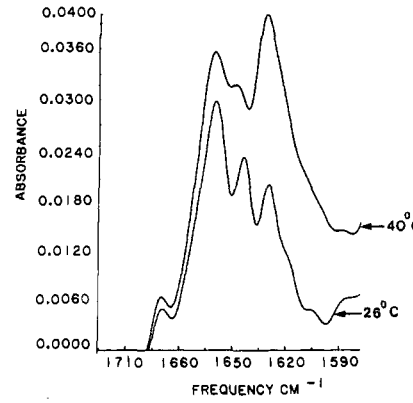

FIGURE 7: Comparison of the deconvoluted amide I bands of collagen at 26 and 40 °C under non-fibril-forming conditions. Lathyritic rat skin collagen at 1 mg/mL in phosphate buffer of ionic strength 0.266. The collagen at 26 °C is "native"; the collagen is nonhelical gelatin at 40 °C.

and 40 °C spectra under non-fibril-forming conditions are compared in Figure 7. One notable feature of these spectra is the considerable broadening of the bandwidths at these temperatures, compared with the data obtained under fibril-forming conditions. A second feature is that at 40 °C where denaturation is occurring under the non-fibril-forming conditions the drastic change in the 1660/1630 cm^{-1} absorbance ratio is evident. At 26 °C, the collagen is still triple-helical.

Discussion

The infrared spectrum of type I collagen is dominated by the contributions arising from vibrations of the amide groups. The amide I and III regions demonstrate the most complex but informative behavior in H_2O. Pyrrolidine ring vibrations related to ring puckering, in the region of 1450 cm^{-1}, are also detectable, but are more prominent in D_2O solutions. The amide I and III and ring puckering spectra are all sensitive to the chain conformations, helix folding, and hydration. Data on the pyrrolidine ring conformationally related spectra will be presented elsewhere. Here we focus primarily on the detailed fine structure of the amide I region. The three carbonyl groups of the Gly-X-Y repeats of each chain within the triple-helix region of the collagen molecule exist in three distinct environments, as depicted in a modified version of the H-bonding model of Ramachandran (1976) (Figure 8).

The Y-position carbonyls are always directed outward from the helix into the solvent environment and are minimally perturbed by other residues of the same molecule. The Gly (G) carbonyls are likewise always exposed to solvent contact. In this case, there may be a modulation in the vibrational frequency of the carbonyl group when the G is in a triplet containing Hyp at Y within the same chain, owing to the G carbonyl interacting with solvent H_2O constrained by other H-bonding interactions as depicted in Figure 8. This would probably not be a very significant perturbation since the G carbonyls are always in solvent contact. The major conformationally dependent carbonyl groups are certainly those at the triplet X positions. Payne and Veis (1988) argued that since the major change in the collagen spectrum on complete denaturation was a marked decrease in the 1660/1630 cm^{-1} absorbance ratio, as depicted in Figure 7, it was likely that the 1660 cm^{-1} band could be ascribed to the X-residue carbonyls. Further, because of the major shift in frequency of −30 cm^{-1}, it was suggested that the 1660 cm^{-1} band was contributed by Pro residues at X. In studies of the poly(L-proline) FTIRS, to be discussed in detail elsewhere, we have

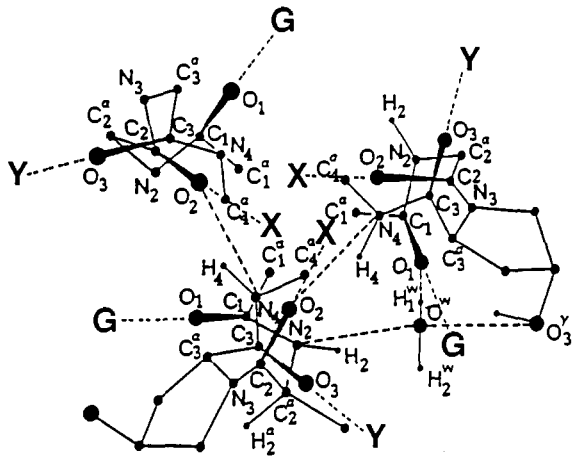

FIGURE 8: Modification of a diagram by Ramachandran showing a cross section of the relative arrangement of the three α chains in the collagen triple helix through a GLY-X-Y triplet in each chain. The large capital letters G, X, and Y depict the aqueous environment associated with each type of backbone carbonyl. It is obvious that the G and Y carbonyls are always exposed to the aqueous environment and are not likely to be conformationally dependent. On the other hand, the X carbonyls are likely to be extremely sensitive to any conformational transition which involves an internal H-bond structure or opens the structure to interaction with the solvent water. As noted under Discussion, a G followed by an hydroxyproline in the same peptide triplet Y position may have its H-bond parameters modified to some extent.

found its principal amide I absorbance peak to be at 1630 cm^{-1}, confirming this assignment.

The carbonyl group stretching frequency depends upon the strength of the H-bonding. For the same amino acid residue, the weaker the H-bond, the higher the band frequency. From the very earliest infrared spectral measurements on collagen films, it has been noted that the helix-related H-bonds are particularly long (Ramachandran & Ramakrishnan, 1976), indicating that they are also quite weak. Thus, the major shift from 1660 to 1630 cm^{-1} upon helix melting indicates that the H-bonding within the triple helix is some 0.8 kcal/mol weaker than the bonding of the same carbonyls to water in the melted state (Payne & Veis, 1988). Following the same line of reasoning, the small shifts in frequency of the deconvoluted "1660" band during the temperature change from 4 to 26 °C, from 1660.1 to 1661.4 cm^{-1} (Figures 4B and 5), can be considered to indicate that there is an increase in the order of the triple helix as compared to the state of maximal solubility at 4 °C. As this increase in order takes place at the expense of decreasing the strength of the internal compound helix H-bonds, the driving force for the gradual helix improvement may be the pyrrolidine ring stacking interactions (Bhatnagar et al., 1988).

As fibrils form at temperatures ≥28–30 °C, there is an abrupt decrease in the frequency of the "1660" band back to the value it had at 4 °C. A similar sharp frequency transition is also evident in the amide II and amide III spectra (Figure 5), and there are shifts in the peak absorbances (Figure 6). When the ionic strength is raised so that fibrils do not form, the data plotted in Figure 5 show that there are no band frequency shifts in the 20–26 °C range. Furthermore, as seen in Figure 6, the temperature dependence of the extinction coefficients under conditions permissive for fibrillogenesis is different from that under conditions not permissive for fibril formation. In the range from 4 to 34 °C under either set of conditions, the collagen configuration remains triple-helical overall. However, these data all demonstrate that changes take place regarding the internal triple-helix hydrogen bonding relating to the X-position carbonyls; thus, the conformations of the residues involved, as specified by their ϕ and ψ values, must have changed. This is equivalent to concluding that a subtle conformational transition-like event occurs in the monomeric collagen prior to the nucleation or growth of the collagen fibrils at higher temperatures in the thermal self-assembly system. The energies involved per mole of H-bonded structure, $\approx$1 cm^{-1} (30 cal/mol), are small and could not be detected in less specific measurements, such as differential thermal analysis. No evidence for intermolecular association has ever been developed for the collagen within the lag phase of in vitro self-assembly, nor at temperatures below the nucleation temperature.

Helseth et al. (1979) have proposed that both specific ionic and hydrophobic interactions are involved in the initial telopeptide–helix region interaction which is the first step in ordered fibril formation. The present data showing, as in Figure 5, that an increase in ionic strength from 0.20 to 0.26 at pH 7.04 abolishes both fibril formation and the 20–26 °C conformational transition may indicate that the salt has an intramolecular effect on the conformation of the collagen molecule as well as shielding some intermolecular interactions. Perhaps fibrillogenesis is inhibited as much by the blockade of the conformational transition as by the shielding of intermolecular electrostatic interactions.

In an aqueous system reaction which has a negative temperature coefficient, it is not surprising to conclude that an improvement in hydrophobic interactions, in this case pyrrolidine ring interactions, stabilizes the triple-helix structure. The data showing a relaxation of this structure on fibril formation, as suggested by the data shown in Figures 5 and 6, were surprising. These data can be explained, however, by the fact that the molecules within a fibril must be folded into a compound supercoiled helix, (Fraser et al., 1987), thus requiring a triple-helix distortion.

The model for the thermally driven collagen self-assembly process which emerges from these considerations is as follows. At 4 °C, the collagen molecules are flexible and have helix imperfections, although they do have intact three-chain structures. As the temperature is raised under fibrillogenesis-permissive conditions of pH and ionic strength, hydrophobic interactions perfect the helix and possibly stiffen the structure, particularly in regions crucial to intermolecular interaction. The perfected structures are in the proper conformation for intermolecular association, and the fibril nucleation process proceeds. As fibrils grow in diameter, however, intermolecular interactions potentiate the structural stability which permits the required helix destablization involved in bending the molecules. Thus, both in solution as monomers and in the final unstressed fibril state, the collagen molecules are not rigid, rodlike structures. Rather they have three-chain structures in which the triple-helix parameters are less than optimal for maximum triple-helix stability and rigidity. Regions with differing contents of proline residues in both X and/or Y triplet positions may have different stabilities. Nonbonded interactions involving prolyl residues in adjacent chains may be the major factor contributing to the stability of the triple-helical conformation. Future studies will concentrate on the contributions of the prolyl residues to helix stability rather than hydrogen-bonding interactions.

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Proton Titration Curve of Yeast Iso-1-ferricytochrome *c*. Electrostatic and Conformational Effects of Point Mutations†

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ABSTRACT: The proton titration curves of yeast iso-1-ferricytochrome *c* and selected point mutants of this protein have been determined between pH 3 and 11 at 10 and 25 °C with a computer-controlled titration system. Initial titration of the wild-type protein to acidic pH followed by subsequent titrations to alkaline and then acidic pH demonstrates hysteresis, with one more group (28.7) titrating between pH 11 and 3 than originally titrated (27.7) between pH 3 and 11. Initial titration to alkaline pH, however, resulted in observation of the same number of groups in both directions of titration (28.7 vs 28.6). At 10 °C, 7.5 fewer groups were found to titrate over the same range of pH. Titration curves obtained for six cytochrome *c* mutants modified at Arg-38, Phe-82, Tyr-48, and Tyr-67 were analyzed by subtraction of the corresponding titration curve for the wild-type protein to produce difference titration curves. In most cases, the effects of these mutations as revealed in the difference titration curves could be accounted for as either the result of introduction of an additional group titrating within this pH range, the result of a change in the pK of a titrating residue, and/or the result of a change in the pK for either the first acidic or the first alkaline protein conformational transition. In addition to demonstration of the electrostatic consequences of the mutations in cytochrome *c* studied here, this study establishes the general usefulness of precise proton titration curve analysis in the characterization of variant proteins produced through recombinant genetic techniques.

The proton titration curve of a protein represents the number of equivalents of hydrogen ions bound or released in response to changes in pH. Consequently, such curves can be regarded as the summed contributions of the titratable groups in a particular protein and the manner in which these groups are influenced by the electrostatic characteristics of their environments (Linderstrøm-Lang, 1924; Tanford, 1962; Nozaki & Tanford, 1967). Unequivocal assignment of individual transitions observed in protein titration curves to specific titratable groups is, however, virtually impossible without additional information. If the ionization state of a particular group can be observed by another technique [e.g., ^{1}H NMR pH titrations (e.g., Meadows, 1972) or UV spectrophotometric pH titrations of tyrosyl residues], then the pK_a of such a group can be assigned unambiguously. Alternatively, a method for simulating the entire titration curve for those instances where the atomic coordinates of the protein are available from crystallographic analysis has been described in detail (Matthew et al., 1985; Matthew, 1986; Matthew & Gurd, 1986). Despite the approximations that are employed in such simulations concerning the nature of the dielectric within the protein and at the protein surface and therefore of the electrostatic in-

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