Equilibrium and Kinetic Studies of the Helix–Coil Transition in α1-CB2, a Small Peptide from Collagen*

Karl A. Piez† and Merry Rubin Sherman‡

ABSTRACT: The helix–coil transition in α1-CB2 a 36-residue peptide from collagen, was studied by optical rotation. Equilibrium rotations after stepwise temperature shifts in the range 1–25°, where helix and coil coexist, show the transition to be fully reversible and concentration dependent, compatible with formulation of the reaction as a monomer–trimer equilibrium. By contrast, the melting of whole collagen is concentration independent and characteristic of a phase transition. The van't Hoff plot for the transition in α1-CB2 is linear ($\Delta C_P \simeq 0$) consistent with evidence that the melting of a collagen-like structure does not expose new hydrophobic residues to the solvent, as occurs with globular proteins. The enthalpy change, $\Delta H°$, is −93 kcal/mole of trimer formed and the entropy change, $\Delta S°$, is −277 cal/°K mole. The corresponding values per residue, assuming the trimer to be 90% helical, are $\Delta H°_{res} = -960$ cal/mole and $\Delta S°_{res} = -2.9$ cal/°K mole. If the helical structure is stabilized largely by backbone hydrogen bonds (one per amino acid triplet), the observed enthalpy change corresponds to −2.9 kcal/mole of hydrogen bonds. This large value suggests either unusually stable hydrogen bonds or the involvement of other stabilizing factors. Initial rate studies of helix formation show the reaction to be third order with respect to peptide chains and to have a heat of activation, ΔH_1*, of −18 kcal/mole of trimer. The negative temperature dependence is characteristic of a conformation-dependent interaction. Kinetic curves of optical rotation during equilibration of solutions of isolated random coil or helical forms have the general shape predicted for a monomer–trimer interconversion, but equilibrium is attained more slowly than expected. A plausible model to explain these results is that there is a rate-limiting interaction of three chains followed by rapid propagation of helix to form a mixture of products of varying helical content. The degree of helicity and stability of a trimer would depend on the initial alignment of the chains. As equilibrium is approached, trimers of low helical content would convert slowly, by way of monomer, into trimers of higher helical content. Trimers of low helical content in the final equilibrium mixture would melt first when equilibrium is disturbed by separating the components or by heating.

The formation of a collagen-type helix requires only the repeating amino acid triplet sequence $(Gly\text{-}X\text{-}Y)_n$, where X and Y can be any amino acid, provided there is an average of at least one residue of proline or hydroxyproline every other triplet. Helix formation may occur whenever three appropriate chain segments come together, whether the segments originate from dissimilar chains, similar or different parts of similar chains, or from a single chain by a folding-over or coiling process. Although the helical structure is apparently always the same at the level of the amino acid triplet (Traub *et al.*, 1969). the molecular structure can vary from a completely intrachain helix to a continuous gel. Reversion to a single molecular structure may be obstructed by small differences in energy levels, kinetic barriers or both.

Early studies of collagen renaturation employed commercial gelatin or denatured whole collagen containing undetermined amounts of cross-linked chains and degraded chains as well as several types of chains (see Harrington and von Hippel, 1961). More recent studies have been made on better-defined natural and synthetic polymers (see von Hippel, 1967). In all cases, however, the reactions have been heterogeneous, leading to an uncertain distribution among many possible products and rendering thermodynamic and kinetic analysis difficult or unreliable.

The renaturation of α1-CB2, a 36-residue segment of the α1 chain of collagen, has been shown to form a collagen-like structure that is largely trimeric and has a helical content of 90 ± 10% (Piez and Sherman, 1970). This system thus provides an excellent model for the thermodynamic and kinetic analysis of triple-stranded helix formation, in which many complicating factors of earlier studies are minimized.

Experimental Section

Preparation of α1-CB2. The isolation, purification, and preparation of solutions of the peptide α1-CB2 in 0.15 M potassium phosphate (pH 4.8) are described in the preceding paper (Piez and Sherman, 1970). Concentrations of standard solutions were determined by hydrolysis and amino acid analysis. The concentration unit c denotes millimoles of monomeric α1-CB2 per liter. The concentration unit C, in milligrams per milliliter, used in calculating molecular rotation, is related to c by $C = 3312c/1000$, where 3312 is the molecular weight of α1-CB2 monomer.

Optical Rotation Measurements. Early studies were made with a Rudolph polarimeter using the 313-nm mercury line as described by Piez and Carrillo (1964). Later studies utilized a Cary Model 60 spectropolarimeter with jacketed cells of 5-, 10- and 20-mm light paths custom designed to

* From the Laboratory of Biochemistry, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20014. *Received June 5, 1970.*

† To whom to address correspondence.

‡ Present address: Howard Laboratory, Sloan Kettering Institute, New York, N. Y. 10021.

have the smallest possible volume (Optical Cell Co., Beltsville, Md.). All measurements were made at 313 nm to facilitate comparison with previous results. Temperatures were measured in the entrance and exit lines to the cell just outside the cell compartment using a digital thermometer and thermistor probes (Yellow Springs Instrument Co.). The average value was taken as the temperature in the cell. By switching from one circulating bath to another, the temperature could be changed rapidly. A micro thermistor probe placed in a cell showed that the largest temperature shift employed, 27° to about 1°, was 90% complete in 5 sec and was within 0.1° of the final temperature in 20 sec. Temperatures were controlled to ±0.1°. To prevent condensation on the cells, dry nitrogen was flushed through the cell compartment.

Analysis of the Rotation Data. The measured rotation in degrees, α, was converted into molecular rotation, $[\phi]$, through the relationship: $[\phi] = 1000\ \alpha M/LC$, where M is the average residue molecular weight (91.5), L is the path length in millimeters, and C is the concentration in milligrams per milliliter. The molecular rotation of the random coil form at 313 nm and 25° was considered equal to the observed molecular rotation of a dilute peptide solution at that temperature, $[\phi]_R^{25} = -915$ deg cm²/dmole. To evaluate the temperature dependence of this parameter, dilute solutions of the random form were chilled rapidly to various temperatures, T (degrees centigrade), and the initial rates of renaturation were observed and extrapolated to zero time, giving $[\phi]_R^T$.

A value for the molecular rotation of the helical form at 313 nm and 5°, $[\phi]_H^5 = -1990$ deg cm²/dmole, was obtained by chromatographic separation of the components of a partially renatured sample (Piez and Sherman, 1970). An alternative estimate of $[\phi]_H^5$ was obtained by systematic variation of the value assigned to this parameter in a least-squares deviation fit of the equilibrium rotation data to the theoretical expression for monomer–trimer interconversion (see Results). The temperature dependence of $[\phi]_H$ is not known, but it was assumed to be the same as that determined for the random form. In any case, it is small and would not significantly affect the calculations.

Theory

Equilibrium Data. Characterization of the equilibrium mixture of random coil, R, and helical forms, H, of α1-CB2 has shown that monomer and trimer are the only species present in measurable amounts (Piez and Sherman, 1970). For development of the theory the number of species is two but the number of chains in the helical form, n, is not specified. The equilibrium can then be written as

$$n(\mathrm{R}) \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (\mathrm{H}) \tag{1}$$

and the equilibrium constant, K, is expressed by

$$K = \frac{k_1}{k_{-1}} = \frac{(\mathrm{H})}{(\mathrm{R})^n} \tag{2}$$

where k_1 and k_{-1} are the rate constants for helix formation and melting, respectively. Since (R) and (H) are molar concentrations of random coil and helical forms, the total concentration, c, of peptide chain is

$$c = n(\mathrm{H}) + (\mathrm{R}) \tag{3}$$

The molecular rotation of a solution at equilibrium, $[\phi]_E$, is assumed to represent the weight-average rotation of the two forms; that is

$$[\phi]_E = \frac{n(\mathrm{H})}{c}[\phi]_H + \frac{(\mathrm{R})}{c}[\phi]_R \tag{4}$$

From the observed rotation and the known molecular rotations of the components (see Methods), K for the coil–helix transition may then be calculated according to

$$K = \frac{1}{n}\frac{[\phi]_E - [\phi]_R}{([\phi]_H - [\phi]_E)^n}\left(\frac{[\phi]_H - [\phi]_R}{c}\right)^{n-1} \tag{5}$$

The temperature dependence of the equilibrium constant is used to obtain the enthalpy change, $\Delta H°$, per mole of helix formed, according to the van't Hoff equation

$$\frac{\mathrm{d}\ln K}{\mathrm{d}(1/T)} = -\frac{\Delta H°}{R} \tag{6}$$

where R is the gas constant and T is the absolute temperature. The concentration dependence of the equilibrium constant as defined by eq 5 can be used to determine the value of n. In the application of these equations to α1-CB2 it was also convenient to calculate n from initial rate studies.

Kinetic Data. If the equilibrium in a solution of peptide is disturbed by changing the temperature or concentration or by separating the components, the molecular rotation will change toward a new equilibrium value at a rate given by

$$\frac{\mathrm{d}[\phi]_t}{\mathrm{d}t} = nk_1\frac{([\phi]_H - [\phi]_t)^n}{([\phi]_H - [\phi]_R)^{n-1}}c^{n-1} - k_{-1}([\phi]_t - [\phi]_R) \tag{7}$$

where $[\phi]_t$ is the molecular rotation at time, t. The initial rate of rotational change in the equilibration of isolated random coil form, v_0, is obtained from eq 7 by setting $[\phi]_t = [\phi]_R$ giving

$$v_0 = nk_1([\phi]_H - [\phi]_R)c^{n-1} \tag{8}$$

The value of n can be calculated from plots of $\ln v_0$ *vs.* $\ln c$ for data obtained at one temperature. A more complete analysis of initial rate data includes the temperature dependence of k_1 as defined by the Arrhenius equation

$$k_1 = k_0 e^{-\Delta H_1^*/RT} \tag{9}$$

where ΔH_1^* is the heat of activation for helix formation and k_0 is a statistical factor. The preceding equations may be combined in the form

$$\ln v_0 - \ln([\phi]_H - [\phi]_R) = -\frac{\Delta H_1^*}{R}\frac{1}{T} + \ln(nk_0) + (n-1)\ln c \tag{10}$$

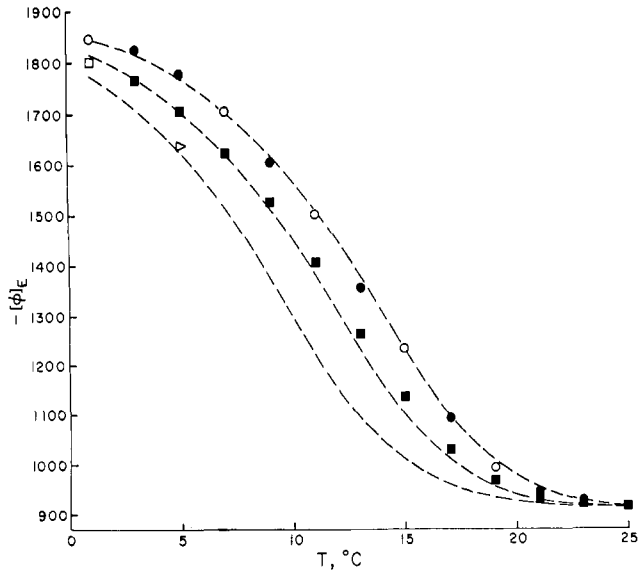

FIGURE 1: Equilibrium curves for the helix–coil transition in α1-CB2 at several concentrations (○ and ●, 1.44 mM; □ and ■, 0.80 mM; △, 0.43 mM). Open symbols denote molecular rotation at equilibrium after a downward temperature shift; closed symbols denote values obtained after an upward temperature shift. Dashed curves were computed for a monomer–trimer equilibrium with $\Delta H^\circ = -93$ kcal/mole of trimer at the above concentrations; see eq 5 and 6.

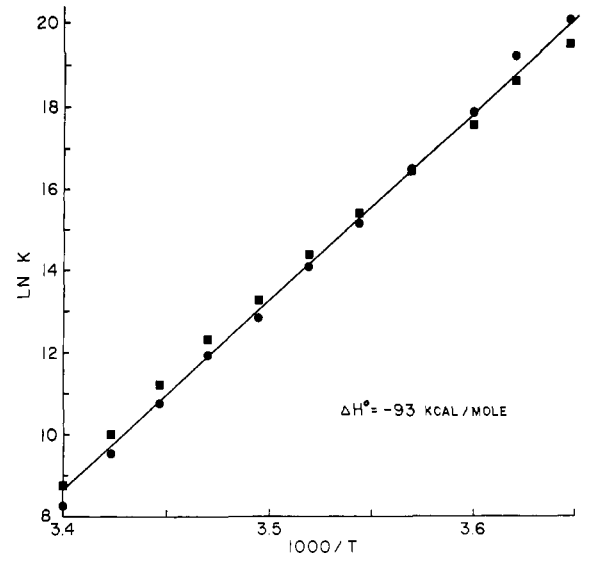

FIGURE 2: Temperature dependence of the equilibrium constant, K, for the helix–coil transition in α1-CB2 at two concentrations (●, 1.44 mM; ■, 0.80 mM). K was calculated from the rotation data in Figure 1 using eq 5 with $n = 3$.

in which data from various concentrations and temperatures may be analyzed in a single matrix solution. The values of ΔH_1^*, n, and k_0 are then readily calculated from the coefficients of the terms $1/T$ and ln c and the constant term $\ln(nk_0)$, and k_1 is obtained from eq 9. From the resutls for K and k_1 at any temperature, the rate constant for helix melting, k_{-1}, is obtained from the definition of the equilibrium constant, eq 2.

Results

Equilibrium Studies. After a solution of α1-CB2 was heated or cooled to a given temperature, the optical rotation approached the same final value regardless of the direction of the temperature shift. The time required to achieve the new equilibrium value varied from less than 1 hr at temperatures over 15° to 2–3 days below 5°, at concentrations above 0.8 mM. In less concentrated solutions equilibration at low temperature took many days, making measurements impractical.

Figure 1 shows the molecular rotation at equilibrium, $[\phi]_E$, for two concentrations of α1-CB2 between 1 and 25°. The reversibility of the rotatory change after each small temperature change and the superposition of the entire curves obtained during stepwise melting and renaturation confirmed that equilibrium was reached at each temperature. At 1°, the lowest temperature used in this study, the value of $[\phi]_E$ increased with concentration, showing that the transition to the helical form was not complete. The percentage of helical form in the most concentrated solution at 1° was estimated to be 95% by comparison of the observed molecular rotation with $[\phi]_H$ determined for best fit as described below. The values of $[\phi]_E$ above 25° showed only the small temperature dependence characteristic of randomly coiled structures.

From the rotation data in Figure 1, the equilibrium constant, K, for the monomer–n-mer interconversion in eq 1 was calculated according to eq 5 as a function of T. Initial calculations utilized the molecular rotation of the random form measured at 25°, the value for the helical form determined chromatographically, and the temperature dependence determined in the initial rate studies (see below). When a value of $n = 1$ or 2 was used in the calculation of K, the van't Hoff plot (ln K *vs.* $1/T$) showed significant curvature. The value of $n = 3$, expected for formation of a collagen-type structure, gave van't Hoff plots that were linear (Figure 2). With $n = 3$ and $[\phi]_R^{25} = -915$ deg cm²/dmole, the estimate of $[\phi]_H^5$ was then defined by minimizing the deviation of the equilibrium data from eq 5. The selected value of $[\phi]_H^5 = -1890$ deg cm²/dmole was not far from the value determined chromatographically, −1990 deg cm²/dmole, and was considered to be more accurate. It was used in all subsequent data analyses and simulations. Theoretical melting curves calculated with these constants gave an excellent fit to the experimental equilibrium data, correctly predicting the concentration dependence (Figure 1). From the slope of the van't Hoff plot in Figure 2, the enthalpy change, ΔH°, was calculated to be −93 kcal/mole of trimer formed. The corresponding equation for K as a function of T is $\ln K = 46{,}600/T - 150$, where K has units of (l./mole)². The value of K would change if other concentration units were used, but the value of ΔH° would not be affected. From the thermodynamic laws, $\Delta F^\circ = -RT \ln K$ and $\Delta S^\circ = (\Delta H^\circ - \Delta F^\circ)/T$, and the value of ΔH°, the entropy change, ΔS°, for the transition was calculated to be −277 cal/°K mole of trimer formed.

Initial Rate Studies. With a sample of α1-CB2 in the cell of a Cary Model 60 spectropolarimeter, and the instrument recording at a fast chart speed, the temperature was dropped rapidly and the increase in negative optical rotation at 313

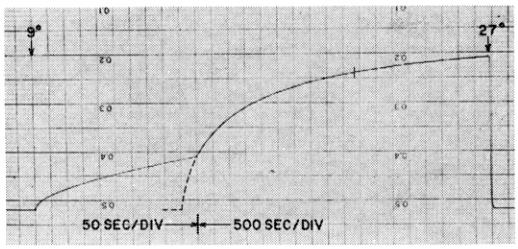

FIGURE 3: Typical chart recording from a Cary Model 60 spectropolarimeter used to calculate initial rates of helix formation in α1-CB2. With a 1.57 mM sample at 27° and the instrument recording a base line, the temperature was quickly changed to 9° and the increase in negative rotation was recorded. After 500 sec the chart speed was decreased. When sufficient data had been collected, the temperature was increased to 27° in preparation for the next experiment.

nm, α, was recorded. The chart from a typical experiment is reproduced in Figure 3. Similar experiments were performed at temperatures from 1 to 17° and concentrations from 0.2 to 1.57 mM. Numerical values for α were read from the chart at 25-sec intervals for 500 sec, omitting the values at 0 and 25 sec. The points were then fitted to a third-order polynomial. A lower order equation could not fit the data, while a higher order polynomial was too sensitive to experimental scatter. From the slope of the smoothed curve at $t = 0$, v_0 was calculated, and from the intercept, $[\phi]_R$ was calculated. From experiments at several temperatures, the variation of $[\phi]_R$ with T was found to be linear within the range studied, with a slope of 1.6 deg cm²/dmole°K.

The data in Figure 4 for ln v_0 as a function of temperature and concentration was analyzed according to eq 10. Data points corresponding to rates of rotational change that were too slow (the two lowest concentrations) or too fast (high concentration at low temperatures) to be accurately measured were omitted from the matrix solution of this equation. In the first solution, the number of chains involved in helix formation was considered to be unknown. The best fit of the data gave a value of $n = 2.8$. When n was assigned a value of 3.0, and the matrix was solved for two unknowns, k_0 and ΔH_1^*, nearly as good a fit to the data was obtained. For $n = 3$, the value of ΔH_1^* is -18 kcal/mole of trimer and the temperature dependence of k_1 is given by $\ln k_1 = 9120/T - 23.6$ (see eq 9). From this relationship and the temperature dependence of the equilibrium constant given above, rate constants for helix melting, k_{-1}, at various temperatures were calculated according to $\ln k_{-1} = \ln k_1 - \ln K$. The temperature dependencies of K, k_1, and k_{-1} are compared in Figure 5. The heat of activation for helix melting, ΔH_{-1}^*, is given by the slope of the line for $\ln k_{-1}$ *vs.* $1/T$, or more simply $\Delta H_{-1}^* = \Delta H_1^* - \Delta H^\circ = -18 + 93 = 75$ kcal/mole of trimer melted. Helix melting has a large positive temperature dependence.

Complete Kinetics. For a more complete kinetic analysis, data were obtained for both helix formation and melting over long time periods. As described above, data for helix formation were easily obtained by rapidly cooling solutions of α1-CB2 random coil. Helix melting was more difficult to measure since solutions containing only the helical form of α1-CB2 could not be easily obtained at the temperatures and concentrations studied. At temperatures below about 5°, however, melting is sufficiently slow to permit separation

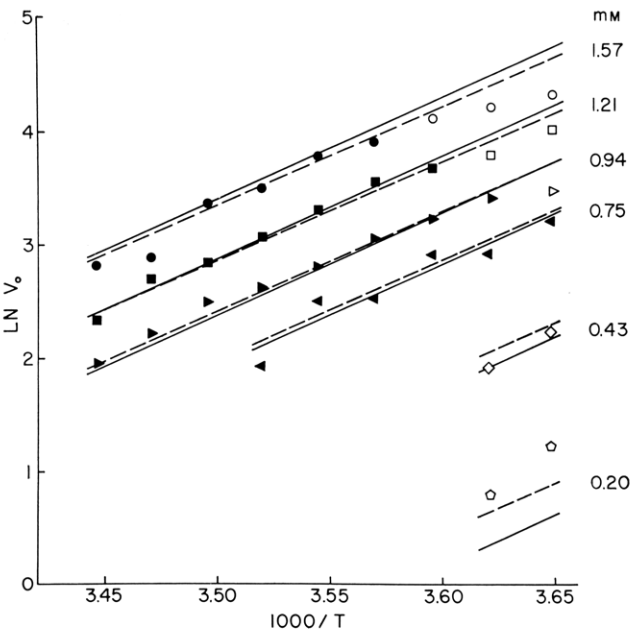

FIGURE 4: Initial rates of rotational change in formation of α1-CB2 helix as a function of temperature and concentration; see eq 8–10. Dashed lines show the best fit of the data to eq 10, which gives $n = 2.8$. Solid lines show the best fit when $n = 3.0$. Data points indicated by open symbols were omitted from the matrix solution.

of the helical and random forms by molecular sieve chromatography (Piez and Sherman, 1970). Such separations were performed and fractions were collected at 5°. Rotations of selected fractions were determined at 5° and the change in rotation was followed as the isolated trimers reverted to a new equilibrium distribution at 5° or a higher temperature.

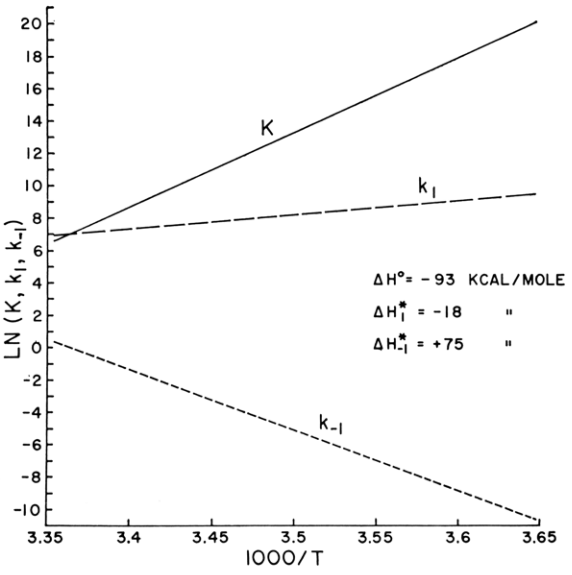

FIGURE 5: Temperature dependence of equilibrium and rate constants for the helix–coil transition in α1-CB2. The line for K was taken from Figure 2. The line for k_1 was calculated from the data in Figure 4 for $n = 3$. The line for k_{-1} was calculated from $\ln k_{-1} = \ln k_1 - \ln K$.

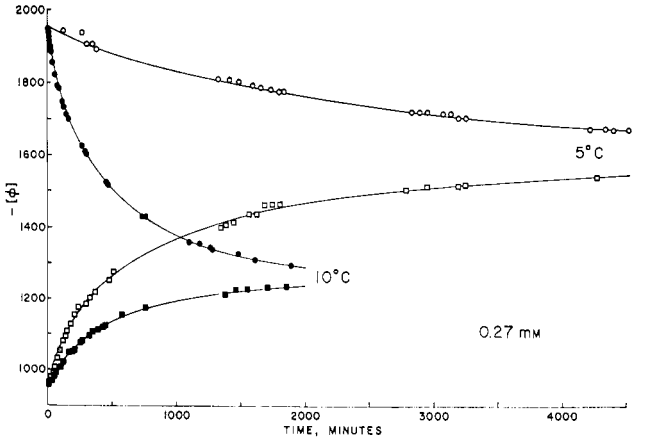

FIGURE 6: Kinetics of equilibration of isolated random coil (■, □) and helical (○, ●) forms of α1-CB2 for one concentration at two temperatures.

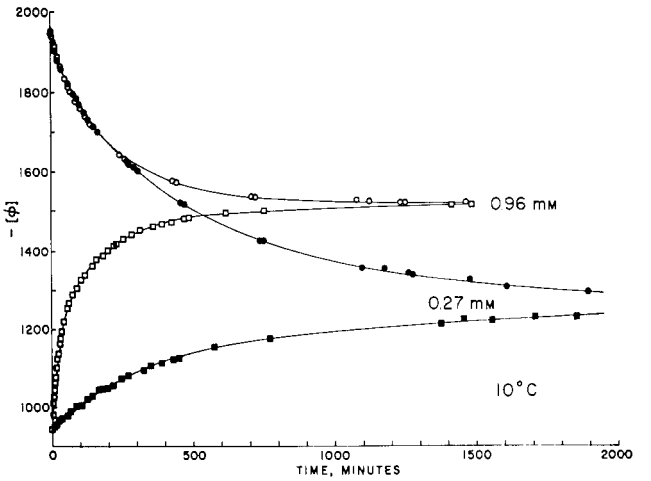

FIGURE 7: Kinetic data, as in Figure 6, for two concentrations of α1-CB2 at the same temperature.

When no further change in rotation with time was detectable, the sample was completely melted by warming to 27°. It was then quickly cooled to the original temperature and the change in rotation was followed during equilibration of the same peptide sample, starting with the random form. In this manner pairs of curves were obtained that showed the approach to equilibrium in the direction of both helix formation and melting.

Kinetics curves are shown in Figure 6 for one concentration, 0.27 mM, at two temperatures, 5 and 10°. The difference in the rates illustrates the negative temperature coefficient for helix formation and the larger positive temperature coefficient for melting. In Figure 7 are shown data obtained at one temperature, 10°, for two concentrations of peptide, 0.27 and 0.96 mM. Readily apparent are the marked concentration dependence of the rates of initial helix formation and the absence of concentration dependence for the initial rate of melting, as expected for a monomer–trimer reaction.

Quantitative comparison of the observed kinetics with the theory was complicated by the complex form of the analytical integral of the rate eq 7 when $n = 3$. The equation was therefore integrated numerically by the fourth-order Runge–Kutta method. A computer program, modified from one written by Mrs. J. Hebbert of the Naval Ordnance Laboratory, was used to produce kinetic curves for assigned values of k_1, k_{-1}, temperature, peptide concentration, and initial rotation.

When the values of k_1 and k_{-1} calculated from the equilibrium and initial rate studies were employed, the simulated curves agreed with the observed equilibrium rotation and initial rates of helix formation and melting and the general form of the curves was correct. However, as illustrated by the comparison of Figures 8 with 6, and 9 with 7, theory predicted that equilibrium from either direction would be approached more rapidly than was observed. By systematically varying the values assigned k_1 and k_{-1} while maintaining the observed value of $K = k_1/k_{-1}$, better overall agreement could be obtained but significant discrepencies persisted.

An alternative approach to the kinetic analysis was attempted by substituting two-point slopes from the experimental curves into the differential rate eq 7, and solving for the best-fitting rate constants with $n = 3$. The scatter in the empirical values of $d[\phi]/dt$, however, prevented the application of this method. The computer program written for slope analysis did provide a check on the theoretical curves. Kinetic data simulated by the numerical integration program was used as input for the slope analysis program to calculate k_1 and k_{-1}. Agreement between the values obtained and the input to the simulation program proved the validity of the integration method.

Discussion

The data reported here for the collagen fragment α1-CB2 must be interpreted differently from data for whole collagen. Aside from the fact that the helix–coil transition in collagen in solution cannot be studied under fully reversible conditions, the extraordinary chain length of collagen chains has important implications. If the enthalpy of melting one residue in a collagen structure, ΔH°_{res}, is about 1 kcal/mole (see below), then ΔH° for a collagen molecule, which contains about 3000 residues, is about 3000 kcal/mole. As pointed out by Flory and Weaver (1960), the equilibrium constant for collagen melting would then have a predicted temperature dependence of about 10^6-fold/deg, which is characteristic of a phase transition. For α1-CB2 by contrast, ΔH° is about 100 kcal/mole of trimer, with a corresponding temperature dependence of the equilibrium constant of about 1.5-fold/deg. Therefore, the helix–coil transition in α1-CB2 is better described as an ordinary chemical reaction than a phase transition. The observable consequence of the great difference in chain length between whole collagen and the segment α1-CB2 is that the helix–coil transition in collagen is temperature dominated while in α1-CB2 the effect of concentration is also important.

The values of ΔH° and ΔS° for the formation of α1-CB2 helix are −93 kcal/mole and −277 cal/°K mole of trimer, respectively. The three peptide chains contain a total of 108 residues. The assumption that the helical content of the trimer is 90% (Piez and Sherman, 1970) reduces the number of residues involved in a conformational transition to 97. The enthalpy and entropy change per residue for helix formation are therefore $\Delta H^\circ_{res} = -960$ cal/mole and $\Delta S^\circ_{res} = -2.9$

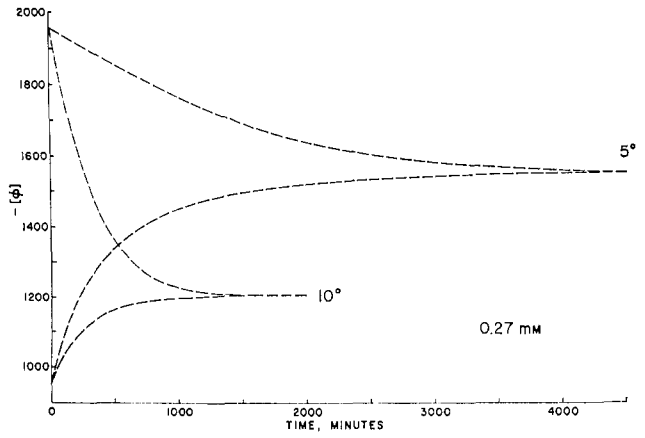

FIGURE 8: Theoretical kinetic curves for equilibration of isolated random coil and helical forms of α1-CB2, assuming a monomer–trimer reaction. Numerical integration of eq 7 utilized values of the rate constants calculated from the equilibrium and initial rate studies according to the equations for k_1 and k_{-1} given in the text. Compare with experimental results in Figure 6.

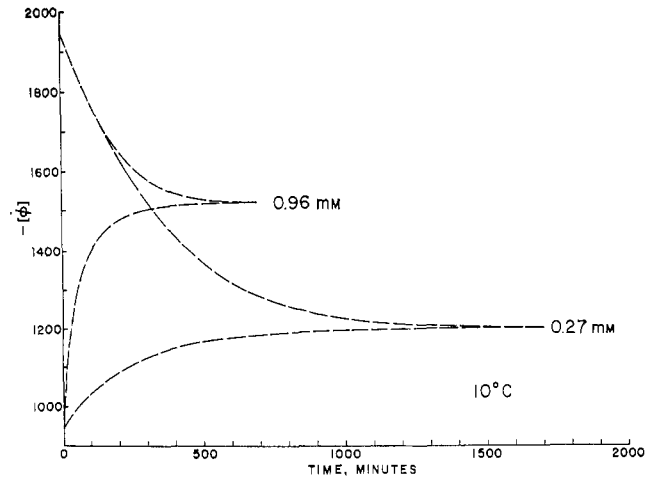

FIGURE 9: Theoretical curves, as in Figure 8, corresponding to the data in Figure 7.

cal/°mole. This value of $\Delta H°_{res}$ may be compared with the value of 1.2 kcal/mole found by Flory and Spurr (1961) for the melting of collagen. If the enthalpy change in these transitions is attributed to the formation or breaking of interchain hydrogen bonds, of which there is one per triplet (Traub *et al.*, 1969), the average enthalpy change for the (formation or) dissociation of hydrogen bonds of the type found in the peptide would be $3\Delta H°_{res} = (-)2.9$ kcal/mole. This suggests a rather stable hydrogen bond, which might result from the exceptionally high basicity of the carbonyl group of a proline residue (Veis and Nawrot, 1970). Alternatively, other factors such as hydrogen bonding to water (Ramachandran and Chandrasekharan, 1968), may contribute significantly to the enthalpy change.

The melting of collagen has been treated as an isothermal phase transition at equilibrium. In this case the melting temperature, T_m, equals $\Delta H°/\Delta S°$ (see Rao and Harrington, 1966). Using the values of the thermodynamic parameters determined for α1-CB2, T_m under standard conditions would be 93/0.277 = 335°K = 62°. Since the major determinant of T_m for whole collagen is the imino acid content, the calculated value of T_m would be expected to apply to a very long polymer containing 33 residue % imino acids, like α1-CB2. Although there is no known collagen with this composition, a T_m of about 57° would be predicted by extrapolation from data on other collagens (Josse and Harrington, 1964), in good agreement with the calculation from the peptide data. The melting of the α1-CB2 trimer was observed at much lower temperatures in these experiments because of concentration effects demonstrable only in short-chain polymers.

In the analysis of the present experiments, a value of $[\phi]_H$ was selected to obtain the best fit of the equilibrium data to a linear plot of ln K *vs.* $1/T$. When the value of $[\phi]_H$ determined by molecular sieve chromatography was used, the van't Hoff plot showed slight downward curvature in the region corresponding to temperatures below 5°. Either estimate of $[\phi]_H$ gave a nearly constant value of $\Delta H°$ for the transition in α1-CB2. In other words the change in heat capacity, ΔC_p, was negligible. This may be contrasted with the denaturation of globular proteins for which graphs of ln K *vs.* $1/T$ have nonlinearity corresponding to ΔC_p in the vicinity of 2 kcal/°K mole (see, for example, Pace and Tanford, 1968). The change in C_p is attributed to the exposure of hydrophobic groups to solvent during unfolding. In the native collagen structure, however, the polypeptide chain backbones form the core of the rod-like molecule and all amino acid side chains are on the outside, apparently fully exposed to the solvent.

The logarithm of the initial rate of α1-CB2 helix formation was proportional to $1/T$, as predicted by the Arrhenius eq 9 for a chemical reaction. For the analogous process in collagen, the Arrhenius equation does not apply, but ln k_1 is proportional to $1/T\Delta T$, where $\Delta T = T_m - T$ (Flory and Weaver, 1960; see also von Hippel, 1967). This latter relationship is thought to derive from the temperature dependence of the size of the nucleus in a nucleated crystallization. While helix formation in α1-CB2 cannot be described as a crystallization (or phase transition), a nucleation step must be involved here as well, and an effect of ΔT would be expected. Indeed, the negative temperature dependence of the forward reaction rate reflects the fact that a given nucleus is more stable at low temperature and is therefore more likely to lead to helix formation. The small magnitude of the temperature dependence suggests that the size of the nucleus changes very little within the temperature range studied and explains the absence of a measurable effect of ΔT. It should also be noted that the appropriate value of T_m for the calculation of ΔT would be that of a long-chain polymer, about 57°, in which case ΔT would not change much in the temperature range studied (1–17°).

Initial rate studies of α1-CB2 helix formation show the reaction to be (close to) third order, as required for monomer–trimer interconversion with no stable intermediates. The renaturation of whole collagen is usually reported to be (close to) second order (see von Hippel, 1967), which probably represents an average of the many reactions which are possible with long chains. When the rotational changes during equilibration of isolated forms of the peptide were computer simulated, the major features of the empirical kinetics were

reproduced, but the equilibrium was approached more rapidly than in the real data. Since the characterization of the helical form of α1-CB2 (Piez and Sherman, 1970), and the equilibrium and initial rate studies are all consistent with monomer–trimer reaction, it is proposed that the theoretical kinetics describe the change in average molecular weight but not the change in helical content. The discrepancy would be accounted for by the initial formation and melting of trimeric products of low helicity.

The proposed kinetic scheme which is compatible with all the observations involves the rate-limiting formation of several species of nucleated trimer followed by rapid helix propagation to the extent permitted by the alignment of chains in the complex. Species with poor alignment could form readily since nucleation involves only a part of each chain. However, some of the resultant products would have marginal stability and would gradually convert, by way of monomer, into species of higher helical content. Helix formation (observed increase in negative rotation) would then lag behind trimer formation (computed kinetic curves). It is unlikely that the different helical forms of the peptide could interconvert directly in an annealing process, since the necessary partial opening of the helix would markedly decrease the stability of these short-chain structures and they would immediately melt. Furthermore, if such annealing were possible, the observed kinetics should be less than third order. In the melting of the helical form of α1-CB2, trimeric species having the lowest helical content (and stability) would melt first, so that helix melting would lag behind the calculated decrease in trimer content.

The preceding scheme provides a qualitative explanation for the kinetic behavior of the collagen fragment α1-CB2. It differs significantly from the schemes required to account for the kinetics of renaturation of long chains which generally include rate-limiting propagation and annealing steps. Furthermore, in interpreting these data, it is not necessary to invoke helix formation in either a stable or an unstable single-chain intermediate (see von Hippel, 1967).

In a recent series of papers, Harrington and his colleagues report detailed studies of renaturation of single and cross-linked collagen chains and have clarified the nature of the helix–coil transition as it occurs in long-chain polymers (Harrington and Karr, 1970; Harrington and Rao, 1970; Hauschka and Harrington, 1970a–c). Our results are completely consistent with their findings. The differences in emphasis arise from the different size of the chains studied. The theory of nucleated crystallization can be applied to the longer protein chains, while the concentration dependence of triple-helix formation is more evident in studies with the short peptide.

Acknowledgments

We are indebted to Drs. William Harrington, Donald Crothers, and George Weiss for helpful discussions.

References

Flory, P. J., and Spurr, O. K., Jr. (1961), *J. Amer. Chem. Soc. 83*, 1308.

Flory, P. J., and Weaver, E. S. (1960), *J. Amer. Chem. Soc. 82*, 4518.

Harrington, W. F., and Karr, G. M. (1970), *Biochemistry 9*, 3725.

Harrington, W. F., and Rao, N. V. (1970), *Biochemistry 9*, 3714.

Harrington, W. F., and von Hippel, P. H. (1961), *Advan. Protein Chem. 16*, 1.

Hauschka, P. V., and Harrington, W. F. (1970a), *Biochemistry 9*, 3734.

Hauschka, P. V., and Harrington, W. F. (1970b), *Biochemistry 9*, 3745.

Hauschka, P. V., and Harrington, W. F. (1970c), *Biochemistry 9*, 3754.

Josse, J., and Harrington, W. F. (1964), *J. Mol. Biol. 9*, 269.

Pace, N. C., and Tanford, C. (1968), *Biochemistry 7*, 198.

Piez, K. A., and Carrillo, A. L. (1964), *Biochemistry 3*, 908.

Piez, K. A., and Sherman, M. R. (1970), *Biochemistry 9*, 4129.

Ramachandran, G. N., and Chandrasekharan, R. (1968), *Biopolymers 6*, 1649.

Rao, N. V., and Harrington, W. F. (1966), *J. Mol. Biol. 27*, 577.

Traub, W., Yonath, A., and Segal, D. M. (1969), *Nature (London) 221*, 914.

Veis, A., and Nawrot, C. F. (1970), *J. Amer. Chem. Soc. 91* (in press).

von Hippel, P. H. (1967), *in* Treatise on Collagen I. Chemistry of Collagen, Ramachandran G. N., Ed., London, Academic, Chapter 6.