Characterization of the Product Formed by Renaturation of α1-CB2, a Small Peptide from Collagen*

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ABSTRACT: Isolated α1 chains of collagen are cleaved by cyanogen bromide into discrete peptides which may be separated chromatographically. One of these peptides containing 36 amino acid residues (α1-CB2) has been utilized to study the helix–coil transition characteristic of collagen. It is reversibly transformed at low temperatures to a helical product which has been characterized by optical rotation, circular dichroism, molecular sieve chromatography, and ultracentrifugation. It consists primarily of trimer but may contain small amounts of higher molecular weight aggregates. The circular dichroism spectrum at 2° is characteristic of a collagen-like structure with a helical content of 90 ± 10%. The incomplete helicity may indicate some misalignment of the peptide chains. Molecular sieve chromatography at 5° reveals the presence of only two forms, monomer and trimer, in concentration-dependent equilibrium. The distribution coefficient for chromatography of the trimer on a standardized polyacrylamide column suggests an asymmetric structure consistent with calculated dimensions of 113 × 11.4 Å for a rod-like molecule similar to collagen.

Studies of the mechanism of formation of the triple-helical structure of collagen have encountered difficulties because of the great chain length and the heterogeneity of available samples. We have attempted to simpify the problem by utilizing a relatively small peptide of known amino acid sequence isolated from cyanogen bromide digests of the α1 chain of rat skin collagen (Bornstein and Piez, 1966). The peptide has the sequence (Bornstein, 1967)

Gly-Pro-Ser-Gly-Pro-Arg-Gly-Leu-Hyp-Gly-Pro-Hyp-
Gly-Ala-Hyp-Gly-Pro-Gln-Gly-Phe-Gln-Gly-Pro-Hyp-
Gly-Glu-Hyp-Gly-Glu-Hyp-Gly-Ala-Ser-Gly-Pro-Hse

The positions shown as containing hydroxyproline contain varying amounts of proline as a result of incomplete hydroxylation (Bornstein, 1967).

Since this sequence is composed of triplets of the form Gly-X-Y and contains one third proline plus hydroxyproline, it would be expected to form a collagen-like helix at sufficiently low temperatures and a random coil[1] at higher temperatures. Preliminary to the detailed thermodynamic and kinetic analysis of this renaturation process reported in the following paper (Piez and Sherman, 1970), it was necessary to determine the homogeneity of the product, the number of chains utilized in its formation, the nature of the helical structure, and the degree of helicity. These properties have been explored using the Archibald technique of ultracentrifugation, molecular sieve chromatography, and circular dichroism spectroscopy.

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[1] The term random coil is used here to denote the denatured form of the peptide, characterized by a temperature-insensitive, low-negative optical rotation at 313 nm, and does not imply a Guassian distribution of chain elements.

Experimental Section

Preparation of α1-CB2. Soluble collagen was obtained from the skin of rats given β-aminopropionitrile to inhibit collagen cross-linking. The peptide was prepared from cyanogen bromide digests of the α1 chain of this collagen by chromatography on phosphocellulose as described by Bornstein and Piez (1966). It was further purified as follows. Fractions from several phosphocellulose column runs containing 5–15 mg of α1-CB2 were combined, lyophilized, redissolved in 1–2 ml of 0.1% acetic acid, and chromatographed at room temperature on a 2 × 72 cm column of Bio-Gel P6 equilibrated with 0.1% acetic acid (Piez, 1968). The absorbancy of the effluent was monitored at 235 nm. Fractions comprising the major peak of absorbance were combined and lyophilized and the peptide was dissolved to give a concentration of 0.5–1% in 1–2 ml of 0.15 M potassium acetate (pH 4.8). This pH was chosen for all the studies reported here since rat skin collagen, which is used for comparison, is not soluble above pH 5 at low ionic strength. The solution was then stored at 1° for 2 days or longer. Under these conditions the peptide was converted largely into the helical form. The sample was then chromatographed at 5° on a 1.5 × 40 cm column of Bio-Gel P10 equilibrated with the acetate buffer by techniques that have been described (Piez, 1968). By this procedure the helical form of the peptide was resolved from the monomeric form and from any contaminants that chromatograph with the monomer (see Figure 1). Only those fractions containing the helical form were used. They were combined and lyophilized.

Final solutions were prepared by dissolving a sample (10–15 mg) together with the buffer salts in 1 ml of water, and passing the solution at room temperature through a 1 × 30 cm column of Bio-Gel P2 equilibrated with 0.15 M potassium acetate (pH 4.8). For circular dichroism spectroscopy, the acetate buffer was replaced by 0.15 M potassium fluoride, adjusted to pH 4.8 with a trace of sulfuric acid. Fractions of 1 ml were collected and the concentration of α1-CB2 was determined from the optical rotation at 313 nm. Since α1-CB2 is

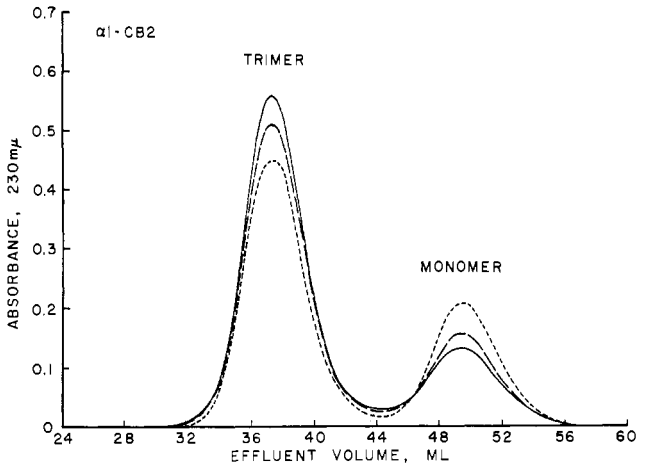

FIGURE 1: Molecular sieve chromatography of equilibrium mixtures of random coil (monomer) and helical (trimer) α1-CB2 at three different peptide concentrations. The samples, each containing 0.7 mg of peptide, were equilibrated at 5° at concentrations of 0.48 mg/ml (solid curve), 0.27 mg/ml (long dash), and 0.14 mg/ml (short dash) before being placed on the Bio-Gel P10 column.

completely excluded from Bio-Gel P2, the peptide is eluted in a sharp peak in which one or two fractions contain 5 mg/ml or more. Lower concentrations were obtained by using other fractions or by dilution. Effluent fractions preceding α1-CB2 and free of peptide were used for dilution and for reference purposes. Samples prepared in this manner had the predicted amino acid composition. The absence (<0.01 residue/mole) from hydrolysates of aspartic acid, threonine, valine, isoleucine, tyrosine, lysine, and histidine, which are not present in α1-CB2, indicated the absence of other peptide or protein material.

Separation of Helical and Random Coil Forms of α1-CB2. Molecular sieve chromatography on Bio-Gel P10 as described above was used to separate and determine the relative amounts of helical and random coil forms in equilibrium mixtures. Samples of α1-CB2 at several concentrations in acetate buffer were kept at 5° for several days to achieve equilibrium. Volumes of each solution containing the same mass of peptide were chromatographed at 5° on the 1.5 × 40 cm column. Absorbance in the effluent at 230 nm was monitored continuously at room temperature using a flow cell with a 1-cm light path in a Beckman DB-G spectrophotometer and recorded on a Beckman 10-in. chart recorder. Denaturation of helical α1-CB2 is sufficiently rapid at 25° that the material isolated as helix on the 5° column would have the extinction coefficient characteristic of the random form by the time it was monitored at room temperature. The relative amounts of random and helical peptide in the original solution are therefore equal to the relative areas of the absorbance peaks as measured by planimetry.

The same column, equilibrated with 0.15 M potassium chloride, was calibrated with ovalbumin (to measure the excluded volume), ribonuclease (a protein of known properties that is partially included), and potassium acetate (to measure the total volume). It was found that ovalbumin was slightly included but the presence of a small amount of aggregate provided a measure of the excluded volume. Using the approach described by Ackers (1964) and assuming a restricted diffusion model for the molecular sieve column, hydrodynamic properties of the helical form of α1-CB2 were determined.

Ultracentrifugation. The molecular weight of α1-CB2 in the helical and random coil forms was determined by the Archibald (approach to equilibrium) method as recommended by Chervenka (1969), except that FC-43 fluorocarbon oil was not used and measurements were made only at the upper meniscus. All photographs were made at a phase-plate angle of 70° without changing the setting between runs. Photographs were taken over a period of about 1 hr beginning about 15 min after reaching speed. The molecular weight of ribonuclease was also measured to establish our ability to apply this technique. The conditions for individual samples are given in Table I. The molecular weight of the helical form of α1-CB2 was measured after filling the cell, placing it in the rotor, and keeping the rotor and cell at 1° for several days to allow equilibration between the random coil and helical forms.

Circular Dichroism Spectroscopy. Samples of α1-CB2 in 0.15 M potassium fluoride adjusted to pH 4.8 with a trace of sulfuric acid were prepared as described above to give a concentration of about 5 mg/ml. This solvent was chosen since it is transparent in the low-ultraviolet region. Although neutral salts can alter the stability of the collagen helix, the low concentrations of salt used in these experiments would have very small effects (von Hippel and Schleich, 1969). It was therefore assumed that the proportions and optical properties of the random coil and helical forms of α1-CB2 in 0.15 M potassium acetate would not be significantly changed in 0.15 M potassium fluoride.

To obtain a circular dichroism spectrum of the helical form of α1-CB2, a sample containing about 5 mg/ml was kept at 1° for several days. The sample, containing about 95% helical form,[2] was then rapidly diluted about 30-fold using cold pipets and maintaining the temperature below 5° at all times. The diluted sample was then placed in a jacketed cell (Optical Cell Co., Beltsville, Md.) at 2° with a 1-mm light path and the circular dichroism spectrum was obtained within 1 hr using a Cary Model 60 spectropolarimeter with a Model 6002 CD accessory. Although an equilibrated solution of α1-CB2 at this concentration would contain a large amount of random coil, the rate of melting of the helix is so slow below 5° that the optical changes are insignificant for several hours (Piez and Sherman, 1970). A value of 93% helical form was obtained from the optical rotation at 313 nm of a sample handled in the same way but placed in a 10-mm cell. The difference between this value and the calculated value of 95% (Piez and Sherman, 1970) may be attributed to some loss of helical form on dilution but is within experimental error. The circular dichroism spectrum of the random coil form was obtained after raising the temperature of the cell to 44° where melting is complete.

For comparison, the circular dichroism spectra of soluble collagen in the same solvent and at 2° (helical) and 4° (random coil) were obtained. The soluble collagen was prepared from the skin of lathyritic rats (Bornstein and Piez, 1966). It is completely melted at about 40° under the conditions employed (Piez and Carrillo, 1964).

[2] The distinction is made between the per cent helical form in a peptide solution, meaning the per cent of molecules in the renatured or trimeric state under the given conditions, and the per cent helicity of the renatured form, as determined by comparison of the optical properties to those of native collagen.

The results were calculated as the molecular ellipticity, $[\theta]$, using the relationship: $[\theta] = 1000\,\theta M/LC$, where θ is the measured ellipticity in degrees, L is the path length in millimeters, C is the concentration in milligrams per milliliter, and M is the average residue molecular weight, 91.5 for the peptide, 91.2 for collagen. The data were not corrected for refractive index.

Molecular Rotation. The optical rotation at 313 nm, α, of a peptide solution in the acetate buffer was measured at 25° where denaturation is essentially complete. From this value the molecular rotation of the random coil form, $[\phi]_R$, was calculated using the expression: $[\phi] = 1000\,\alpha M/LC$, where M and C have been defined above.

The value of C was determined by hydrolysis and amino acid analysis of an ailquot. The specific rotation, $[\alpha]$, is related to $[\phi]$ by the equation $[\phi] = [\alpha]M/100$. No refractive index correction was applied. The molecular rotation of the helical form of α1-CB2 was determined from the molecular sieve chromatograms as described in Results.

TABLE I: Archibald Molecular Weights.

	α1-CB2		
	Random Coil	Helix[a]	RNase
Rotor speed, rpm	36,000	10,000	20,000
Temperature, °C	27	1	25
$\bar{v}$	0.69[b]	0.69[b]	0.71[c]
Concentration, %	0.5	0.5	0.4
M_w, known[b]	3,312	9,936	13,682
M_w, found	3,430	10,800	13,600

[a] 95% helical form, 5% random coil. [b] Calculated from the amino acid composition. The helix is assumed to be a trimer for the molecular weight calculation. [c] An average of the values summarized by Scheraga and Rupley (1962).

Results

Molecular Sieve Chromatography. The melting of helical α1-CB2 is very slow at low temperatures. Renaturation to the helical state is very slow at low concentrations since three chains must come together to form each helical molecule. Therefore, a sample equilibrated at 5° and chromatographed rapidly on a cold molecular sieve column where there is large dilution, can be resolved into its random coil and helical forms. The separation is illustrated in Figure 1 for three concentrations of α1-CB2. As expected from the mass action law for a trimolecular reaction, the more concentrated samples contain a higher proportion of the helical form (see Piez and Sherman, 1970).

Since behavior on a molecular sieve column depends on both molecular weight and shape, it is possible to estimate the physical parameters of a solute by utilizing a calibrated column. The distribution coefficient of the solute, K_D, is defined by

$$K_D = \frac{V_S - V_0}{V_t - V_0}$$

where V_S is the elution volume of the solute, V_0 is the excluded volume of the column, and V_t is the total volume. From K_D for a substance of known Stokes radius, a, one can calculate the effective gel pore radius, r, according to the theoretical treatment of Ackers (1964). The Stokes radius of an unknown substance can then be obtained from its distribution coefficient and the value of r. For the Bio-Gel P10 column utilized in this study, V_0 was 23.0 ml, V_t was 63.5 ml, and ribonuclease appeared in the effluent at 46.5 ml. From the value of $a = 17.3$ Å for ribonuclease (Fawcett and Morris, 1966), an effective gel pore radius of 143 Å was obtained from the table provided by Ackers (1964). The resultant values of a for the two forms of α1-CB2 were 30.2 Å for the helical form and 13.7 Å for the random coil form.

Molecular Rotation. The value obtained for $[\phi_R]$ was −915 deg cm²/dmole at 313 nm and 25°. The separation by molecular seive chromatography also permitted the calculation of a value for the molecular rotation of the helical form of α1-CB2, $[\phi]_H$, from the molecular rotation of an equilibrium mixture of the two forms $[\phi]_E$, the value of $[\phi]_R$, and the relative amounts of the two forms calculated from the areas under the peaks, A_H and A_R, from the equation

$$[\phi]_H = [\phi]_E + \frac{A_R}{A_H}([\phi]_E - [\phi]_R)$$

The values obtained from the three chromatograms shown in Figure 1 and a fourth sample at 0.37 mg/ml were −2000, −1970, −1970, and −2020 deg cm² per dmole giving an average of −1990 deg cm²/dmole at 313 nm and 5°. This value for $[\phi]_H$ and the value of $[\phi]_R$ of −915 deg cm²/dmole at 25° may be compared with the corresponding values for collagen under the same conditions of −2120 and −785 deg cm² per dmole calculated from the data of Piez and Carrillo (1964).

Molecular Weights. The Archibald technique of ultracentrifugation was chosen since measurements could be made rapidly before the chemical equilibrium had shifted significantly. The results in Table I are averages from four to six photographs taken 15–75 min after reaching speed. There was no significant change in the calculated weight with time during this period. The average molecular weight obtained for a ribonuclease standard, 13,600, is well within experimental error of the known value. The molecular weight of 3430 for the random coil form of α1-CB2 agrees well with the value of 3312 calculated from the amino acid composition and with the value of 3230 previously measured by high-speed sedimentation equilibrium (Bornstein and Piez, 1966). The weight-average molecular weight of an equilibrium mixture of the helical and random coil forms, under conditions where 95% by weight would be in the helical form (Piez and Sherman, 1970), was found to be 10,800 with an average deviation of 5%. Correction for the 5% random coil content gives a molecular weight for the helical form of 11,200. The ratio of the molecular weights of the two forms is 3.26. This value differs from the value of 3.0 expected for a trimer by 9% which is at the limit of (but within) the estimated experimental error.

Circular Dichroism Spectra. Figures 2 and 3 show the circular dichroism spectra of α1-CB2 and of collagen in the helical and random coil forms. The close similarity between the spectra is immediately evident. The helical forms show a

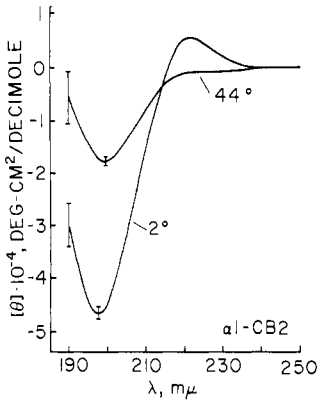

FIGURE 2: Circular dichroism spectra of α1-CB2 measured at 2 and 44° at a concentration of 0.162 mg/ml. The sample at 2° contained 93% helical form and 7% random coil.

positive band at 221 nm (collagen) and 223 nm (α1-CB2) with associated molecular ellipticities of 7200 and 6700 deg cm² per dmole, respectively. Both show a negative band at 197 nm with a molecular ellipticity close to −50,000 deg cm²/dmole. Spectra of the random coil forms, studied at 44°, are also similar with a negative band at 199 nm and a small negative plateau suggesting a minor band centering at about 225 nm. The circular dichroism parameters are summarized in Table II.

Discussion

The similarity of the circular dichroism spectra of collagen and α1-CB2 at 2° clearly indicates that the renatured peptide contains the same polyproline II type helix as collagen, and permits an estimation of the helical content of the renatured peptide. The quantitative interpretation of the large negative band at 197 nm in the low-temperature spectra is complicated by the presence of a negative band at 44° which centers at 199 nm. If the bands observed at high temperatures are attributed to some asymmetric structure other than the collagen helix which is not lost in conversion into the helix, then the helical content should be calculated from the net contribution of the helix, or the difference between the spectra at 2 and 44°. The

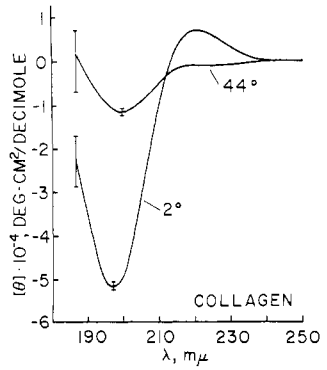

FIGURE 3: Circular dichroism spectra of helical (native) and random coil (denatured) rat skin collagen measured at 2 and 44°, respectively. The concentration of collagen was 0.145 mg/ml.

TABLE II: Circular Dichroism Parameters for Collagen and α1-CB2.

	λ (nm)		[θ][a]	
	Collagen	α1-CB2	Collagen	α1-CB2
Helical form				
+Band	221	223	7,200	6,700[b]
Crossover	213	216		
−Band	197	197	−51,500	−49,200[b]
Random coil form				
−Band 1	~225	~225	~−1,200	~−700
−Band 2	199	199	−11,300	−16,900

[a] deg cm²/dmole, not corrected for refractive index. [b] Corrected for the measured presence of 7% random coil form.

qualitative similarity between the low- and high-temperature spectra for both collagen and the peptide, however, suggests the persistence of some collagen-type asymmetry in the melted forms. In this case, the helical content of the renatured peptide should be calculated from the total ellipticity observed at a given temperature.

The second interpretation is supported by other considerations. Polyproline II type helices are stabilized in part by the stereochemical restrictions of the cyclic imino acids, proline and hydroxyproline. Studies on polyproline indicate that the bond angles at the α-carbon atom of the imino acids imposed by the pyrrolidine ring are not altered by heating (see Carver and Blout, 1967). If the random coil form of a collagen chain contains local structure that arises in this way, the amount would be expected to be proportional to the imino acid content. Comparison of the circular dichroism parameters of collagen and α1-CB2 at 44° confirms this prediction (see Table II). The magnitude of the 199-nm band for the random form of α1-CB2 is 50% greater than the band for whole denatured collagen and it contains 50% more imino acid (33 *vs.* 22 residue %). Further support for the hypothesis of local structure in otherwise randomly coiled chains is given by the circular dichroism spectra of other polypeptides (Tiffany and Krimm, 1969).

We have therefore used the total ellipticity at 197 nm as a measure of helical content. If collagen were assumed to be 100% helical at 2°, then the trimer of α1-CB2 would be 95% helical. Although collagen is probably largely helical, there is at least one region (at the N-terminal ends of the α chains) comprising about 2% of the molecule that is nonhelical (Bornstein and Piez, 1966). A more realistic, but still arbitrary, value for the helical content of collagen might be 95%, resulting in a value of 90% for the helicity of the α1-CB2 trimer. A consideration of the experimental errors involved suggests an uncertainty of ±10%. If the positive band at 221 or 223 nm is used in a similar manner to calculate helical content, a value of 88% helicity is obtained, in good agreement with the results from the negative band.

The helical content of the renatured peptide may also be

estimated from optical rotatory studies. From the values of $[\theta]_H$ and $[\theta]_R$ for α1-CB2 and collagen and the usual assumption that $([\theta]_H - [\theta]_R)$ measures the helicity of collagen-like structures, a helical content of 75% is obtained. This calculation represents a somewhat simplified analysis of the rotation data. The result suggests, however, that the helicity derived from the circular dichroism spectra may be an upper estimate.

The incomplete helicity of renatured α1-CB2 may arise from several causes. The most likely would seem to be the unraveling of the helix at its ends and misalignment of the peptide chains. In the latter case, the product of renaturation would consist of a mixture of trimeric species. Kinetic data reported in the following paper are most easily explained by this hypothesis (Piez and Sherman, 1970).

The ratio of the molecular weights obtained for the helical and random coil forms of α1-CB2, 3.26, is consistent with the identification of the helical form as a trimer. Although data from the Archibald method do not prove the homogeneity of the helical form, the chromatographic data suggest that equilibrium mixtures consist primarily of two molecular weight classes which can be described as monomer and trimer. The presence of a small amount of material larger than a trimer, and having a lower degree of asymmetry, cannot be ruled out. *It is significant that no dimers can be detected* presumably because such forms are intrinsically unstable. This observation is important in interpreting the kinetics of helix formation (Piez and Sherman, 1970).

The determination of the Stokes radius of the α1-CB2 trimer and a knowledge of its molecular weight permit an estimation of its dimensions. The formula relating the Stokes radius, a, to the frictional ratio, f/f_0, is

$$\frac{f}{f_0} = a\left(\frac{4\pi N}{3M(\bar{v} + \delta v_0)}\right)^{1/3}$$

were N is Avogadro's number, M is the molecuoar weight, $\bar{v}$ is the partial specific volume of the anhydrous solute, δ denotes the grams of solvent associated with each gram of solute, and v^0 is the specific volume of pure solvent (Tanford, 1961). The parameter δ is difficult to evaluate and critically affects the value of f/f_0. In the case of a collagen-like molecule, the surface-to-volume ratio is large and the amino acid side chains are on the outside, suggesting that the amount of associated water is relatively large. For globular proteins, the largest value assumed to be reasonable is about 1.0 g of solvent/g of solute (Tanford, 1961). Using this value for α1-CB2 trimer, we obtain a frictional ratio of 1.60, which corresponds to a prolate ellipsoid with a ratio of semiaxes, a/b, of 11 (Schachman, 1959). The equivalent cylinder, which more closely approximates a collagen-like molecule, would have a length, $L = 2a$ and a diameter, $d = 2(2/3)^{1/2}$, $b = 1.636b$. From the preceding relationships, the equation for the volume of a cylindrical molecule

$$\frac{\bar{v}M}{N} = \pi\left(\frac{d}{2}\right)^2 L$$

and the values of $M = 9936$ and $\bar{v} = 0.69$ cm^3/g, the length and diameter are calculated to be 139 and 10.3 Å, respectively. If the associated water is less than the amount assumed, the apparent asymmetry will be greater. For example, if $\delta = 0.5$ g of solvent/g of solute, the length and diameter of the cylinder would be 178 and 9.1 Å, respectively.

While the precise dimensions of helical α1-CB2 calculated above are subject to many uncertainties, the renatured peptide unquestionably chromatographs as a highly asymmetric molecule. Its putative structure may be compared to an ideal triple helix of α1-CB2 based on the known structure of collagen. The glycyl residues in adjacent chains in collagen are displaced by one residue along the axis of the molecule (Ramachandran, 1967). A perfect alignment of three parallel α1-CB2 chains (maximal helix) would therefore have two residues in one chain and one residue in another chain protruding at each end of the triple-stranded helix, or a total length of 39 residues. Introducing the residue spacing of 2.9 Å along the axis of a collagen helix (Traub *et al.*, 1969), and assuming the same value for the protruding residues, one obtains a total length of 113 Å. The corresponding average diameter is 11.4 Å. These dimensions for an ideal collagen-like structure are consistent with those derived from the chromatographic behavior of renatured α1-CB2.

References

Ackers, G. K. (1964), *Biochemistry 3*, 723.

Bornstein, P. (1967), *Biochemistry 6*, 3082.

Bornstein, P., and Piez, K. A. (1966), *Biochemistry 5*, 3460.

Carver, J. P., and Blout, E. R. (1967), *in* Treatise on Collagen, Vol. I, Ramachandran, G. N., Ed., London, Academic, p 441.

Chervenka, C. H. (1969), *in* A Manual of Methods for the Analytical Ultracentrifuge, Palo Alto, Calif., Beckman Instruments, p 64.

Fawcett, J. S., and Morris, C. J. O. R. (1966), *Separation Sci. 1*, 9.

Piez, K. A. (1968), *Anal. Biochem. 26*, 305.

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

Piez, K. A., and Sherman, M. R. (1970), *Biochemistry 9*, 4134.

Ramachandran, G. N. (1967), *in* Treatise in Collagen, Vol. I, Ramachandran, G. N., Ed., London, Academic, p 103.

Schachman, H. K. (1959), Ultracentrifugation in Biochemistry, New York, N. Y., Academic, p 239.

Scheraga, H. A., and Rupley, J. A. (1962), *Advan. Enzymol. 24*, 161.

Tanford, C. (1961), Physical Chemistry of Macromolecules, New York, N. Y., Wiley, Chapter 6.

Tiffany, M. L., and Krimm, S. (1969), *Biopolymers 8*, 347.

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

von Hippel, P. H., and Scleich, T. (1969), *in* Structure and Stability of Biological Macromolecules, Timasheff, S. N., and Fasman, G. D., Ed., New York, N. Y., Marcel-Dekker, p 417.