The Disordered and β Conformations of Silk Fibroin in Solution*

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ABSTRACT: Silk fibroin in water lacks any secondary structure. Its optical rotatory dispersion and circular dichroism resemble those typical of a coiled structure. Several small Cotton effects appeared in the aromatic absorption bands. The molecule contracted in the presence of salts and expanded moderately in 8 M urea solution. The viscosity studies suggested that the molecule was much less extended than that of a polyion such as ionized poly-L-glutamic acid. Addition of more than 30% (v/v) dioxane or methanol to the solution of silk fibroin in water induced a coil-to-β transition (based on the amide I and V bands of the infrared spectra). The optical rotatory dispersion of the β form displayed a large peak at 205 mμ and two troughs at 229–230 and 190 mμ, and the corresponding circular dichroism showed a small negative band at 217–218 mμ and a large positive one at 197 mμ.

The transition was time dependent and its rate varied with the solvent used. The maximum β content, mostly of the cross-β type, was near 50% in all cases studied, although the magnitude of the Cotton effects depended on the solvent used and to some extent on the protein concentration. The b_0 of the Moffitt equation, based on data between 600 and 300 mμ, remained close to zero. With increasing protein concentration, where viscosity studies suggested extensive aggregation, the Moffitt equation could be applied only within a narrow range of wavelength (between 600 and 460 mμ). This resulted in a large positive b_0 up to +400 and a correspondingly large negative a_0.

A decade ago the oligomers of γ-benzyl-L-glutamate in poor solvents, such as chloroform, were found to exist as β aggregates, which are dextrorotatory in the visible region, as contrasted to unaggregated molecules, which are levorotatory (Yang and Doty, 1957). The use of strongly absorbing organic solvents, however, precludes any reliable measurements in the ultraviolet region, where the circular dichroic bands of the peptide chromophores are located. If we are to study β conformation in solution, we must find a polymer that can dissolve in aqueous or organic solvents of low absorbance. We chose silk fibroin because the fibers of this protein are known to form antiparallel pleated sheets (Marsh *et al.*, 1955). We hoped that the molecule would retain its β conformation (intramolecular or intermolecular) in solution under favorable conditions.

Studying the intermolecular β conformation of polypeptides and proteins in solution is difficult because the molecules tend to aggregate and precipitate because they form intermolecular hydrogen bonds. Indeed, silk fibroin in solution (the protein is dissolved in concentrated LiBr and the salt removed by dialysis against water) will re-form fibers if subjected to shear (Iizuka, 1965). However, we have shown that silk fibroin can exist in the β form without precipitation in mixed solvents of water and dioxane or methanol. This β form displays characteristic Cotton effects different from those of helical and coiled forms (Iizuka and Yang, 1966). At the same time, Sarkar (of this laboratory) and Doty (1966), Davidson *et al.* (1966), and Townend *et al.* (1966) worked on the β conformation of poly-L-lysine. It is gratifying to find that the conclusions drawn from studies of silk fibroin and of poly-L-lysine are much alike, thus providing a new means for studying the β conformation of proteins and polypeptides by using optical rotatory dispersion and circular dichroism.

In this study we will first present evidence that silk fibroin in aqueous media has a disordered conformation, but that the molecule is less extended than would be true of a random coil. In the second part we describe in more detail the coil-to-β conformation of silk fibroin in methanol–water and dioxane–water.

Experimental Procedures

Materials. Raw silk of *Bombyx mori L.* was degummed with a mixture of 0.025% $NaCO_3$ and 0.3% Marseilles soap solution at 100° for 1.5 hr, and then boiled with 0.025% Na_2CO_3 for another hour. It was washed thoroughly with distilled water and then with ethanol. The degummed silk can dissolve in 9.3 M LiBr at 37° after about 2 hr. After dialysis against distilled water for 4 or 5 days until the silver nitrate test showed that no bromide ion was left, the solution was clarified by spinning in a Sorvall centrifuge at 9000 rpm for about 30 min. (We could not filter the solution because fibers would form in the capillaries of the filter; Iizuka, 1966.) The concentration of the supernatant was determined spectrophotometrically, using $A^{1\%}_{1\,cm}$ 11.3 in 0.2 M

* From the Cardiovascular Research Institute and the Department of Biochemistry, University of California San Francisco Medical Center, San Francisco, California 94122. *Received January 25, 1968.* This work was aided by grants from the U. S. Public Health Service (GM-10880, K3-GM-3441, and HE-06285).

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‡ Part of this manuscript was completed while J. T. Y. was on leave at the University of Tokyo. Fellowship awardee of the Commonwealth Fund.

NaCl at 276 mμ (based on micro-Kjeldahl nitrogen analysis; N, 18.3). The molecular weight of silk fibroin established by the light-scattering method was about 300,000 (Iizuka, 1963).

All organic solvents used were of spectroquality grade and water was double distilled. Other chemicals were of reagent grade.

The stock solution of silk fibroin was mixed with appropriate solvents to the desired concentration and composition. The apparent pH of the solution was then adjusted to 7.3 unless stated otherwise, using a Radiometer pH-meter 25.

Methods. Optical rotatory dispersion was measured at 27° with a Cary 60 recording spectropolarimeter and the circular dichroism of the same solution in the same cell was measured at room temperature with a Jasco ORD/UV-5 recording spectropolarimeter with circular dichroism attachment. To cover the wavelength range of 600–186 mμ, we used cells of path lengths 100, 10.1, 0.1, and 0.05 mm. In the ultraviolet region the absorbance of the solution was always kept below 2 to avoid any possible artifacts. The optical rotatory dispersion was expressed in terms of the reduced mean residue rotation, $[m']$, which equals $(3/(n^2 + 2))(M_0/100)[\alpha]$, where the mean residue weight, M_0, is taken to be 78 and n is the refractive index of the solvent. The circular dichroism was expressed in terms of the mean residue ellipticity, $[\theta]$, which equals $3300(\epsilon_L - \epsilon_R)$, where the ϵ's are the mean residue absorptivity of the left and right circularly polarized components. Following current convention, we did not apply the Lorentz correction for the refractive index of the medium to the circular dichroism measurements. The dimension of both optical rotatory dispersion and circular dichroism is deg cm^2 dmole^{-1}.

Infrared spectra were measured with a Perkin-Elmer 21 in the wavelength range between 1750 and 1400 cm^{-1} for amides I and II and with a Perkin-Elmer 337 in the wavelength range between 800 and 400 cm^{-1} for amide V. For the measurements between 1750 and 1400 cm^{-1}, the stock solution of silk fibroin was first concentrated in a cellophane tube under a constant stream of air, dialyzed several times against D_2O, and then sandwiched between two polyethylene films, which were about 0.02–0.04 mm apart. (We did not use the cell supplied by the manufacturer because it was so difficult to fill with the highly viscous solution of silk fibroin.) For measurements between 800 and 400 cm^{-1}, the undeuterated solution was cast as a film on an AgCl plate, which was dried under vacuum.

Viscosities were measured in a Ubbelohde-type viscometer at 27°. The flow time for the solvents was always more than 90 sec.

I. The Disordered Conformation of Silk Fibroin in Aqueous Solution[1]

Results and Discussion

To characterize the conformation of silk fibroin in aqueous solution, we studied the effects of salts, urea, and changes in pH on the optical rotatory dispersion, circular dichroism, and viscosity.

Optical Rotatory Dispersion in the Visible Region. The visible rotatory dispersion of silk fibroin always obeys the one-term Drude equation and can also be fitted with the Moffit equation. The results are summarized in Table I and some representative experiments are shown in

TABLE I: Effects of Salts, Urea, and Changes in pH on the Optical Rotatory Dispersion of Silk Fibroin in Aqueous Solution.

	Drude[a]		Moffit[b]	
Solvent and pH	λ_c (mμ)	$-k \times 10^{-6}$ (mμ)^2	b_0	$-a_0$
Salt[c]				
None	212	9.4	0	210
NaCl, 0.20 M	211	8.9	0	200
0.50 M	212	8.5	0	190
1.00 M	208	8.6	+10	190
1.80 M	214	7.4	0	160
KF, 0.20 M	213	8.8	0	190
0.35 M	211	8.9	0	200
LiBr, 0.99 M	215	8.3	0	190
1.91 M	212	7.9	0	180
3.83 M	215	5.5	0	120
5.64 M	214	4.5	0	100
7.44 M	220	4.0	−10	90
Urea[c]				
4 M	210	10.9	+10	250
8 M	209	11.5	+10	260
pH (0.2 M NaCl)				
4.3	217	8.8	−10	200
7.3	211	8.9	0	200
11.2	211	9.7	0	220
12.2	204	10.0	+10	220
13.0	193	9.6	+30	210

[a] Drude equation $[\alpha] = k/(\lambda^2 - \lambda_c^2)$, $\lambda > 300$ mμ. [b] Moffit equation: $[m'] = a_0\lambda_0^2/(\lambda^2 - \lambda_0^2) + b_0\lambda_0^4/(\lambda^2 - \lambda_0^2)^2$, $\lambda_0 = 212$ mμ, $\lambda > 300$ mμ. [c] pH, 7.3.

Figure 1. The levorotations in all cases decreased with increasing concentrations of monovalent salts (NaCl, KF, and LiBr). Thus, the magnitude of k in the Drude equation dropped gradually and reduced to about one-half at the highest salt concentration used (7.4 M LiBr), whereas the dispersion constant, λ_c, remained unchanged with salt except in 7 or 8 M LiBr. The average λ_c was about 213 mμ. Thus, the b_0 of the Moffit equation (with λ_0 preset at 212 mμ) was close to zero in all cases, but the a_0 became less negative with the addition of salts. This behavior is like that of poly-L-glutamic acid in its ionized state (Iizuka and Yang, 1965).

[1] Presented in part at the 150th National Meeting of the American Chemical Society, Atlantic City, N. J., Sept 1965.

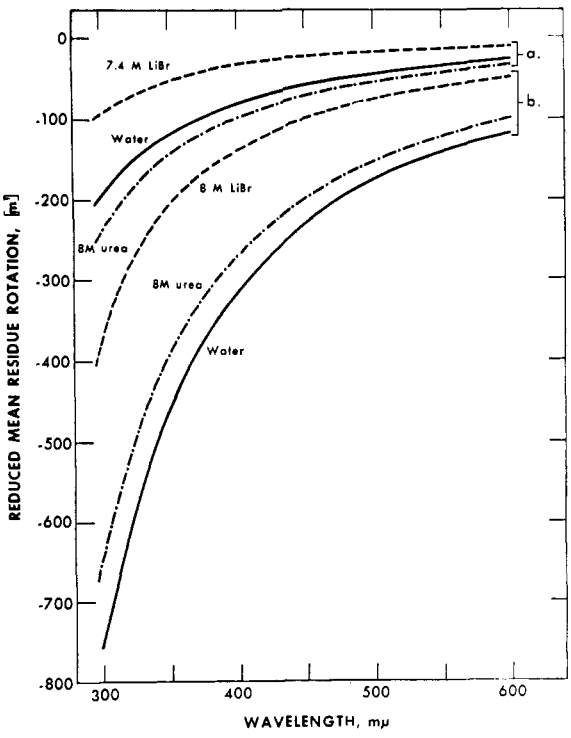

FIGURE 1: Visible rotatory dispersion of silk fibroin in aqueous solutions. The optical rotatory dispersion of the coiled form of poly-L-glutamic acid is included for comparison. Solvent, pH 7.3. (a) Silk fibroin and (b) poly-L-glutamic acid.

The effect of urea was the opposite of that of salts; levorotations increased with increased urea. This is commonly observed when proteins are denatured. Again, the λ_c was close to 213 mμ; thus, the b_0 was essentially zero. The effect of changes in pH was insignificant be-

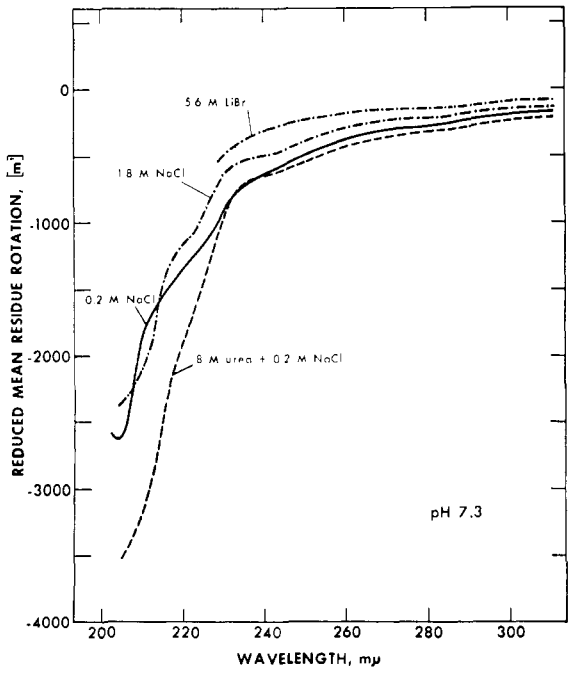

FIGURE 2: Ultraviolet rotatory dispersion of silk fibroin in aqueous solutions.

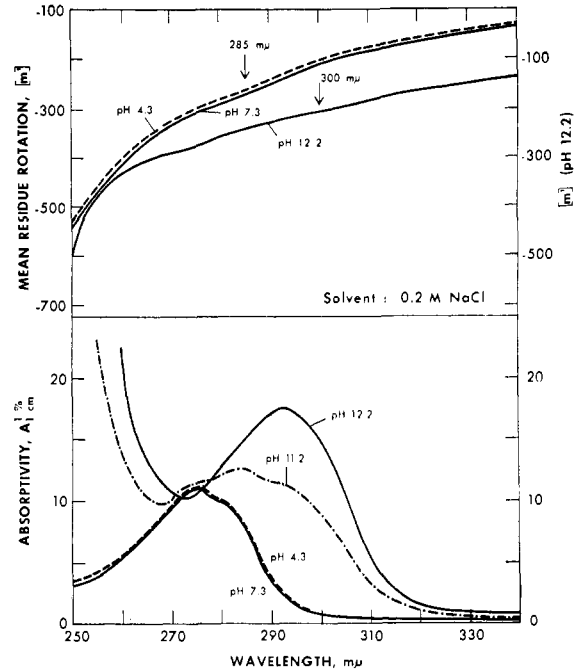

FIGURE 3: Ultraviolet rotatory dispersion and absorption spectrum of silk fibroin solution at different pH values.

tween pH 4.3 and 13, as is evidenced by the nearly constant λ_c and b_0 within this range of pH. These results strongly suggest that the silk fibroin molecules contract in the presence of salts and expand moderately in the presence of urea and that they lack α-helical structure.

When we varied the concentration of the protein from 0.1 to 3% (in the absence of salt or urea), we found that the b_0 remained near zero and that the λ_c was about 212 mμ. The change in a_0 and k was also very small; if anything, both became slightly less negative in high concentrations of protein. Thus, the contraction and expansion of the silk fibroin molecule was comparatively moderate, unlike the behavior of a typical polyelectrolyte, which expands drastically at extreme dilution.

Using the data for amino acid residues by Tanford (1967), we calculated the specific rotations of the disordered form of silk fibroin at three wavelengths: −47, −124, and −307 at 589, 400, and 300 mμ. The experimental rotations obtained in 8 M urea at pH 7.3 or in water at pH 7.3 (in parentheses) were less than the calculated ones: −36(−30), −98(−80), and −242(−197), respectively.

We also measured the optical rotatory dispersion and the infrared spectrum of silk fibroin taken directly from the middle section of matured silk glands, where it is stored prior to its conversion into fibers. The rotations were the same as those of the fibers after their dissolution in water, suggesting the precipitation and redissolution of the silk fibroin molecules do not affect the conformation.

Cotton Effects. The optical rotatory dispersion in the ultraviolet region is summarized in Figures 2–4. In the absorption bands of aromatic groups there appeared Cotton effects (Figure 2) so small that they might have been experimental errors. However, results of repeated

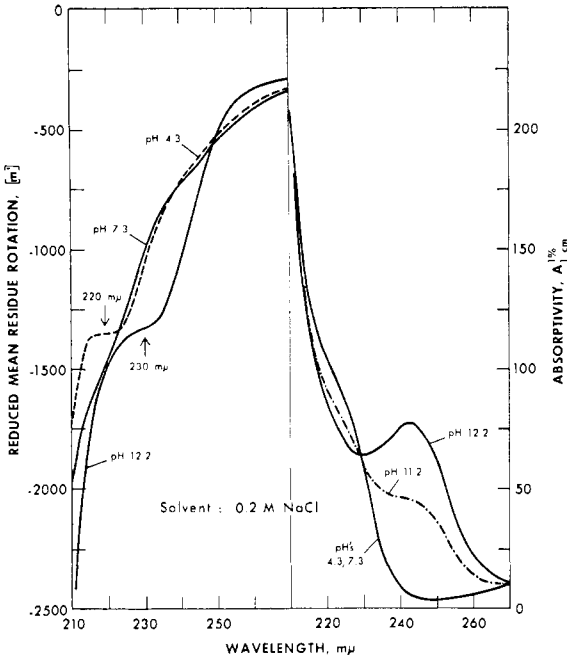

FIGURE 4: Ultraviolet rotatory dispersion and absorption spectrum of silk fibroin solution at different pH values (continuation of Figure 3).

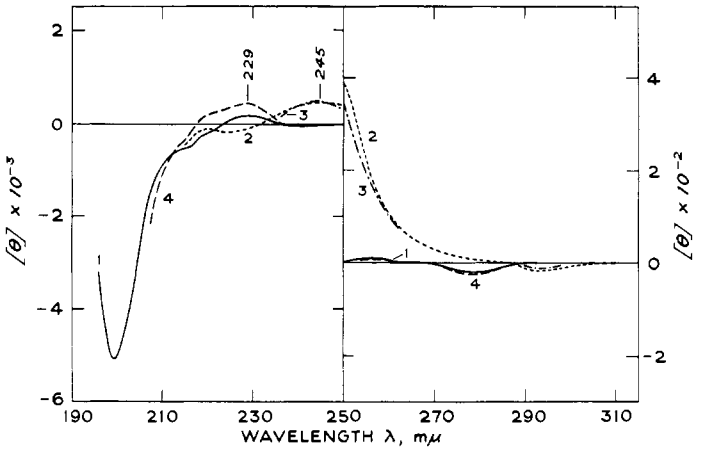

FIGURE 5: Circular dichroism of silk fibroin in aqueous solutions. Curves: 1, water (pH 7.3); 2, water (pH 12.2); 3, 7.4 M LiBr (pH 7.3); 4, 8 M urea.

experiments at various protein concentrations have convinced us that such wavelike profiles are genuine. Even in 8 M urea small inflection points could be detected near 280 and 240 mμ. The optical rotatory dispersion curve in 0.2 M NaCl (solid line in Figure 2) showed a trough near 205 mμ, which is characteristic of the coiled form. The curve obtained in water without salt also had a peak at 190 mμ (to be described in section II), again suggesting the presence of a coiled conformation. (This is further corroborated from the infrared spectrum for the amide V band; see section II.)

The optical rotatory dispersion curves for silk fibroin at pH 4.3 and 7.3 almost coincided (Figure 3). Both had an inflection point near 285 mμ that shifted to about 300 mμ when the pH of the solution was raised to 12.2. (See the circular dichroism spectrum to be described in Figure 5.) For comparison, the absorption maximum of the solution also shifted from 275 to 293 mμ upon raising the pH. The silk fibroin molecule contains about 5 mole % tyrosine residues, which could be responsible for these small Cotton effects. Ionization of the phenolic group of tyrosine residue above pH 12 results in change of both the ultraviolet spectrum and the Cotton effects.

The Cotton effects below 270 mμ were rather complex (Figure 4). In alkaline pH, there was an absorption maximum at 243 mμ; correspondingly, we found a shoulder in the optical rotatory dispersion near 260 mμ. In acid and neutral pH the absorption spectrum showed a shoulder near 225 mμ; correspondingly, there appeared an inflection point near 230 mμ. The dip in the optical rotatory dispersion near 220 mμ in neutral and acid pH shifted to about 230 mμ in alkaline pH, which again agreed with similar red shift in the absorption bands.

To further identify the Cotton effects below 300 mμ, we show in Figure 5 the circular dichroism of silk fibroin in neutral and alkaline pH and also in concentrated LiBr and urea solutions. There was a small negative circular dichroism band at 278 mμ and a positive one near 229 mμ at pH 7.3; these shifted to 293 and 245 mμ when pH was raised to 12.2. The circular dichroism extrema were very close to the absorption maxima shown in Figures 3 and 4. Both bands must be attributable to the aromatic groups of the protein. Figure 5 also shows several small circular dichroism bands, too small to be resolved. The large negative circular dichroism band at 199 mμ is characteristic of a coiled conformation. Because silk fibroin contains over 30 mole % of optically inactive glycine residues, its rotatory power should be smaller than that of most proteins in the disordered state. However, the 199-mμ band was only about one-third as large as that for the coiled form of poly-L-glutamic acid. The same is also evident for the visible rotatory dispersion (Figure 1).

The circular dichroism of silk fibroin in 7.4 M LiBr is nearly identical with its circular dichroism in water at pH 12.2. This might be due to the lowering of the pK value of the phenolic groups in a manner similar to that found for the carboxyl groups of acetic acids in high LiBr solutions (Iizuka and Yang, 1965). The circular dichroism in 8 M urea for the aromatic absorption bands resembled that in water at neutral pH. Although it was not possible to make measurements below 210 mμ, it seemed that the negative band below 200 mμ was larger than it had been in water alone.

Viscosity. Because silk fibroin in solution will so easily form fibrils under shearing stress, especially in pure water or at low ionic strength, it was difficult to obtain reproducible viscosity measurements even in the presence of 0.1 M NaCl. With 0.2 M salt or in urea solution, however, the data are reliable and the Huggins plot of reduced viscosity *vs.* concentration was linear. The intrinsic viscosity, [η], of silk fibroin in 0.2 M NaCl was 0.33 dl/g, decreased to 0.27 dl/g in 7.5 M LiBr, and increased to 0.63 dl/g in 8 M urea. These results are similar to those found in our early study of the coiled form of poly-L-glutamic acid (Iizuka and Yang, 1965).

A quantitative comparison between the two polymers

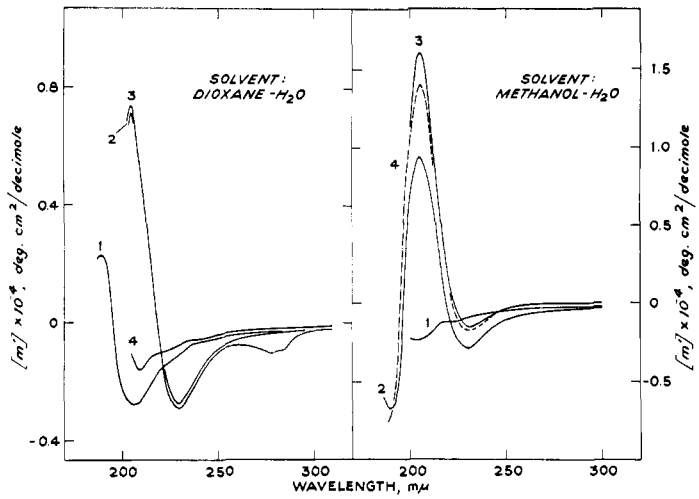

FIGURE 6: Ultraviolet rotatory dispersion of silk fibroin in dioxane–water and methanol–water mixtures. Protein concentration: 0.15 g/dl. Left, percentage dioxane (v/v): 1, none; 2, 50 (with one-tenth solute concentration); 3, 50; and 4, 50 (with water containing 0.2 M NaCl). Right, percentage methanol (v/v): 1, 10; 2, 50; 3, 93 (with one-tenth solute concentration); and 4, 93.

is informative. The intrinsic viscosity of the sodium salt of a poly-L-glutamic acid sample having a molecular weight of about 80,000 was about 1.7 dl/g at neutral pH and in 0.2 M NaCl. This was five times the value of silk fibroin under the same conditions and yet the protein molecule was about four times as large as the poly-L-glutamic acid sample. Likewise, the intrinsic viscosity of poly-L-glutamic acid in 8 M LiBr was only about one-fourth its value in 0.2 M NaCl, whereas that of silk fibroin was decreased only by 25% under these conditions. The gross structure of the silk fibroin molecule

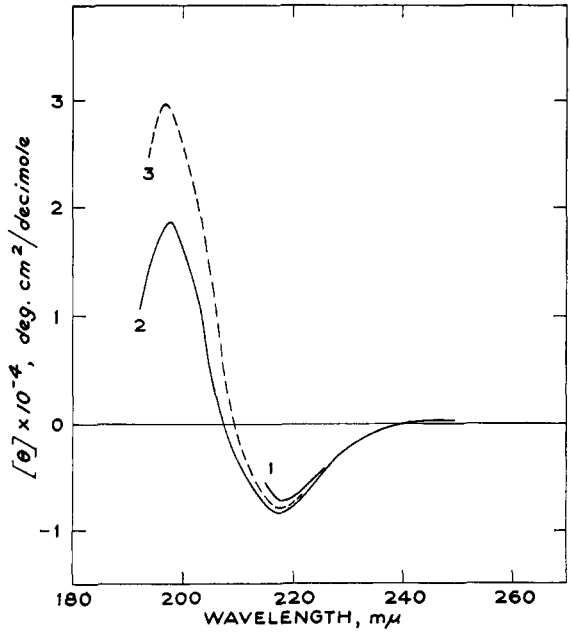

FIGURE 7: Circular dichroism of silk fibroin in dioxane–water and methanol–water mixtures. Curves 1, 0.013% protein in 35% dioxane; and 2 and 3, 0.15% protein in 30 and 93% methanol (v/v).

seems to be "compact" and does not allow the drastic contraction that occurs in poly-L-glutamic acid.

To summarize, the evidence strongly indicates a disordered conformation for silk fibroin in aqueous solution. The molecule behaves to some extent as a polyelectrolyte, that is, it contracts with the addition of salts. The optical rotatory dispersion and circular dichroism results clearly point to the lack of any α-helical structure. On the other hand, the molecule is not as extended as a synthetic polyion such as the sodium salt of poly-L-glutamic acid. It can expand moderately when exposed to concentrated urea solution. The fact that silk fibroin displays Cotton effects in the aromatic absorption bands albeit small, also indicates that the side groups such as tyrosine are not completely free to rotate and perhaps are held together in some way.

II. The Coil-to-β Form Transition of Silk Fibroin in Mixed Solvents[2]

Results

Although silk fibroin in water lacks any α-helical structure, drastic changes in the protein conformation result when dioxane or methanol is added to the aqueous solution.

Optical Rotatory Dispersion and Circular Dichroism. With more than 30% (v/v) of either organic solvent present, the 205-mμ trough typical of the optical rotatory dispersion for the coiled conformation disappears and is replaced by a peak at the same wavelength. In addition, the optical rotatory dispersion displays two troughs at 229–230 and 190 mμ; the position of the former is close to that for an α helix (Blout *et al.*, 1962). Likewise, the large negative circular dichroism band at 199 mμ for the coiled conformation is transformed into a large positive band at 197 mμ, and a new negative circular dichroism band appears at 217–218 mμ. This is not like the double minimum at 222 and 209–210 mμ characteristic of the helix (Holzwarth and Doty, 1965; Yang, 1967b). These new Cotton effects represent the β form, probably the antiparallel type (Iizuka and Yang, 1966; Sarkar and Doty, 1966; Davidson *et al.*, 1966; Townend *et al.*, 1966).

The coil-to-β transition of silk fibroin is time dependent; the rate is faster with higher percentage of dioxane or methanol. Figures 6 and 7 illustrate the optical rotatory dispersion and circular dichroism of silk fibroin in mixed solvents (after it has attained complete or almost complete transition). (Four of the eleven curves in the two figures were taken from the previous publication (Iizuka and Yang, 1966) and are included here for the sake of comparison.) The magnitude of the extrema in both figures varied according to whether methanol or dioxane was used, and with the percentage of methanol. (To some extent they also depended on the concentration of silk fibroin.) A case in point is the data in 50 and 93% methanol (Figure 6, right-hand side). Although the 205-

[2] Presented in part at the 50th Meeting of the Federation of the American Societies for Experimental Biology, Atlantic City, N. J., April 1966 (*Federation Proc. 25*, 411 (1966)).

mμ peak is much larger in 93% methanol, the corresponding trough at 229 mμ actually decreases with increasing methanol concentration. The amplitude, $[m']_{205} - [m']_{229}$, increases with the percentage of methanol. The same finding can be observed in the circular dichroism spectra (Figure 7). We do not attribute the variation with solvent composition to the difference in the β content, as we will show later that the β form is about 50% in each case.

In dioxane–0.2 M NaCl (in water) (1:1) silk fibroin displays Cotton effects strikingly different from those that occur in the absence of salt. Curve 4 in Figure 6 resembles that of the disordered form in water (curve 1). We found no change in rotation with time. Thus, the coil-to-β transition did not seem to occur in the presence of salt. On the other hand, once the β form is formed in dioxane–water (1:1), added salt did not break up the secondary structure. We also observed that silk fibroin in pure water was easily precipitated when the solution was mixed with dioxane–water containing NaCl.

The optical rotatory dispersion profile in the aromatic absorption region (Figure 6) is not smooth and appears to have several small Cotton effects, just as in the case of the disordered form (see Figure 2). The same is true for the circular dichroism spectrum, but these circular dichroism bands were too small to show in Figure 7. In 50% dioxane, silk fibroin (*c*, 0.13) did show pronounced Cotton effects around 280 mμ (Figure 6, curve 3), which we believe are due to strong intermolecular association, probably through tyrosyl–tyrosyl interaction. If the initial protein concentration was one-tenth of that used in curve 3, the large Cotton effects in this region no longer could be measured (Figure 6, curve 2).

That the transition from disordered form to β form is time dependent is illustrated in Figure 8. The $[m']_{229}$ of silk fibroin in 25% dioxane (curve 1, left side) was the same as that in pure water, indicating the absence of conformational change. In 30% dioxane, however, there appeared a latent period in which the magnitude of the 229-mμ trough rose very slowly. This was followed by a sharp rise to a plateau; the whole process took about 10–12 days. As the percentage of dioxane was further increased, the rate of transition became faster and the transition was completed in a few days. For silk fibroin in methanol–water mixtures, the magnitude of the trough increased slowly even in 25% methanol, and the transition was completed in a much shorter period than in dioxane–water mixtures. The rate of transition rose rapidly in 50% methanol, and the transition was completed in 1 or 2 hr for silk fibroin in 93% methanol. The $[m']_{229}$ at the plateau in this case depended strongly on the percentage of methanol used and to some extent on the protein concentration. The magnitude of the trough was larger in 50% methanol than in 25% methanol, but the trend was reversed with higher percentage of methanol. The magnitude in 93% methanol was actually smaller than either of the two other cases.

Figure 9 shows several optical rotatory dispersion curves in the visible region (after completion of transition) and Table II lists the rotatory parameters calculated from both the Drude and Moffit equations of all experiments of silk fibroin in methanol–water and

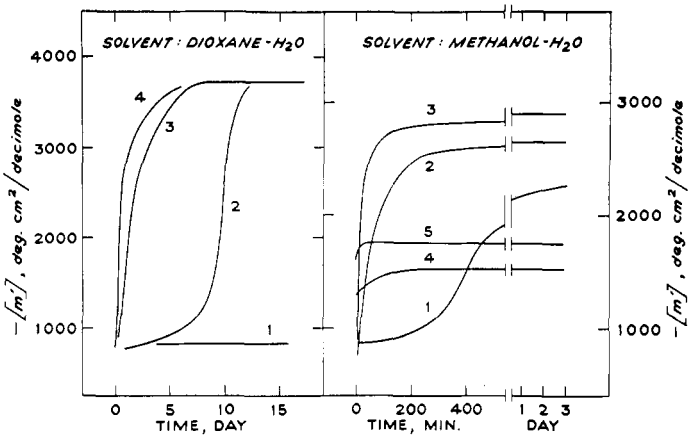

FIGURE 8: Time dependence of the optical rotation (at 229 mμ) of silk fibroin in mixed solvents. Left, percentage dioxane: 1, 25; 2, 30; 3, 35; and 4, 45 (protein concentration: 0.13%). Right: percentage methanol: 1, 25; 2 and 3, 50; and 4 and 5, 93 (protein concentration: 1, 3, and 5, 0.15%; and 2 and 4, 0.015%).

dioxane–water mixtures. The β form of silk fibroin seemed more levorotatory than that of the corresponding disordered form described in I, except in mixtures containing more than 60% methanol. For example, in 93% methanol (Figure 9, top half, curves 3 and 4) the levorotation of the protein was close to zero. Whereas the final $[m']_{400}$ in dioxane–water mixtures appeared to be close to, or slightly more negative than, that in pure water, the levorotation in methanol–water mixtures

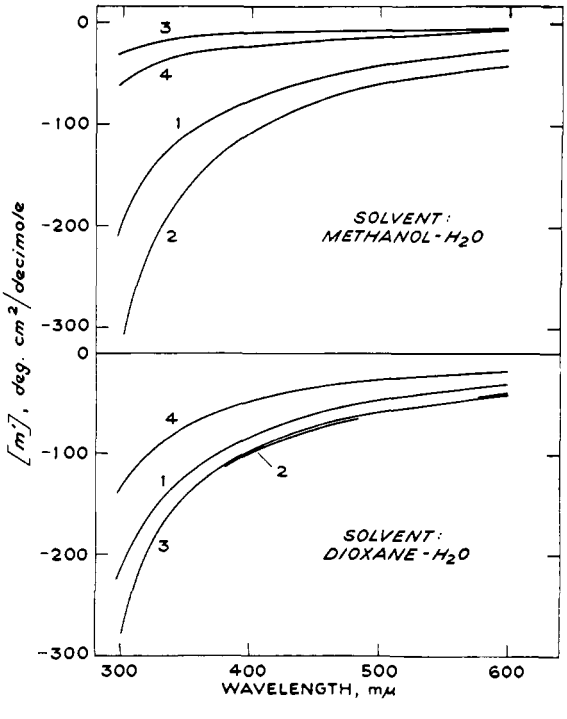

FIGURE 9: Visible rotatory dispersion of silk fibroin in mixed solvents. Upper, percentage methanol: 1, 10; 2, 50; and 3 and 4, 93 (protein concentration: 0.15%, except 3, 0.015%). Lower, percentage dioxane: 1, none; 2 and 3, 45; and 4, 50 (with water containing 0.2 M NaCl) (protein concentration: 0.13%, except 2, 0.013%).

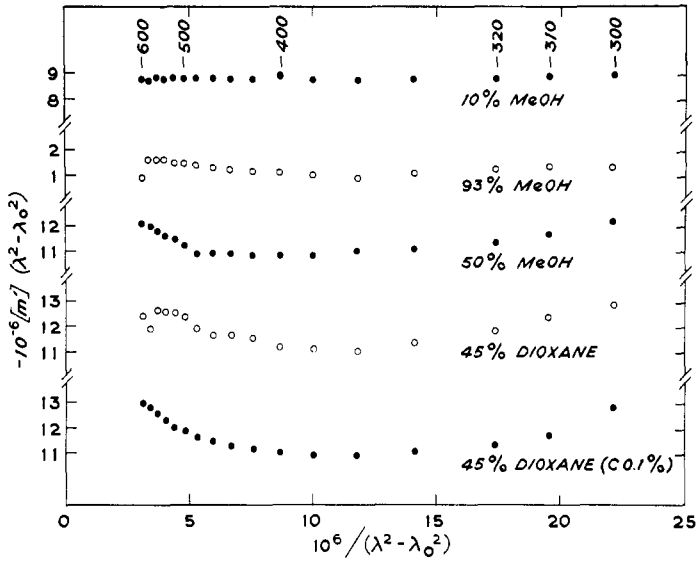

FIGURE 10: Effect of concentration on the linearity of the Moffit equation of silk fibroin in mixed solvents. Protein concentration (from top to bottom): 0.15, 0.015, 0.015, 0.013, and 0.13%.

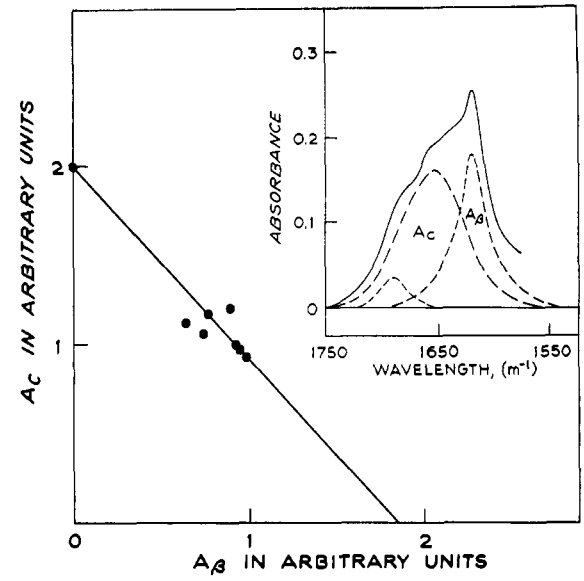

FIGURE 11: Analysis of the amide I infrared band of silk fibroin in dioxane–D_2O (1:1 v/v) mixture. Insert: graphical resolution of three bands for the coiled (A_c) and β (A_β) forms.

first increased with the percentage of methanol up to about 60%, but the trend was reversed when methanol was more than 80%, and dropped (in magnitude) almost to zero in 93%, for instance.

Perhaps more illustrative is the comparison of the Drude and Moffit parameters in various mixed solvents. The visible rotatory dispersion can be fitted with either equation between 600 and 300 mμ, provided the molecules do not aggregate extensively. The b_0 of the Moffit equation in these cases was always close to zero. On the other hand, extensive aggregation, as in the case of 0.1% silk fibroin in 45% dioxane, would usually make the Drude plot nonlinear over the same range of wavelength. Neither could the Moffit equation be applied down to 300 mμ, as illustrated in Figure 10. If a straight line was drawn through the data on the longer wavelength side, say between 600 and 460 mμ, the b_0 invariably became a large positive number varying between 100 and 400 (see Table II). If the solution was diluted to one-tenth its original protein concentration, *i.e.*, about 0.01%, however, the range of wavelength to which the Moffit equation could be applied immediately increased, and the b_0 again dropped to less than +100 and sometimes only +50. This change in b_0 with protein concentration was not accompanied by any significant change in the Cotton effects due to the β form, thus indicating that disaggregation did not destroy its characteristic Cotton effects. Whereas silk fibroin molecules are easily aggregated in dioxane–water mixtures used in this study, the same was true only for 40–60% methanol solutions. Above 80% methanol, the aggregation was less serious, but even here, the use of more concentrated protein solutions, *e.g.*, 0.76% in Table II, resulted in a large b_0, suggesting again an extensive aggregation.

Infrared Spectra. In D_2O the amide I band of deuterated silk fibroin appeared at 1650 cm^{-1} and the amide II band at 1450 cm^{-1} (see Iizuka and Yang, 1966, for the spectra). In dioxane–D_2O (1:1), however, there was a band at 1620 cm^{-1} due to the formation of the β structure. There was also a shoulder around 1690 cm^{-1}, suggesting that the β form is of the antiparallel type (Miyazawa and Blout, 1961). Further evidence came from the study of the amide V band. The film cast from silk fibroin in water showed a broad band at 640 cm^{-1} typical of the coiled form (Miyazawa *et al.*, 1962) and that from dioxane–H_2O (1:1) revealed a new band at 690 cm^{-1}, which Miyazawa *et al.* have assigned to the β form. In either case, no band near 610 cm^{-1} typical of the helix could be identified or at least the amount of helices, if present, was too small to be detected.

To estimate the β content in silk fibroin, we attempted to resolve the infrared spectrum into three bands, one centered near 1650 cm^{-1} representing the coiled form, A_c, and two near 1620 and 1690 cm^{-1} the β form, A_β (Figure 11). Following the procedure of Wada *et al.* (1961) in their study of the β form of oligomers of γ-benzyl-L-glutamate, we plotted the relative areas of A_c *vs.* A_β after normalization for samples in 50% dioxane at various stages of the coil-to-β transition. The maximum β content for silk fibroin was estimated to be about 50%.

Most recently, E. Iizuka (to be published) studied the oriented film of silk fibroin cast from the mixed solvents of dioxane or methanol and water. (To strengthen the film, one to three parts of polyvinyl alcohol was added to one part of solution prior to casting. The film was otherwise too brittle to be stripped off the plate. Polyvinyl alcohol, however, does not absorb strongly in the region of amide I and II bands and therefore does not interfere with the spectrum of interest.) The strong parallel dichroism of the amide I band and perpendicular dichroism of the amide II band suggested the conformation was of the intramolecular cross-β type. This was further supported by a perpendicular dichroism at 1690

TABLE II: Effects of Changes in Solvent on the Optical Rotatory Dispersion of Silk Fibroin at pH (apparent) 7.3.

Solvent (%)[a]	Drude		Moffit[b]		
	λ_c (mμ)	$-k \times 10^{-6}$ (mμ²)	b_0	$-a_0$	$[m']_{100}$
Dioxane in water					
None	212	9.4	0	210	84
35 l	187	14.4	+50	310	113
h	Nonlinear		+360	360	111
45 l	182	12.8	+70	280	97
h	Nonlinear		+250	320	96
50 l	Nonlinear		+90	260	85
Dioxane (in 0.2 M NaCl)					
30 l	217	7.1	−10	160	63
50 h	227	5.2	−20	110	84
Methanol in water					
10 h	214	8.7	0	200	78
20 h	240	9.6	−60	200	90
30 h	236	10.2	−60	230	100
40 h					111
50 l					94
h					109
60 h					106
80 l	147	5.7	+10	130	48
h			+60	220	77
c, 0.39	170	12.2	+70	270	93
c, 0.76			+410	490	133
93 l	132	1.5	+30	40	9
h			+60	120	39

[a] The symbols l and h refer to solutions containing 0.013–0.015 and 0.13–0.15% protein, respectively. [b] See text for the range of wavelength used.

cm^{-1}. In contrast, when the disordered form of silk fibroin in aqueous solution was cast into the oriented film, the sense of dichroism of the amide I and II bands was just the opposite to that of the intramolecular cross-β, implying that in this case the β form was of the intermolecular type.

Viscosity. To further determine the effect of aggregation on the β conformation of silk fibroin, we followed the time dependence of both viscosity and rotation of silk fibroin in 50% dioxane. The obvious feature in Figure 12 is that the change in viscosity does not parallel that of rotation. In dilute solution (*c*, 0.013), the levorotation at 229 mμ rose gradually toward a plateau, whereas the corresponding viscosity changed little with time. At higher concentration (0.12%) the situation was just the reverse; the viscosity increased sharply even after $[m']_{229}$ leveled off. Thus, the β form of silk fibroin was extensively aggregated with increasing concentration. This is also consistent with the appearance of large Cotton effects near 280 mμ (Figure 6, curve 3), which suggested some strong tyrosyl–tyrosyl interaction, probably of the intermolecular type. It is clear from Figure 12 that the observed Cotton effects of the β form cannot be entirely attributed to intermolecular hydrogen bonding. It strongly suggests the presence of a intramolecular-type bond in silk fibroin, although aggregation does affect the final magnitude of the Cotton effects.

Discussion

We have already suggested (Iizuka and Yang, 1966) that additional Cotton effects of the β form may exist below 190 mμ, where we cannot measure them. Our reason for this suggestion is that the circular dichroism bands of the β form (Figure 7) would produce a dextrorotation in the visible region. Our experiments showed that the visible rotatory dispersion in most cases was more negative in dioxane–water and methanol–water mixtures than it was in pure water, unless the methanol content was more than 80%. To further illustrate this point, we compare the experimental optical rotatory dispersion of silk fibroin in 50 and 93% methanol with that calculated from the corresponding circular dichroism spectrum using the Kronig–Kramers transform (Moscowitz, 1960) and assuming a Gaussian form for each band. Figure 13 shows that the experimental values above 300 mμ indicate that there is less dextrorotation

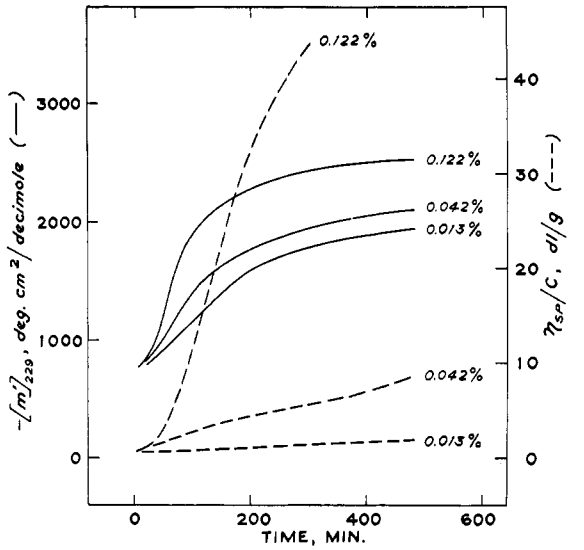

FIGURE 12: Time dependence of the reduced viscosity and optical rotation of silk fibroin in dioxane–water mixture (1:1, v/v) at 27°. The numbers near the curves represent the protein concentration.

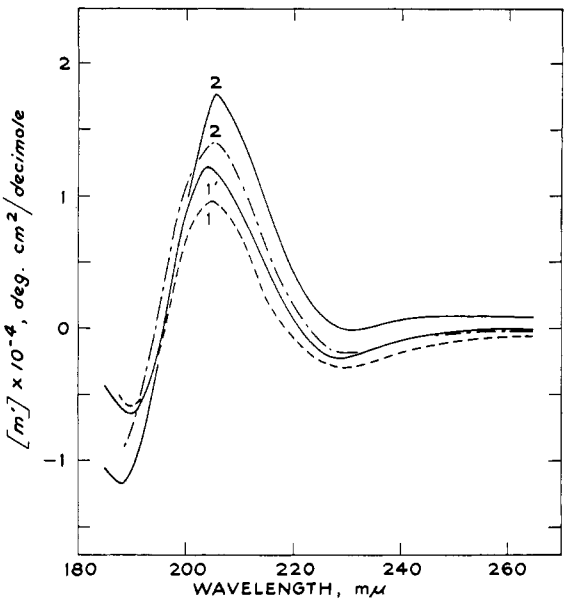

FIGURE 13: Comparison of the experimental and calculated Cotton effects of silk fibroin in methanol–water mixture. Curves 1 and 1′: 50% methanol; curves 2 and 2′: 93% methanol. Broken line: experimental; solid line: calculated from the circular dichroism bands. Protein concentration: 0.15%.

(or more levorotation) than such calculations would indicate. (Below 200 mμ, the experimental measurements were less precise because of the limits of the instrument.) Note that the discrepancy at the 205-mμ peak could be as large as 10^4 deg cm²/dmole. Since silk fibroin contains at most 50% β form, the difference between experimental and calculated rotations would even be larger for 100% β form. Part of this difference might be attributed to background rotations, but background could not reach four orders of magnitude. Thus, we believe that the β form may contain more than the two circular dichroism bands we can now measure.

Using the estimates of the β content given in Figure 11, we can extrapolate to estimate the peak and trough values of 100% β form, which would at least provide an order of magnitude. The average values of $[m']_{229}$ and $[m']_{205}$ for silk fibroin in 50% dioxane, 50% methanol, and 93% methanol were approximately −6000 and +20,000, −5000 and +24,000, and −3000 and +27,000, respectively. While the first set of values was close to that of −6000 and +22,000 reported by Sarkar and Doty (1966), and to the −6000 and +29,000 reported by Davidson *et al.* (1966) for the β form of poly-L-lysine, such agreement is very likely fortuitous. Figure 6 shows that the magnitude of the peak and trough of the β form varied with solvents used, even though the maximum β content in all cases seemed to be near 50% from infrared analysis. Thus the reference values for the β form in 50% dioxane may very well differ from those obtained for poly-L-lysine in pure water. Furthermore, the above estimates were based on data in 0.1% silk fibroin solution, where molecular aggregation was more pronounced. In view of the findings in Figure 12, the extrapolated values for 100% β form could differ when more dilute protein solutions are used. Whether the reference values of the β form of poly-L-lysine are modified by molecular aggregation is not yet reported. The situation is even more complicated than had been expected, as Fasman and Potter (1967) have now reported the existence of two types of optical rotatory dispersion for the β structures. In the case of poly-*S*-carboxymethyl-L-cysteine in water, the largest values obtained for the β form actually had a positive trough with $[m']_{242} = +480$ and a peak of $[m']_{210} = +23{,}000$ (Ikeda and Fasman, 1967). All these findings must be reconciled and clarified before one attempts to calculate the amount of β content in protein molecules.

In spite of its uncertainties and limitations, the b_0 of the Moffit equation has been widely used in the estimation of helical content of proteins. The presence of β form may complicate such interpretation (see Yang, 1967a). Imahori (1960) was the first to find that the visible rotatory dispersion of the β form can be fitted with the Moffit equation. We have, however, shown that the β form of silk fibroin has a b_0 close to zero, but b_0 turns positive upon extensive aggregation. In many of these cases the Moffit equation can no longer be applied, and the positive b_0 was obtained from the data near 600 mμ over a rather narrow range of wavelength. Several proteins such as γ-globulin, which are suspected to contain partial β form and no α helix, also had a b_0 close to zero. Available evidence seems to suggest that the β form in proteins has a zero or close to zero b_0 of the Moffit equation, which becomes positive only when the molecules aggregate extensively. (However, the b_0 of the β form of poly-L-lysine varied from −150 (Sarkar and Doty, 1966) to −240 (Davidson *et al.*, 1966) and needs further investigation.) A zero b_0^β obviously will not affect the estimation of helical content in a protein molecule. On the other hand, a positive b_0^β would underestimate and a negative one overestimate the helical content if both secondary structures exist in the protein mol-

ecule. We have, however, pointed out that the contribution of the helix usually overshadows that of the β form on the basis of current experimental b_0^{β}'s in the literature (Iizuka and Yang, 1966). The same is true in the use of the 233-mμ trough method for estimating the helical content in a protein molecule. In view of the present uncertainty of the reference values for the β form, it also seems premature to attempt any calculations of a combination of the helical, β, and disordered conformations in a protein molecule.

The a_0 of the Moffit equation for silk fibroin in dioxane–water and methanol–water mixtures was usually either close to that or less negative than that found for the disordered form in pure water; the magnitude of a_0 was especially small in 93% methanol. On the other hand, extensive aggregation of the molecules, which resulted in a large positive b_0, also produced a large negative a_0. This finding differs from what has been observed for other β polymers, which are believed to give a positive a_0^{β}. These differences must be resolved or clarified and reconciled when more experimental data on the β form of proteins and polypeptides are accumulated.

While the β form of silk fibroin is shown to be intramolecular, that of poly-L-lysine is still uncertain. Basing their opinion of the results of sucrose gradient centrifugation, Sarkar and Doty (1967) concluded that it was the intramolecular type. Davidson and Fasman (1967), on the other hand, believed that the β structure was favored by an intermolecular association, since the rate of formation of the β structure produced by heating was concentration dependent. The fact that the recovered radioactivity in the β sample used by Sarkar and Doty appeared to be only about 25% of that recovered from the coiled form of poly-L-lysine after centrifugation seemed to imply a loss of β aggregates over a long period of centrifugation according to Davidson and Fasman (1967). The β form of poly-L-lysine did precipitate readily if the concentration of polymer was moderate; even at the 0.01% concentration used by both laboratories, slow precipitation would occur. Clearly, aggregation accompanied the α-to-β transition of poly-L-lysine. However, it is difficult to conclude from the available experiments whether the influence of the intramolecular-type β form on the optical rotation in poly-L-lysine would be more than that of aggregation or whether the aggregates are of the intermolecular β form.

On the basis of infrared spectra we have shown that the maximum amount of the β form for silk fibroin in solution does not exceed 50%. Note that the crystallinity of silk thread is also less than 50% (Iizuka, 1965). This must be related to the amino acid composition and sequence of the protein, even though silk fibroin adopts the intermolecular β form in the thread and the intramolecular β form in solution. Why the silk fibroin molecule favors an intramolecular β structure and not an α helix in the mixed solvents is difficult to answer. The main feature of the chemical composition of *Bombyx mori L.* silk fibroin is its high content of glycine (43 mole %), alanine (31%), and serine (10%) residues. It is known that a large portion of the protein molecule has a sequence of Gly-X-Gly-X..., where X is Ala or Ser. Poly-L-alanine favors the formation of α helix, but poly-L-serine exists in the β form in solution. The X-ray studies indicate that polyglycine I cannot form the α helix but has the β structure, whereas polyglycine II has a structure similar to poly-L-proline II but different from either α helix or β form. We can only speculate that alternating sequence of glycine and alanine (or serine) favors the β form rather than the α helix in solution as well as in solid state.

Acknowledgments

The authors thank Professors J. C. Craig and L. A. Strait for the use of the Jasco ORD/UV-5 spectropolarimeter with circular dichroism attachment and Perkin-Elmer Model 337 spectrophotometer, and Mr. T. Hara of the Sericultural Experimental Station of Japan for the samples of degummed silk. J. T. Yang is indebted to Professor K. Imahori for his valuable discussions and his hospitality.

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Functional Properties of a Hemoglobin Carrying Heme Only on α Chains*

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ABSTRACT: The functional properties of a hemoglobin carrying heme only on the α chains ($\alpha^h\beta$) are described. The protein shows nearly hyperbolic oxygen equilibrium curves with three to ten times higher oxygen affinity than normal hemoglobin, depending on pH. The Bohr effect is present, but is about one-half that of normal hemoglobin. The velocity constant of the reaction between $\alpha^h\beta$ and CO is much higher than in hemoglobin (initial second-order rate constant, $l' \simeq 2 \times 10^6$ M^{-1} sec^{-1}); the same kinetic pattern is obtained in rapid mixing and flash photolysis experiments. Addition of the full heme complement to the $\alpha^h\beta$ compound gives a product with all the properties of natural hemoglobin both in respect to the O_2 equilibrium and to the kinetics of reaction with CO. The functional properties of the $\alpha^h\beta$ compound are different from those of isolated α chains, which indicates that the reactivity of the heme in a given chain is modified by interaction with the partner chain, even if the latter is devoid of prosthetic group.

The existence of compounds resulting from incomplete reconstitution of hemoglobin from globin and heme has been suspected for several years (Winterhalter and Huehns, 1964; Gibson and Antonini, 1966). A compound of this type has been isolated by two groups of investigators (Winterhalter, 1966; Banerjee and Cassoly, 1967). The compounds obtained by the two groups are similar to each other (Banerjee and Cassoly, 1967; Winterhalter and Deranleau, 1967) in many respects. Thus both groups found that the partially saturated compound had, at protein concentrations above 10 mg/ml, a molecular weight corresponding to the hemoglobin tetramer, that it has only two hemes per four polypeptide chains, both attached to the α chains, with the two specific binding sites for heme on the β chains unoccupied. The present paper reports observations on the equilibria and kinetics of the reaction of the partially reconstituted compound obtained by Winterhalter with O_2 and CO, before and after its conversion into hemoglobin by the addition of 2 moles of heme/mole of protein. It may be anticipated that the partially reconstituted compound shows marked changes in functional behavior as compared with hemoglobin. The significance of these results is strengthened by the finding that the addition of heme converts it into functionally normal hemoglobin.

Material and Methods

All chemicals used were of the highest purity available. The compound carrying the heme only on the α chains was obtained by the method previously described (Winterhalter, 1966). The compound referred to before as ICII will be designated here as $\alpha^h\beta$;[1] this does not imply any statement in regard to the actual particle size of the compound (which at protein concentrations above 1% has a molecular weight similar to that of hemoglobin; Winterhalter and Deranleau, 1967). $\alpha^h\beta$ was converted into the compound fully saturated with heme (hemoglobin) by the addition of excess hemin dissolved in potassium phosphate buffer containing 100 mg of KCN/l. (pH 7.5). Cyanide ferric heme in excess over the stoichiometric amount of one per chain was removed by chromatography on a DEAE column (15 × 100 mm) equilibrated with a 0.1 M phosphate buffer (pH 7.0). Elution was carried out with the same buffer. In one experiment $\alpha^h\beta$ was converted into hemoglobin by adding stoichiometric amounts (in re-

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[1] The superscript h denoting one molecule of heme