The Influence of Long-Range Interactions on the Structure of Myoglobin*

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ABSTRACT: The optical rotatory dispersion and circular dichroism of the three isolated cyanogen bromide peptides of apomyoglobin have been examined. From these data, it appears that the helix contents of the peptides in water are markedly lower than they are in the parent protein, although these peptides do assume conformations of higher helix content in 95% aqueous methanol. Sedimentation studies indicate that the N-terminal peptide is aggregated in water, but the other two are not. The results for the two unaggregated peptides suggest that long-range interhelical interactions may play an important role in determining protein conformation. In addition, there may be a connection between the tendency of the N-terminal peptide to aggregate and its possible role in initiating protein folding.

It is generally agreed that the amino acid sequence of a protein determines its conformation in a given solvent, as demonstrated for several proteins such as bovine pancreatic ribonuclease (Anfinsen, 1964) and sperm whale myoglobin (Harrison and Blout, 1965). It is of interest to determine whether the influence of the amino acid sequence on protein conformation is primarily a short-range one, or whether it also involves important contributions from long-range interactions (*i.e.*, between amino acid residues quite far apart in the sequence). In order to obtain experimental evidence on this question, the conformation of apomyoglobin (produced by removal of the heme group from sperm whale myoglobin) has been compared with those of the three large peptides produced by the reaction of cyanogen bromide (Gross and Witkop, 1962) with the two methionine residues of sperm whale myoglobin (Edmundson, 1963).

Myoglobin is largely α helical, both in the crystalline state (Kendrew *et al.*, 1961) and in solution (Beychok and Blout, 1961; Urnes *et al.*, 1961; Urnes, 1963; Samejima and Yang, 1964; Holzwarth and Doty, 1965). Apomyoglobin is also largely α helical in solution (Harrison and Blout, 1965; Breslow *et al.*, 1965). Thus, an examination of the rotatory properties of apomyoglobin, and its three cyanogen bromide peptides, should provide information as to whether the long-range interhelical interactions, present in apomyoglobin, are required in order that the three isolated peptides retain the same degree of α-helical conformation as they possess in the parent protein. In addition, such a study could provide information as to how a protein folds as it is being synthesized, *i.e.*, as to whether an incompletely synthesized protein adopts the conformation of the native molecule.

Experimental Procedure

Reagents. Sperm whale myoglobin was purchased from Calbiochem Co. (lot 71723, purity >98%, salt free). All other reagents were analytical grade, unless otherwise specified.

Preparation of Apomyoglobin. Heme was removed from myoglobin by a modification (Breslow, 1964) of the 2-butanone extraction procedure of Teale (1959).

Preparation of Cyanogen Bromide Peptides. Apomyoglobin was reacted with cyanogen bromide, and the resulting peptides were separated on columns of Sephadex G-75 as described by Edmundson (1963). The effluents were lyophilized and rechromatographed several times on Sephadex G-75 until good separations and good amino acid analyses were obtained. The amino acid analyses of these peptides agreed well with those previously reported (Edmundson, 1963, 1965) (see Table I). Peptide 1 is the C-terminal one containing 22 amino acids, peptide 2 contains 76 amino acids, and peptide 3 is the N-terminal one containing 55 amino acids. The amino acid sequence of these peptides is given by Edmundson (1965).

Amino Acid Analyses. Samples were hydrolyzed at 110° for 22 hr in sealed, evacuated ampoules containing 6 N HCl. The resulting hydrolysate was separated on an automated Technicon amino acid analyzer.

Preparation of Solutions. Solid samples were weighed and dissolved in water to give a pH of about 5.5, measured on a Radiometer pH meter equipped with a scale expander and a Radiometer type GK2021B combined electrode. The instrument was standardized with commercially prepared buffers (Fisher Scientific Co.) having a pH within 0.5 pH unit of that of the sample. The pH was raised to 7 or 9.2 by addition of a dilute solution of sodium hydroxide or sodium borate. Solutions containing methanol were made by first dissolving the solid samples in water and then adding methanol. Optical

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TABLE I: Amino Acid Analysis of Cyanogen Bromide Peptides from Apomyoglobin.

	Peptide 1		Peptide 2		Peptide 3	
	Expt	Theory	Expt	Theory	Expt	Theory
Aspartic acid	1.9	2	2.9	3	2.9	3
Threonine			2.8	3	1.9	2
Serine			3.6	4	2.3	2
Glutamic acid	3.0[a]	3	7.5	7	8.0[b]	9
Proline			3.1	3	1.0	1
Glycine	2.1	2	6.0[a]	6	3.0[a]	3
Alanine	3.3	3	10.0	10	4.6	4
Valine			3.0	3	3.1[b]	5
Isoleucine	1.0	1	4.2[b]	6	2.3	2
Leucine	3.0	3	8.5	8	6.7	7
Tyrosine	1.8	2	1.1	1		
Phenylalanine	1.1	1	2.1	2	3.1	3
Lysine	3.5	4	8.7	10	4.7	5
Histidine			6.4	8	3.7	4
Arginine	1.1	1	1.2	1	2.0	2
Homoserine lactone			0.7[b]	1	0.7[b]	1
Tryptophan					[c]	2
Ammonia	1.7	2	3.0	3	2.6	2

[a] Theoretical number assumed. [b] These values were also found to be lower by Edmundson (1963), probably due to incomplete hydrolysis and side reactions. [c] Not determined.

rotation and circular dichroism measurements were made on solutions in the concentration range of 0.01–0.1%.

Concentration Determination. The concentrations of peptide and protein solutions were determined from their nitrogen content using the simplified Kjeldahl procedure of Lang (1958).

Spectropolarimetry. The optical rotatory dispersion was measured using a Cary Model 60 spectropolarimeter. Water-jacketed quartz cells, with path lengths of 0.1, 1.0, and 2.5 cm, were used. The temperature of the sample was controlled with a Haake type F circulating bath. Solvent base lines were recorded for all sample runs at the corresponding temperatures.

Optical rotatory dispersion data in the ultraviolet region are reported as the reduced mean residue rotation at wavelength, λ, *i.e.*, as

$$[m']_\lambda = \left[\frac{3}{n_\lambda^2 + 2}\right]\left[\frac{\alpha_\lambda M}{cl}\right] \qquad (1)$$

where n_λ is the refractive index of the solvent and α_λ is the observed rotation at wavelength λ; M is the mean residue molecular weight calculated from the amino acid composition of the cyanogen bromide peptides as given by Edmundson (1965), replacing methionine with homoserine, c is the concentration of peptide in units of g/100 cm^3; and l is the path length in decimeters. The reduced mean residue rotation was found to be independent of peptide concentration and path length.

Optical rotatory dispersion data in the range 300–600 mμ were used to calculate the parameters a_0 and b_0 from the intercept and slope of a plot of $[m']_\lambda(\lambda^2 - \lambda_0^2)/\lambda_0^2$ *vs.* $\lambda_0^2/(\lambda^2 - \lambda_0^2)$, with $\lambda_0 = 212$ mμ (Moffitt and Yang, 1956; Urnes and Doty, 1961).

The data for $\lambda^2 - \lambda_0^2$ and $(\lambda^2 - \lambda_0^2)^{-1}$ and for the dispersion of the refractive index of water were obtained from Fasman (1963). Values of n_λ for methanol were calculated from the molar refraction data at 18° (Partington, 1953) by means of the Lorentz equation. These values were corrected for temperature and composition changes by assuming that the change in refractive index with temperature or per cent alcohol at 589.3 mμ (International Critical Tables, 1930) is the same at all wavelengths.

Circular Dichroism. The circular dichroism was measured using a Cary Model no. 6001 attachment tot heir Model 60 spectropolarimeter. Cells of 1- and 2.5-cm path length were employed. Solvent base lines were recorded for all sample runs. Data are reported as the molar ellipticity at wavelength, λ.

$$[\theta]_\lambda = \frac{\theta_\lambda M}{cl} \qquad (2)$$

where θ_λ is the observed ellipticity.

Ultracentrifugal Studies. Sedimentation coefficients and weight-average molecular weights, $\bar{M}_w$, were measured at 23° with a Spinco Model E ultracentrifuge, as previously described (Epand and Scheraga, 1968). The partial specific volume was estimated from the amino acid composition using the procedure described by Cohn and Edsall (1943).

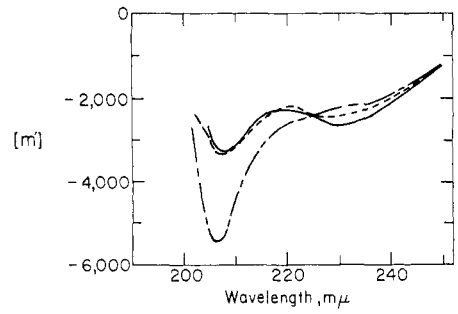

FIGURE 1: Optical rotatory dispersion of peptide 1 at 25°. — – — –, pH 5.1; - - - - -, pH 7.2; ———, pH 9.2.

Results

Using the spectrophotometric titration procedure of Breslow (1964), the apomyoglobin preparation used for reaction with cyanogen bromide was found to combine with 1.0 mole of hematin (Calbiochem lot 71860). The optical rotatory dispersion of this preparation agreed well with that of Breslow *et al.* (1965). In 0.1 N HCl (the solvent in which the reaction with CNBr was performed), apomyoglobin showed a 25% decrease in $[m']_{233}$ relative to its value in neutral solution; however, this change is reversible.

Figures 1–6 show the optical rotatory dispersion and circular dichroism of the cyanogen bromide peptides of myoglobin at 25° at several pH values.

The change in $[m']$ was less than 10% between 210 and 250 mμ when peptide 1 was heated from 7 to 50° and peptide 3 from 6 to 88°. Both peptide 2 and apomyoglobin showed an irreversible change on heating to 60° (Figure 7); no further change in the optical rotatory dispersion was noted on cooling and reheating.

The optical rotatory dispersion between 300 and 600 mμ was fit to the Moffitt–Yang equation, with $\lambda_0 = 212$ mμ. The resulting constants a_0 and b_0 were: −323 and −69, −322 and −133, and −351 and −89 for peptides 1, 2, and 3, respectively, at pH 9.2 (0.01 M sodium borate at 25°.

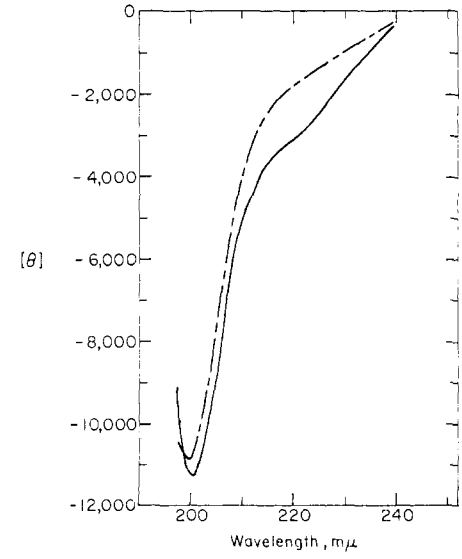

FIGURE 2: Circular dichroism of peptide 1 at room temperature. — – — –, pH 5.1; ———, pH 9.2.

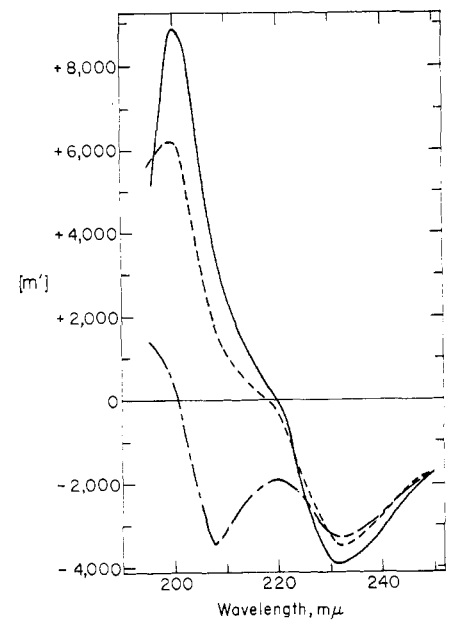

FIGURE 3: Optical rotatory dispersion of peptide 2 at 25°. — – — –, pH 5.8; - - - - -, pH 7.2; ———, pH 9.2.

Large and reversible changes in the optical rotatory dispersion resulted when methanol was added to the aqueous solutions of the peptides and apomyoglobin, the pH being ~5.5 before addition of methanol (Figure 8). The effect observed with peptide 1, ($[m']_{233}$ as a function of methanol concentration) is shown in Figure 9. At an intermediate point in the transition range (35% methanol), there is a marked effect of temperature on the optical rotatory dispersion (Figure 10).

When peptides 1, 2, and 3 were mixed at pH 9.2, 0.01

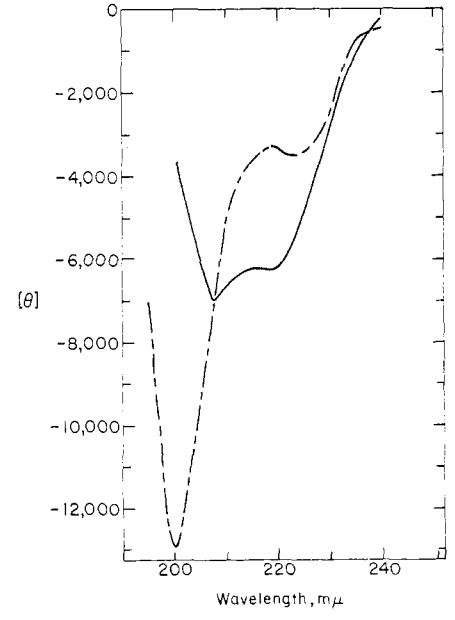

FIGURE 4: Circular dichroism of peptide 2 at room temperature. — – — –, pH 5.8; ———, pH 9.2.

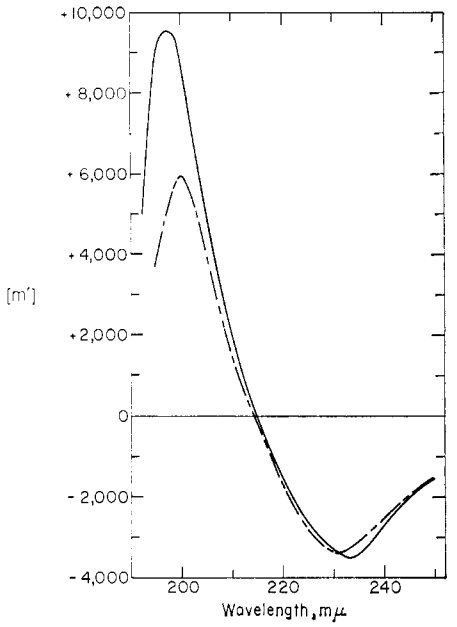

FIGURE 5: Optical rotatory dispersion of peptide 3 at 25°. — — — —, pH 5.5; ———, pH 9.2.

M sodium borate, in equimolar proportions, the resulting optical rotatory dispersion was simply the calculated sum of the curves for the individual peptides.

Ultracentrifuge studies indicate that the N-terminal peptide (peptide 3) forms aggregates in 0.01 M borate buffer (pH 9.2), 23°; two peaks, having sedimentation constants of 8.9 and 2.7 S at a concentration of 4 mg/ml, were observed in sedimentation velocity runs. The same results were obtained at a peptide concentration of 1.3 mg/ml, as well as at pH 5.5 in 0.1 M NaCl at 2 mg/ml. M. J. Crumpton (personal communication) also found S values of 9 and 3.2 for this peptide at a concentration of 12 mg/ml. (We wish to thank Dr. Crumpton for pointing out to us that the N-terminal peptide is aggregated.) Peptides 1 and 2 behave as low molecular weight monomeric species, each showing only one slowly sedimenting peak. The molecular weights of peptides 1 and 2 were obtained from sedimentation equilibrium experiments, using $\bar{v} = 0.75$ as calculated from the amino acid composition according to the procedure of Cohn and Edsall (1943). These data, along with those from the sedimentation velocity runs, are summarized in Table II.

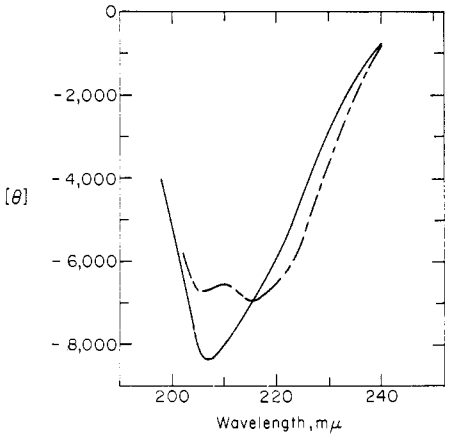

FIGURE 6: Circular dichroism of peptide 3 at room temperature. — · — —, pH 5.5; ———, pH 9.2.

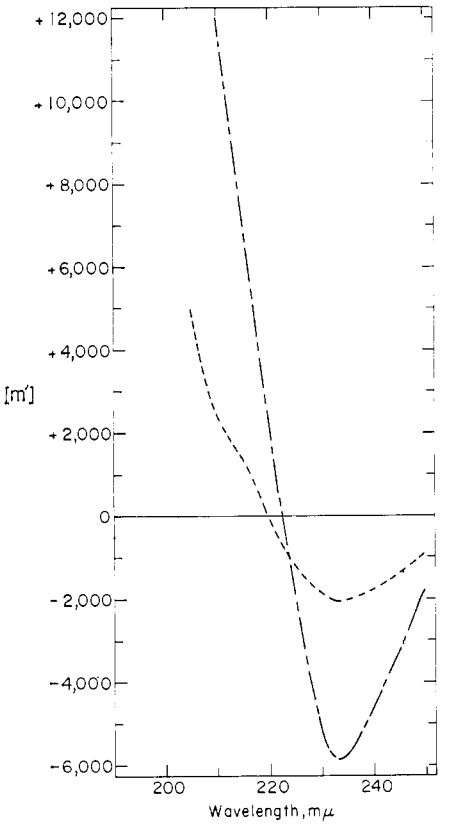

FIGURE 7: Optical rotatory dispersion of apomyoglobin (— — —) and of peptide 2 (- - - - -) after heating to 60° at pH 9.2. These same results were obtained at 60°, and also after cooling this material to 25°.

TABLE II: Ultracentrifuge Studies of Cyanogen Bromide Peptides, 0.01 M Sodium Borate[a] (pH 9.2), 23°, Peptide Concentration 2–4 mg/ml.

Peptide	Sedimentation Coef (S)	$\bar{M}_w$ (from sedimentation equil)	Theor Mol Wt[b]
1	0.5	3500	2556
2	1.1	8000	8237
3	8.9 and 2.7	*c*	6384

[a] This salt concentration is greater than the concentration of charged groups on the peptides, and the buffer is the same as was used for the optical rotatory dispersion and circular dichroism studies. [b] Calculated from the amino acid composition given by Edmundson (1965), replacing methionine by homoserine. [c] Preparation gave interference patterns typical of high molecular weight aggregates, but no exact values could be calculated because of anomolous synthetic boundary results.

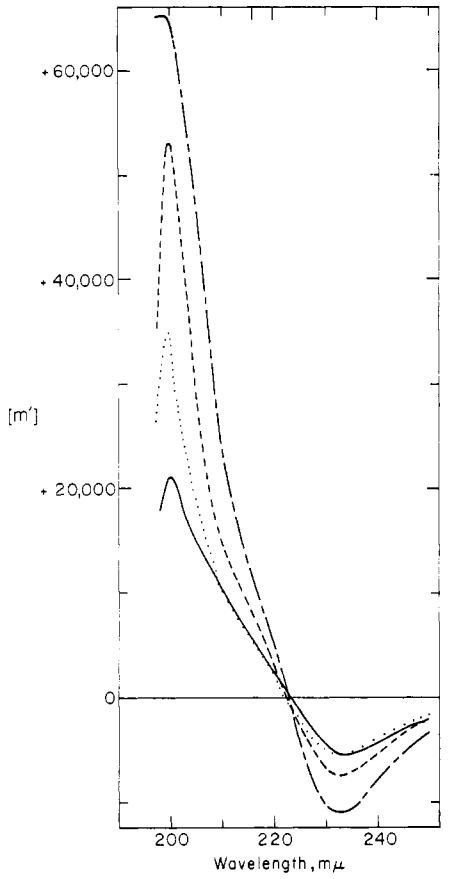

FIGURE 8: Optical rotatory dispersion, at 25°, of peptide 1 (·····), peptide 2 (- - - -), peptide 3 (———), and apomyoglobin (— — — —) obtained by adding methanol (up to a concentration of 95%) to aqueous pH 5.5 solutions. The magnitude of $[m']$ at 200 mμ has an error of about 30%, and thus was not used to calculate per cent helix. These data would lead to a somewhat higher estimate of helix content for all samples except peptide 3.

Discussion

The most detailed knowledge of the structure of sperm whale myoglobin comes from the X-ray crystallographic studies of Kendrew *et al.* (1961), in which 77% of the molecule was shown to be helical. The problem of comparing this structure with that in solution has been extensively studied and reviewed by Urnes (1963), who concluded that the conformation in the crystal is retained in solution. Although this seems to be true as far as the helix content is concerned, it has recently been shown that there is a difference in the reactivity of some histidine residues toward bromoacetate, suggesting a conformational difference between the crystal and solution (Hugli and Gurd, 1968). Conformational changes resulting from the removal of the heme to form the apoprotein must also be considered. Breslow (1964) and Eylar (unpublished results cited by Breslow, 1964) have suggested that the hydrodynamic properties (and, hence gross size and shape) of myoglobin and the apoprotein are the same, although Crumpton and Polson (1965) have detected some differences. Optical rotatory dispersion and circular dichroism studies of Harrison and Blout (1965) and Breslow *et al.* (1965) showed that apomyoglobin has 5–20% less helix than the native protein. Thus, it appears that the apoprotein retains a conformation of high helix content similar to that found in the crystalline state.

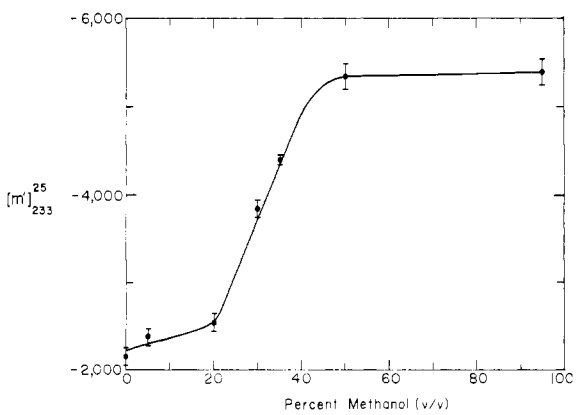

FIGURE 9: Optical rotatory dispersion at 25° of peptide 1 as a function of methanol concentration.

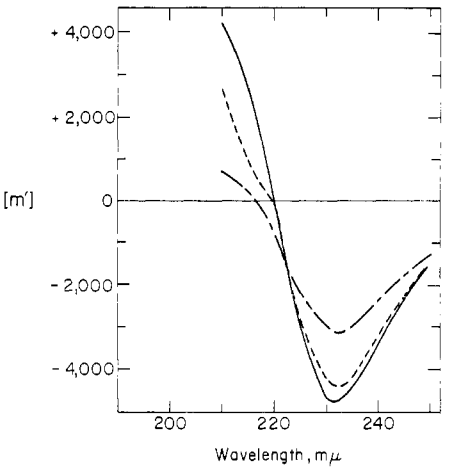

FIGURE 10: Effect of temperature on optical rotatory dispersion of peptide 1 in 35% aqueous methanol. ———, 15°; - - - -, 25°, — — — —, 50°.

Several problems arise in the estimation of the helix content of proteins by means of optical rotation. Among these are the dependence of the rotatory properties upon helix length (Woody and Tinoco, 1967; J., Vournakis, J. F., Yan, and H. A., Scheraga, submitted for publication) and on changes in backbone dihedral angles resulting from variation in the nature of the side chain (J., Vournakis, J. F., Yan, and H. A., Scheraga, submitted for publication). Myoglobin contains short helical segments (Kendrew *et al.*, 1961) and distorted helices (Némethy *et al.*, 1967), both of which could affect the optical rotatory dispersion and circular dichroism at a particular wavelength, or cause shifts in the wavelength of the n–π* transition (Schellman and Lowe, 1968); these effects would influence the estimate of helix content. In addition, aromatic side chains have electronic transitions which may become optically active and complicate rotatory measurements based on the asymmetry at the peptide chromophore (Beychok, 1967). Finally,

there is the problem of assigning rotatory properties to the various secondary structures found in proteins. This can be done for α and antiparallel β structures using synthetic poly-α-amino acids as model systems. However, for the nonhelical regions of myoglobin, the use of the random coil conformation of poly-α-amino acids may not be appropriate. In addition, there may be other conformations, such as the parallel β structure, whose existence has not been demonstrated in solutions of polyamino acids (Kessler *et al.*, 1968). Indeed, when polyamino acid Cotton effects are used to fit the data for myoglobin, the best results are obtained by considering the protein to be 55% helix, 10% coil, and 35% antiparallel β structure (Greenfield *et al.*, 1967; Magar, 1968). However, no β structure has been found in the crystalline state of myoglobin, and there is no other evidence for its presence in solution. For all of the above reasons, it is difficult to give a detailed analysis of the electronic transitions which are responsible for the exact shape and position of the Cotton effects and ellipticities. In spite of all of these complications, rotatory properties have given reliable, though not quantitatively precise, estimates of the helix content of a variety of proteins (Yang, 1967; Beychok, 1967). In particular, the rotatory properties of myoglobin have been studied extensively, and show a very high helix content, in good agreement with the crystal structure.

With these caveats, we have estimated the helix content of the cyanogen bromide peptides of myoglobin using the Cotton effect and circular dichroism of the amide group, as well as the b_0 parameter of the Moffitt–Yang equation. These data are not independent of each other (for example, the circular dichroism can be calculated from the optical rotatory dispersion using the Kronig–Kramers transform); hence, we have measured several properties, since anomalous effects, such as those arising from short or distorted helices or side-chain Cotton effects, would affect each of the measurements differently. It was assumed that the degree of helicity is directly proportional to the measured parameter and that the values for 100% α helix are $[m']_{233} = -14{,}200$, $[m']_{200} = +65{,}000$, $[\theta]_{222} = -32{,}000$, and $b_0 = -630$; the corresponding values for the random coil are $[m']_{233} = -1700$, $[m']_{200} = -3100$, $[\theta]_{222} = 0$, and $b_0 = 0$. These values are the same as those which have been used to calculate the helicity of apomyoglobin (Breslow *et al.*, 1965), except for b_0 which we have changed from -700 and $+100$ for the helix and coil, respectively (Shechter *et al.*, 1964; Harrison and Blout 1965), to the above values which are in agreement with many studies on polyamino acids (Yang, 1967). This change in standard values most greatly affects the quantitative estimation of per cent helix when the latter is low. The data are shown in Table III; the limits of error were estimated from the noise level of the recordings. There are significant decreases in the degree of helicity between the cyanogen bromide peptides and apomyoglobin. The values calculated, using the four different measurements, are in reasonable agreement, indicating that effects from side-chain chromophores, distorted helices, etc., must be of relatively minor importance. In addition, the results in methanol demonstrate that these peptides are capable of possessing rotatory properties indicative of high helix content.

Recently, Crumpton (1968) has reported a value of -8700 for $[m']_{233}$ of peptide 3 in 50 mM phosphate buffer (pH 7.7). This value differs significantly from ours, and the discrepancy is difficult to resolve. His material was prepared and separated in a manner somewhat different from ours, and was measured with a different instrument using different buffers. None of these reasons, however, offers a likely explanation for the discrepancy in the optical rotatory dispersion results. Conceivably the optical rotatory dispersion may be influenced to varying extents by the aggregation.

Myoglobin consists of a series of interacting helical segments; the splitting of the molecule into smaller peptides destroys most of the interhelical interactions which have been predicted to be important in stabilizing the helical conformation of polypeptides in aqueous solution (Poland and Scheraga, 1965). In addition, the splitting may also affect the free energy of the coil form; the number of conformations per amino acid residue which the peptides can attain may be greater than for the protein because of the formers' smaller size and hence smaller excluded volume. Thus, if the helical conformation in the peptides and protein were equally stable, then the protein would achieve a higher helix content because of the decreased stability of its coiled form. A quantitative estimation of this effect, especially for a heteropolymer, is rather complicated and would involve many assumptions. However, we believe that this effect will be relatively small, especially for the larger peptides 2 and 3. This belief is based on the fact that Tanford *et al.* (1966) showed that a variety of proteins ranging in size from $n = 26$ to 1790 amino acid residues per chain had viscosities in 6 M guanidine hydrochloride which obeyed the simple equation $[\eta] = Kn^a$, where $[\eta]$ is the intrinsic viscosity, and a and K are constants. The constancy of a for all of the cases studied suggests that the average freedom of rotation about single bonds and long-range interactions do not vary greatly among a group of randomly coiled proteins with different amino acid composition and chain length. It should also be noted that the effect of long-range interactions has not been taken into account in statistical mechanical theories of the helix–coil transition (Go *et al.*, 1968).

Helix formation in polyamino acids is known to be a cooperative process (Zimm and Bragg, 1959). Hence, a destabilization of a helix may result when a helical segment of a protein is broken into two portions by splitting a peptide bond. Cyanogen bromide cleaves apomyoglobin in the interior of helices D and H (in the nomenclature of Kendrew *et al.*, 1961), which have 7 and 24 helical residues, respectively, in the crystallographic structure of *myoglobin*. For illustrative purpose, let us assume that the helix content of apomyoglobin (and its intact constituent peptides) is 15% less than that of myoglobin. Hence, helices D and H, would be 6 and 20 residues long, respectively, in apomyoglobin. Five or six of the residues of helix H are in peptide 2; hence, the maximum possible length of this helix would be reduced from 20 residues in helix H of apomyoglobin to 15 residues in peptide 1; Since a shorter chain would have a

TABLE III: Rotatory and Dichroic Parameters and Apparent Helix Content of Apomyoglobin and Its Cyanogen Bromide Peptides.[a]

Sample	Conditions	Measurement	Per Cent Helix
Peptide 1	Water, pH 5.1	$[m']_{233} = -2100 \pm 300$	3.5
		$[\theta]_{222} = -1600 \pm 200$	5
	Water, pH 7.2	$[m']_{233} = -2300 \pm 250$	4.5
	0.002 M phosphate buffer, pH 7.2	$[m']_{233} = -3400$	14[b]
	0.01 M sodium borate, pH 9.2	$[m']_{233} = -2500 \pm 250$	6.5
		$[\theta]_{222} = -2750 \pm 150$	8.5
		$b_0 = -69 \pm 6$	11
	95% methanol	$[m']_{233} = -5400 \pm 450$	30
Peptide 2	Water, pH 5.8	$[m']_{233} = -3330 \pm 30$	13
		$[m']_{200} = +350 \pm 200$	5
		$[\theta]_{222} = -3500 \pm 200$	11
	Water, pH 7.2	$[m']_{233} = -3500 \pm 200$	14
		$[m']_{200} = +6300 \pm 600$	14
	0.01 M sodium borate, pH 9.2	$[m']_{233} = -3900 \pm 150$	18
		$[m']_{200} = +8900 \pm 900$	18
		$[\theta]_{222} = -5850 \pm 200$	18
		$b_0 = -133 \pm 8$	21
	95% methanol	$[m']_{233} = -7500 \pm 200$	46
Peptide 3	Water, pH 5.5	$[m']_{233} = -3350 \pm 100$	13
		$[m']_{200} = +6000 \pm 1000$	13
		$[\theta]_{222} = -6200 \pm 100$	19
	Water, pH 7.1	$[m']_{233} = -3100 \pm 200$	11
		$[m']_{200} = +5000 \pm 1700$	12
	0.01 M sodium borate, pH 9.2	$[m']_{233} = -3500 \pm 200$	14
		$[m']_{200} = +8900 \pm 1500$	18
		$[\theta]_{222} = -5800 \pm 100$	18
		$b_0 = -89 \pm 9$	14
	95% methanol	$[m']_{233} = -5500 \pm 450$	31
Apomyoglobin	Water, pH 5.9	$[m']_{233} = -7460$	46[b,c]
	Phosphate buffer, pH 6.1	$[m']_{200} = +35,350$	56[b]
		$[\theta]_{223}$	49[b]
	Borate buffer, pH 9.4	$[m']_{233} = -7330$	45[b,c]
	0.1 M borate buffer, pH 9.2	$b_0 = -310$	49[d]
	95% methanol	$[m']_{233} = -11,000 \pm 800$	74

[a] $[m']$ and $[\theta]$ were measured at 25°, and room temperature, respectively. [b] Data from Breslow *et al.* (1965). [c] We have obtained similar values. [d] Data from Harrison and Blout (1965), recalculated using $b_0 = -630$ and 0 for the helix and coil, rather than -700 and $+100$. Our choice of standard values is based on polyamino acid data summarized by Yang (1967).

smaller per cent helix content, we would expect that the number of helical residues in peptide 1 would be *less* than the maximum value of 15. By similar arguments involving the split of helix D as well as H, the maximum per cent helix (64 and 68%, respectively) in peptides 2 and 3 could be as low as 54 and 60%, if the split portions of the end helices were completely converted into coils; this effect is smaller in peptides 2 and 3 than in peptide 1 because the split helix in the latter peptide is the only helical portion of this fragment. In addition to this dependence of helix content upon chain length, the nature of the end group can also influence the per cent helix; the methionine of apomyoglobin is replaced by homoserine or homoserine lactone, depending upon the pH (Armstrong, 1949), and charged end groups appear, when the peptide bond is split. These end effects would arise in the same regions in the D and H helices, to which we have already attributed a helix-disrupting tendency.

The state of aggregation may also affect the conformation of the preparations. Ultracentrifuge studies indicate that neither apomyoglobin (Theorell and

Åkeson, 1955) nor peptides 1 and 2 (see Table II) aggregate in the buffer in which the optical rotatory dispersion and circular dichroism measurements were made; thus, the conformation of these preparations may be directly compared. However, peptide 3 forms large aggregates, and we therefore cannot compare its conformation as an isolated (but aggregated) peptide with that in the intact apomyoglobin molecule. Peptide 3 is retarded on Sephadex more than peptide 2, suggesting that, in 0.2 M acetic acid, none of the peptides are aggregated. Unfortunately, at lower pH, apomyoglobin forms aggregates (Rumen and Appella, 1962) so that, even at this pH, the conformations of peptide 3 and apomyoglobin could not be compared at the same state of aggregation.

In summary, the disruption of interhelical interactions, the possible increased entropy per residue of the random coil of short peptides, and the dependence of helix content upon chain length and upon the nature of the end groups are all possible reasons as to why the helix contents of the isolated unaggregated peptides 1 and 2 are less than those in apomyoglobin. As discussed above, the long-range interhelical interactions appear to be the most important of these effects.

It has been suggested that, as proteins are synthesized *in vivo* from the N terminus (Dintzis, 1961; Canfield and Anfinsen, 1963), the incomplete peptides begin to fold from the N terminus (Phillips, 1967; Dunnill, 1967). Peptide 3 contains the 55 N-terminal amino acids of apomyoglobin. Because of aggregation its conformation cannot be compared with that of a partially synthesized protein *in vivo*, which may not be aggregated because of its attachment to ribosomes. Of the three cyanogen bromide peptides studied, only peptide 3 aggregated, and it is tempting to suggest that this aggregation is caused by nonpolar groups on the peptide which are also responsible for directing protein folding during synthesis (this has also been suggested by Dr. Crumpton in a personal communication).

Peptide 2 shows a comparatively large and anomalous change in its optical rotatory dispersion properties between pH 5.8 and 7.2. The characteristics of the Cotton effect at pH 5.8 are not interpretable as a simple linear combination of α, β, and random coil structures. The cause of this was not investigated further, but may have been related to the fact that eight histidine residues of this peptide ionize in this pH range. Between pH 7 and 9 there is little change in the optical rotatory dispersion of any of the three peptides or of apomyoglobin itself.

There is an irreversible change in the optical rotatory dispersion of peptide 2 and of apomyoglobin on heating. This change does not seem to be time dependent. It is difficult to definitely assign this change to a conformational transition since some preparations of apomyoglobin can be completely unfolded in urea and will refold reversibly to the native structure (Harrison and Blout, 1965), so that it seems unlikely that a partial unfolding at 60° would be irreversible. The optical rotatory dispersion of peptides 1 and 3 do not show any temperature dependence. The absence of a temperature dependence in the case of peptide 3 seems unusual since it appears to be partially helical; this temperature effect may be related to the state of aggregation of peptide 3. In addition this peptide is small and may have a broad transition as well as a small enthalpy of transition, or a portion of peptide 3 may form a very stable helix whose melting temperature is above 90°. Another possibility is that the conformations of the peptide are not in equilibrium; this is unlikely, since no time-dependent changes in the optical rotatory dispersion are observed and the coil–helix transition induced by methanol is reversible.

We have attempted to regenerate the long-range interactions by simply mixing the separated peptides. However, these interactions were apparently not sufficient to overcome the disaggregation of peptide 3 and the loss of translational entropy of the three peptides which would accompany the recombination. In addition, the replacement of methionine by homoserine and the introduction of charged groups at the ends of the peptides may also have interfered with the recombination.

By using methanol, it has been possible to increase the helix content of the isolated peptides. This solvent is a helix-promoting one for several polypeptides, and chloroethanol has been shown to increase the $-b_0$ of apomyoglobin (Klemperer and Doty, unpublished results cited by Urnes, 1963, p 145). The effect of methanol on the rotatory properties of the cyanogen bromide peptides seems to be caused by a conformational transition rather than by a solvent effect on an asymmetric chromophore since a marked temperature dependence is found at an intermediate alcohol concentration (Figure 10). A large fraction of the amino acid residues of myoglobin are buried in a nonpolar region inside the protein (Kendrew *et al.*, 1961). The fact that methanol is able to regenerate a high helix content suggests that the long-range interactions in the native protein may stabilize the formation of helical structures by relatively nonspecific effects such as bringing the polypeptide chain into a less aqueous environment.

Recent studies on the reaction of antibodies to myoglobin with cyanogen bromide peptides indicate that these peptides can combine with antibody at relatively high concentrations of peptide (Attassi and Saplin, 1968). It was suggested that higher concentrations are needed because of conformational differences between the peptide and the protein. This suggestion is confirmed by the results reported here.

Similar results to ours have been obtained with the S-peptide of ribonuclease (Scatturin *et al.*, 1967) which appears to be essentially a random coil despite the fact that it contains a short helical sequence in the native protein. In this case the helical conformation can be regenerated by binding to the rest of the molecule to form ribonuclease S (Wyckoff *et al.*, 1967).

Two peptides, isolated from the chymotryptic digest of myoglobin, have also been studied by optical rotatory dispersion and have been shown to be less helical than would be expected on the basis of the crystallographic structure of myoglobin (Crumpton and Small, 1967). These results are compatible with ours, but involve the study of incomplete helical segments with no intact ones. The present paper extends these results to larger pep-

tides containing large, unbroken helical sequences. The helices in the cyanogen bromide peptides become less stable in water when removed from interaction with the rest of the protein molecule.

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