Conformational Studies on Modified Proteins and Peptides. Conformation of Peptides with Intact and Overlapping Helices Obtained by Cleavage of Myoglobin at Proline Peptide Bonds*

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ABSTRACT: Cleavage of sperm whale myoglobin at proline peptide bonds has been carried out by reaction with sodium in liquid ammonia. Peptides comprising the sequences 1–36, 37–87, 1–87, 37–119, and 120–153 were isolated and studied by optical rotatory dispersion and circular dichroism. Each of these peptides contained whole intact helices in the three-dimensional structure. In the free state each peptide was much less helical than expected from its conformation in the intact protein. Thus peptide 1–36 (which comprises two intact helices corresponding to 97% helical content) was only 27% helical. Peptide 1–87 (which is 79% helical in the intact protein) showed a helical content of 28%. Peptides 37–87, 37–119, and 120–153 had the following helical contents: 24, 41, and 18%, respectively. Helical content of each of these peptides increased on addition of methanol, leveling off at a methanol concentration around 60%. However, even under these conditions helical content was only a fraction of the values these peptides possess in the native protein and it was highest in peptides in which long-range interactions are feasible.

It may be concluded that an intact whole helix in the protein will be less helical in the free state. Long-range interactions play a major role in stabilizing protein folding. Also, the results may suggest that proteins do not start to assume their native three-dimensional structure during biosynthesis until virtually or entirely completed, depending on the importance of contribution to long-range interactions of the yet-unsynthesized portion.

Conformational studies on peptide fragments from a variety of proteins have been carried out in order to investigate the role played by short-range and long-range interactions in the mode of folding of peptides. This is based on the reasoning that since conformation is a direct consequence of primary structure (Lumry and Eyring, 1954; Anfinsen, 1961, 1964), investigation of the conformation of peptides should aid greatly in understanding interactions responsible for protein conformation. However, by necessity of chemical and enzymic cleavage reactions available, the peptides isolated from a protein and studied have not always been the best models that are desirable for these investigations. Thus fragmentation might have been carried out unavoidably within a helix. Accordingly this decreases the possibility of the isolated portion of a helix to assume helical configuration, especially when it is isolated in conjuction with a bend or a nonhelical peptide portion.

Cleavage at sites located in bends or nonhelical segments and studies of conformation of fragments comprising intact helices should be of great value. Interactions influencing conformation can be better understood when studies are carried out on fragments comprising helices of appreciable length and, if possible, overlaps of adjacent intact helices. For the present work, Mb[1] was chosen as the protein model. This is a highly helical protein (Kendrew *et al.*, 1961; Urnes *et al.*, 1961; Samejima and Yang, 1964; Urnes, 1965). Removal of the heme group results in some unfolding (Harrison and Blout, 1965; Breslow *et al.*, 1965; Atassi, 1966) but the apoprotein is still more than 50% helical. The four proline residues in Mb effect four out of a total of six bends present in the three-dimensional structure (Kendrew *et al.*, 1961). Cleavage at proline peptide bonds should therefore yield intact helices. Also overlapping helices will be obtained when reaction at any proline residue is incomplete. Five fragments were obtained from cleavage of ApoMb at proline peptide bonds (see Atassi and Singhal, 1970a) by reaction with sodium in liquid ammonia (Benisek and Cole, 1965; Benisek *et al.*, 1967). The conformation of these peptides has been studied by optical rotatory dispersion and circular dichroism in order to determine the ability of intact and overlapping helices to assume helical configuration and therefore determine the extent to which this is dependent on long-range interactions.

Materials and Methods

Metmyoglobin and Apomyoglobin. Sperm whale myoglobin used in these studies was the major chromatographic component 10 (MbX) obtained by CM-cellulose chromatography (Atassi, 1964). The apoprotein was prepared from MbX by a procedure similar to that used for the preparation of apohemoglobin (Atassi and Skalski, 1969).

* From the Department of Chemistry, Wayne State University, Detroit, Michigan 48202. *Received February 27, 1970.* This work was supported by a grant (AM-13389) from the National Institute of Arthritis and Metabolic Diseases. National Institutes of Health, U. S. Public Health Service. This is article no. II in the series: Conformational Studies on Modified Proteins and Peptides.

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: Mb, metmyoglobin; ApoMb, apomyoglobin.

Cleavage of Apomyoglobin at the Proline Sites and Isolation of the Fragments. Cleavage at the proline sites was accomplished by reaction with sodium in liquid ammonia (Benisek *et al.*, 1967). Optimum reaction conditions for ApoMb together with methods for separation, exhaustive purification, and characterization of the resultant fragments are described in detail elsewhere (Atassi and Singhal, 1970a), in conjunction with the immunochemical reactivities of these peptides.

Optical Rotatory Dispersion and Circular Dichroism Measurements. Optical rotatory dispersion and circular dichroism studies were carried out at 25° on solutions of proteins or peptides in water (glass double distilled). Solutions contained 0.05–0.4 mg of protein or peptide per ml. Measurements were made with a Cary Model 60 spectropolarimeter, equipped with a Model 6001 circular dichroism accessory.

Measurements on each sample were made at several concentrations employing cells with light paths of 0.5, 1, 5, and 10 mm. For measurements below 220 mμ only 0.5- and 1-mm cells were used with maximum damping (pen period 30), very low-span speeds (30 sec/mμ) and a full range of 0.1 deg. Solvent base-line scans were performed before and after each protein sample. Optical rotatory dispersion results are reported in reduced mean residue rotation, $[m']_\lambda$, corrected for the refractive index dispersion of water, n

$$[m']_\lambda = \frac{(3)}{n_\lambda^2 + 2}\frac{M_R}{100}[\alpha]_\lambda$$

where M_R is the mean residue molecular weight (see Table I for the value of M_R for Mb, ApoMb, and peptides), and $[\alpha]_\lambda$ is the specific rotation at various wavelengths. Also, optical rotatory dispersion data were analyzed quantitatively by means of the Moffitt–Yang equation (Moffitt and Yang, 1956)

$$[m']_\lambda = a_0\frac{\lambda_0^2}{\lambda^2 - \lambda_0^2} + b_0\frac{\lambda_0^4}{(\lambda^2 - \lambda_0^2)^2}$$

The parameter b_0 was determined from the slope of the plot $[m']_\lambda[(\lambda^2 - \lambda_0^2)/\lambda_0^2]$ against $\lambda_0^2/(\lambda^2 - \lambda_0^2)$, with $\lambda_0 = 216$ mμ. The plots were linear in the range 240–270 mμ.

The circular dichroism accessory records data directly in terms of ellipticity, θ, in degrees. In analogy to $[m']$, circular dichroism data are given here as reduced molar ellipticities, $[\theta']$, by correcting for the refractive index dispersion of water. Units of $[\theta']$ are in deg cm² per dmole. Helical contents were estimated from optical rotatory dispersion and circular dichroism data by using $[m']_{233}$ for helical and random coil conformations as −13,500 and −1750, respectively (Fasman, 1963). Values of $[m']_{199}$ for completely helical and completely disordered conformations were taken as +70,200 and 0, respectively (Riddiford, 1966). In circular dichroism, $[\theta']_{220}$ (helix) and $[\theta']_{220}$ (coil) were taken as −29,000 and+3000, respectively (Yang, 1967). For a completely helical conformation, using λ_0 at 216 mμ, b_0 is −535 (Urnes and Doty, 1961).

Effect of methanol on peptide conformation was studied by the addition of increasing amounts of methanol (Analytical grade) to a solution of peptide (0.1–0.3 mg/ml) in water. After each methanol addition, the peptide solution was centrifuged (4000 rpm, 1 hr, 0°) and aliquots were then removed for triplicate nitrogen analyses. Therefore, the exact peptide concentration at each methanol content was known and complications due to any precipitation of peptide on addition of methanol were completely avoided.

TABLE I: Molecular Weights, Nitrogen Contents, and Mean Residue Weights of Peptides.[a]

Protein or Peptide	Mol Wt	Nitrogen Contents (%)	Mean Residue Wt
Mb	17,816	17.36[b]	116.4
ApoMb	17,200	17.66[b]	112.4
1–36	4,082	17.50	113.4
1–87	9,848	17.49	113.2
37–87	5,803	17.38	113.8
37–119	9,481	17.87	114.2
120–153	3,673	17.16	108.0

[a] Values for each peptide were calculated from its amino acid composition. Peptides are referred to by their location in the primary structure of myoglobin. [b] These values are obtained from Atassi and Saplin (1968).

Determination of Protein and Peptide Concentrations. Concentrations of protein and peptide solutions were measured by determination of their nitrogen contents using both a micro-Kjeldahl procedure similar to that described by Markham (1942) and by using Nessler's reagent standardized with ammonium sulfate. Three or four replicate analyses were done on each solution and they varied ±0.3%. The nitrogen contents for Mb, ApoMb, and the present five peptides were calculated from their amino acid composition and are shown in Table I together with the molecular weights.

Results

Optical Rotatory Dispersion Measurements. All peptide samples showed negative rotation with a minimum at 233 mμ and a positive rotation maximum at 199 mμ. Figure 1 shows the optical rotatory dispersion spectra of all the present peptides in the ultraviolet region. Peptide 37–119 showed the highest rotation both at the negative minimum at 233 mμ ($[m']_{233} = -5700$) and at the positive extremum at 199 mμ ($[m']_{199} = +28{,}900$). Peptides 1–36, 1–87, and 37–87 showed only a light decrease in their rotatory behavior in that order (see Figure 1 and Table II). Peptide 120–153 showed appreciably lower rotations both at 233 mμ (−3200) and at 199 mμ (+12,760). The values of $[m']_{233}$ and $[m']_{199}$ for all these peptides, together with b_0 values are summarized in Table II.

The effect of methanol on the rotatory behavior of these peptides was investigated. Figure 2a shows the variations of $[m']_{233}$ with methanol concentration. For each peptide, rotation was increased on addition of methanol. Increase in rotation leveled off when methanol concentration was about 60% (v/v). The increased rotatory power in the presence of methanol was also accompanied by increases in the b_0 values (Figure 2b), suggesting a higher contribution of helical structure when peptides are in methanol. Table II summarizes the values of $[m']_{233}$, $[m']_{199}$, and b_0 for each of these peptides in water and 60% methanol (v/v). The table also shows the corresponding values for Mb and for ApoMb in water.

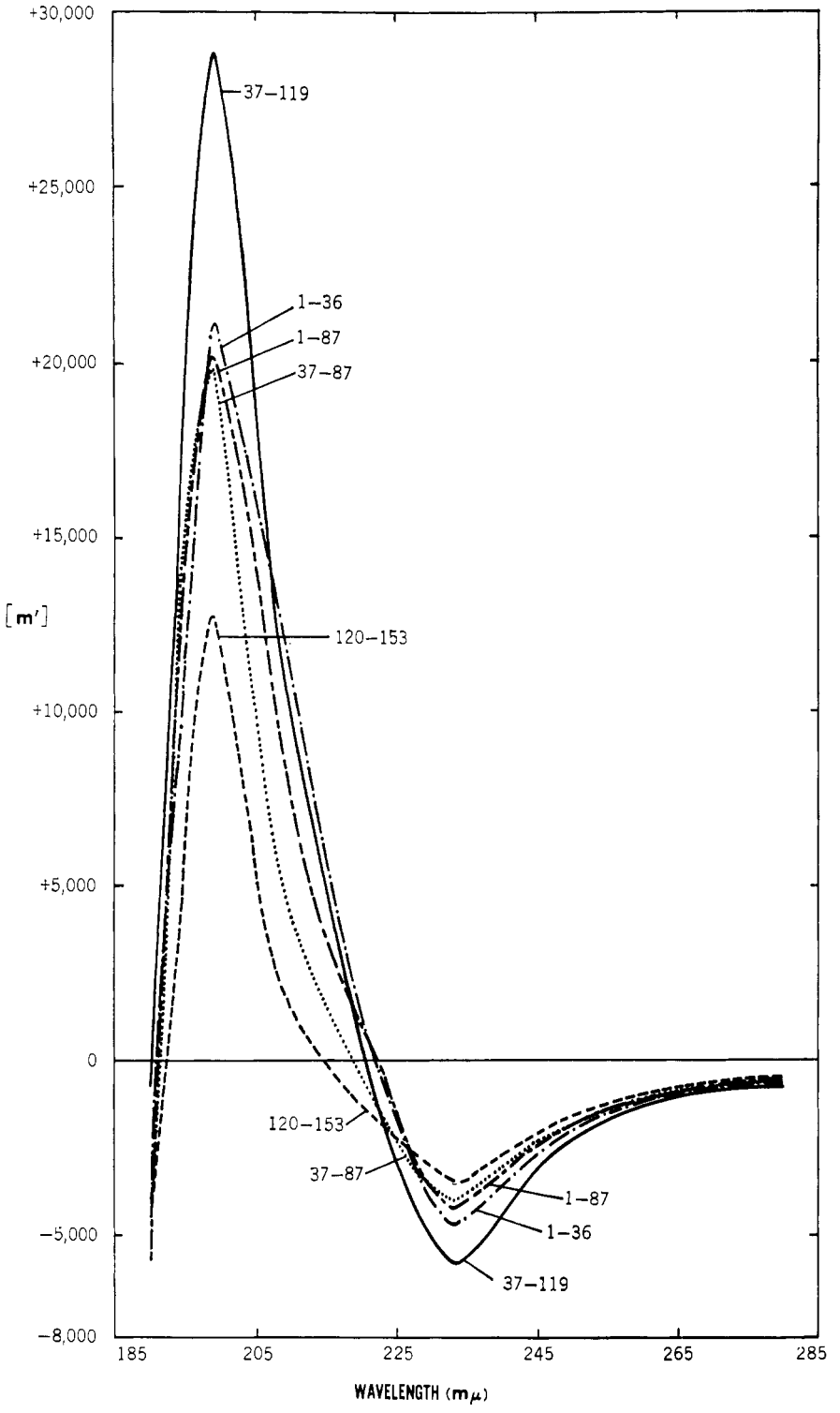

FIGURE 1: Optical rotatory dispersion spectra of peptides 1–36, 37–87, 37–119, and 120–153. Measurements were carried on solutions of each peptide in water. For experimental details, see text.

Circular Dichroism Measurements. All peptide samples in water showed negative ellipticity bands at 208 mμ and at 220–221 mμ. In agreement with optical rotatory dispersion data, ellipticity was also increased in the presence of methanol. Figure 3 shows the circular dichroism behavior of peptide 1–36 in water and 64% methanol (v/v). In the latter solvent only the negative ellipticity band at 220 mμ was prominent and its magnitude (−14,000) was more than twice the corresponding value in water (−6700). The ellipticity band at 208 mμ in water (−7400) disappeared in 64% methanol. In contrast with this, peptide 37–87 (Figure 4) showed discreet negative ellipticity bands both at 220 and at 208 mμ in 63% methanol. These were also appreciably increased (−8100 and −9000) relative to the corresponding values for the same peptide in water (−6500 and −7600 for $[\theta']_{220}$ and $[\theta']_{208}$, respectively). Figure 5 shows the circular dichroism behavior

TABLE II: Optical Rotatory Dispersion Parameters of Peptides, Myoglobin, and Apomyoglobin.

Peptide or Protein	Solvent	$[m']_{199}$	$[m']_{233}$	b_0
1–36	Water	+21,190 ± 300	−4500 ± 100	−164 ± 5
	64% Methanol		−6450 ± 150	−286 ± 20
1–87	Water	+20,180 ± 350	−4230 ± 160	−153 ± 5
	64% Methanol		−6200 ± 100	−275 ± 25
37–87	Water	+19,970 ± 150	−4020 ± 50	−139 ± 4
	63% Methanol		−5950 ± 100	−265 ± 20
37–119	Water	+28,900 ± 250	−5700 ± 130	−243 ± 5
	63% Methanol		−7375 ± 75	−327 ± 15
120–153	Water	+12,760 ± 150	−3200 ± 100	−106 ± 15
	62% Methanol		−5600 ± 175	−240 ± 10
Mb	Water	+46,360 ± 1400	−9320 ± 186	−417 ± 13
ApoMb	Water	+33,100 ± 1500	−7366 ± 100	−311 ± 10

of the overlapping peptide 1–87 comprising segments 1–36 and 37–87. Peptide 1–87 exhibited, in water, higher ellipticity values at 220 mμ (−9900) and at 208 mμ (−11,250) than the corresponding values of either subfragments 1–36 and 37–87 given above. In 64% methanol (v/v), the ellipticity band at 208 mμ disappears while $[\theta']$ at 220 remains prominent and is increased to a value of −13,700. Figure 6 shows the circular dichroism behavior of peptide 37–119. This showed the highest $[\theta']$ values in agreement with its optical rotatory dispersion behavior. The $[\theta']$ minima at 220 mμ (−13,400) and at 208 mμ (−14,700) in water solutions became less discreet in 63% methanol (v/v) with only one band appearing at 218 mμ (−17,300). Finally, the circular dichroism behavior of peptide 120–153 is shown in Figure 7. This peptide showed discreet minima at 220 and at 208 mμ, both in water (−6100 and −8500, respectively) and in 62% methanol (−9200 and −11,000, respectively). With this peptide, also, ellipticity was increased in the presence of methanol. In studies on peptides from the N terminal of ribonuclease, Brown and Klee (1969) showed that helicity increased at low temperatures (1°). In the present work, only the effect of methanol was investigated.

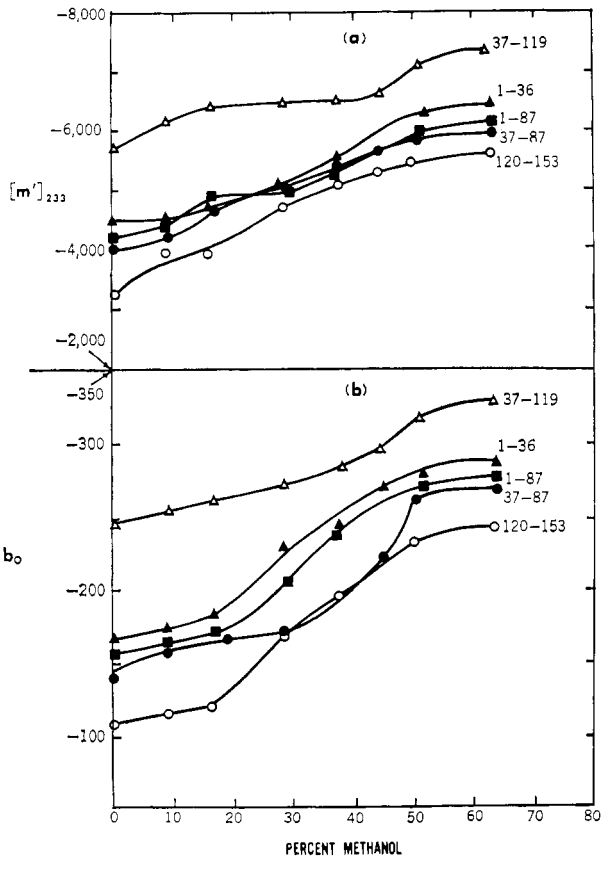

FIGURE 2: Effect of methanol on the optical rotatory dispersion behavior of the various peptides. (a) Variation of $[m']_{233}$ with increase in methanol concentration. (b) Variation of b_0 values with methanol concentration. Peptides 1–36 (▲), 37–87 (●), 1–87 (■), 37–119 (△), and 120–153 (○).

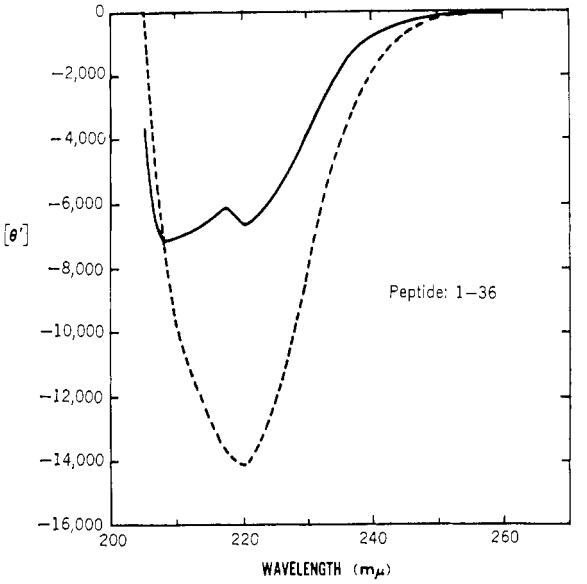

FIGURE 3: Circular dichroism spectra of peptides 1–36 in water (solid line) and in 64% methanol (dashed line).

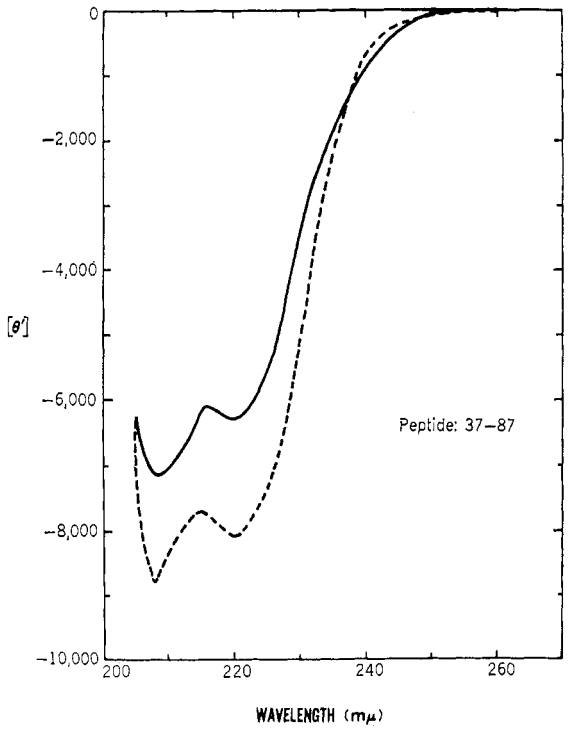

FIGURE 4: Circular dichroism spectra of peptide 37–87 in water (solid line) and in 63% methanol (dashed line).

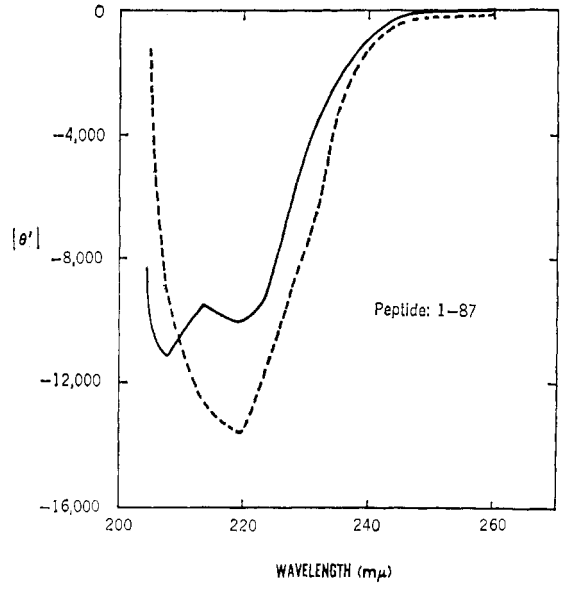

FIGURE 5: Circular dichroism spectra of peptide 1–87 in water (solid line) and in 64% methanol (dashed line).

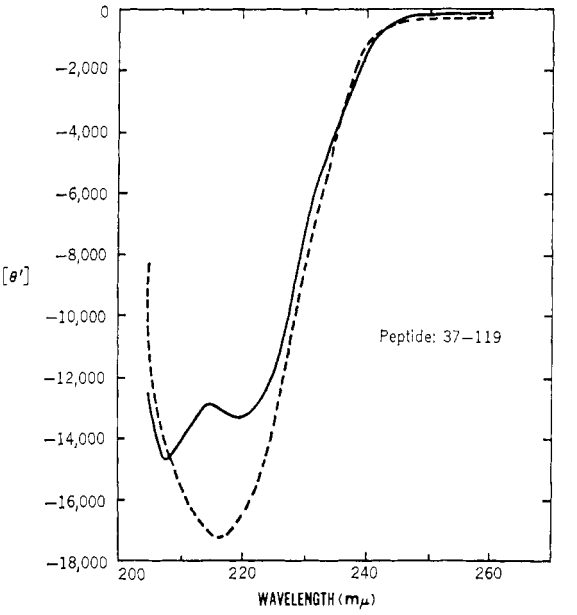

FIGURE 6: Circular dichroism spectra of peptide 37–119 in water (solid line) and in 63% methanol (dashed line).

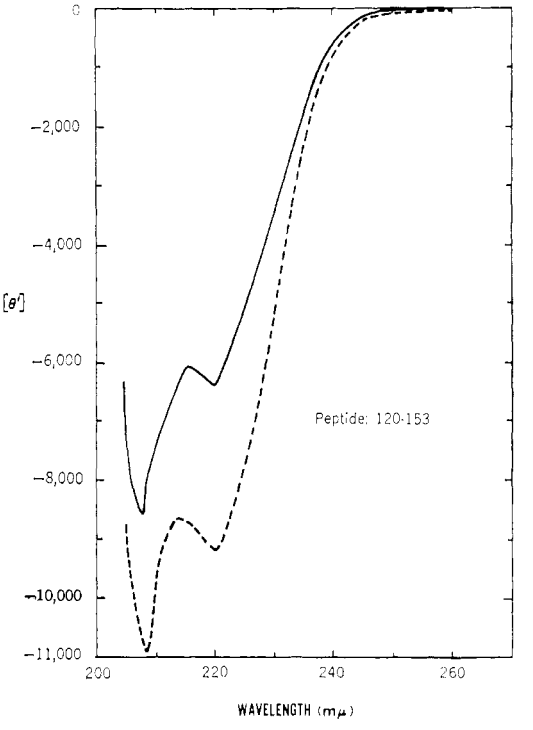

FIGURE 7: Circular dichroism spectra of peptide 120–153 in water (—) and in 62% methanol (----)

Discussion

In the three-dimensional model (Kendrew *et al.*, 1961) peptide 1–36 includes two complete helices A and B. Of the 36 residues 35 of these occupy an α-helical configuration (*i.e.*, 97% helical) in the native molecule. Therefore, it is not surprising that optical rotatory dispersion and circular dichroism measurements indicate the presence of an appreciable helical structure for this peptide in solution. The helical structure of this peptide in solution is probably effected mainly through short-range interactions. Calculations of Kotelchuk and Scheraga (1968) suggest that side-chain backbone interactions determine the preferred time-average conformation of a peptide unit. However, further stability of the conformation of peptide 1–36 through limited long-range

interactions cannot be ruled out. This peptide carries segment 1–16 which, when free, is completely insoluble and in fact forms one of the insoluble core tryptic peptides (Edmundson, 1963). Accordingly, amino acid composition will explain the strong aggregation property of peptides 1–36 (Atassi and Singhal, 1970a). Therefore, we do not think the aggregation of this peptide bears a relationship to a role it might have in initiating protein folding. The aggregation makes interpretation of the results for this peptide difficult. It is pertinent to mention that a longer peptide comprising residues 1–55 has been studied by Epand and Scheraga (1968). The longer peptide 1–55 appears to be less rotatory than the shorter peptide 1–36.

Peptide 37–87 comprises the incomplete helices C, D, E, and the bends between them, together with bend EF. The present results show that this peptide tends to be less helical in solution than peptide 1–36. However in 64% methanol the rotatory behaviors of these two peptides become similar. In peptide 37–87, within the native protein, 34 residues are present in a helical conformation (*i.e.*, 67% helical). Therefore, the appreciable helical character found for this peptide in solution is to be expected, and is probably mainly effected through short-range interactions.

The case of peptide 1–87 is interesting since it overlaps both peptides 1–36 and 37–87 and possesses a theoretical helical content of 79% in the intact protein. This peptide shows an appreciable helical character in solution. Long-range interactions have a good possibility of contributing to the stability of the conformation of peptide 1–87. However, since the optical rotatory dispersion and circular dichroism parameters of this peptide were not appreciably higher than the corresponding parameters of peptide 37–87, it is obvious that backbone interactions play a major factor in this case.

Peptide 37–119 overlaps all of peptide 37–87 and the additional segment 88–119 comprises two complete helices (*i.e.*, F and G in native Mb) and an overall theoretical helical content of 74%. This peptide possesses the highest rotatory power of any Mb peptide so far studied. Its optical rotatory dispersion and circular dichroism parameters are only slightly lower than the corresponding values for ApoMb (see Table II). In this peptide, obviously, long-range interactions contribute appreciably to the stability of its conformation. This peptide occupies a region in the native molecule in such a way that long-range interactions within itself are feasible (Figure 8).

The shortest peptide studied in the present work (peptide 120–153) comprises a whole intact helix (helix H, residues 125–148) and appreciable portions of nonhelical structure (10 residues) with an overall helical content of 71% in the native protein. The rotatory behavior of this peptide suggests a lower contribution of helical structure than any of the other peptides studied here. A shorter peptide which comprises residues 132–153 has been studied by Epand and Scheraga (1968). That peptide contained only about two-thirds of the length of helix H whereas peptide 120–153 contains the whole intact helix. It is therefore significant to compare the behavior of these two peptides in order to investigate the effect of cleavage of a helix on its stability. The parameters $[m']_{233}$, b_0, and $[\theta]_{222}$ for peptide 132–153 (Epand and Scheraga, 1968) were: -2300 ± 250, -69 ± 6, and -1600 ± 200. For the longer peptide 120–153, the corresponding parameters (present work) are -3200 ± 100, -106 ± 15, and -6100 ± 150 (for $[\theta']_{221}$), respectively. Measurements were carried out in water in each case. These differences, which are well outside experimental error, demonstrate that peptide 120–153 achieves a higher helical character than the shorter peptide 132–153. It is therefore suggested here that, on cleavage of a helix into two fragments, neither fragment will be able to attain a high degree of helicity as compared to when the fragments are linked within an intact longer helix.

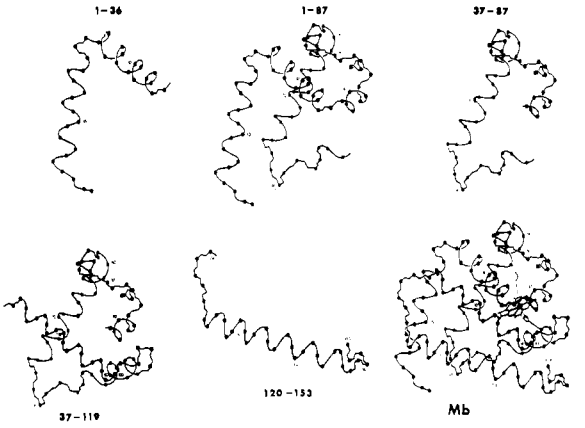

FIGURE 8: Diagram of mode of folding of myoglobin and of peptides, obtained by cleavage at proline, *within* the intact protein. It is not implied in any way that the indicated shape is necessarily the pattern of folding that the peptides will assume in the free state. It is only given to show the original (*i.e.*, before cleavage) conformation of each segment which might help explain any tendency to assume helical structure and the existence of any stabilizing long-range interactions.

Due to many complications that have been outlined by various workers (Beychok, 1968; Schellman and Lowe, 1968; Woody and Tinoco, 1967; Greenfield *et al.*, 1967; Epand and Scheraga, 1968), it is not easy to correlate transitions responsible for optical rotatory dispersion and circular dichroism behavior with helical contents of proteins, even though satisfactory results have been obtained for Mb by many workers (Urnes and Doty, 1961; Urnes, 1965; Breslow *et al.*, 1965; Harrison and Blout, 1965; Andres and Atassi, 1970; Atassi, 1970). The helical contents for the present peptides have been calculated together with those of Mb and ApoMb using our present optical rotatory dispersion and circular dichroism parameters. These helical contents are summarized in Table III. Bearing in mind the foregoing complications, the present helical contents should be considered only as useful estimates. It can be seen that these peptides possessed considerable helical character in water solutions ranging between 30 and 70% that of the helical content of Mb and 40–80% as helical as ApoMb. Perhaps, for the present work, ApoMb may be considered as the proper parent protein. The location of the present peptides and their mode of folding *in the native protein* is shown in Figure 8. It is not implied in any way that the indicated shape is necessarily the pattern of folding the peptides will assume in the free state. However, it is felt that this diagram will be very useful for understanding the reason for tendency to assume helical structure and the existence of any stabilizing long-range interactions.

Perhaps a good way to compare helicity of these peptides is to consider their relative, rather than their observed absolute, helical contents. This will take into account that these

TABLE III: Helical Contents of Various Peptides, Myoglobin, and Apomyoglobin from Optical Rotatory Dispersion and Circular Dichroism Parameters.[a]

Peptide or Protein	Solvent	$[m']_{199}$	$[m']_{233}$	b_0	$[\theta']_{220}$
1–36	Water	30	24	30	30
	64% methanol		40	53	53
1–87	Water	29	21	29	40
	64% methanol		38	51	53
37–87	Water	28	19	26	29
	63% methanol		36	50	34
37–119	Water	41	34	45	50
	63% methanol		49	61	61
120–153	Water	18	12	20	28
	62% methanol		33	45	39
Mb	Water	66	64	77	78
ApoMb	Water	47	48	58	58

[a] Values calculated as described in the text.

TABLE IV: Expected and Found Helical Contents and Ratios of These Contents for Various Peptides.[a]

Solvent:		Water		63% Methanol	
Peptides	A % Helical Content in Intact Protein	B % Helical Content Found	B/A	B % Helical Content Found	B/A
1–36	97	28	0.289	49	0.505
1–87	79	30	0.380	47	0.595
37–87	67	25	0.373	40	0.597
37–119	74	42	0.568	57	0.770
120–153	71	19	0.268	39	0.549

[a] The helical contents found are obtained from Table III and represent the average of the helical contents calculated from various optical rotatory dispersion and circular dichroism parameters.

peptides differ in their theoretical helical contents in the native protein and this might influence the degree of helicity they assume in the free state. The relative helical content will be expressed here by the helical ratio which is the ratio of the observed helical content over that expected from the location of the peptide in the three-dimensional structure of the native protein. These ratios are shown in Table IV for each peptide, both in water and in 63% methanol. From this, it can be seen that the ability of a peptide to assume helical structure is not related to the helicity of the peptide in the native protein. The lowest helical ratios were observed with those peptides in which long-range interactions may not contribute to the stability of their structure (*i.e.*, peptides 1–36 and 120–153). It is noteworthy that these two peptides had similar helical ratios, despite the fact that the helical contents expected from their location in the three-dimensional structure of Mb differed markedly. The highest helical ratio was exhibited by peptide 37–119 and it was almost twice the ratio found for peptides 1–36 and 120–153, the helicity of which is presumably almost entirely due to short-range interactions. This indicates that the conformation of peptide 37–119 is strongly stabilized by long-range interactions. The helicity of each of these peptides increases in methanol giving, therefore, higher helical ratios. The increase in the magnitude of the Cotton effect in methanol may of course be partly due to aggregation.

We have studied the interactions of these peptides with antisera to Mb (Atassi and Singhal, 1970a) and found that larger amounts of peptide (relative to the homologous antigen) were required to achieve maximum interaction with antisera to Mb. Similar behavior was previously observed with peptides obtained by cleavage at the methionine sites (Atassi and Saplin, 1968). As was suggested earlier (Atassi and Saplin, 1968) this can be explained on the basis that the conformations of the peptide in the free state and in the intact protein are different.

Since the helical contents of the N-terminal peptides 1–36 and 1–87 were much lower than the theoretical helical contents expected from their mode of folding in Mb, some conclusions can be made concerning folding during biosynthesis. The results may suggest that proteins will not start to assume their native three-dimensional conformation during biosynthesis until virtually or entirely completed, depending on the extent of contribution to long-range interactions of the yet-unsynthesized portion. Recent studies on the folding of staphylococcal nuclease (Taniushi and Anfinsen, 1969) were in agreement with the present conclusions. These conclusions do not agree with the hypothesis of Phillips (1967). However, one complication arises from the aggregation of peptide 1–36. The environment of residues in an aggregated peptide will not be similar to that they have in the native protein. Also, of course, the environment of a peptide in solution does not resemble that of a growing peptide on the ribosome. Clearly, therefore, it is not possible to test this hypothesis unambiguously at the present time.

In conclusion, the present findings show that by cleavage of myoglobin at proline peptide bonds, intact helices were isolated. Studies of the conformations of these were extremely valuable since complications that arise due to cleavage of a peptide within a helix were avoided. This has been a limiting factor in previous studies since the two separated fragments of a helix might not be able to attain the helical shape. A minimum number of amino acid residues (nine or more) is required before helicity can be detected in synthetic polyamino acids (Goodman *et al.*, 1963). The present work, carried out with peptides comprising intact whole helices and their overlaps thereof, demonstrates the appreciable contribution of short-range backbone interactions, especially from the results on peptide 120–153. Long-range interactions become more important in longer peptides especially with peptide 37–119. This peptide possesses an elaborate mode of folding well-stabilized by long-range interactions. Finally it may be concluded that a helical segment in a protein becomes less helical in the free state when isolated with the helix (or helices) intact. This again points strongly to the stabilizing

effect of long-range interactions on the folding of the protein.

Acknowledgments

The authors thank Mrs. Marie Perlstein and Mr. John Holodnick for their expert assistance.

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