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Reversible Conformational Changes of Myoglobin and Apomyoglobin

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The "thermodynamic" hypothesis of protein conformation (1) states that the sequence of amino acids characteristic of a given protein is sufficient to determine its secondary and tertiary structure, for the molecule will assume the conformation thermodynamically most stable. In an analogous vein, the "conformation" hypothesis (2) proposes that the steric and noncovalent bonding properties of poly-α-amino acid side chains dictate the tendency of these polymers to assume helical, extended, or random coil structures. These hypotheses need little alteration in order to include the possibly significant role of small cofactors and prosthetic groups. Such small molecules may interact with a protein in a specific fashion and thereby influence the relative stability of various folded states.

Myoglobin and apomyoglobin offer an attractive system for evaluating the above hypotheses. A comparison of conformation-dependent parameters of the native and apoproteins will reveal what role, if any, is played by the heme moiety in the folding of myoglobin, while a study of reversible conformational changes of apomyoglobin will reflect the adequacy of the amino acid sequence in dictating the structure. The absence of sulfhydryl side chains or disulfide bridges in these proteins is particularly important. The elegant work of Anfinsen on the reversible unfolding of ribonuclease (3), which has placed the "thermodynamic" hypothesis on a secure experimental basis, depends on the oxidation of reduced disulfide bonds. It is thus not capable of distinguishing the role of the amino acid sequence in general from that of half-cystines in particular. The absence of this complicating factor in the myoglobin-apomyoglobin system therefore provides considerable simplification of interpretation.

As an indication of conformational changes, we have used optical rotatory dispersion. Recent advances in our understanding of this technique as a measure of α-helical secondary structure (4–11) and recent improvements in instrumentation (12) have increased our confidence in the use and interpretations of ordinary rotatory dispersion. In this paper we report data which indicate (*a*) that a small loss in helix content, which is recovered on reconstitution, accompanies removal of the heme prosthetic group from myoglobin; (*b*) that the loss of helical structure of apomyoglobin that occurs on addition of urea can be completely reversed by dialysis against water or buffer; and (*c*) that apomyoglobin, restored in this manner to its native conformation, is capable of combining with heme to form myoglobin.

EXPERIMENTAL PROCEDURE

Reagents

Sperm whale myoglobin, supplied as a paste in saturated $(NH_4)_2SO_4$, was the generous gift of Dr. A. B. Edmundson. Acetone for the apoMb[1] preparation was Mallinckrodt spectral grade. Heme for recombination studies was prepared by dissolving hemin (Nutritional Biochemicals Corporation) in 0.1 N NaOH and diluting into buffer. Urea (reagent grade) was recrystallized from ethanol.

Preparation of Apomyoglobin

ApoMb was prepared by the method of Theorell and Åkeson (13). An aliquot of Mb paste was dissolved in a small amount of water, dialyzed overnight at 4° against a large volume of 0.04 M phosphate buffer, pH 6.8, and then dialyzed exhaustively (again at 4°) against deionized water. The final Mb concentration was 0.5 to 3%. The cold Mb solution prepared in this manner (also used for Mb measurements) was added dropwise and with vigorous (magnetic) stirring to 20 volumes of acetone, which had been acidified by addition of 4 ml of 1 N HCl per liter and chilled to −15°. The precipitated apoMb was then collected by centrifugation at −10°, redissolved in a small volume of chilled, deionized water, and dialyzed in the cold, first against two or three changes of dilute (100 mg per liter) $NaHCO_3$ and then exhaustively against deionized water. Any traces of precipitate present at the end of dialysis were removed by centrifugation. The protein could be stored in ion-free solution in the cold room for several weeks, although most experiments were performed within a week of preparation.

ApoMb was characterized by determining the ratio of optical density at 408 and 280 mμ and by recombination studies. The former test gave an estimate of residual uncleaved Mb (values ranged from less than 1% to about 4%), while the latter served as a criterion for "native" globin structure. Globin preparations which adsorbed more than 1 heme molecule per chain were considered to be partially denatured and were discarded.

Concentration Determinations

Mb and apoMb concentrations were determined spectrophotometrically at 280 mμ on a Cary model 15 spectrophotometer. The molar extinction coefficients, shown in Table I, were calculated on the basis of nitrogen analyses by the Kjeldahl method. Some scatter in the Kjeldahl measurements was experienced, leading to the estimated limits of accuracy shown. Our value for ϵ_{280} of Mb is somewhat higher than those reported by Hermans (14) and by Breslow (15), but only slightly larger than that obtained by Urnes (8).

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[1] The abbreviations used are: Mb, myoglobin; apoMb, apomyoglobin.

TABLE I

Molar extinction coefficients

Globin	ϵ_{408}	ϵ_{280}
Myoglobin	$17.9 \pm 0.2 \times 10^4$	$3.45 \pm 0.05 \times 10^4$
Apomyoglobin		$1.59 \pm 0.08 \times 10^4$

Reconstitution of Myoglobin

Mb was reconstituted from globin and heme by the procedure of Breslow (15). Heme was dissolved in 0.1 N NaOH diluted into 0.1 M borate buffer, pH 9.2, and added immediately in appropriate quantities to globin aliquots that had been dialyzed against or diluted into the same borate buffer. Any delay in addition of heme to globin after the former had been dissolved led to incomplete recombination.

Optical Rotatory Dispersion

Optical rotatory dispersion measurements were performed on a Cary model 60 recording spectropolarimeter. Studies at 5° were performed with the aid of a jacketed cell.

Analysis of Optical Rotatory Dispersion Data

Several alternative methods of analyzing the optical rotatory dispersion data were employed.

"Classical" Moffitt Equation—For rotations at wave lengths greater than 300 mμ, the Moffitt-Yang equation,

$$[R'] = \frac{a_0\lambda_0^2}{\lambda^2 - \lambda_0^2} + \frac{b_0\lambda_0^4}{(\lambda^2 - \lambda_0^2)^2} \quad (1)$$

was applied, based on $\lambda_0 = 212$ mμ, and $[R']$ = the reduced mean residue rotation. a_0 and b_0 were calculated from the intercept and slope, respectively, of the $[R'](\lambda^2 - \lambda_0^2)$ *versus* $1/(\lambda^2 - \lambda_0^2)$ plot. For limiting values of b_0 we have used −700 for a fully helical conformation and +100 for a completely random coil. These values are based on measurements of polypeptide rotatory dispersion and are discussed in the paper of Shechter, Carver, and Blout (11).

"Modified" Moffitt Equation—Urnes (8) has shown that rotatory dispersion data between 240 and 300 mμ can best be fitted by raising λ_0 in Equation 1 to 220 mμ. A similar analysis has been described by Leonard and Foster (16). The limits for b_0 in this "modified" Moffitt treatment are −390 for fully helical substances and +90 for random coils. This calibration is based on the same data used to determine the limits for b_0 in the "classical" Moffitt treatment and the helix content scale for the modified two-term Drude equation discussed below. It differs slightly from the calibration employed by Urnes, but we have used it here to preserve consistency with the other methods of analysis. Since the contribution in the 240 to 300 mμ region from heme Cotton effects in the visible spectrum is small, this approach can be used to compare Mb and apoMb rotations.

Modified Two-term Drude Equation—Shechter and Blout (9) have recently proposed the use of the modified two-term Drude equation,

$$[R'] = \frac{A_{193}\lambda_{193}^2}{\lambda^2 - \lambda_{193}^2} + \frac{A_{225}\lambda_{225}^2}{\lambda^2 - \lambda_{225}^2} \quad (2)$$

for fitting the rotatory dispersion above 280 mμ of polypeptides and proteins which contain α-helix, random coil, or a mixture of both. The parameters A_{193} and A_{225} are obtained by plotting

$$[R']\left(\frac{\lambda^2 - \lambda_{193}^2}{\lambda_{193}^2}\right)$$

against

$$\left(\frac{\lambda_{225}^2}{\lambda^2 - \lambda_{225}^2}\right)$$

and using the relations

$$\text{Slope} = \frac{\lambda_{225}^2 - \lambda_{193}^2}{\lambda_{193}^2} A_{225}$$

and

$$\text{Intercept} = \frac{\lambda_{225}^2}{\lambda_{193}^2} A_{225} + A_{193}$$

In substances which may be regarded as mixtures of helical and random conformations, A_{193} and A_{225} are both linear functions of helix content, and hence they are linearly related to each other. The precise linear relationship depends on the solvent. Two well separated lines have been obtained (10), one for polypeptides and fibrous proteins in water, the other for polypeptides and proteins in various organic solvents of low dielectric constant. The significance of this distinction for globular proteins will be discussed below. The limiting values for fully helical conformations used for A_{193} are +2900 and +3020, and for A_{225} they are −2050 and −1900 (in aqueous and organic solutions, respectively). The limiting values for completely random conformations used for A_{193} are −750 and −600, and for A_{225} they are −60 and zero (in aqueous and organic solutions, respectively).

Far Ultraviolet Cotton Effects

Because of the present lack of adequate calibrations for $[R']_{233}$ and $[R']_{199}$, we have not calculated helix content based on rotations at these wave lengths. We have, however, taken the absence of Cotton effects, other than those reported for helix-random systems (6, 7), as evidence for the absence of structures other than α-helix or random coil.

Calculations

In the expression

$$[R'] = \frac{3}{n^2 + 2}\frac{M}{100}[\alpha]$$

values for the dispersion of the refractive index of water and 8 M urea were obtained from the tables collected by Fasman (17). The refractive index of 8 M urea was extrapolated to 210 mμ by using the Sellmeier formula,

$$n^2 = 1 + \frac{a\lambda^2}{\lambda^2 - \lambda_v^2}$$

M for Mb was taken as 116.4, and for apoMb, as 112.4; these mean residue weights may be calculated from the amino acid composition of sperm whale Mb as determined by Edmundson and Hirs (18).

RESULTS AND DISCUSSION

Optical rotatory dispersion curves for Mb, apoMb, and Mb reconstituted from the apoprotein by addition of heme are dis-

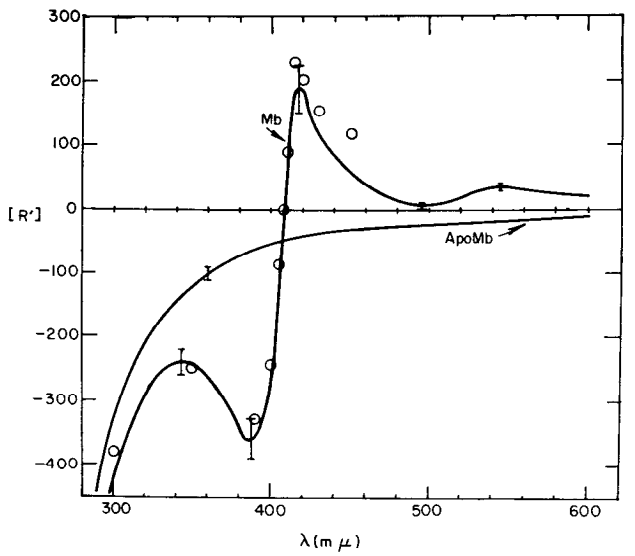

FIG. 1. Optical rotatory dispersion, plotted as *reduced mean residue rotation*, [*R'*], of myoglobin at 30°, apomyoglobin at 5°, and reconstituted Mb at 30°, all in water. The *solid curves* show results for Mb and for apoMb. The *open circles* indicate [*R'*] values for reconstituted Mb.

played in Figs. 1 to 3.[2] A significant difference between Mb and apoMb rotation in the far ultraviolet regions is immediately evident. In particular, the magnitudes of the now familiar peptide Cotton effect are distinctly less with apoMb than with the complete heme protein. The decrease in rotation, effected by extraction of the heme, is fully reversible in good preparations (*circles*, Figs. 1 to 3). Although the curves in these figures are from measurements performed on solutions of the proteins in salt-free water, results identical with those shown were obtained in 0.1 M KCl and in 0.1 M borate buffer, pH 9.2. The measured rotations were also independent of temperature between 5° and 30°.

Since Cotton effects with extrema at 199 and 233 mμ may be associated with the presence of α-helix (4, 6, 7), the observations at these wave lengths indicate a loss in helix content attendant on the removal of the prosthetic group. Analysis of the visible and near ultraviolet rotatory dispersion confirms this conclusion. The various parameters, extracted from our data by the three methods discussed above, appear in Table II. Although the various estimates of helix content differ slightly from one another, they are consistent in showing a smaller degree of helical structure in apoMb as compared with Mb. The value of between 51 and 60% helix for the former is in reasonable agreement with other investigations of this protein (19, 20). The measurements on Mb are also consistent with previous reports (21, 22).

Of some interest is the treatment of the rotatory dispersion of apoMb according to the two-term Drude equation (9, 10). The parameters A_{193} and A_{225} are found to be related in the manner typical of polypeptides and proteins dissolved in *organic solvents*, although the protein in these experiments was of course in an aqueous medium. We interpret this at first somewhat surprising result as a reflection of the local environment of the helix peptide bonds in apoMb. If large portions of the α-helical segments are buried "within" the globular spheroid, they will be, in effect, in small, organic solvent-like regions. In crystalline Mb, many stretches of helix are indeed "buried," at least on one side (23). Since apoMb sediments at nearly the same rate as native Mb (15), it too is probably folded in a compact fashion, with effective local surroundings for many helical regions much as if they were in organic solvent. If this interpretation is correct, then the proper calibrating parameters are those obtained from polypeptides in organic solvents, and the modified two-term Drude estimate of apoMb helix content in Table II has been calculated on this basis. A similar analysis is not possible for Mb, because of the presence of heme Cotton effects at visible frequencies.

The use of the "modified" Moffitt equation at short wave lengths gives a b_0 of −250 and a helix content for Mb of about 71%. This is in excellent agreement with Urnes' (8) b_0 of −258 (uncorrected for Soret Cotton effect); as he has demonstrated, correction for small contributions of the Soret Cotton effect should increase the value for the helix content by only about 2%.

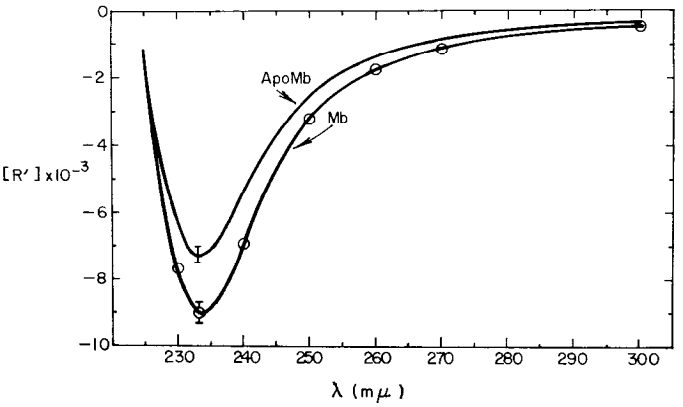

FIG. 2. Optical rotatory dispersion, plotted as *reduced mean residue rotation*, [*R'*], of myoglobin at 30°, apomyoglobin at 5°, and reconstituted Mb at 30°, all in water. The *solid curves* show results for Mb and for apoMb. The *open circles* indicate [*R'*] values for reconstituted Mb.

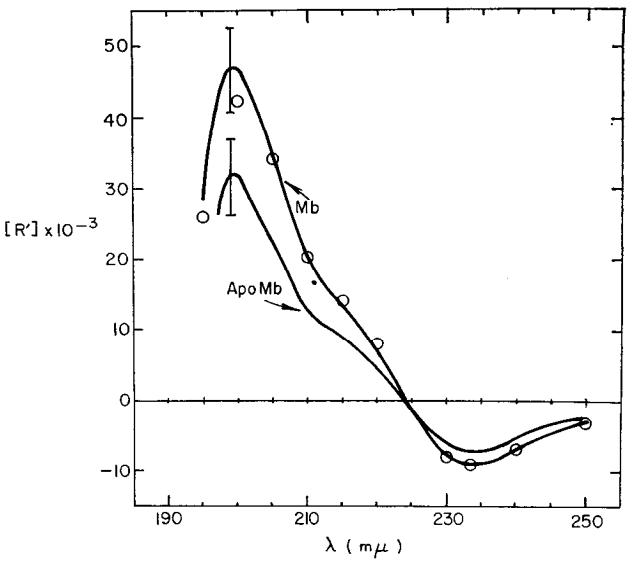

FIG. 3. Optical rotatory dispersion, plotted as *reduced mean residue rotation*, [*R'*], of myoglobin at 30°, apomyoglobin at 5°, and reconstituted Mb at 30°, all in water. The *solid curves* show results for Mb and for apoMb. The *open circles* indicate [*R'*] values for reconstituted Mb.

[2] The asymmetry of the Soret Cotton effect and its failure to return to zero on the negative limb have been substantiated for our Mb sample by numerous measurements, although these observations differ from other published results.

TABLE II

Optical rotatory parameters

Material	"Classical" Moffitt equation (λ_0 = 212 mμ)			"Modified" Moffitt equation (λ_0 = 220 mμ)			"Modified" two-term Drude equation			Far ultraviolet	
	a_0	b_0	Helix[a]	a_0	b_0	Helix[a]	A_{193}	A_{225}	Helix[b]	$[R']_{233}$[c]	$[R']_{199}$[d]
			%			%			%		
Mb				−45	−250	71				−9,000 ± 300	+47,000 ± 6,000
ApoMb	−17	−310	51	−39	−199	60	1,260	−950	51	−7,300 ± 300	+32,000 ± 6,000
ApoMb in 4 M urea	−340	−62	20				280	−490	25	−4,300 ± 300	
ApoMb in 8 M urea	−755	+143	0	(−520	+30	12)[e]	−870	0	0	−2,500 ± 300	
Renatured apoMb	−31	−280	48				1,140	−870	47	−7,000 ± 300	
Reconstituted Mb				−79	−244	70				−9,000 ± 300	+43,000 ± 6,000
Mb, reconstituted after exposure of apoMb to 8 M urea				−97	−230	67				−8,700 ± 300	

[a] Calculated from b_0. For calibration of percentage of helix, see the text.
[b] Calculated according to References 9 and 10. For calibration of percentage of helix, see the text.
[c] Rotation trough.
[d] Rotation peak.
[e] Plot curved.

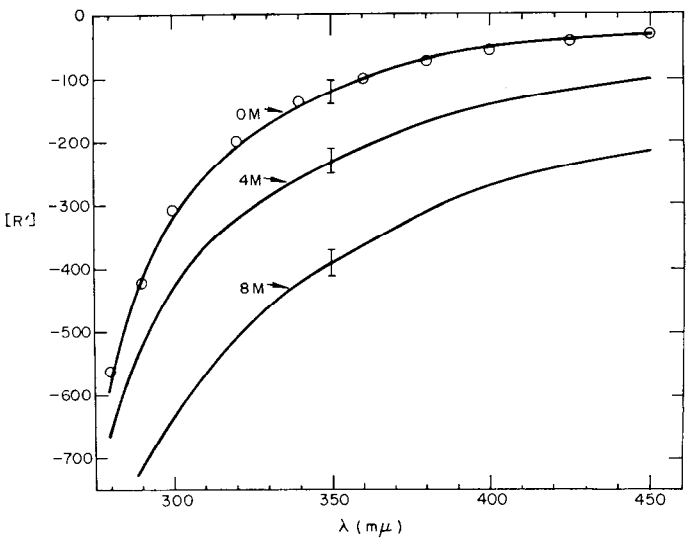

FIG. 4. Optical rotatory dispersion, plotted as *reduced mean residue rotation*, [*R'*], of apoMb in 0, 4, and 8 M urea (*solid curves*) and of apoMb renatured by dialysis against water after exposure to 8 M urea (*open circles*).

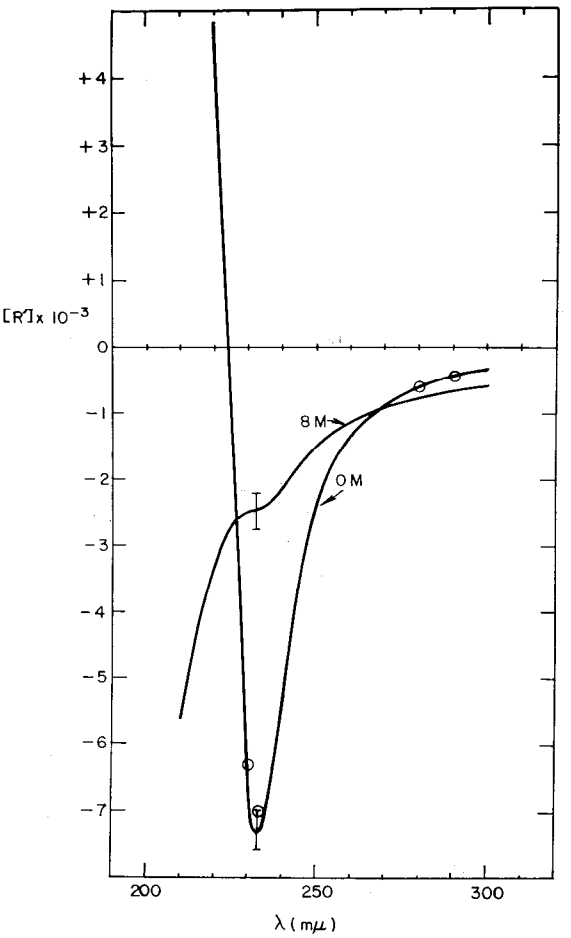

FIG. 5. Optical rotatory dispersion, plotted as *reduced mean residue rotation*, [*R'*], of apoMb in 0 and 8 M urea (*solid curves*) and of apoMb renatured by dialysis against water after exposure to 8 M urea (*open circles*).

The optical rotatory dispersion data, both in the visible and in the far ultraviolet Cotton effects, are therefore consistent with a loss in helix content on removal of the heme from Mb. These data by themselves, however, cannot distinguish between two possible alternatives: (*a*) a relatively small change in helix content occurring in all the molecules, and (*b*) a large change in helix content in some fraction of the molecules while the remainder retains its full helical secondary structure. However, other lines of evidence argue for a homogeneous change. (*a*) Theorell and Åkeson, in their original paper on the preparation of apoMb (13), reported ultracentrifugal homogeneity of the apoprotein. Since loss of a major part of the helix content in a small fraction of the apoMb molecules should result in a considerable expansion of these molecules and hence a polydisperse ultracentrifuge pattern, their observation renders this eventuality unlikely. (*b*) Breslow (15) has shown titration curve and side chain reactivity changes after heme removal which are consistent only with a conforma-

tional difference involving all the protein molecules present. The unmasking to bromoacetate of all but one of four, usually masked, imidazoles, for example, cannot be accounted for by preferential denaturation of some small fraction.

Optical rotatory dispersion curves for apoMb in 4 and 8 M urea are compared with the curve for the untreated protein in Figs. 4 and 5. It is at once evident that a drastic loss of helix content has occurred. Analysis of the visible and near ultraviolet rotation by various methods leads to the results included in Table II. Both the "classical" Moffitt analysis and the modified two-term Drude equation indicate that 8 M urea has destroyed all the helical structure in apoMb. Furthermore, the values of A_{193} and A_{225} obtained in 8 M urea now fall on the line calibrated with polypeptides in *aqueous* solution. This result strengthens our hypothesis above concerning the effect of an organic solvent-like interior in the globular protein. In urea solution, considerable unfolding should bring all the peptide bonds into contact with the aqueous solvent and thereby alter the local dielectric constant.

Removal of urea by overnight dialysis against several changes of water or buffer results in a restoration of the rotatory parameters of native apoMb, as shown by the *open circles* in Figs. 4 and 5 and by the calculated parameters in Table II. The renatured apoprotein is also capable of combining in a 1:1 manner with heme to yield myoglobin. The recombination, as before, was checked both by spectrophotometry and by rotatory dispersion in the visible and ultraviolet regions. The recovery of the Soret absorption, of the associated Cotton effect, and of rotation at 233 mμ all indicate that nearly 100% of the renatured apoMb was capable of recombining with heme. This restoration of combining power on removal of urea is the most stringent test of recovery of the native globin conformation. It therefore demonstrates the true reversibility of the structural changes produced by 8 M urea.

SUMMARY

We have carried out, with essentially complete reversibility, the following sequence of changes on sperm whale myoglobin: heme removal (loss of about 20% helix), urea "denaturation" (complete loss of helix), "renaturation" (recovery of native apomyoglobin helix content), and reconstitution (recovery of full myoglobin helix content). We regard the reversibility of these steps as strong support for the "thermodynamic" and "conformation" hypotheses of protein structure. The total loss of apomyoglobin helix in 8 M urea is of particular importance. Previous attempts to study reversible conformational changes have not included a good estimate of the degree to which native structure has been altered (24, 25). Our results show that all peptide hydrogen bonds in the helical regions have been broken and hence that most or all of the specific side chain interactions in these segments have been destroyed. Furthermore, we have no indication from the rotatory dispersion data that other conformations (such as β structures) have been formed. Apomyoglobin in 8 M urea may therefore be regarded as an entirely disordered chain. Its ability to form again, after removal of urea, the native structure as determined by optical rotatory dispersion and by the specific adsorption of heme validates the conclusion that the amino acid sequence alone is sufficient to determine the conformation assumed by the protein in solution.

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