MEASUREMENT OF LIGAND-INDUCED CONFORMATIONAL CHANGES IN HEMOGLOBIN BY CIRCULAR DICHROISM

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Abstract.—The UV circular-dichroism spectra of human and horse hemoglobins have been determined at various degrees of partial saturation with oxygen. Spectra of the two native hemoglobins were compared with spectra of the corresponding proteins modified with a reagent known to eliminate the conformational rearrangement normally associated with cooperativity. Such comparison indicates that one region, around 260 mμ, is sensitive chiefly to the state of the hemes; changes in another region, around 285 mμ, may be correlated with the conformational transformation linked to cooperative interactions. All circular-dichroism changes are strictly linear with fractional saturation with oxygen. Possible implications of these results to recently proposed mechanisms for cooperativity in proteins are discussed.

The cooperative binding of oxygen to hemoglobin results from interactions among the subunits of the protein. These subunit interactions are mediated through conformational changes in the component α and β polypeptide chains. Significant changes in the relative orientations of the chains upon oxygenation have been detected in crystals of horse oxyhemoglobin,[1, 2] but measurements of the protein in solution have yielded only indirect evidence for these changes as well as for more subtle conformational perturbations within individual subunits.[3] Methods based upon optical rotatory power may be especially sensitive to slight alterations in the asymmetric environment of chromophoric groups and could be very useful for analysis of conformational changes in hemoglobin solutions. We have attempted to sort out from all the changes in the circular-dichroism spectra of hemoglobin accompanying exchange or loss of ligands those changes which might be specifically related to a functionally significant conformational change. In particular, we have tried to distinguish between circular-dichroism changes associated with the state of the hemes, or with conformational perturbations which have no other detectable effects on the protein, and changes associated with the conformational rearrangement essential for cooperative interactions and ligand-induced alterations in the values of linked functions. To facilitate this objective, we have used a chemically modified hemoglobin derivative, in which the conformational rearrangement that is critical for cooperativity has been eliminated. A circular dichroism change correlated with a functionally important conformational change would be a convenient assay for the extent of conformational rearrangement as a function of the ligand bound and could supply evidence for appraising the validity of several schemes recently proposed to account for cooperative interactions in hemoglobin.

Materials and Methods.—Several ultraviolet absorption bands of hemoglobin are associated with optical activity.[4–7] Because of the complexity of the spectrum in this region, measurements of circular dichroism are more readily interpretable than the broad, over-

lapping patterns obtained from optical rotatory dispersion. Several previous circular-dichroism and optical rotatory dispersion studies of hemoglobin in the far ultraviolet have indicated that upon oxygenation, there are large ellipticity changes in the region from 250 to 300 mμ.[4-6] The absorption bands in this region have not been assigned uneqivocally. Circular-dichroism changes also occur in the region below 250 mμ; these probably arise from alterations in the polypeptide backbone, but they are too small to permit statistically valid studies as a function of ligand binding.

Our studies were carried out on a Cary 60 spectropolarimeter eqipped with a circular-dichroism attachment. A three-second time constant and a slit program which maintained a 1-mμ band pass were used for all experiments. The temperature of the cell compartment was kept at 26°C. Normal human and horse hemoglobins were prepared by the method of Drabkin.[8] Hemoglobins were modified by reaction with the bifunctional reagent, bis(N-maleimidomethyl)ether (BME)* according to the procedure of Simon and Konigsberg.[9] Solutions of 4 mg of hemoglobin per ml of 0.1 *M* phosphate buffer, pH 7.2, were equilibrated with various partial pressures of oxygen in a tonometer based upon a design of Spoek *et al.*[10] equipped with a cylindrical cell of 2.0-mm path length at its base. Oxy-, deoxy-, and met-hemoglobin were determined with a Cary 14 spectrophotometer, according to the procedure of Benesch *et al.*[11]

The complexity of the circular-dichroism spectra necessitated extensive processing of the data before meaningful interpretations could be made. First the observed circular-dichroism spectra of human and horse hemoglobins, both normal and BME-modified, were corrected for baseline drift and for the presence of methemoglobin, as assayed from the visible spectrum. Most samples contained less than 10% methemoglobin. The 34 corrected spectra were digitized and converted to values of molar ellipticity ($[\Theta]$)* at each of 22 wavelengths, giving a total of over 700 points. At each wavelength, the points were tested on each of the four hemoglobin samples for simple linear dependence upon Y, the fractional saturation of the protein with oxygen. A least-squares procedure was used to fit the data to this linear model, and all points were scanned for any systematic deviations. Less than 5% of the $[\Theta]$ values fell more than two standard deviations from the computed values based upon strict linearity, and were neglected. These stray points are probably due to instrumental transients, errors in positioning of the sample cell, or contamination of the cell windows with precipitated protein. No systematic deviations from linearity were encountered, for either normal or modified proteins.

Results and Discussion.—A typical set of experimental values, normalized to fractional change in ellipticity, for the four hemoglobin samples is illustrated in Figure 1. The solid line fits a strictly linear dependence of $[\Theta]$ at the selected wavelength upon Y. There is visible scatter of the experimental points, and, if only this single wavelength were used to analyze correlation between Y and $[\Theta]$, the statistical reliability of a linear relationship would be limited. By comparing observed values of Y, determined from visible spectra, with values computed from linear functions of $[\Theta]$ at several wavelengths, we have been able to establish a strictly linear dependence with greater confidence. Conventional statistical tests of the data for the four hemoglobins support this dependence with 99.5–99.8 per cent certainty, except for the case of human BME-hemoglobin, where random scatter reduces the certainty to about 95 per cent. Because of the excellent agreement of the data with a linear model, it was possible to extrapolate the observed ellipticity values to obtain spectra for fully deoxygenated ($Y = 0$) and fully oxygenated ($Y = 1$) hemoglobins. The extrapolated spectra of normal and modified horse and human hemoglobins are shown in Figures 2 and 3. The spectra are all in qualitative agreement with the results reported by Urry[6] and by Beychok *et al.*[4] The spectra are clearly complex, but for purposes

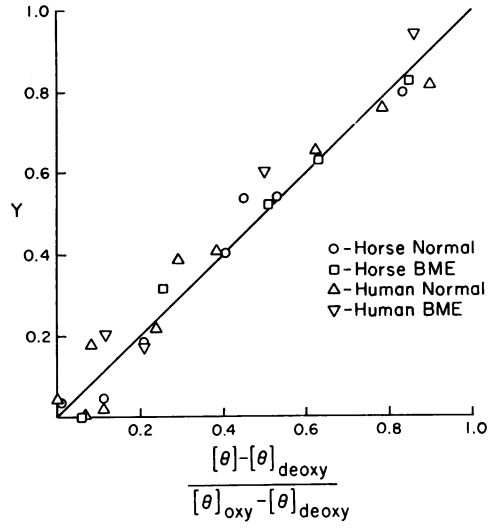

FIG. 1.—Molar ellipticities of four hemoglobin samples at 285.0 mμ, normalized to fraction of total change going from deoxy- to oxyhemoglobin, at different degrees of partial saturation with oxygen. Solid line represents strictly linear relationship.

of discussion, they may be divided into two broad bands. The first, located at around 260 mμ, has a molar ellipticity of $+ 3.5 \times 10^4$ in deoxyhemoglobin, and $+7.5 \times 10^4$ in oxyhemoglobin. The second band, at around 285 mμ, ranges in ellipticity from -1.5×10^4 to $+0.5 \times 10^4$, for deoxy- and oxyhemoglobin, respectively. Both bands are studded with fine structure, which may arise from interactions between the hemes and the aromatic amino acids of the polypeptide pockets in which they lie.[12] This fine structure is seen in all of the 34 spectra used in this study and is well above the signal-to-noise levels of our instrument. The spectra of normal and BME-modified human and horse methemoglobins, shown in Figure 4, are consistent with the spectrum reported by Urry for metmyoglobin.[6] Comparison of the spectra indicates that neither the difference in

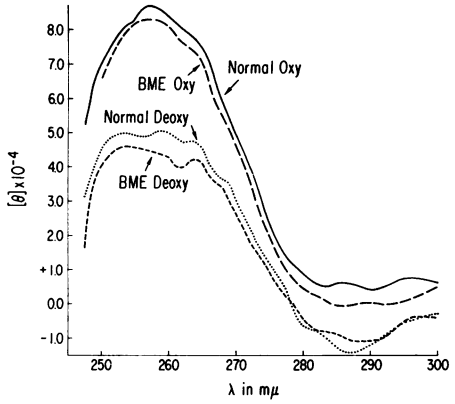

FIG. 2.—Circular-dichroism spectra of oxy- and deoxy-, normal and BME-modified horse hemoglobins, computed as described in text.

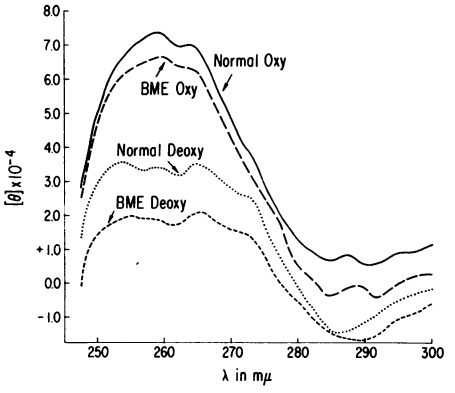

FIG. 3.—Circular-dichroism spectra of oxy- and deoxy-, normal and BME-modified human hemoglobins, computed as described in text.

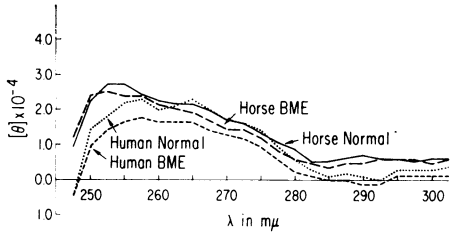

FIG. 4.—Circular-dichroism spectra of human and horse normal and BME-modified methemoglobins, traced directly from the computer plotter output.

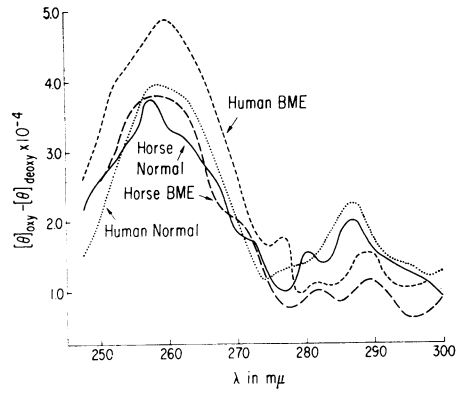

FIG. 5.—Oxy- minus deoxyhemoglobin difference spectra for human and horse normal and BME-modified hemoglobins, computed as described in text.

primary sequence between human and horse hemoglobins, nor the introduction of the bifunctional reagent BME results in a drastic alteration of the protein or its linkage with heme.

Examination of the difference spectra of oxy- minus deoxy- human and horse normal and BME-modified hemoglobins, as illustrated in Figure 5, reveals one region in which both BME-hemoglobins are distinct from their normal counterparts. The maximum range of [Θ] in the 285 mμ band of both BME-hemoglobins is only 55–65 per cent of the range found in normal human and horse hemoglobins. This reduction in the circular-dichroism change is fairly constant from 277.5 mμ to 292.5 mμ. There is also an apparent shift in the fine structure of the spectra of the two modified hemoglobins about 2.5 mμ to the red. The only other difference of any magnitude between the spectra of normal and BME-hemoglobins is an attenuation in the ellipticity of the 260 mμ band for human deoxy-BME-hemoglobin, although the data in this particular region showed the most scatter, and the magnitude of the difference is somewhat uncertain. The ratio of the oxy-minus deoxyhemoglobin difference spectra for BME-modified and normal proteins is close to unity from 257.5 mμ to 275 mμ. Since the ratio of the difference spectra is constant within each of the two regions and undergoes a sharp transition between them, it does not seem likely that the changes which we are observing are due to changes in a band of much greater intensity, centered at another wavelength, and tailing into the region that we have investigated.

To use physical data to evaluate possible mechanisms for cooperative phenomena in proteins, it is essential to establish that changes in experimental parameters are correlated specifically with cooperative processes and are not merely due to subunit changes which are not transmitted to neighboring subunits within the oligomer. The unique properties of BME-hemoglobins facilitate this distinction. Chemical modification of hemoglobin with BME has been shown to eliminate all cooperative interactions.[9] Horse hemoglobin modified in this fashion is constrained into a conformation essentially identical to that of normal horse

oxyhemoglobin, even when the derivative is deoxygenated.[13] The conformation of human BME-hemoglobin has not been studied by X-ray crystallography, but comparison of the solution properties of human and horse derivatives indicates that they may have different conformations: human BME-hemoglobin may maintain a structure more like that of human deoxyhemoglobin, even when oxygenated. In any case, with each derivative, the loss of all interactions is ascribed to the elimination of a functionally critical conformational rearrangement. Since loss of ligand from the constrained molecules is associated with an attenuated change in the circular-dichroism spectrum around 285 mμ, we feel that this region of the spectrum may be correlated with this functionally essential conformational change. A conclusion similar to this was reached by Beychok *et al.*,[4] who compared circular-dichroism spectra of liganded and unliganded isolated α and β chains with the corresponding spectra of intact hemoglobin molecules. In the region around 260 mμ, the observed spectrum of native hemoglobin was no different from a pattern obtained by summing the spectra of the isolated chains, for both oxygenated and deoxygenated protein. Around 285 mμ, on the other hand, the spectrum of normal deoxyhemoglobin could not be duplicated by adding the spectra of deoxygenated α and β chains. The sum of the spectra of oxygenated chains was very similar to that of normal oxyhemoglobin in this region. Since it is believed that no conformational rearrangement accompanies ligand exchange in the isolated chains, Beychok *et al.* concluded that the difference in the circular-dichroism spectra of isolated chains and intact hemoglobin in the region around 285 mμ could most reasonably be correlated with an oxygenation-linked conformation change in the native protein. The isolated chains, however, might be expected to have conformations which are substantially different from those which they assume within the intact hemoglobin molecule,[14] so that quantitative comparison of the circular-dichroism spectral changes of α and β subunits with those of the intact hemoglobin tetramer might not be easily interpreted.

We have also observed, in accord with Beychok *et al.*,[4] that the region of the circular-dichroism spectrum around 260 mμ has apparently only very limited sensitivity to protein conformation. However, this region is very responsive to the state of the heme. As can be seen by comparing the spectra of the BME- and normal hemoglobins in the oxygenated, oxidized, and reduced states—(1) with the possible exception of human deoxy-BME-hemoglobin, in each state the spectrum of the modified protein in this region is virtually identical with that of the parent, unmodified protein; and (2) oxyhemoglobin, in which the heme-complexed iron atoms are in the so-called "low spin" state, has a greater molar ellipticity than the two high spin complexes, viz. deoxy- and methemoglobin. Since crystallographic evidence indicates that oxy- and methemoglobin have the same conformation, this ellipticity difference cannot be ascribed to protein structural changes. However, more drastic conformational alterations, more extensive than those normally associated with ligand exchange, particularly if located in the immediate vicinity of the heme, might be expected to have some effect on this region of the circular-dichroism spectrum. Recent high-resolution X-ray studies on BME-hemoglobin have revealed shifts in the polypeptide backbone in the FG corner of the β chains, a region intimately associated with the heme binding

site.[15] In horse BME-hemoglobin, these conformational alterations are apparently not significant enough to affect the region of the circular-dichroism spectrum around 260 mμ. The anomalous circular-dichroism spectrum of human deoxy-BME-hemoglobin, however, might arise from more extensive distortions in the protein induced by the presence of the reagent. Crystallographic measurements of human BME-hemoglobin should help to clarify the relationship between conformation and this region of the circular-dichroism spectrum.

No definite assignment can be made to the residual difference in the 285 mμ region between the spectra of oxy- and deoxy-BME-hemoglobin. Possibly the state of the heme influences this region of the spectrum. Differences between the 285 mμ circular-dichroism bands of oxy- and deoxy-β chains, which, in the crystal, appear not to undergo any ligand-linked conformational change, are comparable in magnitude to those observed for BME-hemoglobins, although the individual spectra are quite different.[4] Comparison of Figures 2, 3, and 4 indicates only a very slight difference in this region between the spectra of oxy- and methemoglobin, which are also apparently identical in conformation. Certainly the contribution of the heme to the 285 mμ region is not comparable to that to the 260 mμ region of the circular-dichroism spectrum. The two high-spin complexes, methemoglobin and deoxyhemoglobin, give rise to ellipticities of opposite sign at 285 mμ.

A possible alternative source of the residual difference between the circular-dichroism spectra of oxy- and deoxy-BME-hemoglobin in the region around 285 mμ is a nonfunctional conformational change. The wavelength and fine structure associated with this region implicate aromatic amino acids, particularly tyrosine and tryptophan, as possible contributors. Tyrosine C7α and tryptophan C3β are particularly attractive candidates.[1] A slight rotation of one of these aromatic rings induced by ligand loss or exchange could give rise to a change in the circular-dichroism spectrum. However, unless this rotation were coupled to further conformational alterations, culminating in the subunit reorientation vizualized by Perutz *et al.*,[2] no functional interactions would result. According to this interpretation, the ellipticity change around 285 mμ in normal hemoglobin reflects a composite of two conformational changes, one of which is associated with cooperative interactions and changes in the values of linked functions, and one of which has little or no effect on the over-all physical and chemical properties of the molecule. These changes may not be discrete, but merely two degrees of rearrangement of the same residues. This interpretation must remain hypothetical until unambiguous spectral assignments are made.

Regardless of whether the residual circular-dichroism change at 285 mμ in BME-hemoglobin arises from the heme chromophore or from a nonproductive conformational change, the loss, in the BME-derivatives, of 40 per cent of the circular-dichroism change found in normal hemoglobin is consistent with the loss of a functionally critical conformation change. More complex interpretations, invoking additional nonproductive conformational perturbations in normal hemoglobin, are certainly not ruled out by our data. It seems unlikely, however, that only conformational changes which have no effect on cooperativity would be detected by circular dichroism, while the large ligand-linked translational and

rotational movements picked up by crystallographic measurements remain silent. The only difference that our previous experimental results have shown between normal and BME-hemoglobins is the absence, in the modified protein, of a conformational rearrangement essential for cooperativity; the circular-dichroism differences between the two proteins in this region are most reasonably correlated with this conformational change. Since the change in this region of the spectrum is best described as a strictly linear function of degree of oxygenation for both normal and BME-modified proteins, it would follow from this correlation that the functionally critical conformational change is also linear with oxygenation.

Proposed mechanisms for cooperativity in oxygen binding to hemoglobin have placed strong emphasis on conformational changes in the protein. The allosteric theory of Monod *et al.*,[16] in which reaction of hemoglobin with oxygen shifts an equilibrium between only two conformational states of the entire tetrameric molecule, defines a quantitative relationship between extent of conformational change and fractional saturation with ligand. Under the conditions that we used for oxygen binding, the allosteric theory predicts that, for normal hemoglobins, this relationship would deviate somewhat from strict linearity. On the other hand, in a model for cooperativity like those discussed by Koshland *et al.*,[17] conformational rearrangement within individual subunits must necessarily accompany reaction with oxygen. This would predict a simple linear dependence of conformational change upon ligand binding.

The recent studies of Antonini *et al.*,[22] Gibson and Parkhurst,[20] and Guidotti,[19] and the 2.8-Å resolution model of hemoglobin[21] all suggest that neither the original allosteric model nor the simple "square" model of Koshland *et al.* will adequately describe the interactions between α and β chains. A "rectangular" model, as outlined by Wyman,[23, 24] with one plane of strong interactions and one plane of weak interactions seems more consistent with the widely diverse experimental data. If the central postulate of the allosteric theory—that only two conformational states of the hemoglobin tetramer are present in significant proportions at any degree of ligand saturation—is incorporated into a rectangular model of this sort, the deviation of the conformation change-ligand binding relationship from linearity becomes much greater. This deviation is well outside the error of our measurements and would presumably have been detected if such conformational changes were measured, even only in part, by circular dichroism. Our results do agree with a model of hemoglobin cooperativity incorporating a single linear dependence of conformational change upon ligand binding, and are, therefore, consistent with the interpretations in support of models like those of Koshland *et al.*,[17] based upon spin-label experiments of McConnell and his collaborators.[18] We believe that we have subtracted nonfunctional conformational changes by comparing normal and BME-modified protein, and we may have overcome some of the limitations of the spin-label studies.

These results seem quite incompatible with an interpretation of the rectangular model incorporating a concerted transformation of the entire conformations of all four subunits, giving rise to only two conformational states of the hemoglobin tetramer. The multiplicity of conformational states, however, although apparently greater than the two postulated by the original allosteric theory, may

not be so great as predicted by Koshland *et. al.* More complex models would permit a combination of some concerted changes with other changes solely accompanying binding of ligand to the individual subunit. For example, if a concerted conformation change were to occur within the α and β subunits only along each plane of strong interactions, the resulting deviation from linearity in the conformation change-ligand saturation relationship might be so slight as to fit our experimental data. These complex models could not be tested by circular-dichroism measurements on native hemoglobin alone, assuming that all the conformational perturbations could be picked up in the spectra. We are currently appraising such models by further circular-dichroism studies on various modified hemoglobins, in which functional interactions may not have been totally eliminated, but selectively impaired.

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* Abbreviations used: BME, bis(N-maleimidomethyl)ether; [Θ], molar ellipticity, defined as $100\Theta/lc$, where Θ is observed ellipticity in degrees, l is the path length of the cell in cm, and c is the concentration of protein in moles/liter. In this study, the molecular weight of the monomeric subunit of hemoglobin, 16,113 daltons, was used for computing concentration.

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