Circular Dichroism of Hemoglobin in Relation to the Structure Surrounding the Heme

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SUMMARY

Circular dichroism (CD) of various kinds of hemoglobins and the protoporphyrin-globin complex has been measured over the range of 200 to 600 nm. All mammalian hemoglobins so far tested show CD spectra identical with those of human hemoglobin. Although the absorption spectra of lamprey hemoglobin and toad embryonic hemoglobin are similar to those of human hemoglobin, these hemoglobins show various CD spectra different from human hemoglobin in the Soret and visible regions. CD spectra of human γ chains are almost identical with those of β chains, however both are different from those of α chains. These various CD spectra may be attributed to the different structures of heme environments of these hemoglobins.

A protoporphyrin-globin complex exhibits distinctive CD bands both in the Soret and visible regions, indicating that contacts other than the iron-histidine bond make the tetrapyrrole ring optically active. The prominent positive ellipticity of deoxygenated human hemoglobin at 430 nm was decreased by either carboxypeptidase digestion or dialysis against deionized water. These treatments led to a loss of heme-heme interaction and to an increase in the oxygen affinity, without changing the gross structure of the molecule.

CD of hemoglobins and isolated chains are compared in order to obtain a better understanding of the structure of heme environments. The effect of treatments on CD spectra which specifically affect the oxygen-binding property of human hemoglobin are also reported.

EXPERIMENTAL PROCEDURE

Human hemoglobin was prepared according to the method of Rossi-Fanelli, Antonini, and Caputo (6). Crystalline horse heart myoglobin was prepared by the method of Theorell and de Duve (7). Separation of α and β chains was carried out as described previously (2) based on the method of Bucci and Fronticelli (8). Isolated γ chains of fetal hemoglobin were prepared by the method of Kajita, Taniguchi, and Shukuya (9). The γ chains were demercurated by passage through a sulphydryl Sephadex column as described for α and β chains (2), and then reduced by evacuation and addition of a small amount of sodium borohydride in a Thunberg tube. Purity of the isolated chains was checked by starch gel electrophoresis at pH 8.6. Lamprey hemoglobin (1) and toad egg embryonic hemoglobin (10) were prepared as reported previously. Hemoglobin of pig, cow, rabbit, and rat were prepared by hemolyzing washed erythrocytes with no further purifications. Globin was prepared from human hemoglobin by the method of Rossi-Fanelli and Antonini (11). Protoporphyrin was prepared by the method of Grinstein (12). Digestion of human hemoglobin by carboxypeptidase was carried out by incubating 85 mg of the hemoglobin in 5 ml of 0.05 M Tris buffer, pH 8.0, with 2 mg of carboxypeptidase A at 37° for 5 hours.

Deoxygenation was carried out by evacuating the samples in Thunberg-type quartz cells with 1- or 0.1-cm light path with occasional flushing of Q gas (helium-isobutane (99.05:0.95)). In some cases, deoxygenation was performed by adding a few grains of dithionite, which had no effect on CD spectra in the Soret and visible regions. Contamination of methemoglobin was checked by the conversion of methemoglobin into cyanmethemoglobin (13). Methemoglobin derivatives were prepared by adding a 2-fold molar excess of ferricyanide to solutions of oxygenated hemoglobins and the excess reagent was removed by a Sephadex G-25 column. Molar concentrations of hemoglobin and chains were determined spectrophotometrically after conversion into pyridine hemochromate by use of a millimolar extinction coefficient of 32.2 at 557 nm.

Absorption spectra were measured with a Hitachi EPS-2 recording spectrophotometer (Hitachi Electric Company, Ltd., Tokyo, Japan). Amino acid analysis was carried out with a...
Hitachi KLA-3B automatic amino acid analyser. CD measurements were performed at 25° on a JASCO ORD/SP spectropolarimeter (Japan Spectroscopic Company, Ltd., Tokyo, Japan). An aqueous solution of d-lo-camphor-sulfonic acid was used as a standard with an $\varepsilon_L - \varepsilon_R$ of 2.20 at 290 nm. All measurements were performed in 0.1 M phosphate buffer, pH 7.0. The results of CD are expressed in deg cm$^2$ per decimole on a heme basis.

RESULTS AND DISCUSSION

CD Spectra of Human Hemoglobin and Other Tetrameric Hemoglobins—Several features of CD spectra have been reported for human hemoglobin A or its subunits in the ultraviolet region (1, 3), in the Soret region (1-5), and in the visible region (4, 5). There are some disagreements between the spectra reported by different authors, as the CD is difficult to measure, especially in the Soret region because of the strong light absorption by hemoglobin. For example, Ueda, Shiga, and Tyuma (4) reported that their CD spectra did not obey Beer's law and that CD peak wave lengths varied with changes in optical path lengths for a given solution. To eliminate these uncertainties of CD spectra, we have confirmed that, under our experimental conditions, the CD obeys Beer's law with constant peak wave lengths and is reproducible for various concentrations of hemoglobins not exceeding twice of those used in the present experiments. The entire CD curves of hemoglobin A from 200 to 660 nm are given in Fig. 1 with the corresponding absorption spectra. In the Soret and visible regions, CD extrema of oxygenated and deoxygenated hemoglobin correspond in wave length with the absorption maxima. CD extrema of oxygenated hemoglobin are observed at 418, 546, and 578 nm, and absorption maxima are observed at 415, 542, and 578 nm. Deoxygenated hemoglobin exhibits two positive peaks at 552 and 590 nm in the visible region and a complex CD in the Soret region with a positive peak at 432 nm. The absorption maxima are observed at 430 and 555 nm. The CD band at 590 nm appears to correspond to a small shoulder on the absorption spectrum. Methemoglobin shows a positive band in the Soret region but no significant CD peak in the visible region. All CD spectra of mammalian hemoglobin so far tested, namely the hemoglobin of pig, cow, rabbit, and rat, are identical with those of human hemoglobin, reflecting invariance of the amino acid residues surrounding the heme group (14). Toad and chicken hemoglobin exhibit CD spectra similar to those of mammalian hemoglobin except for negative troughs in the Soret region, which are greater than those of mammalian hemoglobin.

CD Spectra of Isolated Chains—We have reported that the CD spectra of isolated chains of human hemoglobin are different from those of native hemoglobin in the Soret region (2). The CD spectra of $\alpha$, $\beta$, and $\gamma$ chains in the Soret and visible regions are shown in Fig. 2. Contamination by methemoglobin is about 5% in $\alpha$ and $\gamma$ chains and undetectable in $\beta$ chains. In the Soret and visible regions, the CD of oxygenated $\alpha$ chains exhibits three positive peaks corresponding to the absorption maxima. A small positive band of deoxygenated $\alpha$ chains at 590 nm is absent in the CD spectra of $\beta$ and $\gamma$ chains. The CD spectra of $\beta$ and $\gamma$ chains are almost identical, but differ from those of $\alpha$ chains, in that $\beta$ and $\gamma$ chains show negative troughs both in the oxygenated and deoxygenated forms. Complex CD curves of $\beta$ and $\gamma$ chains in the Soret region, which arise from the same transitions as the asymmetrical peak of absorption, can be resolved into two Gaussian curves, a negative one centered at a shorter wave length than the absorption maximum and a positive one centered at a longer wave length. A similar splitting of the absorption peaks of oxygenated $\beta$ and $\gamma$ chains at 540 and 578 nm is observed in the CD spectra.

Although a small difference in wave length of absorption maxima was observed between oxygenated $\alpha$ and $\beta$ chains, the molecular extinction of the two types of chains are almost the
same at the absorption peaks. CD measurements indicate the difference between α and β chains more clearly, both in the Soret and in the visible regions, reflecting different environments of the heme.

Hsu and Woody (15) reported that interaction of the Soret transitions with the allowed π-π* transitions in aromatic side chains was a major factor in the rotational strength, and that the coupling of the Soret transitions with excited state of side chains could account for the observed rotational strength of myoglobin in the Soret region. The orientation of the aromatic amino acid residues with respect to the heme, as well as the position of these residues in the primary structure, is considered to have great influence on the CD spectrum. Possible contacts between the globin and heme group have been reported based on the results of x-ray diffraction study at 2.8 Å resolution (14). Comparison of the amino acid sequences of β and γ chains shows that, of 20 amino acid residues which make contact with heme, all but 1 residue which make doubtful contact are common to the two kinds of chains. Fourteen amino acid residues in heme environments are common to α and β chains. Although the number of amino acid residues common to horse heart myoglobin and α chains is smaller than that of α and β chains, the CD spectra of the myoglobin (Fig. 7) and α chains are similar to each other. Among the aromatic amino acid residues which are supposed to give major contribution to the rotational strength (15), the one common to α chains and myoglobin but different in β chains is phenylalanine (B 14), which is substituted by leucine in β chains. It appears that some specific contacts principally determine the CD peaks.

Effects of Recombination on CD Spectra—The CD spectra in the Soret and visible regions of hemoglobin reconstituted from equivalent amount of α and β chains are shown in Fig. 3. No substantial differences are found between native and reconstituted hemoglobin. We have reported that the molar intensity of the CD bands of reconstituted hemoglobin is not simply the arithmetical mean of the intensities of the isolated chains in the Soret region (2). Differences between the CD spectra of reconstituted hemoglobin and the average CD spectra of α and β chains were observed also in the visible region. As shown in Fig. 3, large differences are observed in the deoxygenated forms at around 430 and 580 nm, while the CD spectra of the oxygenated reconstituted hemoglobin is only slightly different. The positive ellipticity of the deoxygenated reconstituted hemoglobin in the Soret region is about twice as great as the value calculated from those of the isolated chains, and the CD peak of reconstituted hemoglobin is at 3-nm shorter wave length than that of the mean of the isolated chains. The positive band of the calculated means of isolated chains is very small and the magnitude of ellipticity is about a quarter of that in the reconstituted hemoglobin. The change caused by recombination was also observed in the absorption spectra of deoxygenated hemoglobin (16). The change consists of increases in peak intensities, about 15% at 430 nm and 10% at 590 nm. It is shown that CD measurements, better than absorption measurements, manifest spectral changes of isolated chains upon recombination.

Carbon monoxide hemoglobin shows complex CD spectra with peaks and troughs by splitting of the α- and β-bands. The spectra are very different from those of oxygenated hemoglobin as shown in Fig. 4. The difference between the bonding of carbon monoxide to heme and that of oxygen to heme was shown by measurements of Mössbauer effect of hemoglobin (17). As in the oxygenated hemoglobin, however, intensities of the CD bands of carbon monoxide α chains are greater than those of β chains, and the spectra of native hemoglobin is approximately the same as an arithmetical mean of the spectra of the isolated chains. Thus it appears that only deoxygenated chains exhibit subunit interactions leading to conformational changes associated with changes in the optical activity.
CD Spectra of Human Hemoglobin Digested by Carboxypeptidase A and Hemoglobin Dialyzed against Water—The heme-heme interaction, a well known feature of oxygenation of hemoglobin, is usually explained by two conformations of deoxygenated subunits in hemoglobin, the one having a lower oxygen affinity than the other in consequence of subunit interactions. A large change in CD ellipticities, observed only in the deoxygenated forms when the isolated chains with high oxygen affinity were recombined to tetrameric hemoglobin with low oxygen affinity, suggests that the CD spectra may reflect conformations related to functional properties.

We measured the CD spectra of human hemoglobin digested by carboxypeptidase A. This modification removes 2 COOH-terminal amino acid residues of β chains (18), namely histidine and tyrosine, which are located far from the heme in the molecule (14), but produce profound changes in the oxygen equilibrium (18). By the digestion, 85% of COOH-terminal two amino acids of β chains were hydrolyzed, the oxygen affinity was increased to about 8-fold of the normal hemoglobin, and the

Hill's n, a parameter of the heme-heme interaction, was decreased to 1.2. The tertiary and quaternary structure of the hemoglobin was not changed by the digestion. The magnitude of the 222-nm trough in CD, which is considered to be a measure of the α helix content, was $2.6 \times 10^6$ deg cm$^2$ per decimole for the digested hemoglobin and $2.7 \times 10^6$ for the native hemoglobin. The sedimentation coefficient was the same for both the native and modified hemoglobin (18). The CD spectra and absorption spectra in the Soret region are shown in Fig. 5. Magnitude of the positive ellipticity for the deoxygenated modified hemoglobin is about 70% of that for the native hemoglobin, while the positive peaks of the oxygenated forms are about the same for both hemoglobins.

Deionization of hemoglobin increases its oxygen affinity, and the addition of an equivalent amount of 2,3-diphosphoglycerate to the deoxygenated hemoglobin decreases its oxygen affinity to the normal value (19). The deionized deoxygenated hemoglobin exhibits the positive CD peak in the Soret region, smaller than that of the native hemoglobin by about 30%, as shown in Fig. 6. By the addition of 2,3-diphosphoglycerate which has no optical activity in this region, the magnitude of the peak for the deionized hemoglobin increased to the value approximately equal to that of the native hemoglobin. The partial oxygen pressures required for half-saturation of the native hemoglobin, the deionized hemoglobin, and the deionized hemoglobin with 2,3-diphosphoglycerate added, were 8.4, 3.2, and 8.3 mm of mercury at 22°, respectively. Absorption spectra of the hemoglobin digested by carboxypeptidase or deionized by dialysis show peaks smaller than native hemoglobin in the Soret region only in the deoxygenated form (Figs. 5 and 6). Differences in the intensities were about 7% in both hemoglobins.

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The assignment of CD bands to specific structures in heme environments might provide an approach to the physicochemical interpretation of the heme-heme interaction.

**CD Spectra of Myoglobin and Monomeric Hemoglobins**—CD spectra of the horse heart myoglobin are given in Fig. 7. They are similar to those of α chains except that the peak at 590 nm in the deoxygenated form is separated from the positive band centered at about 550 nm. The toad embryonic hemoglobin obtained from the egg of *Bufo vulgaris japonicus* is monomeric and has the oxygen-binding property similar to that of the myoglobin and shows absorption spectra similar to those of human hemoglobin (10). The toad embryonic hemoglobin (Fig. 8) exhibits much smaller CD bands than those of the horse heart myoglobin. CD spectra in the visible region are similar in shape with those of myoglobin except for the negative ellipticity around 590 nm. In the Soret region, the embryonic hemoglobin shows complex CD with a positive peak and a negative trough. The CD spectra of lamprey hemoglobin, shown in Fig. 8, are very different from those of other hemoglobins. Negative CD bands of the deoxygenated form are approximately the mirror images of the positive bands of the horse heart myoglobin. Lamprey hemoglobin contains two histidines coordinated to heme at positions 5 and 6 as other hemoglobins (20). It has been reported that water protons are more accessible to the ferriheme iron of lamprey hemoglobin than to the ferrimyoglobin or ferrihemoglobin (21), and that the α helix content of lamprey hemoglobin seems to be less than that of human hemoglobin (1). The structure of lamprey hemoglobin may differ considerably from other hemoglobin. The difference in CD between lamprey and human hemoglobin will be understood on a submolecular basis only when the structure of lamprey hemoglobin is known in atomic detail. These various CD spectra suggest different structures in the heme environments of these hemoglobins.

**CD Spectra of Protoporphyrin-Globin Complex**—Since the combination of heme groups and apoprotein in all of the hemoglobins and myoglobin include the covalent bond between the iron in heme and the histidine residue in globin, the differences observed in the CD of various hemoglobins are considered to be the result of differences in the interaction between heme and globin other than the iron-histidine bond. CD spectra of a protoporphyrin-globin complex, which contains no iron, is given in Fig. 9. The protoporphyrin-globin complex exhibits distinctive CD bands both in the Soret and in the visible regions, and the intensities of the bands are comparable to the intensity of those in human hemoglobin. Thus it is shown that contacts other than the iron-histidine bond make the tetrapyrrol ring optically active and play an important role in the CD of hemoglobins.

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J. Biol. Chem. 1971, 246:383-388.

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