Optically Active Absorption Bands of Hemoglobin and Its Subunits*

(Received for publication, January 23, 1967)

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SUMMARY

Studies of the circular dichroism of the α and β subunits of hemoglobin have shown that while their helix contents are the same as that of hemoglobin A, the intensities of the heme bands at 260 and 410 μ are much greater for the α than for the β chains. In both spectral regions, the molar intensity for hemoglobin A is simply the sum of the intensities of the isolated chains. The ellipticities associated with the 260 μ band are strongly influenced by the attached ligand. A smaller band of opposite sign is observed at 285 μ in deoxyhemoglobin A but is absent in both deoxygenated subunits.

Studies on the isolated α and β subunits of hemoglobin A have shown that the combination of these subunits with ligands differs radically from that of the original hemoglobin (1–3). They are devoid of such allosteric properties as heme-heme interaction or the Bohr effect, and their oxygen affinity resembles that of myoglobin. The method of Tyuma, Benesch, and Benesch (3), based on that of Bucci and Fronticelli (4), has made it possible to prepare α and β subunits which can be recombined to give native hemoglobin as judged by the oxygenation curve and a number of other criteria.

The optical activity associated with peptide and nonpeptide absorption bands of the subunits prepared by this method has now been investigated. Peptide band optical activity in hemoglobin is predominantly due to its very high content of right-handed α-helix. The origin of nonpeptide optical activity may be in side chain or heme absorption bands. Such optical activity is more readily discerned by circular dichroism spectra than by optical rotatory dispersion spectra, and we have primarily resorted to examination of the former. In these, we have observed two bands the parameters of which are particularly responsive to alteration in heme-ligand. One, as expected, is the Soret band near 400 μ; the other, occurring also in all derivatives of hemoglobin and myoglobin, is observed near 260 μ (9). The short wave length band is more easily and precisely examined because the associated extinction coefficient is much lower at these wave lengths than in the Soret spectral interval and therefore more concentrated solutions or longer path lengths may be employed.

In a symmetrical chromophore, such as isolated hemin, binding to the protein may lead to conferral of optical activity through imposition on the heme group of an asymmetric potential due to a particular distribution of static charges in the vicinity of the chromophore. A concurrent requirement is that the chromophore assume a preferred conformation in its bound state. Alteration in optical activity associated with a particular electronic promotion in the chromophore may be brought about by alteration of the asymmetric potential or by alteration in the number of configurations accessible to the bound chromophore or both. The latter may be considered from the point of view of the degree of "wobble" of heme in its binding pocket.

The most striking result of this work is the substantial difference in nonpeptide optical activity between the α and β subunits.

EXPERIMENTAL PROCEDURE

Human and horse hemoglobins were prepared according to methods previously described (6). Several experiments were also performed on horse hemoglobin that had been crystallized as the oxy derivative (7).

Horse blood was kindly provided by Dr. C. Price of Lederle Laboratories, Pearl River, New York. Hemoglobin H was prepared as described previously (3) from the blood of a 17-year-old female patient kindly put at our disposal by Dr. James Wolff. Separation of the α and β chains of human adult hemoglobin A was performed by the method of Tyuma et al. (3). Concentrations of all solutions were determined by spectral analysis after conversion to the cyanomet derivatives.

Methemoglobin was prepared by oxidation with 1 eq of ferricyanide at pH 7.0; cyanmethemoglobin was similarly prepared, but in the presence of 2 eq of cyanide. In the case of the β chains and hemoglobin H, the oxidation was carried out at 37° to avoid oxidation of -SH groups (3). Solutions of deoxyhemoglobin were prepared and transferred to cylindrical cuvettes by an arrangement similar to that described previously (8).

Circular dichroism measurements were performed at 25° on a Jasco dichrograph which had been modified for 10-fold increased sensitivity as described elsewhere (5, 9). Molar ellipticities are
given on a mean residue weight basis, with the use of values of 107.3, 108.7, and 108, respectively, for \( \alpha \), \( \beta \), and \( \alpha_2\beta_2 \) molecules (10).

**RESULTS**

In Fig. 1A are shown the near ultraviolet CD spectra of the oxygenated derivatives of hemoglobin A and of the separated \( \alpha \) and \( \beta \) chains. Fig. 1B shows the CD spectra of the corresponding deoxygenated derivatives. Several features of the spectra are noteworthy. It will be observed that every derivative represented shows a prominent positive band, the maximum of which is located between 255 and 265 mp. In some cases, another band appears near 285 mp. This band is considerably less intense than the shorter wave length band, and it is not always of the same sign. The most striking aspect of the figure is the great difference in intensity between the bands of the \( \alpha \) and \( \beta \) chains. This difference is more pronounced in the deoxy than in the oxy derivatives. The differences are still greater in the areas under the curves, which directly gauge the rotational strength of the transitions (9).

It can be seen from the data given in Table I that the maximum ellipticities of the main band of hemoglobin A and of the recombined chains which give the same CD spectra as hemoglobin A are the arithmetic means of the separated chains values, for both the deoxy and the oxy forms. Thus, the structural features of the chains which generate optical activity near 260 mp do not change on combination of \( \alpha \) and \( \beta \) chains.

The smaller band near 285 mp is also additive in the oxy forms, but it is definitely not additive in the deoxy derivatives. Neither of the separated deoxygenated chains shows a discernible band, but deoxy Hb A, both human and horse (not shown), shows a well resolved negative band at 285 mp. Deoxygenated hemoglobin is thus distinctive in being the only hemoglobin derivative with a negative band near 280 mp and in exhibiting a band which is absent in the separated chains.

In Table I are presented maximum ellipticities of the 260 mp band for several derivatives of the separated and recombined chains of human hemoglobin A and horse hemoglobin. With the exception of the most derivatives, horse and human hemoglobins are seen to agree very well. The 260 mp band of the separated chains is additive in all the derivatives which were studied.

Finally, it should be noted that the values for the separated \( \beta \) chains agree well with those for hemoglobin H.

**Relationship between 260 mp Band and Soret Band**—The Soret ellipticity bands of the separated oxygenated chains and of oxyhemoglobin are positive in sign with integrated intensities about one-third of those of the corresponding 260 mp bands.

In Table II the areas of the 260 mp and Soret CD bands of the oxy derivatives of hemoglobin A and of the separated and recombined chains are compared in arbitrary units. The procedure involved tracing of the bands without smoothing, cutting out of the traced bands, and weighing on a microbalance. The molar concentrations on a heme basis were identical for all entries, although the cell path length used was different for the measurements in the two different spectral intervals. The results show that the ratio of areas for the bands in the \( \alpha \) and \( \beta \) chains are nearly the same for the Soret and 260 mp band and that additivity of the \( \alpha \) and \( \beta \) contributions in the recombined chains is observed in the Soret band areas as well as in the areas under the 260 mp bands.

**Helix Content of Separated and Recombined Chains**—The far ultraviolet CD spectrum of human adult oxyhemoglobin A in the wave length region between 240 and 212 mp closely resembles published curves for metmyoglobin and horse methemoglobin (5, 11). The \( \pi - \pi^* \) negative extremum which is characteristic of right-handed \( \alpha \)-helices occurs at 225 mp as it does in helical proteins.

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**TABLE I**

**Effect of ligands on maximum ellipticity in 260 mp region**

Measurements were made on 0.06\% or 0.12\% solutions of hemoglobin in 0.05 m phosphate buffer, pH 7.0, with the use of cells of 5- or 10-mm light path. Values are reported as \( \Psi_{\text{max}} \) in deg. cm\(^2\) per decimole.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Fe(^{2+})</th>
<th>Fe(^{2+})O(_2)</th>
<th>Fe(^{3+})</th>
<th>Fe(^{3+})CN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horse hemoglobin</td>
<td>285</td>
<td>605, 606, 630</td>
<td>256, 240</td>
<td>405, 420</td>
</tr>
<tr>
<td>Human hemoglobin</td>
<td>240</td>
<td>490, 425, 510</td>
<td>154, 188</td>
<td>490, 440</td>
</tr>
<tr>
<td>Human ( \alpha ) chain</td>
<td>463</td>
<td>630</td>
<td>630</td>
<td></td>
</tr>
<tr>
<td>Human ( \beta ) chain</td>
<td>114</td>
<td>248, 430</td>
<td>195, 234</td>
<td></td>
</tr>
<tr>
<td>Hemoglobin H.</td>
<td>295</td>
<td>394, 365</td>
<td>245, 202</td>
<td></td>
</tr>
<tr>
<td>(( \alpha + \beta ) reconstituted)</td>
<td>255</td>
<td>500</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**TABLE II**

**Areas under ellipticity bands in Soret and 260 mp regions**

Measurements were made on 0.06\% solutions in 0.05 m phosphate buffer, pH 7.0, with path lengths from 1 to 10 mm.

<table>
<thead>
<tr>
<th>Area in arbitrary units</th>
<th>245-265 mp</th>
<th>415-445 mp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxy ( \alpha ) chains</td>
<td>0.0258</td>
<td>0.151</td>
</tr>
<tr>
<td>Oxy ( \beta ) chains</td>
<td>0.0081</td>
<td>0.044</td>
</tr>
<tr>
<td>Oxy Hb A</td>
<td>0.0169</td>
<td>0.098</td>
</tr>
<tr>
<td>Oxy (( \alpha + \beta ) reconstituted)</td>
<td>0.0174</td>
<td>0.097</td>
</tr>
<tr>
<td>Calculated (( \alpha + \beta )/2)</td>
<td>0.0169</td>
<td>0.097</td>
</tr>
<tr>
<td>Ratio ( \alpha/\beta )</td>
<td>3.21</td>
<td>3.45</td>
</tr>
</tbody>
</table>
poly-α- L-glutamic acid. The residue ellipticity at 222 mμ is
-21,000 ± 600 deg. cm² per decimole. Referred to values of
helical and random coil poly-α- L-glutamic acid (5), this corre-
sponds to a helix content of about 69%. The separated β chains
gave an average value about 2% lower than this, and the sepa-
rated α chains a value about 3% greater. These deviations are
roughly within our experimental error. This indicates that sepa-
rated of the oxygenated chains brings about no significant al-
teration in the number of residues which are in helical segments.

**DISCUSSION**

**Assignment of 260 mμ Band**—Several lines of evidence lead to
the conclusion that the major contribution to the 260 mμ band
is made by heme rather than the aromatic residues. Tyrosine
is the least likely candidate since its ellipticity band is at too long
a wave length, i.e. near 275 mμ. Moreover, the tyrosine content
of hemoglobin is about 10 times too small to account for the mag-
nitude of the observed ellipticity at 260 mμ (9). The same argu-
ment is equally applicable to tryptophan and phenylalanine (9).
The dominant role of heme for the 260 mμ band in hemoglobin
is further supported by the negligible ellipticity of native globin at
296 mμ, which is restored to normal values upon recombination
with heme. Finally, at least in the case of the oxy derivatives,
the differences in the CD spectrum at 260 mμ between α and β
chains are paralleled by, and in the same direction as, the differ-
ces observed in the Soret ellipticity band. Since the absorption
bands are almost indistinguishable in this region, the large
difference between the ellipticity of the α and β chains must re-
fold the difference in the heme environment in the two cases (12),
or a difference in tightness of attachment, or both.
The data in Table I show that the low spin complexes exhibit
a larger 260 mμ ellipticity than the high spin ones. In the case
of the β chains, but not the α chains, the ellipticity of the cyano-
met form is lower than that of the oxy form. It is known (13)
that when aside ion binds to metmyoglobin in the crystal, the
molecular axis is not along the z axis referred to the octahedron,
but at an angle to it. Whether or not cyanide may be differently
bound in the α and β chains is not known, but it would be interest-
ing if the cyanide ion were involved in a different interaction in
the α and β chains.

**Assignment of 280 to 290 mμ Bands**—For several of the same
reasons given above, which indicate that side chain optical activ-
ity is not the important feature of the 260 mμ band, it may be
argued that side chain chromophores do generate the longer wave
length bands near 280 to 290 mμ. The apoprotein exhibits a
negative band of magnitude comparable to that found in deoxy-
hemoglobin. The strength of the band is comparable to that
found in model compounds and other proteins (9), and there is
no particular reason to assume that only the aromatic residues
near the heme group must contribute.

Most interestingly, the negative band observed near 280 mμ in
deoxyhemoglobin is not seen in the separated deoxygenated
chains. While this band is thus ligand-dependent, it is also de-
pendent on α, β interaction. In view of the known changes in
α, β contacts accompanying deoxygenation, there is no require-
ment that the responsible chromophore be part of the heme
oxygen. A recent tentative model of hemoglobin constructed by
Perutz (12) implicates both C7α and C15β tyrosine residues
in the α, β contact surfaces.

In summary, it is clear that only the changes in the 280 mμ band
parallel the dramatic change in oxygen binding which occurs
when α and β chains combine to form hemoglobin. By contrast,
the structural features in the α and β chains which are
responsible for the profound differences in the CD spectra at 260
mμ and 405 mμ are preserved upon recombination.
The great difference in ellipticity near 260 mμ among all de-
rivatives of α and β chains should provide a useful diagnostic
tool even in the absence of a thorough knowledge of its under-
lying cause. The measurement is rapid and simple and requires
little material. Its usefulness in studies of hemoglobin biosyn-
thesis requires no elaboration, and it may find wide application
in comparative studies of hemoglobins from different species.

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