Influence of Prosthetic Groups on Protein Folding and Subunit Assembly

RECOMBINATION OF SEPARATED HUMAN α- AND β-GLOBIN CHAINS WITH HEME AND ALLOPLEX INTERACTIONS OF GLOBIN CHAINS WITH HEME-CONTAINING SUBUNITS*

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SUMMARY

Reconstitution of the α and β subunits of human hemoglobin from separated α- and β-globin chains is described. The absorption and circular dichroism spectra, sedimentation coefficients, and sulfhydryl reactivities of the reconstituted subunits are compared with those of native subunits, which are regenerated from the p-mercuribenzoate chains by a novel procedure. The reconstituted subunits specifically recombine with the complementary native ones. Each property of the α subunit agrees, within experimental uncertainty, with the corresponding one of the subunit from which heme was never removed. Thus, markedly disordered and aggregated α-globin is restored to the highly ordered, monomeric α subunit upon recombination with 1 eq of heme or hemin dicyanide. The reconstituted β subunit also compares well with the native subunit, except for a minor difference in a portion of the near-ultraviolet circular dichroism spectrum and a diminished sulfhydryl reactivity. Possible reasons for these differences are discussed. In the met form neither reconstituted subunit forms hemochromogen for several days, whereas the original met subunits become denatured within hours, probably owing to the necessity of exposing the latter, but not the former, to ferricyanide ion in producing the met forms.

It is shown that while α- and β-globins do not combine at 4° (Yip, Y. K., Waks, M., and Beychok, S. (1972) J. Biol. Chem. 247, 7237), recombination does occur if either chain contains heme. The recombination produces half-filled (HF) molecules which are of two kinds, depending on which chain contains heme. The molecular weights of these molecules are different. HFα, the intermediate prepared by mixing α subunits with β-globin, is a dimer; HFβ, the intermediate containing heme on its β chains, is a tetramer. No interchange of heme among chains occurs in the cyanomet forms in either molecule. In each, moreover, the conformation of the heme-free chain differs from that of the same chain when unbound. Both HF molecules bind 1 eq of heme, producing tetrameric hemoglobin.

The most dramatic finding in this work is that the disordered α-globin can be refolded not only by binding heme but also by combining with a β subunit, although the refolding in the latter case is not as complete as in the former.

These results show that the primary sequences of the globin chains generate unstable conformations, which are stabilized by combination with heme. They demonstrate also that refolding of a polypeptide chain can be induced by interaction with a neighboring subunit in an assembly process.

This series of papers deals with the control of protein conformation exercised by prosthetic groups and, particularly, with the entire set of conformational states and processes whereby a functional hemoglobin molecule is assembled from separated globin chains and heme molecules.

In the first paper of the series (1), the preparation of separated α- and β-globins was described, and the conformational differences between these globin chains were examined. It was found that successful preparations of regenerable, isolated chains depended on first separating the parent subunits of human hemoglobin and then removing heme from each. The alternative procedure of removing heme from hemoglobin to form αβ-globin (apohemoglobin), followed by separation of chains in urea or guanidine solutions, or by reaction with p-mercuribenzoate, failed to yield regenerable chains.

Each of the heme-free chains is different from its respective parent subunit in molecular weight, secondary structure, sulfhydrylreactivity, stability to variation in temperature and pH, and other properties. Moreover, two distinctive features of the hemoglobin in relation to their constituent α and β subunits are lacking in the globins. These are (a) that the subunits of hemoglobin have highly similar conformations and (b) that they retain the same over-all folding whether combined or separated. Thus, the separated α-globin chain is substantially unfolded.

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compared to the separated β-globin chain and compared to the combined α-globin in αβ-globin (apohemoglobin). The β-globin, in contrast, appears to have a similar secondary and tertiary structure whether combined or free.

When heme-containing α (monomer) and β (tetramer) subunits are mixed at room temperature, or in the cold, they combine to form tetrameric hemoglobin (2). In contrast, when separated α-globin (dimer or higher aggregates, depending on ionic strength) and β-globin (dimer, independent of ionic strength) are mixed in the cold, no combination occurs. At room temperature, the globins tend to precipitate (1). However, αβ-globin (apohemoglobin, dimer) does not dissociate measurable under these conditions. The separated globin chains and apohemoglobin, thus, do not equilibrate.

The present paper is concerned with several aspects of the re-folding of the globin chains and of reassembly, especially in intermediate stages of hemoglobin formation. Refolding of the individual globin chains upon recombination with heme is studied by comparing the properties of separately reconstituted α and β subunits with native subunits. The primary intent in the measurements, at this point, is to establish that the isolated globin chains are native not only from the point of view of stoichiometric recombination with heme, but in the sense that they can be correctly refolded. To this end, comparisons with native α and β subunits were made by investigating restoration of characteristic absorption spectra, sedimentation coefficients, sulfhydryl reactivity, circular dichroism spectra, and specific recombination with the complementary native subunit. The reconstituted α subunit is indistinguishable in all these characteristics from the original subunit. Thus, the highly disordered α-globin is, by these criteria, fully regenerable with heme. Reconstituted β subunits are identical with the original subunits in all properties except for a minor difference in a small interval of the near-ultraviolet CD spectrum and a difference in sulfhydryl reactivity.

Since the α- and β-globin chains do not bind to each other whereas the heme-containing subunits readily combine, the reassembly studies focused on intermediate states. Thus, it was evidently important to know whether heme-free chains combine with heme-containing subunits and, if they do, whether the nature of the complexes depend on which chain contains heme. It is demonstrated in this work that the globin chains will combine with one another if either chain contains a heme. Moreover, it is shown that the molecular weight of the resultant molecules depends on whether it is the α or β chain that contains the heme.

A dramatic and unexpected finding was that the disordered α-globin is refolded during the combination with a heme-containing β subunit, with an increase of 30% in content of α helix and coincident changes in tertiary structure. While the refolding is, thus, not as complete as that achieved by direct heme binding, the conformational change that takes place in the α-globin chain in the course of binding to its heme-containing partner is nevertheless deep seated. Accordingly, to emphasize the extensive folding process induced by the neighboring subunit, and in anticipation of similar occurrences in other subunit proteins, we refer to the phenomenon as an alloplex interaction.

**MATERIALS AND METHODS**

The determination of sedimentation coefficients, measurement of circular dichroism spectra, gel filtration, and sulfhydryl reactivity were described in the previous paper (1).

1. Alloplex, G. allo, other and L. plex, from plecare, to fold.

The preparations of human hemoglobin, its α and β subunits, apohemoglobin, and α- and β-globins were carried out as described previously (1). A new method was devised for the regeneration of β-SH chains.

**Regeneration of β Chains**—β-PMB2 chains were incubated for 2 hours in 0.1 M phosphate buffer, pH 7.5, and 50 mM in mercaptoethanol. The PMB was removed on a Bio-Gel P-2 column (45 × 5.0 cm) equilibrated with the same buffer. The β-SH chains were then freed of excess mercaptoethanol by another chromatography on a Bio-Gel P-2 column equilibrated with the appropriate buffer.

This method gives complete recovery of β-SH chains, a constant titer of 2.0 ± 0.1 —SH per chain, and a complete absence of oxidation.

**Reconstitution of Subunits**—In a typical experiment, 5 mg of hemin (crystalline type I, Sigma) were dissolved in 25 ml of 0.1 N NaOH. The concentration of the hemin solution was determined by diluting the stock 20-fold with 0.1 N NaOH and measuring the absorbance at 385 nm. A value of 5 × 10⁴ was used as the molar extinction coefficient (3).

For recombination with α- or β-globin either heme or dicyanide heme was added in 1.2 molar excess. During addition of heme to the globins, the pH of the latter was continuously monitored and maintained at 7.5 by adding 1.0 M monobasic potassium phosphate. After standing overnight at 4°C, the samples developed precipitates, which were removed by centrifugation.

**Removal of Excess Heme**—Excess heme was removed by applying the reconstituted heme-protein to a Bio-Gel P-2 column equilibrated with the appropriate buffer. During the course of this investigation it was found that Bio-Gel P-2, a polyacrylamide gel, displays a high affinity for free heme. Subsequently, the column had to be washed with a sample of αβ-globin in order to elute the heme.

The ratio of heme to globin after recombination was ascertained by spectral analysis at 280 and 540 nm. A 1:1 combination of dicyanide heme with globin chains gave an $\lambda_{385}$: $\lambda_{540}$ ratio of 2.44 with α-globin and 2.90 with β-globin, whereas apohemoglobin gave a ratio of 2.60.

**Preparation of Heme-containing Intermediates, αβ and αβPMB**—These molecules, designated as half-filled (HF) intermediates, were prepared at high concentration by mixing equimolar amounts of α-heme chain with β-globin and vice versa. The heme-containing chains were first converted to the cyanmet derivative by the addition of solid potassium cyanide and potassium ferricyanide in sequence followed by chromatography on Bio-Gel P-2 to remove excess small ions. Concentration of the separated α and β subunits was determined by measuring the absorbance of the cyanmet derivative at 540 nm, using extinction coefficient ($E_{1\text{cm}}$) values of 6.99 for the α chain and 6.57 for the β chain (4). For combination with α-globin, the β-cyanmet subunit was first brought to pH 6.7 by dialysis against 20 mM potassium phosphate; the α-cyanmet subunit, which was equilibrated with 0.1 M potassium phosphate at pH 7.5, was added to the β-globin directly. The solutions were allowed to stand at 4°C for periods varying from 2 hours to overnight before further experiments were carried out.

The ratio of heme-containing subunit to heme-free chain was...
calculated from the absorbance at 540 and 280 nm, since absorbance at 540 nm is due to the heme-containing subunits alone whereas that at 280 nm is the sum of that of both chains.

When solutions of the intermediates were used at low concentration, a different method was employed. A mixture of $\alpha^+ + \beta^+$ chains, containing $\alpha$-globin in excess, was chromatographed on a Bio-Gel P-150 column equilibrated with 20 mM phosphate, pH 6.7. The excess of $\alpha$-globin was excluded from the column (1) and the $\alpha^+\beta^+$ intermediate was eluted as a tetramer. In the case of $\alpha^+\beta^-$, the mixture of $\alpha^+ + \beta^-$ contained an excess of $\alpha$-heme subunits. After chromatography, the $\alpha^+\beta^-$ was collected as a dimer and the free $\alpha$ subunits were retarded on the column as monomers. In both cases, spectral analysis displayed a ratio of one heme subunit per one globin chain.

Reconstitution of Hemoglobin—Reconstituted hemoglobin composed of native and reconstituted subunits, $\alpha_{nat}\beta_{nat}$ and $\alpha_{con}\beta_{con}$, were prepared either by mixing equimolar amounts of native and reconstituted subunits or by adding the stoichiometric amount of heme to the IF intermediate molecules. The reaction mixture was allowed to stand at 4°C for periods varying from 2 hours to overnight and any precipitate was removed by centrifugation. The reconstituted hemoglobin was purified as follows. For $\alpha_{nat}\beta_{nat}$, the sample was first equilibrated with 20 mM Tris-HCl, pH 8.5, by filtration through Bio-Gel P-2. It was then loaded onto a DEAE-cellulose column equilibrated with the same buffer, and the column was washed with this buffer until the excess-free $\alpha$ subunits were removed. The reconstituted hemoglobin was then eluted with 0.1 M potassium phosphate, pH 7.5. For $\alpha_{con}\beta_{con}$, the sample was first equilibrated with 20 mM potassium phosphate, pH 6.7, on a Bio-Gel P-2 column. This sample was then loaded onto a CM-cellulose column equilibrated with the same buffer. After washing out excess free $\beta$ subunits with this buffer, the reconstituted hemoglobin was eluted with 0.1 M potassium phosphate, pH 7.5.

Measurement of Spectral Properties—For a comparison of the spectral properties of reconstituted with those of native subunits, absorption spectra from 260 to 600 nm of cyanomet derivatives were recorded on a Cary 14 spectrophotometer. The concentration of the samples was adjusted so that the absorbance at 540 nm showed the same intensity as the corresponding native material. A 10-mm cell was used for scanning the samples in the visible and ultraviolet regions, while for the Soret region, a 1-mm cell was used, so that the same solution could be used for scanning without further dilution. Absorption spectra of the reconstituted subunits in the met form were also measured in this way.

RESULTS

Reconstituted $\alpha$ and $\beta$ Subunits

The restoration of Soret absorption which occurs on stoichiometric recombination of heme or cyanide heme with $\alpha$, $\beta$, and $\alpha\beta$-globins has been found to be a time-dependent process. During the titration of the globins with heme, the Soret absorption increases steadily for 15 to 20 min. The complete recovery of Soret absorption is obtained only after several hours.

Upon the addition of heme to the globins, some precipitation invariably occurs. With $\alpha$-globin, when the reconstitution is carried out with 10-fold more concentrated solution than used for the studies of heme-binding capacity (1), an appreciable amount of precipitation is formed during the addition of heme. Precipitation of reconstituted hemoglobin and of $\beta$ subunits, however, occurs only after several hours. A similar observation has been reported by Rossi-Fanelli et al. (3) for the reconstitution of hemoglobin from $\alpha\beta$-globin.

Spectral Properties—Figs. 1 and 2 show the absorption spectra of the cyanomet derivatives of the reconstituted $\alpha$ and $\beta$ subunits, respectively, compared with those of the native subunits. The overall spectral characteristics of the reconstituted subunits are nearly identical with those of the native materials. The slight variation, which is usually less than 5%, is found in the more sensitive Soret region of the spectrum; this may be due to the fact that the solutions used for the comparison are normalized according to the absorbance at 540 nm. The most distinctive feature of the cyanomet derivative of the reconstituted subunits is that the positions of the Soret absorption maxima, $\alpha$ at 418 nm and $\beta$ at 430 nm, are identical with those of the native subunits. Table I summarizes the spectral characteristics of the

![Fig. 1. Absorption spectra of native (---) and reconstituted (-----) $\alpha$-cyanomet subunits in 0.1 M potassium phosphate, pH 7.5. Concentration normalized according to absorbance at 540 nm.](http://www.jbc.org/)

![Fig. 2. Absorption spectra of native (---) and reconstituted (-----) $\beta$-cyanomet subunits. Conditions as in Fig. 1.](http://www.jbc.org/)
native and reconstituted subunits by comparing their extinction coefficients at 278, 418 or 420, and 540 nm.

The spectral properties of the reconstituted subunits have been further characterized by measuring the absorption of their met derivatives. Fig. 3 shows the absorption of the reconstituted α-met subunit. A similar spectrum was recorded for the reconstituted β-met subunit. Both show characteristic absorption maxima at 630 and 500 nm. Absorption spectra measured at intervals from 2 hours to several days after heme addition do not change. These samples can be converted to the cyanmet derivative by the addition of potassium cyanide, yielding spectra similar to those shown in Figs. 1 and 2.

**Sedimentation Coefficients**—Table II summarizes and compares the sedimentation coefficients of the reconstituted and native subunits, all as the cyanmet derivative. There is good agreement in the $s_{20,w}$ values between reconstituted and native subunits. The $s_{20,w}$ value of 1.85 for the reconstituted α subunit indicates that it is monomeric as is expected for the α subunits. This is a sharp decrease from the $s_{20,w}$ value of 6.61 of α-globin (1). The reconstituted β subunit, in contrast, shows an increase in $s_{20,w}$ value from 2.62 (1), in β-globin, to 3.8 on addition of heme. On the basis of sedimentation behavior, these reconstituted subunits form hemoglobin tetramers when recombined with the alternate native subunits. The $s_{20,w}$ value for $(α_{αt}β_{βt})$ is 3.86, and that of $(α_{αt}β_{βa})$ is 3.80.

**Sulfhydryl Reactivity**—The sulfhydryl reactivities of the reconstituted α- and β-met subunits are summarized in Table III; those of the native subunits and the separated globin chains are included for comparison. The reconstituted α-met subunit shows 0.89 reactive sulfhydryl group per chain, which is con-

### Table I

**Molar extinction coefficients of native and reconstituted subunits**

<table>
<thead>
<tr>
<th>Wave length</th>
<th>$α_{CN met}$</th>
<th>$β_{CN met}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>nm</td>
<td>Native</td>
<td>Reconstituted</td>
</tr>
<tr>
<td>276</td>
<td>$27.4 \times 10^3$</td>
<td>$28.4 \times 10^3$</td>
</tr>
<tr>
<td>420</td>
<td>$118.0 \times 10^3$</td>
<td>$115.0 \times 10^3$</td>
</tr>
<tr>
<td>540</td>
<td>$11.2 \times 10^3$</td>
<td>$11.2 \times 10^3$</td>
</tr>
</tbody>
</table>

*a* Values from Ragatz (4).

### Table II

**Sedimentation coefficients of native and reconstituted cyanmet hemoglobin and its subunits**

<table>
<thead>
<tr>
<th>Cyanmet compounds</th>
<th>Native</th>
<th>Reconstituted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin</td>
<td>4.04 (0.5 mg/ml)</td>
<td>3.86 (0.6 mg/ml)</td>
</tr>
<tr>
<td>$(α_{αt}β_{βt})$</td>
<td>1.88 (0.7 mg/ml)</td>
<td>1.85 (0.6 mg/ml)</td>
</tr>
<tr>
<td>$(α_{αt}β_{βa})$</td>
<td>3.80 (0.6 mg/ml)</td>
<td>3.83 (0.7 mg/ml)</td>
</tr>
<tr>
<td>α</td>
<td>0.89 (0.5 mg/ml)</td>
<td>0.64 (0.7 mg/ml)</td>
</tr>
<tr>
<td>β</td>
<td>0.64 (0.7 mg/ml)</td>
<td>0.45 (0.7 mg/ml)</td>
</tr>
</tbody>
</table>

* The $s_{20,w}$ is 6.61 for α-globin and 2.62 for β-globin. (See text and Ref. 1).

### Table III

**Sulphydryl reactivity of native and reconstituted subunits, separated globin chains, hemoglobin, and heme-containing intermediates**

Sulphydryl reactivity of globin chains was determined by the method of Ellman (6) using 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) at 4°. α-Globin was in 0.1 M potassium phosphate, pH 7.0, and β-globin was in 0.1 M Tris-HCl, pH 8.0. Sulphydryl reactivity of all heme-containing proteins was determined by the method of Boyer (7) using p-mercuribenzoate (PMB) at 25°, except the heme-containing intermediates, which were measured at 4°. The intermediate, $(α_{αt}β_{βa})$, was in 20 mM potassium phosphate, pH 6.7; and $(α_{αt}β_{βa})$ was in 0.1 M Tris-HCl, pH 8.0. All other heme proteins were in 0.1 M potassium phosphate, pH 7.0. SDS refers to 13% sodium dodecyl sulfate and GuCl to 6 M guanidinium HCl in the same buffer systems described above.

<table>
<thead>
<tr>
<th>Compound</th>
<th>By DTNB titration</th>
<th>By PMB titration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Buffer alone</td>
<td>SDS or GuCl</td>
</tr>
<tr>
<td>α</td>
<td>0.90</td>
<td>0.89</td>
</tr>
<tr>
<td>α*</td>
<td>0.97</td>
<td>0.97</td>
</tr>
<tr>
<td>β</td>
<td>1.90</td>
<td>1.90</td>
</tr>
<tr>
<td>β*</td>
<td>0.85</td>
<td>1.06</td>
</tr>
<tr>
<td>α-Globin</td>
<td>0.56</td>
<td>1.90</td>
</tr>
<tr>
<td>β-Globin</td>
<td>0.64</td>
<td>0.64</td>
</tr>
<tr>
<td>$(α_{αt}β_{β})$</td>
<td>0.45</td>
<td>0.45</td>
</tr>
<tr>
<td>$(α_{αt}β_{β})$</td>
<td>1.50</td>
<td>1.50</td>
</tr>
</tbody>
</table>

**Fig. 3.** Absorption spectrum of reconstituted α-met subunit in 0.1 M potassium phosphate, pH 7.5.
sistent with the value found for the native subunit. The reconstituted $\beta$-met subunit, however, shows only 0.85 of the 2.0 reactive sulfhydryl groups per chain. The same reconstituted subunit gives a sulfhydryl titer of 1.06 in 6 M guanidine HCl or in 15% sodium dodecyl sulfate.

Circular Dichroism Spectra—The far-ultraviolet CD spectra of the reconstituted cyanmet $\alpha$ and $\beta$ subunits, in comparison with the corresponding native subunits and globin chains, are shown in Figs. 4 and 5, respectively. The mean residue ellipticity, in degrees cm$^2$/d mole, at 222 nm for the reconstituted $\alpha$ subunit is $-22,000 \pm 500$, which is nearly identical with that of the native $\alpha$ subunit, $-22,600 \pm 500$. The marked increase in negative ellipticity from $-7,750$ of the $\alpha$-globin represents a recovery of helix content from 18 to 65% upon recombination with heme as estimated by the method of Chen and Yang (8). The mean residue ellipticity value of $21,800 \pm 1,000$ for the reconstituted $\beta$ subunit is, on the average, about 5% more negative than that of the native $\beta$ subunit, $-21,000 \pm 300$. The increase in ellipticity in this case is from $-17,750 \pm 200$ of the $\beta$-globin, corresponding to an increase in helix content from 51 to 65%. The negative extrema at 210 nm show similar recovery patterns in both reconstituted subunits.

The recovery of the near-ultraviolet CD spectral characteristics by the reconstituted cyanmet $\alpha$ subunit is shown in Fig. 6. The intensity of the major band at 255 nm in the reconstituted $\alpha$ subunit is $570 \pm 30$, and that for the native $\alpha$ subunit is $620 \pm 30$. The near-ultraviolet CD spectrum of the reconstituted cyanmet $\beta$ subunit, as shown in Fig. 7, exhibits slight variations from that of the native subunit. It has a major ellipticity band of intensity 270 $\pm 20$ at 255 nm while that of the native subunit is 230 $\pm 10$ at 258 nm, and both subunits show a second major ellipticity band of 230 $\pm 10$ at 265 nm. In the spectral range between 280 and 320 nm, a region where the signal to noise ratio is rather low, both reconstituted $\alpha$ and $\beta$ subunits show ellipticity bands slightly lower in intensity, on the average, than those of the corresponding native subunits.

### Heme-containing Intermediates

**Molecular Weights**—In Fig. 8 are shown the results of gel filtration molecular weight determinations of the heme-containing intermediates compared with those of $\beta$-globin, $\beta$-heme subunit, apohemoglobin, and reconstituted hemoglobin. The column was calibrated with hemoglobin A (64,500), apohemoglobin (32,000), and $\alpha$-heme chain (16,000) as molecular weight standards. The exclusion volume was measured using the haptoglobin-hemoglobin complex (150,000). The plot of the ratio of elution volume to exclusion volume, $V_e/V_o$, versus molecular weight shows that the molecular weight of $\alpha^\phi\beta^\varphi$ is about 32,000 and that of $\alpha^\phi\beta^\psi$ is about 60,000. Upon addition of 1 eq of heme to dimeric $\alpha^\phi\beta^\psi$, the molecular weight of this reconstituted hemoglobin increases to a tetramer value, 60,000.
globins with the alternate heme-containing subunits reveals differences in refolding behavior between the two globin chains. Table IV is a summary of the ellipticity of the intermediates and their constituent chains in the far-ultraviolet region. The mean residue ellipticity, in degrees cm$^2$ per dmole, of the intermediate ($\alpha\beta^2$)$_2$ at 222 nm is $-19,250$. This value exceeds the arithmetic mean of the $\alpha$-globin and $\beta$-heme subunit values by $-4,875$. A similar result is observed in the near-ultraviolet CD spectrum. As can be seen in Fig. 9, the mean residue ellipticity value is 236 at 252.5 nm, while the values for $\alpha$-globin, $\alpha\beta$-globin, and $\beta$-cyanmet subunit are 0, 75, and 160, respectively. The mean residue ellipticities of ($\alpha\beta^2$)$_2$ are therefore the sum of those of $\alpha\beta$-globin and the $\beta$ subunit, indicating that when bound to the heme-containing $\beta$ subunit, $\alpha$-globin acquires the optical activity of an $\alpha$-globin within the $\alpha\beta$-globin.

The intermediate ($\alpha\beta^2$) has a mean residue ellipticity value of $-18,500$ at 222 nm, which is slightly lower than the arithmetic mean value of its constituent chains, $-20,175$. Furthermore, its near-ultraviolet CD spectrum, as shown in Fig. 10, has essentially the same intensity as that of the $\alpha$-cyanmet subunit alone. Thus, in contrast to ($\alpha\beta^2$)$_2$, there appears to be a loss of ellipticity, $-1,675$ at 222 nm, when $\alpha$-heme subunit and $\beta$-globin

**Table IV**

Circular dichroism parameters of $\alpha\beta$ and $\alpha\beta^2$ intermediates and their constituent chains

Measurements on globin chains and heme-containing intermediates were carried out at 4°C, and on the heme-containing subunits at 20°C, on a Cary 60 spectropolarimeter equipped with a 6001 CD attachment. The buffers used were the same as described in Table III. The unit of mean residue ellipticity is degrees cm$^2$ per dmole. The mean residue weights used were 108 for $\alpha$-globins and for the heme-containing intermediates, 106 for $\alpha$-globin and 110 for $\beta$-globin.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mean residue ellipticity at 222 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experimental</td>
</tr>
<tr>
<td>($\alpha\beta^2$)$_2$</td>
<td>$-19,250$</td>
</tr>
<tr>
<td>$\alpha$-Globin</td>
<td>$-7,750$</td>
</tr>
<tr>
<td>$\beta$-Heme</td>
<td>$-21,000$</td>
</tr>
<tr>
<td>$\alpha\beta$-Globin</td>
<td>$-17,750$</td>
</tr>
<tr>
<td>($\alpha\beta^2$)</td>
<td>$-18,500$</td>
</tr>
<tr>
<td>$\alpha$-Heme</td>
<td>$-22,600$</td>
</tr>
<tr>
<td>$\beta$-Globin</td>
<td>$-17,750$</td>
</tr>
</tbody>
</table>

$^a$ Calculated taking the mean residue ellipticity value of $\beta$-heme as $-21,000$ and of $\alpha$-globin as $-7,750$.

$^b$ Calculated taking the mean residue ellipticity value of $\alpha$-globin as $-17,750$, which is the average chain value in $\alpha\beta$-globin.

$^c$ Calculated using the values for $\alpha$-heme and $\beta$-globin in the table.
combine to form the (αββ) dimer. However, the full far-ultraviolet intensity of hemoglobin is recovered upon addition of a stoichiometric equivalent of heme to the intermediate.

Sulphydryl Reactivity—The heme-containing intermediate (αbβb), shows 0.64 reactive sulphydryl group per chain, while (αbβb) shows 0.45 per chain (Table III). The sulphydryl titer of these heme-containing intermediates is therefore similar to that of the hemoglobin.

**Discussion**

Reconstituted α and β Subunits—Within the range of experimental uncertainty, the measured properties of the reconstituted α subunits are indistinguishable from those of native α subunits. The tests thus far applied to establish structural identity are limited, to be sure, but they probe different levels of structure. Moreover, in each measurement, the observable undergoes a large change when heme is bound to the isolated globin chain.

Three regions of the absorption spectrum—the visible, between 500 and 630 nm, the Soret band, and the ultraviolet near 280 nm—reflect particularly the heme-binding crevice and the manner of heme attachment. In the met form (Fig. 3), formation of hemochromogen would lead to absence or diminished amplitude of the characteristic peak at 500 nm (0, 10). In both met and cyanmet forms (Figs. 1 and 2), the Soret band would be reduced in intensity and shifted in position if the product of recombination were denatured.

The native conformation of the heme pocket is also assured by the intensity and position of the CD band near 255 nm (Fig. 6). This band, moreover, is especially distinctive in native α subunits and is highly sensitive to the ligand on the heme (11). In all these respects, the reconstituted α subunit shows values in excellent agreement with the original subunit.

At slightly longer wave lengths, 270 to 310 nm, intrinsic protein aromatic contributions to the CD spectrum, reflecting tertiary structure, are discerned, although these are still dominated by heme bands (11). However, the band near 295 nm which is due to the single invariant tryptophan residue (cf. Ref. 12) and is completely absent in the separated α-globin chain (Fig. 6) reappears on heme binding, suggesting that the environment and interactions of that residue, which spaces the A and E helices (13), are regained. This is, however, a spectral region where a low signal to noise ratio generates uncertainty of 25 to 50% in any trace. The restoration of structure in the vicinity of this residue is, accordingly, being investigated by fluorescence spectroscopy.

The most reassuring and prominent feature of the CD spectrum of reconstituted α subunits is the pattern of peptide bands in the region between 200 and 230 nm, representing the secondary structure. At 222 nm, the average value of residue ellipticity at the band maximum agrees with that of the original subunits to better than 3%. This represents a gain of nearly -15,000 deg cm² per dmole (mean residue weight basis) when the α-globin chain binds heme (hemin or dicyanide hemin). A minimum of 70 residues is involved in this event and it is more likely, actually, that the entire chain is restructured.

Reappearance of the full sulphydryl reactivity of cysteine G11α(104) signifies that heme binding has abolished the aggregation of α-globin chains and restored the residue to an exposed position, as occurs in native α subunits. Finally, the sedimentation coefficient is that of a monomer and equals that of the native α subunit, suggesting the same frictional coefficient.

In most respects, equally satisfactory recovery of the characteristic spectral and structural properties of native β subunits is observed on reconstitution of β-globin with heme. Thus, the absorption spectrum agrees over the entire accessible range and the sedimentation coefficient rises to an average value of 3.8 S.

Since the far-ultraviolet CD bands of β-globin are much more intense than those of α-globin, regeneration leads to a less spectacular recovery, but the final values in the peptide region match those of the original subunits.

In the near-ultraviolet, there are occasional minor variations in the CD spectra from preparation to preparation and some slight disagreements of the average values with the averages of native subunits. In the reconstituted subunits, the 255-nm band averages 10% greater intensity in the cyanmet form, there is a slight blue shift in the crossover at short wave length, and the weakly dichroic region between 280 and 310 nm is slightly less positive on average. The Soret CD spectra of reconstituted and native β subunits are the same, with [θ]max = 67,500 in the cyanmet forms, centered at 427 nm.⁴

Among those we have measured, the only property of reconstituted β subunits which does not agree with the corresponding one in the native subunits is the number of reactive sulphydryl groups, which is almost invariably reduced in the former (Table III). In a systematic effort to identify the origin of this problem, we have now been able to establish that it is the addition of hemin itself (or of dicyanide hemin) which is responsible for the low —SH titre. To demonstrate this conclusively required (a) that the original β subunits have 2 sulphydryl eq rather than between 1 and 2. A diminished value is frequently observed with the commonly used methods for regeneration of the subunits from the PM13 chains (14-18). For this reason, we devised a new procedure (sec above) which gives a value of 2 ± 0.1. (b) It also required that the β-globin have 2 sulphydryl eq per mole when measured in SDS or guanidinium chloride, indicating no disulfide formation and (c) that the —SH titre in SDS or guanidinium chloride be diminished directly after hemin binding.

It has been known for many years that hemin catalyzes dissociation of the heme group from sulphydryl in the presence of oxygen, although methemoglobin itself is inactive (19). Sulphydryl groups and free hemin may, then, form an oxidation-reduction couple and it is accordingly possible, although we have only scant and equivocal evidence on this point, that some disulfide formation occurs when hemin is added. The recombination furthermore, occurs at pH 8 with β-globin where the reactive species, ionized sulphydryl, is present to considerable extent.

It should be emphasized, here, that this problem never occurs in the α subunit reconstitution.

With regard, finally, to the reconstituted subunits, it may be noted that the reconstitution procedures utilize hemin or dicyanide hemin directly. This means that the ferric forms are produced without the need to expose the subunits to ferricyanide ion, if the oxidized derivatives of the subunits are desired. An unexpected consequence of this difference is that the reconstituted subunits in the ferric forms appear to be more stable than the native met-subunits. For example, both α and β subunits in the met form undergo transformation to hemochromogens within hours (9), whereas the reconstituted subunits do not undergo such change over extended periods, and the characteristic native methemoglobin absorption spectra persist for several days.

**Properties of the Half-Filled Molecules**—The molecules made by combining separated globin chains with the alternate hemec containing subunit have only half of the heme sites occupied.

⁴ Unpublished observations.
They are of two kinds, designated for convenience in discussing them as HFa and HFβ. HFa has heme only on α chains and is made by combining native α subunits with equivalent amounts of β-globin chains; HFβ has hemes only on β chains and is prepared from native β subunits and α-globin chains.

In describing the distinctive properties of the 2 molecules, it is important to reiterate first that the separated isolated globin chains do not measurably combine under the same conditions where the respective apo- and holo-chains combine to form HFa and HFβ. Furthermore, four experimental features of these latter combinations merit emphasis and enumeration. (a) In the case of each of the HF molecules, one of the reactants has the same molecular weight as the product, but the other has a different molecular weight and the products are homogeneous in each case. (b) When the globins of either kind are mixed with the alternate heme-containing subunit, no precipitation occurs. This is a very important point, and lends special significance to the study of the HFa and HFβ molecules, because the addition of hemin itself to any globin, isolated α, isolated β, or αβ, always leads to some precipitate. It is as if hemin fractionates the globins and this always raises the suspicion that the globin preparation is inhomogeneous, despite experimental tests which suggest otherwise. This difficulty is obviated in solutions of HFa and HFβ and a measured change in the property of the globin chain on binding the subunit averages over all of the globin molecules introduced into the final solution. (c) Each of these intermediate molecules accepts 1 eq of heme with formation of tetrameric hemoglobin. (d) There is no discernible exchange of heme groups between chains within HFa and HFβ, if the heme is in the cyanem form. The evidence comes from the CD bands near 255 nm, the intensities of which are very different in α and β subunits (11). A distribution of heme on the two chains would be readily detected as a departure from the characteristic values. This is difficult to establish in the met form because the bands are weak in both subunits.

As shown in the results, HFa is a dimeric molecule, αββα, and HFβ is a tetramer, (αββα)4. In both molecules, only 0.5 eq of sulfhydryl per monomer react with PMB, the same values as found in hemoglobin and in αβ-globin (90). In the far-ultraviolet, the CD bands of both molecules have intensities that fall between hemoglobin and apohemoglobin. In the case of HFβ, the values are the exact arithmetic means of the β subunit and αβ-globin, indicating that in HFβ, the α-globin has regained the ellipticity which it has when combined in αβ-globin, but in HFa, the intensities are not additive.

Throughout the far ultraviolet the CD intensity is lower in the molecules αββα than in molecules (αββα)4. Additivity would result in the reverse, since α- and β-globins generate equal far-ultraviolet intensities when they are bound together in apohemoglobin, whereas α subunits show greater intensity than β subunits. This suggests that β-globin in αββα is less ordered or differently ordered than in αββα and in free β-globin. With intermediates prepared from apohemoglobin rather than from isolated chains, Cassoly and Banerjee also observed less intensity in the far-ultraviolet bands of molecules containing heme only on α chains than in those of molecules containing heme on β chains (21). In a recent paper, Winterhalter and Wüthrich (22) suggest some unfolding of the heme-free β chain in an intermediate they have characterized, IC II, which contains heme only on α chains.

With respect to quaternary structure, Cassoly and Banerjee (21) recently compared a number of such intermediates made by them and by Winterhalter and Deranleau (23) and found a dependence of molecular weight on method of preparation. Waternman et al. (24) reported a molecular weight of 50,000 for an intermediate, αββα.

The folding of the chains in the intermediate molecules, and the actual interfaces formed, then, depend primarily on which chains carry the heme and, perhaps secondarily, on how the intermediate was formed. In this connection, it is interesting that hemoglobin Köln, a mutant in which heme has been lost from β chains, occurs as a dimer in solution. Moreover, addition of heme to the heme-free β chains increases the sedimentation coefficient to that of a tetramer. This behavior is similar to that of HFa.

αβ Interfaces—Since α-globin does not bind β-globin, but does bind to the heme-containing β subunit, it is necessary to explore first the known differences between β-globin and the β subunit. To begin with, the separated subunit occurs as a tetramer, whereas β-globin is a dimer (1). Moreover, in the subunit both cysteine residues, F9β(93) and G14β(112), are exposed and reactive, whereas in β-globin only 0.5 residue per chain reacts with DTNB. These facts suggest that the interface in the β-globin dimer comprises residues different from any of the β subunit interfaces and that the former interface includes the G14β(112) cysteinyl residues from both chains. The additional diminution of sulfhydryl reactivity in the β-globin dimer probably results from conformational readjustment in the heme pocket brought about by removal of heme.

Conformational changes in the heme pocket were described by Greer (25) in a study of hemoglobin M Hyde Park. Greer showed that the loss of heme from β chains results in the collapse of residues adjacent to the heme binding site into the empty heme pocket. Thus, residues FG3β(96), CD1β(42), CD4β(45), C7β(41), G4β(102), and the other parts of Gβ (probably NH-terminal) and Hβ (probably COOH-terminal) helices move toward and into the pocket. In addition, the segment of the chain from F8β(92) to FG4β(97) changes conformation, and about half of the cysteine residues F9β(93) in molecules which have lost heme suffer oxidation or binding to trace metals. It is now possible to propose qualitative features of the changes to be anticipated when the β subunits are freed of heme. The critical idea is that the interface in the β-globin dimer is closely similar to the α1-α2 interface in hemoglobin and dissimilar to any of the interfaces in the tetramer β-subunit. In the α1-α2 interface in hemoglobin (and presumably in αβ-globin, for the most part), the COOH-terminal part of the G helices, the GH regions and the 1st 9 NH2-terminal residues of the H helices provide about 50% of the atom contacts and two of the three hydrogen bonds. That interface resists disruption in αβ-globin and in β-globin, as well, which remains dimeric over a range of ionic strength and pH. We presume that it is the persistent inaccessibility of residues in that dimer interface which prevents binding of isolated α-globin to β-globin. The presence of heme in β-globin somehow releases contacts in that region or alters the chain conformation in such a way that the complementarity of residues is abolished or weakened, freeing the surface for binding to α-globin chains.

There is, however, a further requirement to be met in the binding of α-globin chains to the β subunit, since the resultant molecule is a tetramer and includes not only an α-β-contact surface, but an α-β-type interface, as well. The latter interface is broken in the formation of αβ-globin for reasons that appear clear from Greer’s results. Thus, the interface is estab-
listed by atoms contributed by C helix, FG and NH₂-terminal G helix residues. Their neighbor residues are those that collapse into the empty heme pocket and the interface is accordingly broken.

The entire C helix and the NH₂-terminal segment of the G helix appear to owe their conformational stability in hemoglobin wholly to the α1-β2 interface and to stabilizing heme contacts. We analyzed the sequences of the two chains by the procedure of Kabat and Wu (26) which tabulates frequencies of helix-breaking residues in proteins of known structures. After the elimination of hemoglobin and myoglobin sequences from their tabulation, the analysis reveals that residues C1, C2, C3, C4, and CD1 in the human α chain are all helix-breaking, and residues C1, C3, and C7 are helix-breaking in the human β chain, likewise, G1, G3, G6, and G7 in the α chain and G1, G3, G5, G6, and G7 in the β chain are all helix breakers. Accordingly, the entire 7-residue C helix and much of the G helix in both chains would not, on the basis of nearest neighbor influence, be expected to adopt helical conformations in either chain.

Thus, the second requirement for binding of the α-globin is the existence of ordered C, FG, and NH₂-terminal G regions which do not occur, or are of transient occurrence, in β-globin. An implied corollary of this conclusion is that an interface mimicking the α1-β2 interface occurs in β subunits, which in the separated state are fully as helical as in hemoglobin itself, and that the absence of such interface stabilization accounts for the decreased order observed in the β-globin chain of the HF (αHβ') dimer.

Alloplex Interaction—We turn now to an analysis of the most prominent result of this work, the refolding of the disordered α-globin chain on binding to the β subunit and the reassembly of the tetramer in this process. Similar refolding phenomena occur in other proteins. For example, ribonuclease S-peptide is refolded on contact with S-protein (27) and complementary fragments of staphylococcal nuclease are refolded upon mixing (28). However, neither of those involves refolding of a subunit brought about by contact with a neighboring subunit, and the reordering in both of those cases is less extensive.

It is not clear, in the first place, why the β-globin remains ordered whereas the α-globin becomes extensively disordered in the isolated states. It is plausible, but not experimentally shown, that the ordered structure of β-globin arises from mutual stabilization due to contacts of structural elements in the dimer. Interestingly, apomyoglobin is as ordered as β-globin although it is monomeric. However, the stabilizing interactions may be quite different in that case, as evidenced by the indiscernibility of molecular weight to heme removal from myoglobin (29). The extent of order is so low in α-globin, that it does not appear worthwhile to suppose that there is a unique conformation in the isolated chain. Rather, it is more likely that α-globin is an ensemble of states, with no particular structural element associated with a particular stretch of residues. The tendency to form loose, high molecular weight aggregates, dependent on pH and ionic strength, is compatible with this idea.

In the alloplex interaction between α-globin and the β subunit, three steps are involved, namely, disaggregation, binding, and refolding. In studies of initial and final states, it is not possible to distinguish between two extreme mechanisms. Specifically, (a) there are two or more conformations of α-globin molecules in rapid equilibrium, only one of which, correctly folded, is bound with subsequent displacement of the equilibrium or (b) an encounter with a disordered molecule at the correct surface leads to a nucleation event which initiates the refolding. The disturbance between these extremes must come from kinetic experiments, several of which are in progress or planned.

The initial event in the alloplex binding must occur at one of the two interfaces which are reformed, in distinction to the more extensive restructuring phenomenon initiated at the heme crevice which takes place when heme is bound. This knowledge allows specific experimental tests of the properties of residues at the proposed contact surfaces. It is important, however, to stress that the conditions of these experiments do not reproduce the complex circumstances that obtain in a reticulocyte during biosynthesis and assembly, with possible involvement of ribosomal and membrane surfaces during folding.

In the next paper of this series, we will discuss possible implications of these distinctions and we will detail certain functional properties of the HF molecules and of the fully reconstituted hemoglobins.

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Influence of Prosthetic Groups on Protein Folding and Subunit Assembly:
RECOMBINATION OF SEPARATED HUMAN α- AND β-GLOBIN CHAINS WITH HEME AND ALLOPLEX INTERACTIONS OF GLOBIN CHAINS WITH HEME-CONTAINING SUBUNITS
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