The Plane of Cleavage in Human Ferrihemoglobin*

I. ULTRAVIOLET DIFFERENCE SPECTROSCOPY

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X-ray diffraction studies have shown that hemoglobin has two predominant interfaces in the tetramer at which dissociation to dimers could occur. These interfaces have been designated as $\alpha^1\beta'$ and $\alpha^1\beta^2$. There are 2 tyrosyl residues and 1 tryptophanyl residue in the $\alpha^1\beta'$ interface but only 1 tyrosyl residue in the $\alpha^1\beta^2$ interface. On dissociation, the ultraviolet absorption spectra of the aromatic chromophores at the interface exposed to the solvent are perturbed. The ultraviolet difference spectrum between ferrihemoglobin dissociated in 1 M NaClO$_4$ and undissociated hemoglobin revealed two negative peaks, one at 292.5 nm and another at 285 nm. This difference spectrum is due to tyrosyl and tryptophanyl residues which reside on the plane of cleavage and were exposed to 1 M NaClO$_4$ upon dissociation. Hence, dissociation must have occurred along the $\alpha^1\beta^2$ interface to yield $\alpha^1\beta^1$ dimers.

The $\Delta F^\circ$ value extrapolated to zero salt concentration calculated on the basis of difference spectroscopy and sedimentation velocity experiments is 8.6 ± 0.7 kcal per mol at pH 7.1 ($K \approx 4.5 \times 10^{-7}$ M).

It is well known that there is an equilibrium between the hemoglobin tetramer and its symmetrical dimers under physiological conditions (2), and that this equilibrium is shifted toward dissociation under conditions of either high or low pH (3, 4) or high ionic strength (5, 6). There are two planes across which this dissociation could occur:

$$\alpha^1 \quad \beta^1$$

$$\beta^2 \quad \alpha^2$$

to give either $\alpha^1\beta^1$ dimers (cleavage along the $\alpha^1\beta^2$ plane represented by the broken line) or $\alpha^1\beta^2$ dimers (cleavage along the $\alpha^1\beta^1$ plane represented by the solid line). The question regarding the interface at which dissociation does occur has not yet been unequivocally resolved.

Mizukami and Lumry (7) studied the spectral changes that occur when horse oxyhemoglobin is dissociated by dilution in 1 M NaCl. These authors attributed the absorbance changes at 293 nm to the exposure of a tryptophan residue to the solvent as tetrameric hemoglobin dissociates to dimers and the changes at 270 nm to the dissociation of dimers to monomers. On the basis of these results and in conjunction with Perutz's report (8) on the contact sites between $\alpha^1$ and $\beta^1$ and between $\alpha^2$ and $\beta^2$ subunits of hemoglobin, Lumry (9) has indicated that the spectral changes at 293 nm occur as a result of perturbation of tryptophan residue, C336, located in the $\alpha^1\beta^2$ interface. If Lumry's (9) interpretation of his spectral data is correct, this would indicate that the dissociation process is $\alpha^1\alpha^2\beta^1\beta^2 \rightarrow \alpha^1\beta^1 + \alpha^2\beta^2$. Unfortunately, this equation was printed incorrectly as $\alpha^1\alpha^2\beta^1\beta^2 \rightarrow \alpha^1\beta^2 + \alpha^2\beta^1$ in the above reference.

Although the dissociation constant that Mizukami and Lumry (7) calculated from their spectral changes for the dissociation of horse oxyhemoglobin to dimers is similar to that calculated by others for human carboxyhemoglobin (5, 6), Kellet (10) recently has shown that human oxyhemoglobin does not dissociate to monomers in 2 M NaCl even at concentrations $\gamma_0$ of that used by Mizukami and Lumry (7). Inasmuch as some effect other than dissociation is responsible for the spectral changes observed by Mizukami and Lumry (7) at 270 nm, it is possible that the spectral changes they observed at 293 nm also are not correlated with dissociation. We have reinvestigated the spectral changes which occur when hemoglobin is subjected to dissociation by high concentrations of salt and have correlated the observed spectral changes with an independent measure of dissociation.

Ferrihemoglobin rather than oxy- or carboxyhemoglobin was used in order to avoid any oxidation of ferrohemoglobin to ferrihemoglobin during the experiment. Also, because of the absence of $\delta$ and $\epsilon$ absorption bands in ferrihemoglobin (11), the contribution of the heme group of ferrihemoglobin to the ultraviolet spectrum in the 270- to 300-nm region is less than that of ferrohemoglobin. Sodium perchlorate was selected as the dissociating reagent because it has been reported by Guidotti (6) to be a very effective dissociating reagent for deoxyhemoglobin, carboxyhemoglobin, oxyhemoglobin, and cyanmethemoglobin.
EXPERIMENTAL PROCEDURE

Materials—Sodium perchlorate (NaClO₄·H₂O) was obtained from Fisher Scientific Co., was recrystallized twice from water, and was kept in a desiccator over a drying agent. Hemolysates of adult human blood were prepared and stored under carbon monoxide as described by Hill et al. (12). The carboxyhemoglobin was isolated from the hemolysates by chromatography on sulfoethyl (SE)-Sephadex (C-50) or SP-Sephadex (C-50) (13) and was concentrated by ultrafiltration (14). The carboxyhemoglobin was converted to ferrihemoglobin in the presence of 4-fold molar excess of potassium ferricyanide (15). The conversion was facilitated by passing air over the mixture of protein and potassium ferricyanide in the presence of intense artificial light. The protein solution was dialyzed against 0.15 M KCl and then was dialyzed exhaustively against water to remove excess potassium ferricyanide. The concentrated ferrihemoglobin then was deionized by passing it through a Dint& column (16). Ferrimyoglobin (whole skeletal muscle, lyophilized and salt-free and obtained from Miles-Seravac (PTY) Ltd., was used without further purification. Excess potassium ferricyanide was added to the commercial sample to ensure complete conversion to ferrimyoglobin. Then the ferrimyoglobin solution was treated to remove excess potassium ferricyanide as in the case of ferrihemoglobin.

Hemoglobin Concentration The concentration of hemoglobin or myoglobin, was measured spectrophotometrically at 540 nm after converting the heme protein to cyanmetheme protein (17) with tnm = 11,000 M⁻¹ cm⁻¹ per heme (18) for the cyanmetheme protein.

Difference Spectra—The difference spectrum measurements were made in an air-conditioned room at 23 ± 1°C using a Cary model 14 recording spectrophotometer with a 0.1-absorbance slide-wire. The procedure used for the difference spectrum measurements was essentially that of Laskowski and Herskovits (19). Prior to the use of the stock solution and its concentration measurements, all of the solutions were filtered through appropriate Millipore filters (pore diameter 0.45 μm) in order to remove any suspended impurities. The concentration used in the experiments was corrected by weight measurements. The final hemoglobin concentration was 0.1% and the final myoglobin concentration was 0.12% in all of the experiments.

Molecular Weight Measurements—A Beckman model E analytical ultracentrifuge equipped with schlieren and interference optics was used for the determination of the molecular weight and the sedimentation coefficient of ferrihemoglobin. Schlieren optics were used for all sedimentation velocity experiments. A He-Ne laser (University Laboratories Inc., model PS200) light source was used with interference optics for sedimentation equilibrium experiments. The initial concentration was determined by the synthetic boundary method.

RESULTS

Salt-dependent Difference Spectrum—The ultraviolet difference spectrum between ferrihemoglobin dissociated into dimers in 1 M sodium perchlorate and tetrameric ferrihemoglobin in water is shown in Fig. 1. It is possible that at least a portion of this difference spectrum could be due to the salt perturbation of the heme spectrum rather than to the exposure of aromatic amino acids on dissociation. In order to evaluate the extent of such an effect, the difference spectrum of ferrimyoglobin under the same conditions was recorded and is shown in Fig. 1. The solid curve was calculated by subtracting 4 times the ferrimyoglobin difference spectrum from the ferrihemoglobin difference spectrum. It is apparent that a portion of the ferrihemoglobin salt-dependent difference spectrum probably is due to the salt perturbation of the heme chromophore, but the major portion of the ferrihemoglobin difference spectrum cannot be attributed to this effect. The minimum at 292.5 nm can be attributed to the perturbation of a tryptophan chromophore, whereas the minimum at 285 nm can be attributed to both tyrosyl and tryptophanyl residues (20, 21). This result would be expected if dissociation occurred at the α1-β2 interface exposing tryptophan residue C837(37)β and tyrosine residues C742(42)α and H233(140)α to the solvent.

This conclusion assumes that ferrihemoglobin is completely dissociated to dimers in 1 M NaClO₄ and predicts that the appearance of the salt-dependent difference spectrum will correlate with this dissociation.

Molecular Weight Measurements—Sedimentation equilibrium experiments were performed with ferrihemoglobin solutions containing either 0.1 M NaClO₄ or 1 M NaClO₄. The molecular weights obtained from these experiments were 62,300 in 1 M NaClO₄. The molecular weight calculated from the amino acid composition of the tetrameric hemoglobin molecule is 64,450 (12). The discrepancy between the true and the calculated molecular weight can be attributed to 6% dissociation in 0.1 M NaClO₄ (Fig. 3). The value of the partial specific volume was taken to be 0.749 ml per g at 20°C at both concentrations of NaClO₄. This was calculated from the data of Kirshner and Tanford (5), which gave β = 0.745 ml per g at 10°C and v = 0.191 ml per g at 25°C at all concentrations of sodium chloride up to 3 M.

Ferrimyoglobin sedimentation coefficients and the results of salt-dependent difference spectra are shown in Fig. 2. The extrapolated values for Sₐₜₜ of 4.55 S without any NaClO₄ and 3.1 S in 1 M NaClO₄ are in good agreement with many previous determinations carried out with different salts under similar conditions (22).

It is obvious from both sedimentation equilibrium and sedimentation velocity data that ferrihemoglobin dissociates to dimer subunits in 1 M NaClO₄. The calculations of the dissociation constant (K) and the free energy (ΔF°) represented in Fig. 3 were done according to the procedure of Kawahara et al. (23). These authors interpreted the curvature in the graph of the values of ΔF° calculated on the basis of sedimentation coefficients to be due to specific ion binding to the dimer subunits which are absent in the tetrameric hemoglobin. The ΔF° value extrapolated to zero salt concentration (Fig. 3) is calculated to be 8.6 ± 0.9 kcal per mol (K = 4.5 × 10⁻⁷ M).

DISCUSSION

On the basis of the above results, it is concluded that dissociation must have occurred along the α1-β2 interface to yield α1β1 dimers.
Fig. 2. Effect of NaClO₄ on the sedimentation coefficient (ΔS) and the molar absorbance at 292.5 nm (●) and 285 nm (■) of terrheminoglobin (3.14 × 10⁻⁵ M and 1.57 × 10⁻⁵ M, respectively). The solid curve was drawn on the basis of ΔS calculated from the propagation of error in the experimental data, assuming an uncertainty of ±0.002 in the measured ΔA values and an uncertainty of ±0.2 S in the measured sedimentation coefficient.

Fig. 3. ΔF° of dissociation as a function of NaClO₄ calculated on the basis of the sedimentation coefficient (ΔS) and molar absorbance at 292.5 nm (●) and 285 nm (■). The error bars were calculated as described in Fig. 2.

Kawahara et al. (23) and Park (24) have shown that the cleavage plane in both liganded and unliganded hemoglobin is the same, α₁β₂ or α₁β₁. Perutz et al. (8), from x-ray diffraction studies, have indicated that the dissociation of tetrameric hemoglobin should occur across the α₁-β₃ interface. Their conclusion depended upon investigating the relationships between point mutations and subunit interactions in hemoglobin (25). The findings of Rosemeyer and Huehns (26) indicated that in liganded hemoglobin the α₁-β₃ interface makes and breaks in a chemically induced association-dissociation process. Their conclusion was the result of studies on hemoglobin modified at the reactive sulphydryl groups by p-chloromercuribenzoate. Briehl and Hobsb (27) studied the pH-dependent ultraviolet difference spectra of oxyhemoglobin. Their results tentatively indicated that the α₁-β₃ interface might break in 3 m KCl resulting in αβ₄ dimers.

Anderson et al. (28) studied the kinetics of the difference spectrum of carboxyhemoglobin and tentatively identified the groups responsible for the spectral changes on dissociation as Tyr C7(42)α and C3(37)β which lie in the α₁-β₃ interface. Sharonova et al. (29) studied the dissociation of deoxyhemoglobin between pH 10 and 11. They calculated that the process was accompanied by the release of about four protons on dissociation due to tyrosyl and lysyl residues. These authors also concluded that the splitting occurs across the α₁-β₃ interface with the formation of αβ₄ dimer subunits.

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REFERENCES
The plane of cleavage in human ferrihemoglobin. I. Ultraviolet difference spectroscopy.
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