The Green Hemoproteins of Bovine Erythrocytes

II. SPECTRAL, LIGAND-BINDING, AND ELECTROCHEMICAL PROPERTIES*

LOUIS J. DEFilippisz and DONALD E. Hultquist

From the Department of Biological Chemistry, The University of Michigan, Ann Arbor, Michigan 48109

The two green hemoproteins isolated from bovine erythrocytes (form I and form II) have been characterized as to spectral, electrochemical, and chemical properties. The absorption spectra of the isolated hemoproteins are typical of high spin ferric states. Reduction of the hemoproteins yields high spin ferrohemoproteins. Complexation of the ferrohemoproteins with CO and the ferrihemoproteins with cyanide yields low spin complexes, demonstrating the presence of an exchangeable weak field ligand in both the ferrous and ferric states of the hemoproteins. The differences in position and intensity of the absorption peaks of the visible spectra allow the two forms to be distinguished from one another.

The midpoint potential of forms I and II were found to be +0.075 and +0.019 V, respectively, at pH 6.4 and +0.038 and -0.005 V, respectively, at pH 7.0. This is consistent with the gaining of 1 proton/electron during the reduction. The Nernst plot reveals an unusual 0.5-electron transfer, whereas a quantitative titration demonstrates a 1-electron transfer. Form I binds cyanide more tightly than form II (K of 84 and 252 PM, respectively).

The observed spectral, electrochemical, and ligand-binding differences between forms I and II can be explained in terms of a greater electron-withdrawing ability of the side chains of the heme of form I relative to the heme of form II.

EXPERIMENTAL PROCEDURES

Materials - Carbon monoxide and nitrogen (both prepurified grade) were obtained from Union Carbide, Linde Division; sodium cyanide from Matheson, Coleman, and Bell; and indigotetrasulfonate from ICN · K&K Laboratories. Other materials used herein are listed in the preceding paper (1).

Spectrophotometry - Spectra were recorded on an Aminco-Chance or Cary model 17 recording spectrophotometer. Oxygen was removed from nitrogen and carbon monoxide in a modified Ace-Burlitch Inert Atmosphere apparatus equipped with BASF catalysts. Reduction of the hemoprotein was performed under the anaerobic atmosphere by adding a few grains of sodium dithionite from the sidearm of a modified Thunberg cuvette. Reduced pyridine hemochrome spectra were obtained by the method of Paul et al. (2).

Photochemical Reduction - Photochemical reduction was performed under an anaerobic atmosphere in the presence of the photosensitizing agents lumiflavin-3-acetate or proflavin, EDTA (3 mM), and light. The molar ratio of lumiflavin-3-acetate or proflavin to hemoprotein was approximately 1:20.

Molar Absorptivities - Extinction coefficients were calculated by correlating iron determinations performed on a Perkin-Elmer 306 atomic absorption spectrophotometer with the spectra of the ferrihemoproteins dissolved in 0.05 M potassium phosphate, pH 7.2. The instrument was calibrated with iron standards prepared in dilute

Fig. 1. Nernst plot of potentiometric titration of indigotetrasulfonate: measured potential (in volts) versus log (oxidized dye/reduced dye). The concentration of indigotetrasulfonate is approximately 25 PM, buffer is 0.1 M potassium phosphate (pH 6.4), T is 25°C and volume of sample is 3 ml. Reduction was performed under strictly anaerobic nitrogen using dilute sodium dithionite as reductant. The sample was mixed until a constant potential was obtained.

undertaken in an attempt to determine a structural basis for these observed similarities and differences of the two hemoproteins. In this paper we report the spectral, electrochemical, and ligand-binding properties of both proteins.

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† Present address, Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, N. Y. 14853.
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**Figure 2.** Absolute absorption spectra of oxidized, reduced, and reduced plus CO states of the green hemoproteins. Plate *a*, form I at a concentration of 4.39 nmol/ml on a heme basis. Plate *b*, form II at a concentration of 4.83 nmol/ml on a heme basis. The buffer is 0.05 M potassium phosphate (pH 7.2). T is 13°C, and volume is 2.5 ml. The reduced species were formed by the addition of a few grains of sodium dithionite under an anaerobic nitrogen atmosphere; the CO complexes were formed in the same fashion but under an atmosphere of anaerobic CO. The region from 475 to 700 nm is shown on expanded scale. The absorbance scale for this region is depicted on the ordinate to the right of the figure while the absorbance scale for the unexpanded region is shown on the ordinate to the left. The numbers in the figure are the wavelength values for prominent maxima and shoulders.
Fig. 3. The absolute absorption spectra of the oxidized and oxidized plus cyanide states of the green hemoproteins. Plate a, form I at a concentration of 4.80 nmol/ml. Plate b, form II at a concentration of 5.89 nmol/ml. The buffer is 0.05 M potassium phosphate (pH 7.2), the temperature is 25°C, and the cyanide concentration is 5 mM. The region from 475 to 700 nm is shown on expanded scale. The absorbance scale for this region is depicted on the ordinate to the right of the figure, while the absorbance scale for the unexpanded region is shown on the ordinate to the left.
nitric acid. Quadruplicate samples of form I and duplicate samples of form II were analyzed. The extinctions of the other derivatives were calculated using the ferrihemoproteins as secondary standards.

Quantitative Reductions—Quantitative reductions were performed using a solution of Na$_2$S$_2$O$_4$ that had been standardized against a solution of lumiflavin-3-acetate ($\Delta_{\lambda_{446}} = 11.300 \text{ m}^{-1} \text{ cm}^{-1}$) containing glucose oxidase, catalase, and 0.1 M glucose in 0.1 M phosphate, pH 7.7, at 20°C. The dithionite was added under an anaerobic nitrogen atmosphere using a 0.5-mi threaded syringe that delivered 5.3 $\mu$l/turn.

Midpoint Potential Determination—The hemoproteins were titrated with sodium dithionite under anaerobic conditions in the presence of the oxidation-reduction dye indigotetrasulfonate which had a reported midpoint potential of pH 7.0 ($E^\circ$) of -46 mV (4). Indigotetrasulfonate was first purified by paper chromatography (Whatman 3MM) in a 95% ethanol/water (1:1) solvent system. Since equilibria of the protein/dye mixture were established quite rapidly, the dithionite titrations were carried out with all possible speed. The oxidized to reduced ratio of the dye was determined at 625 nm and used to correct the absorbance reading at 444 nm from which the oxidized to reduced ratio of the hemoprotein was computed. The correction factor, $y$, is defined as

$$y = \frac{A_{\text{dye,ox},625 \text{ nm}} - A_{\text{dye,red},444 \text{ nm}}}{A_{\text{dye,red},625 \text{ nm}} - A_{\text{dye,red},444 \text{ nm}}}$$

and is equal to 0.241 at pH 7.0 but is essentially zero at pH 6.4. In order to correct for the dye's contribution to the hemoprotein's absorbance at any given point in the titration, $y$ is multiplied by the fraction of the dye reduced times the total change in absorbance at 625 nm and this product is subtracted from the change in absorbance at 444 nm.

The potentiometric data were analyzed by plotting the calculated potential versus the log of the ratio of the oxidized to reduced hemoprotein as per the Nernst equation. The plot for the titration of form I at pH 7.0 was exactly parallel to that obtained at pH 6.4.

At pH 6.4 and 7.0 the dye was found not to have an oxidation-reduction behavior as would be predicted by the Nernst equation. Consequently, the dye was titrated in an anaerobic cuvette fitted with a miniature platinum electrode and reference cell and the measured values plotted to obtain a standard curve (see Fig. 1). Extrapolated values read from the curve were used to determine the potential of the protein/dye mixture at any given point in the titration. The dye's oxidation-reduction behavior on the Nernst plot was found to be of a 1-electron acceptor when mainly in the oxidized state and of a 2-electron acceptor when more than 50% reduced. The electrochemical behavior of the dye at pH 7.0 was predicted from the pH 6.4 data by assuming 1 proton gained for every electron gained. The absorbance changes of the dye in the dye/hemoprotein mixture were monitored at 625 nm, an isosbestic point of the hemoprotein. The buffer was 0.1 M potassium phosphate, pH 7.0, and the temperature was 20°C.

RESULTS AND DISCUSSION

Absorption Spectra of Forms I and II—The absorption spectra of the two forms of the hemoprotein are shown in Figs. 2 and 3. The spectra of the two proteins are readily distinguished from each other but have a number of features in common. In Tables I through IV are summarized the absorption maxima and the molar absorbivities for the absolute and liganded states.

As isolated, both forms I and II possess absorption spectra with two widely separated bands in the visible region, diagnostic of high spin ferric complexes (see Refs. 5 to 7). Relative to form II, the visible bands of ferri-form I (510 to 614 nm) are less pronounced and are found at longer wavelengths.

Reduction with sodium dithionite under strictly anaerobic conditions converts the ferrihemoproteins to high spin ferrohemoproteins. For each protein, reduction is accompanied by

| Oxidation State | Ligand added | $\lambda_{\text{max, nm}}$ | $\lambda_{\text{min, nm}}$ | Extinction coefficient |
|-----------------|--------------|---------------------------|---------------------------|-----------------------|
| Ferrous         | None         | 614(s)                    | 528                       | 9.5                   |
|                 | CO           | 610(s)                    | 569                       | 32                    |
|                 | Pyridine     | 618(s)                    | 573                       | 9.0                   |

* (s) denotes a shoulder rather than a discrete peak.
The ligand exchange with the strong field ligands cyanide and carbon monoxide must take place with the weak field ligand of the hemoproteins and the low spin type spectra of the cyanide-ferrihemoprotein complex and the carbon monoxide-ferrohemoprotein complex, suggest that one strong field ligand and one weak field ligand are bonded to the heme iron. The ligand exchange with the strong field ligands cyanide and carbon monoxide must take place with the weak field ligand of the protein to give the low spin spectra characteristic of complexes with two strong field ligands.

Both forms exhibit peaks in the ultraviolet region at 268 nm with slight shoulders at 261 nm. Since there is not an abnormally large amount of phenylalanine present in the proteins it would appear that this unusually blue-shifted absorbance maximum is due in part to the heme absorbance. Absorbance in this region by hemoproteins has been thought to be due only in part to absorbance by the protein moiety.

In general, the absorption maxima in the visible region of these spectra are found at longer wavelengths for form I than for form II. This supports the conclusion, drawn from the comparison of the reduced pyridine hemochrome spectra of the hemoproteins, that the heme of form I has a more extended resonance pathway.

The distinctive differences between the two forms of the hemoprotein are highlighted in the difference spectra obtained by recording the reduced minus oxidized spectra, the reduced minus cyanide minus oxidized spectra (see Figs. 4 to 6). Dissociation Constant for Cyanide-Ferrihemoprotein Complexes—The two forms of the green hemoprotein were titrated with cyanide as described in the legend to Fig. 7. No assumption was made as to the ionic state of cyanide bound by the hemoproteins. The spectral changes observed upon the stepwise addition of cyanide to forms I and II are shown in Plates a and b, respectively. Upon addition of cyanide, the normal cyanide minus oxidized spectra (see Figs. 4 to 6).
Soret band of the hemoprotein is seen to disappear with the concomitant formation of the Soret and visible peaks of the hemoprotein-cyanide complex. Several clear isosbestic points are present throughout the course of the titration. Analysis of the data by the method of Hill (9) yields a dissociation constant ($K_{d,\text{co}}$) for form I of 84.1 (±0.9 S.D.) μM for form II of 252.2 (±1.9 S.D.) μM. (Data are not shown.) A plot of these data by the method of Scatchard (10) yields a dissociation constant for form I of 80.8 (±3.1 S.D.) μM for form II of 222.9 (±13.5 S.D.) μM (see Fig. 8).

The affinity for cyanide of form I of bovine erythrocyte green hemoprotein is similar to that observed for the human erythrocyte green hemoprotein. Thus the form I of bovine erythrocyte green hemoprotein and the human erythrocyte green hemoprotein are similar not only in terms of the reduced pyridine hemochrome spectrum (1) but also in terms of affinity for cyanide. The value of 90 μM for the $K_{d}$ for the cyanide-human green hemoprotein complex is, within experimental error, identical to values for the cyanide-form I complex presented here.

Electrochemical Properties—Anaerobic titrations of form I revealed the transfer of 1 electron/atom of iron. The spectral changes observed when successive amounts of standardized sodium dithionite solution were added to a solution of the hemoprotein at 20°C are shown in Fig. 9. Upon successive additions of dithionite, the absorbance of the Soret band at 416.5 nm was seen to decrease with a concomitant increase in absorbance of the Soret of the reduced form at 439 nm, and the visible peak at 573 nm. Four sharp isosbestic points were observed, showing that a single spectral species was converted to another single species. The plot of these results (Fig. 9, inset) shows that 0.43 molecule of sodium dithionite was added.

*The apparent $K_{d}$ of 900 μM previously reported for the cyanide complex of the human green hemoprotein (11) is incorrect as a consequence of an error in calculation.
The standard deviation of these readings is approximately 0.0025 V.

consumed for every molecule of hemoprotein converted to its reduced form. Since dithionite is a 2-electron reducing agent, it is evident that 0.86 electron was taken up for each molecule of hemin reduced.

The anaerobic titration of form II also revealed the transfer of 1 electron/atom of iron (data not shown). The spectral changes observed were parallel to those observed for form I. Analysis of data as described for form I demonstrated that 0.54 molecule of sodium dithionite was consumed/atom of iron. Thus, 1.08 electrons were consumed in the reduction of each iron atom. The accuracy of these reductive titrations appeared to be approximately ±10%.

The potentiometric behavior of the two hemoproteins during titration with dithionite in the presence of the indigotetrasulfonate at pH 6.4 is shown in Fig. 10. The data plotted according to the Nernst equation should lie on a straight line if a single species is present. The biphasic behavior of form I is clearly indicative of two species, in an approximate ratio of 4:1. The minor species (of lower, more negative potential) has a behavior identical to form II and is due to contamination of this preparation of form I by form II. The potentiometric titration of form II shows one species to be present. The midpoint potentials for forms I and II at pH 6.4 are +0.075 V and +0.019 V, respectively. At pH 7.0, the midpoint potentials of form I and form II are +0.038 V and −0.005 V, respectively. The standard deviation of these readings is approximately ±0.0025 V.

The experimentally determined values of −0.062 V/pH unit for form I and −0.070 V for form II are in strong agreement with a mechanism which includes the addition of 1 proton for every electron transferred to the oxidized hemoprotein. Theoretically, the transfer of 1 proton/electron results in a potential change of −0.059 V/pH unit.

From the slopes of the lines in Fig. 10 (0.118 V/log(oxlred)) the number of electrons involved in the reduction of forms I and II in the presence of indigotetrasulfonate was calculated to be 0.5. A value of n = 1 would have been predicted for a 1-electron transfer of Fe+++ + e− ⇄ Fe++. This evidence for a one-half electron transfer indicates that the mechanism of electron uptake by the hemoprotein does not occur on a simple 1 electron/heme basis. However, the stoichiometric titration of both forms I and II with a standardized solution of sodium dithionite did give the expected value of near unity.

Several discrepancies of this sort have been reported. Guengerich et al. (12), for example, observed a 2-electron stoichiometry in the reduction of cytochrome P-450, whereas a value of n = 1 was deduced from potentiometric data. This anomaly has been resolved, the stoichiometry of reduction now being unity (13). A possible explanation for the n = 0.5 value for the green hemoprotein would be site-site interactions. Since the hemoproteins in solution exist as monomers with only 1 heme/molecule, this postulate would necessitate transient aggregation of the hemoprotein. No spectral evidence for such aggregation has been observed in either the chemical or photochemical reductions.

**Correlation of Spectral, Electrochemical, and Ligand-binding Properties**—The spectral, electrochemical, and ligand-binding differences between forms I and II can be explained in terms of a greater electron withdrawing ability of the side chains of the heme of form I relative to the heme of form II. Evidence for such a difference in the two hemes was provided by the observation that the pyridine hemochrome spectrum of form I is red-shifted relative to form II (1). Pyridine hemochrome spectra provide direct information concerning the electron withdrawing ability of hemes since the spectra are insensitive to the protein moiety under the protein-denaturing conditions employed. Moreover, the pyridine hemochrome spectra of the protein-free hemes showed the same differences.

As predicted for a more conjugated heme, the visible absorbance maxima of the ferrous, CO-ferrous, ferric, and CN−-ferric species of native form I are found at longer wavelengths than the corresponding maxima of form II; analogous spectral differences are observed when various hemes with known electron-withdrawing capacities are inserted into apo-myoglobin (14, 15). The greater affinity of form I for cyanide is likewise consistent with a more highly conjugated heme; increasing the electron-withdrawing ability of hemes in hemoproteins is known to result in stronger binding of sigma (o−1 electron-withdrawing) donor ligands such as cyanide (14, 16). The more positive oxidation-reduction potential of form I relative to form II (+0.038 V versus −0.005 V) can also be explained in terms of structural differences in the hemes; the presence of electron-withdrawing groups on hemes is known to make the electron-withdrawing potential more positive (17). If there actually are differences between the apoprotein moieties of the two forms, then they are of a subtle enough nature as not to reverse the

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FIG. 7. Spectrophotometric titration with CN⁻. Plate a, form I. The reaction mixture contained 3.69 μM form I and 0.05 M potassium phosphate buffer, pH 7.2. Final volume of reaction mixture is 1.0 ml and T is 25°C. A 0.1-ml sample of an appropriate concentration of neutralized KCN solution (in 0.05 M potassium phosphate buffer) was added to 0.9 ml of protein solution, the resultant spectrum was recorded, and the sample was then discarded. This was repeated with increasing amounts of fresh KCN solution and fresh protein sample. The millimolar concentration of total cyanide in the various samples was: 0, 0; 1, 0.013; 2, 0.028; 3, 0.057; 4, 0.074; 5, 0.134; 6, 0.403; 7, 6.72. Plate b, form II. The reaction mixture contained 3.54 μM form II. The millimolar concentration of total cyanide in the various samples was: 0, 0; 1, 0.013; 2, 0.025; 3, 0.074; 4, 0.149; 5, 0.338; 6, 1.113; 7, 6.8. Procedure and conditions are the same as in Plate a.

FIG. 8. Binding of CN⁻ by form I (•) and form II (○). Analysis by the method of Scatchard (10). \( X_p \) is the concentration of ligand-bound hemoprotein (assuming 1 cyanide is bound/heme), \( X_f \) is the concentration of ligand-free hemoprotein, and \( E_p \) is total concentration of hemoprotein present. All concentrations are expressed in micromoles per liter. Conditions are given in Fig. 7. It was assumed that both hemoproteins were completely converted to the cyanide complex in the presence of 5 mM cyanide and that fractional binding was proportional to fractional changes in absorbance at a given wavelength. Changes in absorbance were monitored at 411 and 410 nm for forms I and II, respectively.

The capacity of the green hemoproteins to exchange ligands and the observed rapid rate of autoxidation of the ferrous form are compatible with the idea that these hemoproteins function as oxidases. Preliminary studies have shown that the hemoproteins can be reduced by solubilized liver microsomal electron transport systems. However, if they do indeed function as oxidases, the substrates which could provide electrons for the hemoproteins in the erythrocyte remain unknown.

L. J. DeFilippi, D. P. Ballou, and D. E. Hultquist, manuscript in preparation.

Unpublished observation.
FIG. 9. Anaerobic titration of form I with dithionite. The reaction mixture contained 4.12 μM form I and 0.05 M potassium phosphate buffer, pH 7.2, in a final volume of 2.5 ml under an anaerobic atmosphere of N₂ at 20°C. Small aliquots of standardized sodium dithionite (1.05 mM in 0.15 M potassium pyrophosphate buffer, pH 8.5) were added under anaerobic conditions and the spectra (Curves a to f) recorded immediately. Curve g shows spectrum after autoxidation. Inset, plot of form I reduced (calculated from changes in absorbance at 439 nm) versus dithionite added. See text for additional information.

FIG. 10. Nernst plot of potentiometric titration of form I and form II at pH 6.4. The reaction mixture contained 1.26 μM form I (later found to be a mixture containing approximately 20% form II) or 1.02 μM form II, 0.10 M sodium phosphate buffer (pH 6.4), and indigotetrasulfonate (approximately 4 μM). Reduction was effected by the addition of small aliquots of dilute sodium dithionite. The lines drawn are theoretical for a 0.5-electron transfer (slope of 0.118 V/log(ox/red)) with a midpoint potential of +0.075 V for form I (●●●) and +0.019 V for form II (▲▲▲).

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