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Oxidized Dimeric *Scapharca inaequivalvis*

CO-DRIVEN PERTURBATION OF THE REDOX EQUILIBRIUM*

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The dimeric hemoglobin isolated from *Scapharca inaequivalvis*, HbI, is notable for its highly cooperative oxygen binding and for the unusual proximity of its heme groups. We now report that the oxidized protein, an equilibrium mixture of a dimeric high spin aquomet form and a monomeric low spin hemichrome, binds ferrocyanide tightly which allows for internal electron transfer with the heme iron.

Surprisingly, when ferricyanide-oxidized HbI is exposed to CO, its spectrum shifts to that of the ferrous CO derivative. Gasometric removal of CO leads to the oxidized species rather than to ferrous deoxy-HbI. At equilibrium, CO binds with an apparent affinity (p_{50}) of about 10–25 mm of Hg and no cooperativity (20 °C, 10–50 mM buffers at pH 6.1). The kinetics of CO binding under pseudo-first order conditions are biphasic ($t_{1/2}$ of 15–50 s at pH 6.1). The rates depend on protein, but not on CO concentration.

The nitrite-oxidized protein is not reduced readily in the presence of CO unless one equivalent of ferrocyanide, but not of ferricyanide, is added. We infer that ferrocyanide, produced in the oxidation reaction, is tightly bound to the protein forming a redox couple with the heme iron. CO shifts the redox equilibrium by acting as a trap for the reduced heme. The equilibrium and kinetic aspects of the process have been accounted for in a reaction scheme where the internal electron transfer reaction is the rate-limiting step.

Hemoglobin I (HbI)[1] of the clam *Scapharca inaequivalvis* is a highly cooperative dimer whose heme groups are in unusually close proximity in both the carbonmonoxy and deoxy conformational states (1, 2). Since there have been a number of reports of intramolecular electron transfer in proteins between relatively distant metals (3–6), we designed experiments to determine if the proximity of the heme groups in *S. inaequivalvis* HbI allows for intramolecular electron transfer between their iron atoms.

The reaction we set out to investigate was the heme-catalyzed, water-gas shift reaction ($CO + H_2O \rightleftarrows CO_2 + 2H^+ + 2e^-$) whereby the oxidation of carbon monoxide provides the electrons for heme reduction (7). Oxidized human hemoglobin, when exposed to one atmosphere of carbon monoxide at 20 °C, becomes reduced very slowly, with a half-time of about 1000 h. We anticipated that the reduction would occur more quickly with *Scapharca* HbI, since a much faster reduction, with a half-time of about 0.5 h occurs for cytochrome a_3 of cytochrome-*c* oxidase where two metal centers (copper and heme iron) are able to act as acceptors for the two electrons liberated in the oxidation of carbon monoxide to carbon dioxide (7).

When we exposed oxidized *Scapharca* HbI to carbon monoxide, we were surprised to find that this cooperative dimer becomes reduced even faster than cytochrome a_3 of cytochrome-*c* oxidase, with a half-time of seconds at pH 6. A more detailed examination convinced us that we were observing a reaction that differed significantly from the water-gas shift reaction. The discriminating factors are that the rapid reduction reaction occurs for ferricyanide-oxidized *Scapharca* HbI and not for nitrite-oxidized *Scapharca* HbI, and that oxidation of CO to CO_2 is not required.

Finding a dependence of the rapid reductive process upon the presence of ferro/ferricyanide, we departed from our initial experimental design and probed the details of the observed reaction. We found that in *Scapharca* HbI a redox couple that allows for reversible electron transfer between protein-bound ferro/ferricyanide and Fe^{2+}/Fe^{3+} of the heme groups is formed. The heme reduction observed in the presence of carbon monoxide can be attributed to a shift of the redox equilibrium due to carbon monoxide binding to ferrous heme. As will be shown, the reversible intramolecular electron transfer process is dependent upon pH, ionic strength, and extent of dissociation of oxidized *Scapharca* HbI into monomers.

The previous papers on intramolecular electron transfer reactions have shown that electrons can travel between metal centers in a protein matrix over appreciable distances (3–6). Although we lack information on the site of ferro/ferricyanide binding in *S. inaequivalvis* HbI, the results obtained clearly show that factors that affect protein structure (*e.g.* state of association) also affect the rate of intramolecular electron transfer in this system.

MATERIALS AND METHODS

HbI from *S. inaequivalvis* was extracted and purified as previously described (8). HbI was oxidized with a 10-fold molar excess (over heme) of ferricyanide or by addition of a few grains of KNO_2. The oxidized protein was then passed through a Sephadex G-25 column equilibrated at pH 6.1 with different buffers as specified in the text.

Human hemoglobin was prepared from outdated blood and freed from organic and inorganic ions by standard procedures. Methemoglobin was obtained by oxidation with a few grains of KNO_2 and subsequent chromatography on a Sephadex G-25 column. Sperm whale myoglobin was purchased from Sigma and dissolved directly in the desired buffer.

Atomic absorption measurements of ferricyanide-oxidized *Scapharca* HbI were carried out on samples subjected to chromatography

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[1] The abbreviations used are: HbI, hemoglobin I; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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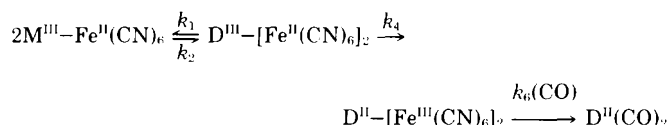
on a Sephadex G-25 column (in 0.5 M NaCl, 0.05 M bis-Tris-HCl buffer, pH 6.1, and subsequently in distilled water) and on a column of Amberlite mixed-bed resin. A Perkin-Elmer atomic absorption spectrometer was employed. The protein concentration at the end of the chromatographic procedure was about 10 μM.

The monomer-dimer equilibrium of the oxidized protein was measured spectrophotometrically making use of known absorbance differences (9). Solutions of ferricyanide- or nitrite-oxidized *Scapharca* HbI were diluted progressively with varied concentrations of bis-Tris buffer at pH 6.1 covering the ionic strength range 10–50 mM. Spectra between 450 and 700 nm were recorded on a Cary 219 instrument, and the absorbance differences between 560 and 620 nm were plotted *versus* protein concentration. The data were fitted to a monomer-dimer equilibrium with a least-squares method (Enzfitter™, F. J. Leatherbarrow).

The kinetics of dimer (D) dissociation into monomers (M) were measured in a Durrum rapid-mixing apparatus by rapid-mix dilution of ferricyanide- or nitrite-oxidized *Scapharca* HbI with the desired buffer. The absorbance decrease following dilution was monitored at 610 nm. The kinetic constants for the reaction were calculated in terms of the relaxation process D $\rightleftarrows$ 2*M*. The following equations have been used: $1/t = k_1 + 2k_2M$; and $K_{eq} = k_1/k_2$ where K_{eq} is the equilibrium dissociation constant, t is the relaxation time of the reaction, k_1 and k_2 are the dissociation and association rate constants, respectively, and M is the equilibrium concentration of monomer.

CO titrations of ferricyanide-oxidized *Scapharca* HbI were carried out in a tonometer. CO gas was added in increments to deoxygenated protein solutions by means of a gas tight syringe. Each CO addition was followed by rotation of the tonometer for 30 min in a water bath at 20 °C prior to recording the spectrum.

The rate of CO binding to oxidized *Scapharca* HbI under varied conditions was measured in stopped-flow experiments performed with a Hi-Tech (Hi-Tech Scientific, Salisbury, England) apparatus coupled to a Cary 219 spectrophotometer or with a Durrum rapid-mixing apparatus coupled to an OLIS data-collecting system. The latter instrument was used in all experiments that were carried out under a nitrogen atmosphere. Protein solutions of *Scapharca* HbI were mixed with CO-containing buffers, and the absorbance increase was monitored at 560 nm. When nitrite-oxidized HbI was used, one equivalent of ferrocyanide per heme was added to the hemoglobin solution just before the stopped-flow experiments. Ferrocyanide solutions were freshly prepared and protected from direct light. The kinetic data were fitted to the following simplified reaction scheme:



$$2M^{III}\text{–}Fe^{II}(CN)_6 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} D^{III}\text{–}[Fe^{II}(CN)_6]_2 \xrightarrow{k_4} D^{II}\text{–}[Fe^{III}(CN)_6]_2 \xrightarrow{k_6(CO)} D^{II}(CO)_2$$

which yields $d[D^{II}(CO)_2]/dt = k_4[D^{III}\text{–}[Fe^{II}(CN)_6]_2$ (see "Discussion"). The rate constants k_1 and k_2 were obtained from the rapid-mixing dilution experiments and the value of k_4 was allowed to vary. Computations were performed on a Microvax-VMS 3500 by means of the Matlab program (copyright by The Math Works, Inc.).

Flash photolysis experiments were performed in parallel on ferrous CO *Scapharca* HbI and on the ferricyanide oxidized protein that had been degassed in a tonometer and equilibrated with 0.1 atm of CO gas. Protein concentrations were 50 μM heme; the absorbance changes were followed between 400 and 450 nm.

Ultrafiltration experiments were carried out at 20 °C using Centricon PM 10 concentrators, which were centrifuged at 2200 × *g* for 30 min. Ultrafiltration was carried out using ferricyanide-oxidized *Scapharca* HbI (in 10 mM bis-Tris-HCl buffer at pH 6.1) equilibrated with air or with 1 atm of CO. In the latter case the concentrator was sealed with a rubber tip. Ferro- and ferricyanide in the ultrafiltrates were determined by using the spectrophotometric method of Dixon (10).

RESULTS

Carbon Monoxide Reactivity of Scapharca HbI Oxidation Products—Oxidation of dimeric *Scapharca* HbI with ferricyanide results in the formation of dimeric methemoglobin, which undergoes a rapidly reversible, pH-dependent dissociation into monomers. The oxidized monomers show the distinctive spectral properties of hemichromes in which the distal heme ligand is provided by the protein. Sodium dithionite readily reduces the oxidized protein to the five-coordinate ferrous derivative. These properties of oxidized *Scapharca* HbI remain unchanged whether the oxidation is by nitrite or by ferricyanide (9–11).

The products formed by nitrite oxidation show no reduction upon exposure to one atmosphere of carbon monoxide during a 1-h period, while those formed by ferricyanide oxidation are rapidly reduced and form the CO adduct. The time-course of the reduction process of the ferricyanide-oxidized protein was followed in a stopped-flow apparatus. At pH 6.1, in 20 mM bis-Tris-HCl buffer, at about 100 μM heme, the half-time of reduction and CO binding is about 20 s. The reaction slows down with an increase in pH. Under comparable conditions at pH 7.0, the protein becomes reduced with a half-time of 100 s. At pH 8.5, only about 10% of the protein becomes reduced and binds CO during a 15-min period. Fig. 1 shows that the time course of reduction and CO binding at pH 6.1 is slower at low protein concentration, where monomers are the predominant species. The figure also shows that the process is independent of CO concentration above 50 μM. The absence of a CO concentration dependence is a clear indication that the reaction is not rate-limited by the bimolecular process of CO binding to the heme group.

Subunit Dissociation of Scapharca HbI Oxidation Products—Among the distinctive characteristics of oxidized *Scapharca* HbI is its pH-dependent, reversible dimer-monomer dissociation. As previously reported (9), the dissociation into monomers is correlated with a change in the visible absorption spectrum. This change is characteristic of a high spin to low

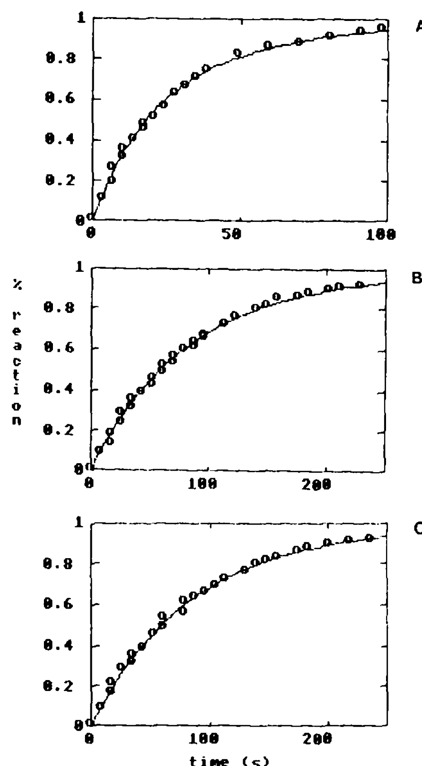


FIG. 1. **Time course of CO reduction of the oxidized *Scapharca* HbI-ferrocyanide complex as a function of protein and CO concentration.** Experiments carried out with a Hi-Tech apparatus on solutions of HbI oxidized with 1.2 equivalents of ferricyanide in 20 mM bis-Tris-HCl, pH 6.1, at 20 °C. Oxidized HbI-ferrocyanide concentration (μM): *A*, 105; *B*, 10; *C*, 10. CO concentration (μM): *A*, 500; *B*, 500; *C*, 50. The time courses, fitted to the simplified reaction scheme given under "Materials and Methods," yield the following values of k_4: *A*, 0.11 s^{-1}; *B*, 0.03 s^{-1}; *C*, 0.03 s^{-1}. The fitted amplitudes were within 5% of the experimental ones.

spin transition. We utilized this spectral change to investigate the relative dissociation of nitrite- and ferricyanide-oxidized *Scapharca* HbI dimers into monomers. The spectral properties of both oxidation products and, by inference, the aggregation state were found to be dependent upon pH and ionic strength. The spectral properties of nitrite- or ferricyanide-oxidized *Scapharca* HbI showed no differences when compared at identical protein concentration. Experiments were performed at pH 6.1 to obtain quantitative data on the monomer-dimer equilibrium. The ionic strength dependence of the dissociation has not been previously documented. The fraction of dimers, $1 - \alpha$, is shown in Fig. 2 to decrease with increasing ionic strength, with no significant difference between ferricyanide- and nitrite-oxidized *Scapharca* HbI. The calculated monomer-dimer dissociation constants for the three buffer strengths studied range from 2.3×10^{-4} to 4.5×10^{-4} M.

The rates of monomer-dimer equilibration are of interest, since they might affect the carbon monoxide reactions observed with the *Scapharca* HbI oxidation products. Accordingly, dilution experiments were performed with a rapid mixing apparatus on nitrite- or ferricyanide-oxidized *Scapharca* HbI. The results given in Table I and those of Fig. 2 lead us to conclude that there are no significant differences in the monomer-dimer equilibrium for nitrite- and ferricyanide-oxidized *Scapharca* HbI.

Binding of Ferrocyanide to Oxidized Scapharca HbI—When *Scapharca* HbI is oxidized by ferricyanide, a complex is produced between the oxidized protein and ferrocyanide formed during the heme oxidation (shown later). The methods typically used to oxidize hemoglobin involve exposure of the protein to the heme oxidant and a subsequent chromatographic step, at high salt concentration, to remove excess oxidant from the sample (14). These conditions remove ferri/ferrocyanide from human hemoglobin, but not from *Scapharca* HbI.

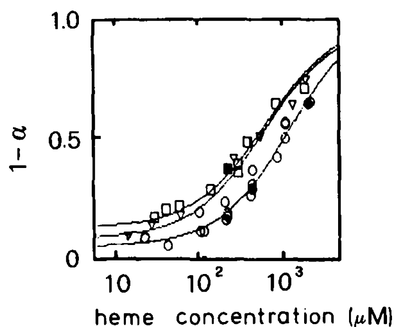


FIG. 2. **Fraction of dimers ($1 - \alpha$) in oxidized *Scapharca* HbI as a function of protein concentration at pH 6.1 at three different ionic strengths.** Temperature: 20 °C. Buffer: 10 (□), 20 (△), 50 (○) mM bis-Tris-HCl or Hepes-HCl. Nitrite- (*open symbols*) and ferricyanide-oxidized (*closed symbols*) HbI. *Lines* represent fitted curves for a monomer-dimer equilibrium and were calculated with the following dissociation constants: 2.3×10^{-4} M (10 mM bis-Tris-HCl); 2.5×10^{-4} M (20 mM bis-Tris-HCl); 4.5×10^{-4} M (50 mM bis-Tris-HCl or Hepes-HCl).

TABLE I

Values of association (k_2) and dissociation (k_1) rate constants in the monomer-dimer equilibrium of oxidized Scapharca HbI at pH 6.1 as a function of buffer concentration

Temperature: 20 °C.

Buffer	Ionic strength	k_2	k_1
	mM	$M^{-1}s^{-1}$	s^{-1}
bis-Tris-HCl	10	3.1×10^5	70
bis-Tris-HCl	20	2.8×10^5	68
bis-Tris-HCl	50	1.4×10^5	58
Hepes-HCl	20	2.8×10^5	68

Scapharca HbI was oxidized by ferricyanide, and unbound ferri/ferrocyanide was removed by gel filtration in 0.5 M NaCl, 0.05 M bis-Tris-HCl, pH 6.1. The sample was then subjected to a chromatographic step on a column of Amberlite™ mixed-bed resin to insure the removal of any unbound or loosely bound ferro/ferricyanide. When analyzed by atomic absorption spectroscopy, *Scapharca* HbI thus treated was found to contain 0.8 ± 0.05 ferri- or ferrocyanide bound tightly per heme group.

The conclusion that it is ferrocyanide, formed during the heme oxidation, that binds tightly to oxidized *Scapharca* HbI was reached based on the following experimental results. No rapid reduction occurred if ferricyanide was added to nitrite-oxidized *Scapharca* HbI under one atmosphere of CO, whereas the rapid carbonmonoxide-driven reduction was observed when increments of ferrocyanide were added. Fig. 3 shows that addition of one equivalent of ferrocyanide to *Scapharca* HbI under CO was sufficient to produce 93% reduction.

Similar CO-driven reduction experiments were carried out on nitrite-oxidized 100 μM human hemoglobin and sperm whale myoglobin in 20 mM bis-Tris-HCl at pH 6.1. Both proteins were exposed to one atmosphere CO after addition of one equivalent of ferrocyanide. The human hemoglobin became reduced with a half-time of about 20 min. Sperm whale myoglobin became 20% reduced in the first 2 h and very little reduction occurred during the next 24 h.

Carbon Monoxide Reactivity of the Oxidized Scapharca HbI-Ferrocyanide Complex—Based on the foregoing results, we hypothesized that the reduction of the oxidized *Scapharca* HbI-ferrocyanide complex under carbon monoxide is due to a shift in the redox equilibrium between the iron atoms of *Scapharca* HbI and bound ferrocyanide. This hypothesis was tested by equilibrium and kinetic experiments.

The oxidized *Scapharca* HbI-ferrocyanide complex (formed by standard procedures of heme oxidation with ferricyanide and removal of excess oxidant) was equilibrated with varying

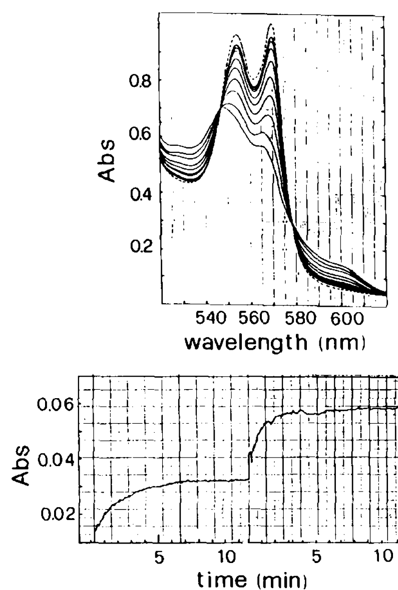


FIG. 3. **Titration of nitrite-oxidized *Scapharca* HbI with ferrocyanide.** The HbI solution (70 μM), in 10 mM bis-Tris-HCl buffer, at pH 6.1, was equilibrated with 1 atm of CO in a tonometer at 20 °C. *Top*, 0.1 equivalents of a degassed solution of 10^{-2} M ferrocyanide were added at each step and the spectrum was recorded after 10 min. Final spectrum (*dotted line*) obtained by addition of dithionite. *Bottom*, time course of reduction (first two steps).

concentrations of carbon monoxide in order to determine the degree of CO-driven reduction under different conditions. Experiments were carried out at pH 6.1 at 20 °C with oxidized *Scapharca* HbI at a concentration of 50 μM (in heme) in varied concentrations of bis-Tris or Hepes buffer. Fig. 4 shows the spectral changes observed in a typical CO binding experiment, and Fig. 5 presents Hill plots of the data obtained. The apparent CO affinity is decreased as the ionic strength was increased. Under all conditions examined, the Hill plots have unit slopes, indicative of noncooperative CO binding. These results are consistent with the existence of an underlying redox equilibrium in which the heme sites are not functionally linked and do not exhibit cooperativity.

Rapid-mixing experiments further support the hypothesis that the rapid CO-driven reduction is due to a shift of an underlying redox equilibrium. In experiments like those shown in Fig. 1, the oxidized *Scapharca* HbI-ferrocyanide complex was mixed with CO under varied conditions. The time course of the observed reaction was always biphasic. Decreasing the protein concentration or increasing the ionic

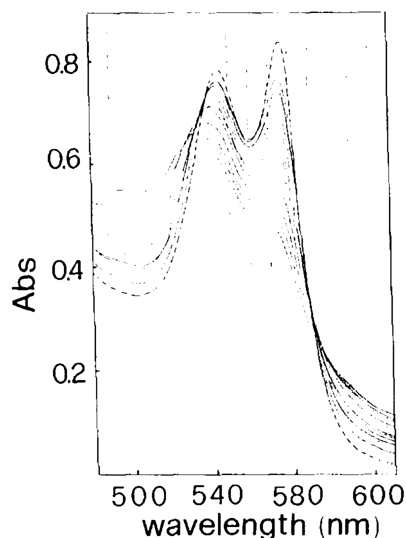


FIG. 4. **Titration of the oxidized *Scapharca* HbI-ferrocyanide complex with CO.** Spectral changes observed upon addition of CO gas to a tonometer containing 50 μM oxidized HbI-ferrocyanide in 20 mM bis-Tris-HCl buffer, at pH 6.1, 20 °C. The final CO-saturated spectrum (*dashed line*) was obtained by addition of dithionite.

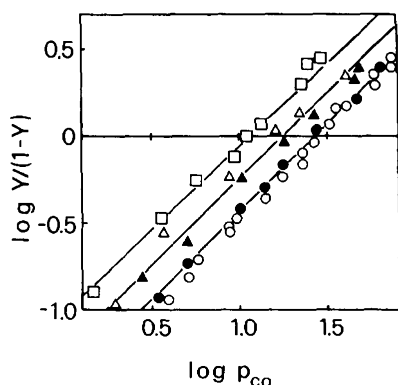


FIG. 5. **Hill plots of CO binding to the oxidized *Scapharca* HbI-ferrocyanide complex.** Experiments carried out at pH 6.1 and 20 °C using protein concentrations of about 50 μM. Buffer ionic strength: □, 10 mM, $\log p_{50}$ = 1.07; △, ▲, 20 mM, $\log p_{50}$ = 1.26; ○, ●, 50 mM, $\log p_{50}$ = 1.45. *Open symbols*, bis-Tris-HCl; *closed symbols*, Hepes-HCl.

strength diminished the amplitude of the fast phase and slowed both phases of the reaction. Table II shows that comparable results were obtained in rapid-mixing experiments with nitrite-oxidized *Scapharca* HbI to which one equivalent of ferrocyanide was added prior to mixing with CO-containing buffers.

There was some variation in the percentage reduction observed when the oxidized *Scapharca* HbI-ferrocyanide complex was mixed with CO-containing buffer, with 60–80% reduction being typical. This variation could be due to there being somewhat less than one ferrocyanide per heme in these experiments. It is also possible that complications affecting the reduction percentage are caused by oxygen binding to the reduced form (as it becomes populated). With respect to this latter point some rapid-mixing experiments were carried out with nitrogen-equilibrated solutions. Greater than 80% reduction was consistently observed when degassed ferrocyanide was added to nitrite-oxidized *Scapharca* HbI under nitrogen prior to rapid mixing with CO. No major differences in rates or time courses of the reaction were observed. On this basis, the consistent increase in reduction under nitrogen might be due to increased stability of the ferrous deoxy form.

The adduct formed upon exposure of the oxidized *Scapharca* HbI-ferrocyanide complex to CO is spectrally identical to that formed by exposure of ferrous *Scapharca* HbI to CO. Degassing the former produces a molecule with the spectrum of the oxidized protein rather than that of ferrous *Scapharca* HbI. Moreover, flash photolysis produces a species with a spectrum like that of the deoxy ferrous *Scapharca* HbI (Fig. 6). These results indicate that the CO-driven reduction is a reversible process and that reoxidation of *Scapharca* HbI in the complex is slow with respect to CO rebinding at CO concentrations above 0.1 atm. However, the equilibrium data shown in Fig. 5 indicate that reoxidation of *Scapharca* HbI in the complex must be an appreciable factor in establishing the redox balance at lower CO concentrations.

Differential Affinity of Ferro- and Ferricyanide for Scapharca HbI—Is the ferrocyanide, which is tightly bound to oxidized *Scapharca* HbI, still bound when the CO adduct is formed by CO-driven perturbation of the redox equilibrium? To investigate this question, the following ultrafiltration experiments were carried out using Centricon PM-10 concentrators. Solutions of the oxidized HbI-ferrocyanide complex at 150 μM (heme) were equilibrated with 1 atm of air or CO. The latter were centrifuged in a concentrator sealed under a CO atmosphere. After centrifugation, ferricyanide was found

TABLE II

Rate constants for the CO-driven reduction reaction of nitrite-oxidized Scapharca HbI after addition of one equivalent of ferrocyanide

Experiments have been carried out in bis-Tris-HCl buffer at pH 6.1 and 20 °C. CO concentration after mixing was 500 μM. Protein concentrations are as indicated.

Buffer ionic strength	k_4 50–100 μM, HbI	k_4 5–20 μM, HbI
mM	s^{-1}	
10	0.20	0.17[a]
20	0.10 ± 0.01	0.03
	0.17 ± 0.02[b]	0.09[b]
	0.19[c]	0.13 ± 0.01[c]
50	0.029 ± 0.003[c]	0.035[c]
	0.021 ± 0.002[b]	0.012[b]

[a] Identical at 50 or 500 μM CO.
[b] Ferricyanide-oxidized, passed through Sephadex G-25.
[c] Experiments carried out in 1 atm of nitrogen.

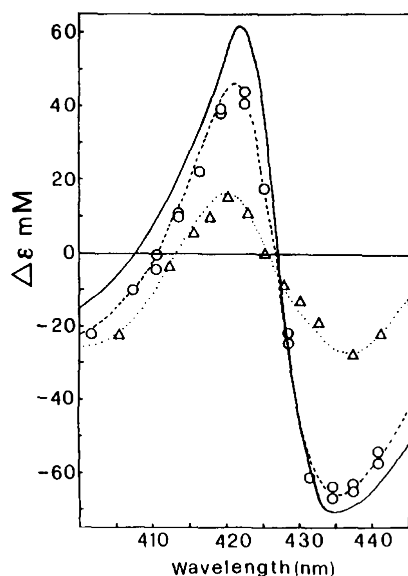
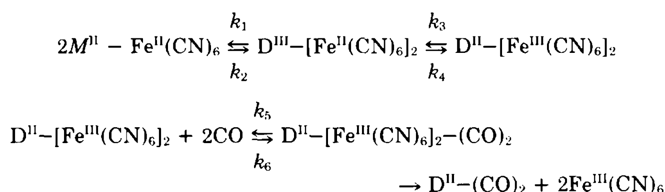


FIG. 6. **Static and kinetic difference spectra of *Scapharca* HbI.** Static difference spectrum of ferrous HbI-CO minus deoxygenated ferrous HbI (——), kinetic difference spectrum of ferrous HbI-CO minus the flash photolysis product in the presence of dithionite (O), and CO-reduced HbI-ferrocyanide complex minus the flash photolysis product (Δ).

in the ultrafiltrate. Its concentration, measured spectrophotometrically, was found to be 115 ± 0.5 μM. The spectrum of the protein corresponded to 130 μM of the CO adduct. In the air-equilibrated solutions, no ferri- or ferrocyanide was released from the oxidized *Scapharca* HbI-ferrocyanide complex as determined by comparable analysis of the ultrafiltrate. On this basis, the affinity of ferricyanide for the CO-reduced protein was estimated to be about 10^3 M^{-1} and that of ferrocyanide for the oxidized protein to be higher than 10^6 M^{-1}.

DISCUSSION

The dimeric hemoglobin from *S. inaequivalvis* displays interesting intramolecular electron transfer reactions made possible by formation of a reversible redox couple between heme iron and tightly bound ferrocyanide. CO, a heme ligand with high affinity for the ferrous form, perturbs the redox equilibrium by promoting formation of the reduced CO-bound species. Consequently, rapid heme reduction is observed when the ferricyanide-oxidized protein is exposed to CO. The reduction half-time is 20–50 s at low pH, low ionic strength, and protein concentrations of 10–100 μM. These aspects of the CO-driven reduction of the oxidized *Scapharca* HbI-ferrocyanide complex can be represented by the following minimal scheme:



$$2M^{II} - Fe^{II}(CN)_6 \underset{k_2}{\overset{k_1}{\leftrightarrows}} D^{III}\text{–}[Fe^{II}(CN)_6]_2 \underset{k_4}{\overset{k_3}{\leftrightarrows}} D^{II}\text{–}[Fe^{III}(CN)_6]_2$$

$$D^{II}\text{–}[Fe^{III}(CN)_6]_2 + 2CO \underset{k_6}{\overset{k_5}{\leftrightarrows}} D^{II}\text{–}[Fe^{III}(CN)_6]_2\text{–}(CO)_2 \rightarrow D^{II}\text{–}(CO)_2 + 2Fe^{III}(CN)_6$$

The first step in the scheme corresponds to the reversible monomer-dimer equilibrium of oxidized *Scapharca* HbI, an equilibrium which can be measured by monitoring the protein concentration dependence of the changes in the visible absorption spectra (9). The position of this equilibrium is not altered by bound ferrocyanide, as indicated by the similarity of the concentration dependence shown by ferricyanide- and nitrite-oxidized *Scapharca* HbI (Fig. 2). At pH 6.1, the monomer-dimer equilibrium constant varies between 2 and 4 × 10^{-4} M depending on the buffer strength. In particular, an increase in ionic strength from 10 to 50 mM favors monomer formation. Kinetically, this effect is manifest in the rate of monomer association, which decreases from 3 to 1 × 10^5 M^{-1} s^{-1} under these conditions (Table I).

The second step in the scheme corresponds to the reversible electron transfer between the heme iron and bound ferrocyanide. The binding site of ferrocyanide on the oxidized *Scapharca* HbI molecule is not yet known. It probably corresponds to the binding site of ferricyanide. If this is so, electrons are transferred to and from the heme iron via the same pathway. The fact that bound ferrocyanide does not influence the monomer-dimer equilibrium strongly suggests that ferri- and ferrocyanide are not bound in the subunit interface. The different affinity of ferricyanide for reduced *Scapharca* HbI (estimated at 10^3 M^{-1}) and of ferrocyanide for the oxidized protein (estimated at $\geq 10^6$ M^{-1}) can be attributed to the change in the net charge of the anion and to the structural rearrangements that accompany oxidation, reflected also in the marked tendency of the oxidized protein to dissociate into monomers.

The third step in the scheme corresponds to the reversible binding of CO to the reduced protein. Carbon monoxide binding perturbs the redox equilibrium involving bound ferrocyanide and the oxidized heme iron (step 2) and acts as a trap for the reduced heme, thereby driving the equilibrium toward a dead-end reaction (step 3). Gasometric removal of CO allows for reversal of step 3 and reestablishment of the unperturbed redox equilibrium.

In light of this minimal scheme, the reaction of the oxidized *Scapharca* HbI-ferrocyanide complex with CO reflects a complicated multistep equilibrium. Tonometric experiments under steady-state conditions (Figs. 4 and 5) show that the reaction is not cooperative (n = 1), consistent with there being no cooperativity in the underlying redox equilibrium. The apparent affinity constant for CO varies between 10 and 25 mm of Hg depending on buffer ionic strength and its effect on the monomer-dimer equilibrium (step 1). The affinity constants for CO can be used to estimate the apparent midpoint potential of the reduction reaction on the basis of the equation proposed by Boelens and Weaver (12) for the reduction of cytochrome-*c* oxidase in the presence of CO. In turn, the value obtained ($E_{1/2}$ = 220–250 mV), by assuming a standard redox potential for the ferro/ferricyanide couple of 360 mV at pH 6.0, can be utilized to calculate the apparent midpoint potential of the heme iron ($E_{1/2}$ = 110–140 mV). This is comparable to the value measured at pH 6.0 in 1 M glycine-HCl buffer for the β chains of human hemoglobin ($E_{1/2}$ = 110 mV) and higher than those characterizing the α chains ($E_{1/2}$ = 60 mV) and sperm whale myoglobin ($E_{1/2}$ = 55 mV) (15, 16).

The CO perturbation of the redox equilibrium of the oxidized *Scapharca* HbI-ferrocyanide complex investigated in rapid-mixing experiments has intriguing features. Notably, the time courses are biphasic under all the experimental conditions employed (*i.e.* buffers of different ionic strength, different mode of preparation of the oxidized *Scapharca* HbI-ferrocyanide complex, air equilibrated *versus* anaerobic solutions). The amplitude of each phase and its corresponding rate constant depend on protein concentration (the slow phase dominates at low protein concentration and high ionic strength), but are independent of the concentration of carbon monoxide between 50 and 500 μM.

The lack of dependence on CO concentration can be explained easily on the basis of the minimal reaction scheme,

since binding of CO to the reduced heme is very fast ($k_5 \simeq 10^5$ $M^{-1} s^{-1}$) (13) with respect to the kinetics of the overall process. In fact, flash photolysis experiments showed that CO binding to the reduced protein (step 3) occurs with a half-time of milliseconds in the CO concentration range covered, while the half-time of the overall process is 15 s or more. Hence, step 3 cannot be rate limiting.

The steps which can contribute to the observed time courses therefore are the monomer-dimer equilibrium and electron transfer. The monomer-dimer equilibrium slows the reaction down as it proceeds since the total concentration of oxidized hemoglobin is decreasing and gives rise in a decelerating time course. It follows that the observed rate can be approximated to k_4 [$D^{III} - Fe^{II}(CN)_6$]. The electron transfer rate constant k_4 thus calculated in various buffer conditions is given in Table II. At 20 mM ionic strength the value obtained is $\simeq$0.15 s^{-1} at protein concentrations $\geq$ 50 μM; at lower protein concentration the value of the rate constant appears to be somewhat lower. On the basis of k_4 and of the redox potentials for heme reduction and for the ferro/ferricyanide couple, which yield a redox equilibrium constant, K_{ox}, of 1.7×10^4, a rough estimate of k_3 can be obtained. From the relationship $K_{ox} = k_3/k_4$, k_3 is around 2.5×10^3 s^{-1}.

The effect of ionic strength on k_4 over the range of 10–50 mM is comparable to that observed in other systems (5). It is intriguing that an increase in ionic strength (from 10–50 mM) or pH (in the range of 6.1–8.5) both lead to a decrease in the overall reaction rate, although the monomer-dimer equilibrium is shifted in opposite directions (Fig. 2 and Ref. 9). As discussed below, the observed decrease in reaction rate may be explained in terms of an effect on one of the three factors governing the electron transfer process.

In general terms, electron transfer rates are determined by three factors: distance between the redox centers, differences in the value of the redox potentials of the two metal complexes, and reorganization energy of the acceptor complex. Structural changes in the protein, caused by changes in buffer strength, might alter one or all of these. In particular, large changes in molecular geometry in the vicinity of the heme group can lead to a high activation energy and hence slow electron transfer. In the oxidized *Scapharca* HbI-ferrocyanide complex the reduction process is coupled to marked structural rearrangements that are ultimately reflected in changes in the association state of the protein. On this basis one would expect electron transfer to be a slow process despite the fact that the driving force, estimated at about 220–250 mV, is relatively high as compared to other systems. Indeed the estimated rate constant of $\simeq$0.15 s^{-1} is in line with the value calculated for sperm whale myoglobin reacted with pentaamino-ruthenium. In this system the electron transfer process from the heme iron to the covalently bound ruthenium complex has a driving force of 65–80 mV and occurs with a rate of about 0.04 s^{-1}, as observed upon flash photolysis in the presence of CO (4, 6). The location of the ruthenium complex on the myoglobin molecule has been determined, and a possible route of electron transfer through the protein has been proposed (4, 6).

Lastly, the behavior of *Scapharca* HbI can be compared with that of human hemoglobin and sperm whale myoglobin. In the case of human hemoglobin, Gibson (17) described a catalytic effect of ferrocyanide when comparing the rate of reduction by ascorbic acid of the ferricyanide- and nitrite-oxidized protein in the presence of CO. In our experimental setup, addition of ferrocyanide to nitrite-oxidized HbA allows it to become reduced with a half-time of about 20 min under conditions where *Scapharca* HbI becomes reduced with a half-time of only 20 s. Under comparable conditions, sperm whale myoglobin becomes only about 10% reduced in 2 h. It should be emphasized that in *Scapharca* HbI the affinity of ferrocyanide for the oxidized protein is higher than for human hemoglobin, where the anion can be removed completely by gel filtration in 0.5 M salt (14, 15). For human methemoglobin with oxidants completely removed by gel filtration procedures, the reduction reaction in the presence of CO occurs at an extremely slow rate ($t_{1/2} \simeq 1000$ h) and follows a pathway that can be accounted for in terms of the water-gas shift reaction: $CO + H_2O \rightleftarrows CO_2 + 2H^+ + 2e^-$ (7).

In conclusion, the unusually fast reduction of the heme iron of ferricyanide-oxidized *S. inaequivalvis* HbI in the presence of carbon monoxide is due to the formation of a tight complex between ferrocyanide and the dimeric hemoglobin. The ferrocyanide binding site allows for internal electron transfer through the protein matrix to and from the heme. The whole process can be represented by a complex multistep equilibrium in which the electron transfer between bound ferrocyanide and the heme iron dominates the experimental situation and CO perturbs the redox equilibrium by acting as a trap for the reduced heme.

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