Unusual Affinity of Cyanide for Ferrous and Ferric *Scapharca inaequivalvis* Homodimeric Hemoglobin. Equilibria and Kinetics of the Reaction[†]

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ABSTRACT: The homodimeric hemoglobin from the mollusk *Scapharca inaequivalvis* (HbI) yields very stable ferrous and ferric cyanide adducts. The stability of the ferrous complex is particularly unusual such that it enabled determination of the spectroscopic properties of the complex and the characterization of the cyanide binding reaction to deoxygenated HbI at equilibrium and kinetically. The absorption spectrum of the ferrous cyanide complex is typical of a low-spin derivative; in the near-infrared region, it displays two bands at 695 and 840 nm attributable to charge transfer transitions. At pH 9.2, cyanide binds to deoxy HbI with no cooperativity and an apparent affinity constant of 17 M^{-1}, which is about 10-fold higher than that for deoxy horse heart myoglobin. The rate of cyanide dissociation from both the ferrous and the ferric HbI adducts is slow relative to those of the other hemoproteins investigated to date and provides the major contribution to the unusual affinity for the ligand. The rate of cyanide binding to the ferric protein, in which the pentacoordinate derivative is the dominant species, is about 100-fold faster relative to that of the ferrous protein. In structural terms, the high affinity for cyanide of *Scapharca* hemoglobin has been ascribed to the decreased overall polarity of the heme pocket which is related to the localization of the heme groups at the subunit interface.

Cyanide is one of the few ligands which is able to interact with both ferric and ferrous heme iron, albeit with a different affinity. The complexes formed with ferric heme are very stable and have been studied extensively in a number of hemoproteins to elucidate the kinetic and equilibrium barriers of the reaction. The association equilibrium constants are around 10^5 M^{-1} in myoglobins and 10^7 M^{-1} in hemoglobins at alkaline pH values and decrease at neutral and slightly acid pH values (Antonini & Brunori, 1971; Awad & Badro, 1967). The difference in stability is determined primarily by the rate of cyanide dissociation since the overall rates of cyanide binding to ferric myoglobins and hemoglobins are similar (k_{app} = 150−300 M^{-1} s^{-1}). The kinetics of cyanide dissociation is extremely slow (1×10^{-3} to 1×10^{-6} s^{-1}) and has not been investigated systematically (Antonini & Brunori, 1971). Cyanide binding to ferric heme comprises several steps. It requires dissociation of the iron-coordinated water molecule and, under physiological conditions where HCN is the predominant species (pK_a of the acid is 9.4), diffusion of HCN into the protein followed by deprotonation of the acid within the distal pocket to yield the cyanide anion which rapidly forms the iron(III)−cyanide bond (Mintorovich et al., 1989; Brancaccio et al., 1994). The major kinetic barrier to cyanide binding at neutral pH is represented by HCN deprotonation, which is influenced by the presence of a distal histidine and by the overall polarity of the pocket. Thus, the rate of cyanide binding is significantly decreased in mutant myoglobins in which distal residues are replaced by apolar amino acids (Brancaccio et al., 1994).

The reaction of cyanide with ferrous hemoproteins has been studied relatively little due to the low stability of the complex even at high pH values (K about 2.5 M^{-1}). Therefore, in most hemoproteins, only the kinetics of cyanide dissociation has been investigated in experiments in which the ferrous cyanide complex is formed as a transient species during reduction of the ferric cyanide complex. In turn, the transient ferrous cyanide adduct dissociates slowly into HCN and the reduced protein (Keilin & Hartree, 1955; Bellelli et al., 1990; Brunori et al., 1992; Antonini et al., 1994). The suggested reaction mechanism involves, as in the case of the ferric cyanide adducts, donation of a proton from the distal histidine to cyanide to form the acid which rapidly dissociates (Bellelli et al., 1990). In HbA, it has been established recently that cyanide dissociation from the ferrous protein displays cooperativity and is sensitive to allosteric effectors, as observed in the case of oxygen (Brunori et al., 1992).

The homodimeric, cooperative *Scapharca inaequivalvis* hemoglobin (HbI)[1] is an ideal candidate for the study of the cyanide binding reaction. The overall polarity of the distal pocket is decreased compared to those of other hemoproteins (Ilari et al., 1995) due to the unique assembly of the globin chains which brings the two heme groups almost in direct contact at the subunit interface and thereby shields them from solvent (Royer et al., 1989; Royer, 1994). The vicinity of the heme groups provides the basis for their functional interaction. It permits information on the ligation state of one heme to be transferred directly to the other across the subunit interface through steric rearrangements of the heme peripheral substituents, a modification in the spatial relationship of the heme relative to the pocket, but no major

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[1] Abbreviations: HbI, dimeric *Scapharca inaequivalvis* hemoglobin; Sw Mb, sperm whale myoglobin; Mb, horse heart myoglobin.

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quaternary changes (Royer et al., 1989; Royer, 1994). In spite of the different structural basis of cooperativity with respect to vertebrate Hbs, the kinetic control of ligand binding is similar. Thus, in the reaction with oxygen, cooperative effects are reflected mainly in the dissociation reaction which proceeds at a different rate depending on oxygen saturation, and in the reaction with CO, cooperativity is evident in the increase of combination velocity as the reaction proceeds (Antonini et al., 1984; Chiancone et al., 1990).

Ferric HbI likewise has interesting properties for the study of cyanide binding since at alkaline pH it provides a unique example of a hemoprotein with a distal histidine and no heme-coordinated water molecule (Boffi et al., 1994). In fact, ferric HbI undergoes a complex pH-dependent equilibrium between three different species: a dimeric aquomet derivative which is present at pH values around neutrality, a dimeric pentacoordinate species which predominates at alkaline pH values, and a monomeric hemichrome which is formed at acidic pH values (Spagnuolo et al., 1994).

The present work shows that the decreased overall polarity of the heme pocket in HbI results, as expected, in a significant increase of the stability of both the ferrous and the ferric cyanide complexes. Therefore, it has been possible for the first time to characterize the equilibrium and kinetic properties of cyanide binding to a ferrous hemoglobin and to compare them with those of the ferric cyanide complex. This comparison suggests a common mechanism at the basis of cyanide binding and release in both derivatives.

MATERIALS AND METHODS

S. inaequivalvis HbI was extracted and purified as described previously (Chiancone et al., 1981). Concentrations are expressed on a heme basis using the molar absorptivity value of 11 500 M^{-1} cm^{-1} for the deoxy derivative at 555 nm. The ferric protein was obtained by addition of excess (50:1 molar ratio) potassium nitrite to the oxygenated protein. The excess oxidant and its byproducts were removed by gel filtration on a Sephadex G-25 column equilibrated with 0.1 M borate buffer at pH 9.2. Horse heart myoglobin (Mb) was obtained from Sigma (Sigma Chemical Co., St. Louis, MO) and filtered before use. Concentration was determined on the reduced deoxy derivative using a molar absorptivity of 11 800 M^{-1} cm^{-1} at 556 nm. The ferric cyanide derivatives of both HbI and Mb were obtained by addition of a slight molar excess of KCN to the ferric protein.

Spectrophotometric titrations of the ferrous derivative were carried out on a Varian Cary 3 instrument at pH 9.2 and 20 °C in a tonometer containing 0.4 mM deoxy HbI under a nitrogen atmosphere. The titrating solution was 1 M KCN buffered at pH 9.2 with KH_2PO_4 (Keilin & Hartree, 1955). The titrating KCN solution was degassed in a stoppered test tube and was added with an air-tight syringe. After each addition, 20 min was allowed to achieve equilibrium.

Spectra of the ferrous cyanide HbI complex in the near-infrared region were measured on a Jasco 570 UV-VIS-NIR spectrophotometer using 1.5 mM ferrous HbI in borate buffer at pH 9.2 containing 1 M KCN and 50 mM dithionite.

The kinetics of cyanide binding was followed on an Applied Photophysics stopped flow apparatus (Applied Photophysics Ltd., Leatherhead, U.K.) by mixing the ferric or ferrous derivatives in 0.1 M phosphate buffer with buffered KCN solutions. In the experiments on the ferrous derivative, a degassed solution of sodium dithionite was added to the protein and to the KCN solution to a final concentration of about 50 mM.

The kinetics of cyanide dissociation from ferrous HbI was measured on a Hewlett-Packard HP 8452 A diode array spectrophotometer equipped with a Hi-Tech SFA-11 rapid mixing apparatus (dead time of about 50 ms). Solutions of ferric HbI−CN (90−100 μM heme) containing a 2.5 M excess of cyanide over heme were mixed with dithionite solutions (25−100 mM), and the reaction was followed between 480 and 620 nm with 2 nm resolution. The time resolution varied between 0.1 and 4 s. The experiments were carried out at pH 9.2 in 0.1 M borate buffer and at pH 8.0, 7.0, and 6.0 in 0.1 M phosphate buffer. The experiments at pH 9.2 and 7.0 were performed also in the presence of CO. In these experiments, the dithionite solution was equilibrated with 1 mM CO before mixing. The data files were collected into rectangular matrices (spectra vs time) and analyzed according to the singular value decomposition (SVD) procedure (Henry & Hofrichter, 1992). The SVD algorithm transforms the data set **A** into the products of three matrices, $\mathbf{A} = \mathbf{USV}^T$, where the **U** columns are the orthonormal basis spectra, the **V** columns correspond to the relative amplitudes as a function of time, and **S** is a diagonal matrix containing the singular values in decreasing order. In all experimental conditions, no more than three significant basis spectra (**U** columns) were obtained from the deconvolution procedure. Hence, the spectra of cyanomet HbI, ferrous cyanide HbI (transient), and deoxy HbI were assumed to be linear combinations of the three basis spectra with nine coefficients. In turn, the time courses relative to the three species are linear combinations (with the same coefficients) of the three **V** columns. The time courses were fitted to two consecutive exponential processes according to the simple scheme

$$\text{HbI(III)}-\text{CN} \xrightarrow{k_1} \text{HbI(II)}-\text{CN} \xrightarrow{k_2} \text{HbI(II)} + \text{CN}^-$$

The two rate constants and the nine coefficients were fitted by a nested loop fitting procedure. In the first iteration, the two constants k_1 and k_2 were obtained which yield the best fit to the three **V** columns; in the second iteration, the nine coefficients of the linear combination of the three **U** columns were obtained which yield the best fit to the experimental data. The guess for the coefficients was made on the basis of the known spectra of ferric cyanide HbI and deoxy HbI and assuming that the spectrum of the transient species is identical to that of ferrous cyanide HbI obtained in the static titration. The MATLAB program (The Math Works Inc., Natick, MA) for a 486 IBM compatible personal computer was used for all calculations.

The kinetics of cyanide dissociation from the ferric proteins was studied using ferric horse heart Mb immobilized on CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) as a cyanide scavenger. Horse heart Mb (about 100 mg) was immobilized on 6 mL of CNBr−Sepharose 4B in 0.1 M sodium bicarbonate as follows. The suspension was stirred at room temperature for 1 h, washed with coupling buffer and subsequently with ethanolamine for 2 h, and lastly equilibrated with the desired buffer. The concentration of immobilized protein, typically $6-7 \times 10^{-4}$ M heme, was determined from the absorption spectrum; the effect of turbidity was minimized by the use of protein free gel in the reference cell as described by Chiancone and

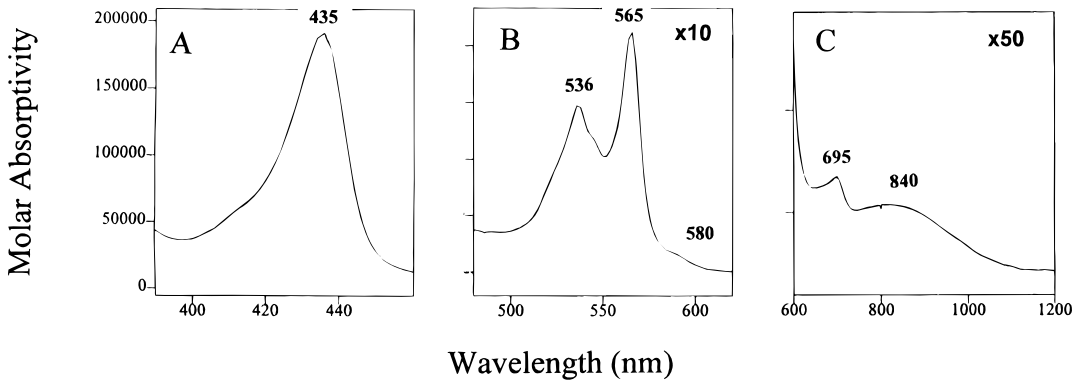

FIGURE 1: Optical absorption spectra of the ferrous HbI cyanide derivative in the visible and near-infrared region. The spectrum was measured at 20 °C in a sealed cell under nitrogen gas in the presence of 50 mM dithionite and 1 M cyanide solution buffered at pH 9.2 with acid potassium phosphate.

Gattoni (1987). The rate of cyanide dissociation was measured by mixing in a test tube 3 mL of ferric cyanide HbI or Mb (10−20 μM heme) at pH 7.0 in 0.1 M phosphate or at pH 9.2 in 0.1 M borate with immobilized Mb (500−1000 M excess over heme) equilibrated in the same buffer. The test tube was placed in a thermostatted water bath at 20 °C and the mixture stirred gently. At established times, a filter sampler (Porex Medical Fireburn, Georgia) was gently pressed into the test tube to separate the immobilized phase from the supernatant whose spectrum was measured in the visible region.

RESULTS

Spectroscopic Characterization. The optical absorption spectrum in the visible, Soret, and near-infrared region of the ferrous HbI cyanide derivative is shown in Figure 1; the molar absorptivity was calculated on the basis of the absorbance of the deoxygenated protein (Chiancone et al., 1981). The Soret and visible spectrum (Figure 1A,B) is typical of a low-spin derivative with peaks of maximum absorbance at 435, 536, and 565 nm and a distinct shoulder at 580 nm. The near-infrared spectrum (Figure 1C) shows a peak at 695 nm and a broad band centered at 840 nm; no further peaks are detectable up to 2500 nm (data not shown). To our knowledge, near-infrared spectra of ferrous cyanide hemoglobin adducts have not been reported before.

The visible spectrum of ferric cyanide HbI displays an absorption maximum at 544 nm as reported by Boffi et al. (1994).

Thermodynamic Characterization. The affinity of cyanide for deoxy HbI was determined at pH 9.2 by titrating the protein at a concentration of 0.4 mM with a cyanide solution. The fractional saturation (Y) was calculated from the absorbance changes at the peak positions of the ferrous cyanide and deoxy derivatives, namely at 565, 536, and 555 nm. The titration curve (Figure 2) gives a linear Hill plot with a slope of 0.96 ± 0.16, indicating that the heme sites are equivalent and independent with no evidence of cooperativity. The apparent affinity constant of deoxy HbI for KCN corresponds to 17 M^{-1}. At pH values lower than 9.2, the apparent affinity constant decreases dramatically (e.g. at pH 7.0 a fractional saturation of 40% is reached upon addition of 1 M KCN). This behavior suggests that the cyanide ion, rather than HCN, binds preferentially to ferrous HbI. The photosensitivity of the ferrous cyanide adduct was assessed during the course of the titration by repeated scanning of the sample at different

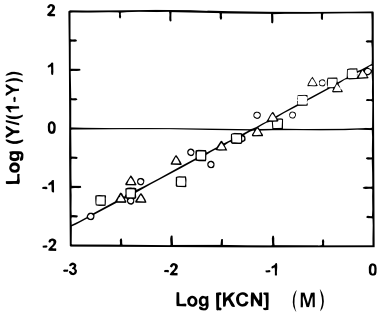

FIGURE 2: Hill plot of cyanide binding to ferrous HbI. Titrations were carried out as detailed in Materials and Methods. The Hill coefficient, n, was 0.96, and the value of the apparent affinity constant was 17 M^{-1}. Different symbols refer to different data sets.

Table 1: Kinetic Data for Cyanide Binding to Ferric HbI and Horse Heart Myoglobin[a]

	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})
HbI	2.3×10^2	6.2×10^{-6}
Mb	3.0×10^2 [b]	2.2×10^{-3}

[a] The experiments have been carried out at pH 9.2 and 20 °C with the procedure outlined in Materials and Methods. [b] Taken from Brancaccio et al. (1994).

scan speeds and slit widths. No photodissociation was observed using a slit width of 0.4 nm and a scanning rate of 200 nm/min.

The affinity constant of cyanide for the ferric protein could not be measured directly over the pH range 7.0−9.2 given the high stability of the complex and the slow rate of the cyanide binding reaction; it was therefore estimated from the ratio of the kinetic constants (see below).

Kinetic Characterization. The kinetics of cyanide binding to ferric HbI has distinct characteristics at different pH values due to the existence of an aquomet derivative, a pentacoordinate species, and a hemichrome in a pH-dependent equilibrium (Spagnuolo et al., 1994). At pH values higher than 8.5, where the pentacoordinate derivative is the dominant species, the cyanide binding reaction proceeds as a simple second-order process. At pH 9.2, the apparent second-order rate constant, obtained from the pseudo-first-order plot, is 2.3×10^2 M^{-1} s^{-1} (Figure 3A and Table 1). Experiments carried out at pH values lower than 8.5 did not yield a linear dependence of the pseudo-first-order rate as a function of cyanide concentration, and the individual time courses were not described by a simple exponential curve. The time courses at pH 8.0 and 7.0 are shown in Figure 4 in

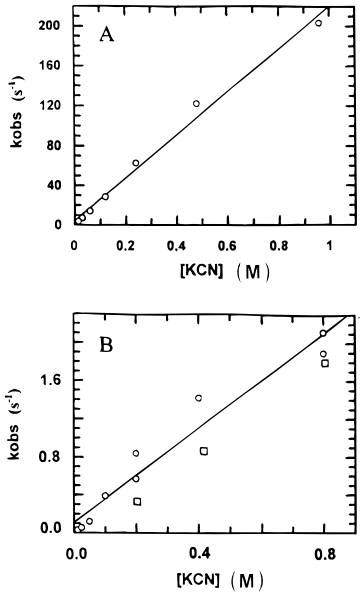

FIGURE 3: Kinetics of KCN combination with ferric (A) and ferrous (B) *S. inaequivalvis* HbI. The observed pseudo-first-order rates are plotted against KCN concentration with the following conditions: temperature, 20 °C; and buffer, 0.1 borate (pH 9.2). The protein concentration was 1.2×10^{-4} M (heme). Experiments on the ferrous derivative were carried out in the presence of 50 mM dithionite. The straight lines represent the best fit to the experimental points. In B, control experiments were carried out on ferrous horse heart Mb (□).

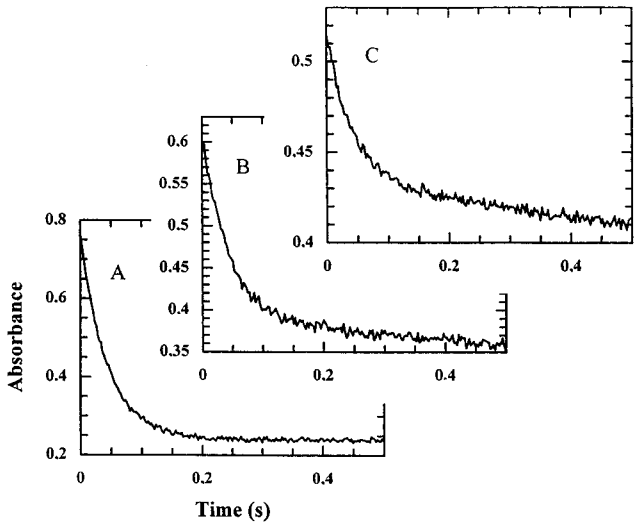

FIGURE 4: Time courses of KCN binding to ferric *S. inaequivalvis* HbI as a function of pH with the following conditions: (A) pH 9.2, 0.1 M borate buffer; (B) pH 8.0, 0.1 M phosphate buffer; and (C) pH 7.0, 0.1 M phosphate buffer. Experimental conditions were as in Figure 3. In B and in C, the end point of the reaction (100 s) was 0.266 and 0.275, respectively.

comparison with the time course measured at pH 9.2. Simple inspection shows that at the lower pH values a slow phase appears whose amplitude increases with a decrease in pH, depends on protein concentration, but is independent of cyanide concentration.

The experiments carried out on ferrous deoxy HbI at pH 9.2 (Figure 3B, Table 2) yield an apparent second-order rate constant of about 2.7 M^{-1} s^{-1}, a value very close to that obtained for horse heart Mb (2.5 M^{-1} s^{-1}). It should be pointed out that the time course for cyanide binding to both proteins is clearly biphasic. The fast phase accounts for more than 80% of the reaction at all cyanide concentrations

Table 2: Kinetic Data for Cyanide Binding to the Ferrous HbI Derivative[a]

pH	k_{on} (M^{-1} s^{-1})	k_1 (s^{-1})	k_2 (s^{-1})
9.2	2.7	0.16 ± 0.004	0.011 ± 0.001
	−	−	0.011 ± 0.001[b]
8.0	−	0.19 ± 0.003	0.013 ± 0.002
7.0	−	0.19 ± 0.004	0.019 ± 0.001
	−	−	0.020 ± 0.002[b]
6.0	−	0.18 ± 0.004	0.024 ± 0.002

[a] The time courses of the cyanide dissociation reaction were fitted to two consecutive exponential processes as indicated in Materials and Methods. Dithionite concentration, 100 mM; protein concentration, 90−100 mM heme before mixing. [b] Experiments carried out in the presence of CO, 1 mM before mixing.

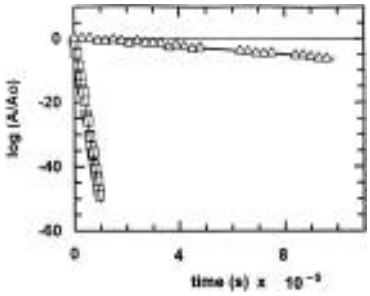

FIGURE 5: Kinetics of CN dissocation from ferric cyanomet HbI as compared to that from cyanomet horse heart Mb with the following protein: (□) horse heart Mb and (△) HbI in 0.1 M borate buffer (pH 9.2). The experimental setup is described in Materials and Methods. Straight lines represent the least squares fit to the experimental points.

investigated and depends linearly on cyanide concentration. The pseudo-first-order plot of Figure 3B was obtained by plotting the rate constant of the fast phase as a function of cyanide concentration. In contrast, the rate of the slow process is independent of cyanide concentration, a finding which points to the occurrence of some photodissociation. In fact, even though the experiments were carried out on a diode array spectrophotometer using a short time interval (0.1 s), the kinetics of cyanide binding requires the protein to be exposed for tens of minutes to white light.

Cyanide dissociation from ferrous and ferric HbI has been followed with different experimental approaches. The rate of cyanide dissociation from the ferric cyanide derivative was measured using a cyanide scavenger, namely immobilized Mb, as outlined in Materials and Methods (Figure 5, Table 1). The methodology was validated first in experiments carried out with Mb; these yielded a value for the cyanide dissociation rate constant of 2.2×10^{-3} s^{-1}, in very good agreement with literature data (Antonini & Brunori, 1971). The rate constant of cyanide dissociation from ferric HbI is 6.2×10^{-6} s^{-1}, roughly 300-fold slower than that from Mb. For both HbI and Mb, the cyanide dissociation rate is pH-independent between pH 7.0 and 9.2. At pH values lower than 7.0, the apparent decrease in cyanide affinity of Mb (Awad & Badro, 1967) renders the use of the immobilized protein as the cyanide scavenger impractical. On the basis of the experimental values of the kinetic constants, the apparent equilibrium association constant of cyanide for ferric HbI and Mb at pH 9.2 is 3.7×10^7 and 1×10^5 M^{-1}, respectively.

The rate of cyanide dissociation from ferrous HbI was measured at pH 9.2 upon mixing cyanomet HbI with a solution containing sodium dithionite in excess over heme

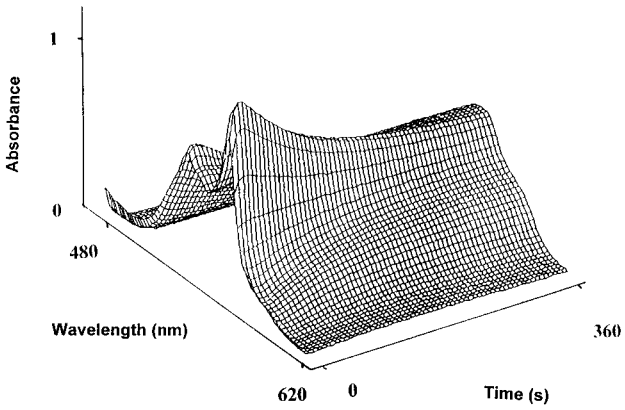

FIGURE 6: Time-resolved spectra following the dissociation of cyanide from ferrous HbI. The spectra were recorded after mixing cyanomet HbI with dithionite in a stopped flow apparatus; the reduction of the complex is completed within the dead time (50 ms). The experimental conditions were as follows: 0.1 M borate buffer (pH 9.2), 45 mM HbI, 50 mM dithionite, $T = 293$ K, and 6 s time interval.

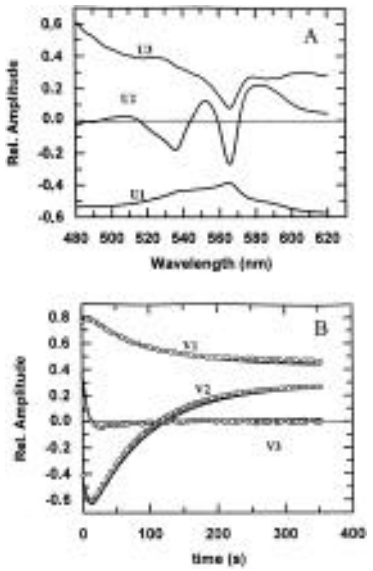

FIGURE 7: SVD analysis of the time-resolved spectra of Figure 6. (A) The first three **U** columns. (B) Fit of the three **V** columns according to the two consecutive exponential schemes. The *S* values for the three significant species were 23.31, 3.81, and 0.46. The rate constants were $k_1 = 0.16$ s^{-1} and $k_2 = 0.011$ s^{-1}.

(Figure 6). The ferric HbI−cyanide complex is rapidly reduced by the excess dithionite, and the ferrous cyanide complex thus generated slowly dissociates to yield cyanide and deoxy HbI (Keilin & Hartree, 1955; Brunori et al., 1992). In control experiments, 1 mM redox mediator methylviologen was added to the dithionite solution (Cox, 1977). An increase in the rate of the reduction process was observed such that most of the fast reduction process occurred in the dead time of the instrument, but no changes in the rate of cyanide dissociation were detected. The analysis of the data carried out with the SVD deconvolution procedure revealed that three significant species contribute to the overall signal (Figure 7A). The time courses relative to the three **U** columns (Figure 7B) were fitted according to the scheme with two consecutive reactions outlined in Materials and Methods. The rate constants obtained from a set of experiments performed as a function of pH and dithionite concentration are summarized in Table 2. The value of the cyanide off rate increases 2-fold (from 0.011 to 0.024 s^{-1}) at pH values lower than 7.0. Table 2 also shows the results of experiments carried out in the presence of 1 mM CO at pH 7.0 and 9.2. In these experiments, as soon as cyanide dissociates from the ferrous derivative, carbon monoxide binds to deoxy HbI, thus keeping the protein in the liganded state throughout the reaction. The observed rates of cyanide dissociation are indistinguishable from those measured in the absence of CO.

DISCUSSION

Spectroscopic Properties. The absorption spectrum of ferrous HbI−CN in the Soret and visible region (Figure 1A,B) is typical of a low-spin hexacoordinate derivative and closely resembles the spectra observed as transients during the reduction of ferric cyanide HbA, sperm whale Mb, *Parascaris* hemoglobin, and *Aplysia* Mb (Belleli et al., 1990; Brunori et al., 1992; Antonini et al., 1994). In HbI, the intensity ratio of the a/β bands (or Q_o/Q_v bands according to Platt's nomenclature) is similar to that observed in *Parascaris* Hb and higher than those in the other hemoproteins studied. The shoulder at about 580 nm is clearly visible in HbI and Sw Mb; in the other hemoproteins investigated, it is covered by the broad Qo band. The 580 nm band can be tentatively assigned to a Q 1 to 0 transition (hot band) involving a low-frequency porphyrin vibrational mode, an assignment that can be validated by analysis of the temperature dependence of the band intensity. In the wavelength region above 600 nm (Figure 1C), two bands are detectable, namely a peak at 695 nm and a broad band centered at 840 nm. In view of their very low intensity, these two bands are most likely due to charge transfer transitions. These may be ascribed to electron promotion from the filled iron d_{xy}, d_{yz}, and d_{xz} orbitals to the empty d_{z^2} and $d_{x^2-y^2}$ orbitals. Alternatively, the broad band at 840 nm may involve charge transfer transitions from the iron orbital to the p or p* orbitals of the ligand, as observed in the case of oxygen (Eaton & Hofrichter, 1976). Indeed, heterogeneity in the orientation of the ligand is likely to broaden the band shape of a charge transfer transition that involves the ligand orbitals.

Thermodynamics of Cyanide Binding. The stability of both the ferrous− and ferric−CN adducts in HbI unusually is high. Thus, at pH 9.2, the apparent affinity constant of cyanide for ferrous HbI is 17 M^{-1}, about 8-fold greater than that for ferrous Sw Mb (Keilin & Hartree, 1955). In ferric HbI, the affinity constant determined from the ratio between the binding and the dissociation rate constants corresponds to about 3.7×10^7 M^{-1} as compared to 1×10^5 M^{-1} in Sw Mb (Antonini & Brunori, 1971). The affinity of cyanide for the heme iron appears to be dictated mainly by electrostatic attraction between cyanide and iron on the one hand and between cyanide and oppositely charged groups in the distal pocket on the other (Brancaccio et al., 1994). This electrostatic factor accounts for the higher affinity of cyanide for ferric than for ferrous heme. The unusual stability of the iron(II)− and iron(III)−cyanide complexes in HbI can be explained in these terms and ascribed to the low polarity of the heme pocket which is related to the shielding of heme groups from solvent (Royer et al., 1989) and is clearly manifest in the fluorescence properties of HbI reconstituted with zinc porphyrin (Ilari et al., 1995).

Cyanide binding to ferrous HbI is characterized by a Hill coefficient of 0.96 ± 0.16 (Figure 2). In HbI therefore,

cyanide does not display cooperativity in binding to ferrous heme at variance with oxygen and carbon monoxide. This point will be discussed below.

Kinetics of Cyanide Binding and Dissociation. The study of the kinetics of cyanide binding to a large number of ferric heme proteins has brought out a complex reaction mechanism which involves (i) dissociation of the heme-coordinated water molecule, (ii) diffusion of HCN into the protein, (iii) deprotonation of the acid within the heme pocket to yield the cyanide anion, and (iv) formation of the bond between the iron atom and cyanide (Mintorovitch et al., 1989; Brancaccio et al., 1994). Experiments on myoglobins with mutations at the distal histidine have indicated clearly that this residue plays an important role in determining the binding rate as it affects both the acid dissociation constant of HCN within the pocket and the stability of the iron-bound water molecule (Brancaccio et al., 1994). Thus, when an apolar or a negatively charged residue is substituted for the distal histidine, a substantial decrease in the cyanide binding rate is observed due to the concomitant decrease in the deprotonation of HCN and destabilization of the iron-coordinated water molecule.

Ferric HbI is unusual in that it is the only hemoglobin which possesses a distal His but is pentacoordinate at alkaline pH values (Boffi et al., 1994). Hence, at pH 9.2, the energy barrier for cyanide binding should be entirely accounted for by HCN deprotonation. Figure 4A shows that the apparent second-order rate constant for cyanide binding is very similar to that reported for Mb at neutral and alkaline pH values ($150-300\ M^{-1}\ s^{-1}$), namely under conditions in which the sixth coordination position is occupied by a water molecule. It follows that destabilization of the heme-bound water molecule must have a limited effect on the overall cyanide binding kinetics. At neutral and acidic pH values, the pentacoordinate, dimeric ferric HbI species is in equilibrium with a dimeric aquomet and a monomeric hemichrome (Spagnuolo et al., 1994). Under these conditions, the time course of cyanide binding to HbI becomes clearly biphasic. In addition to the fast phase, which represents the bimolecular binding reaction, a slow phase appears whose amplitude increases with a decrease in pH, is independent of cyanide concentration, but depends on protein concentration. The rate of the slow phase is at least 2 orders of magnitude slower than the fast one (Figure 4). The observed behavior can be accounted for by assuming that the monomeric hemichrome, which is present at neutral and acidic pH values, cannot bind cyanide. In the monomeric hemichrome, the sixth coordination position is occupied by a protein residue, most likely the distal histidine (Spagnuolo et al., 1994; Boffi et al., 1994), and cyanide appears to be unable to displace it. Therefore, at neutral pH values, the rate of cyanide binding is limited by the rate of assembly of the monomeric hemichrome into the dimeric aquomet and/or pentacoordinate derivative and hence by a second-order association process.

The kinetics of cyanide binding to ferrous hemoproteins to our knowledge has never been reported, possibly due to the experimental difficulties related to the photolability of the complex and to the use of concentrated cyanide solutions at high pH values. The present experiments were carried out in parallel on HbI and Mb and yielded nearly identical binding rates for the two proteins. At pH 9.2, these are about 100 times slower than those pertaining to the ferric derivatives at pH 9.2. The mechanism of cyanide binding to the ferrous protein can be expected to be similar to that operative in ferric pentacoordinate HbI as both derivatives lack the water molecule in the sixth coordination position (Boffi et al., 1994). Hence, also in ferrous HbI, the kinetic barrier should be represented by deprotonation of HCN, which is likely to occur at a similar rate in the two derivatives. On this basis, the 100-fold difference in the rates of cyanide binding to ferrous and ferric HbI reflects a difference in the intrinsic rate of cyanide bond formation.

In HbI, the rate of cyanide dissociation is significantly slower than in Mb both in the ferric and in the ferrous derivative. This finding indicates that there is a common factor which stabilizes bound cyanide irrespective of the oxidation state of the heme iron and must be related to the above-mentioned decreased overall polarity of the pocket. The pH dependence of cyanide release from HbI is similar to that of Sw Mb (Table 1) in that the p*K* of the transition is around 7.0. As for Sw Mb, it may be envisaged that the distal His acts as a proton donor to the bound cyanide molecule, facilitating its dissociation (Bellelli et al., 1990).

Most interesting is the observation that cyanide dissociation from ferrous HbI occurs at the same rate in the absence and in the presence of CO. In contrast, in HbA, the cyanide release process displays kinetic cooperativity as it is described by different constants depending on whether the final product is deoxy or liganded hemoglobin; in other words, the cyanide dissociation process displays kinetic cooperativity (Brunori et al., 1992). The lack of cooperative effects in cyanide binding to ferrous HbI can be ascribed to the unique mechanism of heme−heme interaction operative in this protein. The structural modifications accompanying oxygen or CO binding to one heme are transferred to the other through geometrical changes in the heme peripheral substituents and tertiary changes that are restricted to the heme environment (Royer et al., 1989; Song et al., 1993; Royer, 1994). Upon cyanide binding to deoxy HbI, even though the heme iron becomes low-spin and is pulled toward the heme plane, the stereochemical changes which are a prerequisite for cooperativity do not take place. It may be hypothesized that cyanide is unable to trigger such conformational changes because the iron−ligand bond is weaker than in the case of oxygen and CO and because the electron acceptor power of cyanide is poor. The observation that cyanide binding to HbI is noncooperative, although changes in the Fe−proximal histidine bond most likely accompany the iron atom movement toward the heme plane, confirms the contention that this bond does not represent a major conduit for information transfer among hemes. This conclusion is in accordance with a previous finding that breakage of the Fe−proximal histidine bond does not abolish cooperativity (Coletta et al., 1990).

Lastly, it should be pointed out that the apparent affinity constants for cyanide binding to ferrous Mb ($2.5\ M^{-1}$) and HbI ($17\ M^{-1}$) measured in equilibrium experiments are about 10-fold lower than those obtained from the ratio between the binding and dissociation rate constants, a difference which appears to be beyond experimental error. In order to establish whether the observed difference can be ascribed to the formation of metastable intermediate(s), transient resonance Raman and optical absorption experiments will be carried out and reported in a following paper.

In conclusion, the direct comparison of cyanide binding to ferrous and ferric HbI has shown that the same reaction

mechanism applies and the same factors determine the stability of the two adducts. Among these, the overall polarity of the pocket appears to be of major importance. Structurally, cyanide binding and dissociation are controlled by a proton donor−acceptor mechanism in which the distal histidine is most likely involved. In the ferrous protein, the lack of cooperativity in cyanide binding provides evidence that movement of the iron atom toward the heme plane does not suffice to trigger the necessary conformational changes.

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