CO Binding and Valency Exchange in Asymmetric Hb Hybrids†

Laurent Kiger, Claude Poyart, and Michael C. Marden*

Inserm U473, 84 rue du Général Leclerc, 94276 Le Kremlin Bicêtre Cedex, France

Received March 12, 1998; Revised Manuscript Received June 8, 1998

ABSTRACT: There remains a major controversy concerning the properties of asymmetric hemoglobin hybrids, that is, doubly liganded tetramers consisting of an unliganded dimer and a liganded dimer. Different experimental evidence leads to opposing conclusions. Based on dimer−tetramer equilibrium studies, special "T-like" properties were assigned to this hybrid (species 21), while the other biliganded tetramers were considered as similar to fully liganded Hb [Ackers et al. (1992) *Science 255*, 54−63]. We report here results for three types of experiment. In the first, the asymmetric hybrids are produced by photodissociating CO ligands from [dimer-CO/dimer-azido-met] hybrids. Since the CO association rates differ by over an order of magnitude for the two allosteric states, the CO kinetics are a sensitive probe of the tetramer conformation. The results show mainly rapid R-like kinetics for CO rebinding to the asymmetric hybrids. The second technique employs a stopped-flow apparatus to obtain a higher percentage and a longer equilibration time of the asymmetric hybrid. In this case, sodium dithionite is used to remove oxygen from a solution containing [dimer-oxy/dimer-azido-met] hybrids. After a fixed delay (but before loss of azide ligands), a second mixing with a buffer equilibrated under CO allows observation of CO binding to species 21. As for the flash measurements, the kinetics show predominantly rapid CO binding, typical of the liganded (R-state) tetramer. The rapid CO binding is not in agreement with the predictions of a T-like conformation for species 21. One possible explanation is that the long incubation times used to study the dimer−tetramer equilibrium do not lead to a stable asymmetric hybrid, but rather a random distribution of oxidized subunits due to electron transfer between the iron atoms of the subunits [Shibayama et al. (1997) *Biochemistry 36*, 4375−4381]. We have repeated these experiments and confirm the valency exchange in a mixture of Hb A and S (or C) parent forms, as evidenced by compensating amounts of oxidation or reduction of the Hb parents.

A radical departure from the classic two-state model (*1*) for hemoglobin was proposed by Ackers et al. (*2*, *3*) based on dimer−tetramer equilibrium studies. They observed evidence that the asymmetric hybrid [dimer-deoxy/dimer-metCN] shows an enhanced stability relative to other biliganded tetramers. This effect implies a preferred binding of the second ligand, the symmetry rule (SR), to the dimer with one ligand already bound. The asymmetric hybrid (species 21) was classed as a T-state Hb, similar to the singly liganded tetramers; one would then expect ligand binding to species 21 to occur with the low T-state rate.

In a previous study, we described a method to photoproduce the asymmetric hybrids starting with CO/CN hybrids (*4*). The CO recombination to the asymmetric hybrids did not show T-like kinetics, a result not compatible with the SR model. To explain this discrepancy, one needs to consider the kinetics of transitions for the Hb molecule. A major difference in the techniques is the time of sample incubation. While the dimer−tetramer equilibrium studies require a 48 h incubation to allow for dissociation of the stable deoxy tetramer, only several seconds for mixing of the liganded forms is necessary for the flash photolysis studies.

A second difference is the length of time the Hb molecule has spent in the asymmetric state. In the flash photolysis technique, the Hb tetramers are initially in the fully liganded or R-state conformation, a conformation which binds CO in only a few milliseconds. One can then ask if enough time was allowed for the allosteric transition of the asymmetric hybrid. To observe the slow T-like kinetics after photodissociation, two conditions are required: the T-state must be the favored conformation for the photoproduct, and the allosteric transition must be faster than the R-state recombination rate, which is about 1 ms for samples equilibrated with 0.1 atm CO (100 μM free CO). Much lower CO levels are not an option since the photodissociated CO will maintain a minimum level just after photolysis. Lower Hb and CO concentrations might lead to dimers or unsaturated sites.

Studies of HbCO flash kinetics at 1 atm versus 0.1 atm CO show different amounts of the slow phase. This indicates that the allosteric transition rates are on the same order of magnitude as the CO binding rates (0.1−1 ms). Direct observation of absorption signals near the isosbestic point for ligand binding also indicates a rapid reequilibration requiring about 100 μs (*5*). However, one could conceive of slower rates for the doubly liganded tetramers in question.

† This research was supported by the Institut National de la Santé et de la Recherche Médicale, and the Faculté de Médecine Paris Sud.

* Corresponding author: INSERM U473, 84 rue du Général Leclerc, 94276 Le Kremlin-Bicêtre Cedex, France. Telephone: (33 1) 46-70-89-89. FAX: (33 1) 46-70-64-46. E-mail address: marden@kb.inserm.fr.

S0006-2960(98)00570-4 CCC: $15.00
Published on Web 09/19/1998

In the present study, we have repeated the flash photolysis study with azide as the metHb ligand. We also report an alternate method of producing an asymmetric hybrid. Rather than photodissociation of CO, dithionite is used to remove oxygen from an oxy/ferric hybrid. After a fixed incubation time of the newly formed deoxy/ferric hybrid, CO association kinetics can be measured by stopped flow. The delay time can be as long as permitted by the hybrid stability, which is a few seconds for use of azide. Thus, relative to the flash method, the incubation time is extended over 2 orders of magnitude; the fraction of asymmetric hybrids is also improved with the stopped-flow method since dithionite removes essentially all the oxygen ligands whereas it is difficult to photodissociate more than about two-thirds of the CO ligands with 10 ns laser pulses.

In light of the recent report of electron transfer between Hb subunits (*6*), we repeated this type of experiment under slightly different conditions. This effect could explain why results differ for experiments performed on different time scales. The long incubation times of 48−72 h required for the dimer−tetramer equilibrium studies (*2*, *3*, *7*) could involve other protein transitions.

MATERIALS AND METHODS

It is well established that the liganded forms oxyHb, HbCO, and CN-metHb will rapidly exchange dimers following a binomial distribution. Unlike the deoxy tetramers which are highly stabilized (*8*), the dimer exchange for liganded tetramers occurs within a few seconds (*9*), allowing a rapid preparation of liganded hybrids. A large excess of the metHb parent will minimize the fraction of $Hb(O_2)_4$ parent to ensure that the oxy dimers will be found predominantly as the oxy/met hybrid species. For a binomial distribution of dimers of two types with "f" the fraction of ferrous dimers, then the relative tetramer populations ferrous/ferrous, ferrous/ferric, and ferric/ferric will be f^2, $2f(1 - f)$, and $(1 - f)^2$, respectively; the fraction of ferrous sites occurring within asymmetric hybrids is $(1 - f)$.

The problem of obtaining the deoxy/liganded asymmetric hybrid is removing the ligands from only one dimer. CN and azide are "stable" ligands since they are not photodissociable and dissociate slowly from ferric iron. CO can be photodissociated from CO/met hybrids to generate the asymmetric form; alternatively, sodium dithionite can be used to remove the oxygen from oxy/met hybrids.

Hb Preparation. Hb A was purified from hemolysate of healthy donors, stripped of organophosphates, as previously described (*10*). Hb S or Hb C was separated from other Hb components (mainly Hb A) by preparative isoelectric focusing at 5 °C, using granulated gel (Ultrodex) and ampholines (with a pH gradient from 6 to 8; no. 80-1125-93) purchased from Pharmacia Biotech. Oxidized fractions of Hb were discarded, and the purified oxyHb samples were stored in liquid nitrogen. An additional analytical IEF at 5 °C was performed in polyacrylamide PAGE (Pharmacia Biotech) along a gradient from pH 6.5 to 8.5 with the LKB system to check the purity of each Hb. The electrophoretic migration pattern of Hb variants was compared to standard Hb variants (A, F, S, and A_2) and found to be in agreement with their theoretical p*I*.

Whenever possible, we used the experimental conditions and methods reported in the studies by Ackers et al. (*7*), such as buffer "A": 0.1 M Tris-HCl buffer, 0.1 M NaCl (for a total chloride concentration of 0.18 M), 1 mM Na_2-EDTA, pH 7.4, at 21.5 °C. Hbs were equilibrated against this buffer, by passing the sample through a Sephadex G-25 column.

MetHb A was prepared by addition of a 20% excess of potassium ferricyanide to an oxyHb solution. For an observed bimolecular rate coefficient for cyanide association to ferric Hb of 100 M^{-1} s^{-1} (*11*), formation of cyano-metHb has a time constant of 16 min at 10 μM KCN; a ligand concentration of 1 mM was used to ensure binding within a few minutes. After a 30 min incubation at room temperature, the solution was passed twice through a Sephadex G-25 column to remove the oxidation side products. An absorption spectrum was recorded before and after addition of a large excess of potassium ferricyanide to confirm that the oxidation reaction was achieved. An alternate test is to measure the absorption spectrum before and after addition of CO, to detect a small fraction of ferrous hemes. An aliquot was also injected into a buffered solution equilibrated under CO and photodissociated immediately; this isolates the signal for ferrous, CO hemes, as the ferric ligands (water, CN, azide, OH) do not show signals on this time scale; signals of less than 1 part per 1000 (relative to a HbCO sample) were obtained.

Because ferrocyanide binds tightly to Hb, we tested another method to prepare the cyano-metHb stock. OxyHb A was autoxidized at 37 °C in buffer A in the presence of chloramphenicol (20 μg/mL). After 48 h incubation, the solution was centrifuged (10 000 rpm at 4 °C), and a small precipitate was removed. Over 90% of the hemes were oxidized, so the amount of ferricyanide necessary to complete the reaction was not more than 20% of heme sites. The removal of side products was done following the same procedure as described above. Both methods of oxidation gave the same results concerning the experiments of electron transfer.

Azido-metHb was prepared in the same manner as cyano-metHb, but with buffer "B" (pH 7.2 at 25 °C) consisting of 50 mM Bis-Tris, 100 mM NaCl, 1 mM Na_2EDTA, and 5 mM NaN_3.

UV/Visible Spectra. UV/visible absorption spectra of Hb solutions were recorded with an SLM-Aminco (DW2000) spectrophotometer in a quartz cuvette of 4 mm optical path length. The cyano-met species was used to determine the heme concentration, taking an ϵ value of 11 mM^{-1} cm^{-1} at 540 nm (*12*).

Azide Dissociation Kinetics. To confirm that azide is a stable ligand, the azide dissociation kinetics were monitored with a HP8453 diode-array spectrophotometer. Azido-metHb was mixed with 20 mM sodium dithionite (dtn) in buffer "B" at 25 °C in an optical cuvette equilibrated under 0.1 atm CO gas.

In general, there are two pathways for this reaction (*13*): (1) ligand dissociation followed by reduction by dithionite; and (2) a reduction of the ferric heme−ligand system followed by dissociation of the ligand from the newly formed ferrous iron. For cyano-metHb, the second pathway is kinetically favored; there is a transition from met-CNHb to

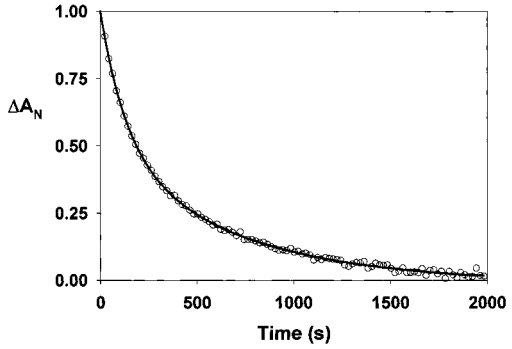

FIGURE 1: Kinetics of azide dissociation from 10 μM metHb. The initial rate is 0.01/s at pH 7.2, 25 °C. The azido-metHb sample was diluted in a buffer containing dithionite and CO; absorption spectra were measured to follow the reaction.

ferrous-CN-Hb whose rate depends on the dithionite concentration and may occur on the millisecond time scale (*14*).

For the case of azide, dissociation is monophasic and requires about 1000 s at low dithionite concentrations (first pathway), but at dithionite concentrations above 1 mM, the second pathway becomes competitive for at least one type of subunit. A spectrum between 350 and 450 nm was recorded every 10 s; isosbestic points were found for the spectral transition from metHb-N_3 to Hb-CO, indicating that no intermediate species were present such as ferrous heme bound to azide. The azide dissociation was biphasic (Figure 1), with the rapid phase requiring about 100 s at 20 mM dithionite.

Cyanide Dissociation Kinetics. CN displacement by azide was monitored spectrophotometrically; analysis of the spectra indicates about a time constant for dissociation of about 6 h with 10 mM azide in buffer A at 21 °C. Without azide, open beakers of metHbCN at room temperature (near 25 °C) were stirred under a continuous flow of air to remove the cyanide; the results showed a larger variation (3−5 h) in the time constant.

Experiment 1. CO Binding to Photoproduced Species 21. CO bimolecular kinetics were measured after flash photolysis with a 10 ns YAG laser (Quantel) pulse delivering 160 mJ at 532 nm (*15*). Samples were equilibrated under 0.1 atm CO in 1 mm optical path length cuvettes closed with a serum cap. With a detection wavelength at 436 nm, a typical kinetic curve was obtained as the average of 10 measurements with pulses repeated every 2 s. Kinetics were first measured for a HbCO sample. Azido-metHb was then added, and the mixture was photodissociated within 30 s.

Experiment 2. CO Binding to $[\alpha^{+N_3}\beta^{+N_3}/\alpha^{deoxy}\beta^{deoxy}]$ *by Stopped Flow*. Kinetic experiments were carried out with a Biologic (SFM-3) stopped flow equipped with three syringes for double mixing. The methodology used to generate the asymmetric hybrid $[\alpha^{+N_3}\beta^{+N_3}/\alpha^{deoxy}\beta^{deoxy}]$ is similar to that described by Sharma (*16*) for oxy/CO hybrids. Three stock solutions at pH 7.2 were prepared: S1, buffer B containing 20 mM dithionite; S2, a solution of 10 μM oxyHb and 160 μM metHb in buffer B; S3, buffer B containing 10 mM dithionite equilibrated under 20 torr CO, which corresponds to about 25 μM in dissolved CO. Solutions S1 and S2 are first mixed to generate the deoxy/met hybrids, which are then mixed with S3 to study CO binding.

Different mixing volumes could be used to study the dependence of the kinetics on the concentration of Hb or CO. The detection wavelength was also varied to make sure the observed reaction was in fact CO binding.

Experiment 3. Detection of Valency Exchange after a 50 h Incubation. The procedure of hybridization used by Ackers' group (*7*) was followed rigorously to obtain the asymmetric species $[\alpha^{+CN}\beta^{+CN}/\alpha^{deoxy}\beta^{deoxy}]$ from the parent species cyano-metHb A and deoxyHb S (or C). Both protein samples were deoxygenated on ice under a humidified stream of nitrogen during 1 h, with gentle shaking. To prevent cyanide dissociation from the liquid to gas phase (*6*), nitrogen gas was humidified with successive bubblers, previously flushed overnight, containing water and buffer A with 100 μM KCN. Typical sample concentrations were 0.8 mM (on a heme basis). In parallel, the enzyme system from Sigma: glucose oxidase (no. G-2133) with β_D(+)glucose (no. G-5250) and bovine liver catalase (no. C-40) used to scavenge the O_2 traces and guarantee anaerobic conditions, was deoxygenated. Note that no KCN was added to the enzyme buffer, since CN can dramatically decrease the enzyme activity of catalase. The final enzyme concentrations, and control of the enzyme activity of the catalase lot, were as suggested by Ackers et al. (*7*). In a glovebag at room temperature (near 22 °C), the enzymes and Hb samples were mixed for a few minutes; near equal amounts of the two parent species were added to a solution of buffer A, leading to a final concentration of cyanide in the anaerobic solution of 40 μM. An aliquot was taken to assess the preincubation absorption spectrum at 21.5 °C. Finally the Hb mixture was incubated in a septum-sealed conical vial, itself protected from oxygen in a dithionite solution.

Spectroscopic Analysis of Hb Species. After 50 h of incubation, the vial containing the sample mixture was removed from the dithionite solution and immediately stored on ice. Then the protein solution was exposed to 1 atm CO gas to stabilize and protect the deoxy hemes from oxidation. The postincubation absorption spectrum was recorded immediately after diluting 10−20 μL of protein solution into 1 mL of buffer A with no KCN. A small amount of dry KCN was then added; from the absorption spectra, with and without added KCN, one can determine the total percent oxidized and also the percentage aquo-metHb at the end of the incubation.

The separation of the parent Hb A species from Hb S (or Hb C) and the measurement of their spectroscopic characteristics are fundamental to assess if electron transfer has occurred during the incubation. We did not use a preparative IEF to separate the Hbs because it generates as much as 10% oxidation of the hemes during the focusing at 5 °C. We chose rather to separate the Hbs by anion exchange chromatography (*17*). A 150 μL aliquot (in the original Tris buffer) was diluted 20-fold, reconcentrated, and diluted again in 0.2 M glycine buffer at pH 7.3, with 1 mM KCN, saturated with CO gas. The sample was then loaded onto a micro column (1 × 5 cm) of DEAE-cellulose equilibrated with the same buffer. Hb S or C was eluted first, and Hb A was finally released from the column with 0.2 M glycine, 0.2 M NaCl, 1 mM KCN buffer at pH 7.3. This method is very convenient and allows a separation of the two parent species in a few minutes without developing a pH or salt gradient. The purity of the two Hb fractions collected by chromatography was checked by analytical IEF on polyacrylamide PAGE gel as described above. The postincubation absorp-

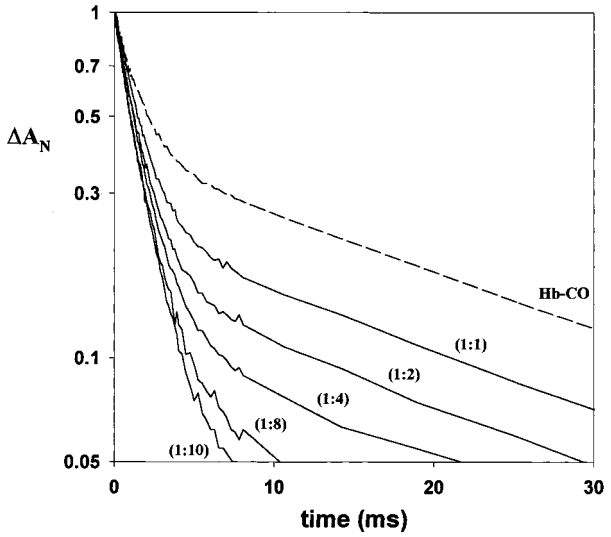

FIGURE 2: CO rebinding kinetics after photodissociation of samples containing a mixture of 12 μM HbCO and azido-metHb; the values indicated are the relative concentrations of HbCO to metHbN_3. The decrease in the fraction of slow phase, upon addition of azido-metHb, confirms the formation of hybrids (through dimer exchange) which have a lower fraction slow phase for the hybrid relative to HbCO. Experimental conditions were 25 °C, pH 7.2.

tion spectrum of each Hb was analyzed by simulating the spectrum as a linear combination of the fully carboxylated ferrous and cyano-ferric spectra. Data simulations were carried out using the nonlinear least-squares routine of the software program Scientist.

Since the goal of these experiments was to detect valency exchange (and not the hybrid population), no attempts were made with the low-temperature isoelectric focusing. The samples were stabilized by addition of CO followed by separation of the two types of Hb. In addition to the spectral properties, the CO rebinding kinetics after flash photolysis were measured before and after addition of an excess of dithionite.

RESULTS AND DISCUSSION

These experiments were designed to take into account transformations in the protein state. The measurements therefore involve different time scales. The simplest and most rapid experiment employs the flash photolysis technique. Starting from solutions of HbCO and metHb, one can study CO binding to the asymmetric hybrids in a few minutes.

Experiment 1. Photoproduction of Asymmetric Hybrids. The kinetics of CO rebinding to Hb are shown in Figure 2, for samples of HbCO before and after addition of azido-metHb. HbCO alone shows nearly equal amounts of the rapid and slow bimolecular phases. The rate of the slow phase (4×10^5 M^{-1} s^{-1}) is characteristic of T-state Hb. The ratio of the rates of the rapid and slow phases was 20, as compared to typical values of 30−50 for the comparison of the binding rates of the fourth to first ligand. Kinetics are rapid for triply liganded tetramers, which provide most of the kinetic signal at low laser energies. Dimers also provide a rapid CO binding signal, considered a static contribution on this time scale. In the photolysis study at 25 °C with 10 ns pulses, only half of the hemes are dissociated at the beginning of the bimolecular phase, due to incomplete dissociation and nanosecond geminate recombination. The curve for HbCO in Figure 2 therefore represents a mixture of the partially liganded forms.

The addition of azido-metHb decreases the fraction of the slow phase (Figure 2), as previously reported for studies with cyano-metHb (*4*). The change in the kinetics confirms the formation of hybrids whose properties are different from those of the parent HbCO.

Because the ferric sites are not photodissociable, there is a decrease in the amounts of deoxy and singly liganded tetramers which normally have the slow T-like rates. At high ratios of metHb to HbCO, the main photoproduct is the asymmetric hybrid, with some deoxy or singly liganded Hb from the small fraction of the $Hb(CO)_4$ parent, and some triply liganded forms since the photodissociation is not 100% efficient. Since the ferric ligands are not dissociated, the signal amplitude remains the same for ratios of metHb to HbCO as high as 10 to 1, before a significant inner filter effect occurs.

If the asymmetric hybrid has R-like CO binding rates, then one would expect a decrease in the fraction of the slow phase relative to the control HbCO. An increase in the slow phase is expected based on the SR model (*3*) which predicts T-like properties for the 21 species. The simulated fractions are shown in Table 1, taking into account dimers and partial photodissociation of the hybrids. The amounts of dimer, HbCO, and CO/azide hybrid were calculated; the hybrid was further decomposed into the amount of [dimer-deoxy/dimer-azide] and triply liganded [(deoxy)(CO)/$(azide)_2$] tetramers just after photodissociation. Dimers and triply liganded forms were taken as rapidly reacting species; the experimentally determined fractions fast and slow of control samples were used for $Hb(CO)_4$ tetramers. Two cases could then be simulated: rapid CO binding to species 21 as for the two-state model (with allosteric parameters that yield less than 50% T-state for the doubly liganded forms) and the SR model which predicts slow T-like properties of species 21. As for the previous study with cyano-metHb (Figure 4 in ref *4*), the present results favor a model with rapid R-like CO binding to species 21. The observed decrease in the fraction of slow phase is therefore not compatible with the SR model.

The higher the ratio of azido-metHb to HbCO, the lower the fraction of slow phase (Table 1); the decrease in the fraction of slow phase versus the ratio of azido-metHb to HbCO is consistent with a binomial distribution of dimers. The large difference in the kinetics allows one to estimate that CO binding to the 21 species has an R-like rate, as expected for an R-state Hb or a mixture of R and T states in rapid equilibrium. In the latter case, one expects the R-state rate modulated by the fraction of R-state. Note that the total Hb concentration increases upon addition of metHb, so the dimer contribution (which has no slow phase) decreases. MetHb with azide as ligand is known to exist as a mixture of high- and low-spin forms; since high-spin ligands (water or F^-) shift the allosteric equilibrium less than the low-spin forms (*18*), use of azide might overestimate the T-like contribution, relative to low-spin ligands such as CN.

Experiment 2. Generation of Species 21 by Stopped Flow. Double mixing experiments were performed to observe CO binding to an asymmetric [deoxy-dimer/liganded dimer]. The experimental scheme is shown in Figure 3. In the first

Table 1: Simulations of Kinetic Data of CO Binding to Asymmetric Valency Hybrids[a]

	[metHb-N_3]/ [HbCO]	% ferrous as hybrid	% signal species 21	% as slow phase		
				observed	rapid 21	slow 21
flash photolysis	0	0	0	40	(40)	(40)
	1	46	23	23	21	44
	2	62	31	17	14	45
	4	75	37.5	11	9	46
	8	85	42.5	7	5	47
	10	87	43.5	5	4	48
stopped flow	0	0	0	90	(88)	(88)
	16	91	91	18	6	97

[a] Simulations consider three species initially containing ferrous hemes: $Hb(CO)_4$, $dimer(CO)_2$, and the hybrid [$dimer(CO)_2$/$dimer(azide)_2$] (second column). For stopped-flow experiments, the ferrous hemes are initially oxygen-bound. Estimated contributions to the CO binding signal assume a binomial distribution of ferrous and ferric dimers, and no signal from ferric subunits. 50% photodissociation was determined, which explains why the flash signal for species 21 [dimer-deoxy/dimer-azide] represents only half of the potential signal for the hybrids. The last two columns are the simulated percentage of slow CO rebinding kinetics, based on rapid binding (two-state model) or slow binding (symmetry rule) of CO to species 21.

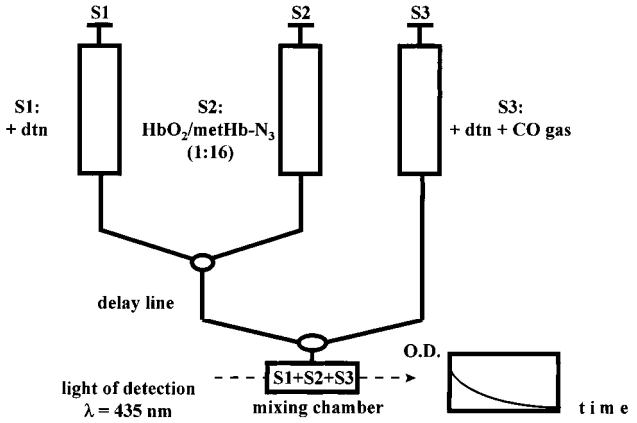

FIGURE 3: Stopped-flow scheme for double-mixing experiments. Solution 2 consists of a mixture of oxyHb and metHb; a large excess of metHb ensures that the oxy dimers occur mainly as part of the oxy/met hybrid. The first mixing with dithionite (dtn) produces the [deoxy-dimer/met-dimer] asymmetric hybrid. After a variable delay, mixing with the third (CO) solution allows observation of CO binding to the asymmetric hybrids.

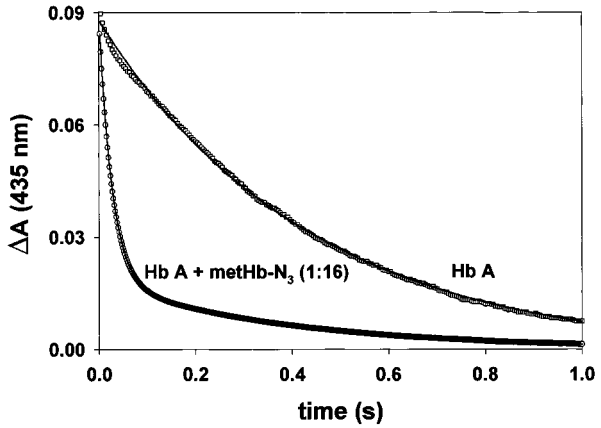

FIGURE 4: Stopped-flow kinetics of CO binding at 25 °C, pH 7.2. For the control (upper) curve, oxyHb was first mixed with dithionite; 100 ms later the resultant deoxyHb was mixed with the CO solution and shows mainly slow phase of rate 2×10^5 M^{-1} s^{-1}. For the hybrid solutions (lower curve), an oxy/azido-met Hb solution was first mixed with dithionite; after a 100 ms delay, the second mixing allowed study of CO binding to the deoxy/azido-met hybrid. The initial [oxyHb] = 10 μM for both cases; the initial [azido-metHb] = 160 μM to ensure that oxy dimers were mainly paired with oxidized dimers; final concentrations were 10 μM CO and 3 μM in ferrous hemes.

mixing stage, solution S1 containing the hybrid [$\alpha^{+N_3}\beta^{+N_3}$/ $\alpha^{O_2}\beta^{O_2}$] is mixed with the buffered dithionite solution. During a delay time of at least 100 ms, the O_2 dissociation from the hybrid is achieved (at 25 °C, $\tau_{off} \approx 20$ ms), the dithionite consuming the free O_2 in <1 ms. After the fixed delay time, the third solution is mixed with the [$\alpha^{+N_3}\beta^{+N_3}$/ $\alpha^{deoxy}\beta^{deoxy}$] hybrids to study their reaction with CO.

The CO binding kinetics are shown in Figure 4. Without the azido-metHb parent, the kinetics are predominantly slow, typical of CO binding to deoxyHb. For the hybrid solutions at 1 ferrous to 16 ferric hemes, 18% slow phase was observed (Figure 4, Table 1). Species 21 represents over 90% of the CO binding signal, higher than for the flash photolysis studies since dithionite efficiently removes oxygen eliminating the triply liganded hybrid species; the remaining contributions are 6% for CO binding to deoxyHb and 3% for CO binding to dimers. After correction for these contributions, the kinetics for the asymmetric hybrid would be about 13% slow phase.

As for the flash photolysis results, the stopped-flow kinetics indicate that the asymmetric hybrid is not predominantly in a T-like conformation. The ratio of fast to slow CO binding rates was 15, slightly less than that observed for the flash experiments. The full amplitude of the rapid phase is more difficult to measure by stopped flow as compared to flash photolysis; a lower CO concentration was used to compensate the lower temporal resolution.

This result is similar to the stopped-flow kinetics showing about 30% slow phase for CO binding to symmetric deoxy/CN hybrids (*19*) and the study of asymmetric deoxy/CO hybrids showing about 20% slow phase for CO binding (*16*). Taken together, these results indicate that there is not a predominance of slow deoxy-like kinetics to hybrids of either type. As previously reported (*19*), the two-state model underestimates the amount of slow phase in the stopped-flow experiment, as if there were some slowly interconverting forms.

Time-dependent changes during the 100−200 ms delay time (between mixings) should be considered. Loss of azide is not expected on this time scale, based on the observed kinetics of azide dissociation (Figure 1) which requires about 100 s. Dissociation of azide ligands would produce additional CO binding sites, since aquo-metHb is rapidly reduced by dithionite. This would lead to an increase in CO binding signal size, which was not observed.

After mixing with dithionite, the dimers and tetramers may reestablish equilibrium under the deoxy conditions. Note that deoxy tetramers require several hours for dissociation, while liganded Hb forms dimers on the order of 1 s; tetramer formation requires about 1 s at a dimer concentration of 1 μM. The parameter of interest here is the dissociation rate of species 21; two cases can be considered. If species 21 is stable as predicted by the symmetry rule (over 100 s for dissociation into dimers), then there could be significant loss of deoxy dimers during 200 ms, but little change in the contribution of species 21 (about 90% of the signal) even at longer times since the final distribution would also be close to binomial. If species 21 does not show an enhanced stability, then one expects a loss of the hybrid due to accumulation of the more stable deoxyHb tetramers; with a tetramer to dimer rate of 1/s, about 10 s is required before deoxyHb accounts for over 50% of the ferrous dimers; one would then expect a time-dependent increase in the fraction of slow CO binding in the stopped-flow experiments. Such an effect was reported for the experiments starting with oxy/CO hybrids, but CO binding reactions were considered as an alternate explanation in that case (*16*).

Experiment 3. Electron Transfer. A modification of the samples during the long 50 h incubation would certainly change the interpretation of the dimer−tetramer equilibrium data, and possibly explain the discrepancy with the ligand binding results. One such transformation through valency exchange has recently been proposed (*6*). To further investigate this effect, we made additional studies of these experiments by incubation of cyano-metHb A with deoxyHb S or deoxyHb C.

Overall Oxidation of Hybrid Mixture. Samples of a mixture of deoxyHbS and cyano-metHb A were incubated for 50 h at final concentrations of 0.4 mM of each Hb (on a heme basis) and an excess of 40 μM KCN. Based on the absorption spectra (before and after incubation) of the mixture, there was little change in the total fraction oxidized. After the incubation, spectra of aliquots before and after addition of KCN showed a small change in absorption corresponding to a transition from aquo- to cyano-metHb for 4% of the total Hb, similar to the result of 3.9% reported by Ackers et al. (*7*). This would imply that about 8% of the ferric hemes were without CN at the end of the incubation. About half of this change was estimated to be CN loss due to the dilution of the aliquot (*7*); however, this assumes that the control sample before dilution was 100% saturated in CN.

Oxidation State of Individual Hbs. After separation of the two types of hemoglobin, the postincubation absorption measurements indicate that the Hb A was about 30% ferrous, while the Hb S decreased to about 70% ferrous form. The Hb A, originally fully oxidized, was therefore partially reduced during the 50 h incubation. The final spectrum of Hb A is shown in Figure 5, with reference cyano-metHb and HbCO spectra, and a linear combination (30% HbCO plus 70% cyano-metHb) of the reference spectra.

In the lower section of Figure 5, the difference spectra are shown. A random mixture of ferric and ferrous hemes would lead to 50% of each, depicted in Figure 5 as the difference (HbCO minus metHbCN) multiplied by 0.5. The difference spectrum for Hb A (after incubation minus preincubation) shows about 30% transition; the opposite is observed for Hb S (preincubation minus postincubation). The gain in ferrous hemes by Hb A is compensated by the loss of ferrous hemes of Hb S.

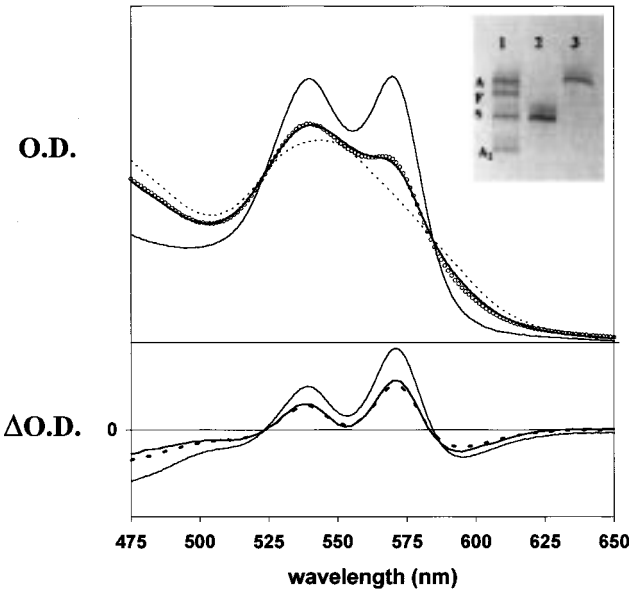

FIGURE 5: Absorption spectra. Samples of cyano-metHb A and deoxyHb S were mixed (final concentrations of each were 400 μM on a heme basis) and incubated for 50 h in buffer A at pH 7.4, 21.5 °C. After incubation, the samples were exposed to CO, and the Hb components A and S were separated. Top section: the initial stock solutions showed the expected spectra for the pure CO (solid line, showing two bands) and CN (dotted line) forms. The small circles show the spectrum of (the initially oxidized) Hb A, after incubation with deoxyHb S and subsequent separation; it shows a significant ferrous CO contribution, compared here with a linear combination of 30% HbCO and 70% cyano-metHb (solid line running through the circles). The IEF (insert) confirms the separation of Hb A (lane 3) and Hb S (lane 2) relative to the controls in lane 1. Difference spectra are shown in the lower section. The largest is the difference spectrum for 50% exchange, that is, half of the difference of the pure forms (HbCO minus cyano-metHb); this would correspond to a complete randomization of the oxidized subunits. The smaller difference spectra are for Hb A (solid line, postincubation minus preincubation) which is the reverse of that for the originally ferrous Hb S (dashed line, preincubation minus postincubation), indicating a valency exchange.

As a second measure of the fraction of ferrous hemes, an aliquot of the samples was exposed to CO and photodissociated; the amplitude of the CO kinetic signal of each type of Hb confirmed the valency exchange of 30% (Figure 6). Hb A (after incubation) showed about 30% of the signal for a fully ferrous HbCO sample, determined on the same sample by addition of dithionite to reduce the ferric hemes. The Hb S sample, after incubation, showed about 70% ferrous form, relative to its control with dithionite (Figure 6).

These results showing a valency exchange are in agreement with those of Shibayama et al. (*6*) and support the hypothesis of an electron transfer between the Hb subunits. The actual transfer rates would depend on whether the electron transfer is only intratetramer and the fraction of hybrid.

In the response to the electron-transfer hypothesis, data were presented showing additional bands in the IEF confirming this mechanism for deoxy/aquo-met hybrids, but the authors insisted that the transfer does not occur for deoxy/CN-met hybrids (*7*). However, their evidence is the presence of only three bands in the IEF of the CO/CN hybrids; this

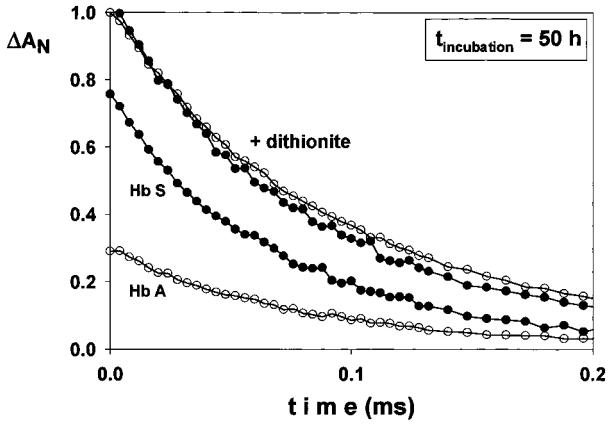

FIGURE 6: Flash photolysis signals. After a 50 h incubation of cyano-metHbA with deoxyHb S, followed by separation of Hb A from Hb S, the Hb A fraction showed about 30% CO binding signal. Similarly, the originally ferrous Hb S lost the same amount of ferrous signal. Note that oxidized subunits show no CO rebinding signal, since the metHb ligands are not photodissociable. As an internal control of the fully ferrous form, both samples were measured again after addition of dithionite.

argument is not valid since CO and met-CN have the same isoelectric point; thus, a single band could consist of both CO and metCN subunits.

Loss of CN. Another observation for the electron-transfer experiments is the loss of CN during the incubation (*6*). This is due to the conflicting requirements of maintaining both deoxy- and CN-bound subunits. Since CN is volatile, purging the sample with nitrogen or argon to remove oxygen will also remove excess CN. Either the gas should contain CN or a solution of CN must be added.

Electron transfer between a deoxy and CN-met subunit implies dissociation of CN due to the low affinity of ferrous iron for CN (*14*). The newly oxidized subunit may then rebind CN; however, since the CN association is very slow (over 15 min at 10 μM free CN), the average time for rebinding may be longer than the time to escape from the solution, which depends on the efficiency of the gas−liquid exchange and sample stirring.

The kinetics of CN binding also demonstrate the difficulty of maintaining Hb permanently bound to CN. Considering the dissociation time constant of less than 6 h, the oxidized subunits would lose their CN ligand many times during the 50 h incubation. Each time the CN reassociation is slow (requiring 1000 s at 10 μM free CN). At high Hb concentrations, the fraction saturation in CN is the critical parameter and corresponds to the fraction of time spent with CN bound. At [metHb] = 1 mM and CN affinity of K_D = 1 μM, one expects about 97% saturation. As mentioned above, the spectra of samples after incubation, before and after addition of KCN, also indicate incomplete CN saturation [92−96% of the ferric hemes with CN depending on the contribution of the dilution step (*7*)]. In other words, the oxidized hemes spend about 3% of the time (over 1 h total) as aquo-metHb, a form where there is agreement that electron transfer occurs (*7*). Electron transfer to aquo-met hemes therefore cannot be neglected. Either the transfer also occurs to CN-met subunits, or the transfer to aquo-met subunits requires less than 1 h.

Conclusion. Based on the dimer−tetramer studies, a shift in the tetramer stability of a factor of 170 was reported for the deoxy/CN-metHb hybrids relative to the other doubly liganded forms (*7*). For the flash photolysis studies with azide or CN (*4*) as ligand, and the stopped-flow studies with azide or CO (*16*) as second ligand, no major shift to a T-like state was observed for CO binding to the asymmetric hybrids. Also no major difference was observed between the symmetric and asymmetric hybrids (*16*, *21*). In the stopped-flow study, the asymmetric hybrid was allowed to "incubate" from 100 ms to a few seconds (*16*) before addition of CO, a time long compared to the physiological oxygen binding processes. Ligand binding studies and theoretical arguments concerning the cooperativity of ligand binding (*20*) have not supported the symmetry rule prediction of a T-like conformation for these hybrids. Cyano-metHb is probably a good model for liganded Hb, but electron transfer which randomizes the location of the oxidized subunits could be an explanation for the near-binomial distribution of asymmetric hybrids at long incubation times, which is the principle evidence for the enhanced stability of the 21 species.

Electron transfer between heme groups is not simply a novel *in vitro* phenomenon. In addition to the electron transport chain of cytochromes, the oxidized subunits of Hb are reduced by metHb reductase (cytochrome b_5), a hemoprotein (*22*). Thus, part of the natural function of Hb is an electron acceptor, in order to maintain a low overall percentage of oxidation. Electron transfer between hemes is thus natural and essential to the correct function of Hb over long periods of time. More studies of this effect (*23*), and new controls on the dimer−tetramer equilibrium studies, seem essential for a complete description of Hb function.

ACKNOWLEDGMENT

We thank G. Caron for technical assistance, and Professors S. Edelstein (University of Geneva), M. Perutz (MRC, Cambridge), and N. Shibayama (Jichi Medical School) for their critical comments.

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BI9805700