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Asymmetric Cyanomet Valency Hybrid Hemoglobin, ($\alpha^{+CN-}\beta^{+CN-}$)($\alpha\beta$): The Issue of Valency Exchange

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ABSTRACT: A new framework for hemoglobin cooperativity was proposed by Ackers and colleagues on the basis of the hyper thermodynamic stability and deoxy (T) quaternary structure of one of diliganded deoxy-cyanomet hybrid hemoglobins, ($\alpha^{+CN-}\beta^{+CN-}$)($\alpha\beta$), studied by hybridization of the equimolar mixture of deoxyhemoglobin and cyanomethemoglobin through a long (70−100 h) dimer exchange reaction [Daugherty et al. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 1110−1114]. Recently, we reported that the published hyperstability of ($\alpha^{+CN-}\beta^{+CN-}$)($\alpha\beta$) is incorrect due to the occurrence of valency exchange between the heme sites of both parental hemoglobins during the long deoxy incubation [Shibayama et al. (1997) *Biochemistry 36*, 4375−4381]. We also noted a difficulty in maintaining both anaerobicity and excess free cyanide of the sample during the long incubation, which led to formation of cyanide-unbound aqometheme in the original deoxyhemoglobin resulting from the electron transfer to cyanometheme. This paper is a response to a recent argument against our work [Ackers et al. (1997) *Biochemistry 36*, 10822−10829]. Ackers et al. have claimed that no appreciable formation of aqomethemoglobin with their methods ensures their sample integrity, based on a supposition that our observed valency exchange may have occurred via *aqometheme*. In this paper, however, we demonstrate that appreciable (>27%) valency exchange really occurs between deoxy and *cyanometheme* sites during 72 h incubation under conditions where both anaerobicity and excess free cyanide of the sample solution are maintained by a continuous flow of humidified N_2 with HCN. This confirms our view that previous experimental data on ($\alpha^{+CN-}\beta^{+CN-}$)($\alpha\beta$) obtained by the long incubations should be subject to reexamination while our earlier estimation of *a lower limit* of free energy of ($\alpha^{+CN-}\beta^{+CN-}$)($\alpha\beta$) (i.e., ≥ -10.1 kcal/mol) by a rapid method (35 min) is still valid. We also suggest a possibility that the T quaternary structure of ($\alpha^{+CN-}\beta^{+CN-}$)($\alpha\beta$) assigned by Ackers and colleagues using the long incubations is an artifact arising from the valency exchange. These results suggest that the putative mechanistic picture for hemoglobin cooperativity inferred from studies on deoxy-cyanomet hybrids is without foundation.

Hemoglobin (Hb)[1] cooperativity arises from less thermodynamic stabilities of the intermediate species relative to the stability of fully deoxyHb or fully oxyHb. Thus, the molecular mechanism of Hb cooperativity must be understood in terms of the nature of the intermediate species. However, there are two difficulties in studying the oxygenation intermediates. First, high cooperativity suppresses relative abundance of the oxygenation intermediates, pre-

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cluding direct study. Second, six asymmetric species cannot be studied in isolation due to dissociation of asymmetric tetramers into dimers with reassociation to form new symmetric tetramers.

In a series of studies by Ackers and his colleagues, these difficulties have been partly circumvented by determining the dimer−tetramer equilibrium constants for all 10 ligation microstates of oxygenation analogue systems using techniques including (i) analytical gel chromatography for fully liganded and symmetric hybrid species (*1, 2*); (ii) stopped-flow kinetics of dimer−tetramer association rates for various deoxyHbs including mutants (*3, 4*); (iii) haptoglobin-trapping kinetics of tetramer−dimer dissociation rates (*3*−*5*); and (iv) cryogenic electrophoresis techniques for determination of the fractional equilibrium populations of the asymmetric species in the presence of the parental species (*6, 7*).

In 1985, Smith and Ackers (*5*) first reported the thermodynamic stabilities of all 10 cyanomet ligation microstates of Hb. Although several other oxygenation analogue systems have been studied in the same laboratory (*8*−*12*), the cyanomet ligation system continues to be the only system in which the ligation microstates have been characterized using structure-sensitive probes (*1, 13*−*16*).

The dimer−tetramer assembly free energies[2] for all 10 cyanomet ligation microstates at pH 7.4, 21.5 °C, were classified into three discrete levels (*14*). The lowest free energy (−14.4 kcal/mol) was found uniquely for deoxyHb, and the highest energy (−8.5 kcal/mol) was observed for six of the 10 species: the fully liganded species (cyanometHb); the two triliganded species [$(\alpha^{+CN-}\beta^{+CN-})(\alpha^{+CN-}\beta)$ and $(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta^{+CN-})$]; and the three diliganded species [$(\alpha^{+CN-}\beta)(\alpha\beta^{+CN-})$, $(\alpha^{+CN-}\beta)_2$, and $(\alpha\beta^{+CN-})_2$]. A third distinct energy level (−11.4 kcal/mol) was found for the two monoliganded species [$(\alpha^{+CN-}\beta)(\alpha\beta)$ and $(\alpha\beta^{+CN-})(\alpha\beta)$] and the one diliganded species [$(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta)$]. The most important feature of this energetic distribution was that the four diliganded species distributed into two distinct energy levels separated by 3 kcal/mol; i.e., the dimer−tetramer association equilibrium constant for $(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta)$ is about 170 times that of the other three diliganded species. Note that this hyperstability of $(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta)$ is a result derived from their experimental finding of approximately binomial (1:2:1) equilibrium distribution of deoxyHb, $(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta)$, and cyanometHb after 70−100 h of deoxy incubation of equimolar mixture of deoxyHb and cyanometHb (*13*).

To determine the nature of $(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta)$ in the third free energy level of particular interest, Daugherty et al. (*13*) have studied the effects of pH and single-site mutations on its assembly free energy. The energetic perturbations induced by pH and single-site mutations were identical for $(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta)$ and deoxyHb. These results were consistent in pointing to the conclusion that $(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta)$ is in a form of the T quaternary structure. This conclusion together with the hyperstability of this key intermediate species led to their proposal of the molecular code mechanism (*13*), which has been somewhat extended so as to include the energetic data of other ligation systems they have studied (*12, 14, 17*).

The molecular code mechanism was, however, incompatible with the widely accepted two-state allosteric model of Monod et al. (*18*). The most remarkable discrepancy between them was that the molecular code mechanism permits strong cooperativity for ligand binding within a dimeric half of a T quaternary tetramer (up to a 170-fold affinity change) (*13*) while the two-state model allows only noncooperative ligand binding within each quaternary structure (*18*). However, direct determination of oxygen equilibrium curves of hemoglobins constrained in the crystals (*19, 20*) or silica gels (*21, 22*) to maintain the T quaternary structure has recently shown that cooperativity within the T structure is absent or very weak, at least in the case of oxygenation. Moreover, Edelstein (*23*) pointed out that the hyperstability of $(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta)$ [thermodynamically equivalent to a hyper (a 170-fold) cooperativity for cyanomet ligation within a half-tetramer] may not be the case for the corresponding oxygenation intermediate, and thus the oxygenation parameters cannot be deduced from the published thermodynamic data on cyanomet ligation system.

Because of the central importance of the thermodynamic stability of $(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta)$ to the mechanistic understanding of Hb cooperativity, we recently reexamined the published binomiality of the hybrid mixture of deoxyHb and cyanometHb by an independent method: A binomial (1:2:1) equilibrium distribution of the equimolar mixture of cyanometHb and fully oxyHb was used as a starting condition, followed by rapid deoxygenation (*24*). Upon deoxygenation for 35 min, the population of the hybrid was decreased to 8.6% of the total, suggesting that the equilibrium distribution of the deoxy-cyanomet hybrid system is fairly far from 1:2:1. Furthermore, we separated by HPLC the two types of parents after long incubations and identified two related problems. One is the occurrence of valency exchange reaction between the parents, which precludes the long deoxy incubations. Second is the problem of evaporation of HCN during necessary deoxygenation and deoxy incubation processes, resulting in appreciable formation of cyanide-unbound aqometheme in the original deoxyHb. Our explanation for the appreciable aqomet formation was that the electron transfer from deoxyheme to cyanometheme would create ferric heme site in the original deoxyHb, which could not be fully saturated with the residual cyanide. This explanation is consistent with the generally held view that the rate of cyanide dissociation from Fe^{3+}Hb is extremely slow. Note that an immediate evaporation of HCN is reasonable for the following reasons: (i) the HCN form is predominant (i.e., >95%) at pH 7.4 [CN^- and H^+ are equilibrated with HCN whose p*K* value is about 9 (*25*)], (ii) the vapor pressure of HCN should be significant at 21.5 °C [the boiling point of HCN is about 26 °C (*26*)], and (iii) to maintain anaerobicity we used an oxygen absorber [A-500HS: a mixture of iron, water, and some inorganic compounds; placed in the gas part of the sample vial (*24*)] that was recently ascertained to absorb HCN gas.

[1] Abbreviations: Hb, hemoglobin; Hb A, human adult hemoglobin; Hb C, mutant hemoglobin C, in which β6-Glu is replaced by Lys; Tris, tris(hydroxymethyl)aminomethane; Na_2EDTA, disodium ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography.

[2] The assembly free energy values (i.e., the free energy changes upon assembly of two dimers into the tetramer) are obtained from the dimer−tetramer association equilibrium constants through the standard relation $\Delta G = -RT \ln K$ (ΔG, free energy; R, gas constant, T, absolute temperature, K, equilibrium constant).

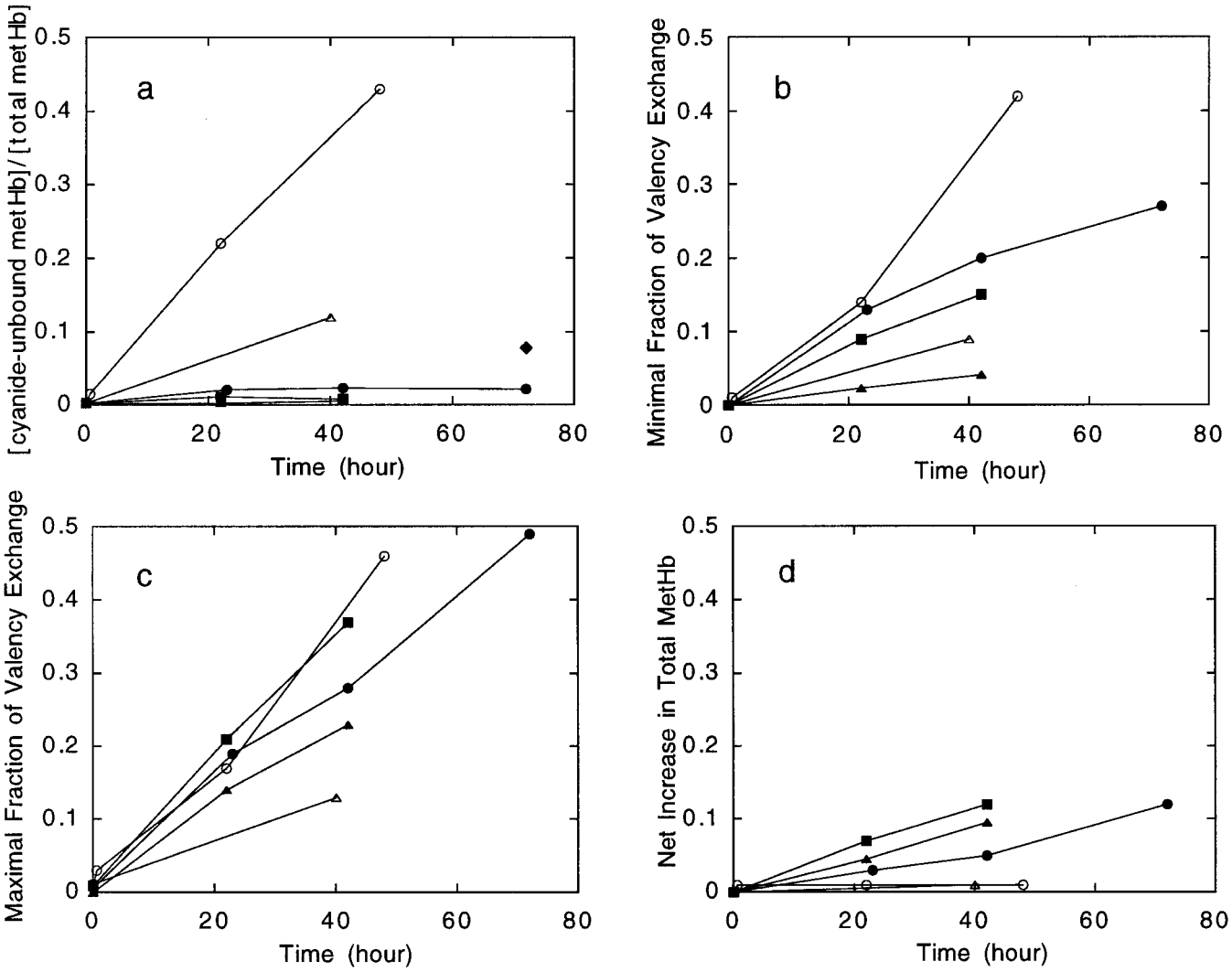

FIGURE 1: Time courses of (a) formation of cyanide-unbound metHb, (b) minimal fraction of valency exchange (formation of Fe^{2+}Hb in the original cyanometHb C), (c) maximal fraction of valency exchange (formation of Fe^{3+}Hb in the original deoxyHb A), and (d) net increase in the total metHb, during the hybridization of equimolar mixture of deoxyHb A and cyanometHb C at 21.5 °C ($t = 0$ is defined as a starting time of deoxygenation). Open circle, pH 7.4 in the absence of free cyanide (taken from ref *24*). Closed circle, pH 7.4 in the presence of 10 μM free cyanide. Closed square, pH 7.4 in the presence of 60 μM free cyanide. Open triangle, pH 9.1 in the absence of free cyanide. Closed triangle, pH 9.1 in the presence of 10 μM free cyanide. A closed diamond in panel a indicates the data at pH 7.4 of Ackers et al. (*17*).

In a recent publication of Ackers et al. (*17*), the following argument against Shibayama et al. (*24*) has been advocated: (i) No appreciable cyanide loss occurs with their methods; (ii) no significant valency exchange (between deoxy and *aqomethemes*) should therefore occur in their sample; (iii) any formation of aqometHb or occurrence of valency exchange (between deoxy and *aqomethemes*) could be readily monitored by their cryogenic isoelectric focusing techniques. However, their reply to our work has been unclear because the above argument has been based on a supposition that our observed valency exchange may have occurred via aqometHb arising from the cyanide dissociation of cyanometHb. This supposition ignores our statement that the aqomet formation should be a result (not a cause) of the valency exchange. Clearly, the real issue in this conflict is whether valency exchange occurs between deoxyheme and *cyanometheme* sites in the hybridizing sample.

In this paper, we present conclusive evidence that appreciable valency exchange occurs between deoxyheme and *cyanometheme* sites of the hybridizing sample. In addition, we demonstrate that most of the previous observations for $(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta)$ by Ackers and colleagues (*1, 2, 5, 13*−*16*) are entirely consistent with behavior predicted from the occurrence of the valency exchange in their samples.

EXPERIMENTAL PROCEDURES

Human blood hemolysate was prepared according to Kilmartin and Rossi-Bernardi (*27*). Hb A and Hb C were purified by ion-exchange chromatography. CyanometHb C was prepared by the method of Shibayama et al. (*24*). The met contents of oxyHb A before hybridization experiments were determined to be 0.6−0.9% of the total by the method of Evelyn and Malloy (*28*). Each experiment was carried out in 0.1 M Tris buffer (at pH 7.4) or 0.1 M glycine buffer (at pH 9.1) containing 0.1 M Cl^- and 1 mM Na_2EDTA in the presence of approximately 10 or 60 μM of free cyanide at 21.5 °C.

In this study, cyanometHb C was first mixed with a stoichiometric amount of oxyHb A in a thermostated reactor at 21.5 °C to give a final concentration of 1.0 mM (on a

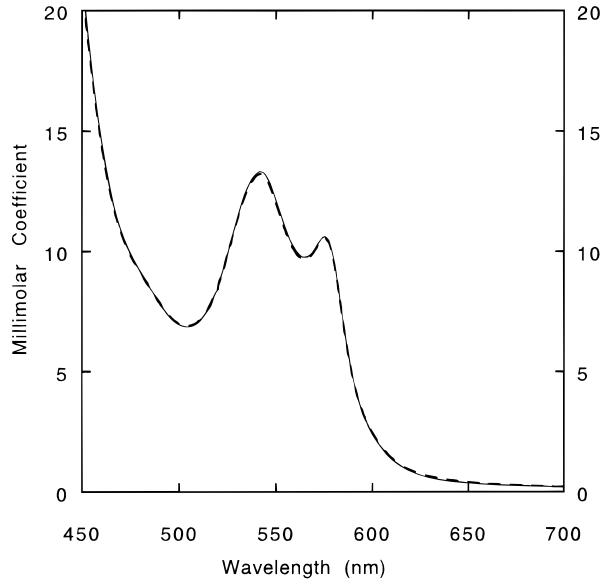

FIGURE 2: Absorption spectra of the equimolar mixture of Hb A and cyanometHb C after 72 h of deoxy incubation. Dashed curve, spectrum immediately after dilution of the Hb mixture into air-equilibrated distilled water without KCN. Solid curve, spectrum after addition of 1 mM KCN. Incubation was carried out in 0.1 M Tris buffer, pH 7.4, containing 0.1 M Cl^-, 1 mM Na_2EDTA, and 10 μM free cyanide, at 21.5 °C, using a continuous flow of humidified N_2 gas with HCN gas. The concentration of each Hb during the incubation was 1 mM on a heme basis.

heme basis) for each parental Hb. Then the hybrid mixture was deoxygenated by a flow of humidified gas mixture of N_2 with HCN, followed by the deoxy incubation using a continuous flow of the same gas mixture. This gas mixture was generated by passage of pure N_2 gas through a bubbler containing either of the following alkaline KCN solutions: 9 mL of 0.5 M KCN in 1 M NaOH with 0.2% sodium dithionite to give approximately 10 μM free cyanide to the sample solution or 50 mL of 0.5 M KCN in 0.3 M NaOH with 0.2% sodium dithionite to give approximately 60 μM free cyanide to the sample solution. The concentration of free cyanide was routinely checked before and after the incubation by using 1 mL of the standard buffer equilibrated with the gas mixture from the outlet of the sample reactor, followed by titration with aqomet myoglobin. It was confirmed that the concentration of free cyanide was not significantly changed during the long deoxy incubations we examined (22−72 h).

Spectrophotometric Analysis of Samples after Incubations. Visible absorption spectra of the equimolar mixtures of deoxyHb A and cyanometHb C at various incubation times were measured after appropriate dilution with air-equilibrated distilled water with and without 1 mM KCN. Immediately after the spectral measurements, the diluted mixtures with 1 mM KCN were subject to HPLC analysis by using DEAE-5PW column (Toso) at room temperature as described previously (*24*). For each parent separated by HPLC, the percentage of hemes in the cyanomet and oxy forms was doubly checked by the spectra and by the capability of CO binding as described in Shibayama et al. (*24*).

RESULTS AND DISCUSSION

Valency Exchange between Deoxyheme and Cyanometheme Sites. In our previous study (*24*), despite the positive verification of occurrence of valency exchange between heme sites of deoxyHb and cyanometHb (open circle in Figure 1b), appreciable formation of aqometHb during the incubations (open circle in Figure 1a) complicated the explanation of the results.[3] In the present study, therefore, we have used a continuous flow of humidified gas mixture of N_2 with HCN to maintain both anaerobicity and excess free cyanide of the sample solution during the incubations. Figure 2 shows the absorption spectra of the equimolar mixtures of Hb A and cyanometHb C after a 72 h deoxy incubation. Solid and dashed curves are the spectra immediately after dilution of the mixture into distilled water with and without excess KCN, respectively. Using a small difference in absorbance at 630 nm between the two spectra, the aqometHb content in the mixture was estimated to be 2.2% of the total metHb (closed circle in Figure 1a).

Table 1: Valency Exchange Reactions between Fe^{2+}Hb and Fe^{3+}Hb

Fe^{2+}Hb	Fe^{3+}Hb	valency exchange	conditions	ref
deoxy	cyanomet	yes	pH 7.4, 21.5 °C	this study
oxy	cyanomet	no	pH 7.4, 21.5 °C	*a*
deoxy	aqomet	yes	pH 7.4, 21.5 °C	*b*
oxy	aqomet	yes	pH 7.18, 37 °C	*c*

[a] Shibayama et al. (*24*). [b] Ackers et al. (*17*). [c] Bunn and Jandl (*34*).

Spectrophotometric analysis of both parents separated by HPLC provide conclusive evidence that appreciable valency exchange occurs between deoxyheme and cyanometheme sites during the deoxy incubation: 27% of the original cyanometHb was reduced to Fe^{2+}Hb during 72 h of incubation in the presence of 10 μM free cyanide (closed circle in Figure 1b) despite a net increase in the metHb level of 12%. Note that the amount of the reduced original cyanometHb gives minimal fraction of the valency exchange (Figure 1b) and that of the oxidized original deoxyHb yields maximal fraction (Figure 1c). Note also here that under aerobic conditions no valency exchange occurs between oxyheme and cyanometheme sites (Table 1). Thus, the exchange during the HPLC separation and spectral measurement can be negligible.

As shown in Figure 1d, net increases in met contents are significantly enhanced by the presence of HCN gas (Figure 1d). This is probably due to the effects of free cyanide on oxidation, because of the fact that 61 μM free cyanide significantly accelerates overall oxidation (closed square in Figure 1d). Interestingly, the valency exchange is significantly slowed at pH 9.1 (Figure 1b). Also, significant overall oxidation is observed at pH 9.1 in the presence of HCN gas (Figure 1d), presumably due to large fraction of CN^- form at this pH.

A recent argument of Ackers et al. (*17*) against Shibayama et al. (*24*) appears to be beside the issue since they have dealt only with a possibility of electron transfer to *aqometheme* (neglecting the possibility to *cyanometheme*). Note that their claim regarding the ability of their isoelectric focusing

[3] Quantitative comparison of the amounts of the valency exchange and aqometHb verified the validity of our statement that the aqomet formation is a result (not a cause) of the valency exchange. If only aqometHb were the electron acceptor for deoxyHb, formation of aqometHb should always exceed the amount of the valency exchange. However, this was not the case for our previous results (*24*) (also see Figure 1).

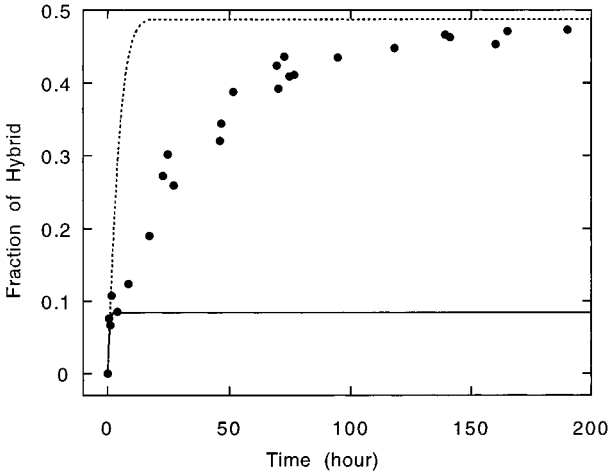

FIGURE 3: Comparison of observed and simulated results for time course for hybrid formation through dimer exchange reaction of the equimolar mixture of deoxyHb and cyanometHb at pH 7.4, 21.5 °C. Closed circle, observation of Huang and Ackers (*2*). Dotted line, simulation assuming published tetramer−dimer dissociation rate constants of deoxyHb (ref *3*), cyanometHb (ref *5*), and $(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta)$ (ref *5*). Solid line, simulation assuming the published tetramer−dimer dissociation rate constants of deoxyHb (ref *3*), cyanometHb (ref *5*), and $(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta)$ (ref *24*). A consensus dimer−tetramer reassociation rate constant of 1.1×10^6 M^{-1} s^{-1} (*4*) is adopted for the simulations.

techniques to detect the occurrence of valency exchange is only valid for aqometheme, not for cyanometheme, since cyanometHb and COHb (as well as any $Fe^{2+}CO$-cyanomet hybrid species) migrate to the same isoelectric point. Clearly, their studies do not provide evidence against occurrence of the valency exchange between deoxyheme and *cyanometheme* sites in their samples. It is worth pointing out that their observed aqometHb content in the hybrid mixture after a 72 h incubation (closed diamond in Figure 1a) was significantly larger than our present value (closed circle in Figure 1a) (i.e., 7.8% vs 2.2% of the total met Hb).[4]

Time Course of Formation of $(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta)$ *Suggests the Occurrence of Valency Exchange.* Figure 3 shows a time course of formation of $(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta)$ during hybridization of equimolar mixture of cyanometHb and deoxyHb reported by Huang and Ackers (*2*) (replotted from their Figure 5). Interestingly, their observed time course is biphasic. The hybrid was formed (7−11%) within 1 h of incubation, and then the population of the hybrid increased slowly with time and approached a plateau population of 45−48% in approximately 100 h (*2*).

We would like to point out here that (i) the rate-limiting step of the hybrid formation is slow dissociation of deoxyHb with a half-time of 7.7 h (*3*), (ii) the concentration of dissociated unliganded (deoxy) dimer is much lower than that of dissociated liganded (cyanomet) dimer, and (iii) the values of the dimer−tetramer reassociation rate constants are believed to be little affected by the ligation states of the dimers [i.e., a consensus value of 1.1×10^6 M^{-1} s^{-1} is generally accepted (*4*)]. From these points, it is reasonable to expect that the majority of the dissociated unliganded dimer would immediately reassociate with the liganded dimer and make the asymmetric hybrid tetramer. For example, the time required for establishment of a binomial (1:2:1) distribution can be approximated by the half-time of the dissociation of deoxyHb (i.e., 7.7 h).

To further confirm this point, a simulation was carried out using their published tetramer−dimer dissociation rate constants of cyanometHb (*5*), deoxyHb (*3*), and $(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta)$ (*5*). As expected, their observed time course is too slow to be modeled by their own results (dotted line in Figure 3). A similar simulation was carried out except that the dimer to tetramer assembly free energy for $(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta)$ is assumed to be −10.1 kcal/mol, which is our estimated lower limit of free energy for $(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta)$ (*24*) (also see below). To a first approximation, this calculation can simulate the rapidly hybridizing component in their observation (solid line in Figure 3). Therefore, the most straightforward explanation for the observed biphasic time course is that the rapidly hybridizing component is attributed to the formation of $(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta)$ reflecting true hybridization equilibrium while the slow phase is an artifact arising from the valency exchange. This explanation is reinforced by experimental evidence that the rate of the valency exchange (closed circle in Figure 1b) is similar to that of the observed slow phase in Figure 3. We should emphasize here that the valency exchange plays a role in randomizing the oxidized subunits in the samples and thus finally brings the system close to a binomial (1:2:1) distribution (a nearly random mixture at valency exchange equilibrium).

Relevance of Our Earlier Estimation of an Upper Limit of the Thermodynamic Stability of $(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta)$. Apparently the protocol to avoid the effects of the valency exchange requires short deoxy incubation (Table 1). Therefore, in our previous study (*24*) we used a binomial (1:2:1) equilibrium distribution of cyanometHb, $(\alpha^{+CN-}\beta^{+CN-})$-$(\alpha^{O2}\beta^{O2})$, and oxyHb as a starting condition, followed by rapid (35 min) deoxygenation using an oxygen-scavenging enzyme system. It was found that the population of the hybrid was reduced from the original 47% of the total in the presence of oxygen to 8.6% in its absence. At that time, neither significant valency exchange nor aqomet formation was detected. We thus considered the 8.6% as an upper limit for the population of the hybrid at equilibrium, since equilibrium may have not been reached within the 35 min

[4] The following points should be noted in the data of Ackers et al. (*17*). (i) Their expression for aqometHb content was the percentage of aqometHb in the total Hb (including Fe^{2+}Hb) while our expression is the percentage of aqometHb in the total metHb. Thus, factor 2 is necessary for comparison with our results. (ii) To estimate the amount of cyanide dissociated during sample dilution and spectral measurement, they evaluated aqometHb content of an equimolar mixture of oxyHb and cyanometHb (without incubation) after dilution into cyanide-free distilled water. The estimated aqometHb content in this control experiment was 4.6% of the total metHb, and then this value was subtracted from the sample value for correction (i.e., 7.8% − 4.6% = 3.2%). However, this approach appears to be invalid because of the fact that their control value (i.e., 4.6%) is larger than our sample value (2.2%). Their large control value is likely to reflect aqometHb originally present in their control mixture. (iii) They used a gas mixture of N_2 with HCN, which was generated by passage of N_2 through the standard buffer (probably with 10 μM KCN) for deoxygenation of the parents [see Methods in Ackers et al. (*17*); not described in their previous publications]. The deoxygenated parents were mixed together, and an oxygen-scavenging enzyme system was added. Then, the samples were transferred into vials and incubated. However, even using the above procedures, it would be difficult to maintain 10 μM free cyanide during the long incubation. The main reason for this is that unavoidable slight overall oxidation of Hb at 1 mM (in heme) would consume free cyanide during the incubation. Also, the enzyme system contains 0.3−0.6 mg/mL catalase (corresponding to 5−10 μM heme), which binds cyanide.

(*24*). This observation was in marked contrast to the report of an approximately binomial distribution created under the long incubation conditions (*2, 13, 14*). The discrepancy corresponds to at least 10-fold reduction in the dimer-tetramer assembly equilibrium constant[5] of $(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta)$ (or at least 1.3 kcal/mol higher assembly free energy compared to the published value of −11.4 kcal/mol).

Nevertheless, Ackers et al. (*17*) have asserted that our observed 8.6% cannot be used as even an upper limit for the equilibrium population of the hybrid since the time dependence of the concentration of the hybrid (upon deoxygenation of the hybrid mixture of cyanometHb and oxyHb) was nonmonotonic (*29*) (meeting abstract; details unknown). However, their assertion neglects the effects of the valency exchange on the concentration of the hybrid species. We should emphasize that their claimed nonmonotonicity is reasonably attributed to the effect of the valency exchange: upon deoxygenation of the hybrid mixture the fractional population of the hybrid was rapidly decreased to approach the true equilibrium value, and then it was gradually increased to a binomial value by the relatively slow valency exchange. This view is consistent with the simulation results of Figure 3 (as discussed above). Therefore, our earlier estimation of a lower limit of free energy for $(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta)$ [i.e., ≥ -10.1 kcal/mol (*24*)] is still valid.

Previous Data Supporting the Hyperstability of $(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta)$. Smith and Ackers (*5*) first reported the hyperstability of $(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta)$ using the haptoglobin-trapping kinetics of $(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta)$ in the presence of the parents, deoxyHb and cyanometHb. In that kinetic experiment, three distinct phases were observed. On the basis of the analysis of a small intermediate kinetic phase with a rate constant of 3.9×10^{-3} s^{-1}, assembly free energy for $(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta)$ was assigned to be −11.4 kcal/mol, in close agreement with the value later determined by the equilibrium method. This agreement has been the strongest evidence supporting the hyperstability of $(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta)$. However, it should be noted that in that study a similar intermediate kinetic phase with a rate constant of 2.4×10^{-3} s^{-1} was observed for $(\alpha^{+CN-}\beta)(\alpha\beta^{+CN-})$ in the presence of the parents, $(\alpha^{+CN-}\beta)_2$ and $(\alpha\beta^{+CN-})_2$, leading to incorrect assignment for the free energy of $(\alpha^{+CN-}\beta)(\alpha\beta^{+CN-})$. Considering the valency exchange, the observed intermediate kinetic phases for both asymmetric species can be explained by small amounts of contaminating monoliganded species, $(\alpha^{+CN-}\beta)(\alpha\beta)$ and $(\alpha\beta^{+CN-})(\alpha\beta)$, with approximately halfway free energy between the values of deoxyHb and cyanometHb, i.e., those could be created by the valency exchange among the heme sites of the parents during the necessary preincubation in both cases.

Doyle and Ackers (*15*) measured the oxygen dissociation equilibrium curves of the mixture of oxyHb and a large excess of cyanometHb. These authors reported that the oxygen binding by vacant sites of $(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta)$ shows very high cooperativity with a Hill coefficient of 1.9−2.0, consistent with the hyperstability of $(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta)$. Since they used the mixture of oxyHb and cyanometHb as a starting condition and the measurements are relatively short (∼1 h), no significant valency exchange is expected to occur in that study. However, their estimation of the fraction of the hybrid was based on the incorrect hyperstability of $(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta)$ [i.e., approximately 95% of ferrous hemes was assumed to be carried by the hybrid when 4.65 mM cyanometHb and 0.169 mM deoxyHb were used (*15*)]. Instead, if our estimated lower limit of the free energy of $(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta)$ (i.e., −10.1 kcal/mol) is adopted, the percentage of ferrous hemes in the hybrid is less than 50%, and the remainder (>50%) is carried by Hb A. Therefore, their claimed high cooperativity of the hybrid should be due merely to their underestimation of the fractional population of highly cooperative Hb A in the hybrid mixture.

Previous Data Suggesting the T Quaternary Structure of $(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta)$. Ackers and colleagues have attempted to assign the quaternary structure of the key intermediate, $(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta)$ (*1, 13*−*16*). In reaching their conclusion that $(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta)$ is in a form of the T quaternary structure, however, they have neglected considerable effects of the valency exchange on their experimental results. We demonstrate here that most of the previous observations for $(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta)$ are entirely consistent with behavior predicted from occurrence of the valency exchange.

Daugherty et al. (*1, 13*) reported that the pH dependence of the assembly free energy of $(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta)$ resembles that of deoxyHb and contrasts sharply with that of fully liganded species, suggesting the T quaternary structure of $(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta)$. According to Daugherty et al. (*1*), the fractional equilibrium population of $(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta)$ in the hybrid mixture became smaller as pH raised in the high pH region (8.0−9.5), but was a nearly binomial value (45−50% of the total) below pH 8.0. As seen from Figure 1b, the valency exchange reaction is slowed at pH 9.1. Moreover, Daugherty et al. (*1*) adopted short incubations in the high pH region (e.g., only 2.25 h incubation at pH 9.5) probably because they considered the pH dependence of the tetramer−dimer dissociation rate of deoxyHb. Therefore, it is extremely likely that their observed instability of $(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta)$ in the high pH region reflects the true nature of this species while the hyperstability at neutral pH is an artifact arising from the valency exchange. Note that at pH 9.5 the energy of $(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta)$ is almost same as that of the fully liganded species (cyanometHb) and there is only 0.84 kcal/mol energy difference between the four diliganded species (*1*).

Daugherty et al. (*13*) and LiCata et al. (*16*) measured the dimer−tetramer equilibrium constants for a series of single-mutant $(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta)$, each bearing a single modified amino residue in an α or β subunit across the interface from the cyanomet-liganded dimer (denoted herein as CD*; C and D imply the cyanomet dimer and deoxy dimer, respectively, and the symbol * indicates the existence of a single modified residue on the dimer). The mutationally induced perturbations in the assembly free energy for $(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta)$ (i.e., $\Delta G_{CD^*} - \Delta G_{CD}$ where ΔG_X is the free energy for a tetrameric species X relative to the dissociated dimers) were almost identical to those for deoxyHb ($\Delta G_{DD^*} - \Delta G_{DD}$) but were radically different from the corresponding perturbations found for cyanometHb ($\Delta G_{CC^*} - \Delta G_{CC}$), leading to their conclusion that $(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta)$ has the T quaternary

[5] The percentage of the hybrid observed (8.6%) can be converted to a dimer-tetramer association equilibrium constant of 2.9×10^7 M^{-1} through eq 4 of LiCata et al. (*7*). This equilibrium constant was approximately one-tenth of the value reported by Ackers and colleagues [i.e., 2.8×10^8 M^{-1} (*14*)].

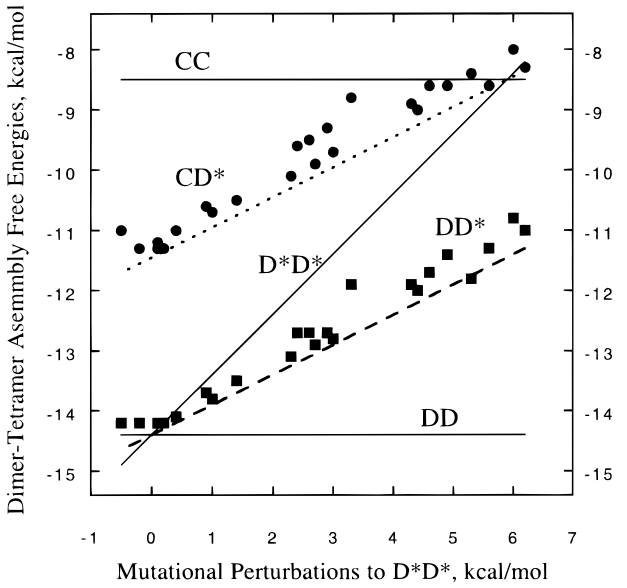

FIGURE 4: Plots of the published dimer−tetramer assembly free energy values for single-mutant $(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta)$ (CD*, closed circle) and single-mutant deoxyHb (DD*, closed square) against the energetic perturbations induced by the individual mutations to their common parental molecule, naturally occurring double-mutant deoxyHb (D*D*). The free energy data on D*D* are taken from Turner et al. (*4*), and those on CD* and DD* are from LiCata et al. (*16*). The diagonal solid line indicates the free energies for D*D*, and the upper and lower horizontal lines show the free energies for unmodified cyanometHb (CC) and unmodified deoxyHb (DD), respectively. Dotted line with a slope of 0.5 represents the mean energies of CC and D*D*. Dashed line with a slope of 0.5 indicates the mean energies of DD and D*D*.

structure. However, it is reasonable to expect that artifacts arising from the valency exchange between CC and D*D* may have seriously affected the free energy determination on CD*.

To further clarify the reason why apparent correlation between the free energy values of CD* and DD* could emerge from their data, the experimental free energy values reported for CD* (closed circle) and DD* (closed square) by LiCata et al. (*16*) are plotted against $\Delta G_{D*D*} - \Delta G_{DD}$ [i.e., the perturbations induced by the individual mutations to the common parental molecule, D*D*, taken from Turner et al. (*4*)] in Figure 4. The diagonal solid line with a slope of unity represents the energies for the common parent, D*D*, and the upper and lower horizontal solid lines show the free energies for the other parental molecules, CC and DD, respectively. In Figure 4, the parental mean energies for CD* and those for DD* are also drawn in parallel by dotted and dashed straight lines, respectively. As expected from occurrence of the valency exchange between CC and D*D*, the free energy for CD* is correlated with the parental mean value. Also, the free energy for DD* is correlated with the parental mean value. This correlation reflects the small nonadditivity of mutational effects as suggested by LiCata et al. (*7*), although the nonadditivity was established as significant in about half of the same series of mutants (i.e., <0.6 kcal/mol in all but Hb St. Claude). Due to the above two independent factors, CD* and DD* behave alike in response to the mutations[6] regardless of the quaternary structure of $(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta)$.

Molecular Code Mechanism. Ackers and colleagues proposed a new framework for Hb cooperativity (*13, 14, 17*) in which ligand binding onto subunits within both the symmetry-related half-tetramers specifically promotes T−R quaternary transition (i.e., the symmetry rule for quaternary switching) while the ligation at only a single half-tetramer generates positive conformational free energy of tertiary constraint without triggering the global T−R transition (i.e., cooperativity within the T quaternary structure). This molecular code mechanism was originally deduced from the T quaternary structure and hyperstability of $(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta)$ (*13*). Although other ligation systems have been so far studied in the same laboratory, the experimental data on $(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta)$ have provided the strongest available evidence supporting the molecular code mechanism. Of particular importance is the fact that the relevance of the symmetry rule depends solely upon the T quaternary structure of $(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta)$, because of a lack of available *structural data* on the corresponding diliganded species in other ligation systems.

As discussed in the above section, the published T quaternary structure of $(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta)$ is likely to be an artifact arising from the valency exchange. In addition, a recent CO rebinding kinetics study on the equilibrium mixture of HbCO and a large excess of cyanometHb has suggested that the photoproduct, $(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta)$, could not be more than 50% T state (*30*). Moreover, previous data of Ackers and colleagues do not provide conclusive evidence that the other three diliganded deoxy-cyanomet hybrids are in a pure R state. Rather, many experiments carried out in other laboratories have suggested the presence of allosteric equilibria in $(\alpha^{+CN-}\beta)_2$ and $(\alpha\beta^{+CN-})_2$ (*31*−*33*). Overall, there is no positive evidence that $(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta)$ especially favors the T quaternary structure compared to the other diliganded species. Therefore, the validity of the symmetry rule appears to be extremely doubtful. Finally, our revised free energy for $(\alpha^{+CN-}\beta^{+CN-})(\alpha\beta)$ (i.e., ≥ -10.1 kcal/mol) as well as the ambiguity of the previous quaternary assignment indicates that cooperativity within the T quaternary structure is, if any, not so strong as emphasized by Ackers et al. (*14*).

[6] One can see that the deviation of the free energy for CD* from its parental mean value is correlated with that found for DD*. This correlation is not surprising in view of the fact that the small nonadditivity of mutational perturbations deviates (reduces) the fractional population of CD* from a binomial value (50%) even at valency exchange equilibrium (where the distributions of ferric hemes within its parents, CC and D*D*, would be random). Note that such mutational effects are negligible when Hb C (or S) is used as one of the parents (the other is Hb A) because Hb C (or S) and Hb A have identical dimer−tetramer equilibrium constants, independent of the ligation (*6, 7*).

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