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Reexamination of the Hyper Thermodynamic Stability of Asymmetric Cyanomet Valency Hybrid Hemoglobin, ($\alpha^{+CN^-}\beta^{+CN^-}$)($\alpha\beta$): No Preferentially Populating Asymmetric Hybrid at Equilibrium

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ABSTRACT: It has been reported that hybridization of the equimolar mixture of cyanomethemoglobin and deoxyhemoglobin through dimer exchange reaction results in establishment of an approximately binomial (1:2:1) equilibrium distribution of these parental hemoglobins and their hybrid molecule, ($\alpha^{+CN^-}\beta^{+CN^-}$)($\alpha\beta$), within several days under anaerobic conditions at pH 7.4, 21.5 °C, leading to a hyper (*i.e.*, about 170 times) thermodynamic stability of ($\alpha^{+CN^-}\beta^{+CN^-}$)($\alpha\beta$) relative to the stability of the other diliganded species at pH 7.4, 21.5 °C [Daugherty, M. A., Shea, M. A., Johnson, J. A., LiCata, V. J., Turner, G. J., & Ackers, G. K. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 1110−1114]. To examine whether the published "binomiality" for this deoxy−cyanomet hybrid system really reflects the thermodynamic stability of ($\alpha^{+CN^-}\beta^{+CN^-}$)($\alpha\beta$), we used a binomial (1:2:1) equilibrium distribution of the equimolar mixture of cyanomethemoglobin and fully oxygenated hemoglobin as a starting condition, and then this system was rapidly deoxygenated. We found that the relative population of the hybrid was reduced to 8.6% of the total upon deoxygenation. It was also found that the hybridization experiment under anaerobic conditions was not allowed to continue for a long time due to a valency exchange reaction between deoxy and cyanomet derivatives. For instance, a 48 h incubation resulted in the oxidation of 44% of Fe^{2+} to Fe^{3+} hemes in the original deoxyhemoglobin and the reduction of 42% of Fe^{3+} to Fe^{2+} hemes in the original cyanomethemoglobin. These results suggest that a real distribution of the deoxy−cyanomet hybrid system at equilibrium is fairly far from 1:2:1 (binomial distribution), and the thermodynamic stability of ($\alpha^{+CN^-}\beta^{+CN^-}$)($\alpha\beta$) is less than one-tenth of the hyperstability previously reported. In addition, most of the previous results on deoxy−cyanomet valency hybrids placed under long anaerobic conditions should be subject to reexamination due to possible valency exchange reactions.

Even though human adult hemoglobin (Hb A)[1] is the most thoroughly studied example of an allosteric protein, the molecular mechanism of cooperative oxygenation of Hb is not fully understood. Since Hb cooperativity arises from less thermodynamic stabilities of the intermediate species

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[1] Abbreviations: Hb A, human adult hemoglobin; Hb, hemoglobin; Hb C, mutant hemoglobin C, in which β6-Glu is replaced by Lys; XL-($\alpha^{Ni}\beta^{Ni}$)($\alpha^{Fe}\beta^{Fe}$), asymmetric hybrid Hb in which the $\alpha1\beta1$ dimer containing Ni^{2+} protoporphyrin IX and the complementary $\alpha2\beta2$ dimer containing ferrous protoporphyrin IX were cross-linked between Lys-82β1 and Lys-82β2 by reaction with bis(3,5-dibromosalicyl) fumarate, Tris, tris(hydroxymethyl)aminomethane; Na_2EDTA, disodium ethylenediaminetetraacetic acid; IHP, inositol hexaphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography.

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relative to that of fully deoxyHb or fully oxyHb, an important step in understanding the molecular mechanism of the cooperativity is the characterization of intermediate species in the course of oxygenation. However, because of the cooperativity, the oxygenation intermediates are present in low concentrations at equilibrium so that stable models for the intermediates are indispensable for the study of Hb cooperativity.

Deoxy−cyanomet valency hybrid Hbs have been most widely used as models for oxygenation intermediates. Because of the chemical similarity of the $Fe^{3+}CN^-$ and $Fe^{2+}O_2$ adducts, it has been generally accepted that deoxy−cyanomet valency hybrid Hbs are useful models for oxygenation intermediates. Symmetric deoxy−cyanomet valency hybrids, in which cyanomet hemes are located exclusively in the α or the β subunits, have been extensively studied (Banerjee & Cassoly, 1969; Ogawa & Shulman, 1972; Cassoly & Gibson, 1972). On the other hand, the properties of the other six asymmetric hybrids, including mono- and triliganded species, are less clear, because dimer−dimer rearrangement may lead to a complex hybrid equilibrium and this makes it difficult to characterize asymmetric species in isolation.

In a series of pioneering studies of Ackers and his colleagues (1992), this difficulty has been partly circumvented by determining the dimer−tetramer equilibrium constants for all ligation microstates of Hb. Briefly, (1) the dimer−tetramer equilibrium constants for deoxyHbs were determined by the haptoglobin kinetics method (Ip & Ackers, 1977); (2) the tetramer−dimer dissociation rates of asymmetric hybrid Hbs were kinetically resolved from haptoglobin kinetics time courses for the hybrid mixtures in the presence of the parental Hbs (Smith & Ackers, 1985); (3) the fractional populations of asymmetric hybrids and their parents at equilibrium were determined by quenching at low temperature and a subsequent cryogenic isoelectric focusing procedure (LiCata et al., 1990; Daugherty et al., 1991). Combining these methods with independent determinations of the dimer−tetramer equilibrium constants for fully liganded Hb and symmetric hybrid Hbs by a conventional gel chromatography, it has become feasible to investigate the relative thermodynamic stabilities of all hybrid species with reference to the dissociated $\alpha\beta$ dimers.

In 1985, Smith and Ackers (1985) first reported the thermodynamic stabilities of all 10 ligation cyanomet microstates of Hb. Although several metal−iron hybrid Hbs have been later studied in the same laboratory (Smith et al., 1987; Speros et al., 1991; Huang & Ackers, 1996; Huang et al., 1996a, b), the cyanomet ligation system continues to be the most extensively studied system, in which all ligation microstates have been energetically and structurally characterized by a variety of experiments (Smith & Ackers, 1985; Perrella et al., 1990, 1994; Daugherty et al., 1991, 1994; Ackers et al., 1992; Doyle & Ackers, 1992; LiCata et al., 1993; Huang & Ackers, 1995; Jayaraman & Spiro, 1995; Huang et al., 1996c).

The dimer−tetramer assembly free energies for all 10 cyanomet ligation microstates at pH 7.4, 21.5 °C were classified into three discrete levels. 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 ten 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; *i.e.*, the dimer−tetramer 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 the result derived from an approximately binomial distribution of [cyanometHb]:[$(\alpha^{+CN^-}\beta^{+CN^-})(\alpha\beta)$]:[deoxyHb] ≈ 1:2:1 found in the deoxy−cyanomet hybrid system at equilibrium: the "binomiality" implies that the hybrid has the mean stability of both parents.

Using as a reference species the dissociated $\alpha\beta$ dimers which are expected to bind ligands noncooperatively, the ligand binding free energies for the cyanomet system can be estimated as the difference in the assembly free energy between each species and fully deoxyHb. The striking result is that the $\alpha1-\beta1$ (or $\beta1-\alpha1$) ligation pathway shows hypercooperativity represented by a 170-fold affinity change, due to the hyperstability of $(\alpha^{+CN^-}\beta^{+CN^-})(\alpha\beta)$, whereas the other $\alpha1-\alpha2$, $\beta1-\beta2$, and $\alpha1-\beta2$ ligation pathways are essentially noncooperative (Daugherty et al., 1991).

Because of the central importance of the thermodynamic stability of $(\alpha^{+CN^-}\beta^{+CN^-})(\alpha\beta)$ to the mechanistic understanding of Hb cooperativity, we have reexamined the published binomiality of this deoxy−cyanomet hybrid system by using an independent method. Hybridization of cyanometHb with deoxyHb through dimer exchange usually requires a long-time deoxy incubation to approach its equilibrium (*i.e.*, approximately 70 h) since the slow tetramer−dimer dissociation of deoxyHb is the rate-limiting step of hybridization (Ip & Ackers, 1977). Such long-time deoxy incubations are the most troublesome part of the previous experiments. In this study, therefore, we have designed a hybridization experiment without a long incubation: binomial (1:2:1) equilibrium distribution of the oxy−cyanomet hybrid system was used as a starting condition, and then this hybrid system was rapidly deoxygenated. This method requires a relatively short time (<1 h) to determine whether or not the binomiality holds in the deoxy−cyanomet hybrid system at equilibrium: if the binomial distribution were preserved upon deoxygenation, the binomiality of the deoxy−cyanomet hybrid system would be validated. In this reexamination, we found that the population of the hybrid was markedly reduced upon deoxygenation, suggesting that the equilibrium distribution of the deoxy−cyanomet hybrid system is fairly far from 1:2:1 (binomial distribution). In addition, the deoxy incubation was not allowed to continue for long time, since a considerable valency exchange reaction occurred between deoxy and cyanomet derivatives during the incubation.

EXPERIMENTAL PROCEDURES

Human blood hemolysate was prepared according to Kilmartin and Rossi-Bernardi (1971). Hb A and Hb C were purified by ion-exchange chromatography. CyanometHb was prepared by the method of Daugherty et al. (1994) with a minor modification. To remove ferrocyanide, which is bound tightly by Hb, we adopted the method of Benesch et

al. (1964) followed by passage through a column of Amberlite MB-3 (Rohm & Hass). The removal of ferrocyanide is essential for the present study, because the residual ferrocyanide could be an electron mediator which accelerates valency exchanges. The met contents of oxyHb A before hybridization experiments were determined to be 1.1% of the total by the method of Evelyn and Malloy (1938). Experiments were carried out in 0.1 M Tris buffer, pH 7.4, containing 0.1 M Cl^-, 1 mM Na_2EDTA, and 10 μM or 1 mM KCN, at 21.5 °C, analogous to the standard conditions of Ackers et al. (Smith & Ackers, 1985).

Hybridization Experiments for Preparing ($\alpha^{+CN^-}\beta^{+CN^-}$)-($\alpha\beta$). In this study, cyanometHb C was first mixed with a stoichiometric amount of oxyHb A to attain a binomial (1:2:1) equilibrium distribution rapidly; the dimer exchange of the liganded tetramers occurs on the order of seconds (Smith & Ackers, 1985; Marden et al., 1996), and then the hybrid mixtures were deoxygenated by using pure N_2 gas. The final concentration of each parental Hb in the mixture was 0.8−1.4 mM on a heme basis. Anaerobic incubations at 21.5 °C were carried out in one of the following two ways: (1) When the times for incubations were shorter than 1 h, the initial concentration of free KCN was set to 10 μM. The sample was first deoxygenated by using a flow of humidified pure N_2 for 25 min, followed by adding deoxygenated glucose oxidase (Sigma), bovine catalase (Sigma), and β-D-glucose (Sigma) under Ar atmosphere. The final concentrations of glucose oxidase, bovine catalase, and β-D-glucose were 1.5 mg/mL, 0.75 mg/mL, and 0.5%, respectively. Note that this oxygen-scavenging enzyme system cannot be used at 1 mM KCN, because catalase would be inactivated by cyanide. (2) When the times for incubations were longer than 1 h, the initial concentration of free KCN was set to 1 mM. Oxygen Absorbing System (A-500HS) purchased from ISO (Yokohama, Japan) was placed in the gas part of a glass vial which contained the oxy sample in its bottom, and then the atmosphere in the vial was replaced with pure N_2. To further maintain anaerobicity, the sample vial was sealed in a small K-nylon/polyethylene bag (ISO) with another Oxygen Absorbing System and Oxygen Indicator (ISO). The anaerobicity in this bag (<0.1% O_2) could be simply monitored by the color of the indicator with eye.

Reduction of Hb under Anaerobic Conditions. After the incubation, the dissociation of the tetramers in each sample was rapidly quenched by reduction of cyanomet hemes with dithionite. By means of a gas-tight syringe, 20−25 μL of the sample was mixed with 200 μL of pH 6.45 quench buffer containing 0.5% sodium dithionite, 10 mM phosphate, and 2 mM IHP in a thermostated reactor at 21.5 °C. The quench buffer is continuously stirred under Ar atmosphere. Finally, nitrogen-saturated 10 μL of 2 M Tris was added to the sample 1 min after quenching, to raise the pH above 8 for subsequent anaerobic isoelectric focusing. Note that this method is also effective for quenching the oxy−cyanomet hybrid system because the oxy derivatives can be rapidly reduced along with the cyanomet ones.

Anaerobic Isoelectric Focusing. Anaerobic isoelectric focusing in cylindrical gels was carried out according to the method of Bunn (1981) with some modifications. We used slightly wider Pyrex glass tubes (5 mm i.d. and 100 mm long), containing 4% polyacrylamide (w/v), 0.16% bis(acrylamide) (w/v), and 2.5% pH 6−8 ampholyte. Acrylamide and bis(acrylamide) were purchased from Nacalai (Kyoto, Japan). Ampholyte was purchased from Pharmacia (Ampholine pH 6−8). The gels were poured to a height of 75 mm in the glass tubes. The anolyte and the catholyte used were N_2-saturated 0.1 M HEPES and N_2-saturated 0.01 M 2-aminoethanol with 1.4 mM dithionite, respectively. Anaerobicity is maintained by continuously passing a stream of pure N_2 into the catholyte, on top. The apparatus was kept at 1 °C with a Haake F3 water bath. Gels were prerun for 30 min prior to loading samples. The samples (up to 150 μL) containing about 0.5% dithionite were then loaded onto the cylindrical gels. Both the prerun and the run itself were performed at 500 V. About 60 min after loading samples, the Hb bands had usually focused. We confirmed that IHP binds tightly to deoxyHbs during our isoelectric focusing procedure and modifies their p*I* values more anodic. The image scanning was made on a color photograph of the glass tubes containing the gels by using an EPSON GT6500 image scanner. The quantification of each band was carried out on a Macintosh personal computer with NIH Image (a public domain image processing and analysis program). Band-to-band color standardization is not necessary, because all species are in fully deoxy forms in our anaerobic isoelectric focusing gels.

Spectrophotometric Analysis of Hybrid Mixtures and Both Parental Hbs. Visible absorption spectra of the equimolar mixtures of deoxyHb A and cyanometHb C at various incubation times (0−48 h) were measured after appropriate dilution with air-equilibrated distilled water without and with 1 mM KCN. Immediately after the spectral measurements, the diluted mixtures with 1 mM KCN were subject to further HPLC analysis by using DEAE-5PW column (Toso) at room temperature. The column was pre-equilibrated with buffer A (0.015 M Tris acetate buffer with 10 μM KCN; pH titrated to 8.1 by adding acetate). Elution was performed by using a gradient from 100% buffer A to 70% buffer A and 30% buffer B (0.015 M Tris acetate buffer with 0.2 M sodium acetate and 10 μM KCN; pH titrated to 7.75 by adding acetate) in 30 min at a flow rate of 1 mL/min. Although Hb A and Hb C were well resolved by HPLC, no contamination in each Hb component was routinely checked by rechromatography using the same gradient. For each Hb, the percent of hemes in the cyanomet and oxy forms was doubly checked by the following two methods. (1) The ratio of the absorbance at 542 and at 576 nm was compared with the calculated values for the mixtures of oxyHb and cyanometHb in various proportions (Miura & Ho, 1982; Perrella et al., 1994). (2) The concentration of the ferrous hemes in the sample was estimated from the absorption change upon CO binding to the sample, and then the concentration of cyanomet hemes in the sample was obtained by subtracting the spectral contribution of the ferrous hemes from the original spectrum. In all cases, both methods yielded the identical results.

RESULTS

Oxy−Cyanomet Hybrid System as a Control. OxyHb A was hybridized with a stoichiometric amount of cyanometHb C under aerobic conditions for 10 min. It is known that the dimer exchange of liganded tetramers occurs on the order of seconds (Smith & Ackers, 1985; Marden et al., 1996) so that a binomial (1:2:1) equilibrium distribution of this oxy−cyanomet hybrid system can be obtained rapidly. Then, the resulting oxy−cyanomet hybrid system at equilibrium was

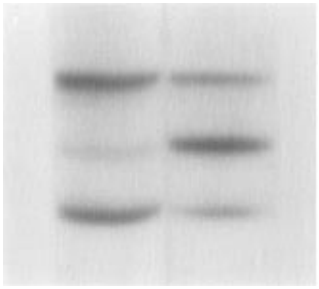

FIGURE 1: Photograph of the gels after anaerobic isoelectric focusing of the equimolar mixtures of Hb A and cyanometHb C hybridized under aerobic (on the right) and anaerobic (on the left) conditions. Hb C is the uppermost of the three bands (most cathodic), and the hybrid is the centermost band. Incubations were carried out in 0.1 M Tris buffer, pH 7.4, containing 0.1 M Cl^-, 1 mM Na_2EDTA, and 10 μM KCN, at 21.5 °C.

rapidly quenched by reduction with ditionite in the presence of IHP at 21.5 °C under an Ar atmosphere. This procedure converts the all-existing species, namely, oxyHb A, $(\alpha^{+CN^-}\beta^{+CN^-})_C(\alpha^{O2}\beta^{O2})_A$, and cyanometHb C, into corresponding deoxyHbs and stabilizes the tetramers and allows them to be separated by anaerobic isoelectric focusing. As shown in Figure 1 (right column), a prominent and stable middle band of the asymmetric hybrid appeared. The amount of the hybrid is 47% of the total, attributable to an approximately binomial (1:2:1) distribution, in good agreement with the previous report [47.9 ± 0.9% hybrid estimated from published energetic data on the oxy−cyanomet hybrid system by Doyle and Ackers (1992)]. This finding indicates that no significant dissociation of the asymmetric hybrid occurs during dithionite reducing and anaerobic isoelectric focusing processes.

Another control was run to check that no hybridization occurs between both parents during these processes. For this purpose, a sample of oxyHb A and another sample of cyanometHb C were injected into the quench buffer at the same time, and no significant formation of the hybrid was detected by our usual procedures.

Deoxy−Cyanomet Hybrid System. An oxy−cyanomet hybrid system at equilibrium was then deoxygenated by using humidified pure N_2 gas for 25 min, followed by addition of an oxygen-scavenging enzyme system. After an additional 10 min to approach equilibrium, the resulting deoxy−cyanomet hybrid system was quenched similarly as in the experiment for the oxy−cyanomet hybrid system, and subjected to anaerobic isoelectric focusing. Figure 1 shows a comparison between anaerobic isoelectric focusing of the oxy−cyanomet hybrid system and its deoxygenated product, the deoxy−cyanomet hybrid system. As shown in Figure 1, the fractional population of the asymmetric hybrid is dramatically decreased upon deoxygenation, *i.e.*, 47−8.6% hybrid of total Hbs, suggesting that the equilibrium distribution of this deoxy−cyanomet hybrid system is fairly far from 1:2:1 (binomial distribution). It should be mentioned here that the fractional population of $(\alpha^{+CN^-}\beta^{+CN^-})(\alpha\beta)$ was slightly dependent upon the method for deoxygenation. For example, comparative controls (30−40 min deoxy incubation) performed without an oxygen-scavenging enzyme system yielded slightly larger populations of the hybrid (*i.e.*, 10−15% of the total). The method presented here gave the lowest fractional population of the hybrid ever examined.

The present finding is in marked contrast to the previous report of an approximately binomial (1:2:1) equilibrium

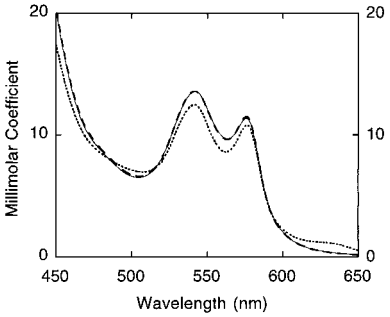

FIGURE 2: Absorption spectra of the equimolar mixture of Hb A and cyanometHb C before and after 48 h of deoxy incubation, before incubation without adding excess KCN (broken line), after incubation without adding excess KCN (dotted line), and with adding 1 mM KCN (solid line). Absorption spectra were measured after dilution with air-equilibrated distilled water without and with 1 mM KCN at 25 °C and were normalized by assuming that 533 nm is one of the isosbestic points of oxy and cyanomet derivatives. Incubations were carried out in 0.1 M Tris buffer, pH 7.4, containing 0.1 M Cl^-, 1 mM Na_2EDTA, and 1 mM KCN (before incubation), at 21.5 °C, under anaerobic conditions. The concentration of each Hb during the incubation was 0.8 mM on a heme basis.

distribution of the same deoxy−cyanomet hybrid system except using long-time deoxy incubations (Daugherty et al., 1991, 1994; Ackers et al., 1992; LiCata et al., 1993; Huang & Ackers, 1995). The discrepancy corresponds to an approximately 10-fold reduction in the dimer−tetramer association equilibrium constant of $(\alpha^{+CN^-}\beta^{+CN^-})(\alpha\beta)$ (or approximately 1.3 kcal/mol higher dimer−tetramer assembly free energy).

Spectrophotometric Analysis of Hybrid Mixtures and Both Parental Hbs. Figure 2 shows the absorption spectra of the equimolar mixtures of Hb A and cyanometHb C before and after 48 h of deoxy incubation. The spectra were measured after appropriate dilution with air-equilibrated distilled water without and with 1 mM KCN. As shown in Figure 2, the hybrid mixture after the incubation contained a significant amount of aquomet derivatives (*i.e.*, 43% of the total met contents). By adding excess (1 mM) KCN, the aquomet derivatives were fully converted to cyanomet form and the spectrum became indistinguishable from that measured before the incubation (Figure 2). This finding indicates that the total met contents of the mixture were not significantly altered during 48 h of deoxy incubation, but the concentration of free KCN in the sample solution, initially set to 1 mM, was markedly decreased during the incubation. When incubation was shorter than 1 h, no appreciable amount of aquomet derivatives was observed. Overall, the longer the incubation, the more formation of aquomet derivatives was observed.

To check a possibility of a valency exchange reaction, the diluted hybrid mixtures with 1 mM KCN were separated into the parental species, Hb A and Hb C, by using HPLC under aerobic conditions. During HPLC, the asymmetric hybrid nearly dissipates and only the two parents in fully liganded forms can be collected, because the time-scale of HPLC separation is much longer (approximately 10 min) than that of the dimer exchange between the fully liganded tetramers (on the order of seconds). Spectrophotometric analysis of both parents revealed that valency exchange occurs during deoxy incubation (see Figure 3). For example, a 48 h incubation resulted in the oxidation of 44% of Fe^{2+} to Fe^{3+} hemes in the original deoxyHb A and the reduction of 42%

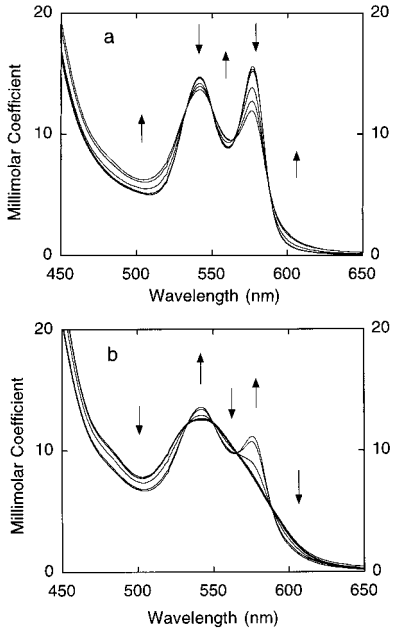

FIGURE 3: Valency exchange reaction between deoxyHb A and cyanometHb C during deoxy incubation: Absorption spectra of (a) Hb A (originally in deoxy form) and (b) Hb C (originally in cyanomet form) at various incubation times: 0, 0.5, 4, 22, 43, and 48 h. The arrows indicate the increase in time. After separation of the equimolar mixtures of Hb A and Hb C by HPLC, absorption spectra of both Hbs were measured under aerobic conditions at 25 °C, and were normalized by assuming that 533 nm is one of the isosbestic points of oxy and cyanomet derivatives. Incubations were carried out in 0.1 M Tris buffer, pH 7.4, containing 0.1 M Cl^-, 1 mM Na_2EDTA, and 10 μM (for 0 and 0.5 h) or 1 mM KCN (for 4, 22, and 43 h), at 21.5 °C, under anaerobic conditions. The concentration of each Hb during the incubation was 0.8 mM on a heme basis.

of Fe^{3+} to Fe^{2+} hemes in the original cyanometHb C. As seen in Figure 3, the valency exchange is gradual rather than instantaneous, and the amounts of the oxidation and the reduction are always approximately equivalent. Accordingly, the amounts of exchanged met hemes were usually as great as the formation of the aquomet derivatives (*e.g.*, 44 *vs* 43% of the total met hemes after 48 h of deoxy incubation).

Controls were run to check that (1) no valency exchange reaction occurs between oxyHb A and cyanomet Hb C and (2) cyanomet Hb cannot be reduced by itself under anaerobic conditions. For point 1, cyanometHb C was incubated with 20-fold excess of oxyHb A (*i.e.*, the final Hb concentrations of Hb C and Hb A were 0.127 and 2.54 mM, respectively) under aerobic conditions for 43 h, and no Fe^{2+} hemes (0%) were found in the original cyanometHb C after the incubation. For point 2, cyanometHb A was incubated by itself under anaerobic conditions for 43 h, and no Fe^{2+} hemes (0%) were detected after the incubation.

DISCUSSION

Valency Exchange. An underlying requisite for determination of the fractional populations of cyanometHb, $(\alpha^{+CN^-}\beta^{+CN^-})(\alpha\beta)$, and deoxyHb at equilibrium is that the valences of the hemes in all these species remain unchanged. However, our results showed that the incubation of deoxyHb A and cyanometHb C for 30 min resulted in formation of detectable amounts of Fe^{3+} and Fe^{2+} hemes in the original deoxyHb A and cyanomet Hb C (*i.e.*, 3 and 1%), respectively, and the exchange equilibrium, corresponding to 50% exchange, was almost attained in 48 h (Figure 3). Parallel incubation of the mixture of cyanometHb and oxyHb under aerobic conditions resulted in no exchange. Thus, the extent of the valency exchange is dependent on the length of deoxy incubation.

The present study also revealed that a significant amount of aquomet derivatives was formed in the deoxy−cyanomet hybrid system during 48 h of deoxy incubation (Figure 2), indicating that free KCN in the sample solution was consumed during the incubation. The disappearance of free CN^- in the sample solution can be expected from the following chemical properties of cyanide: (1) CN^- and H^+ are equilibrated with HCN whose p*K* value is around 9, so that the HCN form is predominant at pH 7.4 (the CN^- form is less populated) (National Research Council of the U.S.A., 1930); (2) the boiling point of HCN is about 26 °C so that the vapor pressure of HCN is significant at 21.5 °C (National Research Council of the U.S.A., 1928). Thus, it is likely that most of free CN^- in the sample solution was consumed by evaporation of HCN during necessary deoxygenation and subsequent deoxy incubation processes.[2]

The amounts of the exchanged met hemes during deoxy incubations were usually as great as the formation of the aquomet derivatives, indicating that the valency exchange parallels the formation of the aquomet derivatives. It is of interest that the formation of the aquomet derivatives, when cyanometHb was by itself incubated under anaerobic conditions, was not so significant as that in the case of the deoxy−cyanomet hybrid system. The simplest interpretation of these observations is that the dissociation of cyanide from cyanometHb is so slow that no significant aquomet derivatives appeared in the case of cyanometHb alone, whereas, in the presence of deoxyHb, the valency exchange between cyanometHb and deoxyHb generated an appreciable amount of met derivatives in the original deoxyHb, which could not be, however, fully saturated with the residual free cyanide.

We presume that a similar valency exchange reaction has occurred in the previous hybridization experiments using deoxyHb and cyanometHb (Smith & Ackers, 1985; Perrella et al., 1990, 1994; Daugherty et al., 1991, 1994; Ackers et al., 1992; LiCata et al., 1993; Huang & Ackers, 1995; Jayaraman & Spiro, 1995; Huang et al., 1996c), because our incubation conditions are almost the same as previously used, and the published data themselves do not provide any evidence against the occurrence of the valency exchange between deoxyHb and cyanometHb. The previous observation of an approximately binomial distribution could be explained by postulating that the valency exchange really reached its equilibrium.

Determination of Fractional Populations of Hb Species by Anaerobic Isoelectric Focusing after Rapid Reduction.

[2] Experimental conditions in previous studies seem to be more disadvantageous to persistence of free cyanide. Ackers and his colleagues have used throughout a standard buffer initially containing 10 μM free KCN, which would be consumed by only the presence of 1% met hemes in the original deoxyHb at approximately 1 mM (Daugherty et al., 1991, 1994). Moreover, to maintain anaerobicity, they have usually added to their samples an oxygen-scavenging system, including 0.3−0.6 mg/mL (about 5−10 μM in heme) catalase which could bind cyanide. Perrella et al. (1994) used solutions initially containing 1 mM free KCN and no oxygen-scavenging enzyme system. However, anaerobicity of the samples was maintained by a continuous flow of humidified N_2 gas, which could significantly accelerate the evaporation of HCN from the sample solutions.

To determine the fractional tetramer populations in the hybrid mixture, we utilized an approach which takes advantage of the fact that deoxyHb dissociates into its dimers far less readily than liganded Hbs (Bunn, 1981). This approach depends upon reducing Hbs rapidly and maintaining them in the fully reduced deoxy forms during isoelectric focusing. Our method enables to reduce oxyHb rapidly to deoxyHb before dimer−dimer rearrangement, since the O_2 dissociation rate of oxyHb is 13−21 s^{-1} at pH 7, 20 °C (Sawicki & Gibson, 1977) while the dimer exchange of liganded tetramers occurs on the order of seconds (Smith & Ackers, 1985; Marden et al., 1996). In the case of cyanometHb, however, the reduction leads to the formation of an intermediate, cyanide-bound (low-spin) ferrous Hb, which dissociates to unliganded deoxyHb with a rate constant of about 0.25 s^{-1} at pH 7 at 20 °C (Brunori et al., 1992). This difficulty could be circumvented by addition of IHP to the quench buffer at low pH. Under these conditions, the cyanide release from the intermediate is significantly accelerated (Brunori et al., 1992) and the tetramer−dimer dissociation of $(\alpha^{+CN^-}\beta^{+CN^-})(\alpha\beta)$ is markedly inhibited (Marden et al., 1996). Thus, it is possible in principle, by using our method, to study the deoxy−cyanomet and oxy−cyanomet hybrid equilibria. To further validate our approach in practice, we have checked that (1) no significant dissociation of the asymmetric hybrid occurs during anaerobic reducing and isoelectric focusing processes and (2) no hybridization of parental Hbs occurs during these processes (see Results).

Equilibrium Distribution of DeoxyHb, $(\alpha^{+CN^-}\beta^{+CN^-})(\alpha\beta)$, and CyanometHb. In this study, we utilized a rapid method for determining whether or not the relative concentrations of deoxyHb, $(\alpha^{+CN^-}\beta^{+CN^-})(\alpha\beta)$, and cyanometHb at equilibrium are 1:2:1 (binomial distribution). Our results clearly showed that the equilibrium distribution of the deoxy−cyanomet hybrid system at pH 7.4, 21.5 °C, is fairly far from 1:2:1 (Figure 1). However, because full attainment of equilibrium is questionable after 25 + 10 min of our deoxy incubation, our present value of 8.6% should be considered as an upper limit of the fractional equilibrium population of $(\alpha^{+CN^-}\beta^{+CN^-})(\alpha\beta)$. Moreover, at that time, there was a small amount of Fe^{3+} hemes (*i.e.*, about 3%) in the original deoxyHb A, which arose from the valency exchange reaction (about 1%), autooxidation during deoxygenation (about 1%), and initial met contents (about 1%). This factor also tends to increase the apparent fractional population of the hybrid. Therefore, the real population of $(\alpha^{+CN^-}\beta^{+CN^-})(\alpha\beta)$ formed in the equimolar mixture of both parents at equilibrium is not more than 8.6% of the total, and the thermodynamic stability of $(\alpha^{+CN^-}\beta^{+CN^-})(\alpha\beta)$ is less than one-tenth of the hyperstability previously reported (or the dimer−tetramer assembly free energy is at least 1.3 kcal/mol higher than the reported value of −11.4 kcal/mol).

Implications of the Present Results for Cyanomet Ligation System. Because of the chemical similarity of the $Fe^{3+}CN^-$ and $Fe^{2+}O_2$ adducts, it has been generally accepted that deoxy−cyanomet valency hybrid Hbs are useful models for oxygenation intermediates. Spectroscopic and thermodynamic studies of symmetric deoxy−cyanomet valency hybrids, $(\alpha^{+CN^-}\beta)_2$ and $(\alpha\beta^{+CN^-})_2$, have supported this idea (Banerjee & Cassoly, 1969; Ogawa & Shulman, 1972; Cassoly & Gibson, 1972).

Recently, Ackers and his colleagues (1992) have reported that one of the asymmetric diliganded hybrid, $(\alpha^{+CN^-}\beta^{+CN^-})(\alpha\beta)$, shows a hyper thermodynamic stability relative to the other diliganded species (Daugherty et al., 1991; Ackers et al., 1992). Due to the hyperstability of $(\alpha^{+CN^-}\beta^{+CN^-})(\alpha\beta)$, the $\alpha1-\beta1$ (or $\beta1-\alpha1$) ligation must show hypercooperativity represented by a 170-fold affinity change at the first and the second ligation steps, whereas the other $\alpha1-\alpha2$, $\beta1-\beta2$, and $\alpha1-\beta2$ ligation pathways are essentially noncooperative. Further structural characterizations of $(\alpha^{+CN^-}\beta^{+CN^-})(\alpha\beta)$ have revealed that this intermediate has a deoxy (T) quaternary structure (Daugherty et al., 1991; Ackers et al., 1992; Doyle & Ackers, 1992; LiCata et al., 1993; Jayaraman & Spiro, 1995). These highly distinct features of $(\alpha^{+CN^-}\beta^{+CN^-})(\alpha\beta)$ led to the proposal of a "molecular code" for cooperative switching, which translates configurations of the 10 ligation states into switch points of quaternary transition according to a "symmetry rule"; namely, T to R quaternary structure change is governed by the presence of at least one heme-site ligand on each of the $\alpha\beta$ dimeric half-molecules within the tetramer (Daugherty et al., 1991; Ackers et al., 1992).

The molecular code and symmetry rule of Ackers et al. (1992) have broadly influenced recent studies of Hb cooperativity, since their theory has provided the most extensive and complete description of the cooperative process for Hb in terms of the all intermediate ligation states. On the other hand, it has been revealed that their fundamental data on cyanomet ligation system (Daugherty et al., 1991; Ackers et al., 1992), however, lead to a striking and uncommon feature of cyanomet ligation (Ferrone, 1986; Straume & Johnson, 1988; Shibayama et al., 1993, 1995; Edelstein, 1996): the relative magnitudes of macroscopic Adair constants, K_i ($i = 1, 2, 3,$ or 4) for cyanomet ligation at pH 7.4, 21.5 °C, are in the order of $K_1 \leq K_3 < K_2 \leq K_4$ (K_1:K_2:K_3:K_4 = 1:57:3:170), in contrast to $K_1 \leq K_2 < K_3 \leq K_4$ (K_1:K_2:K_3:K_4 = 1:2.8:14:160) for oxygenation at pH 7.4, 25 °C (Imai, 1982). If this were the case, cyanomet ligation would be a poor model for oxygenation. In a recent article, Edelstein (1996) concluded that, from quantitative analysis, most of the specialties in cyanomet ligation arise from the hyperstability of $(\alpha^{+CN^-}\beta^{+CN^-})(\alpha\beta)$.

Our conclusion that the stability of $(\alpha^{+CN^-}\beta^{+CN^-})(\alpha\beta)$ is not more than one-tenth of the hyperstability previously reported resolves, at least partly, the mystery of the previous results. Taking into account the decreased stability of $(\alpha^{+CN^-}\beta^{+CN^-})(\alpha\beta)$, the $\alpha1-\beta1$ (or $\beta1-\alpha1$) ligation shows less than 17-fold affinity change, which is not much different from our direct observation of a 5.6-fold increase in oxygen affinity at the first and the second oxygenation steps of XL-$(\alpha^{Ni}\beta^{Ni})(\alpha^{Fe}\beta^{Fe})$ (Shibayama et al., 1993) and does not violate the requirement for monotonous behavior of the macroscopic Adair constants. Therefore, cyanomet ligation is still a good model for oxygenation.

A remaining question is whether the molecular code is relevant or not. Our direct determination of the first two-step microscopic Adair constants for Hb oxygenation did not show the distinct feature of the $\alpha1\beta1$ oxygenation, conflicting with the prediction of the molecular code (Shibayama et al., 1995). Moreover, our previous finding of significant spectral changes of Ni^{2+} protoporphyrin accompanied by CO (or oxygen) binding to XL$(\alpha^{Ni}\beta^{Ni})(\alpha^{Fe}\beta^{Fe})$ at pH 7.4 indicates that the key intermediate with two ligands

bound is not in a pure T quaternary structure, in contrast to the assessment of the symmetry rule (Shibayama et al., 1993). More recent CO rebinding kinetics study on the equilibrium mixture of HbCO and a large excess of cyanometHb suggested that the photoproduct, ($\alpha^{+CN^-}\beta^{+CN^-}$)($\alpha\beta$), is not predominantly in the T quaternary structure (Marden et al., 1996), raising a question of the published structural assignment for this key intermediate prepared by long-time deoxy incubations (Daugherty et al., 1991; Ackers et al., 1992; Doyle & Ackers, 1992; LiCata et al., 1993; Jayaraman & Spiro, 1995).

Our revised dimer−tetramer assembly free energy for ($\alpha^{+CN^-}\beta^{+CN^-}$)($\alpha\beta$), *i.e.*, −10.1 kcal/mol or above (less stable), approaches significantly the energy for the other diliganded species, *i.e.*, −8.5 kcal/mol. Thus, the previous emphasis on the highly distinct feature of ($\alpha^{+CN^-}\beta^{+CN^-}$)($\alpha\beta$) is invalid. However, the present results cannot rule out a possibility that ($\alpha^{+CN^-}\beta^{+CN^-}$)($\alpha\beta$) is slightly more stable than the other three diliganded species. One reason for this is that our electrophoresis results give only an upper limit of the stability of ($\alpha^{+CN^-}\beta^{+CN^-}$)($\alpha\beta$), due to the limitation of rapid deoxygenation. Another reason is that the previous data on the other deoxy−cyanomet hybrids are unreliable, because the valency exchange reactions may have occurred in most of the previous studies using deoxy−cyanomet hybrids under long anaerobic conditions, not only in the hybridization studies on ($\alpha^{+CN^-}\beta^{+CN^-}$)($\alpha\beta$) but also in the gel-permeation studies on $(\alpha^{+CN^-}\beta)_2$ and $(\alpha\beta^{+CN^-})_2$, in the hybridization studies on ($\alpha^{+CN^-}\beta$)($\alpha\beta$) and ($\alpha\beta^{+CN^-}$)($\alpha\beta$), and in the structural assignments of ($\alpha^{+CN^-}\beta^{+CN^-}$)($\alpha\beta$), ($\alpha^{+CN^-}\beta$)($\alpha\beta$), and ($\alpha\beta^{+CN^-}$)($\alpha\beta$) (Smith & Ackers, 1985; Perrella et al., 1990; 1994; Daugherty et al., 1991, 1994; Ackers et al., 1992; Doyle & Ackers, 1992; LiCata et al., 1993; Huang & Ackers, 1995; Jayaraman & Spiro, 1995; Huang et al., 1996c). At present, therefore, the molecular code for cooperative switching cannot be validated by the available data on cyanomet ligation system.

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