Thermodynamic Stability of the Asymmetric Doubly-Ligated Hemoglobin Tetramer ($\alpha^{+CN}\beta^{+CN}$)($\alpha\beta$): Methodological and Mechanistic Issues†

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Received June 10, 1997; Revised Manuscript Received July 14, 1997⊗

ABSTRACT: Free energy contributions to cooperativity by the eight ligation intermediates of human hemoglobin (Hb) have been characterized extensively using six oxygenation analogs [cf. Huang et al. (1996) *Biophys. J. 71*, 2094−2105, Table 2]. These unprecedented data bases have strongly supported the *molecular code mechanism* of Hb cooperativity [Ackers et al. (1992) *Science 255*, 54−83]. The present study addresses a recent argument against this work [Shibayama et al. (1997) *Biochemistry 36*, 4375−4381] based on "free energy" determinations for a doubly-ligated species of the CN−met analog. Shibayama et al. (1997) have claimed that, in the hybridization experiments that have been used to determine free energy of the asymmetric "species [21]" tetramer, a portion of the bound cyanide is allegedly released from CN−met Hb during the incubation with deoxy Hb that is used to achieve hybrid equilibrium. These authors have claimed that cyanide release has resulted in extensive electron exchange between heme sites of the hybridizing sample, leading to incorrect evaluation of the equilibrium species population by the cryogenic techniques that have been employed. In this report, we demonstrate that neither appreciable cyanide loss nor electron exchange occurs with the methods that have been used extensively by our two laboratories for these equilibrium determinations [Perrella et al. (1990) *Biophys. Chem. 35*, 97−103; Daugherty et al. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 1110−1114]. An alternative experiment, which Shibayama et al. (1997) have carried out to illustrate their claim, does not evaluate a thermodynamic equilibrium property of the species [21] hybrid. The relevance of their newly-estimated "free energy" is therefore unclear. Nevertheless, Shibayama et al. (1997) have claimed that their proposed "free energy" (which is ∼1.3 kcal more positive than the free energy of −11.4 kcal found independently by our two laboratories) renders invalid the *molecular code mechanism* of hemoglobin cooperativity. This representation is utterly without foundation since a free energy even more positive than suggested by Shibayama et al. (1997) would be fully consistent with the *molecular code mechanism*.

A new model for hemoglobin (Hb)[1] cooperativity was proposed in 1992 in which ligand binding onto subunits within both of the symmetry-related half-tetramers specifically promotes quaternary T→R transition (Figure 1), while ligation at only a single half-tetramer ($\alpha^1\beta^1$ or $\alpha^2\beta^2$) generates positive conformational free energy of "tertiary constraint" without triggering the global T→R switch (*1*). This new framework (herein denoted the "*molecular code mechanism*") was deduced from an extensive data base comprising (a) measured thermodynamic cooperativity terms for all eight partially-ligated intermediates using three well-established oxygenation analogs[2] ($Fe^{2+}/Fe^{3+}CN$, $Co^{2+}/Fe^{2+}CO$, and Fe^{2+}/Mn^{3+}) and (b) structure-sensitive probes that indicated T→R quaternary switching specifically accompanies the ligation steps which yield tetramers having ligated subunits on both dimeric half-molecules.

The equilibrium free energy contribution to cooperativity by each ligation intermediate (microstate) was determined from its relative dimer−tetramer assembly free energy[3] (*2*, *3*). Six of the intermediates (Figure 1) were formed by hybridization of their respective "parent" tetramers, while the problem of O_2 lability was circumvented by usage of nondissociating O_2 analogs. Assembly free energies were

† This work was supported by NIH Grants R37-GM24486 and PO1-HL51085 and by NSF Grant MCB9405492.

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⊗ Abstract published in *Advance ACS Abstracts,* August 15, 1997.

[1] Abbreviations: Hb, human hemoglobin; CN−met, cyanomet; Na_2-EDTA, disodium ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; met Hb, methemoglobin; HbA, hemoglobin A; HbS, hemoglobin S; HbC, hemoglobin C; HbO_2, oxyhemoglobin.

[2] Oxygenation analog systems are designated as [M_1/M_2X] where M_1 is the native Fe^{2+} or another heme-substituted metal ion that mimics the "deoxy" heme site (e.g., Co^{2+}, Zn^{2+}) and M_2X likewise mimics stereochemistry of the $Fe^{2+}O_2$ heme site (e.g., $Fe^{3+}CN$, $Fe^{2+}CO$, Mn^{3+}).

[3] Relationship to the equilibrium binding curve: Each assembly free energy ${}^{ij}\Delta G_2$ reflects the contribution to cooperativity from ligating *i* heme sites in the specific configuration *ij* (Figure 1), since each equilibrium constant K_{4i} of the Adair binding isotherm [cf. Chu et al. (1984) and Doyle et al. (1997)] is

$$K_{4i} = \sum g_{ij}(k_\alpha)^p(k_\beta)^q \exp[-({}^{ij}\Delta G_2 - {}^{01}\Delta G_2)/RT]$$

where g_{ij} is the statistical degeneracy of species *ij*, k_α and k_β are binding constants of subunits *within the dissociated dimers*, and *p* and *q* are numbers of α and β subunits ligated *within the tetramer*. The tetramer binding partition function, *Z*, is

$$Z = \sum_{i=0}^{4} K_{4i}(x)^i$$

where (*x*) is the thermodynamic activity of unreacted ligand X, and the traditional binding curve of oxygenation is

$$\bar{Y} = {}^1/_4[\mathrm{d} \ln Z/\mathrm{d} \ln(x)]$$

where $\bar{Y}$ is the fraction of occupied binding sites at ligand activity (*x*).

S0006-2960(97)01382-2 CCC: $14.00

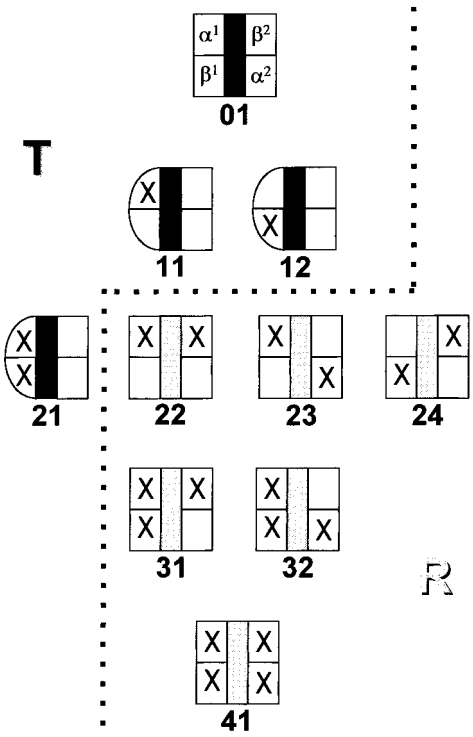

FIGURE 1: Ligation species (microstates) of tetrameric Hb. Symmetry-related dimeric half-molecules ($\alpha^1\beta^1$ and $\alpha^2\beta^2$) are depicted left and right of the dimer−dimer interface, which mediates the quaternary switch (i.e., a global rotation between dimers; cf. ref 39). Initial binding (denoted by X) generates tertiary conformation change of the ligated subunit, leading to enhanced affinity at the second binding step which forms species [21], i.e., without quaternary transition. Binding the second ligand onto either subunit of the opposite dimer promotes quaternary T→R switching. A "symmetry rule" is indicated by the dashed line. Studies leading to this mechanism (*1*) analyzed the equilibrium free energies of dimer−tetramer assembly for each of these microstates and correlated their properties with data from structure-sensitive probes. In these studies the six "asymmetric" species (i.e., species [11], [12], [21], [22], [31], and [32]) were characterized as hybrids in equilibrium with their "symmetric" parent tetramers, each contributing one of its (identical) dimeric halves.

determined for the microstate tetramers using techniques that were specifically optimized for this purpose, including (i) analytical gel chromatography (*4*−*6*); (ii) stopped-flow kinetics of subunit association rates (*7*−*10*); (iii) haptoglobin-trapping measurements of dissociation rates (*3*, *7*, *11*, *12*); and (iv) cryogenic electrophoresis techniques that were pioneered by Perrella (*13*−*18*). Specific implementation of these techniques by the Ackers laboratory for quantitation of hybrid species are detailed in refs 8, 10, 11, and 19−23. Our two laboratories, which have determined thermodynamic stability of the species [21] hybrid, have applied the cryogenic methodology using different protocols and obtained essentially identical results (e.g. refs 24 and 25).

The Hb Molecular Code. Studies on the partially-ligated intermediates which led to the molecular code mechanism (*1*) utilized three oxygenation analog systems for which data are shown in Table 1, columns 2, 4, and 5. These and subsequent results demonstrated that the dimer to tetramer assembly free energy[3] may be controlled by the configuration of occupied heme sites within the doubly-ligated tetramers, not solely by the total number of sites ligated. The distribution of assembly free energies required by the concerted allosteric model (*26*) for the eight partially-ligated Hb species (Figure 1) had been formulated earlier (*27*) but was not followed by the three analogs (*28*). For each analog system, (a) the asymmetric doubly-ligated "species [21]" molecule exhibited a different free energy from the remaining doubly-ligated tetramers (Table 1), and (b) ligation onto vacant sites of the species [21] tetramer led to a different increment in free energy compared with ligating the vacant sites of species [22], [23], or [24]. This feature was also observed when the doubly-ligated CN−met tetramers were reacted with O_2 at their vacant heme sites (*10*), suggesting common mechanistic rules for the two ligands. A consensus binding partition function was deduced from the strikingly similar distributions of free energy terms within each analog system (Table 1). This consensus function was then used (*1*, *10*) to resolve the independently-determined stoichiometric assembly constants of native HbO_2 (*29*) into their microstate components (Table 1, columns 1 *vs* 6) under the premise of common mechanistic rules for O_2 and the three analogs (cf. ref *30*, for abbreviated review of the 1992 strategy).

Subsequent determinations with additional analogs (Table 1, columns 3 and 8) have confirmed the combinatorial distribution for doubly-ligated tetramers, and have led to explicit free energy distributions for the $Fe^{2+}/Fe^{2+}CO$ and Fe^{2+}/FeO_2 microstates[2] (Table 1, columns 7 and 9). These resolved free energies for the CO and O_2 intermediates were used to calculate binding curves[3] that agreed closely with the experimentally measured ones and also with the 1992 molecular code analysis (*21*). It is of particular significance that the microstate free energies predicted (*1*) for native HbO_2 (Table 1, column 6) on the basis of columns 2, 4, 5, and 1 were subsequently determined independently of the columns 2, 4, and 5 analogs and of the stoichiometric O_2 data (*21*). This outcome of the eleven-year sequence of studies (1985−1996) demonstrated the soundness and validity of the strategy, techniques, and model systems that were employed.

In a recent publication, Shibayama et al. (*31*) have asserted that the values of assembly free energy for CN−met species [21] reported by the laboratories of Ackers and Perrella (*3*, *6*, *11*, *12*, *15*, *22*, *25*, *30*, *32*) are incorrect due to the presence of undetected electron exchange among the heme sites prior to isoelectric focusing. In addition, Shibayama et al. (*31*) suggested that such artifacts might also have compromised the free energy determinations on species [22], [23], and [24] and hence the true species [21] free energy might be indistinguishable from those of the other doubly-ligated tetramers. They allege that their establishment of these possibilities casts doubt on the applicability of all prior CN−met data that have provided thermodynamic support for the molecular code mechanism.

In this report we present data which demonstrate and re-establish that any formation of met Hb, or occurrence of electron exchange, is readily monitored using the cryogenic isoelectric focusing protocols. We demonstrate that neither significant cyanide loss nor electron exchange occurs in the Hb hybridization reactions under conditions that have been used for our studies and which differ critically from the experiment presented by Shibayama et al. (*31*). We also show that, even if the value for assembly free energy of CN−met species [21] proposed by Shibayama et al. were correct, it would not carry the implication they have claimed with respect to validity of the Hb molecular code mechanism. Their proposed value would, in fact, be totally consistent with this mechanism.

Table 1: Experimental Assembly Free Energies $^{ij}\Delta G_2$ of Hemoglobin Ligation Analogs[a]

i	-1-[b] $\frac{Fe^{2+}}{Fe^{2+}O_2}$	Microstates, *ij*	-2-[b,c] $\frac{Fe^{2+}}{Fe^{3+}CN}$	-3-[c,d] $\frac{Co^{2+}}{Fe^{3+}CN}$	-4-[a,c] $\frac{Fe^{2+}}{Mn^{3+}}$	-5-[a,c] $\frac{Co^{2+}}{Fe^{2+}CO}$	-6-[e] $\frac{Fe^{2+}}{Fe^{2+}O_2}$ (predicted 1992)	-7-[a,d] $\frac{Fe^{2+}}{Fe^{2+}CO}$	-8-[b,d] $\frac{Zn^{2+}}{Fe^{2+}O_2}$	-9- $\frac{Fe^{2+}}{Fe^{2+}O_2}$ (resolved 1996)
0	−14.4	01	*−14.4*	*−10.6*	*−14.4*	*−10.6*	*−14.4*	*−14.4*	*−14.4*	*−14.4*
1	−11.5	11	*−11.3*	*−8.9*	*−11.5*	*−9.0*	*−11.5*	*−11.1*	*−11.6*	*−11.6*
		12	*−11.2*	*−8.5*	*−10.7*	*−8.4*	*−11.5*	*−11.0*	*−11.6*	*−11.6*
2	−8.9	21	*−11.4*	*−9.0*	*−11.0*	*−8.3*	*−9.2*	*−10.2*	*−9.4*	*−9.4*
		22	−8.3	−7.5	−7.8	−7.4	−7.2	−7.9	−7.7	−7.7
		23	−8.2	−7.7	−7.6	−7.5	−7.2	−7.8	−7.6	−7.6
		24	−8.5	−7.5	−8.2	−7.4	−7.2	−7.9	−7.8	−7.8
3	−7.3	31	−8.6	−7.7	−7.9	−7.7	−7.2	−7.9	−7.5	−7.5
		32	−8.4	−7.9	−7.9	−7.6	−7.2	−7.8	−7.5	−7.5
4	−8.1	41	−8.5	−8.3	−7.5	−8.0	−8.0	−8.0	−8.1	−8.1

[a] Microstate species [*ij*] of six ligation analogs (columns 2−5, 7, and 8). [b] Stoichiometric assembly free energies of the native HbO_2 system (column 1). [c] The microstate free energies predicted (*1*) from the consensus distributions of columns 2, 4, and 5 which were constrained to also conform with stoichiometric terms (column 1) of the native system. [d] Additional analogs that were subsequently characterized (columns 3, 7, and 8) and [e]microstate distribution for the native HbO_2 system which was determined independently of data from the initial three analogs that were used, with data of column 1, to predict column 6. Specific references: [column 1] (*29*); [column 2] (*3*, *15*); [column 3] (*21*); [column 4] (*28*, *45*); [column 5] (*8*, *21*); [column 6] (*1*); [column 7] (*21*); [columns 8, 9] (*22*).

METHODS AND RESULTS

Procedures used in this study for equilibration, anaerobic incubation, and cryogenic isoelectric focusing of CN−met Hb hybrids were the same as employed in previously published works from the Ackers laboratory (cf. introduction), with one exception: to purposefully promote met Hb formation and electron exchange, excess KCN was routinely omitted from the hybridization mixture. Our purpose was to demonstrate the ability of our methods to have detected the compromising effects which Shibayama et al. (*31*) have alleged were present in our data. Comparisons will be made which demonstrate the integrity of our published results against these allegations.

Hybridization of deoxy Hb, met Hb, and CN−met Hb To Form Species [*21*]. Oxy Hb and CN−met Hb were deoxygenated under a flow of N_2 at 0−2 °C for at least 1 h in "standard buffer" composed of 0.1 M Tris HCl, 0.1 M NaCl (total chloride = 0.18 M), 1 mM Na_2EDTA, 10 μM KCN, at pH 7.4, 21.5 °C. The N_2 was humidified by passage through two bubblers containing H_2O and standard buffer, respectively, followed by passage through a vapor trap. The deoxygenated samples were then transferred into an anaerobic chamber. An O_2-removing enzyme system was added to deoxy Hb to give final concentrations of 1.8 mg/mL glucose oxidase (Sigma), 0.6% glucose, and 0.3 mg/mL *Aspergillus niger* catalase[4] (Sigma). After 10 min incubation at room temperature, the solutions were mixed in proportional volumes to give 50% deoxy HbS, 25% met Hb, and 25% CN−met Hb at a total Hb concentration of 1 mM in heme. This mixture was aliquoted into 100 mL septum-sealed conical vials. The vials were inverted and immersed in 0.1% sodium dithionite solution within crimp-sealed serum vials per standard procedure (*19*). The double-sealed Hb samples were incubated at 21.5 °C for 48 h. Three other hybrid mixtures (deoxy HbS/CN−met HbA, deoxy HbA/CN−met HbS, deoxy HbA/CN−met HbC) were also investigated in this study.

Cryogenic Isoelectric Focusing. Formation of the hybrid tetrameric species in each mixture was determined by quantitative cryogenic isoelectric focusing (cryo-IEF) (*14*, *19*). The hybridization reaction was first quenched at −35 °C by mixing 10 μL of hybrid mixture with 150 μL of quench solution containing 50% ethylene glycol and 50% standard buffer. To minimize oxidation during subsequent manipulation, the quenched mixture was placed under a flow of CO for 10 min at −35 °C. An aliquot of the quench mixture was then loaded onto an isoelectric focusing gel tube (pH gradient was 6−8; for samples containing HbC, the gradient was pH 7−9). These IEF tubes were maintained below −25 °C while focusing for 24 h. Finally, each tube was optically scanned at multiple wavelengths (typically 420 and 540 nm).

Spectroscopic Analysis of Deoxy Hybrid Mixture. The dithionite quench used by Shibayama et al. results in essentially complete reduction of the met-, and CN−met heme sites prior to isoelectric focusing, precluding any analysis by electrophoretic methods of the extent of met Hb formation and its possible redistribution among the Hb species. To estimate met Hb formation, Shibayama et al. used an indirect approach in which the reaction mixture was not quenched but simply diluted and measured spectrophotometrically. Their spectra showed a large abundance of met Hb (their Figure 2). To evaluate features of the Shibayama et al. methodology we performed a corresponding experiment with our system.

A hybrid mixture containing a 1:1 ratio of deoxy Hb and CN−met Hb (following deoxy incubation for 72 h) was placed under a flow of humidified CO for 10 min at 0−2 °C to minimize the availability of ferrous hemes that could become oxidized during dilution at the time of spectroscopic

[4] The level of catalase activity is crucial for preventing oxidation of Hb to met Hb by H_2O_2 generated from the glucose oxidase-catalyzed reaction between O_2 and glucose. Such met Hb formation was routinely monitored by cryogenic isoelectric focusing during the present study. In cases where significant met Hb was detected, we repeated the hybrid experiments with satisfactory results, either by replacing the sample of catalase or by increasing the amount of catalase in the mixture.

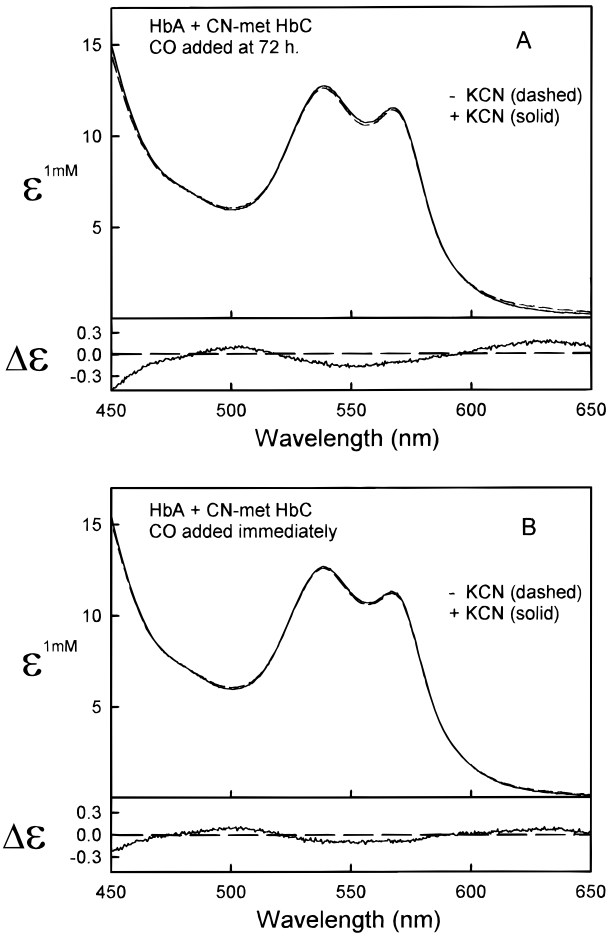

FIGURE 2: Absorption spectra for hybrid mixture of HbA and CN−met HbC tetramers. (A) An equimolar mixture of deoxy HbA and CN−met HbC was incubated anaerobically for 72 h at 21.5 °C and then saturated with CO. Dashed curve: spectrum immediately after dilution of the Hb mixture into H_2O. Solid curve: spectrum after addition of 1 mM KCN. (B) An equimolar mixture of oxy HbA and CN−met HbC was saturated with CO immediately prior to measurement of spectra. Dashed curve: Spectrum immediately after dilution of the Hb mixture into H_2O. Solid curve: Spectrum after addition of 1 mM KCN. Lower parts of A and B show difference spectra for the presence and absence of added KCN.

measurement. The mixture was then diluted into CO-saturated distilled H_2O at 21.5 °C to a final concentration of 10−20 μM heme. A sample spectrum was taken immediately, and KCN was added to a final concentration of 1 mM, followed by a second spectral scan (Figure 2A). Using the difference in absorbance at 540 nm between the two measurements (after correcting for dilution due to addition of KCN), the met Hb in the mixture was estimated to be 3.9%.

Control experiments showed that dilution of CN−met Hb into H_2O to a final concentration of approximately 10 mM resulted in gradual dissociation of cyanide from the CN−met Hb. Thus, to correctly evaluate met Hb content in the original deoxy hybrid mixture, the amount of cyanide dissociated during sample dilution and spectral measurement was estimated by the following control experiment (Figure 2B). Oxy HbA was mixed with CN−met Hb in a 1:1 molar ratio at approximately 1 mM heme, and the mixture was immediately placed under humidified CO for 10 min at 0−2 °C. The mixture was then diluted into distilled H_2O to a concentration of 10−20 μM for spectral measurement. KCN was added (concentration, 1 mM), and the spectrum was

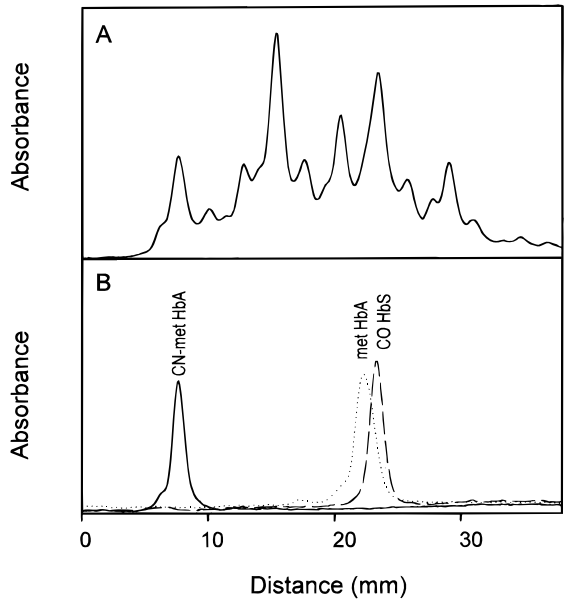

FIGURE 3: Cryogenic isoelectric focusing scans of Hb hybrid mixtures. (A) Mixture comprising 50% deoxy HbS, 25% CN−met HbA, and 25% met HbA, which was incubated anaerobically for 48 h before quenching at −35 °C. The sample was then saturated with CO and subjected to cryo-IEF. The curve shows an absorption scan at 420 nm. Oxidation of HbS by met HbA resulted in formation of met HbS/deoxy HbS hybrid tetramers which are shown as a series of bands on the right side of the CO HbS (Fe^{2+}) peak. Hybrids of met HbS/CN−met HbA yielded additional peaks, focusing between those of HbS and CN−met HbA. No attempt was made to identify each peak. (B) Scans show the focusing positions of CN−met HbA, met HbA, and CO HbS. The three Hb species were focused individually in separate tubes which were scanned at 420 nm (*19*).

measured again. The difference in 540 nm absorbance, after correction for dilution from the addition of KCN, was used to estimate met Hb content arising from the cyanide dissociation that occurred upon dilution into CN-free H_2O as well as from met Hb originally present in the sample. The estimated met Hb content in the control mixture was 2.3% of total Hb. Therefore, the true met Hb content generated during deoxygenation of HbA plus CN−met HbC and from the species [21] hybrid incubation was 1.6% of the total Hb (i.e., the total met Hb content for the deoxy hybrid mixture minus that for the control experiment). This value may be compared with the 42%−46% oxidation reported by Shibayama et al. (*31*) in their hybridization experiment.

Is Electron Exchange Present but Undetected in the Anaerobic Incubations Prior to Cryo-IEF? The claim of Shibayama et al. (*31*) is as follows: During anaerobic incubation of the hybridization mixture of deoxy Hb and CN−met Hb, cyanide is lost, resulting in electron exchange among the met and deoxy heme sites, which presumably is not detected by our cryoisoelectric focusing analyses. A demonstration that this is *not* the case is presented in Figure 3. First, deoxy HbS was incubated with met HbA and CN−met HbA in the absence of excess KCN (always an essential component in protocols of our two laboratories). Results of a 48 h anaerobic incubation are shown in Figure 3A: electron exchange between deoxy Hb and met Hb has resulted in the formation of met/deoxy hybrid tetramers with varying degrees of oxidation, shown as a series of bands on the right side of the CO HbS (Fe^{2+}) band. Hybrids of met/CN−met give rise to additional peaks focusing between CO

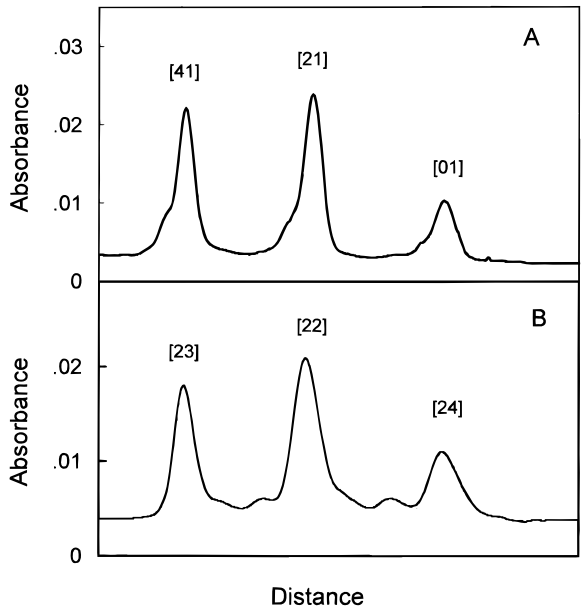

FIGURE 4: Isoelectric focusing of hybrid mixtures forming CN−met species [21] (panel A) and [22] (panel B) at pH 7.4, 21.5 °C (6). Species [21] was formed by mixing deoxy HbS (species [01]) with CN−met HbA (species [41]), and the mixture was incubated for 119 h. Species [22] was formed by mixing CN−met HbA species [23] with CN−met species HbS [24], and the mixture was incubated for 46 h.

HbS and CN−met Hb. The band positions of the three (unreacted) parent tetramers are illustrated in Figure 3B.

DISCUSSION

In contrast with the foregoing demonstration, the experiments used routinely and reported extensively by our laboratories (in which met Hb was not present as a parent species and excess KCN was maintained in the anaerobic incubation mixture) have been free of significant oxidation, as shown in Figures 4−6 for a wide range of conditions. The methods for elimination of electron exchange, and for its detection by cryogenic electrophoresis techniques that were established by the pioneering studies of Perrella (*14*, *15*, *33*), have been employed routinely in the Ackers laboratory (*6*, *8*, *10*−*12*, *19*−*23*, *25*, *30*, *32*). Extensive experience of our respective laboratories with this technique has found that spurious banding will appear whenever the strict conditions of the experimental protocol are not met. The isoelectric focusing experiment is thus a sensitive indicator of problems in the hybridization reaction and has provided essential quality control for the published studies from both laboratories. It should also be noted that the ability of cryofocusing to separate partially-oxidized species has enabled Perrella and collaborators to trap, isolate, and identify intermediates in the reaction between Hb and CO (*17*, *18*, *33*). It is also significant that both of the laboratories that have determined thermodynamic stability of the CN−met species [21] hybrid have applied the cryogenic methodology using different protocols and obtained similar results.

Symmetric Species [22], [23], and [24]. Shibayama et al. have also insinuated that the CN−met free energies for species [22], [23], and [24] (Table 1, column 2) may be erroneous and that all doubly-ligated species have essentially identical assembly free energies. However, as documented herein, the CN−met free energies for species [21] *vs* [22] have been determined in different laboratories, using both kinetic and equilibrium techniques (*3*, *15*, *20*, *22*), over wide ranges of conditions and with heterotropic effectors (*6*, *10*−*12*, *16*, *30*), yielding a large body of consistent results. Figure 4 shows the comparative data for species [21] and species [22] in the cryogenic data base of Huang and Ackers (*6*).

Accurate equilibrium constants for dimer−tetramer assembly of doubly-ligated CN−met species [23] and [24] have been determined extensively by analytical gel permeation chromatography (*4*, *34*) and their dissociation curves were presented in Doyle and Ackers (*10*), Huang and Ackers (*6*), and Huang et al. (*12*, *30*). In these determinations, the presence of excess KCN prevented cyanide loss from the

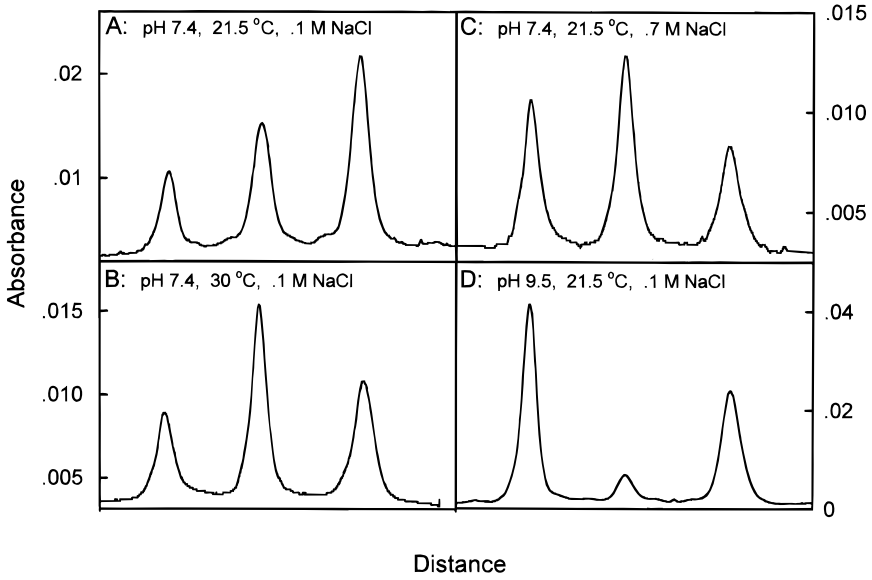

FIGURE 5: Cryogenic isoelectric focusing scans of CN−met species [21] hybrid mixtures studied previously at different solution conditions. (A) Hybrid mixture incubated anaerobically for 48 h at pH 7.4, 21.5 °C, and 0.1 M NaCl (*6*). Under these conditions, an additional 24 h incubation was needed to reach hybrid equilibrium (see Figure 6). (B) Hybrid mixture incubated for 85 h at 30 °C, pH 7.4, 0.1 M NaCl (*6*). (C) Hybrid mixture incubated for 95 h at pH 7.4, 21.5 °C, 0.7 M NaCl (12). (D) Hybrid mixture incubated for 2.3 h at pH 9.5, 21.5 °C, 0.1 M NaCl (*11*).

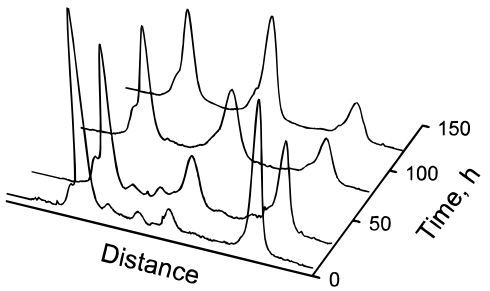

FIGURE 6: Species distribution for the hybrid mixture of deoxy HbA and CN−met HbS as a function of anaerobic incubation time. Species were separated by cryogenic isoelectric focusing, and the gel tubes were scanned at 430 nm. For each scan the left and right peaks represent HbA and CN−met HbS, respectively. The middle peak shows the relative population of the hybrid species [21]. The hybrid mixtures shown were incubated anaerobically at 21.5 °C for 0.6, 23, 71, and 119 h. Each scan was normalized to total Hb for comparison.

occupied heme sites, as evaluated by cryoisoelectric focusing. The incorrect claim by Shibayama et al. (*31*) that Ackers et al. have not published results that would demonstrate integrity of their experiments against effects of spurious oxidation and electron exchange appears to result from a lack of awareness that the cryogenic methodology provides these controls.

The Experiment of Shibayama et al. The experiment upon which Shibayama et al. (*31*) have based their claims regarding the CN−met Hb species [21] free energy differs critically from the protocol discussed above. They mixed CN−met Hb with oxygenated Hb forming an O_2/CN−met hybrid sample which reaches equilibrium within several minutes. Their sample mixture was then deoxygenated by 25 min of N_2 exposure, followed by addition of O_2-scavenging enzymes, and incubated under anaerobic conditions for an additional 10 min *but not to equilibrium* (*31*). The reaction was quenched at 21.5 °C using dithionite and IHP, followed by isoelectric focusing at 1 °C.

The results of this experiment, remarkably, are presented only after short incubation periods (*31*) and without any objective evidence that hybrid equilibrium has been achieved in their anaerobic Hb incubation. By contrast, the previously characterized time course of hybridization (*6*, *15*, *19*, *20*) has dictated an inescapable necessity for the longer incubations. Since these incubations can be conducted without compromising sample integrity (as demonstrated by Figures 4−6), it is surprising that the Shibayama et al. anaerobic incubations and isoelectric focusing results were not followed for time periods corresponding to the ones found necessary by previous researchers for the attainment of hybrid equilibration [see also ref 15, Figure 2 in ref 5, and Figure 5 in ref 6]. The hybrid mixture of HbA and CN−met HbC conforms to a statistical distribution of species under oxy conditions, as shown by the experiment of Shibayama et al. (*31*) and as it was shown nearly twenty years earlier by the cryogenic technique (*35*). Why did Shibayama et al. not observe the same binomial distribution of species after deoxygenation of their mixture by nitrogen tonometry? Since O_2 removal by this procedure is a slow process (25 min as reported by the authors) and the tetramer dissociation rate of deoxyHb is very slow ($t_{1/2}$ = 7.5 h) as compared with tetramer dissociation rates of the ligation intermediates (*3*, *20*), their finding can be partially explained by the same factors previously noted in the failure to isolate hybrid by isoelectric focusing at temperatures above zero. Under these conditions the hybrid may be detected only as a transient species (*36*). Since the time dependence of the concentration of species [21] is nonmonotonic (*20*), the nonequilibrium value obtained by Shibayama et al. does not measure any equilibrium parameter of the system and thus cannot be used to estimate an upper (nor a lower) limit for the species [21] assembly free energy.

Would an Assembly Free Energy of $^{21}\Delta G_2 = -10.1$ kcal Invalidate the Molecular Code Mechanism of Hb Cooperativity? The claim by Shibayama et al. that a $^{21}\Delta G_2$ value of −10.1 kcal/mol would provide evidence against the molecular code mechanism is incorrect and without foundation. Such a value would actually support the molecular code by revealing the same combinatorial rule of free energy distribution among doubly-ligated CN−met tetramers that was discovered using $^{21}\Delta G_2 = -11.4$ kcal/mol. This same rule was found among doubly-ligated intermediates of the other resolved O_2 analog systems: for all six O_2 analogs of Table 1 (columns 2−5, 7, and 8), the doubly-ligated tetramers were found to assemble with two distinct free energies, according to whether both ligated subunits were on the same symmetry-related half-tetramer (species [21]) or on opposite half-tetramers (species [22], [23], and [24]). This combinatorial rule is also manifested by the native Fe/FeO_2 system (Table 1, column 9). It implies that contributions to Hb cooperativity may be controlled by site-specific configurations of the ligated hemes and not solely by the number of sites ligated.

The characteristic free energy difference between species [21] and the other doubly-ligated tetramers was an important clue which led to discovery of other differences in functional and structural properties of the microstate tetramers, including their site-specific contributions to the Bohr effect (*11*, *16*); their enthalpic and entropic roles (*6*); their modulation by NaCl (*12*, *30*); and their differences in quaternary structure (*10*, *32*, *37*). *These subsequently discovered "symmetry rule responses" to a variety of structure-sensitive probes would have yielded the same mechanistic picture even if the doubly-ligated microstates had identical assembly free energies and regardless of the initial clues that motivated their investigation.*

It is reasonable to expect that analogs which conform to normal heme site stereochemistry and also execute the overall T→R quaternary response to ligation, as defined by Perutz (cf. refs 38 and 39), would also follow rules of the native HbO_2 system at the intermediate states of ligation. Such analogs are *generally expected* to show *quantitative* deviations from the behavior of native HbO_2 (as with enzyme−substrate analogs that manifest altered K_m and k_{cat} parameters while mimicking the native catalytic mechanism). The strategy of the Ackers laboratory was to analyze a range of chemically diverse analogs known to execute the T→R transition upon overall ligation. *It was not assumed that mechanistically-valid analogs must exhibit functional responses that are quantitatively identical to the native system.* The strategy employed (*1*) was (a) to determine the common functional characteristics among microstates of the various analog systems; (b) to formulate a binding function that reflected their consensus functional and structural properties; and (c) to evaluate native HbO_2 parameters by constraining the consensus relationships to conform with Adair constants from direct O_2 binding. Using this strategy a 36-fold

intradimer cooperativity was deduced for species [21] of the native $Fe^{2+}/Fe^{2+}O_2$ system. By contrast, a 170-fold value had been found with $Fe^{2+}/Fe^{3+}CN$, while that found for $Co^{2+}/Fe^{2+}CO$ was only 2.5-fold. In spite of the published record of these findings (*1*), it has been claimed by Shibayama et al. (*39*, *40*), Eaton and colleagues (*40*−*44*), and Edelstein (*43*) that an intradimer cooperativity of 170-fold was proposed for native HbO_2. These authors have argued that the molecular code must be invalid because such high intradimer cooperativity would be incapable of accommodating the behavior of native HbO_2. By contrast, however, the 1992 molecular code parameters (Table 1, column 6) *which were evaluated from the oxygenation curves of native HbO_2* (*1*) are in close agreement with those determined independently (column 9), while each set of cooperativity parameters predicts the HbO_2 binding curve to within its experimental accuracy (*22*). It was emphasized in Ackers (*28*) that "in our choice of the CN−met system for the first complete resolution of the intermediate species, we may have serendipitously forced the molecule to reveal, in bold caricature, a state that is usually manifested with more subtlety in other ligands." While it is important to continue developing experimental tests for perturbations in functional behavior of oxygenation analogs (*20*−*23*, *30*), there is now a sufficient body of consistent data over a range of chemically diverse O_2 analogs and with the O_2 ligand itself (Table 1) to indicate that CN−met ligation has not "tricked" the Hb tetramer into an aberrant set of allosteric rules from those it uses with O_2.

Conclusions. (i) Shibayama et al. (*31*) have failed to establish their claim that the equilibrium assembly free energy of CN−met Hb species [21] has been incorrectly measured by the cryogenic electrophoresis protocols employed. In reaching their conclusion that our experiments were invalidated by a presumed loss of cyanide and undetected electron exchange, these authors have ignored the extensive literature (beginning with the pioneering studies of Perrella's laboratory) that has established the cryogenic quenching and electrophoresis methods for accurate quantitation of oxidation and electron exchange. Most remarkably, Shibayama et al. (*31*) have represented their assembly "free energy" for species [21] (i.e., ≤ -10.1 kcal, error unknown) as an equilibrium value without presenting any documenting evidence that equilibrium was attained. Such a "free energy" cannot be regarded comparably with the actual equilibrium value of -11.4 ± 0.2 kcal/mol that has been extensively documented for the same conditions they used (cf. introduction and refs therein). (ii) Shibayama et al. (*31*) have also failed to establish that their claimed value for the species [21] assembly free energy would provide evidence against the molecular code mechanism of Hb cooperativity. Their claim appears to be based on an incorrect supposition regarding the logic by which CN−met data were used in conjunction with data of the other analogs (*1*) to deduce the microstate free energies of native HbO_2 intermediates (Table 1, column 6). By alleging that the molecular code mechanism was based solely on the data of a single analog, which was assumed to have *quantitatively* identical cooperativity properties as O_2, Shibayama et al. have misrepresented the strategy, logic, and data bases that were documented in the published record. A species [21] value of −10.1 kcal in Table 1, column 2, would have led to the identical molecular code partition function that was deduced (*1*, *10*) and also to identical free energy values for the microstate intermediates of native HbO_2 that are listed in Table 1, column 6, of this report.

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BI971382X