Oxygen Equilibrium Properties of Nickel(II)−Iron(II) Hybrid Hemoglobins Cross-Linked between 82β1 and 82β2 Lysyl Residues by Bis(3,5-dibromosalicyl)fumarate: Determination of the First Two-Step Microscopic Adair Constants for Human Hemoglobin†

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ABSTRACT: We have previously reported that cross-linked asymmetric Ni(II)−Fe(II) hybrid hemoglobin, XL[α(Fe)β(Fe)][α(Ni)β(Ni)], in which the $\alpha1\beta1$ dimer containing ferrous protoporphyrin IX and the adjacent $\alpha2\beta2$ dimer containing nickel(II) protoporphyrin IX were cross-linked between Lys-82β_1 and Lys-82β_2 by reaction with bis(3,5-dibromosalicyl)fumarate, represents an adequate model for determination of the $\alpha1\beta1$ oxygenation properties of native hemoglobin [Shibayama, N., Imai, K., Morimoto, H., & Saigo, S. (1993) *Biochemistry 32,* 8792−8798]. To extend the approach using cross-linked Ni(II)−Fe(II) hybrids to all possible pathways for initial-half oxygenation of hemoglobin, we have prepared three other types of cross-linked Ni(II)−Fe(II) hybrids, carrying nickel(II) protoporphyrin IX in two subunits and ferrous protoporphyrin IX in the other two subunits, and have determined the two-step oxygen equilibrium curves of the ferrous subunits within these cross-linked hybrids. For the first step of oxygenation, the α subunit shows about 3-fold higher affinity than the β subunit at all pH values examined, indicative of a significant functional heterogeneity of the subunits in deoxyhemoglobin. For the second step of oxygenation, the cooperativity represented by the Hill coefficient (n_{max}) increases in the order of $\beta1\beta2$ (n_{max} = 1.36), $\alpha1\beta1$ (n_{max} = 1.41), $\alpha1\beta2$ (n_{max} = 1.64), and $\alpha1\alpha2$ (n_{max} = 1.72) at pH 7.4 in the presence of 0.1 M Cl^- at 25 °C. In all hybrids, the oxygen affinities and cooperativities are pH-dependent, the Hill coefficients becoming larger as pH increases. These results imply that [α(Fe)β(Fe-O_2)]$_2$ is the only minor diliganded intermediate in the oxygen equilibrium of tetrameric hemoglobin. The implications of these findings for the mechanism of hemoglobin cooperativity are discussed.

Tetrameric human adult hemoglobin (Hb A)[1] has served as a model for understanding how subunit assemblies can utilize intersubunit interactions to regulate the biological functions. Accurate oxygen equilibrium curves of Hb A have allowed us to determine the equilibrium constant at each oxygenation step (Imai, 1981a,b, 1982, 1994). These four equilibrium constants, namely, Adair constants, have been good measures of cooperativity and oxygen affinity of Hb tetramer. However, once we take into account the functional heterogeneity of the α and β subunits and the molecular symmetry of the Hb tetramer, there are various possible oxygenation pathways leading from fully deoxygenated Hb to fully oxygenated Hb. In consequence, the total number of microscopic oxygen equilibrium constants becomes 16. Although these 16 microscopic equilibrium constants are very important for an understanding of the detailed molecular mechanism of Hb, it is theoretically impossible to resolve these parameters from oxygen equilibrium curves of native Hb A.

Many attempts have been made for resolution of such microscopic parameters by using metal-substituted hybrid Hbs (Imai et al., 1980; Blough & Hoffman, 1982; Shibayama et al., 1986a). Among them, Ni(II)−Fe(II) hybrids can provide a powerful means for this purpose, because it has been shown that Ni-PP, which binds neither oxygen nor CO, mimics a fixed deoxyheme with respect to its effect on oxygen equilibrium properties of the counterpart ferrous subunits within both symmetric Ni(II)−Fe(II) hybrid Hbs under various solution conditions (Shibayama et al., 1986a). Moreover, validity of Ni-PP as a model for a fixed deoxyheme has been confirmed by other physicochemical measurements (Alston et al., 1984; Shibayama et al., 1986b, 1987).

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[1] Abbreviations: Hb A, human adult hemoglobin; Hb, hemoglobin; Fe^{2+}-PP, ferrous protoporphyrin IX (ferrous heme); Fe^{3+}-PP, ferric protoporphyrin IX; Fe^{3+}-DP, ferric deuteroporphyrin IX; Ni-PP, nickel(II) protoporphyrin IX; XLHb, hemoglobin cross-linked between Lys-82β_1 and Lys-82β_2 by reaction with bis(3,5-dibromosalicyl)-fumarate, where XL signifies the presence of cross-linking; XL[α(Fe)β-(Ni)]$_2$, cross-linked symmetric hybrid hemoglobin containing ferrous protoporphyrin IX in the α subunits and nickel(II) protoporphyrin IX in the β subunits; XL[α(Ni)β(Fe)]$_2$, cross-linked symmetric hybrid hemoglobin complementary to the preceding one; XL[α(Fe)β(Fe)]-[α(Ni)β(Ni)], cross-linked asymmetric hybrid hemoglobin, containing ferrous protoporphyrin IX in the $\alpha1\beta1$ dimer and nickel(II) protoporphyrin IX in the adjacent $\alpha2\beta2$ dimer; XL[α(Fe)β(Ni)][α(Ni)β(Fe)], cross-linked asymmetric hybrid hemoglobin, containing ferrous protoporphyrin IX in the $\alpha1$ and $\beta2$ subunits and nickel(II) protoporphyrin IX in the $\alpha2$ and $\beta1$ subunits; Tris, tris(hydroxymethyl)aminomethane; Bistris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol; IHP, inositol hexaphosphate; 4-PDS, 4,4′-dithiopyridine.

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A limitation of our experimental approach using hybrid Hbs had been that asymmetric Ni(II)−Fe(II) hybrid Hbs cannot be studied in isolation because un-cross-linked asymmetric tetramers may dissociate into the $\alpha\beta$ dimers, which then reassociate to form not only the former asymmetric tetramers but also the symmetric molecules.

Smith and Ackers (1985) used a deoxy−cyanomet hybrid system, in which ligation is mimicked by cyanide binding to the ferric heme, and demonstrated that the microscopic ligation pathways leading from deoxyHb to cyanometHb are not equivalent. According to the thermodynamic coupling between dimer dissociation and ligand binding equilibria, the free energy change induced by each cyanomet ligation was indirectly estimated from the tetramer−dimer equiibrium constants of corresponding deoxy−cyanomet hybrid Hbs. This experimental strategy enabled them to characterize un-cross-linked asymmetric intermediates as equilibrium mixtures with their parent molecules. Ackers and his colleagues have claimed that the ligation properties along the $\alpha1-\beta1$ pathway are greatly different from those along the other initial-half ligation pathways, $\alpha1-\alpha2$, $\beta1-\beta2$, and $\alpha1-\beta2$ (Perrella et al., 1990a; Daugherty et al., 1991; Doyle & Ackers, 1992; LiCata et al., 1993).

Another novel experimental approach to ligation intermediates has been developed by Perrella et al. (1986, 1990b), who have determined the concentrations of the intermediates in reaction equilibrium between Hb and CO by use of rapid quench and quantitative cryogenic isoelectric focusing techniques. In their study, a marked difference between the symmetric and asymmetric diliganded intermediates was observed: the symmetric diliganded intermediates are absent while the asymmetric ones, [α(Fe-CO)β(Fe-CO)][α(Fe)β(Fe)] and/or [α(Fe-CO)β(Fe)][α(Fe)β(Fe-C)], are present at 50% CO saturation. Unfortunately, this experimental approach has not been applicable to oxygenation intermediates, because of the difficulty in trapping the oxygenation intermediates by selectively oxidizing the deoxyhemes within the intermediates.

Recently, we demonstrated that the oxygen equilibrium properties of highly purified XLHb, in which $\alpha1\beta1$ dimer and $\alpha2\beta2$ dimer are cross-linked between Lys-82β_1 and Lys-82β_2 by reaction with bis(3,5-dibromosalicyl)fumarate, are very similar to those of unmodified Hb with respect to overall oxygen affinity, the first and the fourth Adair constants, cooperativity, and the alkaline Bohr effect (Shibayama et al., 1991). Therefore, it may reasonably be assumed that the cooperative mechanism of the Hb molecule is not altered by this $\beta-\beta$ cross-linking. On the basis of these findings, we have applied this $\beta-\beta$ cross-linking to preparation of cross-linked asymmetric Ni(II)−Fe(II) hybrid, XL[α(Fe)β(Fe)]-[α(Ni)β(Ni)] (Shibayama et al., 1993). Our direct oxygen equilibrium measurements on highly purified cross-linked hybrid showed that the $\alpha1-\beta1$ cooperative interaction in the initial oxygenation stage of Hb is significant, but not so strong as in the case of the cyanomet ligation system.

In this paper, we report on oxygen equilibrium properties of four types of cross-linked Ni(II)−Fe(II) hybrid Hbs, containing two Ni-PP and two Fe^{2+}-PP. These results provide a general mechanistic feature for initial-half oxygenation of Hb. Finally, we use our present data to compare with previous data on other ligation systems using the non-oxygen heme site ligands mentioned above.

EXPERIMENTAL PROCEDURES

Materials. Hb A was prepared as previously described (Shibayama et al., 1991). Bis(3,5-dibromosalicyl)fumarate was synthesized according to the method of Walder et al. (1979) and recrystallized twice from ethanol.

Preparation of Cross-Linked Ni(II)−Fe(II) Hybrid Hbs. Symmetric Ni(II)−Fe(II) hybrid Hbs were prepared as previously reported (Shibayama et al., 1986a). A stoichiometric amount of bis(3,5-dibromosalicyl)fumarate was added to each symmetric hybrid Hb in 0.1 M borate−NaOH buffer, pH 8.95, and allowed to incubate for 2 h at 30 °C under a CO gas atmosphere. After passage through a column of Sephadex G-25 equilibrated with 0.02 M Tris-HCl buffer, pH 7.4, un-cross-linked derivatives were removed by gel-filtration column chromatography on Sephacryl S-100 HR (Pharmacia) in the presence of 1 M $MgCl_2$. The fraction of the tetramer was passed through a column of Sephadex G-25 equilibrated with 0.01 M phosphate buffer, pH 6.85, and then applied to a column of CM52 cellulose (Whatman) equilibrated with the same buffer. The column was eluted by a linear gradient from 0.01 M phosphate buffer, pH 7.10, to 0.015 M phosphate buffer, pH 7.47. The major peak was collected. Kinetic measurements of sulfhydryl reactivity of Cys-93β in both cross-linked symmetric hybrids toward 4-PDS under a deoxygenated condition revealed the presence of an electrophoretically silent impurity with high reactivity; *e.g.*, about 7% impurity in the XL[α(Fe)β(Ni)]$_2$ sample and about 3% impurity in XL[α(Ni)β(Fe)]$_2$. Finally, this impurity was removed by utilizing its much-increased sulfhydryl reactivity as reported by Shibayama et al. (1991).

In our recent paper (Shibayama et al., 1993), one of the asymmetric hybrids, XL[α(Fe-CO)β(Fe-CO)][α(Ni)β(Ni)], was prepared in the following three steps: (i) preparation of asymmetric mixed-valency hybrid, XL[α(Fe^{2+}-PP-CO)β(Fe^{2+}-PP-CO)][α(Fe^{3+}-DP)β(Fe^{3+}-DP)], (ii) substitution of Fe^{3+}-DP by Ni-PP through the heme-exchange reaction, and (iii) removal of the electrophoretically silent impurity. In this study, another asymmetric hybrid, XL[α(Fe-CO)β(Ni)]-[α(Ni)β(Fe-CO)], was prepared by similar three steps except for using [α(Fe^{2+}-PP-CO)β(Fe^{3+}-DP)]$_2$ and [α(Ni)β(Fe-CO)]$_2$ as the parent molecules of this asymmetric hybrid.

On analytical isoelectric focusing using Pharmalyte, pH 6−8 (Pharmacia), each cross-linked hybrid showed a single band that migrated toward the lower pH region compared to unmodified Hb A due to the loss of two positive charges of two Lys-82β residues (data not shown). By sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) analysis, each product showed two bands with nearly equal intensities, one corresponding to the α monomers and the other corresponding to the β dimers (data not shown). Kinetic time courses of sulfhydryl groups of Cys-93β in deoxygenated samples toward 4-PDS are all monophasic, suggesting the absence of high-oxygen-affinity impurity. These data together with our data on highly purified XLHb (Shibayama et al., 1991) strongly indicate that purified samples are cross-linked between Lys-82β_1, and Lys-82β_2.

Oxygen Equilibrium Measurements. Oxygen equilibrium curves were determined with an improved version (Imai, 1981a) of an automatic oxygenation apparatus of Imai et al. (1970) interfaced to a microcomputer (Nippon Electric, Tokyo) for on-line data acquisition, storage, and analysis (Imai, 1994). The oxygen saturation was monitored at 470

nm with a Cary Model 118C spectrophotometer (Varian). It is important to note here that the absorption spectrum of Ni-PP in the α subunit undergoes relatively large changes upon oxygenation of the ferrous subunit of hybrid Hbs. Therefore, we measured the oxygen equilibrium curves of these hybrids at 470 nm, where the absorbance change of Ni-PP is negligible (Shibayama et al., 1993).

To minimize the metheme levels, catalase and superoxide dismutase were added to the Hb samples (Lynch et al., 1976; Winterbourn et al., 1976). In all cases, both the deoxygenation and reoxygenation curves agreed well with each other. Thus, the deoxygenation data were used for two-step Adair analysis. The best-fit values of the first and second intrinsic Adair constants, $K_i[i = 1$ and 2, in reciprocal millimeters of mercury (1 mmHg = 133.3 Pa)], were obtained by fitting a two-step Adair equation to each deoxygenation curve through a least-squares procedure (Imai, 1981b). Oxygen affinity at each step of oxygenation was expressed by K_1 and K_2. Overall oxygen affinity was expressed by partial pressure of oxygen at half-oxygen saturation, P_{50} (in millimeters of mercury). Cooperativity of oxygenation was expressed by the maximal slope of the Hill plot, n_{max}. The P_{50} and n_{max} values were calculated from the K_1 and K_2 values.[2]

The methemoglobin contents were determined according to the method of Evelyn and Malloy (1938) immediately after each measurement.

RESULTS

The Hill plots of the oxygen equilibrium curves of purified XL[α(Fe)β(Ni)]$_2$, XL[α(Ni)β(Fe)]$_2$, and XL[α(Fe)β(Ni)]-[α(Ni)β(Fe)] at various pH values are shown in Figure 1, which also includes published oxygen equilibrium curves of XL[α(Fe)β(Fe)][α(Ni)β(Ni)] (Shibayama et al., 1993). The oxygen equilibrium parameter values, P_{50}, n_{max}, K_1, and K_2, of these four cross-linked Ni(II)−Fe(II) hybrids are listed in Table 1. In Table 1, we also present the parameter values for XLHb (Shibayama et al., 1991) and native HbA (Imai, 1982). Note, that P_{50} and n_{max} values for XLHb and Hb A in Table 1 are calculated from the following formulas: $P_{50} = (K_1K_2)^{-1/2}$ and $n_{max} = 2/[1 + (K_1/K_2)^{1/2}]$, to express the first one-half oxygenation in these tetramers and make direct comparisons with the hybrid easier. The values of log K_1 and log K_2 for both symmetric hybrids, XL[α(Fe)β(Ni)]$_2$ and XL[α(Ni)β(Fe)]$_2$, are plotted against pH in Figure 2A. A similar comparison between both asymmetric hybrids, XL[α(Fe)β(Ni)][α(Ni)β(Fe)] and XL[α(Fe)β(Fe)][α(Ni)βNi)], is shown in Figure 2B.

The lines in Figure 1 were calculated from the best-fit values of two-step Adair constants listed in Table 1. Unlike native Hb A, any hybrid Hbs with only two oxygen binding sites should give symmetric Hill plots of the oxygen equilibrium curves. As seen in Figure 1, the fits are nearly perfect in all the cases, indicating that the experimental plots are symmetric and all our preparations are free from high-oxygen-affinity impurity.

All hybrid Hbs exhibit very low affinity for oxygen. As shown in Table 1, the P_{50} values for these hybrids are comparable to those for XLHb and Hb A, implying that our model can be representative of initial-half oxygenation of Hb A. By comparison of XL[α(Fe)β(Ni)]$_2$ with XL[α(Ni)β(Fe)]$_2$, significant functional heterogeneity of the α and β subunits is observed. With regard to the first oxygen, XL[α(Fe)β(Ni)]$_2$ shows about 3-fold higher affinity than XL[α(Ni)β(Fe)]$_2$ at all pH values examined. For the second step of oxygenation, XL[α(Fe)β(Ni)]$_2$ exhibits about 10−20-fold higher affinity than XL[α(Ni)β(Fe)]$_2$, due to relatively strong α1−α2 cooperative interaction. The mean K_1 values of both symmetric hybrids are 0.0051 mmHg^{-1} at pH 6.4, 0.010 mmHg^{-1} at pH 7.4, and 0.022 mmHg^{-1} at pH 8.4, which are not significantly different from the corresponding K_1 values of XL[α(Fe)β(Ni)][α(Ni)β(Fe)], XL[α(Fe)β(Fe)][α(Ni)β(Ni)], XLHb, and Hb A. This result reinforces the validity of Ni-PP as a model for a fixed deoxyheme.

The cooperativity represented by the Hill coefficient increases in the order of β1β2 ($n = 1.36$), α1β1 ($n = 1.41$), α1β2 ($n = 1.64$), and α1α2 ($n = 1.72$) at pH 7.4 in the presence of 0.1 M Cl$^-$ at 25 °C. In all hybrids, these cooperative interactions are pH-dependent, the Hill coefficients becoming larger as pH increases. Judging from the slope of the plots in Figure 2, the number of protons released at the first oxygenation step for each hybrid is similar to that of XLHb or Hb A. However, slightly more protons are released at the second oxygenation stage for each hybrid compared to XLHb or Hb A. Unlike Hb A, the oxygenation parameters of these cross-linked hybrids are insensitive to IHP (Table 1), due to the fact that the binding site for the phosphate group is occupied by the fumaryl group (Walder et al., 1980; Shibayama et al., 1991).

By use of the oxygen equilibrium parameters in Table 1, the relative populations of the intermediate species at the first and the second oxygenation steps are tentatively evaluated (shown in Table 2). At the first step of oxygenation, the relative population of the α monoliganded intermediate is 3-fold greater than that of the β monoliganded intermediate because of a functional heterogeneity of the subunits in deoxyHb. At the second step of oxygenation, the most striking feature is that one of the diliganded symmetric intermediates, [α(Fe)β(Fe-O$_2$)]$_2$, is almost absent (about 1% of the total diliganded molecules). The population of each asymmetric intermediate is about 20% of the total diliganded molecules, and thus the other symmetric intermediate, [α(Fe-O$_2$)β(Fe)]$_2$, becomes predominant in oxygen equilibrium (about 50−60% of the total diliganded molecules). These features are preserved at all pH values examined.

DISCUSSION

Cross-Linked Symmetric Hybrid Hbs. XL[α(Fe)β(Ni)]$_2$ shows about 3-fold higher affinity for the first oxygen molecule than XL[α(Ni)β(Fe)]$_2$ at all pH values examined.

[2] Since the present oxygen equilibrium curves were obtained from a single preparation of highly purified cross-linked hybrid Hbs, we are not able to report the standard errors of their oxygenation parameters originating from sample-to-sample variations. Instead, we have considerable experience with Hb A sample, so that we can show the standard deviations of K_i ($i = 1-4$), P_{50}, n_{max}, and metHb contents for 60 μM Hb A from 22 measurements in 0.05 M Bistris buffer, pH 7.4, in the presence of 0.1 M Cl$^-$ at 25 °C (Imai, 1994). Mean values and standard deviations (shown in parentheses) of these parameters are as follows: $K_1 = 0.0517$ mmHg^{-1} (37.9%); $K_2 = 0.0398$ mmHg^{-1} (41.5%); $K_3 = 0.453$ mmHg^{-1} (55.8%); $K_4 = 6.9$ mmHg^{-1} (29.4%); $P_{50} = 4.14$ mmHg (6.8%); $n_{max} = 2.99$ (3.3%); metHb contents = 5.5% (42%). Since two-step Adair parameters of cross-linked hybrids (in the present study) can be more restrictively determined than the four-step parameters of Hb A as listed above, it is reasonable to consider that the errors in the present study are much smaller than those of Hb A.

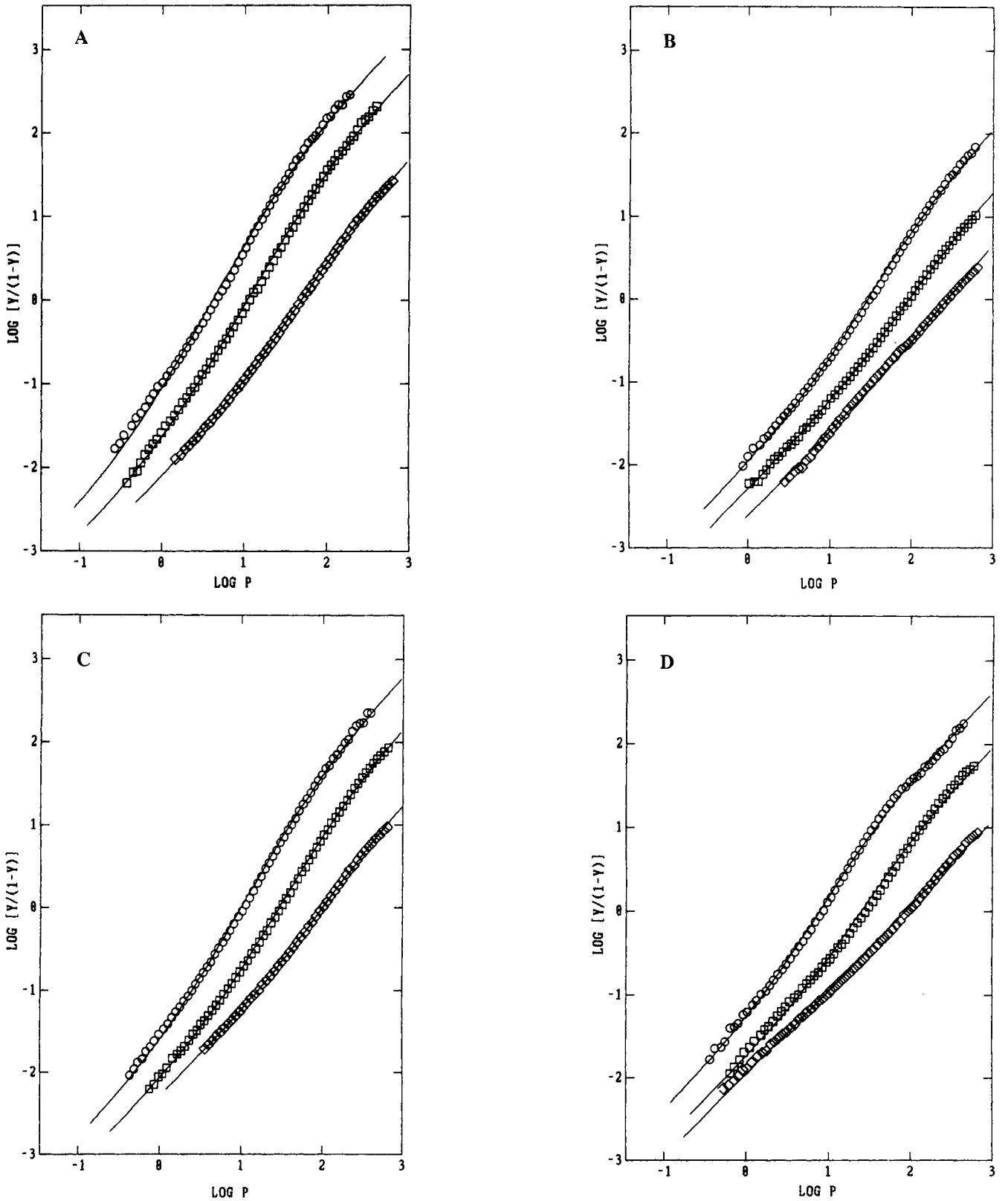

FIGURE 1: Hill plots of oxygen equilibrium curves for XL[α(Fe)β(Ni)]$_2$ (A), XL[α(Ni)β(Fe)]$_2$ (B), XL[α(Fe)β(Ni)][α(Ni)β(Fe)] (C), and XL[α(Fe)β(Fe)][α(Ni)β(Ni)] (D) at pH 6.4 (◇), pH 7.4 (□), and pH 8.4 (○). Other conditions are described in Table 1. Lines were calculated from the best-fit values of the two-step Adair constants listed in Table 1.

This result indicates a significant functional heterogeneity of the α and β subunits in deoxyHb.

According to the stereochemical mechanism, Perutz (1970) predicted a higher affinity of the α subunit relative to the β subunit within deoxyHb, because the steric hindrance of the Val(E11)-β67 to the bound oxygen may play a critical role in lowering the affinity of the β subunit in deoxyHb. There have been several lines of experimental evidence which indicate that the α subunit shows higher oxygen affinity than the β subunit in deoxyHb. Huestis and Raftery (1972) suggested that initial oxygen molecules bind preferentially to the α subunit from ^{19}F nuclear magnetic resonance (NMR)

Table 1: Oxygen Equilibrium Parameters of Cross-Linked Ni(II)−Fe(II) Hybrid Hemoglobins[a]

sample	pH	anion	P_{50}[b] (mmHg)	n_{max}[c]	K_1 (mmHg^{-1})	K_2 (mmHg^{-1})	metHb[d] (%)
XL[α(Fe)β(Ni)]$_2$	6.4	0.1 M Cl^-	49.2	1.45	0.0077	0.054	2.9
	7.4	0.1 M Cl^-	10.9	1.72	0.015	0.55	0.9
	8.4	0.1 M Cl^-	4.17	1.75	0.034	1.7	1.0
	7.4	0.1 M Cl^-, 2 mM IHP	11.5	1.70	0.015	0.49	1.8
XL[α(Ni)β(Fe)]$_2$	6.4	0.1 M Cl^-	274	1.20	0.0024	0.0054	15.5
	7.4	0.1 M Cl^-	93.5	1.36	0.0051	0.023	4.6
	8.4	0.1 M Cl^-	28.9	1.55	0.0099	0.12	2.9
	7.4	0.1 M Cl^-, 2 mM IHP	95.3	1.33	0.0052	0.021	5.8
XL[α(Fe)β(Ni)][α(Ni)β(Fe)]	6.4	0.1 M Cl^-	101	1.33	0.0050	0.019	5.9
	7.4	0.1 M Cl^-	29.4	1.64	0.0074	0.16	2.1
	8.4	0.1 M Cl^-	10.1	1.73	0.016	0.64	1.3
	7.4	0.1 M Cl^-, 2 mM IHP	30.7	1.60	0.0082	0.13	2.1
XL[α(Fe)β(Fe)][α(Ni)β(Ni)][e]	6.4	0.1 M Cl^-	86.2	1.03	0.011	0.012	7.0
	7.4	0.1 M Cl^-	24.9	1.41	0.017	0.096	4.3
	8.4	0.1 M Cl^-	7.7	1.53	0.040	0.42	1.5
	7.4	0.1 M Cl^-, 2 mM IHP	25.4	1.39	0.017	0.090	3.2
XLHb[f]	6.4	0.1 MCl^-	71.9	1.25	0.0084	0.023	2.4
	7.4	0.1 MCl^-	19.0	1.27	0.030	0.092	2.0
	8.4	0.1 M Cl^-	6.85	1.20	0.097	0.22	1.8
	7.4	0.1 M Cl^-, 2 mM IHP	29.5	1.55	0.031	0.37	2.2
Hb A[g]	6.5	0.1 M Cl^-	87.4	0.98	0.0119	0.011	2.0
	7.4	0.1 M Cl^-	27.2	1.26	0.0218	0.062	2.5
	8.4	0.1 M Cl^-	12.6	1.05	0.072	0.0881	3.1
	7.4	0.1 M Cl^-, 2 mM IHP	124	1.23	0.00502	0.013	2.2

[a] Experimental conditions are as follows: temperature, 25 °C; Hb concentration, 60 μM (on a metal basis) for crosslinked hybrid Hbs and XLHb and 600 μM for Hb A; buffer, 0.05 M Tris-HCl or Bistris-HCl. [b] P_{50} values for XLHb and Hb A are calculated by using the formula $P_{50} = (K_1K_2)^{-1/2}$. [c] n_{max} values for XLHb and Hb A are calculated by using the formula $n_{max} = 2/[1 + (K_1/K_2)^{1/2}]$. [d] Methemoglobin contents after measurements. [e] Data from Shibayama et al. (1993). [f] Data from Shibayama et al. (1991). [g] Data from Imai (1982) except for the data at pH 8.4, which are unpublished.

measurements on Hb trifluoroacetylated at Cys-β93. Asakura and Lau (1978) showed that the α subunit exhibits higher oxygen affinity than the β subunit, from electron paramagnetic resonance measurements on Hb in which the heme group of either the α or β subunit was spin-labeled. More quantitative experimental data were reported by Sawicki and Gibson (1977), who performed a laser photolysis experiment on partially saturated Hb in equilibrium with a low concentration of oxygen. According to their assignment of the two observed kinetic components (corresponding to the α and β subunits), the oxygen affinity of the α subunit is higher than that of the β subunit by a factor of about 3 in deoxyHb, *e.g.*, K_1 = 0.024 $mmHg^{-1}$ and 0.0071 $mmHg^{-1}$ for the α and β subunits, res ly, in 50 mM phosphate buffer, pH 7.0, at 20 °C. T sults are entirely consistent with our oxygen equilibr ta on cross-linked Ni(II)−Fe(II) hybrids.

In addition, a quite similar functional heterogeneity of the subunits was recently confirmed by measurements on direct oxygen equilibrium curves for single crystals of Hb A grown in poly(ethylene glycol) with the deoxy quaternary structure (Rivetti et al., 1993). Since the α and β hemes have different orientations with respect to the *a* and *c* crystal axes, the oxygen binding to each subunit could be experimentally distinguishable. According to Rivetti et al. (1993), the α subunit has about 3−5-fold greater affinity than the β subunit in crystals with the deoxy quaternary structure. Such subunit heterogeneity in crystals is in agreement with the present results in solution.

We found that the cooperative interaction between two α subunits is significantly stronger than that between two β subunits (Table 1, Figure 2A). A simple structural explanation for this difference is that the two β subunits within the tetramer have no intersubunit contact in deoxyHb, while the two α subunits share an intersubunit contact in either deoxyHb or oxyHb (Fermi, 1975; Shaanan, 1983). Moreover, it is well-known that the α1−α2 salt bridges formed in deoxyHb play an important role in stabilizing the deoxy quaternary structure. On the other hand, the oxygen-induced structural change in the β1 subunit cannot be directly transmitted to the β2 subunit, so that the β1−β2 interaction should arise from indirect effects. Since we found relatively strong cooperativity along the α1−β2 or α1−β1 pathway, it is possible that the relatively inert β1−β2 cooperativity arises from indirect interaction transmitted through β1−α1−β2 or β1−α2−β2.

Previously, we have reported oxygen equilibrium properties of un-cross-linked symmetric Ni(II)−Fe(II) hybrid Hbs under various solution conditions (Shibayama et al., 1986a). Both hybrids showed noncooperative oxygenation at pH 6.5. Upon raising pH, [α(Fe)β(Ni)]$_2$ showed cooperativity but [α(Ni)β(Fe)]$_2$ did not show cooperativity even at pH 8.5. At all pH values examined, oxygen affinity of [α(Fe)β(Ni)]$_2$ was always higher than that of [α(Ni)β(Fe)]$_2$. There previous findings are qualitatively in agreement with the present results on cross-linked symmetric hybrids. Moreover, our detailed oxygen equilibrium study of highly purified XLHb has revealed that this cross-linking causes little perturbation on the oxygenation properties of unmodified Hb with respect to oxygen affinity, cooperativity, and alkaline Bohr effect (Shibayama et al., 1991). Thus, it is unlikely that the mechanism of the Hb cooperativity is altered by this β−β cross-linking.

By detailed comparison, however, we found several quantitative differences in oxygenation properties between cross-linked and un-cross-linked hybrids [Table 1, values in Table 2 of Shibayama et al. (1986a)]. First, the K_1 values of both cross-linked hybrids are found to be smaller (lower affinity) than those of un-cross-linked hybrids. Second, the Hill coefficients of both cross-linked hybrids are significantly

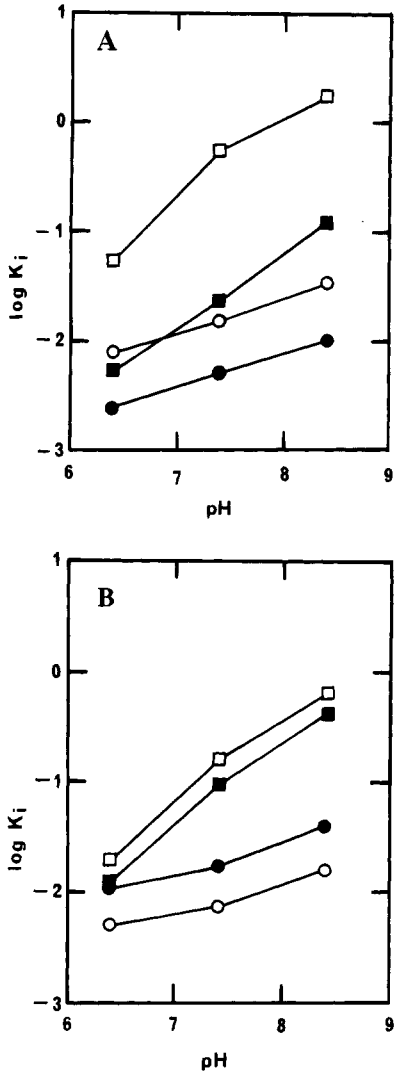

FIGURE 2: (A) pH dependence of Adair constants of cross-linked symmetric Ni(II)−Fe(II) hybrid Hbs, XL[α(Fe)β(Ni)]$_2$ (open symbols) and XL[α(Ni)β(Fe)]$_2$ (closed symbols). (B) pH dependence of Adair constants of cross-linked asymmetric Ni(II)−Fe(II) hybrid Hbs, XL[α(Fe)β(Ni)][α(Ni)β(Fe)] (open symbols) and XL[α(Fe)β(Fe)][α(Ni)β(Ni)] (closed symbols). The data in Table 1 are plotted. ○ and ●, log K_1; □ and ■, log K_2.

larger (more cooperative) than those of un-cross-linked hybrids. It should be noted that these discrepancies are partly due to the presence of dissociated dimers in the earlier study, where we used relatively low concentrations of un-cross-linked hybrids for oxygen equilibrium measurements; *e.g.*, about 16 μM (on a metal basis) (Shibayama et al., 1986a). In fact, our preliminary oxygen equilibrium data on un-cross-linked hybrids at various protein concentrations suggest significant effects of the dimers on oxygenation properties of both un-cross-linked symmetric hybrids. As the protein concentration increases, the oxygen affinities of both un-cross-linked hybrids significantly decrease and the cooperativity of un-cross-linked [α(Fe)β(Ni)]$_2$ becomes more significant (unpublished data). However, another finding of noncooperative oxygenation of un-cross-linked [α(Ni)β(Fe)]$_2$ even at 145 μM suggests the possibility that the cross-linking used in the present study can strengthen the β1−β2 interaction. Even if this were the case, such an effect of the cross-linking could not impair our principal conclusion that [α(Fe)β(Fe-O_2)]$_2$ is the only minor diliganded intermediate

Table 2: Estimation of Equilibrium Populations for the Intermediate Species at the First and Second Oxygenation Steps of Tetrameric Hemoglobin[a]

oxygen-bound subunit(s) within mono- or diliganded intermediate	relative percent population pH 6.4	pH 7.4	pH 8.4	hybrid molecule used for estimation
First Oxygenation Step[b]				
α1	76	75	77	XL[α(Fe)β(Ni)]$_2$
β1	24	25	23	XL[α(Ni)β(Fe)]$_2$
Second Oxygenation Step[c]				
α1α2	47.1	58.9	60.0	XL[α(Fe)β(Ni)]$_2$
β1β2	1.5	0.8	1.2	XL[α(Ni)β(Fe)]$_2$
α1β2	21.5	17.0	21.3	XL[α(Fe)β(Ni)][α(Ni)β(Fe)]
α1β1	29.9	23.3	17.5	XL[α(Fe)β(Fe)][α(Ni)β(Ni)]

[a] Oxygen equilibrium parameters for hybrid Hbs are shown in Table 1. [b] Concentration of each monoliganded intermediate is given by CK_1, where K_1 is the first Adair constant of symmetric hybrid and C is a normalization constant. [c] Concentration of each diliganded intermediate is given by CK_1K_2 (for symmetric hybrid) or $2CK_1K_2$ (for asymmetric hybrid), where K_1 and K_2 are the first and second Adair constants of hybrid and C is a normalization constant.

in oxygen equilibrium of Hb, because the β1−β2 is still the weakest interaction in our cross-linked system.

Cross-Linked Asymmetric Hybrid Hbs. By detailed comparison of the oxygen equilibrium properties between XL[α(Fe)β(Fe)][α(Ni)β(Ni)] and XL[α(Fe)β(Ni)][α(Ni)β(Fe)], the K_1 values of XL[α(Fe)β(Fe)][α(Ni)β(Ni)] are found to be about 2-fold larger than those of XL[α(Fe)β(Ni)]-[α(Ni)β(Fe)], whereas the K_2 values of XL[α(Fe)β(Fe)]-[α(Ni)β(Ni)] are slightly smaller than those of XL[α-(Fe)β(Ni)][α(Ni)β(Fe)] at all pH values (Figure 2B). In consequence, the α1β2 oxygenation is always more cooperative than the α1β1 oxygenation at all pH values (Table 1). It should be pointed out that the α1β2 (or α1β1) is composed of two functionally different subunits. If we take into account a 3-fold functional difference between the subunits as discussed above, the magnitude of intrinsic α1−β2 (or α1−β1) interaction should be stronger than appeared in the Hill coefficient.

If Ni-PP is a perfect model for fixed deoxyheme, the K_1 values of both asymmetric hybrids must agree well each other (the K_2 values need not). Thus, our finding of slight (about 2-fold) differences in the K_1 values is indicative of a quantitative limitation of our model. However, we wish to emphasize here that, in spite of slight discrepancy, our model is probably the most adequate representative of initial oxygenation of Hb A.

Comparison with Other Ligation Systems. Since the CO equilibrium curve exhibits a shape very similar to that of the oxygen equilibrium curve, it has been widely accepted that CO ligation at each subunit modulates the binding affinities of other subunits in a similar way to oxygenation (Anderson & Antonini, 1968). Perrella et al. (1986, 1990b) have determined the concentrations of intermediates in the reaction equilibrium between Hb A and CO at 20 °C, pH 7.0, in the presence of 0.1 M KCl. They found that the diliganded intermediate(s) existing in detectable amounts can be [α(Fe-CO)β(Fe-CO)][α(Fe)β(Fe)] and/or [α(Fe-CO)β(Fe)]-[α(Fe)β(Fe-CO)], because the concentrations of the symmetric intermediates are below the detection limit of their method [*e.g.*, < 0.5% of the total Hb at 50% CO saturation (or <3% of the total diliganded Hbs)] (Perrella et al., 1990b). Our present results indicate that one of the symmetric

intermediates, $[\alpha(Fe)\beta(Fe\text{-}O_2)]_2$, is a negligible diliganded intermediate in the oxygen equilibrium of tetrameric Hb A (Table 2). This finding is in agreement with the previous data on CO ligation. However, our results also indicate that another symmetric intermediate, $[\alpha(Fe\text{-}O_2)\beta(Fe)]_2$, should exceed both the asymmetric intermediates in equilibrium concentration (Table 2). This finding sharply contradicts the previous observation on CO intermediates. If CO ligation is a close analog to oxygenation, why was $[\alpha(Fe\text{-}CO)\beta(Fe)]_2$ almost absent in the previous study? The most likely interpretation of this discrepancy is that functional heterogeneity of the α and β subunits in CO ligation is different from that in oxygenation, since the functional heterogeneity of the subunits should depend on the nature of heme site ligand. In fact, Perrella et al. (1986) reported that more CO bound to the β subunit than to the α subunit in monoliganded species at 50% CO saturation. This indicates that the β subunits show higher affinity for CO than the α subunits in deoxyHb. This inverse relation in subunit heterogeneity might be due to less steric hindrance of distal Val(E11)-β67 to CO than to oxygen. As a result of elevated affinity of the β subunit, the equilibrium concentration of $[\alpha(Fe\text{-}CO)\beta(Fe)]_2$ relative to the other diliganded intermediates should be decreased, even if significant α1−α2 interaction is preserved in CO ligation. Also, we wish to point out here an experimental difficulty in detection of $[\alpha(Fe\text{-}CO)\beta(Fe)]_2$ as well as $[\alpha(Fe)\beta(Fe\text{-}CO)]_2$ by their cryogenic analysis. Even though all of the possible CO ligation pathways were functionally equivalent, the ratio of equilibrium concentrations of the diliganded intermediates, namely, $[\alpha(Fe\text{-}CO)\beta(Fe)]_2$:$[\alpha(Fe\text{-}CO)\beta(Fe\text{-}CO)][\alpha(Fe)\beta(Fe)]$:$[\alpha(Fe\text{-}CO)\beta(Fe)][\alpha(Fe)\beta(Fe\text{-}CO)]$:$[\alpha(Fe)\beta(Fe\text{-}CO)]_2$, would be 1:2:2:1 (not 1:1:1:1). Because of the inability of the cryogenic technique to resolve both asymmetric diliganded molecules, the expected ratio of the three resolved peaks is 1:4:1 for $[\alpha(Fe\text{-}CO)\beta(Fe)]_2$:$[\alpha(Fe\text{-}CO)\beta(Fe\text{-}CO)][\alpha(Fe)\beta(Fe)]$ + $[\alpha(Fe\text{-}CO)\beta(Fe)][\alpha(Fe)\beta(Fe\text{-}CO)]$:$[\alpha(Fe)\beta(Fe\text{-}CO)]_2$. Accordingly, this inherent disadvantage should also make it difficult to resolve $[\alpha(Fe\text{-}CO)\beta(Fe)]_2$ in the previous CO equilibrium studies (Perrella et al., 1986, 1990b).

Ackers and his colleagues have determined the free energies of tetramer−dimer equilibria for all 10 ligation states of Hb, using deoxy−cyanomet hybrid Hbs as the ligation intermediates (Smith & Ackers, 1985; Smith et al., 1987; Perrella et al., 1990a, Daugherty et al., 1991; Speros et al., 1991; Doyle & Ackers, 1992; Ackers et al., 1992; LiCata et al., 1993). Since there is a thermodynamic linkage between the dimer dissociation and ligand binding equilibria, the free energy change induced by each ligation was indirectly estimated. The most remarkable result obtained is that the free energy of $[\alpha(Fe^+CN)\beta(Fe^+CN)][\alpha(Fe)\beta(Fe)]$ is distinctly different from those of the other three diliganded intermediates at pH 7.4, at 21.5 °C in the presence of 0.1 M NaCl.[3] According to a tetramer−dimer equilibrium study on the cyanomet ligation system by Perrella et al. (1990a), cyanomet ligation along the α1−β1 pathway shows remarkable cooperativity with a Hill coefficient of 1.9 (corresponding to a 170-fold change in affinity) while those along the other pathways, α1−α2, β1−β2, and α1−β2, exhibit noncooperativity with a Hill coefficient of unity (no change in affinity). As a result of considerable coomerativity along the α1−β1 pathway, the equilibrium population of $[\alpha(Fe^+CN)\beta(Fe^+CN)][\alpha(Fe)\beta(Fe)]$ relative to the total diliganded species goes to about 99%. Clearly, our present results do not support such an outstanding feature of α1β1 ligation (Table 2). Also, an apparent discrepancy is seen in macroscopic behavior between cyanomet ligation and oxygenation of Hb A: the relative magnitudes of macroscopic stepwise binding constants are in the order of $K_1 \leq K_3 < K_2 \leq K_4$ for cyanomet ligation vs $K_1 \leq K_2 < K_3 \leq K_4$ for oxygenation of native Hb A (Imai, 1982). This striking feature of cyanomet ligation results in unusual negative cooperativity and considerable accumulation of the diliganded intermediates in equilibrium condition. Moreover, Perrella et al. (1990b) demonstrated that the free energy of $[\alpha(Fe\text{-}CO)\beta(Fe\text{-}CO)][\alpha(Fe)\beta(Fe)]$ cannot be significantly different from those of the other diliganded intermediates in CO ligation. Therefore, they have argued whether the cyanomet ligation system is satisfactory as a representative of oxygenation or CO ligation (Perrella et al., 1990b, 1992).

It should be important here to point out a plausible origin of these puzzling results of the cyanomet ligation system. The main difficulty in treating cyanomet ligation is that the free energy change induced by cyanomet ligation arises from both heme oxidation (from Fe^{2+} to Fe^{3+}) and cyanide binding (to the ferric heme). It has been well-known that the oxidation−reduction equilibrium of Hb is a cooperative process (Brunori et al., 1969), so that a significant part of the cooperative free energy could originate from a "cooperative" oxidation reaction of Hb. In contrast to oxygenation of Hb, however, equilibrium and kinetic measurements of the oxidation reaction of Hb have revealed that the difference in electron affinities of the subunits is very large and is significantly affected by pH, subunit assembly, and molecular forms (T or R) (Brunori et al., 1968, 1969; Perrella et al., 1993). Furthermore, Ackers and his colleagues have assumed that dissociated dimers bind two ligands noncooperatively with a very high affinity in a similar manner to the dimeric oxygenation, although virtually nothing is known about the functional properties of the dimers in the cyanomet ligation system. Since the indirect estimates of the ligation free energy by Ackers and colleagues are critically dependent on the assumptions on the dimeric properties, the validity of these assumptions should be experimentally assured.

Features of Initial-Half Oxygenation of Hb. The finding of approximately 3-fold affinity of the α subunits in deoxyHb together with relatively strong α1−α2 interaction makes the α1−α2 oxygenation pathway thermodynamically advantageous. Although such functional heterogeneity of the subunits masks some of the intrinsic cooperativities along the asymmetric pathways, α1−β1 and α1−β2, both asymmetric interactions are still significant. Therefore, both asymmetric pathways are also of importance for oxygenation

[3] Daugherty et al. (1992) suggested that $[\alpha(Fe^+CN)\beta(Fe^+CN)][\alpha(Fe)\beta(Fe)]$ is in the T-state while the other diliganded deoxy-cyanomet hybrids are in the R-states. On the basis of this finding, Ackers et al. (1992) have proposed a combinatorial switching mechanism of Hb; *i.e.*, quaternary switching from T to R is governed by the presence of at least one heme site ligand on each of the αβ dimeric half-molecules within the tetramer. This symmetry rule predicts that $[\alpha(Fe\text{-}O_2)\beta(Fe\text{-}O_2)][\alpha(Fe)\beta(Fe)]$ is in the T-state while the other diliganded oxygenation intermediates are in the R-state (Ackers et al., 1992). Since the present studies do not provide any information regarding the quaternary structures of the diliganded cross-linked Ni(II)−Fe(II) hybrids, it is difficult to discuss the relevance of the present results to the symmetry rule. To test the symmetry rule, sulfhydryl reaction kinetics and nuclear magnetic resonance studies on the cross-linked Ni(II)−Fe(II) hybrid Hbs are now in progress.

of Hb (Table 2). In contrast, lower affinity of the β subunits in addition to relatively weak $\beta1-\beta2$ interaction results in remarkable reduction of thermodynamic stability of $[\alpha(Fe)\beta(Fe\text{-}O_2)]_2$. In consequence, $[\alpha(Fe)\beta(Fe\text{-}O_2)]_2$ is the only minor diliganded intermediates in oxygenation of Hb (Table 2). In addition, special care must be taken to compare with other ligation systems using non-oxygen ligands, because ignorance of possible ligand-dependent functional heterogeneity of the subunits often leads to quantitatively different expressions of the same mechanism.

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