Oxygen Equilibrium Properties of Asymmetric Nickel(II)–Iron(II) Hybrid Hemoglobin†

Naoya Shibayama,*,‡ Kiyohiro Imai,§ Hideki Morimoto,‖ and Satoshi Saigo‡

Department of Physics, Jichi Medical School, Minamikawachi, Tochigi 329-04, Japan, Department of Physiology, Medical School, Osaka University, Suita, Osaka 565, Japan, and Department of Biophysical Engineering, Faculty of Engineering Science, Osaka University, Toyonaka, Osaka 560, Japan

Received February 17, 1993; Revised Manuscript Received May 19, 1993

ABSTRACT: Asymmetric Ni(II)–Fe(II) hybrid hemoglobin, XL[α(Fe)β(Fe)][α(Ni)β(Ni)], in which the $\alpha 1\beta 1$ dimer containing ferrous protoporphyrin IX and the complementary $\alpha 2\beta 2$ dimer containing Ni(II) protoporphyrin IX were cross-linked between Lys-82β_1 and Lys-82β_2 by reaction with bis(3,5-dibromosalicyl) fumarate, was synthesized and characterized. We have previously shown that (i) Ni(II) protoporphyrin IX, which binds neither oxygen nor carbon monoxide, mimics a fixed deoxyheme with respect to its effect on the oxygen equilibrium properties of the counterpart iron subunits in both symmetric Ni(II)–Fe(II) hybrid Hbs [Shibayama, N., Morimoto, H., & Miyazaki, G. (1986) *J. Mol. Biol. 192*, 323–329] and (ii) the cross-linking used in this study little affects the oxygen equilibrium properties of hemoglobin [Shibayama, N., Imai, K., Hirata, H., Hiraiwa, H., Morimoto, H., & Saigo, S. (1991) *Biochemistry 30*, 8158–8165]. These remarkable features of our model allowed us to measure the oxygen equilibrium curves for the first two steps of oxygen binding to the $\alpha 1\beta 1$ dimer within the hemoglobin tetramer. At all pH values examined, the affinities of this asymmetric hybrid for the first oxygen molecule are as low as those of native hemoglobin. The hybrid did not show cooperative oxygen binding at pH 6.4, while significant cooperativity was observed with rising pH; *i.e.*, the Hill coefficient was increased from 1.41 to 1.53 upon a pH change from 7.4 to 8.4. The electronic absorption spectrum of Ni(II) protoporphyrin IX in the $\alpha 2$ subunit was changed upon carbon monoxide (or oxygen) binding to the $\alpha 1\beta 1$ dimer. This change provides additional information about the interaction between liganded and unliganded dimers within the asymmetric tetramer.

Even though cooperative oxygenation of human adult Hb (Hb A)[1] has been studied extensively as a paradigm for regulatory actions of allosteric proteins, the molecular mechanism of Hb is still an elusive problem. This is mostly due to the strong cooperativity of Hb oxygenation, which makes it difficult to characterize the oxygenation intermediates. Thus, the majority of the information available has been limited to the two end-state species, namely, deoxyHb and oxyHb, and little is known about the oxygenation intermediates.

One of the most specific methods for studying the oxygenation intermediates is to substitute the heme in one or more of the four subunits with another metalloprotoporphyrin (Ikeda-Saito et al., 1977; Blough & Hoffman, 1982; Simolo et al., 1985; Shibayama, 1986a; Inubushi et al., 1986). Since the properties of the metal-substituted hybrid Hbs appeared to be directly affected by the electronic configuration of substituted metal ion, the successful execution of such studies should depend upon the choice of metalloporphyrin.

We have chosen Ni-PP to be an adequate model for a fixed deoxyheme among various metalloprotoporphyrins containing iron-series transition-metal ions, as judged from the oxygen equilibrium properties of both symmetric hybrid Hbs, α_2(Ni)β_2(Fe) and α_2(Fe)β_2(Ni), under various solution conditions (Shibayama et al., 1986a). Moreover, the validity of Ni-PP as a model for the deoxyheme has been confirmed by other physicochemical measurements (Shibayama et al., 1986b, 1987; Alston et al., 1984). Another advantage of using Ni(II)–Fe(II) hybrid Hbs has been that the electronic absorption spectrum of the α(Ni) subunit can be a good structural marker for the hybrid Hbs, since the coordination states of Ni-PP in the α subunits are dependent upon the structure of Hb; *i.e.*, the four-coordinated state is dominant in a deoxy-quaternary structure, while in an oxy-quaternary structure the five-coordinated state is significantly increased (Shibayama et al., 1986a,b, 1987).

Relatively weak interaction within the $\alpha 1\beta 1$ dimer has been generally accepted as a feature of Hb tetramer. Crystallographic studies on deoxyHb and fully liganded Hb have shown that ligand binding causes large changes at the $\alpha 1\beta 2$ interface with minor variations at the $\alpha 1\beta 1$ interface (Baldwin & Chothia, 1979). Moreover, there have been several pieces of experimental evidence against cooperativity within the dis-

† This work was supported by a research grant from the Ministry of Education, Science and Culture of Japan (01780314 to N.S.) and by a research fund from Ajinomoto Co., Tokyo (to K.I.).

‡ Jichi Medical School.
§ Osaka University Medical School.
‖ Osaka University Faculty of Engineering Science.

[1] Abbreviations: Hb A, human adult hemoglobin; Hb, hemoglobin; XLHb, hemoglobin cross-linked between Lys-82β_1 and Lys-82β_2 by reaction with bis(3,5-dibromosalicyl) fumarate; 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; NiHb, hemoglobin containing nickel(II) protoporphyrin IX in both the α and β subunits; α_2(Fe)β_2(M), symmetric hybrid hemoglobin containing ferrous protoporphyrin IX in the α subunits and metalloprotoporphyrin IX in the β subunits; α_2(M)β_2(Fe), symmetric hybrid hemoglobin complementary to the preceding one; XL[α(Fe^{2+}-PP-CO)β(Fe^{2+}-PP-CO)][α(Fe^{3+}-DP)β(Fe^{3+}-DP)], asymmetric mixed-valency hybrid hemoglobin, in which the $\alpha 1\beta 1$ dimer containing carbon monoxide complex of ferrous protoporphyrin IX and $\alpha 2\beta 2$ dimer containing ferric deuteroporphyrin IX were cross-linked between Lys-82β_1 and Lys-82β_2 by reaction with bis(3,5-dibromosalicyl) fumarate; XL[α(Fe)β(Fe)][α(Ni)β(Ni)], asymmetric hybrid hemoglobin, in which the $\alpha 1\beta 1$ dimer containing ferrous protoporphyrin IX and the complementary $\alpha 2\beta 2$ 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; Tris, tris(hydroxymethyl)aminomethane; Bistris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol; IHP, inositol hexaphosphate; 4-PDS, 4,4′-dithiopyridine.

0006-2960/93/0432-8792$04.00/0

sociated $\alpha 1\beta 1$ dimer (Hewitt et al., 1972; Chu et al., 1984). From these findings, it has been generally believed that the $\alpha 1\beta 2$ interface plays an essential role in heme–heme interaction of Hb, whereas the $\alpha 1\beta 1$ interface does not. However, this idea has been recently questioned by several investigators showing experimental evidence for a significant role of the $\alpha 1\beta 1$ interface within the tetramer (Kawamura-Konishi & Suzuki, 1988; ElAntri et al., 1989; Daugherty et al., 1991; Levy et al., 1992; Ackers et al., 1992). Especially, Ackers and his colleagues have measured the tetramer–dimer equilibrium of deoxycyanomet hybrid Hbs as the ligation intermediates and have suggested the presence of an extremely strong cooperative interaction within the $\alpha 1\beta 1$ dimer prior to the quaternary transition from T to R (Daugherty et al., 1991; Ackers et al., 1992).

Another novel experimental approach has been developed by Perrella and his co-workers, who have resolved the populations of the intermediates at equilibrium and during approach to equilibrium (Perrella et al., 1986, 1990a,b). By comparing data on the intermediates with different ligation systems, such as CO, NO, and cyanomet ligation systems, they have shown that the properties of the $\alpha 1\beta 1$-liganded intermediate depend upon the nature of the heme ligand. Unfortunately, neither the experimental approach by Ackers et al. (1992) nor that by Perrella et al. (1990b) has necessarily been applicable to oxygenation intermediates. We have prepared an asymmetric Ni(II)–Fe(II) hybrid Hb, [α(Fe)β-(Fe)][α(Ni)β(Ni)], to study its oxygen equilibrium properties.

Such an asymmetric hybrid Hb could not be studied in an isolated form, because asymmetric tetramers without cross-linking may dissociate into the $\alpha\beta$ dimers which then reassociate to form not only the former asymmetric tetramers but the symmetric molecules. A strategy for circumventing this difficulty was developed by Miura and Ho (1982) using an intramolecular cross-linking technique. This method has made it possible to isolate cross-linked asymmetric cyanomet valency hybrids (Miura & Ho, 1982; Miura et al., 1987), cross-linked asymmetric chemically modified hybrids (Miura & Ho, 1984), and cross-linked asymmetric Co(II)–Fe(II) hybrids (Inubushi et al., 1986; Kitagishi et al., 1988; Kaminaka et al., 1989). In these preparations, the bifunctional cross-link agent, bis(3,5-dibromosalicyl) fumarate, which cross-links Lys-82β_1 and Lys-82β_2 in oxyHb (Walder et al., 1980), was commonly used to lock the tetrameric forms. Until quite recently, it has been believed that this cross-linking causes significant perturbations on Hb oxygenation, which result in a remarkably high affinity for the first oxygen molecule, significantly decreased cooperativity, and a reversal of the order of K_1 and K_3 values (Walder et al., 1980; Miura et al., 1987). Due to such perturbations, it may not be appropriate to use the cross-linked hybrid Hbs for the study of intermediate molecules.

Recently, we have solved this problem of cross-linking by removal of an electrophoretically silent impurity which has contaminated all previous samples cross-linked between Lys-82β_1 and Lys-82β_2 by bis(3,5-dibromosalicyl) fumarate (Shibayama et al., 1991). To remove this impurity, we have introduced a unique purification method, which utilizes a difference in reactivity of the sulfhydryl groups of cysteine-93β between the authentic XLHb and the impurity under a deoxygenated condition. After this procedure, the oxygen equilibrium properties of purified XLHb became very similar to those of unmodified Hb with respect to oxygen affinity, cooperativity, and the alkaline Bohr effect. Therefore, the cross-linking between Lys-82β_1 and Lys-82β_2 by the fumaryl group has proved to be a good chemical modification for preparing asymmetric hybrid Hbs.

In this paper, we have developed a novel preparation method for cross-linked asymmetric Ni(II)–Fe(II) hybrid Hb, XL-[α(Fe)β(Fe)][α(Ni)β(Ni)], which may be an adequate model for studying the oxygenation properties of the $\alpha 1\beta 1$ dimer within the tetramer. Oxygenation cooperativity of XL[α-(Fe)β(Fe)][α(Ni)β(Ni)] is a good measure of the $\alpha 1\beta 1$ interaction, while the absorption change of Ni-PP in the $\alpha 2$ subunit accompanying the ligation of the $\alpha 1\beta 1$ dimer provides additional information for the interaction between the liganded dimer and the unliganded dimer within the asymmetric tetramer.

EXPERIMENTAL PROCEDURES

Materials. Hb A was prepared as described in our recent paper (Shibayama et al., 1991). ApoHb A was prepared according to the method of Yonetani (1967). Ferric deuteroHb A was prepared as reported by Antonini et al. (1964) with minor modifications. 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 Asymmetric Ni(II)–Fe(II) Hybrid Hb. 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; (iii) removal of the electrophoretically silent impurity.

(*i*) XL[α(Fe^{2+}-PP-CO)β(Fe^{2+}-PP-CO)][α(Fe^{3+}-DP)β-(Fe^{3+}-DP)] was prepared according to the method of Miura and Ho (1982) with the following modifications. For the dimer-exchange reaction, HbCO and equimolar ferric deuteroHb in 0.1 M borate–NaOH buffer, pH 8.95, were incubated for 1 h at 0 °C in the presence of 0.1 M NaF under a CO gas atmosphere. Then, a stoichiometric amount of bis-(3,5-dibromosalicyl) fumarate was added to the reaction mixture 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, uncross-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 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 of 0.01 M phosphate buffer, pH 7.10, and 0.015 M phosphate buffer, pH 7.47. Three major peaks, which correspond to XLHb-(Fe^{2+}-PP-CO), XL[α(Fe^{2+}-PP-CO)β(Fe^{2+}-PP-CO)][α(Fe^{3+}-DP)β(Fe^{3+}-DP)], and XLHb(Fe^{3+}-DP), were eluted in the order of increasing number of ferric deutero subunits. The second peak corresponding to the asymmetric valency hybrid, XL[α(Fe^{2+}-PP-CO)β(Fe^{2+}-PP-CO)][α(Fe^{3+}-DP)β(Fe^{3+}-DP)], was collected.

(*ii*) For heme-exchange reaction, a 10-fold stoichiometric amount of Ni-PP dissolved in a minimal amount of *N,N'*-dimethylformamide was added to the XL[α(Fe^{2+}-PP-CO)β-(Fe^{2+}-PP-CO)][α(Fe^{3+}-DP)β(Fe^{3+}-DP)] in 1 M Gly–NaOH buffer, pH 8.50, and the mixture was incubated for 2 h at 30 °C under a CO gas atmosphere. After passage through a column of Sephadex G-25 equilibrated with 0.01 M phosphate buffer, pH 6.85, Hb was applied to a column of CM52 cellulose equilibrated with the same buffer. The column was eluted in a linear gradient manner with 0.01 M phosphate buffer, pH

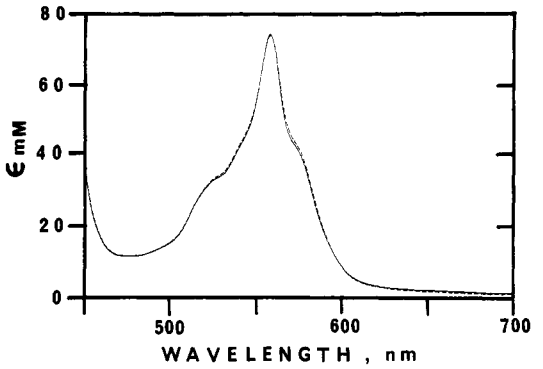

FIGURE 1: Comparison of electronic absorption spectrum of deoxygenated XL[α(Fe)β(Fe)][α(Ni)β(Ni)] (—) with a computer-simulated spectrum for an equimolar mixture of deoxy FeHb and NiHb (- - -) in 0.1 M phosphate buffer at pH 7.0, at 10 °C. ϵ_{mM} is a millimolar coefficient of tetrameric Hb.

6.91, and 0.015 M phosphate buffer, pH 7.35. A main peak corresponding to XL[α(Fe-CO)β(Fe-CO)][α(Ni)β(Ni)] was collected.

(*iii*) Kinetic measurement of sulfhydryl reactivity of Cys-93β in XL[α(Fe)β(Fe)][α(Ni)β(Ni)] toward 4-PDS under a deoxygenated condition revealed about 3% impurity with high reactivity (data not shown). This impurity was removed by utilizing its much increased sulfhydryl reactivity as reported in our recent paper (Shibayama et al., 1991).

Identification of XL[α(Fe)β(Fe)][α(Ni)β(Ni)]. On analytical isoelectric focusing using Pharmalyte, pH 6–8 (Pharmacia), purified XL[α(Fe-CO)β(Fe-CO)][α(Ni)β(Ni)] 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, the 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). A kinetic time course of sulfhydryl groups of Cys-93β in deoxygenated XL[α(Fe)β(Fe)][α(Ni)β(Ni)] toward 4-PDS is monophasic, suggesting the absence of a high-oxygen-affinity impurity. These data together with our recent data on highly purified XLHb (Shibayama et al., 1991) strongly indicate that purified XL[α(Fe)β(Fe)][α(Ni)β(Ni)] is cross-linked between Lys-82β_1 and Lys-82β_2.

To assure the composition of Ni-PP and Fe^{2+}-PP in purified XL[α(Fe)β(Fe)][α(Ni)β(Ni)], we compared the electronic absorption spectrum of deoxygenated XL[α(Fe)β(Fe)][α(Ni)β(Ni)] with a computer-simulated spectrum for an equimolar mixture of deoxy FeHb and NiHb. As shown in Figure 1, two spectra are indistinguishable from each other, indicative of the hybrid with 1:1 Ni-PP:Fe^{2+}-PP content. We have previously reported that in a deoxy-quaternary structure the absorption spectrum of the α(Ni) subunit quite differs from that of the β(Ni) subunit because Ni-PP in the α subunit is predominantly four-coordinated in a deoxy-quaternary structure, while that in the β subunit is always five-coordinated (Shibayama, 1986a). Therefore, the nearly perfect agreement between the two absorption spectra in Figure 1 suggests not only 1:1 Ni-PP:Fe^{2+}-PP content but also 1:1 α(Ni):β(Ni) content in the asymmetric hybrid. These results are entirely consistent with the expected compositions of XL[α(Fe)β(Fe)]-[α(Ni)β(Ni)].

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. 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 α2 subunit undergoes relatively large changes upon oxygenation of XL[α(Fe)β(Fe)][α(Ni)β(Ni)]. Therefore, we measured the oxygen equilibrium curves of this hybrid at 470 nm, where the absorbance change of Ni-PP is negligible (see Figure 4B).

To minimize the methemoglobin 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 $mmHg^{-1}$ (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 mmHg). 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.

Electronic Absorption Spectra. Electronic absorption spectra of XL[α(Fe-CO)β(Fe-CO)][α(Ni)β(Ni)] under CO gas atmosphere were recorded on a Model U-3210 spectrophotometer (Hitachi, Tokyo). Measurements on XL[α(Fe)β-(Fe)][α(Ni)β(Ni)] and Hb A under a deoxygenated condition were carried out by using a tonometer without dithionite as described previously (Shibayama et al., 1991).

RESULTS

Oxygen Equilibrium Properties of XL[α(Fe)β(Fe)][α-(Ni)β(Ni)]. The Hill plots of the oxygen equilibrium curves of purified XL[α(Fe)β(Fe)][α(Ni)β(Ni)] at various pH values are shown in Figure 2. The oxygen equilibrium parameter values, P_{50}, n_{max}, K_1, and K_2, are listed in Table I, which also includes K_1 values of XLHb (Shibayama et al., 1991) and of native Hb A (Imai, 1982) for comparison. The values of log K_1 and log K_2 of XL[α(Fe)β(Fe)][α(Ni)β(Ni)] are plotted against pH in Figure 3.

[2] Since the present oxygen equilibrium curves were obtained from a single preparation of highly purified hybrid Hb, we are not able to report the standard errors of its oxygenation parameters originating from sample-to-sample variations. Instead, we have considerable experience with Hb A samples, so that we can show the standard deviations of K_i (i = 1–4), P_{50}, n_{max}, and metHb contents for 60 μM of Hb A from 22 measurements in 0.05 Bistris buffer, pH 7.4, in the presence of 0.1 M Cl^- at 25 °C (Imai, 1993). 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 the hybrid (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. Moreover, we have determined oxygen equilibrium curves of several preparations of the hybrid, which still contained high-oxygen-affinity impurity, and found a good correlation between the oxygenation parameter values and the impurity contents: as the impurity contents decreased, the K_1 value was consistently decreased and the n_{max} value was consistently increased. This observation also indicates relatively small standard deviations of the oxygenation parameters for the hybrid Hb.

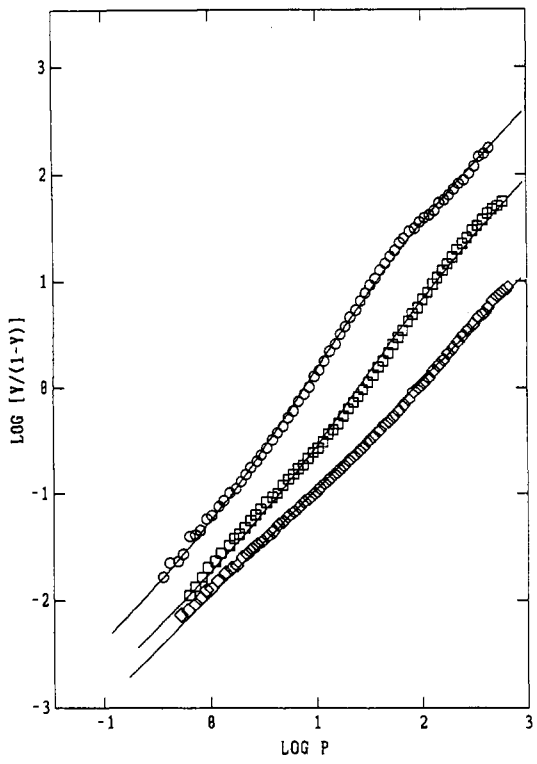

FIGURE 2: Hill plots of oxygen equilibrium curves for XL[α(Fe)β(Fe)][α(Ni)β(Ni)] at pH 6.4 (◇), 7.4 (□), and 8.4 (○). Other conditions are described in Table I. Lines were calculated from the best-fit values of the two-step Adair constants listed in Table I.

Table I: Oxygen Equilibrium Parameters of XL[α(Fe)β(Fe)][α(Ni)β(Ni)]

conditions[a]		P_{50}	n_{max}	K_1	K_2	metHb[b]
pH	anion	(mmHg)		($mmHg^{-1}$)	($mmHg^{-1}$)	(%)
6.4	0.1 M Cl^-	86.2	1.03	0.0110 XLHb: 0.0084[c] Hb A: 0.0119[d] (at pH 6.5)	0.0122	7.0
7.4	0.1 M Cl^-	24.9	1.41	0.0170 XLHb: 0.030[c] Hb A: 0.0218[d]	0.0957	4.3
8.4	0.1 M Cl^-	7.7	1.53	0.0400 XLHb: 0.097[c] Hb A: 0.0720[d]	0.424	1.5
7.4	0.1 M Cl^- + 2 mM IHP	25.4	1.39	0.0173 XLHb: 0.031[c] Hb A: 0.0052[d]	0.0897	3.2

[a] Other conditions were as follows: temperature, 25 °C; Hb concentration, 60 μM (on a metal basis) for XL[α(Fe)β(Fe)][α(Ni)β(Ni)] and XLHb and 600 μM (on a metal basis) for Hb A; buffer, 0.05 M Tris-HCl or Bistris-HCl. [b] Methemoglobin contents after measurements. [c] Data from Shibayama et al. (1991) under similar conditions. [d] Data from Imai (1982) under similar conditions.

The lines in Figure 2 were calculated from the best-fit values of two-step Adair constants listed in Table I. 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 2, the fits are excellent at all pH values, indicating that the experimental plots are symmetric and our preparations are free from high-oxygen-affinity impurity.

In the absence of IHP, the K_1 values of XL[α(Fe)β(Fe)][α(Ni)β(Ni)] are similar to those of XLHb and Hb A at all pH values (Table I; Figure 3). Unlike Hb A, the oxygenation parameters of the present cross-linked asymmetric hybrid are insensitive to IHP (Table I), due to the fact that the binding site for the phosphate group is occupied by the fumaryl group

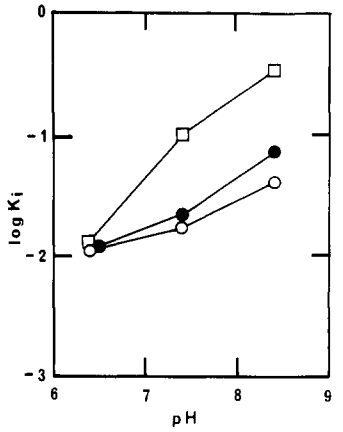

FIGURE 3: pH dependence of Adair constants of XL[α(Fe)β(Fe)][α(Ni)β(Ni)] (open symbols) and Hb A (solid symbols). The data in Table I are plotted. (○ and ●) log K_1; (□) log K_2.

(Walder et al., 1980; Shibayama et al., 1991).

At pH 6.4, XL[α(Fe)β(Fe)][α(Ni)β(Ni)] shows nearly noncooperative oxygenation (n_{max} = 1.03), while it exhibits significant cooperativity at pH 7.4 and 8.4; *i.e.*, n_{max} = 1.41 at pH 7.4, and n_{max} = 1.53 at pH 8.4.

Electronic Absorption Spectrum of XL[α(Fe-CO)β(Fe-CO)][α(Ni)β(Ni)]. Since the oxygen affinity of XL[α(Fe)β(Fe)][α(Ni)β(Ni)] is as low as that for deoxyHb A, complete saturation of the hybrid cannot be attained even under an atmospheric oxygen pressure. Since saturation with CO could be easily achieved even at acidic pH value, CO was used as the heme ligand for the spectral measurements of liganded hybrid.

Figure 4A shows pH-dependent electronic absorption spectra of XL[α(Fe-CO)β(Fe-CO)][α(Ni)β(Ni)]. Since the absorption spectrum of deoxygenated XL[α(Fe)β(Fe)][α(Ni)β(Ni)] is not affected by solution conditions, the spectral change of Ni-PP induced by CO ligation should be pH dependent. These spectral changes of Ni-PP accompanied by CO binding to XL[α(Fe)β(Fe)][α(Ni)β(Ni)] were obtained by subtracting the spectra of the α(Ni)β(Ni) within XL[α(Fe)β(Fe)][α(Ni)β(Ni)] from those within XL[α(Fe-CO)β(Fe-CO)][α(Ni)β(Ni)] at various pH values (Figure 4B).

We have previously reported that Ni-PP in the β subunits is always five-coordinated, while the coordination state of Ni-PP in the α subunits depends on the structural state of Hb (Shibayama et al., 1986a). Thus, it is reasonable to assume that the spectral changes observed in Figure 4A,B originate mostly from the changes of coordination state of Ni-PP in the α2 subunit. As seen in Figure 4B, the coordination state of Ni-PP is converted from a four-coordination state (corresponding to the negative peak at 557 nm) to a five-coordination state (corresponding to the positive peaks at 539 and 576 nm) upon CO binding to the ferrous subunits of the hybrid Hb, indicating that cooperative interaction is transmitted from the liganded dimer to the unliganded dimer via the interdimer contacts even at pH 6.53.

Our preliminary spectral data on partially oxygenated hybrid Hb under an atmospheric oxygen pressure implied that similar pH-dependent spectral changes of the α2(Ni) subunit also occurred upon oxygenation (data not shown).

DISCUSSION

Preparation of Cross-Linked Asymmetric Ni(II)–Fe(II) Hybrid Hb. The previous preparation method of Miura and Ho (1982) could not be adopted straightforwardly in our

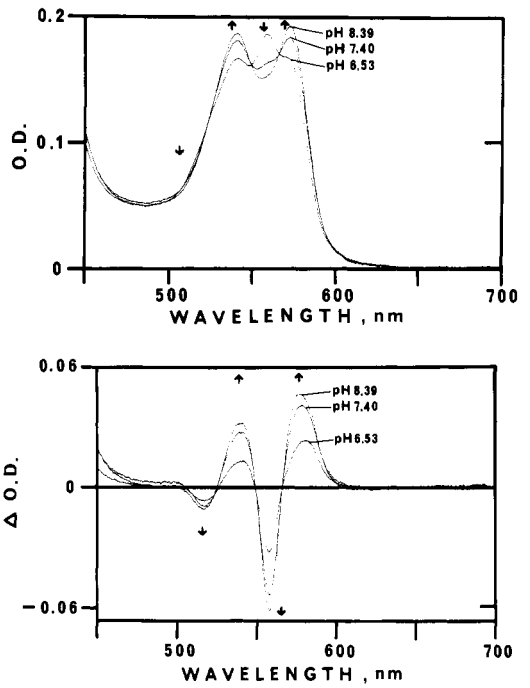

FIGURE 4: (A, top) pH dependence of electronic absorption spectrum of XL[α(Fe-CO)β(Fe-CO)][α(Ni)β(Ni)] in 0.05 M Tris-HCl or Bistris-HCl buffer at 25 °C. The protein concentration is 3 μM for tetrameric Hb. Arrows indicate the absorbance change with rising pH: 6.53, 7.40, 8.39. (B, bottom) Calculated absorption changes of the α(Ni)β(Ni) dimer within XL[α(Fe)β(Fe)][α(Ni)β(Ni)] accompanying CO binding to the ferrous subunits of the hybrid, using the spectrum of the α(Ni)β(Ni) dimer within CO-bound hybrid as a reference. The protein concentration is 3 μM for tetrameric Hb. Arrows indicate the absorbance change with rising pH: 6.53, 7.40, 8.39.

preparation, because both parent molecules of their samples (cross-linked mixed-valency hybrid Hbs) take the oxy-quaternary structure, while NiHb, one of the parent molecules of our sample (XL[α(Fe-CO)β(Fe-CO)][α(Ni)β(Ni)]), takes a deoxy-quaternary structure (Alston et al., 1984). If NiHb and HbCO were used as the parent molecules of [α(Fe-CO)β-(Fe-CO)][α(Ni)β(Ni)], the equilibrium concentration of [α-(Fe-CO)β(Fe-CO)][α(Ni)β(Ni)] presumably would differ from a binomial value. Furthermore, Chatterjee et al. (1986) reported that the reaction of bis(3,5-dibromosalicyl) fumarate with deoxyHb produces a mixture of two cross-linked species, namely, Hb cross-linked between Lys-99α_1 and Lys-99α_2 in addition to the Hb cross-linked between Lys-82β_1 and Lys-82β_2. Such cross-linking may occur in the reaction of bis-(3,5-dibromosalicyl) fumarate with NiHb or possibly with [α(Fe-CO)β(Fe-CO)][α(Ni)β(Ni)].

To avoid these difficulties, we first prepared XL[α(Fe^{2+}-PP-CO)β(Fe^{2+}-PP-CO)][α(Fe^{3+}-DP)β(Fe^{3+}-DP)] according to the method of Miura and Ho (1982) with modifications, and then Fe^{3+}-DP in XL[α(Fe^{2+}-PP-CO)β(Fe^{2+}-PP-CO)]-[α(Fe^{3+}-DP)β(Fe^{3+}-DP)] was replaced by Ni-PP through the heme-exchange reaction. Other notable improvements over the previous preparation method are as follows: (i) the present method requires no mutant Hb, such as Hb C (Glu^--β6 → Lys^+) or Hb S (Glu^--β6 → Val), which has been needed to produce an electric charge difference for all previous preparations (Miura & Ho, 1982, 1984; Inubushi et al., 1986; Kitagishi et al., 1988; Kaminaka et al., 1989); (ii) the present cross-liking condition at pH 8.95 produced only 3% of the electrophoretically silent impurity of total cross-linked Hbs, while approximately 20% of the impurity was formed under

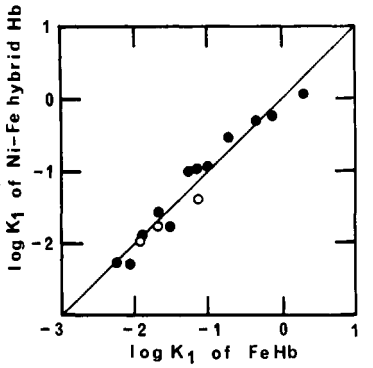

FIGURE 5: Comparison of K_1 values of various Ni(II)–Fe(II) hybrid Hbs with those of corresponding FeHbs at 25 °C under various solution conditions. The data in Table I of this paper and the previous data on symmetric Ni(II)–Fe(II) hybrid Hbs by Shibayama et al. (1986b) are plotted. (○) K_1 values of XL[α(Fe)β(Fe)][α(Ni)β(Ni)] vs those of FeHb at various pH values; (●) mean K_1 values of α_2(Fe)β_2(Ni) and α_2(Ni)β_2(Fe) with or without chemical modifications vs K_1 values of corresponding FeHbs in various solution conditions.

the previous cross-linking condition at pH 7.0 (Shibayama et al., 1991).

For the metal substitution, we have utilized the heme-exchange reaction (Bunn & Jandl, 1968). Since the heme-exchange rate was much reduced in the XLHb, presumably due to its structural stability (Benesch & Kwong, 1990), we used Fe^{3+}-DP in place of Fe^{3+}-PP to increase the exchange rate (Rossi-Fanelli & Antonini, 1960). After the heme-exchange reaction, heme-exchanged product, XL[α(Fe-CO)β-(Fe-CO)][α(Ni)β(Ni)], was isolated from unexchanged derivatives, if any, by ion-exchange chromatography which distinguished the difference in numbers of remaining ferric deutero subunits.

Validity of Cross-Linked Asymmetric Ni(II)–Fe(II) Hybrid Hb as a Model for an Oxygenation Intermediate. We have previously reported that mean K_1 values of α_2(Fe)β_2(Ni) and α_2(Ni)β_2(Fe) are in excellent agreement with those of native Hb A under various solution conditions (Shibayama et al., 1986a). This suggests that Ni-PP mimics a deoxyheme with respect to its effect on the oxygen equilibrium properties of counterpart ferrous subunits in α_2(Fe)β_2(Ni) and α_2(Ni)β_2-(Fe) (Shibayama et al., 1986a). This statement has been extended into various chemically modified hybrid Hbs with various structures, implying that Ni-PP mimics a fixed deoxyheme irrespective of the Hb structure which accommodates it (Shibayama et al., 1986b). This finding is especially important for the study of the intermediate ligation model whose structure is yet unknown.

Recently, we have reported that the cross-linking between Lys-82β_1 and Lys-82β_2 by bis(3,5-dibromosalicyl) fumarate little affects the oxygenation properties of Hb and is a chemical modification particularly useful for preparing asymmetric hybrid Hbs (Shibayama et al., 1991).

In this study, we have combined the two modifications mentioned above to synthesize cross-linked asymmetric Ni-(II)–Fe(II) hybrid Hb. The validity of this model is confirmed by agreement in the K_1 values of XL[α(Fe)β(Fe)][α(Ni)β-(Ni)] and native Hb A at various pH values (Figure 3). A comparison of the K_1 values of XL[α(Fe)β(Fe)][α(Ni)β(Ni)] with those of FeHb at various pH values is shown in Figure 5, which also includes the previous data on symmetric Ni-(II)–Fe(II) hybrids with or without chemical modifications and those of corresponding FeHbs under various solution conditions (Shibayama et al., 1986b). This plot shows a close linear correlation between the hybrid Hbs and FeHbs.

α1β1 Interaction within the Tetramer. The finding of significant cooperativity in the α1β1 oxygenation of XL[α(Fe)β(Fe)][α(Ni)β(Ni)] at pH 7.4 or 8.4 raises the possibility that there is a direct heme–heme interaction between the α1 and β1 subunits. Although it has been widely believed that the α1β2 interface serves as a more important pathway for heme–heme interaction than the α1β1 interface, it may also be plausible that relatively small changes in the interface between tightly packed complementary subunits could be energetically more important to transmit the heme–heme interaction.

The presence of a cooperative interaction across the α1β1 interface was suggested by observations for Hb San Diego [Val-β109(G11) → Met] with mutation at the α1β1 interface. Loukopoulos et al. (1986) showed that Hb San Diego has significantly decreased cooperativity as compared to normal Hb A, while X-ray crystallographic analysis on mixed crystals of deoxyHb San Diego and deoxyHb A did not clearly show significant differences at the α1β2 interface (Anderson, 1974). Moreover, ElAntri et al. (1989) suggested a significant role of the α1β1 interface by comparison of the sulfhydryl vibrational absorption of Cys-β112 in Hb San Diego with that in normal Hb A. Kawamura-Konishi and Suzuki (1988) measured the reconstitution kinetics of chemically modified Hbs from the modified α and β chains. Their kinetic data on circular dichroism (CD) changes in the Soret region suggested that structural transition of Hb tetramer accompanies changes in the interaction between the α1 and β1 subunits. Levy et al. (1992) investigated the electron paramagnetic resonance of valency hybrid Hbs with oxygen or CO bound to the ferrous subunits. The comparison of different types of valency hybrids led to the conclusion that the distal perturbations induced by CO binding are transmitted across the α1β1 interface within an oxy-quaternary structure. Miura and Morimoto (1980) investigated the effects of chemical modifications of particular subunits in [α(Fe-NO)β(Fe-NO)][α(Fe)β(Fe)] on its triplet hyperfine structure in the electron paramagnetic resonance spectrum, which is known to arise from the nitrosyl heme of the α subunits within the deoxy-quaternary structure. By comparing the relative intensity of the triplet structure, they concluded that the intersubunit interaction is increased in the order α1α2, α1β2, and α1β1. Imai (1982) found a significant cooperativity in the binding of oxygen with [α(Fe^{2+})-β(Fe^{2+})][α($Fe^{3+}CN^-$)β($Fe^{3+}CN^-$)] from measurements on oxygen equilibrium curves of mixtures of oxyHb and cyanometHb at 25 °C in 0.1 M phosphate buffer, pH 7.4. These previous data and the present direct oxygen equilibrium data on XL[α(Fe)β(Fe)][α(Ni)β(Ni)] imply that the α1β1 interaction plays an important role in the cooperative interactions within the Hb tetramer.

It should be noted here that there remains another possibility for the interpretation of significant α1β1 cooperativity within the tetramer. This is by an indirect interaction that does not involve the α1β1 interface change, *e.g.*, α1 → α2 → β1 or β1 → α2 → α1. Unfortunately, from only the present oxygen equilibrium data of the hybrid, it is difficult to discriminate between such an indirect effect and a direct one. The noncooperative nature of dissociated dimers indicates the importance of interdimer contacts for generating the cooperativity of Hb. Therefore, we must accept the importance of the α1β2 and α1α2 contacts for cooperativity of Hb. However, it is also true that the noncooperative nature of dissociated dimers would not in principle conflict with the possibility for direct α1β1 interaction within the tetramer setting. To resolve the individual pathways for the structural interaction of Hb, it should be necessary to characterize the structure of liganded asymmetric hybrid Hb investigated in the present study as well as those of the other types of di-liganded and mono-liganded hybrid Hbs.

Another experimental approach aiming for information about the ligation intermediates has been exploited by Ackers and his colleagues (Smith & Ackers, 1985; Smith et al., 1987; Perrella et al., 1990a; Daugherty et al., 1991; Speros et al., 1991; Ackers et al., 1992). They have determined the tetramer–dimer equilibrium constants for 10 cyanomet ligation species at pH 7.4 in the presence of 0.1 M NaCl at 21.5 °C. According to the thermodynamic coupling between dimer dissociation and ligand binding equilibria, the free energy change accompanied by each ligand binding to the tetramer was indirectly estimated (Smith & Ackers, 1985; Perrella et al., 1990a; Daugherty et al., 1991). A remarkable result obtained was the existence of an extremely strong heme–heme interaction within the α1β1 dimer prior to the quaternary transition from T to R (Daugherty et al., 1991).

Although our finding of significant cooperative interaction between the α1 and the β1 subunits at pH 7.4 or 8.4 is qualitatively consistent with the data on cyanomet ligation, the modest 5.6-fold increase in the stepwise binding affinity for the α1β1 oxygenation at pH 7.4 (Table I) is much smaller than the factor of 170 for the corresponding reaction of cyanomet ligation (Daugherty et al., 1991). Moreover, the finding of significant spectral changes of Ni-PP accompanied by CO (or oxygen) binding to XL[α(Fe)β(Fe)][α(Ni)β(Ni)] at pH 7.4 indicates that some structural interactions are transmitted from the liganded dimer to the unliganded dimer within the asymmetric intermediate. This finding is in marked contrast with the assessment of Daugherty et al. (1991) that the ligation of the α1β1 dimer does not affect the functional properties of unliganded α2β2 dimer within [α($Fe^{3+}CN^-$)-β($Fe^{3+}CN^-$)][α(Fe^{2+})β(Fe^{2+})].

Oxygen equilibrium data on native Hb A of Imai (1982) gave K_1 = 0.0218 $mmHg^{-1}$ and K_2 = 0.062 $mmHg^{-1}$ at pH 7.4 in the presence of 0.1 M Cl^- at 25 °C, which provide only a 3-fold increase in stepwise oxygen affinity when the first and second oxygen molecules are bound. Note that in this case the value of 3-fold increase in affinity is the macroscopic mean value of α1α2, β1β2, α1β2, and α1β1 oxygenation pathways. According to the data set on the cyanomet ligation system by Perrella et al. (1990a), the macroscopic mean affinity change for the first two ligands becomes as large as 60-fold, suggesting that there is a significant difference between oxygenation and cyanomet ligation. Moreover, the data set of the cyanomet ligation system indicated a different order of the relative magnitudes of macroscopic stepwise binding constants from that of oxygenation, *e.g.*, $K_1 \leq K_3 < K_2 \leq K_4$ for cyanomet ligation vs $K_1 \leq K_2 < K_3 \leq K_4$ for oxygenation,

[3] It is also possible to speculate that there has been a breakdown of their fundamental assumption that dissociated dimers bind two ligands noncooperatively. In the case of cyanomet ligation, this assumption is probably not exactly correct because of the following features of met-systems: (i) the free energy of cyanomet ligation is separable into two energy terms from heme oxidation ($Fe^{2+} \rightarrow Fe^{3+}$) and from cyanide binding; (ii) the oxidation–reduction equilibrium of tetrameric Hb is a cooperative system (Brunori et al., 1969); (iii) the dimeric Hb (dimeric Hb–haptoglobin complex) shows substantial heterogeneity of subunits in the oxidation–reduction equilibrium by a factor of about 10 at pH 7.0, at 30 °C (Brunori et al., 1968). Thus, these special properties of met-systems may result in substantial apparent negative cooperativity for the dimeric ligation.

[4] Smith et al. (1987) also investigated the Mn(II)–Fe(II)–CO system. In this case, however, the free energy values of mono- or tri-liganded species have not been reported.

which results in a considerably large population of di-liganded intermediates in the cyanomet ligation system. Such an apparent discrepancy between the oxygenation and the cyanomet ligation can be interpreted, at least in part, by the difference in heme ligand[3] as suggested by Perrella et al. (1990b).

Ackers and his colleagues also reported the tetramer–dimer equilibrium data on Mn(III)–Fe(II) (metmanganese) and Co(II)–Fe(II)–CO hybrid systems[4] (Smith et al., 1987; Speros et al., 1991). In the case of the Mn(III)–Fe(II) system, the same results were obtained as those for the cyanomet system (Smith et al., 1987). However, the properties of 10 ligation species of the Co(II)–Fe(II)–CO system were found to be different from those of the cyanomet and metmanganese systems (Speros et al., 1991). Ackers et al. (1982) tentatively calculated tetramer–dimer equilibrium constants for eight oxygenation intermediates from experimentally resolved macroscopic values by Chu et al. (1984), assuming the same distribution pattern as found in the Co(II)–Fe(II)–CO system. Their estimated tetramer–dimer equilibrium constants for the oxygenation intermediates gave only a 2.8-fold increase in affinity change for the $\alpha 1\beta 1$ oxygenation pathway at pH 7.4 and 21.5 °C (Ackers et al., 1992). This value is not much different from our direct observation of a 5.6-fold increase in oxygen affinity but is significantly different from the factor of 170 for the corresponding reaction of cyanomet ligation. These results imply that the $\alpha 1\beta 1$ interaction in the initial oxygenation stage of Hb is neither inert, as predicted by crystallographic studies, nor strong, as in the case of the cyanomet ligation system.

REFERENCES

Ackers, G. K., Doyle, M. L., Myers, D., & Daugherty, M. A. (1992) *Science 255*, 54–63.

Alston, K., Schechter, A. N., Arcoleo, J. P., Greer, J., Parr, G. R., & Friedman, F. K. (1984) *Hemoglobin 8* (1), 47–60.

Anderson, L. N. (1974) *J. Clin. Invest. 53*, 329–333.

Antonini, E., Brunori, M., Caputo, A., Chiancone, E., Rossi-Fanelli, A., & Wyman, J. (1964) *Biochim. Biophys. Acta 79*, 284–292.

Baldwin, J. M., & Chothia, C. (1979) *J. Mol. Biol. 129*, 175–220.

Benesch, R. E., & Kwong, S. (1990) *J. Biol. Chem. 265*, 14881–14885.

Blough, N. V., & Hoffman, B. M. (1982) *J. Am. Chem. Soc. 104*, 4247–4250.

Brunori, M., Alfsen, A., Saggese, U., Antonini, E., & Wyman, J. (1968) *J. Biol. Chem. 243*, 2950–2954.

Brunori, M., Taylor, J. F., Antonini, E., & Wyman, J. (1969) *Biochemistry 8*, 2880–2883.

Bunn, H. F., & Jandl, J. H. (1968) *J. Biol. Chem. 243*, 465–475.

Chatterjee, R., Welty, E. V., Walder, R. Y., Pruitt, S. L., Rogers, P. H., Arnone, A., & Walder, J. A. (1986) *J. Biol. Chem. 261*, 9929–9937.

Chu, A. H., Turner, B. W., & Ackers, G. K. (1984) *Biochemistry 23*, 604–617.

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.

ElAntri, S., Zentz, C., & Alpert, B. (1989) *Eur. J. Biochem. 179*, 165–168.

Evelyn, K. A., & Malloy, T. H. (1938) *J. Biol. Chem. 126*, 655–662.

Hewitt, J. A., Kilmartin, J. V., Ten Eyck, L. F., & Perutz, M. F. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 203–207.

Ikeda-Saito, M., Yamamoto, H., & Yonetani, T. (1977) *J. Biol. Chem. 252*, 8639–8644.

Imai, K. (1981a) *Methods Enzymol. 76*, 438–449.

Imai, K. (1981b) *Methods Enzymol. 76*, 470–486.

Imai, K. (1982) *Allosteric Effects in Haemoglobin*, Cambridge University Press, Cambridge, England.

Imai, K. (1993) *Methods Enzymol.* (in press).

Imai, K., Morimoto, H., Kotani, M., Watari, H., Hirata, W., & Kuroda, M. (1970) *Biochim. Biophys. Acta 200*, 189–196.

Inubushi, T., D'Ambrosio, C., Ikeda-Saito, M., & Yonetani, T. (1986) *J. Am. Chem. Soc. 108*, 3799–3803.

Kaminaka, S., Ogura, T., Kitagishi, K., Yonetani, T., & Kitagawa, T. (1989) *J. Am. Chem. Soc. 111*, 3787–3794.

Kawamura-Konishi, Y., & Suzuki, H. (1988) *Biochem. Biophys. Res. Commun. 156*, 348–354.

Kitagishi, K., D'Ambrosio, C., & Yonetani, T. (1988) *Arch. Biochem. Biophys. 264*, 176–183.

Levy, A., Sharma, V. S., Zhang, L., & Rifkind, J. M. (1992) *Biophys. J. 61*, 750–755.

Loukopoulos, D., Poyart, C., Delanoe-Garin, J., Matsis, C., Arous, N., Kister, J., Loutradi-Anagnostou, A., Blouquit, Y., Fessas, P., Thillet, J., Rosa, J., & Galacteros, F. (1986) *Hemoglobin 10*, 143–159.

Lynch, R. E., Lee, G. R., & Cartwright, G. E. (1976) *J. Biol. Chem. 251*, 1015–1019.

Miura, S., & Ho, C. (1982) *Biochemistry 21*, 6280–6287.

Miura, S., & Ho, C. (1984) *Biochemistry 23*, 2492–2499.

Miura, S., & Morimoto, H. (1980) *J. Mol. Biol. 143*, 213–221.

Miura, S., Ikeda-Saito, M., Yonetani, T., & Ho, C. (1987) *Biochemistry 26*, 2149–2155.

Perrella, M., Sabbioneda, L., Samaja, M., & Rossi-Bernardi, L. (1986) *J. Biol. Chem. 261*, 8391–8396.

Perrella, M., Benazzi, L., Shea, M. A., & Ackers, G. K. (1990a) *Biophys. Chem. 35*, 97–103.

Perrella, M., Colosimo, A., Benazzi, L., Ripamonti, M., & Rossi-Bernardi, L. (1990b) *Biophys. Chem. 37*, 211–223.

Rossi-Fanelli, A., & Antonini, E. (1960) *J. Biol. Chem. 235*, PC4-PC5.

Shibayama, N., Morimoto, H., & Miyazaki, G. (1986a) *J. Mol. Biol. 192*, 323–329.

Shibayama, N., Morimoto, H., & Kitagawa, T. (1986b) *J. Mol. Biol. 192*, 331–336.

Shibayama, N., Inubushi, T., Morimoto, H., & Yonetani, T. (1987) *Biochemistry 26*, 2194–2201.

Shibayama, N., Imai, K., Hirata, H., Hiraiwa, H., Morimoto, H., & Saigo, S. (1991) *Biochemistry 30*, 8158–8165.

Simolo, K., Stucky, G., Chen, S., Bailey, M., Scholes, C., & McLendon, G. (1985) *J. Am. Chem. Soc. 107*, 2865–2872.

Smith, F. R., & Ackers, G. K. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 5347–5351.

Smith, F. R., Gingrich, D., Hoffman, B. M., & Ackers, G. K. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 7089–7093.

Speros, P. C., LiCata, V. J., Yonetani, T., & Ackers, G. K. (1991) *Biochemistry 30*, 7254–7262.

Walder, J. A., Zaugg, R. H., Walder, R. Y., Steele, J. M., & Klotz, I. M. (1979) *Biochemistry 18*, 4265–4270.

Walder, J. A., Walder, R. Y., & Arnone, A. (1980) *J. Mol. Biol. 141*, 195–216.

Winterbourn, C. C., McGrath, B. M., & Carrell, R. W. (1976) *Biochem. J. 155*, 493–502.

Yonetani, T. (1967) *J. Biol. Chem. 242*, 5008–5013.