Considerable controversy remains as to the functional and structural properties of the asymmetric αβ half-oxygenated intermediate of human hemoglobin, consisting of a deoxygenated and an oxygenated dimer. A recent dimer-tetramer equilibrium study using [Zn(II)/Fe(II)-O₂] hybrid hemoglobins, in which Zn-protoporphyrin IX mimics a deoxygenase, showed that the key intermediate, [α(Fe-O₂)β(Fe-O₂)][α(Zn)β(Zn)], exhibited an enhanced tetramer stability relative to the other doubly oxygenated species. This is one of the strongest findings in support of distinctly favorable intra-dimer cooperativity within the tetramer. However, we present here a different conclusion drawn from direct O₂ binding experiments for the same asymmetric hybrid, [α(Fe)β(Fe)][α(Zn)β(Zn)], and those for [α(Fe)β(Zn)]₂ and [α(Zn)β(Fe)]₂. In this study, the O₂ equilibrium curves for [α(Fe)β(Fe)][α(Zn)β(Zn)] were determined by an O₂-jump stopped-flow technique to circumvent the problem of dimer rearrangement, and those for [α(Fe)β(Zn)]₂ and [α(Zn)β(Fe)]₂ were measured by using an Imai apparatus. It was shown that the first and second O₂ equilibrium constants for [α(Fe)β(Fe)][α(Zn)β(Zn)] are 0.0209 mmHg⁻¹ and 0.0276 mmHg⁻¹, respectively, that are almost identical to those for [α(Fe)β(Zn)]₂ or [α(Zn)β(Fe)]₂. Therefore, we did not observe large difference among the asymmetric and symmetric hybrids. The discrepancy between the present and previous studies is mainly due to previously observed negative cooperativity for [α(Fe)β(Zn)]₂ and [α(Zn)β(Fe)]₂, which is not the case in our direct O₂ binding study.

Although cooperative oxygenation of human adult hemoglobin (HbA) has been studied extensively as a paradigm for regulatory actions of allosteric proteins, the functional and structural properties of its eight partially oxygenated intermediates remain elusive. This is mostly due to the strong cooperativity of Hb, which suppresses relative abundance of the intermediates, precluding direct study. One of the most specific methods for studying the oxygenation intermediates has been to substitute the heme in one or more of four subunits with another metalloprotoporphyrin IX, which does not bind O₂ while mimicking either normal deoxyheme or oxyheme (1–6). However, there has been a limitation to this approach: Six asymmetric forms cannot be studied in isolation, because the asymmetric hybrid tetramers dissociate into dimers, which then reassociate to form not only the former asymmetric tetramers but also symmetric ones (i.e. dimer rearrangement).

Ackers and his colleagues (7) have partly circumvented this difficulty by measuring the dimer-tetramer equilibrium constants for symmetric hybrid species in the presence of the symmetric parental species. In 1985, this methodology was first applied to resolution of the tetramer stabilities of all ten ligation intermediates of the deoxy-cyanomet [Fe(II)/Fe(III)-CN⁻] hybrid system, in which cyanoemheme mimics a fixed oxyheme (8). The most striking finding was that the dimer-tetramer association equilibrium constant for (α⁺CN⁻β⁺CN⁻)(αβ) was about 170 times that of the other three doubly liganded species at pH 7.4, which implies a hypercooperativity in specific ligation steps in the αβ₁ dimer within the tetramer (i.e. a 170-fold affinity change). Subsequently, the effects of pH, temperature, and single-site mutations on the dimer-tetramer equilibrium of (α⁺CN⁻β⁺CN⁻)(αβ) were studied (7, 9, 10). It was suggested that this key intermediate assumes a form of the deoxy-T-quaternary structure as judged from the nature of the dimer-dimer interface.

Based on the hyperthermodynamic stability and T quaternary structure of (α⁺CN⁻β⁺CN⁻)(αβ), Ackers’ group proposed a new framework for Hb cooperativity, called a symmetry rule (SR), in 1991 (9). The key features of the SR model are: (i) The two ligation steps leading to the asymmetric αβ₁ half-liganded intermediate show distinctly favorable cooperativity while those leading to the other three doubly liganded intermediates exhibit no favorable cooperativity; (ii) The asymmetric αβ₁ half-liganded intermediate assumes a deoxy-T-quaternary structure while the other three doubly liganded intermediates exhibit an oxy-R-quaternary structure.

Recently, we showed that both the published hyperstability and T structure assignment for (α⁺CN⁻β⁺CN⁻)(αβ) were artifacts arising from valency exchange between the deoxy and cyanomethe species during the long deoxy incubation that had been used routinely in the laboratory of Ackers (11, 12). Ackers et al. (13) have now accepted the valency exchange artifacts in their previous studies using deoxy-cyanomet hybrids. Nevertheless, they still advocate the SR model based on their dimer-tetramer study using [Zn(II)/Fe(II)-O₂] hybrid system in which Zn-protoporphyrin IX mimics a fixed deoxyheme (13, 14). It was reported that [α(Fe-O₂)β(Fe-O₂)][α(Zn)β(Zn)] exhibited an enhanced (i.e. 7.5- to 15-fold) tetramer stability relative to the other three doubly oxygenated species, providing evidence for distinctly favorable intra-dimer cooperativity.
In this study we have investigated the oxygenation properties of [Zn(II)/Fe(II)] hybrid Hbs in a direct way. The O₂ equilibrium curves for [α(Fe)/β(Zn)]₂ and [α(Zn)/β(Fe)]₂ were measured by Imai apparatus (15), and those for [α(Fe)/β(Fe)][α(Zn)/β(Zn)] were determined by an O₂-jump stopped-flow technique (16, 17) to circumvent the problem of dimer rearrangement. Our present results show that the oxygenation parameters of [α(Fe)/β(Fe)][α(Zn)/β(Zn)] are almost identical to those of [α(Fe)/β(Zn)]₂ or [α(Zn)/β(Fe)]₂, in contrast to the prediction of the SR model.

**EXPERIMENTAL PROCEDURES**

**Preparation of HbA, ZnHbA, and Symmetric Zn(II)/Fe(II) Hybrid Hbs**—HbA was prepared as previously described (18). Isolated α- and β-chains were prepared by one column method of Gerard et al. (19) with some modifications. Zinc(II)-protoporphyrin IX was prepared according to the method of Adler et al. (20). ZnHbA was prepared by the method of Shibayama et al. (3) for preparing Ni(II)-substituted HbA. To further purify the ZnHbA sample, it was passage through a column of Sephadex G-25 equilibrated with 20 mM Tris/HCl buffer, pH 8.8, and then applied to a column of DE52 cellulose (Whatman) equilibrated with the same buffer. The column was eluted by a linear gradient of 20 mM Tris/HCl buffer, pH 8.8, and 20 mM Tris/HCl buffer, pH 8.2, with 100 mM NaCl. Both symmetric Zn(II)/Fe(II) hybrid Hbs, [α(Fe)/β(Zn)]₂ and [α(Zn)/β(Fe)]₂, were prepared as previously described (6).

**Determination of O₂ Equilibrium Curves of HbA and Symmetric Zn(II)/Fe(II) Hybrid Hbs**—O₂ equilibrium curves of HbA and symmetric Zn(II)/Fe(II) hybrids were determined by Imai apparatus (15). The O₂ equilibrium curves of HbA and [α(Zn)/β(Fe)]₂ were measured at 560 nm, whereas the measurements for [α(Fe)/β(Zn)]₂ were carried out at 470 nm where the spectral changes of the Zn-containing β subunits were negligible (6). The O₂ equilibrium curves of both symmetric Zn(II)/Fe(II) hybrid Hbs were analyzed by a two-step Adair equation as described previously (4). All the measurements were carried out at 25 °C in 50 mM Tris plus 50 mM bis-Tris buffer with 100 mM Cl⁻, pH 7.4. Catalase and superoxide dismutase were added to Hb samples to minimize methHb formation (21, 22).

**Preparation of [α(Fe)/β(Fe)][α(Zn)/β(Zn)]—**A 15-fold molar amount of ZnHbA was mixed with oxyFeHbA to give the Zn and Fe concentrations of 13 and 0.87 mM, respectively. The buffer used was 50 mM Tris plus 50 mM bis-Tris with 100 mM Cl⁻ at pH 7.4 (at 25 °C). The resulting hybrid mixture was deoxygenated using pure N₂ gas, followed by deoxy incubation at 10 °C for 72 h to reach equilibrium. According to Ackerman et al. (13), equilibrium time for this hybrid system was ~120 h at pH 7.4, 21.5 °C, and a ratio of fifteen ZnHbA per FeHbA would give 0.880:0.115:1 ratio of [FF], [ZZ], and [FZ], respectively. Among the six rate constants, k₁, k₃, and k₄ were fixed at 1.2 × 10⁶ M⁻¹ s⁻¹ (which was calculated from the value of 1.1 × 10⁶ M⁻¹ s⁻¹ at 21.5 °C and the enthalpy for this reaction, ΔH = 3.7 kcal/mol (24)); the k₂ value was fixed at 1.1 s⁻¹ which was assumed to be identical to the value of 1.1 s⁻¹ at 21.5 °C because of very small enthalpy for this reaction (24)). At the end of each numerical integration process, the Kₑ value of the hybrid was fixed at 0.0209 mm Hg⁻¹ (which could be determined by the O₂-jump experiments at low concentrations of O₂⁻), whereas the K₂ value was treated as a variable and the four Adair constants of HbA determined by Imai apparatus under the same experimental conditions were used.

**RESULTS AND DISCUSSION**

**O₂ Equilibrium Properties of [α(Fe)/β(Fe)][α(Zn)/β(Zn)]—**Direct determination of the O₂ equilibrium curves for asymmetric hybrid Hbs in the presence of the parent has been difficult, because dimer rearrangement reaction coupled with oxygenation during the measurement alter the proportions of the hybrids. To circumvent this problem, we have devised an O₂-jump stopped-flow method in which a fully deoxygenated hybrid mixture, consisting of ZnHbA, [α(Fe)/β(Fe)][α(Zn)/β(Zn)], and FeHbA, is rapidly mixed with a known amount of O₂ and
Fig. 1. Schematic representation of the molecular events that occur after mixing the deoxy hybrid mixture, consisting of Zn-HbA, [α(Fe)/β(Fe)]/[α(Zn)/β(Zn)], and Fe-HbA, with O₂. The squares and circles represent low and high affinity conformations of the subunits, respectively, and bound O₂ is denoted by O₂. The open and gray-shaded symbols indicate Fe and Zn-containing subunits, respectively. For simplicity, T → R transition of Hb is assumed to occur at the second O₂ binding step.

Fig. 2. Normalized time courses for the reactions of the hybrid mixture, consisting of Zn-HbA, [α(Fe)/β(Fe)]/[α(Zn)/β(Zn)], and Fe-HbA, with (>) 6.31 μM, (>) 30.0 μM, (>) 65.5 μM, (>) 131 μM, and (>) 625 μM O₂. Reactions were initiated at 25 °C using Orii’s (23) stopped-flow apparatus, and subsequent absorbance changes were measured at 470 nm. The buffer used was 50 mM Tris plus 50 mM bis-Tris with 100 mM Cl⁻, pH 7.4. Y is fractional saturation of O₂. All the time courses consist of two distinct kinetic phases, corresponding to T → R conformational transition and the subsequent dimer rearrangement (see text and Fig. 1). The solid lines are the time courses for dimer rearrangement reactions that were obtained by either a linear extrapolation of the dimer rearrangement kinetic phases to zero time (> and <) or simulations using a numerical integration program combined with a nonlinear least-squares fitting routine (see text and Fig. 1). The asymmetric half-oxygenated Hb

Asymmetric Half-oxygenated Hb

the fractional O₂ saturation for the hybrid mixture is determined prior to dimer rearrangement.

Fig. 1 is a schematic representation of the molecular events that occur following the O₂-jump: (i) O₂ binding to [α(Fe)/β(Fe)]/[α(Zn)/β(Zn)] and deoxyFeHbA still in their original low affinity T conformations (within the mixing dead time), (ii) transition from the low affinity T to high affinity R conformation, favoring the doubly liganded high affinity R form of the hybrid (<1 s), and (iii) dissociation of [α(Fe-O₂)/β(Fe-O₂)]/[α(Zn)/β(Zn)] into dimers to form the more stable parents on a much slower time-scale (>1 s). Note that because the rate constants for O₂ dissociation from mono-ligated species are 200–800 s⁻¹ under the same experimental conditions (25), the half-times for O₂ binding to the T-state molecules are less than 4 ms. Consequently, process i should complete within the mixing dead time (<18 ms). Note also that in this study [α(Fe)/β(Fe)]/[α(Zn)/β(Zn)] was prepared by hybridization of deoxyHbA with a 15-fold molar excess of ZnHbA to reduce the relative concentration of HbA as small as possible.

Fig. 2 shows the O₂ binding time courses for the reactions of the deoxy hybrid mixture with 6.31, 30.0, 65.5, 131, and 625 μM O₂. At 6.31 and 30.0 μM O₂, the time courses of dimer rearrangement were very slow and well separated from those of the T-R conformational transition. In such cases, the Y values of the hybrid mixture after the T-R transition and prior to the dimer rearrangement can be determined by a linear extrapolation of the dimer rearrangement kinetic phases to zero time (> and <) or simulations using a numerical integration program combined with a nonlinear least-squares fitting routine (see “Experimental Procedures”). For the nonlinear curve fitting, the experimental points > 2 s (<), those > 3 s (<), and those > 20 ms (<) were used to eliminate the effects of T-R transition phase.

Before discussing the O₂ equilibrium curve of [α(Fe)/β(Fe)]/[α(Zn)/β(Zn)], the O₂ equilibrium curves of HbA in the absence of IHP at pH 7.4 and in the presence of IHP at pH 6.5 determined by the present technique are compared with those obtained by Imai apparatus (15) to assure the accuracy of the present O₂-jump technique (open and closed circles versus dotted lines, Fig. 3A). As seen in Fig. 3A the comparison gave good agreement, suggesting that the present O₂-jump technique can be applied to the precision determination of the O₂ equilibrium curves of Hb.

Fig. 3B shows the Hill plot for the O₂ equilibrium curve of [α(Fe)/β(Fe)]/[α(Zn)/β(Zn)] at pH 7.4 and 25 °C determined by the O₂-jump technique (open squares, Fig. 3B). The asymmetric hybrid exhibits a low affinity for O₂ and slight cooperativity (nHill = 1.1). The O₂ association equilibrium constants K₁ and K₂ for [α(Fe)/β(Fe)]/[α(Zn)/β(Zn)] were determined to be 0.0209 and 0.0276 mm Hg⁻¹, respectively, by fitting all of the data points in Fig. 3B (solid line is the best-fit curve).

Fig. 3B also presents the previously reported O₂ equilibrium curve for this hybrid at pH 7.4 and 21.5 °C, which was calcu-
Asymmetric Half-oxygenated Hb

![Diagram](image)

**FIG. 3.** Hill plots for $O_2$ equilibrium curves of (A) HbA and (B) [α(Fe)/β(Fe)][α(Zn)/β(Zn)] determined by $O_2$-jump technique. $P$ is the partial pressure of $O_2$ (in mm Hg) and $Y$ is the fractional saturation of $O_2$. In panel A, open and filled circles are experimental points for HbA at 25 °C in the absence of IHP at pH 7.4 with 100 mM Cl− and those in the presence of 2 mM IHP at pH 6.5 with 100 mM Cl−, respectively. Two dotted lines are the results obtained by Imai apparatus (15) under the same experimental conditions. In panel B, squares are experimental points for [α(Fe)/β(Fe)][α(Zn)/β(Zn)] at pH 7.4 and 25 °C in the presence of 100 mM Cl−, and the solid line is the best-fit curve. The dotted line (without experimental points) is the observation of Ackers et al. (13) for [α(Fe)/β(Fe)][α(Zn)/β(Zn)] at pH 7.4 and 21.5 °C.

lated from the dimer-tetramer equilibrium constants of deoxy, mono-ligated, and doubly liganded [Zn(II)/Fe(II)-$O_2$] hybrids (dotted line, Fig. 3B) (13, 14). The previously observed $K_c$ value is approximately twice as large as the present observation (see Table I). This discrepancy is probably due to insufficient $O_2$ saturation of the hybrid in the previous dimer-tetramer experiments (13). According to Ackers et al. (13), ZnHbA and FeHbC were hybridized under 1 atm of $O_2$, and then the fractional population of the asymmetric AC hybrid formed was determined. However, because the asymmetric hybrid cannot be fully saturated with $O_2$ under 1 atm of $O_2$ (see Fig. 3B), the hybridized sample would be contaminated by deoxy- and mono-ligated hybrids, leading to overestimation of the fractional population of [α(Fe-$O_2$)/β(Fe-$O_2$)][α(Zn)/β(Zn)].

$O_2$ Equilibrium Properties of [α(Fe)/β(Zn)]$_2$ and [α(Zn)/β(Fe)]$_2$—Fig. 4 shows the Hill plots for our $O_2$ equilibrium curves of [α(Fe)/β(Zn)]$_2$ and [α(Zn)/β(Fe)]$_2$ determined by Imai apparatus (15) (circles and squares, Fig. 4), along with the previous data by the dimer-tetramer experiments (dotted and dashed lines, Fig. 4) (13, 14). Striking discrepancy is seen in the present and previous results. Our results indicate that both hybrids exhibit slight cooperativity ($n_{50} = 1.1–1.2$) whereas significant negative cooperativity was found in the previous study ($n_{50} < 1$).

To eliminate the possibility of negative cooperativity, we carried out an $O_2$-jump experiment in which fully deoxy- genated [α(Fe)/β(Zn)]$_2$ was mixed with 131 μM $O_2$. If the hybrid displays positive cooperativity, $O_2$ binding to the original low affinity conformation (which completes within the mixing dead time) should be followed by transition from the low affinity to high affinity conformation, which increases the fractional saturation of $O_2$. If negative cooperativity were the case, on the contrary, dissociation of once bound $O_2$ from doubly liganded hybrid should take place. As expected, a significant amount of $O_2$ binding was observed within 500 ms (inset, Fig. 4), providing additional evidence for positive cooperativity. It should be noted that the observed $O_2$ binding can quantitatively account for the affinity transition from $K_1$ to $K_2$ of [α(Fe)/β(Zn)]$_2$.

The Contribution of the Asymmetric α1β1 Half-ligated Intermediate to Hb Cooperativity—Our direct $O_2$ binding study demonstrated that the oxygenation parameters of [α(Fe)/β(Fe)][α(Zn)/β(Zn)] are almost identical to those for [α(Fe)/β(Zn)]$_2$ or [α(Zn)/β(Fe)]$_2$ (see Table I). Therefore, $K_2$ and previous results. Our results indicate that both hybrids exhibit slight cooperativity ($n_{50} = 1.1–1.2$) whereas significant negative cooperativity was found in the previous study ($n_{50} < 1$).

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Perrella et al. (26–28) have used a cryogenic technique for trapping the reaction intermediates of Hb and CO, which in many aspects is a close approximation to $O_2$. In this method the intermediate compounds between Hb and CO at various CO saturation levels are trapped by rapidly quenching an aqueous solution of Hb into a cryosolvent containing ferricyanide. The valency hybrids formed by oxidation of the unliganded hemes of the intermediates are then separated and quantified by isoelectric focusing at low temperature. According to their analysis, the distribution of all the intermediates at pH 7.0 and 20 °C (27), no preferable cooperative interaction was found in the α1β1 ligation. Rather, the strongest cooperativity was found in the α1α2 ligation pathway.

Shibayama et al. (29) have determined the first two-step microscopic $O_2$ equilibrium constants of Hb using cross-linked Ni(II)/Fe(II) hybrid Hbs, carrying Ni(II)-protoporphyrin IX, which binds neither $O_2$ nor CO, in two subunits and normal hemes in the other two subunits. The cross-linked Ni(II)/
Asymmetric Half-oxygenated Hb

Experimental conditions are described in the legends to Figs. 3 and 4.

|                | $K_1$ $\pm$ SE | $K_2$ $\pm$ SE | $n_{0.5}$ $\pm$ SE | Reference |
|----------------|----------------|----------------|---------------------|-----------|
| $\alpha(\mathrm{Fe})\beta(\mathrm{Fe})[\alpha(\mathrm{Zn})\beta(\mathrm{Zn})]$ | 0.0209 (0.018) | 0.0276 (0.061) | 1.1 (1.3) | This study (13) |
| $\alpha(\mathrm{Fe})\beta(\mathrm{Zn})_2$ | 0.1119 (0.018) | 0.031 (0.00304) | 1.2 (0.58) | This study (13, 14) |
| $\alpha(\mathrm{Zn})\beta(\mathrm{Fe})_2$ | 0.0127 (0.018) | 0.0219 (0.006) | 1.1 (0.73) | This study (13, 14) |

$^a$ Association equilibrium constant for the first binding $O_2$ (mm Hg$^{-1}$).

$^b$ Association equilibrium constant for the second binding $O_2$ (mm Hg$^{-1}$).

$^c$ Slope of Hill plot at half saturation.

Fe(II)-O$_2$] system is an excellent analog of native [Fe(II)/Fe(II)]-O$_2$, because Ni(II)-protoporphyrin IX mimics a fixed deoxyheme with respect to its effect on the oxygenation properties of the counterpart Fe(II) subunits within the same tetramer (3, 30, 31) and because the cross-link used has little effect on the oxygenation properties of HbA (18). At pH 7.4 and 25 °C, the cooperativity represented by the Hill coefficient increased in the order of $\beta_2\beta_1$ (n$_{50} = 1.36$), $\alpha_1\beta_1$ (n$_{50} = 1.41$), $\alpha_1\beta_2$ (n$_{50} = 1.64$), and $\alpha_1\alpha_2$ (n$_{50} = 1.72$), indicating no favorable cooperative interaction in the $\alpha_1\beta_1$ ligation pathway. Interestingly, the observation that the $\alpha_1\alpha_2$ ligation pathway exhibits the greatest cooperativity is consistent with the results of the CO ligation intermediates (27) and with the present data of Zn(II)/Fe(II) hybrids (see Table I).

Is there any positive evidence for distinctly favorable intra-dimer cooperativity? The original evidence for this preferred ligation pathway was the hyper (170-fold) stability of $(\alpha^\infty\text{CN}^-\beta^-\text{CN}^-)\beta(\alpha\beta)\beta(\alpha\beta)$ relative to the other three doubly liganded species (7–9). However, this evidence has been proven to be an artifact arising from valency exchange between the deoxy and cyanomet-hemes during the long deoxy incubation that had been used routinely in the laboratory of Ackers (11, 12). Recently, Ackers et al. (13) attempted to minimize the valency exchange artifact by using a relatively short deoxy incubation and the oxidation of unliganded heme. Thus, one may expect the same difficulty as previously reported for deoxy-cyanomet hybrid studies (12).

The reported kinetics for formation of $[\alpha(\mathrm{Fe}-\text{CO})]\beta(\mathrm{Fe}-\text{CO})[[\alpha(\mathrm{Co})]\beta(\mathrm{Co})]$ and $[\alpha(\mathrm{Fe}^\infty\text{CN}^-)\beta(\mathrm{Fe}^\infty\text{CN}^-)]\\beta(\mathrm{Co})\beta(\mathrm{Co})$ followed distinct biphasic behavior (see Fig. 5 of
The dimer-tetramer equilibrium constant for the previously observed slow phases are not clear. These hybrids were considerably overestimated, although the simulations using the published dimer-tetramer rate constants for these hybrids showed that both hybridizations should rapidly reach equilibrium within a few minutes (data not shown). Thus, it is likely that the published tetramer stabilities of these hybrids were considerably overestimated, although the origins of the previously observed slow phases are not clear.

(iii) The dimer-tetramer equilibrium constant for $[\alpha(Fe-CO)\beta(Fe-CO)](\alpha(Fe)\beta(Fe))$ was calculated using incorrect tetramer stabilities of $[(\alpha+CN^-\beta+CN^-)(\alpha\beta)\beta(Fe-CO)\beta(Fe-CO)](\alpha(Fe)\beta(Fe))$, and $[\alpha(Fe+CN^-)\beta(Fe+CN^-)](\alpha(Fe)\beta(Fe))$ as discussed above (34).

Conclusion—This work presents direct measurements of $O_2$ binding to asymmetric hybrid $Hb$ that has significant implications for the nature of $Hb$ cooperativity. The data presented here show that, in the case of $[Zn(II)/Fe(II)-O_2]$ hybrid system, the first two-step $O_2$ binding to $Hb$ molecule is roughly pathway-independent and shows only slight cooperativity regardless of ligation pathway (see Table I). These findings refute directly the claims by Ackers’ group about the same hybrids (13, 14) and resolve the long standing controversy of whether or not the asymmetric $\alpha1\beta1$ half-oxygenated intermediate makes a unique contribution to human $Hb$ cooperativity. Furthermore, our results, eliminating the negative cooperativity previously observed (13, 14), are more consistent with the two-state allosteric model of Monod et al. (36). The present observations are also qualitatively consistent with previous data on the $[Fe(II)/Fe(II)-CO]$ system by Perrella and Di Cera (27) and those for the cross-linked $[Ni(II)/Fe(II)-O_2]$ system by Shibayama et al. (29), in that the greatest cooperative interaction is found in the $\alpha1\sigma2$ ligation pathway, although the magnitudes of cooperativity are system-dependent. One plausible interpretation for the observed small cooperativity in the $[Zn(II)/Fe(II)-O_2]$ system is that the substitution of $Zn(II)$ protophyrin IX for deoxyheme leads to further stabilization of the lowest affinity state of $Hb$ (6).

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