MEMOIRS OF THE FACULTY OF SCIENCE, KYOTO UNIVERSITY, SERIES OF BIOLOGY
Vol. VI, pp. 1–14, December 1972

Subunit Conformation during the Allosteric Transitions in Hemoglobin

By

Toyozo MAEDA

Department of Biophysics, Faculty of Science,
Kyoto University

(Received August 31, 1972)

ABSTRACT The effects of 2, 3-diphosphoglycerate on the oxygen equilibria of the half-cyanmet hybrid hemoglobins $\alpha_2^+(CN)\beta_2(O_2)$ and $\alpha_2(O_2)\beta_2^+(CN)$ and the reaction of *p*-mercuribenzoate with the β93 SH groups of the half-met hybrid hemoglobins, $\alpha_2^+(H_2O)\beta_2(O_2)$ and $\alpha_2(O_2)$-$\beta_2^+(H_2O)$ have been studied to investigate the subunit conformation during the allosteric transitions in hemoglobin. Although DPG decreases the oxygen affinity of $\alpha_2^+(CN)\beta_2(O_2)$ and $\alpha_2(O_2)\beta_2^+(CN)$, the effects are several times larger for $\alpha_2^+(CN)\beta_2(O_2)$ than for $\alpha_2(O_2)\beta_2^+(CN)$. The oxygen binding of the stripped hybrids are essentially non-cooperative. On the addition of DPG, the oxygen binding to $\alpha_2^+(CN)\beta_2$ becomes cooperative, while the phosphate has no effect on the cooperativity in $\alpha_2(O_2)\beta_2^+(CN)$. The response of $\alpha_2(O_2)\beta_2^+(CN)$ to DPG suggests that the cyanmet β subunits change their conformation when partner α subunits bind the oxygen molecules, *i.e.*, there exists propagation of conformational changes from the α subunits to the β subunits. On the other hand, the second order rate constants for the reaction of *p*-mercuribenzoate with the β93 SH groups have been evaluated from kinetic curves obtained by the stopped flow method. It is found, from comparison of the rate data, that the reactivity of the β subunit is primarily dependent on ligation of the β subunit and also dependent on ligation of the neighboring α subunits. With an assumption that some changes in the rate constant reflect alterations in conformation of the β subunit, the reactivity of the β93 SH groups suggests that the β subunit takes four different conformations, named O, P, Q, and R, depending on the state of the α subunit. Deoxy β subunit takes R conformation with deoxy α and Q with met α. The conformation of met β subunit is O with oxy and met α and changes into P when the α subunit becomes deoxygenated. Thus, ligation of the α subunit affects the conformation of the β subunit. The present results do not follow both the original simple allosteric models of Monod, Wyman and Changeux and of Koshland, Némethy and Filmer and lead to some modifications of both the models.

Introduction

Two different models have been presented for the molecular mechanism of the cooperative binding of substrate to allosteric proteins. In the Monod-Wyman-

Changeux model, the subunits of a protein molecule take two different conformations, arranged in a symmetrical fashion, and the protein molecule is assumed to maintain symmetry during the conformational changes.[1] The Koshland-Némethy-Filmer model, on the other hand, assumes progressive or sequential changes in the subunit conformation, only the subunit binding a ligand being able to transform its conformation.[2]

Both the models can equally well explain the experimental saturation curves of hemoglobin. In order to obtain further insights in the allosteric interactions, it is necessary to investigate the subunit conformation during the allosteric transitions and several studies have already appeared using some physical techniques as well as some chemical methods.[3-8]

X-ray[9] and biochemical studies[10-12] have elucidated that an unique allosteric effector, 2, 3-diphosphoglycerate (DPG*) specifically cross-links the β subunits in the deoxy quaternary structure across the dyad axis of symmetry and profoundly lowers the oxygen affinity of hemoglobin. On the other hand, the rate of reaction of various sulfhydryl reagents with the β93 SH groups have been known to depend greatly on the conformations of hemoglobin, *i.e.*, the oxy and deoxy conformations.[13] Thus, since the sensitivity of hemoglobin to DPG and the reactivity of the β93 SH groups depend on its tertiary and quaternary structure, in the present report we have studied the effects of DPG on the oxygen equilibria of the half-cyanmet hybrid hemoglobins and the reaction of PMB with the β93 SH groups of the half-met hybrid hemoglobins to investigate the subunit conformation during the allosteric transitions. Our results indicate that the conformation of the β subunit is primarily dependent on ligation of the β subunit and is also dependent on ligation of the neighboring α subunits and lead to some modifications of the MWC and KNF models.

Experimental Section

Isolation of α and β Chains

Human adult hemoglobin was prepared from fresh blood by lysing washed red cells with 1–1.5 volumes of water. The mercurated α and β chains, α_{PMB} and β_{PMB} were obtained according to the procedure described by Bucci and Fronticelli[14] or Geraci *et al.*[15] The α chains were rendered free of mercury by washing the α_{PMB} chains adsorbed on CM-cellulose column, equilibrated with 0.01 M phosphate buffer (pH 6.7), with 0.015 M mercaptoethanol. Removal of mercury from the β_{PMB} chains was carried out by the method of Tyuma *et al.*[16] The completeness of the

---

* Abbreviation used are: DPG, 2, 3-diphosphoglycerate; Bis-tris, bis (2-hydroxyethyl) iminotris (hydroxymethyl)-methane; PMB, *p*-mercuribenzoate. The MWC and KNF models are those presented by Monod, Wyman and Changeux and by Koshland, Némethy and Filmer, respectively. Molecular species of hemoglobin are represented by the formulae such as $\alpha_2^+(CN)\beta_2(O_2)$, $\alpha_2\beta_2(H_2O)$, $\alpha_2\beta_2^+(CN)$, *etc.* where $\alpha(O_2)$ and $\beta(O_2)$, α and β, $\alpha_2^+(H_2O)$ and $\beta_2^+(H_2O)$, $\alpha_2^+(CN)$ and $\beta_2^+(CN)$ indicate the oxygenated, deoxygenated, met, and cyanmet subunits, respectively.

regeneration of sulfhydryl groups from the mercurated subunits was confirmed by means of a disc-electrophoresis using pH 8.3 buffer system or by the spectrophotometric titration of the free SH groups as described by Boyer[17] and Benesch and Benesch.[18]

Preparation of Hybrid Hemoglobins

The isolated chains were oxidized to the cyanmet form at 30° for 15 min in the presence of 1.3 equivalent amounts of potassium ferricyanide and 10 equivalent amounts of potassium cyanide. After the complete oxidation of the chains had been spectrophotometrically confirmed, the excess potassium ferricyanide was removed through Sephadex G-25 column equilibrated with 0.05 M bis-tris buffer, pH 7.4 at 4°. The half cyanmet hybrid hemoglobin was obtained by mixing the cyanmet chains with equivalent amounts of the partner oxy chains. The disc-electrophoregrams of the reconstituted $\alpha_2^+(CN)\beta_2(O_2)$ and $\alpha_2(O_2)\beta_2^+(CN)$ showed that the reconstitutions were complete, no bands for the isolated chains being recognizable. The half-met hybrid hemoglobins were prepared in the same manner except that the chains were oxidized to the ferric form at 6° for 0.5–1 min with 20 equivalent amounts of potassium ferricyanide and 0.1 M phosphate buffer, pH 7.2 and horizontal starch gel electrophoresis were used. The proportion of the met chains in the met-hybrid hemoglobin was estimated by the method of Evelyn and Malloy.[19] The concentration of hemoglobin was determined spectrophotometrically at 540 mμ after conversion into cyanmet hemoglobin (1.15×10^4 $M^{-1}cm^{-1}$ per heme).

Ferrihemoglobin was obtained by adding 10 equivalent amounts of potassium ferricyanide at 6° for 0.5–1 min to oxyhemoglobin followed by removal of excess reagent through Sephadex G-25 column.

Measurements of Oxygen Equilibrium Curves

The oxygen equilibrium curves of hemoglobin were measured on 5×10^{-5} M (heme equivalent) hybrid hemoglobin in 0.05 M bis-tris buffer, pH 7.4, containing 2.5×10^{-4} M potassium cyanide, either in the absence or presence of 0.1 M NaCl at 25° by the automatic recording method of Imai *et al.*[20] The measurements also performed on human adult hemoglobin at the same experimental conditions except without potassium cyanide. The measurement of a single curve was completed within 40 to 60 min. The exchange between cyanmet and ferrous hemes can be considered insignificant during that period because Bunn and Jandle[21,22] showed that heme exchange in cyanmet hemoglobins, deoxyhemoglobins or oxyhemoglobins is negligible for that period at 25°. The content of the cyanmet chains in the hybrid hemoglobins, determined after measurements of oxygenation curves, was from 55% to 65%.

The oxygen equilibrium curves were expressed as conventional y *vs.* log p plot (y, percentage oxygen saturation; p, oxygen pressure) and further analyzed by the Hill plots (log $[Y/(1-Y)]$ *vs.* log p plot, where Y is fractional oxygen saturation). The oxygen affinity and the extent of cooperativity in oxygen binding are expressed by the oxygen pressure at $y=50$, P_{50}, and the slope of the Hill plots, n, respectively.

Kinetic measurements

The reaction of PMB with hemoglobin was followed by displaying the optical absorption at 255 mμ (slit width 2 mμ) on a memory scope with a commercial stopped flow apparatus (Model SPU-1, Yanagimoto Co. LTD). All experiments were carried out at 20° in 0.1 M phosphate buffer (pH 7.2). In each run, about 20 kinetic curves were photographed within 20 min and the average value of the rate constant was obtained from the curves. The concentration of hemoglobin and PMB was kept constant in the present investigation, 5×10^{-5} M in heme and 1×10^{-4} M, respectively. Deoxygenation of hemoglobins was carried out by successive evacuations and introductions of nitrogen gas. The second-order rate constant was evaluated by the equation,

$$k=\{(a-b)\ (t_1-t_2)\}^{-1}ln\{(a-x_1)\ (b-x_2)/(b-x_1)\ (a-x_2)\},$$

where a and b are the initial concentration of the SH groups and PMB, respectively, and x_1 is the concentration of the reacted SH groups during time $t=0$ to t_1.

The time for which the hemoglobin solutions were kept at 20° was 20–40 min. Using the rate constant of heme exchange reported by Bunn and Jandle,[21)22)] the percentage heme exchange between ferrihemoglobin at 20° for 40 min was calculated to be 2.8%. The exchange between the α chains and the non-α chains would be much less, being maximum one-ninth of the total exchange. The sum of the percentages of the heme and electron exchanges between ferrihemoglobin and oxyhemoglobin was of the same order of magnitude as the heme exchange in ferrihemoglobins. The contaminations of the hybrid half-methemoglobins due to heme and electron exchanges would therefore be small in the present experimental conditions. We made a preliminary experiment to check the exchanges. The deoxy $(\alpha^{+}_{PMB}+\beta_{PMB})$ was incubated at 20° for 2 hr in 1 M glycine-0.1 M phosphate buffer (pH 7.2). The aerated solution was then added by about 10 equivalent amounts of potassium cyanide to convert the met chains to the cyanmet form. The β chains were separated by the method of Bucci and Fronticelli[14)] and were analyzed for the ratio of the cyanmet and the oxy chains by the spectrophotometric method. The contents of the cyanmet form in the β chains before and after the incubation were 9 and 20%, respectively. Control experiment to check the autoxidation showed that the cyanmet contents increased from 11 to 17% after the incubation. Considering experimental errors in the determinations (5%), the results suggest that the contaminations of the half-met hemoglobins due to the exchanges are slight.

The content of the met chains in the hybrid hemoglobins, measured after the stopped flow experiments, was found to be 56% for $\alpha^{+}_{2}(H_2O)\beta_2(O_2)$ and 58% for $\alpha_2(O_2)\beta^{+}_{2}(H_2O)$.

Results

Interaction of DPG with the Half-Cyanmet Hybrid Hemoglobins

Effects of DPG on the Oxygen Affinity

Effects of DPG on the oxygen equilibrium curves of $\alpha^{+}_{2}(CN)\beta_2(O_2)$ and $\alpha_2(O_2)$-

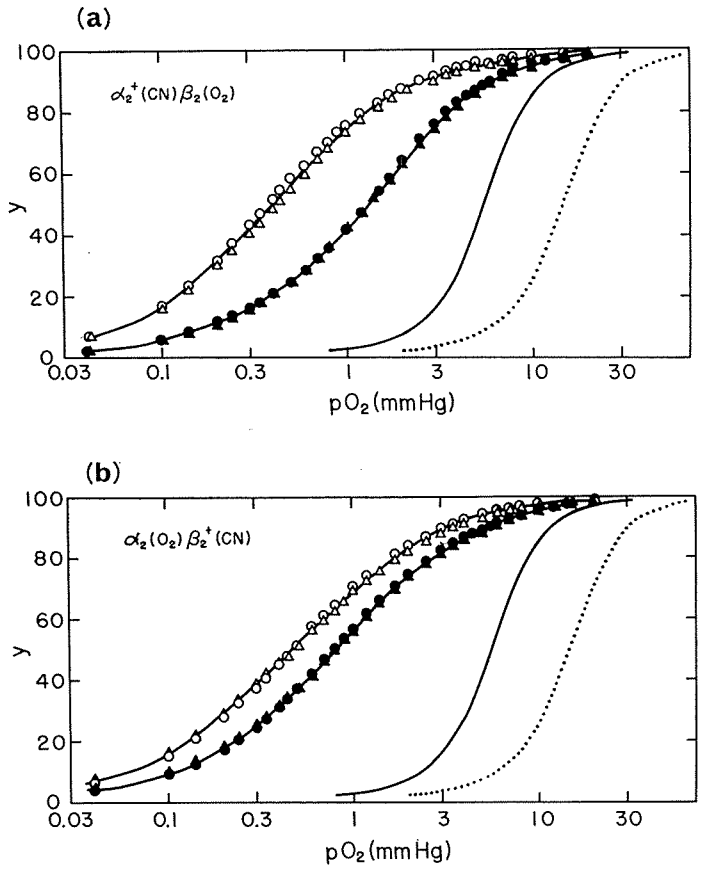

Fig. 1. Effects of DPG on the oxygenation curves of $\alpha_2^+(CN)\beta_2(O_2)$ and $\alpha_2(O_2)\beta_2^+(CN)$. Hemoglobin concentration, 5×10^{-5}M (heme equivalent); in 0.05M bis-tris buffer (pH 7.4), 2.5×10^{-4}M KCN and 0.1M NaCl; temperature, 25°. (a) $\alpha_2^+(CN)\beta_2(O_2)$, (b) $\alpha_2(O_2)\beta_2^+(CN)$. Open symbols, stripped; filled symbols, in 2 mM DPG. The curves for $\alpha_2(O_2)\beta_2(O_2)$ are given for comparison. Solid line, stripped; dotted line, in 2 mM DPG.

$\beta_2^+(CN)$ are shown in Fig. 1. DPG evidently shifts the curves to the right-hand side in both the hybrids and the effect on $\alpha_2^+(CN)\beta_2(O_2)$ is larger than that on $\alpha_2(O_2)$-$\beta_2^+(CN)$ in both the absence and presence of 0.1 M NaCl. Although the effect of DPG expressed by the ratio of P_{50} in the presence of 2 mM DPG to P_{50} for stripped hemoglobin is larger for normal hemoglobin, $\alpha_2(O_2)\beta_2(O_2)$, than for both the hybrids in NaCl-free buffer, it is larger for $\alpha_2^+(CN)\beta_2(O_2)$ than for $\alpha_2(O_2)\beta_2(O_2)$ in the presence of 0.1 M NaCl as summarized in Table 1, which is consistent with the result of Haber and Koshland.[23] Table 1 also includes the data of the isolated α and β

Table 1. Summary of the Effects of DPG on the Oxygen Equilibrium Functions of the Hybrid and Normal Hemoglobins and the Isolated Chains.

Hemoglobin Samples	Condition	P_{50} (mmHg)		P_{50}^{DPG}/P_{50}^{st}*	n	
		Stripped	in 2 mM DPG		Stripped	in 2 mM DPG
$\alpha_2(O_2)\beta_2(O_2)$	NaCl-free	1.9	15.3	8.1	2.52	3.02
	in 0.1 M NaCl	5.6	15.1	2.7	2.95	2.93
$\alpha_2^+(CN)\beta_2(O_2)$	NaCl-free	0.30	1.41	4.7	1.08	1.41
	in 0.1 M NaCl	0.40	1.38	3.5	1.17	1.52
$\alpha_2(O_2)\beta_2^+(CN)$	NaCl-free	0.40	0.83	2.1	1.15	1.13
	in 0.1 M NaCl	0.47	0.79	1.7	1.10	1.14
α Chain	in 0.1 M Phosphate Buffer	0.63	—	—	1.0	—
β Chain	in 0.1 M Phosphate Buffer	0.24	—	—	1.0	—

* ; the ratio of P_{50} in 2 mM DPG to P_{50} of stripped hemoglobin.
Experimental conditions are as described in Fig. 1.

chains for comparison.[24]

Since P_{50} of the stripped hybrids hardly changes by 0.1 M NaCl, that has large effects on the stripped normal hemoglobin, non-specific salt effects on the hybrids seem to be small. Accordingly, the effects of DPG on the hybrids are considered to be due to specific interactions of the phosphate with the protein molecules.

Effects of DPG on the Cooperativity

All the Hill plots of oxygenation for the hybrids show a tendency to deviate slightly from the straight line at the very high range of Y as shown in Fig. 2. The Hill plots for $\alpha_2^+(CN)\beta_2(O_2)$ given by Haber and Koshland[23] also seem to have the same tendency. The origin of the deviation is still unknown. The Hill plot for $\alpha_2^+(CN)\beta_2(O_2)$ in the presence of DPG exhibits another deviation from the straight line at the lower part of the plot. This deviation may be ascribable to the Darling-Roughton effect due to the inevitable contamination of the cyanmet β chains formed from the oxy β chains in the hybrid, *i.e.*, an increase in oxygen affinity and a decrease in the Hill coefficient of hemoglobin caused by the partial oxidation[25] or by the partial formation of cyanmet hemes.[26] Accordingly, we have confined the analysis and discussion to the main parts of the Hill plots giving a straight line.

The oxygen binding of the stripped hybrids is essentially non-cooperative with

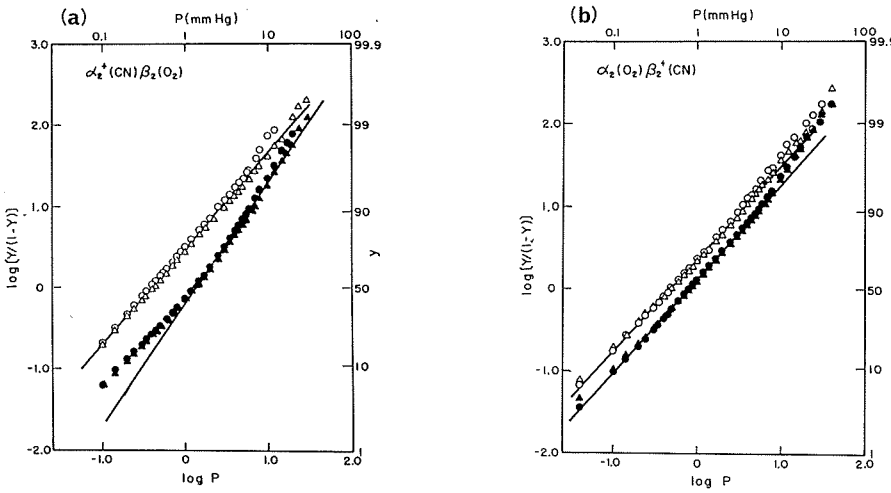

Fig. 2. Hill plots of the oxygenation of $\alpha_2^+(CN)\beta_2(O_2)$ and $\alpha_2(O_2)\beta_2^+(CN)$ in the absence and presence of DPG. Experimental conditions and symbols are as described in Fig. 1. (a) $\alpha_2^+(CN)\beta_2(O_2)$, (b) $\alpha_2(O_2)\beta_2^+(CN)$.

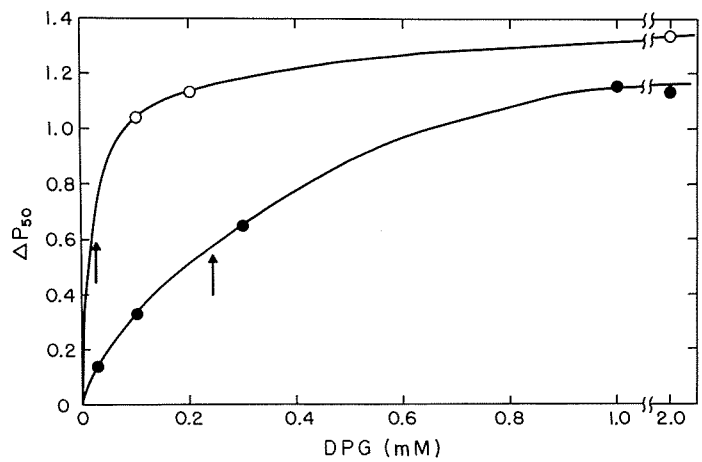

Fig. 3. Dependence of the oxygen affinity of $\alpha_2(O_2)\beta_2(O_2)$ and $\alpha_2^+(CN)$-$\beta_2(O_2)$ on the concentration of DPG. The ordinate is the shift of P_{50} from that of stripped hemoglobin and given by $\times 10$ mmHg for $\alpha_2(O_2)\beta_2(O_2)$ and mmHg for $\alpha_2^+(CN)\beta_2(O_2)$. Open circles, $\alpha_2(O_2)\beta_2(O_2)$; filled circles, $\alpha_2^+(CN)\beta_2(O_2)$. Arrows show the values of the maximum P_{50} shift. The concentration of DPG giving those values is 3×10^{-5} M for $\alpha_2(O_2)\beta_2(O_2)$ and 2.3×10^{-4} M for $\alpha_2^+(CN)\beta_2(O_2)$. Experimental conditions are as described in Fig. 1. except in NaCl-free buffer.

the Hill coefficient, n, 1.08 to 1.17 as summarized in Table 1. Even in the presence of DPG the oxygen binding of $\alpha_2(O_2)\beta_2^+(CN)$ is essentially non-cooperative. However, the addition of DPG makes the oxygen binding of $\alpha_2^+(CN)\beta_2(O_2)$ significantly cooperative. These circumstances remain unchanged in the presence of 0.1 M NaCl.

On the other hand, it has been found that 2 mM DPG increases the Hill coefficient of normal hemoglobin from 2.5 to 3.0 only in the absence of NaCl.[24]

Dependence of the Oxygen Equilibrium Curves of $\alpha_2^+(CN)\beta_2(O_2)$ on the DPG concentration

The oxygen affinity of $\alpha_2^+(CN)\beta_2(O_2)$ gradually decreases with increasing DPG concentration as shown in Fig. 3. In NaCl-free buffer the effect of DPG in lowering the oxygen affinity, however, is weak for $\alpha_2^+(CN)\beta_2(O_2)$ compared with that for $\alpha_2(O_2)\beta_2(O_2)$. The concentration of DPG giving the half value of the maximum P_{50} shift is 2.3×10^{-4} M for $\alpha_2^+(CN)\beta_2(O_2)$ and 3×10^{-5} M for $\alpha_2(O_2)\beta_2(O_2)$. Moreover, the slope of the main part of the Hill plots gradually increases with DPG concentration (0.03 to 2 mM).

The Reaction of PMB with the β93 SH groups of the Half-met Hybrid Hemoglobins

The kinetic curves for the reaction of PMB with the β93 SH groups of the re-

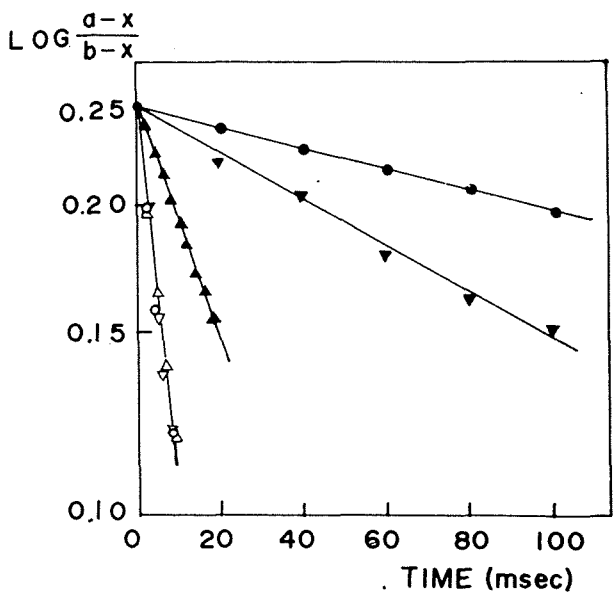

Fig. 4. Kinetic plots for the reaction of PMB with the β93 SH groups of the half-met hemoglobins. a and b are the initial concentration of the SH groups and PMB, respectively, and x is the concentration of the reacted SH groups during time t=0 to t. In all theexperiments, a and b are kept constant, 2.5×10^{-5} M and 1×10^{-4} M, respectively. ● and ○ : deoxy and oxy $\alpha_2\beta_2$, ▼ and ▽ : deoxy and oxy $\alpha_2^+(H_2O)\beta_2$ and △ and ▲: deoxy and oxy $\alpha_2\beta_2^+(H_2O)$.

constituted $\alpha_2(O_2)\beta_2(O_2)$, $\alpha_2^+(H_2O)\beta_2(O_2)$, $\alpha_2(O_2)\beta_2^+(H_2O)$, and ferrihemoglobin were measured both in the deoxy and in the oxy states and some typical plots of the kinetic results were reproduced in Fig. 4. In a few experiments the kinetic polt became biphasic, but further experiments gave monophasic straight line. We discarded the biphasic data because such curves are considered to originate from mixtures contaminated with some impurities. The rate constants are calculated from the kinetic plots and the obtained average values are given in Table 2.

Table 2. Second-order rate constant of the reaction of PMB with the β93 SH groups of the half-methemoglobins at 20° in 0.1 M phosphate buffer (pH 7.2).*

Hemoglobin	Second-order rate constant (M^{-1} sec^{-1}) Oxy	Deoxy
$\alpha_2\beta_2$	$(1.5\pm0.5)\times10^6$	$(3.3\pm0.8)\times10^4$
$\alpha_2+\beta_2$	$(1.5\pm0.2)\times10^6$	$(9.9\pm1.3)\times10^4$
$\alpha_2\beta_2^+$	$(1.5\pm0.4)\times10^6$	$(3.5\pm0.5)\times10^5$
$\alpha_2^+\beta_2^+$	1.5×10^6	

* The concentration of PMB and hemoglobin, after mixing was 1×10^{-4} and 5×10^{-5} M in heme, respectively. The average values of more than eight measurements with several independent preparations are given.

Discussion

Cooperativity in Oxygen Binding of the Half-cyanmet Hybrid Hemoglobins

It has been found that 2 mM DPG increases the Hill coefficient of normal hemoglobin from 2.5 to 3.0 in the absence of NaCl.[24] The phosphate also increases the cooperativity in oxygen binding of $\alpha_2^+(CN)\beta_2(O_2)$. In $\alpha_2^+(CN)\beta_2(O_2)$ and $\alpha_2(O_2)$-$\beta_2^+(CN)$ the oxygen binding is essentially non-cooperative in the absence of DPG, while in the former cooperativity appears on the addition of 2 mM DPG. This cooperativity would be ascribable to the stabilization of the deoxy state through the cross-linkage between the β subunits by DPG. On the other hand, oxygen binding in $\alpha_2(O_2)\beta_2^+(CN)$ is essentially non-cooperative even in the presence of DPG though it lowers the oxygen affinity of the hybrid. It may be due to weaker binding of DPG to $\alpha_2\beta_2^+(CN)$ than to $\alpha_2^+(CN)\beta_2$.

The present result that DPG is capable of interacting with the hybrid hemoglobins is consistent with the results of NMR studies by Ogawa and Shulman,[27] a study of the binding of a spin-labeled triphosphate with hemoglobin by Ogata and McConnell[28] and a recent report of the effect of DPG on oxygen equilibrium of $\alpha_2^+(CN)\beta_2(O_2)$ by Haber and Koshland.[23] Especially, the result obtained by Ogata and McConnell that a spin-labeled triphosphate binds more strongly to $\alpha_2^+(CN)\beta_2$ than to $\alpha_2\beta_2^+(CN)$ is in good agreement with the present result that DPG has larger effect on the oxygen affinity of $\alpha_2^+(CN)\beta_2(O_2)$ than on that of $\alpha_2(O_2)\beta_2^+(CN)$.

On the other hand, the observation of Haber and Koshland on the cooperativity in oxygen binding of $\alpha_2^+(CN)\beta_2(O_2)$ is in quite conflict with our results. They have found that DPG decreases the oxygen affinity of $\alpha_2^+(CN)\beta_2(O_2)$ without affecting the slope of the Hill plot ($n=1.3$) in 0.2 M N-2-hydroxyethylpiperazine-N'-2-aminoethanesulfonic acid buffer, pH 7.2 containing 0.06 M NaCl and 5×10^{-4} M EDTA. The origin of this discrepancy in the conclusions on the cooperativity in $\alpha_2^+(CN)\beta_2(O_2)$ is unknown at present.

The Reactivity of the β93 SH groups of the Half-met Hybrid Hemoglobins

Several conclusions can be drawn from comparison of the rate constants for various hemoglobins summarized in Table 3. (1) The ferric β subunit is equivalent to the oxy subunit with respect to the reactivity. The rate constants for $\alpha_2(O_2)\beta_2(O_2)$,

Table 3. Conformation of the β subunit in relation to the state of neighboring α subunit.*

State of β subunit	State of neighboring α subunit		
	Deoxy	Met	Oxy
Deoxy	R	Q	
Met	P	O	O
Oxy		O	O

* The β conformations designated as O, P, Q, and R are the conformations in which the β subunit has the rate constant for the reaction with PMB of 1.5×10^6, 3.5×10^5, 9.9×10^4, and 3.3×10^4 M^{-1} sec^{-1}, respectively.

$\alpha_2^+(H_2O)\beta_2(O_2)$, $\alpha_2(O_2)\beta_2^+(H_2O)$, and $\alpha_2^+(H_2O)\beta_2^+(H_2O)$ are all the same. Evidences for such equivalence of the met to the oxyhemoglobin have been accumulated by various methods; X-ray crystal analysis, for example.[29] (2) The reactivity of the β subunit is primarily dependent on ligation of the β subunit. The rate constant for $\alpha_2(O_2)\beta_2(O_2)$ or $\alpha_2^+(H_2O)\beta_2^+(H_2O)$ is some 50 times larger than that for $\alpha_2\beta_2$. (3) The reactivity of the β subunit is also dependent on ligation of the neighboring α subunits. The rate constants for $\alpha_2(O_2)\beta_2^+(H_2O)$ is different from that for $\alpha_2\beta_2^+(H_2O)$ (about 5 times larger). Similar differences are observed between $k(\alpha_2^+(H_2O)\beta_2)$ and $k(\alpha_2\beta_2)$, and also between $k(\alpha_2\beta_2^+(H_2O))$ and $k(\alpha_2^+(H_2O)\beta_2^+(H_2O))$.

Influence of the State of α Subunit on the Conformation of β Subunit.

If we assume that some changes in the rate constant of the reaction of PMB with the β93 SH groups can be taken as a measure for conformational changes in the β subunit, the above results suggest that the β subunit takes four different conformations depending on the state of the α subunit. One is the conformation in fully oxygenated hemoglobin, named O, giving $k=1.5\times10^6$ M^{-1} sec^{-1} and the second is the conformation in fully deoxygenated hemoglobin, named R, giving $k=3.3\times10^4$ M^{-1} sec^{-1}.

In addition to the known conformations, the β subunit takes two more intermediate conformations, P($k=3.5\times10^5\ M^{-1}\ sec^{-1}$) and Q($k=9.9\times10^4\ M^{-1}\ sec^{-1}$). The conformation of deoxy β subunit is R with deoxy α and changes into Q when the α subunit become ferric. The met β subunit takes O conformation with oxy and met α subunit and the conformation changes into P when the α subunits becomes deoxygenated (see Table III). Ligation of the α subunits does affect the conformation of the neighboring β subunits. The conclusion is consistent with recent physicochemical studies of hemoglobin.[4),27),28),30)]. Moreover, in $\alpha_2(O_2)\beta_2^+(CN)$ the response of the oxidized β subunits to DPG also implies that the β subunits change the conformation with the oxygenation of the α subunits. Otherwise, the oxygen affinity of $\alpha_2(O_2)\beta_2^+(CN)$ would be insensitive to DPG. The conformational changes propagating to neighboring subunits over a subunit where ligation occurs, would contribute to the cooperativity in oxygen binding of hemoglobin.

Mechanism of Oxygen Binding in Hemoglobin

On the assumption that the oxidation of subunit to the cyanmet or met form is equivalent to the oxygenation, it is interesting to consider which model, the MWC or the KNF model better fits to the present results.

The MWC model predicts that $\alpha_2^+(CN)\beta_2$ and $\alpha_2\beta_2^+(CN)$ are in the equivalent state and respond to DPG in the same manner. On the other hand, if we accept that DPG exclusively binds to the β subunits in the deoxy quaternary structure, the KNF model requires that DPG decreases only the oxygen affinity of $\alpha_2^+(CN)\beta_2$ and not that of $\alpha_2\beta_2^+(CN)$ since the β subunits in $\alpha_2^+(CN)\beta_2$ should be still in the deoxy conformation and those in $\alpha_2\beta_2^+(CN)$ should have already transformed into the oxy conformation. Neither prediction, however, accords with the present results since DPG decreases the oxygen affinity of both the hybrids and the magnitude of the effects is larger for $\alpha_2^+(CN)\beta_2(O_2)$ than for $\alpha_2(O_2)\beta_2^+(CN)$.

Here two explanations corresponding to the two models with some modifications will be possible. One of them is the generalized MWC model,[28)] in which the α and β subunits have different intrinsic affinities for ligand and the ligation of them affects the allosteric equilibrium between two states unequally. The introduction of the non-equivalence of the α and β subunits into the MWC model permits the oxygen equilibrium curves of $\alpha_2^+(CN)\beta_2(O_2)$ and $\alpha_2(O_2)\beta_2^+(CN)$ to be affected by DPG in different manners. The alternative is the KNF model with a modification that the conformation of a subunit depends not only on the ligation of the subunit itself, but also partially on that of neighboring subunits. According to this model the β subunits are able to take at least four different conformations corresponding to $\alpha_2\beta_2$, $\alpha_2(O_2)\beta_2$ (or $\alpha_2^+(CN)\beta_2$), $\alpha_2\beta_2(O_2)$ (or $\alpha_2\beta_2^+(CN)$) and $\alpha_2(O_2)\beta_2(O_2)$ (or $\alpha_2^+(CN)\beta_2(O_2)$ and $\alpha_2(O_2)\beta_2^+(CN)$). Assuming that $\alpha_2\beta_2(O_2)$ (or $\alpha_2\beta_2^+(CN)$) can bind DPG with a smaller binding constant than that for $\alpha_2(O_2)\beta_2$ (or $\alpha_2^+(CN)\beta_2$), the modified KNF model can also well account for the present results on the effects of DPG on the oxygen affinity of both the hybrids. Accordingly, although the present results fail to fit to the original simple MWC and KNF models, both the modles with

the above modification can account for them equally.

Haber and Koshland regarded the demonstration of cooperativity in oxygen binding of $\alpha_2^+(CN)\beta_2(O_2)$ and the response of the hybrid to DPG as evidence that ligand binding to hemoglobin follows a mechanism of sequential conformational changes rather than a concerted pathway and analyzed the oxygen equilibrium curves of $\alpha_2^+(CN)\beta_2(O_2)$ in the absence and presence of DPG on the basis of the simple KNF model. However the cooperativity in oxygen binding of $\alpha_2^+(CN)\beta_2(O_2)$ and the response of the hybrid to DPG observed by them do not necessarily exclude the MWC model and moreover the simple KNF model is inadequate to account for the present results on the response of both the hybrids to DPG.

On the other hand, the results on the reactivity of the β93 SH groups of the half-met hybrids indicate that the conformation of the β subunit is primarily dependent upon ligation of the β subunit and is also dependent on ligation of the neighboring α subunit. The conformational change in the α subunit does propagate to the β subunit. Effect of the propagation is not to change the β conformation to the R or O state but to some other P or Q state. These results also are interpreted successfully by the modified MWC and the KNF models, discussed above, rather than the original simple ones as well as effects of DPG on the oxygen equilibria of the half-cyanmet hybrid hemoglobins. Because the simple MWC model does not hold in its symmetry conserving form for the present system of hemoglobin and the simple KNF model allowing the symmetry breaking does not take the intermediate conformations except the oxy and deoxy conformations and propagation of conformational changes into account.

Recent studies on the binding of a spin-labeled triphosphate to hemoglobin by Ogata and McConnell[28] have supported their generalized MWC model rather than the simple KNF model. They have shown that the electron resonance spectra of the spin-label are only the superposition of the signals of free labels and those bound to hemoglobin and have sharp isobestic points at the various stages of ligation. The results suggest that the spin-label bound to hemoglobin experiences only one enviroment and there exists only one conformation in deoxyhemoglobin and partialy liganded hemoglobins available for DPG binding as far as observed by the spin-label technique. Ogawa and Shulman[27] and Cassoly *et al.*[31] showed by nuclear magnetic resonance studies that the cyanmet subunits in the deoxygenated hybrids apparently are in an equilibrium between different conformations and organic phosphates shift the conformational equilibrium. Although this result also supports the MWC model, in order to discriminate the modified MWC and KNF model and to establish an unique model it is necessary to obtain information on conformational aspects in the α subunit of the hybrid hemoglobins.

Acknowledgement

The author is indebted to Professor S. Ohnishi for his continuing guidance and encouragement.

References

1) Monod, J., J. Wyman and J.-P. Changeux, On the nature of allosteric transitions: A plausible model. J. Mol. Biol. **12:** 88, 1965
2) Koshland, D. E., Jr., G. Némethy, and D. Filmer, Comparison of experimental binding data and theoretical models in proteins containing subunits. Biochemistry **5:** 365, 1966
3) Ogawa, S. and H. M. McConnell, Spin-label study of hemoglobin conformations in solution. Proc. Natl. Acad. Sci. U. S. **58:** 19, 1967
4) Hayashi, A., T. Suzuki, A. Shimizu, H. Morimoto and H. Watari, Changes in EPR spectra of M-type abnormal haemoglobins induced by deoxygenation and their implication for the haem-haem interaction. Biochim. Biophys. Acta **147:** 407, 1967
5) Ogawa, S., H. M. McConnell, and A. Horwitz, Overlapping conformation changes in spin-labelled hemoglobin. Proc. Natl. Acad. Sci. U. S. **61:** 401, 1968
6) Shulman, R. G., S. Ogawa, K. Wüthrich, T. Yamane, J. Peisach, and W. E. Blumberg, The absence of heme-heme interactions in hemoglobin. Science **165:** 251, 1969
7) Antonini, E. and M. Brunori, On the rate of a conformational change associated with ligand binding in hemoglobin. J. Biol. Chem. **244:** 3909, 1969
8) Brunori, M., G. Amiconi, E. Antonini, J. Wyman, and K. H. Winterhalter, Artificial intermediates in the reaction of hemoglobin. J. Mol. Biol. **49:** 461, 1970
9) Perutz, M. F., Stereochemistry of cooperative effect in hemoglobin. Nature **228:** 734, 1970
10) Benesch, R., and R. E. Benesch, Intracellular organic phosphates as regulators of oxygen release by hemoglobin. Nature **221:** 618, 1969
11) Benesch, R. E., R. Benesch, and C. I. Yu, The effect of pyridoxal phosphate on the oxygenation of hemoglobin. Fed. Proc. **28:** 604, 1969
12) Bunn, H. F., and R. W. Briehl, The interaction of 2, 3-diphosphoglycerate with various human hemoglobin. J. Clin. Invest. **49:** 1088, 1970
13) Benesch, R. and R. E. Benesch, Some relations between structure and function in hemoglobin. J. Mol. Biol. **6:** 498, 1963
14) Bucci, E. and C. Fronticelli, A new method for the preparation of α and β subunits of human hemoglobin. J. Biol. Chem. **240:** PC 551, 1965
15) Geracci, G., L. J. Parkhurst, and Q. H. Gibson, Preparation and properties of α and β chains from human hemoglobin. J. Biol. Chem. **244:** 4664, 1969
16) Tyuma, I., R. E. Benesch, and R. Benesch, The preparation and properties of the isolated α and β subunits of hemoglobin A. Biochemistry **5:** 2957, 1966
17) Boyer, P. D., Spectrophotometric study of the reaction of protein sulfhydryl groups with organic mercurials. J. Amer. Chem. Soc. **76:** 4331, 1954
18) Benesch, R., and R. E. Benesch, Determination of SH groups in protein. Methods Biochem. Anal. **10:** 43, 1962
19) Evelyn, K. A. and H. T. Malloy, Microdetermination of oxyhemoglobin, methemoglobin and sulfhemoglobin in a single sample of blood. J. Biol. Chem. **126:** 655, 1938
20) Imai, K., H. Morimoto, M. Kotani, H. Watari, W. Hirata and M. Kuroda, Studies on the function of abnormal hemoglobins. I An improved method for automatic measurement of the oxygen equilibrium curve of hemoglobin. Biochim. Biophys. Acta **200:** 189, 1970
21) Bunn, H. F. and J. H. Jandle, Exchange of heme among hemoglobin molecules. Proc. Natl. Acad. Sci. U. S. **56:** 975, 1966
22) Bunn, H. F. and J. H. Jandle, Exchange of heme among hemoglobins and between hemoglobin and albumin. J. Biol. Chem. **243:** 465, 1968

23) Haber, J. E. and D. E. Koshland, Jr., The effect of 2, 3-diphosphoglyceric acid on the changes in β–β interactions in hemoglobin during oxygenation. J. Biol. Chem. **246:** 7790, 1970
24) Tyuma, I., K. Shimizu, and K. Imai, Effect of 2, 3-diphosphoglycerate on the cooperativity in oxygen binding of human adult hemoglobin. Biochem. Biophys. Res. Commun. **43:** 423, 1971
25) Darling, R. C. and F. J. W. Roughton, The effect of methemoglobin on the equilibrium between oxygen and hemoglobin. Amer. J. Physiol. **137:** 56, 1942
26) Benesch, R. E., R. Benesch, and G. Macduff, Subunit exchange and ligand binding. Proc. Natl. Acad. Sci. U. S. **54:** 535, 1965
27) Ogawa, S. and R. G. Shulman, Observation of allosteric transition in hemoglobin. Biochem. Biophys. Res. Commun. **42:** 9, 1971
28) Ogata, R. T. and H. M. McConnell, The binding of a spin-labeled phosphate to hemoglobin. Cold Spring Harbor Symp. Quant. Biol. **36:** 325, 1971
29) Muirhead, H., J. M. Cox, L. Mazzarella, and M. F. Perutz, Structure and function of hemoglobin. III A three-dimensional fourier synthesis of human deoxyhemoglobin at 5.5 Å resolution. J. Mol. Biol **28:** 117, 1967
30) Asakura, T., and H. R. Drott, Evidence of heme-heme interaction in heme-spin-labeled hemoglobin. Biochem. Biophys. Res. Commun. **44:** 1199, 1971
31) Cassoly, R., Q. H. Gibson and R. G. Shulman, Effects of phosphate upon CO binding kinetics and NMR spectra of hemoglobin valency hybrids. Biochem. Biophys. Res. Commun. **44:** 1015, 1971