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# Kinetic Evidence for Propagation of Conformational Changes in the $\alpha$ Subunit to the $\beta$ Subunit of Hemoglobin*

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ABSTRACT: The reaction of *p*-mercuribenzoate with the $\beta$93 SH groups of the artificial half-methemoglobins has been studied by the stopped-flow method, to investigate propagation of conformational change in a subunit to the neighboring subunits. The second-order rate constants for the reaction have been evaluated from kinetic plots. It is found, from comparison of the rate data, that the reactivity of the $\beta$ subunit is primarily dependent on ligation of the $\beta$ subunit and is also dependent on ligation of the neighboring $\alpha$ subunits.

With an assumption that some changes in the rate constant reflect alterations in conformation of the $\beta$ subunit, the present results suggest that the $\beta$ subunit takes four different conformations, named O, P, Q, and R, depending on the state of the $\alpha$ subunit. Deoxy $\beta$ subunit takes R conformation with deoxy $\alpha$ and Q with met $\alpha$. The conformation of met $\beta$ subunit is O with oxy and met $\alpha$ and changes into P when the $\alpha$ subunit becomes deoxygenated. Thus, ligation of the $\alpha$ subunit affects the conformation of the $\beta$ subunit. Some implications are presented on the allosteric mechanism.

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 (Monod *et al.*, 1965). 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 (Koshland *et al.*, 1966).

Both 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 (Ogawa and McConnell, 1967; Hayashi *et al.*, 1967; Ogawa *et al.*, 1968; Shulman *et al.*, 1969; Antonini and Brunori, 1969; Brunori *et al.*, 1970). All the results indicated that the conformation of a subunit changed when the subunit was liganded. On the possibility of propagation of the conformational changes to the neighboring subunits, however, there have been published two different conclusions. Most authors did not observe such propagation while some reported existence of the propagation. To study this problem, we measured, in the present paper, the rate constant of the reaction of PMB[1] with the $\beta$93 SH groups of the artificial half-methemoglobins, since the rates of reactions of various sulfhydryl reagents with the $\beta$93 SH groups of oxyhemoglobin have been known to be greatly different from those for deoxyhemoglobin (Antonini and Brunori, 1969, and the literatures listed therein). Our results have suggested propagation of conformational changes in the $\alpha$ subunit to the $\beta$ subunit.

## Experimental Section

*Isolation of $\alpha$ and $\beta$ Chains.* Human adult hemoglobin was prepared from fresh blood by lysing washed red cells with 1–1.5 volumes of water. The mercurated $\alpha$ chains, $\alpha_{PMB}$, were prepared by the method of Geraci *et al.* (1969) and the mercurated $\beta$ chains, $\beta_{PMB}$, were obtained according to the procedure described by Bucci and Fronticelli (1965). The $\alpha$ chains were rendered free of mercury by washing the $\alpha_{PMB}$ 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 $\beta_{PMB}$ chains was carried out by the method of Tyuma *et al.* (1966).

*Preparation of Hybrid Half-Methemoglobins.* The $\alpha$ chains

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[1] Abbreviation used is: PMB, *p*-mercuribenzoate. The superscripts +, $O_2$, and +CN, written to the right of $\alpha_2$ and $\beta_2$, represent that the indicated subunits are in the met, in the oxygenated, and in the cyanmet form, respectively.

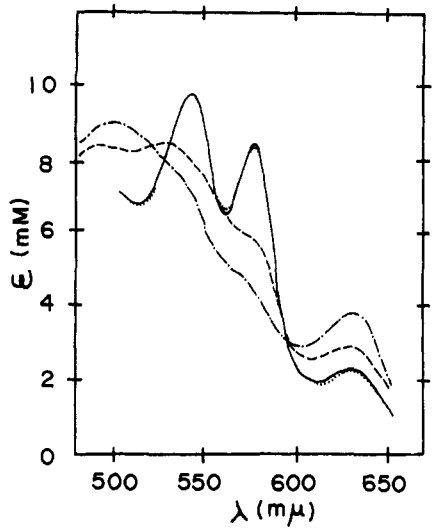


FIGURE 1: Absorption spectra of the isolated $\alpha^+$ chains (-···-), the $\beta^+$ chains (-----), the hybrid hemoglobin $\alpha_2^+\beta_2$ (——), and the $\alpha_2\beta_2^+$ (. . . . . ) in 0.2 M phosphate buffer (pH 5.9). $\epsilon$(mM) is expressed on heme basis.

were oxidized to the ferric form at 6° for 0.5–1 min with 20 equivalent amounts of potassium ferricyanide and the excess reagents were removed immediately after the oxidation through Sephadex G-25 column equilibrated with 0.1 M phosphate buffer (pH 7.2). The half-methemoglobin $\alpha_2^+\beta_2$ was obtained by mixing the $\alpha$ chains, immediately after the preparation, with equivalent amounts of the $\beta$ chains. The $\alpha_2\beta_2^+$ was prepared in the same manner. The preparations of the hybrid half-methemoglobins were carried out at low temperatures (0–6°) to avoid the heme and electron exchanges among the chains and the kinetic measurements were performed quickly after the preparations. The proportion of the met chains in the hybrid hemoglobin was estimated by the method of Evelyn and Malloy (1938). The concentration of hemoglobin was determined spectrophotometrically at 540 m$\mu$ after conversion into cyanmethemoglobin ($\epsilon$ 1.15 $\times$ $10^4$ $M^{-1}$ $cm^{-1}$ (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.

Horizontal starch gel electrophoresis was carried out with Connaught hydrolyzed starch by the method of Smithies (1955) using the discontinuous buffer system of Poulik (1957). The electropherograms of the reconstituted $\alpha_2^+\beta_2$, $\alpha_2\beta_2^+$, and $\alpha_2\beta_2$ showed that the reconstitutions were essentially complete, no bands for the isolated chains being recognizable.

The free SH groups of the hemoglobins were determined spectrophotometrically with PMB as described by Boyer (1954) and Benesch and Benesch (1962).

*Kinetic Measurements.* The reaction of PMB with hemoglobin was followed by displaying the optical absorption at 255 m$\mu$ (slit width 2 m$\mu$) 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 $\times$ $10^{-5}$ M in heme and 1 $\times$ $10^{-4}$ 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, $k = \{(a-b)(t_1-t_2)\}^{-1} \ln\{(a-x_1)(b-x_2)/(b-x_1)\cdot(a-x_2)\}$, 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 the 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 Jandl (1966, 1968), the percentage heme exchange between ferrihemoglobins at 20° for 40 min was calculated to be 2.8%. The exchange between the $\alpha$ chains and the non-$\alpha$ 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 the 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 $\beta$ chains were separated by the method of Bucci and Fronticelli (1965) 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 $\beta$ 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-methemoglobins due to the exchanges are slight.

## Results

*Some Properties of the Hybrid Half-Methemoglobin.* The optical absorption spectra at pH 5.9 of the isolated ferric chains and the hybrid half-metHbs are shown in Figure 1, and are essentially identical with those reported by Banerjee *et al.* (1969). The ferric chains were relatively unstable and the spectra gradually changed with decrease of the peak at 630 m$\mu$ and accompanying appearance of a peak at 530 m$\mu$. Therefore, the ferric chains were mixed with the partner ferrous chains immediately after preparations. The hybrid hemoglobins were fairly stable.

The free SH groups of the half-methemoglobins were titrated and the obtained numbers per tetramer were 2.3 for $\alpha_2^+\beta_2$ and 2.2 for $\alpha_2\beta_2^+$. The corresponding value for reconstituted $\alpha_2\beta_2$ was 2.2. These values agree well with that for intact hemoglobin tetramer. The content of the met chains in the hybrid hemoglobins, measured after the stopped flow experiments, was found to be 56% for $\alpha_2^+\beta_2$ and 58% for $\alpha_2\beta_2^+$.

*Second-Order Rate Constant.* The kinetic curves for the reaction of PMB with the $\beta$93 SH groups of the reconstituted $\alpha_2\beta_2$, $\alpha_2^+\beta_2$, $\alpha_2\beta_2^+$, and ferrihemoglobin were measured both in the deoxy and in the oxy states and some typical plots of the kinetic results were reproduced in Figure 2. In a few experiments the kinetic plot 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 I.

TABLE I: Second-Order Rate Constant of the Reaction of PMB with the $\beta$93 SH Groups of the Half-Methemoglobins at 20° in 0.1 M Phosphate Buffer (pH 7.2).[a]

| Hemoglobin | Second-Order Rate Constant ($M^{-1}$ $sec^{-1}$) Oxy | Deoxy |
|---|---|---|
| $\alpha_2\beta_2$ | $(1.5 \pm 0.5) \times 10^6$ | $(3.3 \pm 0.8) \times 10^4$ |
| $\alpha_2^+\beta_2$ | $(1.5 \pm 0.2) \times 10^6$ | $(9.9 \pm 1.3) \times 10^4$ |
| $\alpha_2\beta_2^+$ | $(1.5 \pm 0.4) \times 10^6$ | $(3.5 \pm 0.5) \times 10^5$ |
| $\alpha_2^+\beta_2^+$ | $1.5 \times 10^6$ | |

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

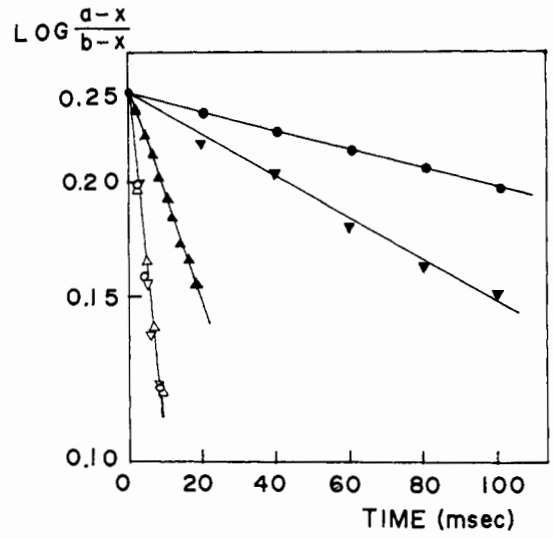


FIGURE 2: Kinetic plots for the reaction of PMB with the $\beta$93 SH groups of the hybrid half-met hemoglobins. $a$ and $b$ are the initial concentration of the SH groups and PMB, respectively, and $x$ is the concentration of the reacted SH groups during time $t = 0$ to $t$. In all the experiments, a and b are kept constant, $2.5 \times 10^{-5}$ and $1 \times 10^{-4}$ M, respectively. ● and ○: deoxy and oxy $\alpha_2\beta_2$, ▼ and ▽: deoxy and oxy $\alpha_2^+\beta_2$, and ▲ and △: deoxy and oxy $\alpha_2\beta_2^+$.

## Discussion

Several conclusions can be drawn from comparison of the rate constants for various hemoglobins. (1) The ferric $\beta$ subunit is equivalent to the oxy subunit with respect to the reactivity. The rate constants for $\alpha_2^{O_2}\beta_2^{O_2}$, $\alpha_2^+\beta_2^{O_2}$, $\alpha_2^{O_2}\beta_2^+$, and $\alpha_2^+\beta_2^+$ are all the same. Evidences for such equivalence of the met- to the oxyhemoglobins have been accumulated by various methods; X-ray crystal analysis, for example (Muirhead *et al.*, 1967). (2) The reactivity of the $\beta$ subunit is primarily dependent on ligation of the $\beta$ subunit. The rate constant for $\alpha_2^{O_2}\beta_2^{O_2}$ or $\alpha_2^+\beta_2^+$ is some 50 times larger than that for $\alpha_2\beta_2$. (3) The reactivity of the $\beta$ subunit is also dependent on ligation of the neighboring $\alpha$ subunit. The rate constant for $\alpha_2^{O_2}\beta_2^+$ is different from that for $\alpha_2\beta_2^+$ (about 5 times larger). Similar differences are observed between $k(\alpha_2^+\beta_2)$ and $k(\alpha_2\beta_2)$, and also between $k(\alpha_2\beta_2^+)$ and $k(\alpha_2^+\beta_2^+)$.

*Influence of the State of $\alpha$ Subunit on the Conformation of $\beta$ Subunit.* If we assume that some changes in the rate constant of the reaction of PMB with the $\beta$93 SH groups can be taken as a measure for conformational changes in the $\beta$ subunit, the above results suggest that the $\beta$ subunit takes four different conformations depending on the state of the $\alpha$ subunit. One is the conformation in fully oxygenated hemoglobin, named O, giving $k = 1.5 \times 10^6$ $M^{-1}$ $sec^{-1}$ and the second is the conformation in fully deoxygenated hemoglobin, named R, giving $k = 3.3 \times 10^4$ $M^{-1}$ $sec^{-1}$. In addition to the two known conformations, the $\beta$ subunit takes two more intermediate conformations, P ($k = 3.5 \times 10^5$ $M^{-1}$ $sec^{-1}$) and Q ($k = 9.9 \times 10^4$ $M^{-1}$ $sec^{-1}$). The conformation of deoxy $\beta$ subunit is R with deoxy $\alpha$ and changes into Q when the $\alpha$ subunit becomes ferric. The met $\beta$ subunit takes O conformation with oxy and met $\alpha$ subunit and the conformation changes into P when the $\alpha$ subunit becomes deoxygenated (see Table II). Ligation of the $\alpha$ subunits does affect the conformation of the neighboring $\beta$ subunits.

Various data have been published on the propagation of conformational changes to the neighboring subunits. Hayashi *et al.* (1967) measured electron spin resonance signal at −196° of the ferric ion in the $\beta$ subunit of an abnormal hemoglobin, Hb $M_{Hyde\ Park}$, and found a change in the signal on oxygenation of the $\alpha$ subunit. This change, however, was not observed in other Hb M samples. Ogawa *et al.* (1968) looked at electron spin resonance signals of a spin label attached to the $\beta$93 SH groups of the half-cyanmethemoglobin and observed a slight but definite difference between $\alpha_2\beta_2^{+CN}$ and $\alpha_2^{O_2}\beta_2^{+CN}$. With another spin label, however, they observed no change in the electron spin resonance spectrum when the $\alpha$ chains were oxygenated and deoxygenated. Shulman *et al.* (1969) reported that the nuclear magnetic resonance spectra of the heme protons in the mixed-state hemoglobins, $\alpha_2\beta_2^+$ and $\alpha_2\beta_2^{+CN}$, were found to be only a superposition of the spectra of $\alpha_2\beta_2$ and $\alpha_2^+\beta_2^+$, and $\alpha_2\beta_2$ and $\alpha_2^{+CN}\beta_2^{+CN}$, respectively. Antonini and Brunori (1969) suggested that the rate of the reaction of PMB with the $\beta$93 SH groups of hemoglobin was dependent exclusively on the presence of ligand on the $\beta$ chains and was independent of the state of the $\alpha$ chains. In a more recent study with the artificial half-cyanmethemoglobins, which has been done independently of us, they again confirmed the above suggestion (Brunori *et al.*, 1970). They obtained the same reaction curve of PMB for $\alpha_2^{O_2}\beta_2^{+CN}$ and $\alpha_2\beta_2^{+CN}$. However, if we compare the rate constant for $\alpha_2^{+CN}\beta_2$ calculated from the given curve with that for $\alpha_2\beta_2$ given in their previous paper, we notice a difference between the constants.

Examination of these various results, which sometimes look contradictory among them, leads to the following suggestions. The propagation of conformational changes, if any, takes

TABLE II: Conformation of the $\beta$ Subunit in Relation to the State of Neighboring $\alpha$ Subunit.[a]

| State of $\beta$ Subunit | State of Neighboring $\alpha$ Subunit Deoxy | Met | Oxy |
|---|---|---|---|
| Deoxy | R | Q | |
| Met | P | O | O |
| Oxy | | O | O |

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

place to various extents depending on the state of hemoglobin chains. For example, comparison of the result of Brunori *et al.* showing the same reaction curve of PMB for $\alpha_2^{O_2}\beta_2^{+CN}$ and $\alpha_2\beta_2^{+CN}$ with our result of the different rate constant of the reaction of PMB for $\alpha_2^{O_2}\beta_2^{+}$ and $\alpha_2\beta_2^{+}$ would lead to a suggestion that the cyanmet $\beta$ chains ($Fe^{3+}$–$CN^-$) are more rigid and restrict the propagation and the met $\beta$ chains ($Fe^{3+}$–$H_2O$) are more flexible and allow the propagation. Another point to be noted is that the detection of conformational changes may depend on the method used and the site looked at. A relatively small difference in the spin labels caused successful detection, on one hand, and failure in the detection, on the other, of the propagated conformational change in the half-cyanmethemoglobin.

*Implications on the Allosteric Mechanism.* The results on the half-methemoglobins indicate that the conformation of the $\beta$ subunit is primarily dependent upon ligation of the $\beta$ subunit and is also dependent on ligation of the neighboring $\alpha$ subunit. The conformational change in the $\alpha$ subunit does propagate to the $\beta$ subunit. Effect of the propagation is not to change the $\beta$ conformation to the R or O state but to some other P or Q state. The MWC model, therefore, does not hold in its symmetry conserving form for the present system of hemoglobin. Rather, the results seem to support the KNF model allowing the symmetry breaking, although the model does not take explicitly propagation of conformational changes into account.

To say further on the mechanism of the cooperative binding of oxygen to hemoglobin, we must assume equivalence of the met to the oxy state. We would then have the following implications. The conformation of the $\alpha$ subunit changes into O upon oxygenation and this conformational change propagates to the surface of the subunit, affecting the subunit interactions. The modulation of the interaction energy itself could cause the allosteric binding of oxygen. In addition, the modulation of the subunit interactions alters the $\beta$ conformation into Q and this alteration could result in a change in the oxygen affinity of the $\beta$ subunit. Such an effect would also be related to the allosteric interactions of proteins.

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