The Structure of Partially Oxygenated Hemoglobin

A HIGHLY REACTIVE INTERMEDIATE TOWARD A SULFHYDRYL TITRANT*

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It is known that Cys β89 in hemoglobin A reacts with sulfhydryl reagents more rapidly in the oxy than in the deoxy form. In this study, the reaction of the residues toward 4,4′-dithiodipyridine was measured at various degrees of saturation with O₂.

Analysis of initial rates of the reaction revealed that an intermediate, which reacts even more rapidly than the oxy form, occurs in the course of O₂ binding to hemoglobin A, especially at higher degrees of saturation. Its occurrence was independent of the overall O₂ affinity of hemoglobin.

Time courses of the reaction were measured at pH 8.0, where the change in O₂ saturation of hemoglobin by the 4,4′-dithiodipyridine reaction is negligible, and they were analyzed by the curve-fitting procedure. It was found that the rapidly reacting species appears in parallel with the Adair intermediate carrying three O₂ molecules, and their close relationship was suggested.

Furthermore, the analysis of the time course indicated the existence of another molecular species with an intermediate reactivity between those of oxy and deoxy forms.

It was concluded that the ligand-linked structural changes in hemoglobin take place through several steps.

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Materials and Methods

Materials—Striped Hb A was prepared by the method of Benesch et al. (16) with slight modifications. For the measurement of initial rates of 4-DTP reaction, Hb A was further purified by the chromatography on DE-52 cellulose (Whatman Biochemicals) column equilibrated with 0.01 M potassium phosphate buffer at pH 6.7. 4-DTP was of a specially prepared grade from Nakarai Chemicals, Ltd. (Kyoto). The Hb preparation contained 1.8 mol of 4-DTP reactive Cys/mol of tetramer. For the preparation of 4-DTP-modified Hb, Hb was incubated with 4-DTP (2.8 mmol/mol of Hb tetramer) for 30 min on ice, and the unreacted 4-DTP was removed with a Sephadex G-25 column.

Kinetic Measurements—The reaction between 4-DTP and Hb A was measured with a stopped flow apparatus, Model RA-401 (Union Giken Co., Ltd., Hirakata, Japan) equipped with a data processor, Model 71. Stopcocks on the reservoirs have been modified for anaerobic experiments. In order to obtain the partially oxygenated Hb, 4 ml of Hb solution (50 μM in tetramer) was equilibrated in a stopped Erlenmeyer flask with gentle shaking under the flow of N₂ gas containing a given proportion of O₂. The gas was prepared with an "oxygen pump" (Model SEP-104 from Toray Co., Tokyo). For fully oxy or deoxy Hb, Hb was equilibrated with pure O₂ or N₂ gas, respectively. The out flowing gas from the flask was led through a Tygon tube and was bubbled into a 4-DTP solution (1 or 6 mM) in one of the two reservoirs of flow apparatus, so that the O₂ concentration of the two solutions should be exactly the same. After the equilibrium was attained (usually after 20 min), the flask was turned upside-down, and the partially oxygenated Hb solution was transferred anaerobically through the Tygon tube into the other reservoir by a slightly positive gas pressure. The reaction was started by mixing the 4-DTP and Hb solutions, and the absorbance change due to the formation of 4-thiopyridone was followed with a cylindrical cell of 2-mm diameter (1.56 mm in effective light path) at 25 °C. The wavelength of observation was set at 341 nm, an isoelectric point for oxy and deoxy Hb, which was determined with the flow apparatus under
the same conditions. Dead time of the mixing cell was 1.3 ms, and the slit width was 7 nm in the wavelength of measuring light.

After the measurement, the 4-DTP solution in the reservoir was replaced with a sodium dithionite solution (0.1 to 1%, depending on the O$_2$ concentration). Absorbance change at 578 nm due to the deoxygenation of Hb by dithionite was measured with the flow apparatus. The fractional O$_2$ saturation of Hb before the deoxygenation reaction was calculated from the absorbance change by comparing with that of fully oxy Hb.

**Kinetic Analyses.—**Initial rates of the reaction between 4-DTP and Hb A were estimated by extrapolating the rate to the starting time by the second order regression. For the extrapolation, the rates (ΔA/Δt) were calculated at 1-s intervals (from 1- to 19-s) at pH 7.0, and at 0.25-s intervals (from 0.5- to 4.75-s) at pH 8.0. Nonlinear regression analyses of time courses of the reaction were carried out as described previously (7, 8).

O$_2$ Equilibrium of Hb.—p$_o$ and n$_h$ values of native and modified Hb were calculated from the O$_2$ equilibrium data, which was obtained by the polarographic method, as reported previously (7, 17).

**RESULTS AND DISCUSSION**

**Dependency of 4-DTP Reaction Rate on O$_2$ Saturation of Hb—**Cys β3 of Hb A reacts more slowly in deoxy than in oxy form, but still much more rapidly than the “masked” Cys residues (18). The property makes kinetic analyses simple. However, when Hb is partially oxygenated, the kinetics may form, but still much more rapidly than the “masked” Cys residues (18). It is anticipated that the time course of the reaction should reflect not only the distribution of molecular species at the starting point, but also the conformational changes due to the chemical modification. In order to avoid the ambiguity, we attempted at first to analyze the initial reaction rate instead of examining the full range of the time course. It is thought that only the rate for the “first” reagent molecule is observed at the initial stage of reaction.

Since the reaction system is expected to contain multiple Hb species with different SH reactivities, the initial rate (ν) can be expressed as a sum of the rates, as follows.

\[ v = c_R c_I \sum_{i=1}^n f_k k_i \]

where $c_R$ and $c_I$ denote the concentrations of Hb and reagent, respectively, and $f_i$ and $k_i$ are the fractional concentration and second order rate constant for ith Hb species, respectively. As seen from Equation 1, the initial rate, $v$, reflects the distribution of the components, though they are weighted with the rate constants, and its dependence on $Y$ will give some information about the process of conformational changes. For example, from the two state allosteric model (4), it is predicted that the $v$ *versus* $Y$ curve is concave upward considerably, whereas the curvature should be smaller in the sequential model (3).

Fig. 1A shows initial stages of the reaction between 4-DTP and Hb A at various O$_2$ saturation levels. Direct determination of the initial rates was difficult, because the time courses were exponential and most of them showed a considerable heterogeneity. Therefore, the initial rates were estimated by extrapolating the observed rates to zero time by the least square method, as shown in Fig. 1B. The plots of rate *versus* time were well fitted by quadratic curves, and the initial rates could be obtained from the intercepts.

Table II summarizes the initial rates of 4-DTP reaction for oxy and deoxy Hb at pH 7.0 in the presence and absence of IHP, and at pH 8.0. In the table, the rates are shown as

**Table I.** O$_2$ equilibrium parameters of native and 4-DTP modified Hb A.

| Conditions | Hb A | 4-DTP-Hb |
|------------|------|----------|
| pH 7.0     | p$_{oa}$ | n$_{oa}$ | p$_{ow}$ | n$_{ow}$ |
| pH 7.0 + 2 mM IHP | 11.2   | 3.0      | 7.4     | 2.9     |
| pH 8.0     | 49.5   | 27.0     | 26.3    | 2.5     |
| pH 8.0     | 3.8    | 2.9      | 2.8     | 2.8     |

* In 0.1 M potassium phosphate buffers, at 25°C. Hb concentration was 25 μM as a tetramer.

**Fig. 1.** Some examples of the initial stage of reaction between 4-DTP and Hb A at various degrees of O$_2$ saturation. A, absorbance change at 341 nm was measured. Fractional O$_2$ saturation of Hb (Y) are shown in the figure. Hb, 25 μM as tetramer; 4-DTP, 500 μM (after mixing). In 0.1 M potassium phosphate buffer, pH 7.0, at 25°C. B, estimation of the initial rate by the second order regression. The rates (ΔA/Δt) were calculated from the absorbance values at given time intervals. The plots from the same data as in A are shown.
apparent first order rate constants, i.e. \( v/c_{Hb} \). At pH 7.0 in the absence of IHP, the rate for oxy form was about 6 times as large as that for deoxy. At higher pH, the rates were higher, and the ratio for oxy/deoxy was smaller owing to the relatively large increase in deoxy rate. IHP caused a slight decrease in the reactivity.

In Figs. 2–4, the initial rates were plotted as a function of fractional \( O_2 \) saturation of Hb under the different conditions. In the figures, the initial rates have been converted to relative values \( (v_r) \) according to the equation

\[
v_r = \frac{v - v_{deoxy}}{v_{oxy} - v_{deoxy}}
\]

where \( v_{oxy} \) and \( v_{deoxy} \) are the initial rates of oxy and deoxy Hb, respectively. Therefore, \( v_r = 0 \) for deoxy, and \( v_r = 1 \) for oxy Hb. The plots for the three different conditions showed similar patterns, though the \( O_2 \) affinities of Hb much differ. The plots deviated considerably upward from the diagonal line. Furthermore, in the range of \( Y > 0.7 \), the \( v_r \) value was significantly greater than 1, that is, Hb reacts more rapidly than oxy form. The rate apparently reached a maximum value around 90%

**Table II**

| Conditions | Oxy Hb | Deoxy Hb |
|------------|--------|----------|
| pH 7.0     | 0.928 ± 0.034 | 0.146 ± 0.018 |
| pH 7.0 + 2 mM IHP | 0.827 ± 0.031 | 0.090 ± 0.013 |
| pH 8.0     | 3.86 ± 0.094  | 0.85 ± 0.06  |

* In 0.1 M potassium phosphate buffers, at 25 °C. Hb concentration was 25 \( \mu M \) as a tetramer.

Shown as apparent first order rate constants at 500 \( \mu M \) 4-DTP.

Average ± S.D. of about 10 determinations.

The results shown in Figs. 2–4 are unexpected and obviously do not fit to the cooperativity models noted above. The results indicate the occurrence of a unique molecular species that differs from either oxy or deoxy form with respect to the SH reactivity. It is noteworthy that the shape of the \( v_r \) versus \( Y \) curve did not change appreciably with the overall \( O_2 \) affinity of Hb. This implies that the highly reactive species is related to the degree of ligand saturation, but not to the affinity state of Hb. From the position of the peak, the molecular species seems to appear at a final stage of the ligand-binding sequence.

**Analysis of Time Courses of 4-DTP Reaction**—Although the initial reaction analysis is theoretically unequivocal as noted above, the information obtained is considerably limited; it is difficult to deduce quantitative properties of the molecular species such as the absolute SH reactivity and the concentration in the equilibrium mixture. That may be possible from analysis of the time course of reaction, if the perturbation of the equilibrium by the reagent is negligible.

Although Hb A shows 1.3- to 1.9-fold increase in \( O_2 \) affinity by 4-DTP reaction (Table I), it is expected that the change in \( Y \) should not be so large. It is thought that, in a system closed to \( O_2 \), increase in \( O_2 \) binding due to the affinity increase of Hb results in a decrease in the concentration of free \( O_2 \), which now counteracts the \( O_2 \) binding to Hb. The effect should be larger at higher Hb concentration, and also at higher pH, where Hb shows a higher ligand affinity and, consequently, equilibrium concentration of free \( O_2 \) is relatively low. Numerical calculations from the equilibrium curves (of which parameters are shown in Table I) showed that the maximum increase in \( Y \) are 20 and 6% at pH 7.0 in the presence and absence of IHP, respectively, under the experimental conditions. On the other hand, at pH 8.0, the increase in \( Y \) value was calculated to be less than 2% for the whole range of \( Y \). That is readily
understandable, because the \( p_{50} \) value of 3.8 torr corresponds
to 6.2 \( \mu \)M concentration of \( O_2 \) at 25 °C, and it is much lower
than the Hb concentration employed (100 \( \mu \)M in heme).

Since the 2% change in \( Y \) is thought to be small enough for
detailed analyses, we examined the time course of 4-DTP
reaction at pH 8.0 as a function of \( Y \). A higher 4-DTP
concentration (3 mM) was used to facilitate the measurement
and analysis. \(^2\) Some of the results are shown in Fig. 5.

The reaction of 4-DTP with oxy Hb followed a second order
kinetics with rate constant of 95 \( M^{-1} s^{-1} \). However, other time
courses showed more or less kinetic heterogeneity, and they
were analyzed by the numerical curve-fitting procedure. If we
assume, as described above, that the fractional concentrations
of components, \( f_n \) are constant throughout the reaction, the
time course can be formulated as a sum of exponential terms,
as follows, since 4-DTP is in large excess over Hb.

\[
A_0 / A_t = \sum \left[ f \exp(-k_n t) \right]
\]

where \( A_0 \) and \( A_t \) are the concentrations of unreacted Hb at
time 0 and \( t \), respectively.

For deoxy Hb, the assumption of two components \( (n = 2) \)
was found to give a sufficient fit. The fitted values of rate
constants were \( k_1 = 6.8 \ M^{-1} s^{-1} \) \( (f_1 = 0.94) \) for the major
component and \( k_2 = 82 \ M^{-1} s^{-1} \) \( (f_2 = 0.06) \) for the minor
component. The major component presumably corresponds
to the deoxy-like structure. The presence of the minor component,
which has a reactivity comparable to that of oxy form,
may be due to the met Hb formation.

As seen in Fig. 5, the half-time of the reaction decreased
with increasing \( Y \), but the time courses of partially oxygenated
Hb were considerably heterogeneous. In order to find system-
atically the minimum number of kinetic species involved in
the equilibrium solution, we tried a simultaneous curve fitting
to multiple sets of the progress curve. The rate constants \( (k_i) \)
and fractional concentrations \( (f) \) of kinetic components were
set free, and Equation 3 was fitted simultaneously to 10 data
sets with the \( Y \) values ranging from 0 to 1. In order to ensure
the convergence, two of the rate constants were fixed at 6.8
and 95 \( M^{-1} s^{-1} \), which are the characteristic values of deoxy
and oxy forms, respectively. The fitting was tested for an
increasing number of components \( (n) \) from 2 to 4. As was
expected, the two-state assumption did not give sufficient fits
to the data, especially at \( Y = 0.1 \) to 0.5. When three states are
assumed, the fitting was much improved, but it still failed to
fit to the rapid phase at higher \( O_2 \) saturation. Finally, it was
found that the assumption of four components with different
SH reactivities can reproduce the progress curves satisfac-
tory. The results showed that an intermediately SH reactive
component is present in the equilibrium system in addition to
the deoxy-like, oxy-like, and SH more reactive components.
The \( k \) values for the components are listed in Table III. The
fitted curves are also shown in Fig. 5 as solid lines.

The calculated fractional concentrations of the components
 corresponding to the above \( k \) values were plotted against \( Y \) in
Fig. 6A. The component 2, which has an intermediate SH
reactivity, was present in considerable amounts in the system,
and it cannot be attributed to an artifact arising from the

\[\text{FIG. 5. Time courses of the reaction between 4-DTP and partially oxygenated Hb A. Fractional O}_2\text{ saturations of Hb are shown in the figure. Circles represent the experimental data, and solid lines are the fitted curves on the assumption of four kinetic components (see text). Not all the data sets used for the curve fitting are shown. The line for } Y = 1 \text{ was shifted downward to avoid confusion. Hb, 25 } \mu \text{M as tetramer; 4-DTP, 3 } \mu \text{M. In 0.1 M phosphate buffer, pH 8.0, at 25 °C.}\]

\[\text{TABLE III}\]

Rate constants for the 4-DTP reaction of the components occurring in partially oxygenated Hb in 0.1 M phosphate buffer, pH 8.0, at 25 °C

| Component | Rate constants\(^a\) |
|-----------|---------------------|
| \(1\)      | \(6.8\) \( M^{-1} s^{-1}\) \(\text{ (fixed)}\) |
| \(2\)      | \(27 \pm 3\)         |
| \(3\)      | \(450 \pm 150\)      |
| \(4\)      | \(95\) \(\text{ (fixed)}\) |

\(^a\) (Best fit) \(\pm\) (95% confidence range in F-test).

\(^b\) \(k_1\) and \(k_2\) were fixed at the values for deoxy and oxy Hb, respectively.
small equilibrium displacement due to the 4-DTP reaction. The amount of the component 3 was less than 10% of total, but it still affects considerably the time course and the initial rate of the reaction because of its markedly high reactivity.

Of course, the possibility remains that there may exist other molecular species which are buried in the error of the measurements. It would be more safely stated that at least four molecular species with different SH reactivities exist in the equilibrium system of Hb. Actually, component 2 may represent more than one species, because the fitted curves yet showed a slight deviation from the data points for \( Y = 0.3 \) to 0.5, as seen in Fig. 5.

The pattern shown in Fig. 6A indicates the sequence of the intermediate molecules occurring in the course of \( O_2 \) binding to Hb; component 1 (deoxy-like species) is followed by component 2 and then by 3 (with most reactive Cys), and finally component 4 (oxy-like species) appears. The sequence leads us to the thought that the molecular species detected may have some kind of relation with the stepwise binding of \( O_2 \) to Hb. If we neglect the possible differences in the structure and function between the \( \alpha \) and \( \beta \) subunits, the distribution of Hb species with different numbers of the bound ligand can be calculated from the Adair constants. For the calculation, we employed the values determined by Imai and Yonetani (21) under similar conditions. Although statistical errors in determining the Adair constants have been subjected to some arguments, the errors do not affect the calculation seriously, and they are thought to be accurate enough for our purpose. Results of the calculation are shown in Fig. 6B. When the two kinds of patterns in Fig. 6, A and B are compared, we notice close correlations between component 3 and species \( H_4 \), and between component 4 and \( H_4 \) (fully oxy form), where the suffixes denote the number of \( O_2 \) molecules bound to Hb. Component 1 seems to correspond to \( H_0 \) (deoxy form), though the former decreased more quickly with increasing \( Y \). It should be noted here that the correspondence between the molecular species cannot be very accurate because we do not know at present about the chain difference in \( O_2 \) affinity in the whole course of the ligand binding and also about the sequence of the structural changes in Hb tetramer. The discrepancy between component 1 and \( H_0 \) may imply that the affinity of \( \beta \)-subunits, which reacts with 4-DPT, is higher at the initial stage of ligand binding, and that their structural changes take place prior to those of \( \alpha \)-subunits. Component 2 was rather difficult to assign, but we tentatively assigned it to \( H_1 + H_2 \), since the shapes of the two curves were qualitatively similar.

In order to test validity of the above assignment, we calculated the initial rate of 4-DTP reaction as a function of \( Y \) from the distribution of the Adair intermediates and their assigned values of rate constants. As shown in Fig. 4 with a solid line, the calculated curve accorded well with the experimental values. Especially, it could reproduce the presence of a maximum at a high oxygenation level (approximately \( Y = 0.5 \)). Of course, the result may not necessarily prove the validity of all the above assignment. But, since the calculated curve was considerably sensitive to the amount and the rate constant of \( H_3 \) (component 3), it can be inferred that the rapidly reacting Hb is closely related to the molecular species \( H_3 \). Theoretical curves can not be obtained for the results at \( pH 7.0 \), because the rate constants of the species are not available. Nevertheless, it is supposed that the situation is similar at \( pH 7.0 \) from the similarity in the dependence of \( v_c \) on \( Y \) (Figs. 2 and 3).

More generally, it is concluded that, in going from deoxy to oxy form, Hb undergoes a unique structural change involving the \( \beta \)-chain, very possibly at a later step of the ligand-binding sequence. Besides the peculiar change, another transient structure change (or changes) also seems to take place in the middle of the sequence. These structural changes seem to be related to the ligand-binding sequence, but not to the affinity state of Hb.

Gibson (15) has examined the time course of \( p \)-mercuribenzoate reaction with Hb A during the kinetic binding of CO, but a highly reactive species such as found in the present study was not detectable in the CO-binding reaction. Seemingly, the result indicates that the molecular species is not formed in a detectable amount when CO is the ligand or generally in the kinetic process of binding of ligands.

**Structural and Functional Implications of the SH Reactivity**—Two of the four Hb species, which were found in the 4-DTP reaction, are identified as the well established oxy and deoxy (or R and T) structures, respectively, whereas the other two have not been known before. Cys 293, of which reactivity was used as a structural probe in this study, is located next to the proximal His 992. From the x-ray analysis of the crystal structure of Hb (23, 24), it was shown that in deoxy the SH group of the residue points out into solution, while in oxy it is buried in the Tyr pocket between helix F and helix H. In deoxy, reaction of the SH group is blocked by the hydrogen bond between the side chains of His 146 and Asp 984, and the bond is broken in oxy form. The carboxyl-terminal vicinity including His 146 is one of the sites for \( \alpha \beta \)-contact, and its movement is proposed by Perutz (25) to be involved in the cooperative mechanism. Therefore, it is thought that the
reactivity of the Cys residues reflects the cooperative conformational changes more or less. The present kinetic results indicate that, in partially oxygenated Hb, the SH group can be more reactive than in oxy form. The results may be interpreted as that the SH group can remain free in solution while the hydrogen bond is broken in the intermediate structure. Also, from the presence of kinetic species with an intermediate reactivity between those of oxy and deoxy forms, it is thought that Hb changes its structure stepwise, but not by a one-step process.

We tentatively assigned the highly SH reactive species as a Hb molecule carrying three O₂ molecules. When the Adair scheme is fitted to the O₂ equilibrium curve, the intrinsic association constant of the 4th step, $K_4$, is much greater than others, $K_i$ through $K_3$, under the physiological conditions. Imai (26) showed by the thermodynamic study that the sharp increase in affinity at the final step of O₂ binding is ascribed to the decrease in enthalpy-entropy compensation. From our results, in structural terms, it is considered that the Hb intermediate carrying three O₂ molecules has a unique structure, which is characerized by the high SH reactivity and also by the high affinity for O₂.

In our previous study with Hb M Milwaukee (7), which can combine with O₂ only on the $\alpha$-subunits, it was shown that the singly oxygenated intermediate is different from either the fully oxy or deoxy form in spectral and kinetic properties. Fung et al. (6) have shown that the intermediate is unique also in the proton NMR spectrum. Although it is not yet clear whether or not the intermediary structure of the abnormal Hb corresponds to any of the components found in partially oxygenated Hb A, these results give the same line of evidence on the cooperative mechanism of Hb.

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