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Kinetic study on oxygenation of *Lingula unguis* hemerythrin using the stopped-flow O_2-jump method

Y. Tachi'iri [1,2,*], K. Ichimura [1], T. Yamamura [1,], K. Satake [1], K. Kurita [3], T. Nagamura [3], and H. Kihara [2]**

[1] Department of Chemistry, Faculty of Science, Science University of Tokyo, Kagurazaka, Shinjuku-ku, Tokyo, 160 Japan
[2] Department of Physics, Jichi Medical School, Minamikawachi, Yakushiji, Tochigi, 329-04 Japan
[3] UNISOKU Inc., Ohmine-motomachi, Hirakata, 573-01 Japan

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Abstract. O_2-jump experiments with an improved stopped-flow apparatus have been used to study oxygenation and deoxygenation processes in *Lingula unguis* hemerythrin. With an O_2 electrode set in the observation cell, O_2 concentration could be obtained directly. The reliability of this method has been compared with other conventional methods.

O_2-jump (up and down) experiments were carried out with *L. unguis* hemerythrin at pH 6.8 (non-cooperative pH) and at pH 7.6 (cooperative pH). At pH 6.8, both O_2-jump (up) and O_2-jump (down) experiments showed single exponential processes which were consistent with the following scheme: $Hr + O_2 \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} HrO_2$. The value of k_{on} was estimated to be $(4.4 \pm 0.5) \times 10^5\ M^{-1}\ s^{-1}$, and k_{off} was $(15 \pm 5)\ s^{-1}$. These values are consistent with those obtained by the temperature-jump method (Zimmer et al. 1986). At pH 7.6, O_2-jump (up) experiments showed two relaxation processes, whereas O_2-jump (down) experiments showed a single exponential process. The faster process in the O_2-jump (up) experiments could be attributed to the same process as that seen in the temperature-jump experiments (Zimmer et al. 1986). The slower process in the O_2-jump (up) experiments corresponds to the process obtained in the O_2-jump (down) experiments. The results are discussed in terms of a state with intermediate affinity in O_2-binding and with the possible existence of a slow step in O_2-binding.

Key words: Hemerythrin – Oxygenation – *Lingula unguis* – Stopped-flow

Introduction

Hemerythrins from a group of non-heme iron, oxygen-carrying proteins in the erythrocyte-like cells of the coelomic fluid in four phyla of marine invertebrates (Wilkins and Harrington 1983; Kurtz et al. 1977; Loeher and Loeher 1974; Klotz et al. 1976; Kurtz 1986; Klipenstein 1980). Hemerythrins consist of a monomer (so-called myohemerythrin), a dimer, a trimer, a tetramer, or an octamer with identical or nearly identical subunits. Molecular weights of the subunits of these hemerythrins are approximately 13,500. Each subunit contains two iron atoms, comprising a binuclear active center which binds molecular oxygen.

Oxyhemerythrin shows a specific absorption band at 500 nm, whereas deoxyhemerythrin shows no absorption band in the visible region (Klotz et al. 1976). Therefore, the oxygen binding properties of hemerythrin can be monitored using the absorbance change at 500 nm as a probe.

Most hemerythrins do not show cooperativity in oxygen binding or any Bohr effects (Wilkins and Harrington 1983). Recently, it was reported that hemerythrins from *Lingula unguis* (Zimmer et al. 1986; Manwell 1960; Imai et al. 1986) and *Lingula reevii* (Richardson et al. 1983, 1987) show cooperativity in O_2-binding and a pH-dependent Bohr effect. To elucidate molecular mechanisms for this cooperativity, kinetics of oxygen-binding to *L. unguis* hemerythrin were performed with the temperature-jump (T-jump) method (Zimmer et al. 1986). In order to extend this analysis, we have improved a stopped-flow apparatus for the measurement of O_2-jump (up and down) experiments. This paper describes the performance of the apparatus and its application to oxygenation and deoxygenation processes of *L. unguis* hemerythrin.

Materials

1. *Lingula unguis hemerythrin*

L. unguis were collected from the Ariake Sea in Japan. They were deshelled and the bodies were washed with

Abbreviations: Hr, hemerythrin; Hb, hemoglobin; CM, carboxymethyl; T-jump, temperature-jump

Offprint requests to: H. Kihara
* *Present address*: Hamamatsu Photonics, Ichino-cho, Hamamatsu, 435 Japan
** *Present address*: International Research Laboratory, CIBA-GEIGY Japan Limited, Miyuki-cho 10-66, Takarazuka, 665 Japan

0.1 M phosphate buffer (pH 8.0 containing 0.25 M Na_2SO_4) at 4 °C. The solution containing the insoluble fraction was collected and passed through a grass wool column to remove solids. The resulting solution included erythrocyte-like cells and these were collected by centrifugation. These cells were then washed several times with the same buffer and hemolyzed. Hemerythrin was purified using CM-Sephadex C-50 chromatography on the supernatant of the hemolyzed solution according to the method of Joshi and Sullivan (1973). The sample obtained was rapidly frozen in liquid N_2, and stored at −80 °C.

Protein concentration of an octameric hemerythrin of *L. unguis* was expressed in terms of subunits and was estimated using a molar absorption coefficient of 1,800 M^{-1} cm^{-1} at 500 nm for the oxy form (Joshi and Sullivan 1973).

2. *Human hemoglobin*

The concentration of hemoglobin was estimated using an absorption coefficient of $\varepsilon_{1cm} = 14{,}600\ M^{-1}$ at 576 nm for the oxy form (Di Iorio 1981).

3. *Other reagents and buffers*

All reagents used were analytical grade. CM-Sephadex C-50 was obtained from Pharmacia. Measurements were done at 17 °C ± 0.5 °C in 0.1 M sodium phosphate buffer. Buffers at pH 7.6 and 6.8 were prepared by mixing 0.1 M Na_2HPO_4 and 0.1 M NaH_2PO_4.

Instrumentation

1. *Stopped-flow apparatus for direct measurement of oxygen in the observation cell*

It is essential to observe kinetic processes in O_2-binding proteins with direct measurement of O_2 concentration throughout the reaction. For this purpose, we have constructed a stopped-flow apparatus with three oxygen electrodes; one in each reservoir and one in the observation cell. Figure 1 shows the block diagram of the apparatus. O_2 concentration is monitored at three points; two reservoirs ("A" in Fig. 1) and the observation cell. To keep O_2 pressure at a reproducibly low level the main part of the apparatus is covered with a plexiglass case and this is filled with N_2 gas. A view of the O_2 electrode is shown in Fig. 2, it consists of Pt and Ag parts which are connected using saturated KCl solution. The surface of the electrode is covered with a teflon membrane of 20 μm thickness to preserve the KCl solution. Sensitivity and response time are very much dependent on the thickness and pore size of the membrane. Teflon of this size is the best of the membranes we have tested (cellulose acetate, Mylar and Kapton). Details of the O_2 electrode can be found elsewhere (e.g. Hagihara 1965).

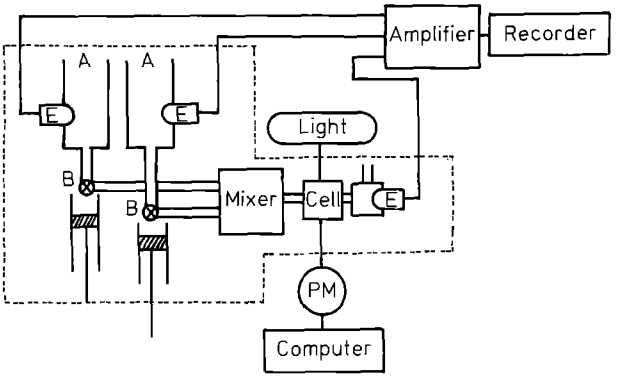

Fig. 1. Block diagram of stopped-flow apparatus for O_2-jump experiment. E; O_2 electrode, B; stop cock to change through channels between reservoir syringes and mixer syringes. PM; photomultiplier. Initially the stop cock position is set so as to connect reservoirs (A) and mixer syringes. Solutions, which are initially stored in two reservoirs (A), are drawn into the two syringes. Then the stop cock position is changed to connect the mixer syringes and the mixer. The two solutions are then mixed. The absorbance change is monitored at the cell, and the O_2 level in the cell is monitored just behind the observation cell. All these operations are computer-controlled. At each shot, a sufficient amount of flow is used to remove reacted solution in the observation cell. O_2 concentration in the two reservoirs can be controlled separately. All the parts in dotted line are covered with a plexiglass box, and nitrogen gas is blown in to avoid any small leak of oxygen from the outside

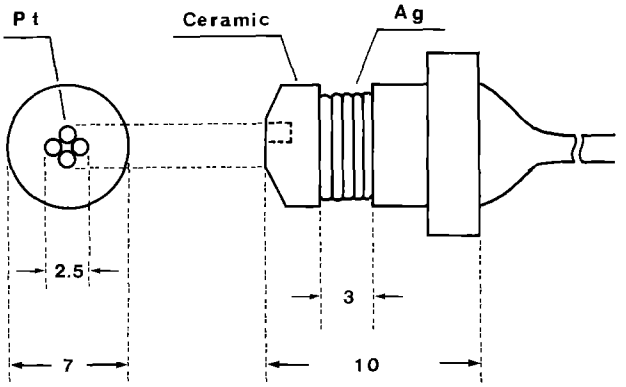

Fig. 2. Outline of oxygen electrode. Unit of scale in the figure is millimeter

2. *O_2 measurement in the observation cell*

Measurement of O_2 results in O_2 consumption (Hagihara 1965) and this means that the apparent O_2 concentration decreases on the surface of the electrode. Figure 3 shows a typical stopped-flow experiment. The flat part (A) shows the O_2 concentration which falls (B) as the electrode consumes O_2. The flat part (A) was measured in every experiment. O_2 concentration was measured by monitoring the voltage between the two metals, Pt and Ag, and the O_2 concentration, x, was calculated from the following equation:

$$x = C(V - V_d)/(V_a - V_d) \quad (1)$$

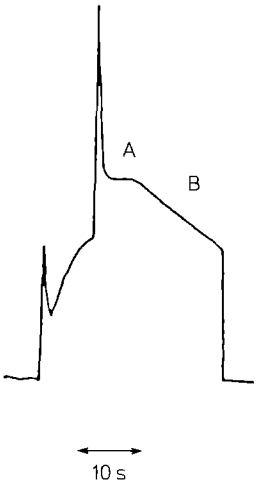

Fig. 3. A typical signal for the measurement of O_2 concentration in the observation cell after a stopped-flow shot. *A*) detected value; *B*) apparent decrease of O_2 concentration due to O_2 consumption by the oxygen electrode

where V is the measured voltage, V_a and V_d are air and O_2-free (dithionite saturated) voltages [1], respectively, and C is a constant equal to 2.855×10^{-4} M (20 °C) or 3.016×10^{-4} M (17 °C) (Data from Kagaku Bin'ran 1980).

3. *Comparison of O_2 concentration measurements*

The accuracy of the O_2 concentration measurement was checked in three ways:

(i) A solution containing x mm Hg O_2 was mixed with O_2-free water. The O_2 concentration observed with the O_2 electrode in the cell ($[O_2]_{cell}$) was plotted against the average ($=x/2$) of the O_2 concentration in the two reservoirs ($[O_2]_{ave}$) (Fig. 4). The figure clearly shows good coincidence of the two measured values.

(ii) O_2-free Hb solution was mixed with a solution containing a known amount (x mm Hg) of O_2. Apparently the data deviated significantly from the expected straight line of 45 deg (Fig. 5). However, a calibration should be done according to Eq. (2), because the O_2 electrode measures freely dissolved O_2 concentration in the cell and does not measure the O_2 bound to hemoglobin.

$$[O_2]_{free} = [O_2]_{total} - [Hb] \cdot y \qquad (2)$$

where $[O_2]_{total}$ is the total O_2 concentration ($[O_2]_{total} = x/2$). $[O_2]_{free}$ is the O_2 concentration dissolved in the solution (in the observation cell). [Hb] is the concentration of hemoglobin and y is the degree of saturation estimated from an oxygen equilibrium curve at a particular $[O_2]_{free}$ (for the estimate of y, see next paragraph). In

[1] Output voltages for O_2-free and dithionite-saturated conditions were found to be the same within experimental error. Therefore, V_d was usually measured for dithionite-saturated conditions to save the time required for the complete replacement of O_2 with N_2

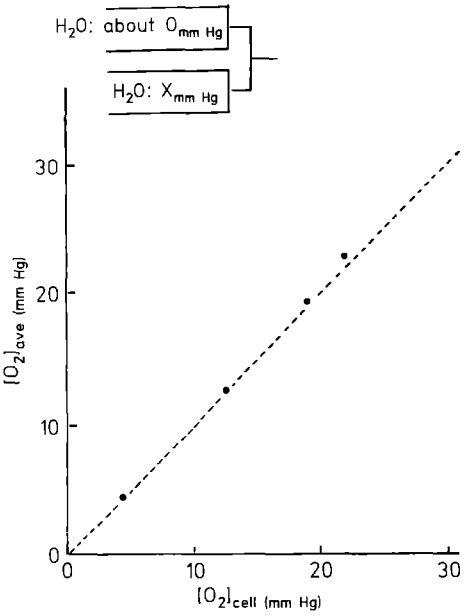

Fig. 4. Average of O_2 concentration in the two reservoirs ($[O_2]_{ave}$) detected by O_2 electrodes vs. O_2 concentration in the observation cell ($[O_2]_{cell}$) detected by the other O_2 electrode. O_2-free water is mixed with water with an $[O_2]$ of x mm Hg

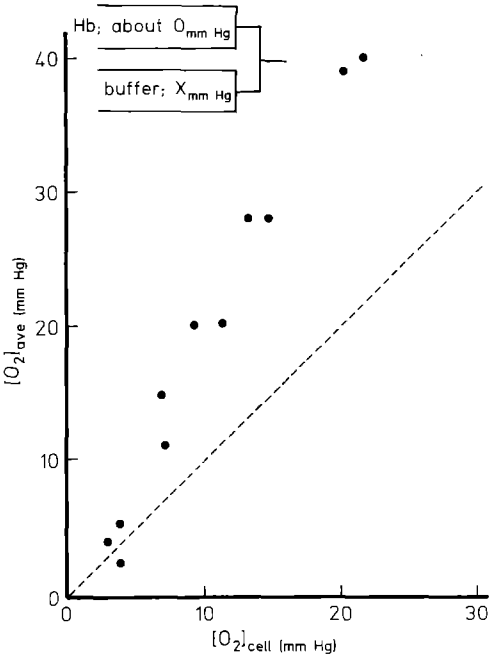

Fig. 5. O_2-free hemoglobin solution is mixed with the buffer with an $[O_2]$ of x mm Hg. The ordinate shows averaged $[O_2]$ value of two reservoirs. The abscissa represents $[O_2]$ in the cell observed with the O_2 electrode

Fig. 6, free O_2 concentration thus calculated was plotted against the value measured with the O_2 electrode in the cell. The experimental values now agree well.

(iii) In the same set of experiments as described in the previous paragraph, the O_2 concentration was estimated

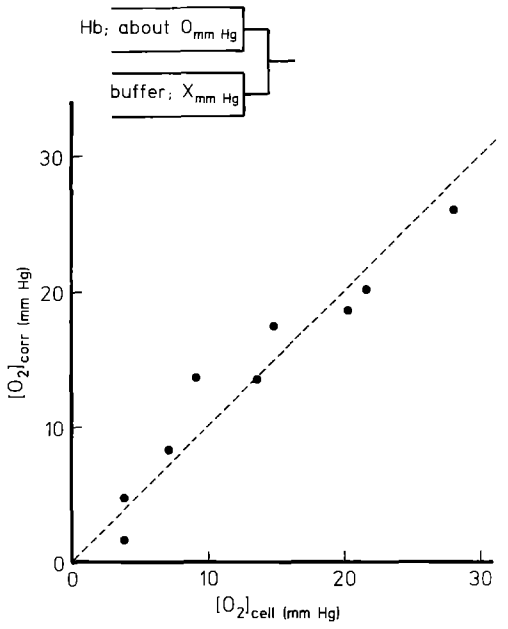

Fig. 6. The ordinate is $[O_2]_{free}$ calculated according to (2). Data are the same as those shown in Fig. 5

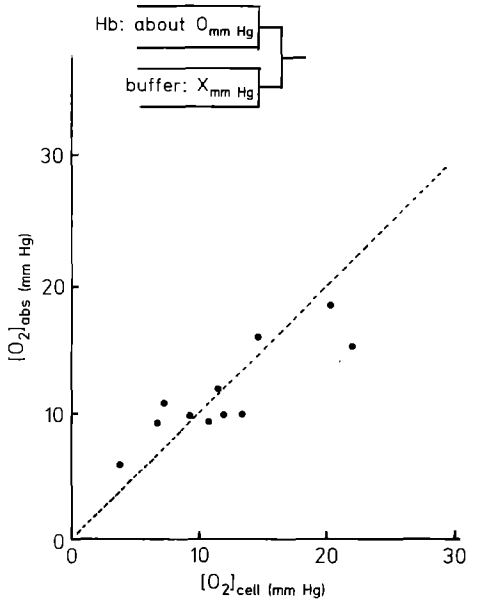

Fig. 7. O_2-free hemoglobin solution is mixed with buffer with an $[O_2]$ of x mm Hg. Oxygen concentration for the reaction was measured with the O_2 electrode in the cell and estimated from absorbance at 576 mm. They are plotted against each other

from the absorbance at 576 nm, the characteristic peak of the oxy form of human hemoglobin. O_2 concentration estimated from the absorbance value and the oxygenation curve was plotted against O_2 concentration measured directly in the observation cell (Fig. 7). They agree reasonably well but not so well as the data of Fig. 6.

From these experiments we conclude that the direct measurement of O_2 concentration agrees well with values obtained with other methods.

Analysis

At pH 6.8, data were analyzed according to scheme (3) which assumes a bimolecular reaction,

$$\mathrm{Hr}+\mathrm{O_2} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \mathrm{HrO_2} \tag{3}$$

where k_{on} and k_{off} are rate constants for the oxygenation and deoxygenation (backward) reactions. Hr denotes the subunit concentration of hemerythrin.

Absorbance change ΔA, through the reaction, is derived as

$$A = \Delta\varepsilon\,\alpha\left(1-\frac{\gamma}{\alpha-\beta\exp(-k_{app}t)}\right) \tag{4}$$

where

$$\alpha = \tfrac{1}{2}([\mathrm{Hr}]_0+[\mathrm{O_2}]_0+K+\gamma) \tag{5}$$

$$\beta = \tfrac{1}{2}([\mathrm{Hr}]_0+[\mathrm{O_2}]_0+K-\gamma) \tag{6}$$

$$\gamma = \sqrt{([\mathrm{Hr}]_0+[\mathrm{O_2}]_0+K)^2-4([\mathrm{Hr}]_0[\mathrm{O_2}]_0-K[\mathrm{HrO_2}]_0)} \tag{7}$$

$$k_{app} = k_{on}\gamma \tag{8}$$

$$K = k_{off}/k_{on} \tag{9}$$

and $\Delta\varepsilon$ denotes the change of extinction coefficient as

$$\Delta\varepsilon = \Delta\varepsilon(\mathrm{HrO_2})-\Delta\varepsilon(\mathrm{Hr})-\Delta\varepsilon(\mathrm{O_2}) \tag{10}$$

Subscript 0 indicates an initial concentration. As oxygen concentration is measured after the reaction, $[O_2]_0$ should be calculated as

$$[\mathrm{O_2}]_0 = [\mathrm{O_2}]+\frac{[\mathrm{Hr}]_0[\mathrm{O_2}]-K[\mathrm{HrO_2}]_2}{K+[\mathrm{O_2}]} \tag{11}$$

where $[O_2]$ denotes oxygen concentration in the observation cell after the reaction. From these equations it can be seen that ΔA is a function of $[Hr]_0$, $[HrO_2]_0$, $[O_2]$ and t with unknown parameters $\Delta\varepsilon$, k_{on} and K. These parameters were best-fitted by the non-linear Simplex method (Nelder and Mead 1964). See Appendix for details.

Results

1. O_2-jump (up) experiment

Nearly O_2-free hemerythrin was mixed with buffer containing y mm Hg O_2 and the subsequent oxygenation of hemerythrin was monitored at 500 nm. Figure 8 shows a typical time course for oxygenation of deoxyhemerythrin at pH 6.8, this is clearly monophasic. Analyses were done, based on the bimolecular reaction, according to the procedure described in *Analysis* and in more detail in *Appendix*. k_{on} and k_{off} values were estimated and were plotted against O_2 concen-tration (Fig. 9). The figure demonstrates that k_{on} and k_{off} are apparently independent of O_2 concentration. Averaged k_{on} and k_{off} values were $4.4\pm0.5\times10^5\ \mathrm{M^{-1}\,s^{-1}}$ and $15\pm5\ \mathrm{s^{-1}}$, respective-

Table 1. Rate constants for oxygenation and deoxygenation of hemerythrins of various species

Species	$k_{on} \times 10^{-6}$ ($M^{-1} s^{-1}$)	k_{off} (s^{-1})	$K_{diss} \times 10^{6}$ (M)	Condition (apparatus)	Ref.
Lingula unguis (octamer)	0.44	15	34	0.1 M phosphate, pH 6.8 at 17 °C (stopped-flow)	This work
	0.63	61	96	0.08 M phosphate + 0.1 M NaCl, pH 6.8 and/or pH 7.6 at 15 °C (T-jump)	Zimmer et al. (1986)
Siphonosoma cumanense (trimer)	11.3	9.1	0.81	0.08 M phosphate + 0.1 M NaCl, pH 6.8 at 15 °C (T-jump)	Zimmer et al. (1986)
Themiste zostericola (monomer)	78	315	4.0	Tris, pH 8.2, I=0.1 M, at 25 °C (T-jump)	Petrou et al. (1981)
(octamer)	7.5	82	11.0	Tris, pH 8.2, I=0.1 M, at 25 °C (T-jump)	Petrou et al. (1981)
Golfingia gouldii (octamer)	7.4	51	6.9	Tris, pH 8.2, I=0.015 M, at 25 °C (T-jump)	De Waal et al. (1976)
Siphonosoma nudus (octamer)	26	120	4.6	0.1 M phosphate, pH 7.0, at 25 °C (T-jump)	Bates et al. (1968)

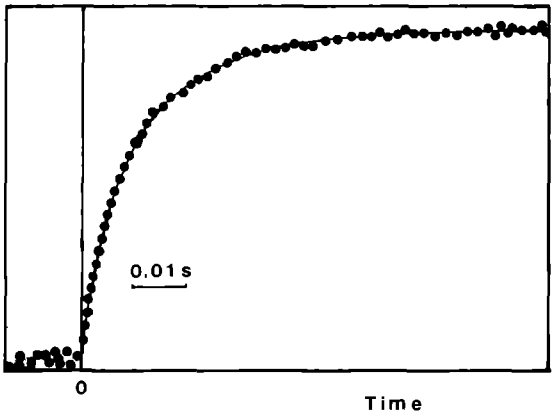

Fig. 8. Time course of hemerythrin absorbance change at 500 nm after mixing by stopped-flow. O_2-free hemerythrin solution was mixed with the buffer containing a particular amount of O_2. The reaction was carried out at pH 6.8. Hemerythrin at 4.5×10^{-5} M and O_2 at 15.2×10^{-5} M were mixed with a ratio of 1 vol/vol. Ordinate represents absorbance in arbitrary units

ly. $[HrO_2]_0$ in (7) and (11) was taken to be zero in this procedure. The rate constants are in good agreement with data obtained by the T-jump method (Zimmer et al. 1986), as summarized in Table 1.

At pH 7.6, in contrast, the time course of oxygenation of hemerythrin is clearly biphasic as shown in Fig. 10. Analyses were done with the following equation:

$$A = -A_1 \exp(-k_1 t) - A_2 \exp(-k_2 t) + A_3 \qquad (12)$$

where k_1 and k_2 are the apparent rate constants of the fast and slow phases. A, A_1, A_2, and A_3 are the absorbance at time t, the absorbance change of the fast and slow phases and the total absorbance change ($=A_1+A_2$). Apparent rate constants of the fast process are in qualitative agreement with those obtained by the T-jump method (Zimmer et al. 1986), but the S/N ratio was not good enough to allow simultaneous determination of both apparent rates. Subsequent analyses were then performed with the assumption that the rates of the fast process were equal to the rate of the process obtained by the T-jump method (Zimmer et al. 1986). Rate constants of the slow phase, k_2, were thus estimated and plotted in Fig. 11 against O_2 concentration[2]. Apparent rate constants, k_2, of the slow phase clearly show concentration dependence.

2. O_2-jump (down) experiment

Hemerythrin at a particular O_2 concentration was mixed with O_2-free buffer. At pH 6.8, the absorbance decreased as a monophasic decay. k_{on} and k_{off} were estimated according to the procedure in *Analysis*, where $[HrO_2]$ was calculated from the absorbance of the initial state. The apparent rate constants were calculated and are plotted

[2] Strictly speaking, (12) does not hold. The fast process corresponds to the process detected by the T-jump method, as assigned in this study. It is a concerted bimolecular reaction as shown in (4) of our previous paper (Zimmer et al. 1986). Then the fast process does not obey a single exponential decay. Moreover, although the T-jump method gives a single exponential decay, the reciprocal relaxation time of the T-jump method is different from k_{app} of the stopped-flow method. Notwithstanding these difficulties, (12) was employed here and the reciprocal relaxation time of the T-jump method was used for k_1 in (12) in further analysis because: *(i)* The signal amplitude of the fast process was small and noisy. Therefore a reliable estimate of k_1 was not possible. *(ii)* Although the definitions of reciprocal relaxation time for T-jump and k_{app} for the stopped-flow method are different, the difference in their values is not very large. In particular, they converge to the same value for a large excess of O_2. *(iii)* The main purpose here is to assign the fast process, and to estimate the contribution of the fast process to the evaluation of the slow process. For this purpose, the approximation employed here is acceptable

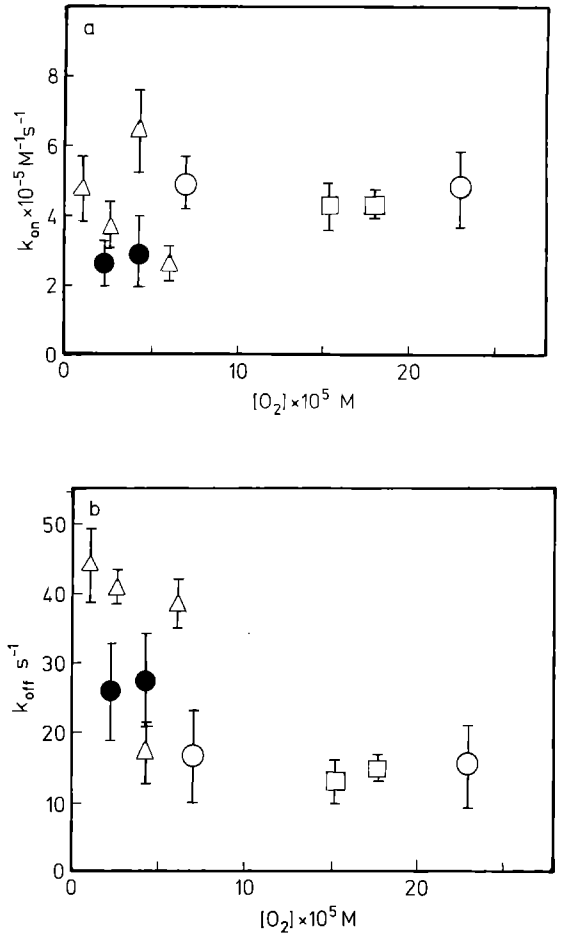

Fig. 9a, b. Rate constants, k_{on} (oxygenation) and k_{off} (deoxygenation) at pH 6.8 as a function of O_2 concentration in the observation cell. Total concentration of hemerythrin, ○; 4.2×10^{-5} M, △; 3.6×10^{-5} M, □; 4.5×10^{-5} M, ●; 4.2×10^{-5} M (data from O_2-jump (down) experiments)

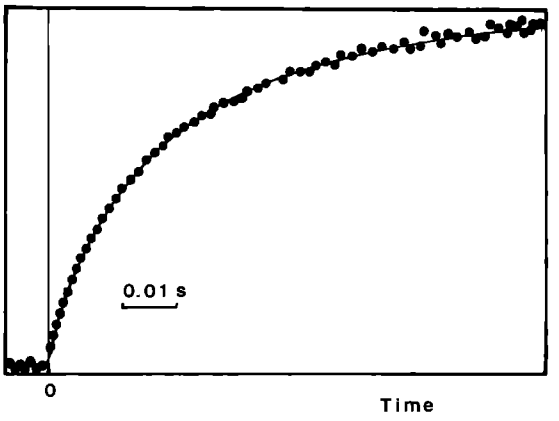

Fig. 10. Time course of hemerythrin absorbance change at 500 nm after mixing with stopped-flow. O_2-free hemerythrin solution was mixed with the buffer containing a particular amount of O_2. The reaction was carried out at pH 7.6. Hemerythrin at 4.9×10^{-5} M and O_2 at 2.5×10^{-5} M were mixed with a ratio of 1 vol/vol. Ordinate represents absorbance in arbitrary units

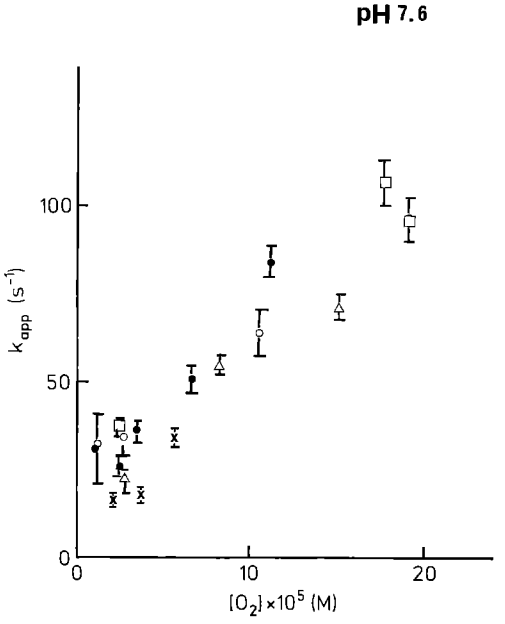

Fig. 11. Apparent rate constants, k_{app} of oxygenation and deoxygenation at pH 7.6 as a function of O_2 concentration in the observation cell. Total concentrations of hemerythrin, ●; 4.1×10^{-5} M, ○; 6.4×10^{-5} M, △; 3.1×10^{-5} M, □; 4.9×10^{-5} M, ×; 4.2×10^{-5} M (data from O_2-jump (down) experiments)

in Fig. 9, this shows that there are no significant differences between O_2-jump (up) and O_2-jump (down) experiments.

At pH 7.6, the absorbance also decreased monophasically, as shown in Fig. 12. The rates calculated were $16 \pm 2.5\ s^{-1}$ at O_2 2.3×10^{-5} M, $18 \pm 2\ s^{-1}$ at O_2 3.8×10^{-5} M and $34 \pm 3\ s^{-1}$ at O_2 5.7×10^{-5} M ([Hr] $= 4.2 \times 10^{-5}$ M). These values corresponded to those from the slow phase of the O_2-jump (up) experiments. At both pH 6.8 and 7.6, the calculated apparent rate constants, k_{app}, agree well with those from O_2-jump (up) experiments.

Discussion

1. At pH 6.8, values of the oxygenation rate constant, k_{on}, and the deoxygenation rate constant, k_{off}, estimated from stopped-flow O_2-jump methods agree well with those obtained by T-jump methods (Zimmer et al. 1986; Petrou et al. 1981; De Waal and Wilkins 1976; Bates et al. 1968) as summarized in Table 1. Rates are consistent with a simple bimolecular reaction such as

$$Hr + O_2 \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} HrO_2$$

which demonstrates that subunits, consisting of octamer bind and release O_2 independently of each other without any allosteric interactions.

2. In our previous paper (Zimmer et al. 1986), reduction of oxyhemerythrin by dithionite was reported. The rate constant was $14.8 \pm 0.8\ s^{-1}$, independent of dithionite concentration. This value is significantly smaller than those obtained by T-jump (Zimmer et al. 1986), but is in excellent agreement with that obtained in this paper.

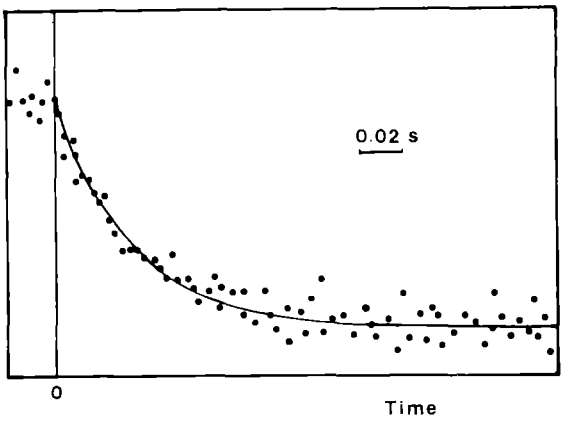

Fig. 12. Time course of deoxygenation reaction of hemerythrin monitored at 500 nm after mixing with stopped-flow. The reaction was carried out at pH 7.6. Hemerythrin at 2.7×10^{-5} M and buffer containing O_2 at 0 mm Hg were mixed with a ratio of 1 vol/vol. Ordinate represents absorbance in arbitrary units

3. At pH 7.6, two processes are observed. The fast process in the O_2-jump (up) experiment corresponded to the process analyzed by the T-jump method, and the slow one corresponded to the process observed in the O_2-jump (down) experiment. With the T-jump experiment, only a single fast process was observed. This process showed positive cooperativity against O_2 concentration, which strongly suggests that it is this process that is responsible for the allostericity of the molecule (Zimmer et al. 1986). On the other hand, there are no strong indications to show that the slow process is related to an allosteric mechanism. The dependence of k_2 on $[O_2]$ is practically linear, which suggests that the slow process reflects a bimolecular reaction without any subunit-subunit interactions. Moreover, if k_2 values are analyzed according to (3)[3], k_{off} and k_{on} estimated from the k_2 vs. $[O_2]$ plot agree quite well with those obtained at pH 6.8. These findings strongly suggest that the O_2 binding process of the T state has nearly the same values for both k_{on} and k_{off}. The difference in O_2 binding between pH 6.8 and 7.6 is simply that there is no allosteric transition at pH 6.8, whereas there is a transition at pH 7.6. The situation is different from that for hemoglobin, where O_2 binding can be assumed to be too fast to be detected by the stopped-flow technique.

In our previous work we assumed a rapid equilibrium for O_2 binding (see derivation of (4) in Zimmer et al. 1986). This was done in order to simplify the model. If this assumption does not hold, results for the T-jump experiment should be re-calculated, although the qualitative results do not change significantly.

4. In principle, the slow process we observed with the stopped-flow technique should be detected in the T-jump study as well. In practice, however, we have often encountered such a case. It could be that the enthalpy of the slow process is so small that this process cannot be detected by the T-jump method.

[3] The analysis employed here is not strictly rigorous. However, as it holds for a large excess of $[O_2]$, the analysis was done as a reasonable approximation

Appendix

Analysis of the second-order reaction

Let us assume a second-order reaction defined by:

$$\mathrm{Hr} + \mathrm{O_2} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \mathrm{HrO_2} \tag{A1}$$

$$t = 0 \quad [\mathrm{Hr}]_0 \quad [\mathrm{O_2}]_0 \quad [\mathrm{HrO_2}]_0$$
$$t = t \quad [\mathrm{Hr}]_0 - x \quad [\mathrm{O_2}]_0 - x \quad [\mathrm{HrO_2}]_0 + x$$

where subscript 0 denotes concentrations at $t=0$. Then,

$$\frac{dx}{dt} = k_{on}([\mathrm{Hr}]_0 - x)([\mathrm{O_2}]_0 - x) - k_{off}([\mathrm{HrO_2}]_0 + x)$$
$$= k_{on}(x-\alpha)(x-\beta) \quad (\alpha > \beta) \tag{A2}$$

where

$$\alpha = \tfrac{1}{2}([\mathrm{Hr}]_0 + [\mathrm{O_2}]_0 + K + \gamma)$$
$$\beta = \tfrac{1}{2}([\mathrm{Hr}]_0 + [\mathrm{O_2}]_0 + K - \gamma)$$
$$\gamma = \sqrt{([\mathrm{Hr}]_0 + [\mathrm{O_2}]_0 + K)^2 - 4([\mathrm{Hr}]_0[\mathrm{O_2}]_0 - K[\mathrm{HrO_2}]_0)}$$

and

$$K = k_{off}/k_{on}$$

Then (A2) is

$$\frac{1}{\alpha-\beta}\left(\frac{1}{\alpha-x} - \frac{1}{\beta-x}\right)dx = k_{on}\,dt$$

$$\frac{1}{\alpha-\beta}\ln\left|\frac{x-\alpha}{x-\beta}\right| = k_{on}t + \text{const.} \qquad \text{As } x=0 \text{ at } t=0$$

$$x = \alpha\left(1 - \frac{\gamma}{\alpha - \beta\exp(-k_{on}\cdot\gamma\cdot t)}\right) \tag{A3}$$

The absorbance change A at time t is defined as

$$A = -\Delta\varepsilon(\mathrm{Hr})x - \Delta\varepsilon(\mathrm{O_2})x + \Delta\varepsilon(\mathrm{HrO_2})x$$
$$= (\Delta\varepsilon)x$$
$$= (\Delta\varepsilon)\alpha\left(1 - \frac{\gamma}{\alpha - \beta\exp(-k_{app}t)}\right) \tag{A4}$$

where

$$\Delta\varepsilon = \Delta\varepsilon(\mathrm{HrO_2}) - \Delta\varepsilon(\mathrm{Hr}) - \Delta\varepsilon(\mathrm{O_2})$$

$$k_{app} = k_{on}\cdot\gamma$$
$$= k_{on}\sqrt{([\mathrm{Hr}]_0 + [\mathrm{O_2}]_0 + K)^2 - 4([\mathrm{Hr}]_0[\mathrm{O_2}]_0 - [\mathrm{HrO}]_0 K)}$$

and $\Delta\varepsilon(\mathrm{Hr})$, $\Delta\varepsilon(\mathrm{O_2})$ and $\Delta\varepsilon(\mathrm{HrO_2})$ are molar extinction coefficients. Thus ΔA vs. t can be analysed by the non-linear least squares method, by keeping K, k_{on} and $\Delta\varepsilon$ as parameters. In practice, however, $[O_2]_0$ is more difficult to determine. Instead, $[\overline{O_2}]$ (O_2 concentration in equilibrium after the reaction) is easier to measure. Then with the following equations, $[O_2]_0$ was estimated from $[\overline{O_2}]$,

$[Hr]_0$, K and $[HrO_2]_0$, as

$$K = \frac{\overline{[Hr]}\,\overline{[O_2]}}{\overline{[HrO_2]}}$$

$$\overline{[HrO_2]} - [HrO_2]_0 = [Hr]_0 - \overline{[Hr]} = [O_2]_0 - \overline{[O_2]}$$

By rearranging,

$$[O_2]_0 = \overline{[O_2]} + \frac{[Hr]_0\,\overline{[O_2]} - K\,[HrO_2]_0}{K + \overline{[O_2]}} \qquad (A\,5)$$

Thus with the observed values of $[Hr]_0$, $[HrO_2]_0$ and $\overline{[O_2]}$, parameters K, k_{on} and $\Delta\varepsilon$ were best-fitted by the non-linear least squares Simplex method (Nelder and Mead 1964).

When $k_{app}\,t \gg 1$ and/or $\alpha \gg \beta$ (which essentially means that one of the reactants ($[Hr]_0$ or $[O_2]_0$) is in large excess), ΔA is approximated as

$$\Delta A = (\Delta\varepsilon)\,\beta\left(1 - \frac{\gamma}{\alpha}\exp(-k_{app}\,t)\right) \qquad (A\,6)$$

which means that ΔA obeys a single exponential decay. Then, except at very short times, the second-order reaction approaches a single exponential process. As an example, if $[O_2]_0 \gg [Hr]_0$, (A 6) is approximated as

$$\Delta A = (\Delta\varepsilon)\,[Hr]_0\,\{1 - \exp(k_{on}\,[O_2]_0\,t)\} \qquad (A\,7)$$

which is normal pseudo first-order.

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