THERMODYNAMICAL ANALYSIS OF OXYGEN EQUILIBRIUM OF STRIPPED HEMOGLOBIN

Kiyohiro IMAI and Itiro TYUMA

Department of Physicochemical Physiology, Medical School,

Osaka University, Osaka, Japan

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SUMMARY: Precise oxygen equilibrium curves of hemoglobin stripped of phosphates were determined at pH 7.4 and five different temperatures. The data were thermodynamically analyzed according to Adair's stepwise oxygenation theory and the allosteric model of Monod et al. Heat of oxygenation of $Hb(O_2)_3$ was significantly larger than that of Hb, indicating that the shape of oxygen equilibrium curve is not invariant with the change of temperature. The results do not support the idea that the cooperative effects are essentially entropic in nature, suggesting that the allosteric transition from the unliganded T-state to the unliganded R-state is an endothermic process, during which the hemoglobin molecule gains entropy.

One of the most useful ways to clarify the mechanism of allosteric action of hemoglobin will be a study of temperature dependence of oxygen equilibrium. However, only few data are available on the temperature dependence which can be subjected to detailed thermodynamical analyses. In this study, precise oxygen equilibrium curves of hemoglobin were determined at five different temperatures from 10° to 30°, and the equilibrium data were analyzed according to Adair's stepwise oxygenation theory (1) and the allosteric model of Monod, Wyman, and Changeux (MWC) (2).

In order to make the equilibrium system simpler, oxygen equilibrium curves were determined in the absence of phosphates which cause heterotropic interactions. Hemoglobin A prepared from human blood was stripped of phosphates as described by Benesch et al. (3). The oxygen equilibrium curves were determined by the automatic recording method of Imai et al. (4) in 0.05 M bis-Tris buffer containing 0.1 M NaCl at 10, 15, 20, 25, and 30°. Several techniques were devised to determine the curves over a wide range of oxygen saturation (0.01 to 0.999) as described elsewhere (5). The pH of the buffer system was adjusted to 7.4 at the respective temperatures, which were maintained within $\pm 0.05^{\circ}$ during oxygen equilibrium measurements. The met-

hemoglobin contents determined by the method of Benesch et al. (6) were less than 4 % after the measurements.

According to Adair (1), the fractional oxygen saturation of hemoglobin, Y, is expressed as follows;

$$Y = (a_1p + 2a_2p^2 + 3a_3p^3 + 4a_4p^4)/4(1 + a_1p + a_2p^2 + a_3p^3 + a_4p^4),$$

where p is oxygen pressure and a's are constants. On the assumption that the four oxygen binding sites are equivalent, a's are given by $a_1 = 4k_1$, $a_2 = 6k_1k_2$, $a_3 = 4k_1k_2k_3$, and $a_4 = k_1k_2k_3k_4$, where k_i is the intrinsic oxygen association constant for $Hb(O_2)_{i-1}$. On the other hand, the MWC model (2) describes Y as;

$$Y = \left[Lc\alpha(1 + c\alpha)^3 + \alpha(1 + \alpha)^3\right]/\left[L(1 + c\alpha)^4 + (1 + \alpha)^4\right],$$

where L is the allosteric constant, i.e. the equilibrium constant for $R \rightleftarrows T$ in the absence of ligands; c is the ratio of the intrinsic oxygen association constant for the T-state, K_T*, to that for the R-state, K_R*; and $\alpha = K_R \cdot p$. The parameters, k's, L, c, and K_T (or K_R) were estimated by the least squares method as described elsewhere (5).

The oxygen equilibrium data expressed by the Hill plots are shown in Fig. 1, where the experimental points are compared with the simulated curves constructed from the MWC parameters. The fit of the experimental points and the simulated curves is very good. The simulated curves obtained from the Adair parameters also showed similar fits. The estimated values of the Adair parameters with the coefficients of variation are summarized in Table 1, which also includes the oxygen pressure at half oxygen saturation, P_{50}, median ligand activity, P_m, maximum slope of the Hill plots, n_{max}, and overall free energy of interaction among oxygen binding sites, ΔF_I, estimated from the Adair parameters as described elsewhere (5). The values of P_{50} are

* In this paper, the oxygen equilibrium constants for the T- and R-states are expressed in terms of intrinsic association constants, K_T and K_R, respectively, instead of intrinsic dissociation constants which were used by Monod et al. The parameter, c, defined by K_T/K_R, however, gives the identical value with that used by Monod et al.

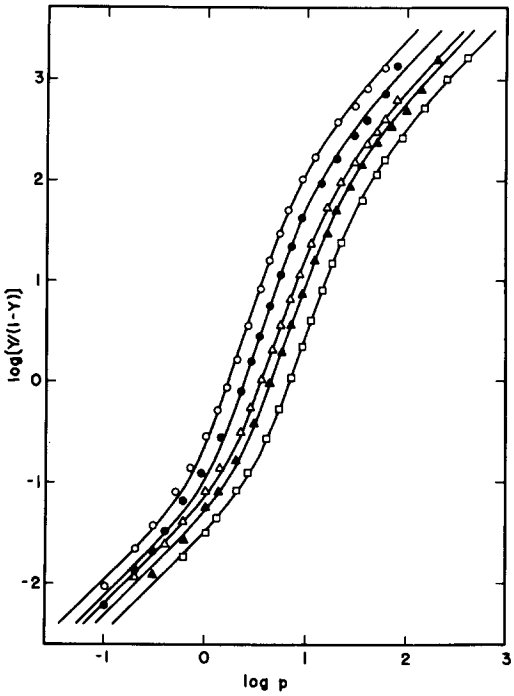

Fig. 1. The Hill plots of oxygen equilibrium of stripped hemoglobin A. Y, fractional oxygen saturation of hemoglobin; p, oxygen pressure (mm Hg). Hemoglobin concentration, 6.0×10^{-5} M on heme basis; in 0.05 M bis-Tris buffer (pH 7.4) containing 0.1 M NaCl. O—O, 10°; ●—●, 15°; Δ—Δ, 20°; ▲—▲, 25°; □—□, 30°.

Table 1. Oxygen equilibrium parameters, k_i (mm Hg^{-1}), P_{50} (mm Hg), P_m (mm Hg), n_{max}, and ΔF_I (cal/mole of O_2) at different temperatures. Figures in parenthesis are the coefficient of variation of the k's.

Temperature	k_1	k_2	k_3	k_4	P_{50}	P_m	n_{max}	ΔF_I
10°	0.107 (7 %)	0.141 (29 %)	0.443 (30 %)	26.7 (11 %)	1.64	1.54	3.02	3100
15°	0.0610 (9 %)	0.266 (21 %)	0.142 (23 %)	19.0 (15 %)	2.30	2.18	2.84	3290
20°	0.0599 (4 %)	0.0998 (14 %)	0.156 (15 %)	9.67 (7 %)	3.49	3.24	2.81	2960
25°	0.0401 (8 %)	0.117 (17 %)	0.0958 (17 %)	8.10 (10 %)	4.32	4.07	2.76	3140
30°	0.0296 (3 %)	0.0511 (8 %)	0.0950 (8 %)	4.45 (4 %)	6.73	6.29	2.81	3020

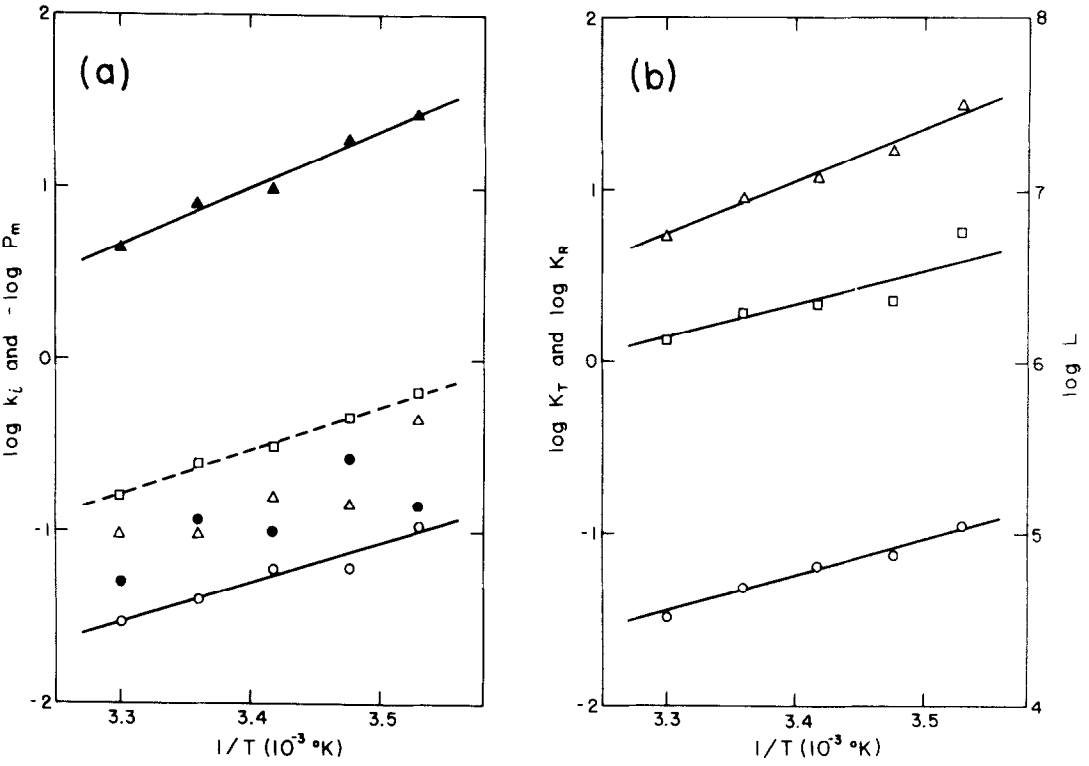

Fig. 2. Dependence of the oxygen equilibrium parameters on the reciprocal temperature. (a) temperature dependence of the Adair parameters and the median ligand activity. O—O, log k_1; ●—●, log k_2, Δ—Δ, log k_3; ▲—▲, log k_4; □—□, -log P_m. (b) temperature dependence of the MWC parameters. O—O, log K_T; Δ—Δ, log K_R; □—□, log L.

Table 2. The MWC parameters, K_T (mm Hg^{-1}), K_R (mm Hg^{-1}), c, and L at different temperatures.

Temp.	K_T	K_R	c	L
10°	0.111 ± 0.014	31.9 ± 9.4	0.00348 ± 0.00027	(5.87 ± 2.21) × 10^6
15°	0.0748 ± 0.0121	17.3 ± 2.1	0.00434 ± 0.00047	(2.29 ± 1.13) × 10^6
20°	0.0646 ± 0.0062	11.8 ± 0.9	0.00548 ± 0.00033	(2.19 ± 0.66) × 10^6
25°	0.0487 ± 0.0065	8.99 ± 0.92	0.00541 ± 0.00046	(1.93 ± 0.78) × 10^6
30°	0.0324 ± 0.0018	5.37 ± 0.23	0.00603 ± 0.00021	(1.33 ± 0.23) × 10^6

slightly larger than those of P_m at all the temperatures. It is uncertain whether the cooperativity measured by n_{max} and ΔF_I depends on temperature or not. In Fig. 2(a), logarithm of the k's and $1/P_m$ is plotted against 1/T

where T is the absolute temperature. The temperature dependences of log k_1, log k_4, and -log P_m are clear, whereas those of log k_2 and log k_3 are uncertain. The heat of oxygenation and the entropy change of oxygenation were estimated from the straight lines, which were determined by the least squares method, giving the following results:

ΔH_1 = -10.2 ± 1.4 kcal/mole of O_2, ΔS_1 = -27.3 ± 4.6 e.u.;

ΔH_4 = -15.0 ± 1.5 kcal/mole of O_2, ΔS_4 = -32.6 ± 4.6 e.u.;

$\Delta H_{overall}$ = -11.7 ± 0.6 kcal/mole of O_2, $\Delta S_{overall}$ = -32.6 ± 1.9 e.u.;

where the subscripts, 1, 4, and overall, stand for the ΔH and ΔS calculated from the temperature dependences of log k_1, log k_4, and -log P_m, respectively. The standard state for ΔS is 1 atm of gaseous oxygen. The value of $\Delta H_{overall}$ is close to -10.7 kcal/mole of O_2 which was previously obtained by Benesch et al. (7). There is no significant difference between ΔS_1 and ΔS_4. ΔH_4 is, however, significantly larger in absolute value than ΔH_1 in contrast with the result of Roughton et al. (8) who showed, using sheep hemoglobin, that ΔH_4 is smaller in absolute value than ΔH_1. In general the estimates of ΔH's are composed of the intrinsic heat of oxygenation, the heat of solution of oxygen, and the heat of the ionization of oxygen-linked acid groups (9). However, the contribution of the last heat (positive in sign (9)) seems to be negligible in ΔH_4, but not in ΔH_1, since k_4 is insensitive to pH change up to pH 9 whereas k_1 increases with the pH increase (10). It has been reported that ΔH estimated from the temperature dependence of P_{50} is -10 kcal/mole of O_2 at pH 7.3 (11) and it approaches -14 kcal/mole of O_2 above pH 9 where the Bohr effect disappears (9, 12). The difference between ΔH_1 and ΔH_4, therefore, may disappear above pH 9, implying that the intrinsic ΔH is the same for the 1st and 4th oxygenation steps.

The significant difference between ΔH_1 and ΔH_4 indicates that the shape of oxygen equilibrium curve is not invariant with the temperature change. As seen in Fig. 1, the temperature-invariance of the shape of oxygen equilibrium curve holds in the middle region but it fails to hold in the lower and higher

regions. The heat of interaction, ΔH_I, and the entropy change of interaction, ΔS_I, which are defined by $\Delta F_I = \Delta H_I - T\Delta S_I$, are obtained from the relations, $\Delta H_I = \Delta H_1 - \Delta H_4$ and $\Delta S_I = \Delta S_1 - \Delta S_4$, respectively, giving $\Delta H_I = 4.8 \pm 1.9$ kcal/mole of O_2 and $\Delta S_I = 5.3 \pm 6.5$ e.u. Since $T\Delta S_I = 1.6 \pm 1.9$ kcal/mole of O_2 is not greater than the ΔH_I, the present result does not support the previous conclusion of Wyman (9, 13) that the cooperative effects in hemoglobin are essentially entropic in nature.

Table 2 summarizes the estimates of the MWC parameters. The logarithm of K_T, K_R, and L is plotted against 1/T in Fig. 2(b). The heat of oxygenation and the entropy change of oxygenation for the T- and R-states were obtained as follows:

$\Delta H_T = -9.8 \pm 0.9$ kcal/mole of O_2, $\Delta S_T = -25.8 \pm 3.0$ e.u.;

$\Delta H_R = -14.3 \pm 1.0$ kcal/mole of O_2, $\Delta S_R = -30.7 \pm 3.5$ e.u.;

where the subscripts, T and R, stand for the ΔH and ΔS calculated from the temperature dependences of log K_T and log K_R, respectively. ΔH_R is significantly larger in absolute value than ΔH_T, whereas there is no significant difference between ΔS_T and ΔS_R.

The heat of the allosteric transition in the absence of oxygen, ΔH_{trans}, and the entropy change of the transition, ΔS_{trans}, estimated from the temperature dependence of log L were as follows:

$\Delta H_{trans} = 10.8 \pm 1.9$ kcal/mole of hemoglobin, $\Delta S_{trans} = 7.8 \pm 6.3$ e.u.

The above results clearly indicate that the transition from the unliganded T-state to the unliganded R-state is an endothermic process and the hemoglobin molecule gains entropy during the transition. The latter finding is consistent with the X-ray data that deoxyhemoglobin assumes a tense conformation whereas the oxy form takes a relaxed conformation (14). The free energy change of the allosteric transition in the absence of oxygen, ΔF_{trans}, is obtained from the relation, either $\Delta F_{trans} = \Delta H_{trans} - T\Delta S_{trans}$ or $\Delta F_{trans} = RT \ln L$. Thus, we have

$\Delta F_{trans} = 8.5 \pm 2.6$ kcal/mole of hemoglobin (at 20°).

According to the model of Perutz (14), deoxyhemoglobin takes a constrained conformation which is stabilized by six-salt-bridges between the subunits and two salt-bridges within the β subunits, whereas oxyhemoglobin is free of these constraints. If we assume that these eight salt-bridges are responsible for the stability of the T-state relative to that of the R-state and that the bond energy per salt-bridge is the order of 1 to 2 kcal/mole (14), then the ΔF_{trans} is expected to be 8 to 16 kcal/mole. The observed value of ΔF_{trans} is within the expected values.

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