THERMODYNAMICS OF EQUILIBRIA OF HEMOGLOBINS M Milwaukee-I AND Saskatoon AND ISOLATED CHAINS OF HEMOGLOBIN A WITH CARBON MONOXIDE

Takao Nakamura[1], Yoshiki Sugita[2,4], Ken Hashimoto[1,5], Yoshimasa Yoneyama[2] and Anthony V. Pisciotta[3]

[1]Department of Biology, Faculty of Science, Osaka University, Toyonaka, Osaka 560, Japan, [2]Department of Biochemistry, Kanazawa University School of Medicine, Kanazawa 920, Japan and [3]Medical College of Wisconsin, Milwaukee, Wisconsin 53226, U.S.A.

Received March 8,1976

SUMMARY-The thermodynamic parameters of the CO-equilibria of isolated chains of hemoglobin A and of two α-chains in hemoglobins M Milwaukee-I and Saskatoon at 25°, pH 7.0 were determined. The parameters for the binding of the first CO molecule to the hemoglobins M were $\Delta H'$=-17 and -18 kcal/mole heme and $\Delta S'$=-30 and -29 e.u. for hemoglobins M Milwaukee-I and Saskatoon, respectively. In contrast to this the characteristics of the second step of the binding were $\Delta H'$=+5.9 and +4.3 kcal/mole and $\Delta S'$=+51 and +49 e.u. These values for the second step were also significantly different from those of the isolated α-chain ($\Delta H'$=-15 kcal/mole and $\Delta S'$=-11 e.u.).

In hemoglobin M Milwaukee-I ($\alpha_2^{3}{}_2^{67\ Glu}$) and hemoglobin M Saskatoon ($\alpha_2^{3}{}_2^{63\ Tyr}$) (1) the 3-chains contain ferric hemes, so that only the α-chains can bind CO or O_2 molecules. Perutz et al. showed by X-ray analysis (2) that the deoxy-form of hemoglobin M Milwaukee-I is isomorphous with deoxy-hemoglobin A. They suggested from spectroscopic measurements (2) that a structural change, similar to that observed in the transformation of the deoxy- to oxy-form of hemoglobin A, took place in the hemoglobin M Milwaukee-I molecule when CO bound to its α-chains. Several other results also suggest a conformation change of the hemoglobin M Milwaukee-I molecule during oxygenation (3). In the binding of two molecules of CO to the α-chains in these hemoglobins M, a heme-heme inter-

Present address: [4]Division of Biochemistry, Tsukuba University school of Medicine, Sakura, Ibaraki 300-31, Japan and [5]Department of Biology, Faculty of Science, Kyushu University, Fukuoka 812, Japan.

action was observed and this interaction can be understood as a stepwise increase of the ligand affinity of the hemoglobin. However, the difference in the nature of the first and second steps of ligand binding to the hemoglobins M molecule has not yet been quantitatively analyzed. The present paper reports studies on the thermodynamic parameters of the two equilibrium steps of these hemoglobins M with CO and a comparison of the values with those of isolated chains of hemoglobin A.

Materials and Methods

Preparation of Hemoglobins M Milwaukee-I and Saskatoon and Isolated Chains of Hemoglobin A--These hemoglobins M were obtained from the blood of heterozygous patients and purified and prepared in the half ferrous and half ferric ($\alpha_2^{2+}\beta_2^{3+}$) form by the method of Nishikura et al. (4). These preparations had a CO binding capacity of 2.0 mole/mole hemoglobin (4). The ferrous heme in α-chains in these hemoglobins M may undergo slow autoxidation in the presence of molecular oxygen at low pressures, but the oxidation could be avoided in the equilibrium experiments with non-oxidizing ligand CO. The α- and β-chains of hemoglobin A were isolated as described previously (5).

CO-Equilibrium of Hemoglobins M--A large (volume: 350 ml) tonometer cell was used. Hemoglobin solution in the deoxy-form (100 μM in heme, 3 ml) was placed in the cell and a small amount of CO gas was introduced quantitatively. After equilibration at a given temperature, fractional saturation of the hemoglobin with CO was determined spectrophotometrically at 570 nm. In calculating the CO pressure in the tonometer cell a correction was made for the decrease caused by partial binding of CO to the hemoglobin in solution.

Calculation of the Association Constants of Hemoglobins M with CO--For estimation of the intrinsic microscopic association constants K_1 and K_2 in the ligand equilibrium of a dimeric protein (like the α-chains in hemoglobins M), we used the following method. (The method of Edsall et al. (6) to estimate K_1 and K_2 in a Scatchard plot could not be used in the present measurements (see Fig. 1)).

The mechanism of cooperative ligand binding to a protein molecule containing two identical sites can be described by the following consecutive equilibrium reactions:

$$P + L \overset{K_1}{\rightleftharpoons} PL, \quad PL + L \overset{K_2}{\rightleftharpoons} PL_2$$

where P and L denote the protein and ligand, respectively. Then

$$K_1=[PL]/2[P][L], \quad K_2=2[PL_2]/[PL][L]$$

and the fractional saturation (Y) is given by

$$Y=([PL] + 2[PL_2])/2[P]_o=(K_1[L] + K_1K_2[L]^2)/(1 + 2K_1[L] + K_1K_2[L]^2),$$

where $[P]_o$ represents the total concentration of protein. From the last equation, $[L]_{1/2}$, the free ligand concentration which gives half saturation of the binding sites (Y=1/2) will be given by

$$[L]_{1/2}=1/(K_1K_2)^{1/2} \qquad (1)$$

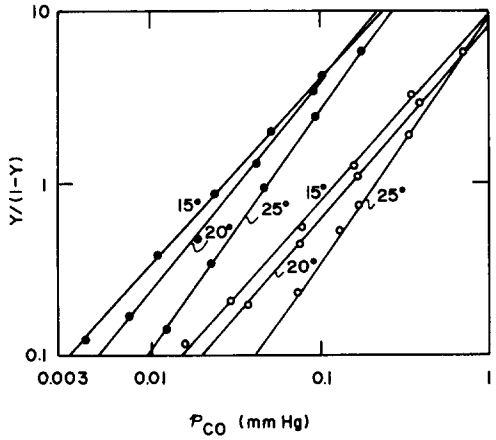

Fig. 1. Hill Plots of CO-Equilibria of Hemoglobins M Milwaukee-I (O) and Saskatoon (●) At pH 7.0. Temperatures are shown in the figure. The solid lines in the figure are theoretical curves calculated by using association constants given in Table II. The lines are almost indistinguishable from straight lines within the present experimental range of Y/(1 - Y) of 0.1-6. With lower CO partial pressures than those examined in the present study ($p_{CO} < 0.003$ mm Hg) it was considerably difficult to determine accurate saturation values of the hemoglobins with CO, since the equilibrium took too long time (> 2 hr).

According to Wyman (7) the value of n_{max} in the Hill plot of this binding reaction will be given by

$$n_{max}=2/(1 + (K_1/K_2)^{1/2}). \quad (2)$$

By combining equations 1 and 2, we obtain

$$K_1=(2 - n_{max})/n_{max}[L]_{1/2} \quad (3)$$

$$K_2=n_{max}/(2 - n_{max})[L]_{1/2} \quad (4)$$

Equations 3 and 4 were used to estimate K_1 and K_2 of the CO-equilibrium of the hemoglobins M from the values of n_{max} and $[L]_{1/2}$ determined experimentally.

CO-Equilibria of Isolated Chains--Isolated chains of hemoglobin A showed extremely high affinity for CO and volumetric determination of the CO-equilibrium, as with the hemoglobins M, was not possible. Assuming a simple bimolecular equilibrium reaction between CO and a chain, the ratio of the rate constant of CO-binding to that of CO-release was taken as the association constant of the chain and CO. It is known that the β-chain exists in a tetrameric form when separated from the α-chain, but it was treated as a monomeric protein in determination of the association constants since there is no heme-heme interaction in the CO-equilibrium of the β-chain tetramer. The rate of CO-binding was measured at 430 nm by the stopped flow method by mixing 10 μM chain with 25 μM CO, and that of CO-release was measured by NO-replacement (8). Further details of the methods have been described elsewhere (9).

Buffer--All the equilibrium and kinetic experiments were carried out in 0.1 M phosphate buffer, pH 7.0.

Table I. Reaction Rate Constants and Association Constants of Isolated Chains of Hemoglobin A with CO and the Thermodynamic Parameters of the Equilibria[a] at 25°, pH 7.0.

Chain	Temp. (°C)	k_+^b ($10^6 M^{-1}sec^{-1}$)	k_-^c (sec^{-1})	$K=k_+/k_-$ ($10^8 M^{-1}$)	$\Delta G'$ (kcal/mole)	$\Delta H'$ (kcal/mole)	$\Delta S'$ (e.u.)
α	10	2.9	0.0041	7.1			
	15	3.1	0.0065	4.8			
	20	2.9	0.011	2.7			
	25	3.4	0.017	2.0	-11±1	-15±1	-11±7
β	10	3.1	0.0018	17			
	15	3.4	0.0035	9.9			
	20	4.6	0.0067	6.9			
	25	5.5	0.011	5.0	-12±1	-14±1	-5.7±3.1

[a] 1 M CO solution was taken as the standard state. Values were obtained by least square analysis of the van't Hoff data with standard deviations.
[b] Rate constant of the CO-binding reaction. Values are averages of five measurements.
[c] Rate constant of the CO-releasing reaction.

Results and Discussion

CO-Equilibria of Isolated Chains of Hemoglobin A--The association constants of CO-binding of the isolated chains were obtained at 10-25°, and the thermodynamic parameters were calculated using van't Hoff's equation (Table I). Brunori et al. (10) reported the association constant only at 20° (3-3.5x$10^8 M^{-1}$ for the α-, and 4.8-5.7x$10^8 M^{-1}$ for the β-chain), and our values at the same temperature are in fairly good agreement with theirs. The present measurements showed that the high affinity of the isolated chains for CO is due to the large exothermic $\Delta H'$. The values of $\Delta H'$ in Table I are in good agreement with that estimated by Gaud et al. (11) (-14.5 kcal/mole for both chains) in an indirect calculation. De Renzo et al. (12) examined the effect of temperature on the O_2 equilibria of the isolated chains. They did not report the $\Delta S'$ values of the reaction but the following values may be estimated from their data: $\Delta G'$=-8.2 kcal/mole, $\Delta H'$=-11 kcal/mole and $\Delta S'$= -9.7 e.u. at 25°, corrected by taking 1 M O_2 solution as the standard state, for both chains. The higher affinity of the chains for CO than for O_2 is due to a more negative value of $\Delta H'$. Present results on CO-binding with isolated α- and β-chains are also comparable

Table II. CO Equilibria and Their Thermodynamic Parameters[a] of Hemoglobins M Milwaukee-I and Saskatoon at 25°, pH 7.0.

Hemoglobin M	Temp. (°C)	$[CO]_{1/2}$ (μM)	n_{max}	K_1 ($10^6 M^{-1}$)	K_2 ($10^6 M^{-1}$)	$\Delta G'$ (kcal/mole)	$\Delta H'$ (kcal/mole)	$\Delta S'$ (e.u.)
Milwaukee-I	15	0.20	1.09	4.2	6.0	K_1 -8.5±2.2	-17±2	-30±9
	20	0.25	1.18	2.6	6.5			
	25	0.28	1.41	1.5	8.5	K_2 -9.5±2.7	+5.9±1.8	+51±11
Saskatoon	15	0.044	1.10	19	28	K_1 -9.4±2.6	-18±2	-29±10
	20	0.050	1.22	12	31			
	25	0.065	1.40	6.6	36	K_2 -10±1	+4.3±0.8	+49±4

[a] 1 M CO solution was taken as the standard state. Values are given per mole of heme in the α-chain.

with those reported for the equilibrium of myoglobin and CO ($\Delta G'$= -10.1 kcal/mole, $\Delta H'$=-17.7 kcal/mole and $\Delta S'$=-25.8 e.u. at 25°, pH 8.00 (13)) but the binding of the isolated chains of hemoglobin A with CO is entropically slightly less unfavorable than that of myoglobin.

CO-Equilibria of Hemoglobins M Milwaukee-I and Saskatoon--Figure 1 shows Hill plots of the CO-equilibria of hemoglobins M Milwaukee-I and Saskatoon at different temperatures. The values of n_{max} and $[CO]_{1/2}$ were determined from the figure. It was observed that these values increased with an increase of temperature (Table II). A similar temperature dependence of n_{max} and $[O_2]_{1/2}$ values of hemoglobin M Milwaukee-I was observed for its O_2 equilibrium (14). The microscopic association constants of CO to these hemoglobins M in the first and second steps of binding were calculated according to equations 3 and 4, and the results are summarized in Table II. It is apparent from the data that significant differences exist between the $\Delta H'$ or $\Delta S'$ values of the first and second steps of the equilibrium: e.g. the binding is exothermic in the first step and endothermic in the second step for both hemoglobins M. The first step was found to be entropically unfavorable compared to the second step, and this fact may suggest that the splitting of interchain salt bridges, which was suggested to occur when deoxygenated hemo-

globins M are fully liganded (3), took place mainly in the first step. The fact that the enthalpy and entropy changes for the second (last) step of the binding of CO to these hemoglobins M differ significantly from those of the isolated α-chain suggests that the formation of ligand between the last vacant chain and CO cannot be considered to be analogous with that of the reaction of the free α-chain. In the half ferrous form of hemoglobin M Iwate, an α-chain mutant, only β-chains are capable of binding to CO. In connection with the fact that no heme-heme interaction was observed with the hemoglobin M Iwate, it is interesting to note that the heat of CO binding to the two β-chains in the hemoglobin was reported to be the same for the first and second steps of the reaction and the value was closely comparable to that of isolated β-chain of hemoglobin A (11).

References

1. Gerald, P.S., and Efron, M.L. (1961) Proc. Natl. Acad. Sci., U.S., 47, 1758-1767.
2. Perutz, M.F., Pulsinelli, P.S., and Ranney, H.M. (1972) Nature New Biol., 237, 259-263.
3. Udem, L., Ranney, H.M., Bunn, H.F., and Pisciotta, A. (1970) J. Mol. Biol., 48, 489-498.
4. Nishikura, K., Sugita, Y., Nagai, M., and Yoneyama, Y. (1975) J. Biol. Chem., 250, 6679-6685.
5. Sugita, Y., Bannai, S., Yoneyama, Y., and Nakamura, T. (1972) J. Biol. Chem., 247, 6092-6095.
6. Edsall, J.T., Felsenfeld, G., Goodman, D.S., and Gurd, F.R.N. (1954) J. Am. Chem. Soc., 76, 3054-3061.
7. Wyman, J.Jr. (1948) Advances in Protein Chemistry, vol. 4, pp.407-531, Academic Press, New York.
8. Gibson, Q.H., and Roughton, F.J.W. (1957) Proc. Roy. Soc. London B. Biol. Sci., 147, 44-56.
9. Nakamura, T., Sugita, Y., and Bannai, S. (1973) J. Biol. Chem., 248, 4119-4122.
10. Brunori, M., Noble, R.W., Antonini, W., and Wyman, J. (1966) J. Biol. Chem., 241, 5238-5243.
11. Gaud, H.T., Gill, S.J., Barisas, B.G., and Gersonde, K. (1975) Biochemistry, 14, 4584-4589.
12. De Renzo, E.C., Ioppolo, C., Amiconi, G., Antonini, W., and Wyman, J. (1967) J. Biol. Chem., 242, 4850-4853.
13. Rudolph, S.A., Boyle, S.O., Dresden, C.F., and Gill, S.J. (1972) Biochemistry, 11, 1098-1101.
14. Hayashi, A., Suzuki, T., Imai, K., Morimoto, M., and Watari, H. (1969) Biochim. Biophys. Acta, 194, 6-15.