The Reactions of the Isolated α and β Chains of Human Hemoglobin with Oxygen and Carbon Monoxide*

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SUMMARY

The values of the equilibrium and kinetic constants for the reactions with oxygen and carbon monoxide of the isolated α and β chains of human hemoglobin, both in the form with sulfhydryl groups blocked by p-hydroxymercuribenzoate and in the form with freely titratable sulfhydryl groups, have been determined.

The data show clearly that the behavior of the isolated chains is not only unlike that of hemoglobin A, but also differs markedly from that of myoglobin.

Since the isolated chains behave as simple systems without heme-heme interaction ($n = 1$ in the O_2 equilibrium), they have been used to test the proposition that the binding of a ligand is correctly expressed as a single step reaction. In at least one case it appears that it is not.

Mammalian hemoglobins contain two types of polypeptide chain, α and β, which are arranged, under normal conditions, in the form of a tetramer containing two chains of each type. The functional behavior of the tetramer, which is characterized by the presence of strong cooperative interactions in the binding of ligands to the hemes, appears to be the result of the presence of two different kinds of polypeptide chain in the molecule. When the chains are isolated, their behavior is very different from that of the intact molecule: the O_2 equilibrium curve shows no heme-heme interaction, there is no significant Bohr effect, and the ligand affinity is greatly increased. When the α and β chains are combined, a striking change occurs, and all of the qualitative properties of the original hemoglobin molecule are restored (1, 2).

A major problem is how the interaction between the two kinds of chain can modify so drastically the behavior of the single units. Since it appears that the full hemoglobin molecule exists in at least two different conformations, with which different reactivities of the hemes are associated, it is of interest to know which of these reactivities most closely approximates that of the isolated chains. An answer to this question requires, of course,

a detailed knowledge of the equilibrium and kinetic constants of the reaction of the isolated chains with ligands.

Apart from this, the study of the behavior of the isolated chains is of interest as a test for the validity of the simple reaction scheme

$$\text{Fe} + X \underset{x}{\overset{x'}{\rightleftharpoons}} \text{Fe}X, \qquad X = \frac{x'}{x} \qquad \text{(A)}$$
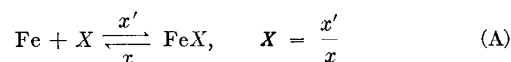
where Fe is a heme protein, X is the ligand, X is the equilibrium constant, and x' and x are the combination and dissociation velocity constants, respectively. This scheme has been commonly applied to single heme proteins, such as myoglobins, or to multiple heme proteins in which no cooperative effects are present. An attempt to analyze in detail the applicability of Model A to the case of horse myoglobin has recently been made.[1] The results indicate that even so simple a system as myoglobin does not conform completely to this elementary scheme in its reaction with oxygen or carbon monoxide.

In view of these considerations, the equilibrium and kinetic constants for the reaction of the isolated hemoglobin chains with oxygen and carbon monoxide have been studied.

MATERIALS AND METHODS

Preparation of Reagents

Hemoglobin Chains—The α and β chains were obtained by treating human oxyhemoglobin with an excess of p-hydroxymercuribenzoate, following the procedure of Bucci and Fronticelli (4) and Bucci *et al.* (5). In the course of this work, several modifications of the original pH gradient described by Bucci and Fronticelli were used. It was found that, so long as separation as monitored by starch gel electrophoresis was complete, the properties of the final products were independent of the gradient used. The best separation was obtained with a continuous gradient from pH 6.5 (0.01 M phosphate) to pH 8.5 (0.02 M K_2HPO_4), and this was the gradient normally used. The total volume of the gradient was 1200 ml. Regeneration of the —SH groups of both chains was accomplished by treatment with cysteine or thioglycolate. The latter procedure, which was the one most commonly used, consisted of a 12-hour dialysis of both chains against 0.05% thioglycolate in 0.01 M phosphate buffer, pH 7.4, containing 0.02 M NaCl and EDTA at a concen-

[1] E. Antonini, Q. H. Gibson, and J. Wyman, unpublished observations quoted by Antonini (3).

tration of 5×10^{-4} M. Afterward the chains were dialyzed for 24 to 48 hours against several liters of the same buffer without thioglycolate. As already reported (5), these procedures gave complete regeneration of —SH groups in the α chains (1 —SH per heme), but only partial restoration in the β chains, with the number of free —SH varying between 1.0 and 1.6 per heme instead of the theoretical value of 2. The number of free —SH groups was determined by the method of Boyer (6) after the last traces of thioglycolate were removed by passing the chains through a Dintzis column (7).[2] The removal of the last traces of thioglycolate is, of course, critical in these analytical measurements. It should be pointed out, however, that the presence of thioglycolate has no effect on the equilibrium and kinetic constants of the chains with free —SH, as shown by the fact that dialyzed and undialyzed solutions behaved identically within experimental error. The α^{PMB},[3] β^{PMB}, and $\beta^{-\mathrm{SH}}$ chains were stored in the cold in dilute phosphate buffer (0.01 M) at pH 7.4; the $\alpha^{-\mathrm{SH}}$ chains were found to be more stable in dilute NaCl (0.02 M) at about the same pH.

The concentration is expressed in heme equivalents and was determined spectrophotometrically in the visible region with the use of extinction coefficients determined by the pyridine-hemochromogen method (8). These were found to be the same as those reported for hemoglobin A (3).

Gases—Oxygen, carbon monoxide, and argon were obtained commercially; nitric oxide was prepared according to the method of Farkas and Melvelle (9), following the description of Gibson and Roughton (10), and was stored in a tonometer. Gas mixtures were made volumetrically by mixing known volumes of different gases at atmospheric pressure. Solutions of the different gases were obtained by equilibration of a small volume of deoxygenated water with a large gas phase at 20°; the concentrations were calculated from published solubility coefficients.

Experimental Conditions—All of the experiments were performed in 0.1 M phosphate buffer at pH 7.0 and at 20°. The protein concentration varied in the different cases from 10^{-4} to 10^{-6} M.[4]

Determination of Constants

Equilibrium Constants for O_2 and CO—The data on the O_2 equilibrium were taken from a previous study (1); they were obtained at a protein concentration of 1 to 2×10^{-4} M in 0.1 M phosphate buffer, pH 6.5 and 7.5, at 20°. In view of the fact that the data indicate the absence of any significant Bohr effect in any of the chains (see Table I of Reference 1), a mean of the published values was used.

The value of M, the partition constant between O_2 and CO, was determined spectrophotometrically at a protein concentra-

[2] This column was prepared by layering approximately 5 cm of Rexyn RG 501 mixed bed resin (Fisher) in deionized water over 1 to 2 cm of AG 1-X4 resin (Bio-Rad), in the hydroxyl form, also in deionized water. (This latter resin is to assure chloride ion removal.)

[3] The abbreviations used are: $\alpha^{-\mathrm{PMB}}$ and $\beta^{-\mathrm{PMB}}$, chains with the sulfydryl groups blocked by p-hydroxymercuribenzoate; $\alpha^{-\mathrm{SH}}$ and $\beta^{-\mathrm{SH}}$, chains with freely titratable sulfydryl groups.

[4] In view of the high dilution at which many of the measurements were made, it should be pointed out that we have obtained no evidence to suggest that our results might be significantly affected by surface denaturation. Two of the criteria used to rule out this possibility are the apparent homogeneity of both the kinetic and equilibrium results and the spectroscopic stability of the material during the course of the measurements.

tion of 6×10^{-5} M. The hemoglobin solutions were equilibrated with a gas mixture containing O_2 and CO in known proportions (the O_2:CO ratio varied from 180 to 200 in the different experiments) with the use of an anaerobic spectrophotometric cuvette with 1-cm path length. Equilibration was obtained by allowing the gas mixture to flow over the surface of the solution while the tonometeric cuvette was shaken in a thermostated bath in the dark; any bubbling was carefully avoided. The minimum time necessary to reach equilibrium under these conditions was found to be about 15 min. The affinity constant for CO was calculated from the experimentally determined values of K, the equilibrium constant for O_2, and M.

Kinetic Constants for O_2 and CO—The velocity constant for the dissociation of O_2 was determined in the stopped flow apparatus at a protein concentration of 6×10^{-5} M. A solution of the oxygenated chains, in the presence of a small excess of O_2, was mixed with a solution of $Na_2S_2O_4$ ($\sim0.5\%$) in deoxygenated buffer, and the absorbance change was followed at $\lambda = 555$ mμ. In other experiments the dithionite solution was equilibrated with CO at 1 atm and the rate of replacement of O_2 by CO was measured. In the case of hemoglobin, these two methods are conceptually different, because the latter involves only the dissociation velocity constant of fully liganded hemoglobin (11). In the present study on the isolated chains, for which the value of n in the O_2 equilibrium is equal to 1, the two methods give, as expected, identical results. The second method, which leads to the immediate formation of the carbon monoxide derivative as the final product of the reaction, was also useful in eliminating the possibility of side reactions with the oxidation products of the dithionite (11).

The velocity constant for the combination of CO with deoxygenated chains (l') was determined under the conditions previously used by Antonini *et al.* (1). The data were obtained at a protein concentration of 2×10^{-6} M, in 0.1 M phosphate buffer, pH 7.0, at 20°.

The velocity constant for the dissociation of CO (l) was determined in a Beckman DK-1 recording spectrophotometer at two different protein concentrations, 1 and 6×10^{-5} M. In these experiments a solution of the carbon monoxide derivative of the chains equilibrated with argon was mixed with a solution containing nitric oxide, and the absorbance change was followed at 421 or at 572 mμ. If we assume that the partition coefficient for NO and CO for the chains is large and that under the experimental conditions the rate of combination with NO is much larger than the rate of dissociation of CO, as would be expected from the results on sheep hemoglobin (10), then the rate of the displacement reaction gives a direct measure of the "off" constant for CO. That this is so was confirmed by the fact that changing the ratio NO:CO by a factor of 100 did not affect the results.

Displacement of O_2 by CO—The rate of displacement of O_2 by CO was determined with an apparatus originally designed for a study of quantum yield measurements.[5] The protein solutions, at a concentration of 1×10^{-6} M, were equilibrated, at 20°, with a gas mixture containing O_2 and CO in known proportion (p_{CO}: $p_{\mathrm{O}_2} = 0.01$ to 0.150), the total pressure being 1 atm. The solutions were then transferred, by means of gas-tight syringes, into a water-jacketed spectrophotometer cell having a 5-cm path length. After temperature equilibration the solution was illuminated with the 546 mμ band of a mercury lamp at an in-

[5] R. W. Noble, M. Brunori, E. Antonini, and J. Wyman, manuscript in preparation.

TABLE I

Equilibrium and kinetic constants for reaction between O_2 or CO and isolated α and β chains of human hemoglobin at pH 7.0 and 20°

Values for human hemoglobin chains are given with ±95% confidence limits.

Chain	1. $p(O_2)_{\frac{1}{2}}$, experimental	2. $K \cdot 10^{-5}$, experimental	3. $k' \cdot 10^{-7}$, calculated from Columns 2 and 4	4. k, experimental	5. Partition constant, M (Equation 1), experimental	6. $p(CO)_{\frac{1}{2}} \cdot 10^3$, calculated from Columns 1 and 5	7. $L \cdot 10^{-7}$, calculated from Column 6	8. $l' \cdot 10^{-6}$, experimental	9. $l \cdot 10^3$, experimental
	mm Hg	*M^{-1}*	*$M^{-1} sec^{-1}$*	*sec^{-1}*		*mm Hg*	*M^{-1}*	*$M^{-1} sec^{-1}$*	*sec^{-1}*
α^{-SH}	0.46 ± 0.16	12 ± 4.2	3.36	28 ± 6	181 ± 29	2.5 ± 0.56	30 ± 6.7	45.8 ± 3.7	13 ± 1.7
α^{-PMB}	0.45 ± 0.17	12.5 ± 4.7	3.87	31 ± 10	210 ± 30	2.1 ± 0.45	36 ± 7.8	39.0 ± 8.7	16 ± 2.3
β^{-SH}	0.40 ± 0.15	14 ± 5.2	2.24	16 ± 5	248 ± 64	1.6 ± 0.46	48 ± 13.9	45.5 ± 5.9	8 ± 0.93
β^{-PMB}	2.65 ± 0.66	2.1 ± 0.52	3.27	156 ± 32	165 ± 35	16 ± 3.6	4.8 ± 1.1	24.2 ± 4.6	27 ± 2.2
Horse myoglobin	0.70	8.0	1.4[a]	11	37	19	4.0	5	17
Aplysia myoglobin	2.65	2.1	1.5[a]	70	106	25	3.0	5	20

[a] Values obtained directly or by replacement procedure (3, 14).

tensity sufficient to photodissociate most (>90%) of the CO compound. On removal of the light the rate of replacement of O_2 by CO was followed in the Beckman DK-1 recording spectrophotometer.

Statistical Analysis of Data

The data were analyzed statistically (12), and the 95% confidence limits listed in the tables were obtained from the standard errors with the use of the Student's t distribution. The significance of the difference between two means is expressed by the probability (p) that the two means are of the same population; this also was evaluated from the Student's t distribution.
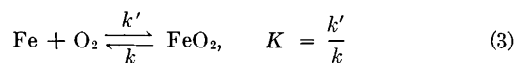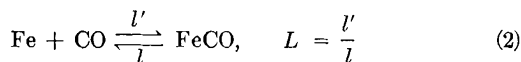
The slopes of the linear functions and the standard error of the slope were computed by the linear regression method.

Analysis of Displacement Reaction

The partition of any ferrous heme protein (Fe) between oxygen and carbon monoxide under equilibrium conditions can be described (13) by a constant, M, defined by

$$M = \frac{FeCO}{FeO_2} \cdot \frac{p_{O_2}}{p_{CO}} \tag{1}$$

where p_{O_2} and p_{CO} are partial pressures of O_2 and CO, respectively. Equation 1 assumes the existence of two simple competing equilibria, *i.e.*

$$Fe + CO \underset{l}{\overset{l'}{\rightleftharpoons}} FeCO, \qquad L = \frac{l'}{l} \tag{2}$$

$$Fe + O_2 \underset{k}{\overset{k'}{\rightleftharpoons}} FeO_2, \qquad K = \frac{k'}{k} \tag{3}$$

where L and K are the equilibrium constants for CO and O_2, respectively; l' and k' are the combination (or "on") constants; and l and k are the dissociation (or "off") constants for CO and O_2, respectively. The rates of these reactions are, respectively,

$$\frac{d(FeCO)}{dt} = l'(Fe) \cdot p_{CO} - l(FeCO) \tag{4}$$

and

$$\frac{d(FeO_2)}{dt} = k'(Fe) \cdot p_{O_2} - k(FeO_2) \tag{5}$$

If the concentration of O_2 and CO is high enough so that the concentration of free binding sites at any moment is negligible in respect to that of the liganded hemes, then

$$k(FeO_2) + l(FeCO) - k'(Fe) \cdot p_{O_2} - l'(Fe) \cdot p_{CO} = 0 \tag{6}$$

Solving Equation 6 for Fe and substituting into Equation 5 gives

$$\frac{d(FeO_2)}{dt} = l \left(\frac{k' \cdot p_{O_2}}{k' \cdot p_{O_2} + l' \cdot p_{CO}} \right) (FeCO)$$
$$- k \left(\frac{l' \cdot p_{CO}}{k' \cdot p_{O_2} + l' \cdot p_{CO}} \right) (FeO_2) \tag{7}$$

If the concentration of O_2 and CO is high enough to be considered unchanged during the course of the reaction, the expressions in parentheses in Equation 7 can be considered as constants. Then, provided $l' \cdot p_{CO} \ll k' \cdot p_{O_2}$, Equation 7 yields

$$\frac{d(FeO_2)}{dt} = l(FeCO) - l' \frac{k \cdot p_{CO}}{k' \cdot p_{O_2}} (FeO_2) \tag{8}$$

This simplification can be applied in view of the fact that $k' \approx 10\, l'$ and $p_{O_2} \approx 10\, p_{CO}$, so that $k' \cdot p_{O_2} \approx 100\, l' \cdot p_{CO}$. Setting

$$\frac{(FeO_2)}{\sum Fe} = z$$

and

$$\frac{(FeCO)}{\sum Fe} = 1 - z$$

and dividing Equation 8 by $\sum$ Fe, we have

$$\frac{dz}{dt} = l(1 - z) - l' \frac{k \cdot p_{CO}}{k' \cdot p_{O_2}} z \tag{9}$$

Solving this differential equation and introducing z_0 as the value of z at $t = 0$, and z_∞ as the value of z as $t \to \infty$, we can write the result in the form

$$l + l' \frac{k \cdot p_{CO}}{k' \cdot p_{O_2}} = \frac{1}{t} \ln \frac{z_0 - z_\infty}{z_t - z_\infty} \equiv R \tag{10}$$

It will be seen that R represents the first order rate constant for the approach to the dark equilibrium.

RESULTS

The values of the equilibrium and kinetic constants for the reaction of O_2 and CO with the isolated α and β chains of human hemoglobin, both in the PMB and —SH forms, are reported in Table I. Results for myoglobins from horse and *Aplysia lima-cina* obtained previously under the same conditions are also given for comparison (14).[1]

Reaction with Oxygen—Since, as shown by Antonini *et al.* (1), the isolated chains have a hyperbolic oxygen equilibrium curve, the values for k', the combination velocity constant for O_2, were calculated from the values of K and k. (No direct experimental results were practical, owing to the very high value of the constant.)

The O_2 dissociation rate follows first order kinetics for all the compounds; the results obtained in a typical experiment are reported in Fig. 1 for both the PMB and the —SH chains. The reaction was followed at $\lambda = 555$ and 578 mμ; the results agreed at the two wave lengths within experimental error. The values reported in Table I are the means of eight determinations made on three different preparations. The "off" constants for α^{-SH}, α^{PMB}, and β^{-SH} chains are similar, and not very different from those of horse myoglobin; that of the β^{PMB} chain is very different, being about 7 times greater than all the others.

Reaction with Carbon Monoxide—Because of the very high affinity of CO for the chains, a direct measurement of the equilibrium constant, L, for CO was not possible. This was therefore obtained indirectly from K and M. The values of M reported in Table I are the means of five experiments performed under the same conditions on three different preparations of the chains.

The kinetics of the reaction between CO and the deoxy derivative of the chains was that of a second order process, as regards both the time course and the dependence of rate on concentration; the second order velocity constants (l') reported in Table I for the reaction with CO are the means of the data reported by Antonini *et al.* (1) and those obtained in this work. The newer data agreed with the old ones, within experimental error.
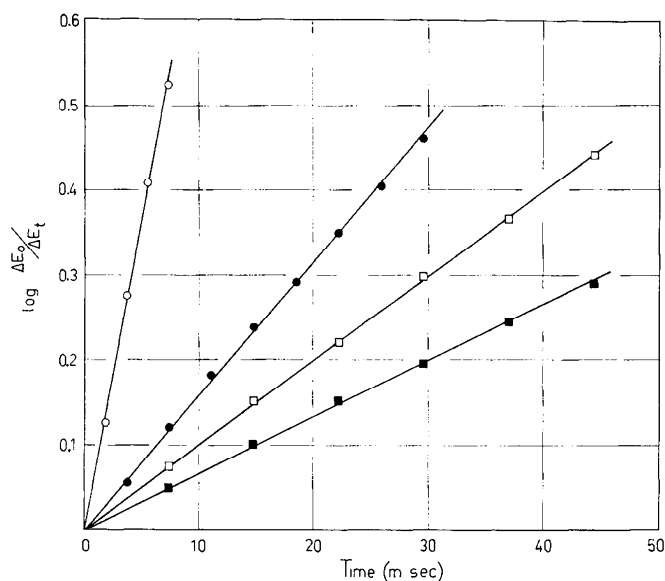

FIG. 1. Time course of O_2 dissociation from the isolated α and β chains of human hemoglobin in 0.1 M phosphate buffer at pH 7.0 and 20°. Time zero corresponds to 3 msec after mixing. O, β^{PMB}; ●, α^{PMB}; □, α^{-SH}; ■, β^{-SH}.
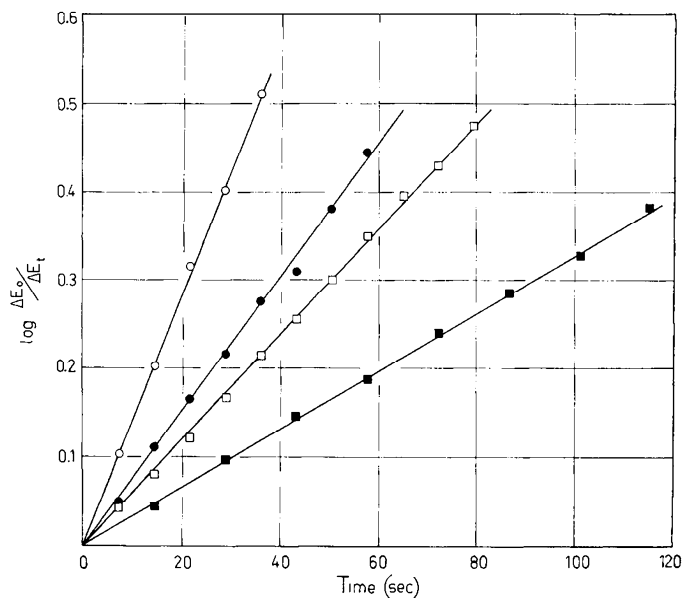

FIG. 2. Time course of CO dissociation from the isolated α and β chains of human hemoglobin in 0.1 M phosphate buffer at pH 7.0 and 20°. O, β^{PMB}; ●, α^{PMB}; □, α^{-SH}; ■ β^{-SH}.
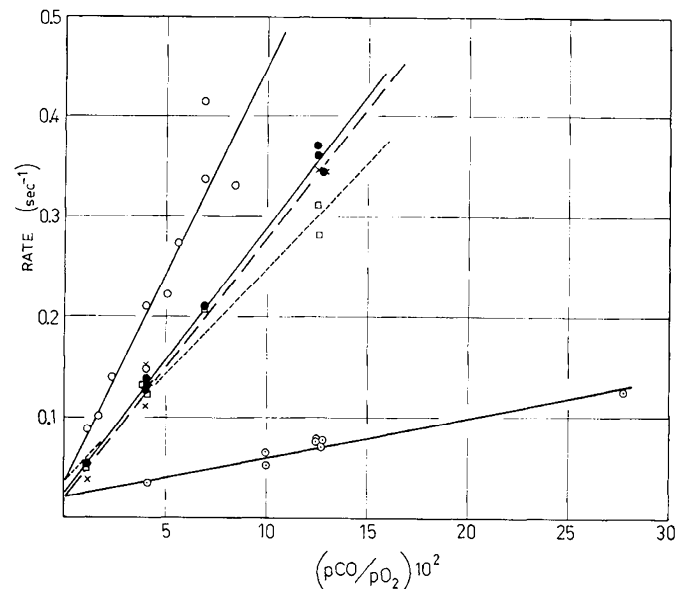

FIG. 3. Rate of replacement of O_2 by CO as a function of the $CO:O_2$ ratio for the isolated α and β chains of human hemoglobin and horse myoglobin in 0.1 M phosphate buffer, at pH 7.0, 20°. O——O, β^{PMB}; ●——●, α^{PMB}; ×– – –×, β^{-SH}; □-----□, α^{-SH}; ⊙——⊙, horse myoglobin.

Each of the values for the dissociation velocity constant for CO (l) reported in Table I is the mean of 10 experiments performed on three different chain preparations. The reaction is a first order process, as can be seen from the experiment reported in Fig. 2.

Displacement of O_2 by CO—The displacement of O_2 by CO was found to be an exponential process, as would be expected from Equation 10. The results obtained for the chains at different $CO:O_2$ ratios are plotted in Fig. 3; the figure also includes the data obtained under the same conditions for horse myoglobin. The straight lines of the plots of the relaxation rate, R, with

TABLE II

Comparison of equilibrium constant with ratio of two kinetic constants in reaction of isolated α and β chains of human hemoglobin with carbon monoxide

Data for horse myoglobin are included for comparison. The values ±95% confidence limits are given. p, as defined under "Materials and Methods," indicates the fractional probability that the two means are of the same population.

Chain	$L \cdot 10^{-7}$	$l'/l \cdot 10^{-7}$	p
	M^{-1}	M^{-1}	
α^{-SH}	30 ± 6.7	35 ± 4.3	0.15
α^{PMB}	36 ± 7.8	24 ± 5.1	0.05
β^{-SH}	48 ± 13.9	57 ± 8.3	0.20
β^{PMB}	4.8 ± 1.1	9.0 ± 1.3	<0.01
Horse myoglobin	4.0	3.0	

TABLE III

Comparison of slope in replacement reaction with experimental value of l'/K for α and β chains of human hemoglobin

Data for horse myoglobin are included for comparison. The values ±95% confidence limits are given. p, as defined under "Materials and Methods," indicates the fractional probability that the two means are of the same population.

Chain	Slope, $l'k/k'$	l'/K	p
α^{-SH}	2.10 ± 0.50	3.8 ± 0.63	<0.01
α^{PMB}	2.65 ± 0.21	3.10 ± 0.81	0.20
β^{-SH}	2.58 ± 0.39	3.25 ± 0.68	0.10
β^{PMB}	4.15 ± 1.37	11.5 ± 2.5	<0.01
Horse myoglobin	0.387 ± 0.087	0.625	

respect to $p_{CO}:p_{O_2}$ were calculated by linear regression, as described under "Materials and Methods."

According to Equation 10 the value of l can be obtained from the intercept of such a plot on the ordinate axis. Although such values are subject to a very large experimental error, they were in agreement with the values reported in Table I, which were obtained with the use of NO as a displacing ligand.

DISCUSSION

It had been shown that the isolated α and β chains of human hemoglobin, in both the PMB and —SH forms, are alike in exhibiting no heme-heme interactions and no Bohr effect in the binding of oxygen (1). This previous work had already suggested that the main kinetic change associated with the loss of heme-heme interaction and with the increase in ligand affinity involves the combination velocity constant. The results of the present experiments give additional support to this view; all the isolated chains, either in the PMB or —SH form, show a dramatic increase of the "on" constant, for both oxygen and carbon monoxide, in comparison with the over-all combination constants of hemoglobin.[5]

It might be asked how similar the behavior of the chains is to that of myoglobin, since x-ray work (15, 16) shows that the conformation of the polypeptide chain and the location of the heme group are very similar for myoglobin and the hemoglobin α and β chains. Comparison of the results reported in Table I shows that, notwithstanding this, the isolated chains are very different from horse myoglobin in the experimental constants M and l'.

The present results show that there is a great similarity in the behavior of the α^{PMB}, α^{-SH}, and β^{-SH} chains, although there are statistically significant differences among them. The β^{PMB} chains, on the other hand, have a very different behavior, and the results reported here show that their low O_2 affinity arises primarily from the high value of the "off" constant for oxygen. A similar situation was found to apply to the behavior of *Aplysia* myoglobin when compared with horse myoglobin (14); in this case too the nearly 5-fold difference in affinity for O_2 can be explained on the basis of a difference in the value of the "off" constant for O_2. The β^{PMB} chains also differ from the others in their affinity for CO. In this case, however, the change in affinity cannot be accounted for solely on the basis of the difference in the value of the dissociation velocity constant.

The question arises of whether β^{-SH} is really the same as native hemoglobin H, as has generally been assumed. Unfortunately, available data on Hb H, which include $p(O_2)_{\frac{1}{2}}$, k', and l', are not sufficiently exact or extensive to provide an answer. Thus, values of $p(O_2)_{\frac{1}{2}}$ given by different authors vary widely (from 0.03 mm of Hg at 10° as given by Ranney, Briehl, and Jacobs (2) to 0.26 mm of Hg at 20° as obtained by Benesch *et al.* (17). The value, $k' = 3 \times 10^7$ M^{-1} sec^{-1}, was measured at 3° (18), and, in the absence of a knowledge of the activation energy, can hardly be compared with our values at 20°, which, however, are about the same. In these two respects, therefore, clear similarities or differences cannot be established. On the other hand, reported values of l' (1.5 to 2.0 $\times 10^7$ M^{-1} sec^{-1}) (18) are between 3 and 5 times larger than our values. It may be noted that a few measurements of ours made on aged β^{-SH} gave values of l' of over twice those reported in the table, namely, $\sim 1 \times 10^7$ M^{-1} sec^{-1}, which approach those of Hb H.

Drastic changes in the O_2 equilibrium properties have practically no effect on the value of M, as is shown in the case of the isolated chains of human hemoglobin or in that of hemoglobin digested by carboxypeptidase A.[6] In both instances, the value of M remains very near to that of normal hemoglobin (~ 240) and different from that of horse myoglobin ($M = 37$). On the other hand, the β^{PMB} chains have a partition constant, M, which is significantly different from that of β^{-SH} and from hemoglobin A. It may be recalled in this connection that the β_{93} residue, to which one of the PMB molecules is attached, is very near to the heme group (15). This might suggest that chemical alterations in this region around the heme group can influence to a different extent the reactivity of the protein for the two ligands; if so, the identity of the ligand attached to the heme would be expected to affect specifically the reactivity of adjacent groups, as has indeed been established (19). Moreover, there are indications that the differences in the behavior of the β^{PMB} and β^{-SH} chains are determined by the presence (or absence) of the PMB molecule on a particular one of the two cysteinyl residues in the β chains of human hemoglobin (β_{93} and β_{112}). Thus, different β^{-SH} preparations were found to contain a variable number of titratable —SH groups (from 0.7 to 1.6 —SH per heme). All preparations containing one or more freely titratable —SH groups were found to be identical in their reactions with oxygen or carbon monoxide, and no kinetic heterogeneity was detectable. On the other hand, the preparations containing less than one titratable —SH group clearly showed two kinetic components whose velocity constants were similar to those found for β^{PMB} and β^{-SH}. These effects were particularly striking in the case of

[6] E. Antonini, J. Wyman, and R. Zito, unpublished data.

the O_2 dissociation, for which the velocity constants differ by a factor of 10. The kinetic homogeneity of preparations containing only one titratable —SH group per heme is a very strong argument that such preparations contain only chains with one —SH group free and one —SH group covered. We conclude, therefore, that whichever of the 2 —SH groups is the more difficult to regenerate, it has no effect on the kinetic behavior of the β^{-SH} chains.

The second problem dealt with in this paper was the applicability of the simple model (A) to the reaction of the isolated α and β chains of human hemoglobin with O_2 and CO.

The kinetics of the reactions with both ligands seems to follow the expected behavior, the combination and dissociation reactions being, respectively, second and first order for both O_2 and CO. The evaluation of the ratio of the two directly determined velocity constants is only possible in the case of the carbon monoxide reaction. Table II gives a comparison of the ratio l'/l with the equilibrium constant for CO as calculated from K and M. As can be seen, there are some discrepancies between the two sets of data, but these became really significant only in the case of the β^{PMB} chains, for which L is about 0.5 l'/l. For the other chains, as well as for myoglobin, the differences are less striking.

The comparison of the results obtained directly with those calculated from the replacement reaction are reported in Table III. Here again, all of the compounds show some differences, which become particularly large in the case of the ^{PMB} chains.

In conclusion, there seems to be evidence that the simple scheme (A) does not apply in every case to the binding of gaseous ligands by simple heme proteins. It should be recalled in this connection that the α chains are always monomeric, the β^{PMB} chains are dimeric or monomeric, depending on the concentration, and the β^{-SH} chains, like hemoglobin H, are tetrameric at the concentrations used in this paper (5). Similar deviations of these reactions from the predicted behavior have already been shown by Antonini, Gibson, and Wyman[1] for the case of horse myoglobin, which is always a monomer. The meaning of such observations is not fully understood, but whenever such discrepancies are established, we are forced to conclude that some additional steps must be added to the original scheme, which is probably oversimplified. It might be necessary to postulate the existence of conformational intermediates in the reaction path.

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