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Kinetics of Carbon Monoxide Binding to the Cooperative Dimeric Hemoglobin from *Thyonella gemmata*. Analysis of Carbon Monoxide Equilibrium Results†

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ABSTRACT: The CO association reaction for the dimeric hemoglobin of *Thyonella gemmata* is biphasic with rate constants of 2×10^3 and 1×10^4 M^{-1} s^{-1}, among the slowest known for a hemoglobin. The kinetic heterogeneity almost certainly derives from differences in the α- and β-chain heme environments since 90% of the hemoglobin migrated as a single electrophoretic component and the more rapid kinetic phase contributed about 48% to the absorbance change. The activation enthalpies for the CO association reaction are 7.5 kcal/mol (rapid heme) and 9.0 kcal/mol (slow heme). The value for horse hemoglobin is 8.3–8.6 kcal/mol corresponding to a CO association constant of 1.7×10^5 M^{-1} s^{-1}. These findings suggest that the CO binding site in *Thyonella* hemoglobin is even more restricted than that in horse hemoglobin. Stopped-flow and flash-photolysis results were identical. Although the hemoglobin is cooperative in CO binding, there is no evidence for a quickly reacting (Hb*) form of the protein. Tandem flash-photolysis experiments show that the heme that is rapidly reacting toward CO is also the more rapid in oxygen association and dissociation. Stopped-flow studies on partially CO-saturated hemoglobin show that intermediate liganded forms are present in significant amounts and allow all equilibrium constants to be determined in a heterogeneous Adair model. The Adair model can account satisfactorily for all existing CO equilibrium and kinetic results and predicts that the dissociation of CO from half-liganded forms should be about 10 times faster than that from Hb-$(CO)_2$. A heterogeneous allosteric model using observed rate constants assumed to pertain to the R form gave $L = 150$ and $c = 0.0207$ and predicted a 48-fold enhancement in the CO off rate (T form vs. R form). If there exists a quickly reacting CO form (Hb*) with an association constant only 10 times that which is observed, but which reverts to the T form more rapidly than CO binds to Hb* (thus accounting for its nonappearance), $L = 15479$, $c = 0.00242$, the enhancement in the CO dissociation rate is 41, and the equilibrium ratio of R to T for half-liganded hemoglobin is 0.02. A larger value for the hypothetical Hb* association constant leads to an even larger value for the allosteric constant L.

The study of the kinetics of CO binding to a cooperative hemoglobin usually provides the clearest kinetic evidence for cooperativity. For tetrameric hemoglobins, evidence for a quickly reacting form (Hb* or R form) can be obtained by flash photolysis (Gibson, 1959a). By the same method, the rate of the Hb* to Hb transition can be measured (Gibson, 1959b; Sawicki & Gibson, 1977) and tetramer–dimer equilibrium constants can be obtained (Edelstein et al., 1970; Steinmeier & Parkhurst, 1975). For cooperative dimeric heme proteins, there may be no detectable Hb* form (Yang, 1974; Geraci et al., 1977). In the preceding paper (Steinmeier & Parkhurst, 1979), a study of the deoxygenation of HbO_2 in the presence of saturated CO (observed at the HbO_2–HbCO isosbestic wavelength) showed that the oxy-R form of *Thyonella* hemoglobin was not a quickly reacting form for the ligand CO or that the R → T process depopulated the R form of half-oxygenated hemoglobin before it could bind rapidly with CO. The present paper reports CO kinetic studies by stopped-flow and flash-photolysis experiments, which, in addition to the more conventional experiments, entailed the study of CO binding to intermediates generated by flash photolysis (tandem flash experiments) and to intermediates prepared by equilibration with CO. Carbon monoxide dissociation was studied by oxygen replacement.

Experimental Procedures

Procedures not dealt with in the preceding paper will be described below.

Carbon monoxide binding by stopped-flow experiments employed 10 μM hemoglobin, heme basis, before mixing, and CO concentrations which ranged, before mixing, from 90 to 940 μM. The reaction was studied in 50 mM potassium

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Table I: CO Association Kinetic Data for *Thyonella gemmata* Hemoglobin[a]

method	% rapid	l' (rapid) (M^{-1} s^{-1})	E_a (kcal/mol)	l' (slow) (M^{-1} s^{-1})	E_a (kcal/mol)
stopped flow	44.1	0.95×10^4		2.0×10^3	
flash photolysis	47.9	1.0×10^4	7.5 ± 0.4	2.1×10^3	9.0 ± 0.2

[a] Measurements were at pH 7, 50 mM potassium phosphate buffer, at 20 °C. Flash-photolysis studies employed 10 μM hemoglobin (heme basis) and 910 μM CO; for stopped-flow studies, the concentrations were hemoglobin, 10 μM, and CO, 940 μM, both before mixing.

phosphate buffer, pH 7, and in 50 mM borate, pH 9. Observing wavelengths were 430 and 440 nm. The temperature was 20 °C.

Carbon monoxide binding by flash photolysis was followed at 440, 430, and 420 nm in 50 mM potassium phosphate buffer, pH 7. The protein concentration varied from 1 μM in heme to 100 μM, and the CO concentration ranged from 90 to 910 μM. The temperature varied from 4 to 23 °C; the percent photolysis varied from 2.5% to better than 99%.

CO binding to partially saturated intermediates was studied in the stopped-flow apparatus. *Thyonella* hemoglobin already partially saturated with CO was mixed in the stopped-flow apparatus with buffer containing additional carbon monoxide. Solutions of hemoglobin partially saturated with CO were made up as described for direct equilibrium determinations (Steinmeier & Parkhurst, 1979). Experiments were carried out in 50 mM potassium phosphate buffer, pH 7, at 20 °C; observing wavelength was 440 nm. The hemoglobin was 5.7 μM before mixing, and the CO solution in the ligand syringe was 960 μM.

Tandem flash-photolysis studies of CO binding. When flash photolysis is carried out on HbCO in the presence of oxygen, following the initial rapid binding of oxygen, there occurs a slow relaxation step in which oxygen is replaced by CO. At varying times after the initial photolysis pulse, the solution contains varying amounts of hemoglobins with two bound oxygens, with one oxygen and one carbon monoxide bound, and with two carbon monoxide molecules bound. Firing a second flash during the relaxation phase allows one to study O_2 binding to species generated by removal of CO from the relaxation intermediates. The time interval between the initial photolysis flash and the second flash was measured with a Heath universal digital instrument (D. C. Heath Co., Benton Harbor, MI 49022) operated in the Time A–B mode, with five decimal readout. These experiments were carried out to aid in the assignments of rapid and slow rate constants for CO binding and oxygen association and dissociation to the two different heme sites.

Carbon monoxide dissociation was studied by observing the relaxation reaction as oxygen replaced CO as the bound ligand. This was feasible since the affinities for the two ligands are comparable (Steinmeier & Parkhurst, 1979). HbCO for these experiments was prepared by passing water-saturated CO gas over an aliquot of oxyhemoglobin (650 μM). The sample was contained in a tube capped with a serum stopper pierced by two syringe needles. The stream of CO gas entered through one needle and exited through the other. Prior to use, the carboxyhemoglobin was diluted with argon-saturated buffer to about 145 μM (heme basis). To initiate the replacement reaction, 0.1 mL of the HbCO solution was added to a 1-cm path length cuvette containing 3.5 mL of oxygen-saturated buffer. After mixture, the solution was covered with a layer of mineral oil, and changes in absorbance at 420 nm were monitored in a Cary 14 recording spectrophotometer. The final hemoglobin concentration was thus about 4 μM. The $(O_2)/(CO)$ ratio was at least 50:1. The reaction was followed for two half-lives and then driven to completion by photolysis of the remaining HbCO by using a photographic flash positioned inside the Cary sample compartment. The absorbance at the end point was then measured, and the absorbance changes vs. time were plotted to determine the relaxation rate constant and then the CO dissociation constant.

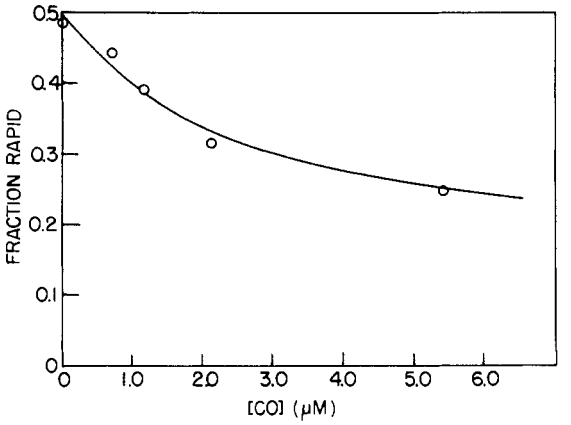


FIGURE 1: A plot of the fraction rapid in CO combination stopped-flow experiments on partially CO-saturated hemoglobin. The abscissa labels the free CO concentration in the various hemoglobin solutions immediately prior to reaction with concentrated CO. The curved line is a two-parameter fit to the data (eq 3) which can be interpreted in terms of either Adair or allosteric models.

Results

Carbon monoxide association rate constants are listed in Table I for both flash-photolysis and stopped-flow measurements along with the activation energies. The numbers given are for pH 7 where the most extensive studies were carried out; however, the rate constants do not change significantly from pH 7 to pH 9. There is no concentration dependence of the rate constants from 1 μM in heme to 100 μM. The activation energy measurements are for eleven experiments, two preparations, one measured at six temperatures and the other measured at five temperatures, from 4 to 23 °C. The fractional photolysis of HbCO varied from 2.5% to better than 99%. Even at 4 °C and saturated CO, there was no evidence for a quickly reacting hemoglobin; the recombination kinetics were invariant with fractional photolytic breakdown.

Figure 1 shows the results of CO combination studies carried out in a stopped-flow device on 5.7 μM (heme, before mixing) partially CO-saturated hemoglobin (fraction rapid, or "F", study). The CO concentration on the x axis is the concentration of free CO in the hemoglobin solution before mixing with the 960 μM CO solution contained in the other syringe. All points refer to the fraction of rapid component at time zero with respect to the stopped-flow experiment. Thus, the y intercept corresponds to an experiment in which Hb (no CO present) is flowed against CO. As the hemoglobin is increasingly liganded prior to stopped-flow reaction, the fraction of the rapid component decreases, showing that the rapidly reacting sites also have the higher affinities for CO. In all cases, however, the two rate constants measured agreed exactly

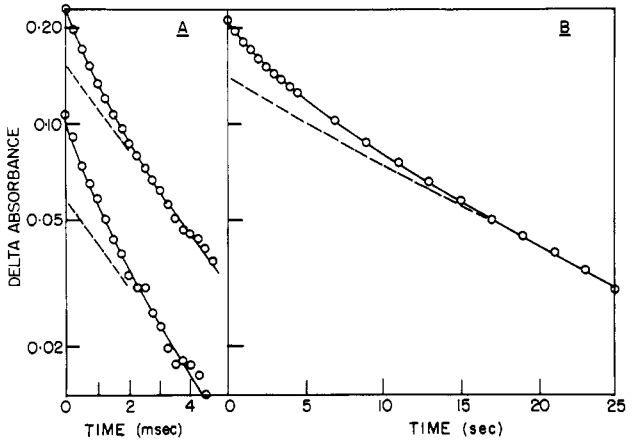


FIGURE 2: CO–O_2 replacement and tandem flash experiments. Panel A shows oxygen binding to *Thyonella* hemoglobin following photolysis of HbCO in the presence of oxygen. The upper trace is for combination following the first flash; the lower trace is oxygen binding following a second flash fired 5.31 s after the first flash. The observing wavelength was 432 nm. Panel B shows the slow relaxation phase where CO replaces oxygen as the bound ligand until the initial equilibrium conditions are reestablished. For these experiments, the protein ws 7.5 μM in heme, CO concentration = 618 μM, (O_2) = 91 μM, buffer was 50 mM potassium phosphate, pH 7, temperature = 20 °C, and, for the relaxation process, the observing wavelength was 421 nm. The dashed lines are computed extrapolations of the slow phases of the reactions to zero time.

with those reported in Table I when the data were fit according to a two-exponential decay model. There was no evidence for a rapid (Hb*) form reacting with a rate constant *faster* than 1×10^4 M^{-1} s^{-1}. The solid curve is a theoretical curve which will be treated in the section on CO equilibrium binding. The last data point corresponds to 86% saturation with CO before mixture with CO in the stopped-flow apparatus.

The CO dissociation rate constant was measured by mixing oxygen-saturated buffer with a solution of HbCO and observing the relaxation rate constant. The relaxation rate constant (R) for the replacement mechanism has been given elsewhere (LaGow & Parkhurst, 1972). The half-time for the relaxation was 12 min. The actual value of the dissociation constant,[1] l_R (dissociation from the R form) calculated, depends on which values are chosen for the oxygen association rate constant. If we use a minimum value obtained from the previous flash-photolysis analysis for oxyhemoglobin, 1.5×10^6 M^{-1} s^{-1}, then l_R is 0.25×10^{-3} s^{-1}. A larger value will cause l_R to increase toward 0.96×10^{-3} s^{-1}. There is also a small uncertainty in the concentration of CO free in solution; however, these two values span the possible range of values for l_R. A similar analysis for the slowly reacting heme site gives l_R between 0.86×10^{-3} and 0.96×10^{-3} s^{-1}. The fact that the relaxation is monophasic for over two half-lives suggests that the two dissociation rate constants are very similar and do not differ by more than a factor of 2; a value of $(0.9 \pm 0.1) \times 10^{-3}$ s^{-1} would appear to be a reasonable value for these rate constants.

[1] In this paper, we follow the convention that the rate constant l pertains to the binding of CO, a prime denotes an association constant, and the absence of a prime denotes a dissociation rate constant. The calculated dissociation constant for CO from oxygen replacement studies is denoted l, and the *observed* association CO rate constant is denoted l'. It is assumed that l is that for the liganded or R form and thus equal to l_R. A flash intensity dependent form, Hb*, was not observed for this hemoglobin. In terms of an allosteric model, we assume $l = l_R$ and denote the R or T form of the protein by subscripts R and T. R′ and T′ pertain to the lower affinity subunit, R and T pertain to the higher affinity subunit, and thus $l_R' > l_R'$. Equilibrium constants are denoted by capital K's for the allosteric model so as not to be confused with the conventional allosteric parameter L. $\bar{X}$ is used to denote the ligand activity at half-saturation.

Table II: Possible Combinations of Kinetic Constants in the CO–O_2 Replacement Reaction

ratio	possible values			
	case I	case II	case III	case IV
R_1/R_2	5	5	5	5
l_1'/l_2'	5	0.2	5	0.2
k_1'/k_2'	5	5	0.2	0.2
k_1/k_2 (calcd)	5	125	0.2	5

Flash photolysis performed on solutions of carboxyhemoglobin in the presence of oxygen usually results in a biphasic reaction. The first very rapid phase corresponds to the binding of oxygen; the second slower phase corresponds to a relaxation process in which HbO_2 is converted to the original equilibrium mixture of HbO_2 and HbCO. In Figure 2, panel A shows the rapid phase of the process; panel B shows the relaxation phase for *Thyonella* hemoglobin. For this hemoglobin, the relaxation phase is itself markedly biphasic, the rapid phase having an apparent relaxation rate constant of 0.35 s^{-1} and the slower phase having a relaxation rate constant of 0.061 s^{-1}. The dashed lines in Figure 2 are extrapolations of the slow phases to zero time and show the initial distribution between rapid and slow components. Because of subunit heterogeneity in this hemoglobin, an equation of the form

$$Y = A_1 \exp(-R_1 t) + A_2 \exp(-R_2 t) \qquad (1)$$

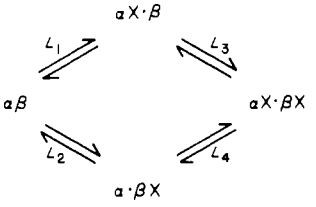
is needed to describe the relaxation process. Here, A_1 and A_2 specify the relative contributions of each component to the absorbance change at $t = 0$ vs. $t = \infty$, and $R_1 = k_1 l_1'(CO)/k_1'(O_2)$ and $R_2 = k_2 l_2'(CO)/k_2'(O_2)$ under the conditions of the experiment (see the legend to Figure 2). Let us arbitrarily designate R_1 as the rapid relaxation rate constant. This "rate constant" is itself made up of three rate constants which are labeled with subscripts to denote a particular subunit in the hemoglobin. Since measurements of each of these rate constants show that biphasic kinetics are always observed, it is not obvious how the various rate constants should be paired to give a given relaxation rate constant. That is, we have numbers for k_f', k_s', l_f', l_s', k_f, and k_s where now the subscripts pertain to the fast and slow components, and we wish to determine in each case which subscript should be "1" and which should be "2", i.e., which heme site is more rapid in CO binding, which in O_2 binding, and which in O_2 dissociation. We may write the ratio $k_1/k_2 = (R_1/R_2)(k_1'/k_2')(l_2'/l_1')$ and construct a table of possibilities (Table II). There are four possibilities. Case II can be ruled out since it predicts a ratio of 125 whereas the observed ratio is either 5 or 0.2. In order to distinguish it further, we carried out the tandem flash experiments. In these experiments, one photolytic flash was used to initiate the reaction, and then the replacement process (relaxation phase) was allowed to proceed for a few seconds. This allowed subunits characterized by R_1 to be converted to HbCO to a greater extent than those characterized by the slower R_2. A second flash was then fired to dissociate the HbCO present, and the reduced hemoglobin thereby generated reacted rapidly with O_2. This time, however, the population of sites which reacted with O_2 was more heavily weighted with type 1 subunits. If k_1' were greater than k_2', then the O_2 binding phase following the second flash should then show an increased proportion of the more rapid phase. In Figure 2, panel A, the top curve corresponds to oxygen binding following the initial flash. The lower curve is for oxygen binding following a second flash set off 5.31 s after the first flash. Both

Table III: Results of Tandem Flash-Photolysis Experiments--Oxygen Binding following the Second Flash

time of second flash (s) after first flash	obsd % rapid	predicted % rapid	
		case I	cases III and IV
3.65	53 ± 7	64.8	18
4.27	55 ± 7	63.9	18.9
5.31	55 ± 7	62.4	20.4
5.93	52 ± 6	61.5	21.3
6.21	52 ± 7	61.1	21.7
6.35	50 ± 5	60.9	21.9
8.60	51 ± 6	57.9	24.8

curves were observed at 432 nm. For the top curve, the fraction rapid was 0.414; for the lower curve, it was 0.548. Table III summarizes the results of the tandem flash experiments and gives the predicted percent rapid phase according to the various classes from Table II. The experimental percent rapid values are probably slightly low owing to the dead time in the flash experiments. It is clear that only class I can be correct. This shows that the *same* subunit (1) is the faster subunit in oxygen binding, oxygen dissociation, CO association, and therefore in the replacement relaxation process.

Adair Model Treatment of CO Binding. Models of the Adair (Adair, 1925) type have been used extensively to fit ligand binding equilibria for various tetrameric hemoglobins. Most treatments have made the simplifying assumption of subunit equivalence, since a heterogeneous (i.e., nonequivalent subunits) Adair model for a tetramer involves 10 hemoglobin species and 32 rate constants. For a dimer hemoglobin, a heterogeneous Adair model for the binding of ligand (X) would be



where $L_1 = (\alpha X{\cdot}\beta)/[(\alpha\beta)(X)]$, $L_2 = (\alpha{\cdot}\beta X)/[(\alpha\beta)(X)]$, $L_1/L_2 = L_4/L_3$, and ligand is represented by X. Assignment of equilibrium constants to actual α,β subunits is arbitrary.[2] The expression for the average number of sites occupied is

$$\bar{\nu} = \frac{(L_1 + L_2)(CO) + 2[(CO)/(\overline{CO})]^2}{1 + (L_1 + L_2)(CO) + [(CO)/(\overline{CO})]^2} \quad (2)$$

where $(\overline{CO})$ is the half-saturation ligand concentration and is 1.3×10^{-6} M. The binding curve does not permit a determination of L_1 and L_2 separately. The fraction rapid, however, in the partially saturated studies, is given by (see Figure 1)

$$F = \frac{1 + L_1(CO)}{2 + L_1(CO) + L_2(CO)} \quad (3)$$

where we *arbitrarily* assign the β heme as being the more rapidly reacting. The data of Figure 1 were fit in terms of the above equation for F by using a grid minimization technique (Becsey et al., 1968) which yielded $L_1 = 1.1 \times 10^5$ M^{-1} and $L_2 = 6.6 \times 10^5$ M^{-1}. When these values were used in the binding equation for $\bar{\nu}$, the Hill number calculated was

[2] The designation of the hemoglobin chains by α and β is arbitrary and is not meant to imply any relationship to mammalian α and β chains.

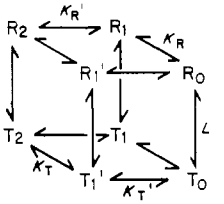
Table IV: Equilibrium and Kinetic Constants for CO Binding in Terms of a Heterogeneous Adair Model

$L_1 = 1.1 \times 10^5$ M^{-1}
$L_2 = 6.6 \times 10^5$ M^{-1}
$L_3 = 1/[L_1(\overline{CO})^2] = 5.4 \times 10^6$ M^{-1}
$L_4 = L_1(L_3/L_2) = 0.9 \times 10^6$ M^{-1}

$l_1' = l_4' = 2.1 \times 10^3$ M^{-1} s^{-1} [$=l'$(slow)]
$l_2' = l_3' = 1.0 \times 10^4$ M^{-1} s^{-1} [$=l'$(fast)]
$l_{-1} = l_1/L_1 = 1.9 \times 10^{-2}$ s^{-1}
$l_{-2} = l_2/L_2 = 1.5 \times 10^{-2}$ s^{-1}
$l_{-3} = l_3/L_3 = 1.8 \times 10^{-3}$ s^{-1}
$l_{-4} = l_4/L_4 = 2.3 \times 10^{-3}$ s^{-1}

1.35, within experimental error of 1.40 ± 0.08 (Steinmeier & Parkhurst, 1979). From $(\overline{CO})$, L_1, and L_2, values can be calculated for L_3 and L_4. Since this model assumes there is no Hb* form (that is, quickly reacting form toward CO), all rate constants can also be calculated from the equilibrium constants and available kinetic data. The Adair model results are tabulated below (Table IV). The values for l_{-3} and l_{-4} correspond to l_R and are about twice the observed values.

Allosteric Model Treatment of CO Binding. An allosteric model (Monod et al., 1965) for CO binding which incorporates subunit heterogeneity can be depicted by



where the subscripts for the R and T forms denote the number of ligands bound and primed forms have ligand bound to the lower affinity sites. The various parameters are

$$K_T = (T_0)(X)/(T_1)$$
$$K_T' = (T_0)(X)/(T_1')$$
$$\rho = K_T/K_T'$$
$$K_R = (R_0)(X)/(R_1)$$
$$K_R' = (R_0)(X)/(R_1')$$
$$\rho\sigma = K_R/K_R'$$
$$c = K_R/K_T$$
$$L = (T_0)/(R_0)$$

where ligand is represented by X. The binding expression in terms of average number of sites occupied ($\bar{\nu}$) is (for $\sigma = 1$)

$$\bar{\nu} = \frac{A(X) + 2B(X)^2}{1 + A(X) + B(X)^2} \quad (4)$$

where

$$A = \frac{1 + \rho}{K_R}\frac{c + \lambda}{1 + \lambda};\ B = \frac{\rho}{K_R^2}\frac{c^2 + \lambda}{1 + \lambda};\ \lambda = 1/L$$

In order to use this result for data fitting, we must assign a value to K_R and thus to l_R', that is, the rate constant for CO binding to the R form of the protein, rapid component. We employed two plausible values, the first, that which was directly measured (10^4 M^{-1} s^{-1}) both by flash and stopped-flow, and a second value 10 times greater, corresponding to a hypothetical rapidly reacting form which reverts to the T form too rapidly to be observed. The best fitting allosteric parameters for the two cases are given below in Table V. It should be

Table V: Equilibrium Constants and Allosteric Parameters for CO Binding in Terms of Heterogeneous Allosteric Models[a]

case I	case II
$K_R = l_R/l_{R'} = 1 \times 10^{-7}$ M	$K_R = 1 \times 10^{-8}$ M
$K_{R'} = K_R/\rho = 4.8 \times 10^{-7}$ M	$K_{R'} = 4.8 \times 10^{-8}$ M
$\rho = 0.21$	$\rho = 0.21$
$\sigma = 1$	$\sigma = 1$
$L = 150.2$ (104-210)	$L = 15\,479$ (10 787-21 285)
$c = 0.02076$ (0.014-0.0300)	$c = 0.00242$ (0.00177-0.00330)
$K_T = K_R/c = 4.82 \times 10^{-6}$ M	$K_T = K_R/c = 4.13 \times 10^{-6}$ M
$K_{T'} = K_{R'}/c = 2.31 \times 10^{-5}$ M	$K_{T'} = K_{R'}/c = 1.93 \times 10^{-5}$ M
$l_T/l_R = 1/c = 48.2$	$l_T/l_R = 1/(10c) = 41.3$

[a] The hemoglobin concentration was 5 μM, heme basis; temperature = 20 °C. The Hill number = 1.40 ± 0.08; $(\overline{CO}) = 1.3 \pm 0.2$ μM. For case I, observed rate constants l and l', respectively, were used for l_R and $l_{R'}$; for case II, $l_{R'}$ was taken to be 10^5 M^{-1} s^{-1} for a hypothetical Hb* form. Ranges for L and c are for ±one standard deviation from the means for the Hill number and $(\overline{CO})$.

noted that these two cases correspond respectively to assuming that the cooperativity arises solely from a difference in the CO dissociation rate constant between the T and R forms and to a change in that rate constant with conformation but with a 10-fold increase in the association constant for the R form over the T form. The function F (eq 3) can also be written for the allosteric model in terms of these two assumptions, for the latter case equilibria being such that the rapidly combining R forms are not observed. For assumption I (l_R and l_T differ but $l_{R'}$ and $l_{T'}$ do not), F is the same as above with

$$K_1 = \left(\frac{c + \lambda}{1 + \lambda}\right)\frac{\rho}{K_R}; \qquad K_2 = K_1/\rho \tag{5}$$

For case II, $l_{R'} = 10 l_{T'}$ and K_1 and K_2 are now

$$K_1 = \frac{\rho c}{K_R}; \qquad K_2 = K_1/\rho \tag{6}$$

For case I, it can be shown that the F experiment gives

$$c = \frac{K_2(1 + \lambda)}{K_R} - \lambda \tag{7}$$

where K_2 is 6.6×10^5 M^{-1}; similarly, for case II

$$c^2 = \frac{4\lambda\rho(\bar{X}K_2)^2}{1 + \lambda - 4\rho(\bar{X}K_2)^2} \tag{8}$$

For data fitted according to eq 4, a value of $\rho = 0.21$ was used. Equation 4 was used to fit the CO equilibrium data according to the two assumptions for K_R and for $\sigma = 1$. For case I, the value of c obtained was 0.0207; $L = 150$. For case II, c is 0.00242 and L is 15 479. For both assumptions, the F experiments lead to values of the allosteric parameter c (eq 7 and 8), which differ from the optimum values for fitting equilibrium data by a factor of about 3.

Discussion

The two most unusual aspects of the CO binding kinetics are the nonappearance of a quickly reacting form (Hb*) in flash photolysis and the slowness of the binding reaction. The Adair model adequately describes the existing body of kinetic and equilibrium data, fitting the equilibrium data and the partial saturation (F function) results. The CO dissociation rate constants are about twice the observed value of l. Since these are calculated from K_3 and K_4 which are derived from $(\overline{CO})$ values and from K_1 and K_2 obtained from fitting the F experiment, this discrepancy is not significant. In this model, the cooperativity must derive from changes in the dissociation constant for CO. Experiments are in progress to test this prediction by using the method of Sharma et al. (1975). According to this model, there is no need for the Hb* form. The partial saturation kinetic studies show clearly that kinetically distinct intermediates can be studied in the CO binding reaction. The detection of these intermediates was possible because of the kinetic heterogeneity in this dimeric hemoglobin. The concentrations of these intermediates must place additional restrictions on the values of parameters in more complex binding models than those in the Adair model. For two allosteric models, the value of c can be obtained in terms of L according to the fit of the data for partial saturation kinetics to a two-constant model. These values for c are given above (eq 7 and 8). The values for L and c in Table V were calculated from fits of the equilibrium data according to eq 4. Both allosteric models agree in assigning an important role in determining the cooperativity to changes in the dissociation constants for CO binding. Both allosteric models are based on the assumption that the heme kinetic heterogeneity is the same (0.21) in the R and T forms of the hemoglobin. The allosteric model II, which assumes a quickly reacting Hb* form with a binding rate 10 times greater than that for $l_{T'}$, predicts a value for L of ~15 000; a larger value for $l_{R'}$ would increase L even further. These values appear to be unusually large for a dimeric protein. This latter model also predicts a large value for $l_T/l_R = 41$. On the other hand, the first allosteric model using only the observed rate constants for the protein and which assumes $l_{R'} = l_{T'}$ predicts $l_R/l_T = 48.3$, for $L = 150$ and $c = 0.0207$. Thus, the Adair and both allosteric models predict that a major part of the cooperativity must derive from differences in the CO dissociation rate constant between the R and the T forms. It is interesting to pursue the results of the allosteric model II further. The ratio of R_1' to $T_1' = 1/(Lc) = 0.0267$. Flash-photolysis measurements on HbCO at low fractional breakdown gave no evidence for the Hb* form. We estimated that the width of the oscilloscope trace would so limit our detection that R_1'/T_1' must be less than 0.05. Thus, if equilibrium were rapidly established, we would not observe this form. The lower limit on the R → T transformation must then be ~5×10^3 s^{-1}, assuming that $l_{R'}$ is 10^5 M^{-1} s^{-1} and that the R → T rate constant must be about 50 times greater than the CO association rate to the Hb* form. This latter rate was maximal for the saturated CO solutions [(CO) = 910 μM] employed in some studies.

The extraordinary slowness of the CO combination reaction (l' values of 2.1×10^3 and 1×10^4 M^{-1} s^{-1} for the two hemes) is not associated with a large activation energy. These activation energies (9.0 and 7.5 kcal/mol, respectively) can be compared to the 8.6 kcal/mol found for horse hemoglobin ($l_{T'} = 1.7 \times 10^5$ M^{-1} s^{-1}; Parkhurst & Gibson, 1967). These values show that the CO binding sites in *Thyonella* are more inaccessible for the binding of CO than are the sites in horse hemoglobin. Perhaps conformational changes are required before the CO can bind. The slowness of the CO binding is in contrast to the oxygen kinetics which are not at all unusual. We have previously (Steinmeier & Parkhurst, 1979) shown that the M determinations, oxygen − CO difference spectra, and oxygen binding kinetics from an initial CO conformation all support a striking difference between oxy and CO forms of the protein. In a recent analysis of horse HbCO, Heidner et al. (1976) showed that the N_ϵ of His-E7 and the $C_{\gamma 2}$ of Val-E11 apparently force the CO into an off-axis orientation in that protein and concluded that the heme cavity is constructed to facilitate the binding of oxygen (in a bent orientation) but not CO and CN which are *forced* into bent

orientations. It appears that the restrictions for CO binding in *Thyonella* hemoglobin must be even more severe.

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Nuclear Magnetic Resonance Conformational Studies on the Chemotactic Tripeptide Formyl-L-methionyl-L-leucyl-L-phenylalanine. A Small β Sheet†

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ABSTRACT: Previous work by several groups has shown that the combination of spin–spin coupling constants and spectral density components (derived from spin–lattice relaxation and/or nuclear Overhauser measurements) may aid in the task of conformational determination of peptides in solution. Using the peptide formyl-L-methionyl-L-leucyl-L-phenylalanine, which is a potent specific chemotactic agent for leucocytes, we show the following: (a) that $^3J_{NHCH}$ coupling constants are consistent with a high degree of rigidity in the peptide backbone in solution, (b) that ^2H isotopic substitution in combination with relaxation data taken at different Larmor frequencies enables spectral density, and thence conformational, information to be obtained, (c) that side-chain conformations for this molecule mirror, in some aspects, those found in the solid state for other peptides containing the same residues, and (d) that temperature dependence of amide chemical shifts does not have direct implication concerning the existence of intramolecular hydrogen bonds in peptides. We are able to propose a family of conformations which appear to interchange rapidly on the NMR time scale and are characterized by a distribution of side-chain rotamers. The basic backbone conformation is, or closely approximates, a small β antiparallel pleated sheet and as such suggests a possible mode of receptor–chemotactic peptide interaction.

The phenomenon of leucocyte chemotaxis, that is, the directed motion of leucocytes along a concentration gradient of certain substances, has occupied the attention of pathologists and immunologists for many years. Historically, a large number of substances have been observed to cause chemotactic behavior in neutrophil leucocytes (Wilkinson, 1974). However, recently interest has focused on small peptides as chemotactic factors. Indeed, recently it was found that several formylated methionyl peptides show chemotactic behavior (Schiffman et al., 1975). Further work (Showell et al., 1976) has shown, via extensive structure–activity peptide synthesis, that the tripeptide CHO-Met-Leu-Phe-OH is a specific chemotactic factor for neutrophil leucocytes. These cells are sensitive to the tripeptide in amounts less than 2400 molecules of peptide per cell. Additional work (Aswanikumar et al., 1977) has shown that the interaction of the peptide with neutrophil cells is via specific receptors. Structure–activity studies have shown strict correlation between covalent structural changes in the peptide and the rank order of pharmacological activity seen in four assay procedures (Showell et al., 1976; Day et al., 1977). This group of peptides is therefore of particular importance since it represents a means of studying cellular immune responses by using a well-defined pure material in contrast to ill-defined chemotactic "factors" of unknown composition (Wilkinson, 1974).

Because of its simplicity and the future possibility of isolating receptor apparatuses from neutrophils, it is clear that this tripeptide is a good system to try to apply contemporary methods involving through-bond and through-space measurements as far as gathering information on its solution

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