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Double-Mixing Kinetic Studies of the Reactions of Methyl Isocyanide and CO with Diliganded Intermediates of Hemoglobin: $\alpha_2^{CO}\beta_2$ and $\alpha_2\beta_2^{CO}$ †

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ABSTRACT: Kinetics of the reactions of CO and methyl isocyanide with two diliganded intermediates of hemoglobin, $\alpha_2^{CO}\beta_2$ and $\alpha_2\beta_2^{CO}$, have been studied by double-mixing and microperoxidase methods. The valency hybrids were prepared by high-pressure liquid chromatography. The reaction time courses of ligand combination and dissociation with both of the ligands were biphasic, and in CO combination reaction the zero-time amplitudes of the two phases were independent of the protein concentration. In the presence of 2 M urea the reaction time course was clearly dependent on protein concentration, as the zero-time amplitude of the fast phase increased at lower protein concentrations. These two observations indicate that little dissociation of tetramers into dimers occurs in the absence of urea. Consistent with this, the kinetic data for the reactions of CO best fit a reaction model consisting of two tetrameric species not in rapid equilibrium with each other. Various considerations, however, suggest that the reaction model is more appropriately described as 2D $\rightleftarrows$ R $\rightleftarrows$ T. The reaction of triliganded species ($Hb_4(CO)_2Me_1$) with methyl isocyanide was monophasic, and the reaction model suggested a fast T $\rightleftarrows$ R structural change after the binding of the third ligand. Although the precise structural nature of the two species remains undefined, it is concluded that the biphasicity in the reactions of the two hybrids is characteristic of the diliganded species only and is independent of the nature of the ligand.

Valency hybrids $\alpha_2^{+CN}\beta_2$ and $\alpha_2\beta_2^{+CN}$, also known as symmetrical hybrids, have been extensively studied as models of diliganded hemoglobin intermediates. Cassoly and Gibson (1972) observed that the time course of CO binding to both of the hybrids was biphasic and the zero-time amplitudes of the two phases did not depend on the protein concentration. In their NMR studies of the valency hybrids, Ogawa and Shulman (1971) observed that at pH 7.3 these hybrids existed in two structures: one similar to deoxyHb and the other to oxyHb. These studies were made at heme concentrations of 2 mM. It was also observed that the lifetime of the two states was longer than 6 ms. Lack of concentration dependence of the relative amplitudes of the fast and slow phases suggests that the fast phase [$l' = (4 \pm 1) \times 10^6$ M^{-1} s^{-1}] cannot be assigned to the reaction of dimers. If the fast phase is assigned to the quaternary R structure and the slow phase to the quaternary T structure, then the interpretation of the kinetic and NMR data mentioned above would include slow rates of T $\rightleftarrows$ R transitions. This is contrary to the generally held view that the quaternary structural changes are fast. Contrary to the findings of Ogawa and Shulman (1971) and Cassoly and Gibson (1972), which suggest that the fast phase is tetrameric, the studies of Smith and Ackers (1985) indicate extensive dissociation into dimers of deoxy hybrids ($\alpha_2^{+CN}\beta_2^{deoxy}$ and $\alpha_2^{deoxy}\beta_2^{+CN}$). These researchers studies the rates of dissociation of deoxy hybrids into dimers by the haptoglobin method. Since this method is based on differences in the absorption coefficients of the R-state tetramer (or dimer) and the T-state tetramer, the results obtained in this study imply that the tetrameric form of deoxy hybrids is in the T state and is dissociated into dimers almost as much as normal oxyHb (oxyHb $k_{4,2}^1 = 1$ s^{-1}; deoxy hybrids $k_{4,2} = 0.8$–0.6 s^{-1}). Unfortunately, the presence of more than one tetrameric species may make it difficult to assign the k_{obsd} to $k_{4,2}$ (i.e., rate

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[1] Abbreviations: HPLC, high-pressure liquid chromatography; valency hybrids, $\alpha_2^L\beta_2^{+H_2O}$ or $\alpha_2^{+H_2O}\beta_2^L$; deoxy valency hybrids, $\alpha_2\beta_2^{+H_2O}$ or $\alpha_2^{+H_2O}\beta_2$; ferro or reduced hybrids, $\alpha_2^L\beta_2$ or $\alpha_2\beta_2^L$; $k_{4,2}$, rate constant for the dissociation of tetramer into dimers; Mp, microperoxidase; Me, methyl isocyanide; Bistris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; IHP, inositol hexaphosphate; symbols R and T are used to denote fast and slow reacting species.

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constant for the dissociation of tetramers into dimers). Nagel and Gibson (1972) followed the time course of tetramer → dimer dissociation not by absorbance changes at 430 nm but by the quenching of aromatic amino acid fluorescence of haptoglobin by the heme of hemoglobin. They observed that the deoxy hybrids dissociated into dimers much more slowly than the oxy hybrids, and the reaction time course was no longer exponential. No numerical values of dissociation rate constant $k_{4,2}$ were given, but the decrease in the reaction rates was significant; on the basis of the reaction time course the authors concluded that the deoxy hybrids can exist in a conformation that resembles deoxyHb, and the complex kinetics of haptoglobin suggested the possibility of two deoxy forms not in rapid equilibrium.

In all of these studies NMR as well as kinetic, the liganded chains were in CN-met form. The hybrids were prepared by a multistep procedure extending over several days and, therefore, it was difficult to rule out the possibility of sample heterogeneity. Rifkind et al. (1976), following a different approach, prepared one of the hybrids by oxidation of human hemoglobin by copper ions. A few years back we prepared the valency hybrids by an HPLC method in which the two hybrids were obtained in good yields within 2 h without much manipulation of hemoglobin solution, and we were able to make kinetic studies the same day (Sharma, 1988). Using a double mixer, we were able to reduce the ferric hemes just before studying the kinetics of CO combination or CO dissociation by the microperoxidase method (Sharma et al., 1976). These studies confirmed the results of earlier kinetic and NMR studies: the reaction time course was biphasic and independent of the protein concentration. Unfortunately, the reaction model used by us for data analysis was based on the tetramer–dimer dissociation rate constants reported by Smith and Ackers (1985) for valency hybrids, which may not be valid for ferro hybrids. The biphasic nature of CO combination reaction time course of these hybrids has important implications regarding the mechanism of cooperativity and the rates of quaternary structural changes in the reactions of hemoglobin (Parkhurst, 1979; Tan & Noble, 1973; Gray & Gibson, 1971). Here we report the results of our further studies on the kinetic properties of ferro hybrids ($\alpha_2^{CO}\beta_2$ and $\alpha_2\beta_2^{CO}$; i.e., all heme in a tetramer are in ferro form). The HPLC method of preparing these hybrids has been extended to larger scales so that we were able to prepare millimolar amounts of each of the hybrids in less than 2 h. These studies were designed to answer the following questions specifically: (1) Is the biphasic nature of the reaction time course observed in the reactions of CO with $\alpha_2^{CO}\beta_2$ and $\alpha_2\beta_2^{CO}$ characteristic of CO only or is it observed with other ligands as well? (2) Is the biphasicity of the reaction time course observed when the diliganded species are prepared by some other method? (3) If the lack of concentration dependence is due to the absence of dimers, then are there any conditions in which dimers are formed and the expected concentration dependence is observed? This would rule out insensitivity or some undefined flaw in the method of double mixing used in this study and add confidence to reaction models which assume the presence of two tetrameric species not in rapid equilibrium. Finally (4), is there a point in the sequential reactions of the hybrids when the fast and slow reacting species are in a rapid equilibrium? To answer these questions, the kinetics of reactions with methyl isocyanide and carbon monoxide in the presence and absence of urea have been studied. Partial flash photolysis studies were made to study the kinetic behavior of ferro subunits in $\alpha_2^{CO}\beta_2^{+H_2O}$ and $\alpha_2^{+H_2O}\beta_2^{CO}$.

Materials and Methods

HPLC separation of the two hybrids was carried out as described previously with the following changes (Sharma, 1988):

(1) A synchropak column CM 300 (250 × 22 mm i.d.) was used. We were able to load 1.75–2 mL of ≈12 g/100 mL hemolysate, 60% oxidized by ferricyanide, and obtained about 1–1.5 mL of each hybrid at a concentration of more than 1 mM heme.

(2) Buffer A was 0.03 M potassium phosphate, pH 6.8; buffer B was 0.015 M potassium phosphate, pH 7.5. The gradient was obtained as follows: 10 min, 100% buffer A, flow rate 4 mL/min; at 20 min 60% A, 40% B, flow rate 4 mL/min; at 80 min 20% A, 80% B, flow rate 8 mL/min; at 85 min 0% A, 100% B. The hybrid $\alpha_2^{CO}\beta_2^{+}$ eluted in 47 min and $\alpha_2^{+}\beta_2^{CO}$ in 82 min. The hybrid solutions were concentrated first by an Amicon stirring ultrafiltration unit with minimum stirring to yield 10–20 mL of sample volume and finally by Centricell 60 (Polyscience, Inc., 400 Valley Road, Warrington, PA) to give 1–2 mL of 1–2 mM hybrid samples. The percent of ferric heme in each concentrated hybrid sample was calculated by the method of Bannerjee and Cassoly (1969) and in most cases was 50 ± 2%. All other experimental details were as described previously (Sharma, 1988).

Double-Mixing Experiments. Kinetic experiments were carried out with phosphate-free Hb solutions at 20 °C by using a Durrum stopped-flow spectrophotometer equipped for double mixing. The hybrid solutions were exposed to CO gas for 10 min and applied to a Bio-Gel-P4 (mesh 50–100, exclusion limit 400 Da) column in a 5-mL disposable plastic syringe from which most of the equilibrating buffer (0.1 M Bistris, pH 7.0) had been removed by spinning for 3–5 min at 3000 rpm. Further spinning for 3 min yielded the hybrid solution free from excess CO and PO_4^{3-} without much dilution of the protein. For kinetic experiments a calculated volume of the hybrid solution was added to a known volume of deoxygenated buffer solution in a gastight syringe to give the desired protein concentration. Dithionite (0.1%) and CO solutions were prepared in the usual manner. Methyl isocyanide (Me) was prepared from the corresponding formamide by the method reported in the literature (Reisberg & Olson, 1980a). A calculated volume of methyl isocyanide was added to a known volume of deoxygenated buffer in a gastight syringe.

In a typical kinetic experiment in the first step of mixing, the hybrid solution was mixed with an equal volume of dithionite solution and aged for a known time (A_t in seconds). The products of the first mixing were then mixed with an equal volume of CO solution or methyl isocyanide. The aging time (A_t) was varied in the range 10 ms–11 s. After the second mixing, the reaction observed was

$$Hb_4(CO)_2 + 2L \rightarrow Hb(CO)_2L_2$$

The time taken for complete reduction of ferric hemes was determined by making a kinetic run in which only the reduction reaction was observed; it was <2 s for both hybrids at pH 7.0. The reaction time course was recorded at 437.8 nm unless otherwise mentioned.

CO dissociation rates were studied at 592 nm by the microperoxidase (Mp) method as described earlier (Sharma et al., 1976).

Results

(*A*) *CO Combination and Dissociation Reactions.* (*i*) When 10 μM solution of $\alpha_2^{CO}\beta_2$ was reacted with CO under pseudo-first-order conditions, the reaction time course at A_t = 2

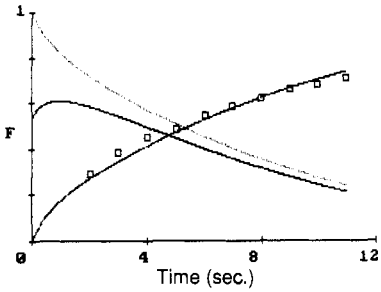


FIGURE 1: Fraction of the slow phase versus aging time (A_t). $[\alpha_2^{CO}\beta_2]$ = 10 μM; [CO] = 16 μM in 0.1 M Bistris, pH 7.0. All concentrations are on heme basis and before mixing. At t = 0, (top) ([D] + 2[R])/$[Hb_{tot}]$, (middle) 2[R]/$[Hb_{tot}]$, (bottom) (2[T] + 4$[\alpha_2\beta_2]$)/$[Hb_{tot}]$; line calculated, □ observed.

s was biphasic. The zero-time amplitudes of the fast (ΔA_f) and slow (ΔA_s) phases were 71 and 29 ± 5%, respectively.

(*ii*) At 10-fold dilution of the protein ($[\alpha_2^{CO}\beta_2]$ = 1 μM before mixing) the zero-time amplitude of the slow phase was 26%. This small variation in ΔA_s (from 29% at 10 μM protein concentration to 26% at 1 μM concentration) is within the accuracy with which we were able to determine the zero-time amplitudes.

(*iii*) In the CO dissociation reaction of $\alpha_2^{CO}\beta_2$ the total protein concentration before mixing was 70 μM. It was mixed with excess reduced microperoxidase. The reaction time course was biphasic and zero-time amplitude of the fast phase (which corresponds to the slow phase in CO combination reaction) was 27% which is within the range of error of ΔA_s (29%) observed in the CO combination reaction. This is in spite of the fact that these two reactions were studied at different protein concentrations and cover very different time domains. The average value of ΔA_s in (i)–(iii) is 30 ± 5% over the range of 1–70 μM protein concentration.

(*iv*) In contrast to the behavior in (i)–(iii), in the presence of 2 M urea the ΔA_s, which was 16% at 10 μM protein concentration, was totally abolished at 1 μM.

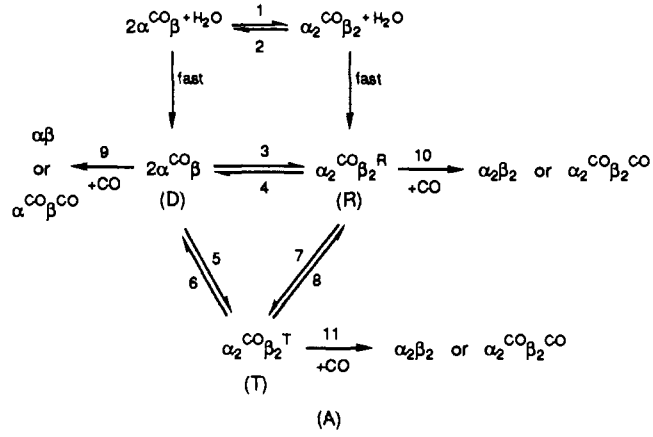
(*v*) We also studied the evolution of the slow phase as a function of the aging time at 10 μM protein concentration. In the range 2–11 s, the zero-time amplitude of the slow phase increased with an increase in the aging time. These data are shown in Figure 1. As reported earlier, if 0.2 mM IHP were added to the CO solution, ΔA_s at A_t = 2 s was increased by ~20%; at A_t = 11 s the effect was small or none.

(*vi*) In the presence of 0.2 mM IHP (in protein solution), the reaction time course was monophasic and slow at all aging times.

(*B*) *Reactions of Methyl Isocyanide with* $\alpha_2^{CO}\beta_2$ *and* $\alpha_2\beta_2^{CO}$. The reaction of methyl isocyanide with the two hybrids was studied at 20 μM protein concentration (before mixing) and 100–1500 μM methyl isocyanide concentration. The reaction time course was biphasic at all ligand concentrations studied for both of the hybrids. This is in contrast to the monophasic reactions of deoxyHb in mixing experiments and the reaction of the last methyl isocyanide [$Hb_4(Me)_3$ + Me → $Hb_4(Me)_4$] in partial flash photolysis experiments.

(*C*) *Partial Flash Photolysis Kinetic Studies on* $\alpha_2^{CO}\beta_2^+$ *and* $\alpha_2^+\beta_2^{CO}$. In these experiments an 85 μM solutions of the valency hybrid ($\alpha_2^{CO}\beta_2^+$ or $\alpha_2^+\beta_2^{CO}$) containing 945 μM CO was partially photolyzed (<2% photolysis) and absorbance changes were recorded at 440 nm. One hundred reaction time courses, each composed of 1024 data points, were electronically digitized and summed. For both hybrids the reaction time course was strictly monophasic and the second-order rate constants were for $\alpha_2^{CO}\beta_2^+$, l'_4 = 2.3 × 10^7 M^{-1} s^{-1}, and for

Scheme I



$\alpha_2^+\beta_2^{CO}$, l'_4 = 3 × 10^7 M^{-1} s^{-1}.

Data Treatment

As there is no significant change in the zero-time amplitudes of the fast and slow phases (observations i–iii, Results) when varying the protein concentration, we exclude the presence of any significant amount of dimers in the reaction solution. Since even in the much slower reactions of CO dissociation the values of ΔA_f and ΔA_s were the same as in CO combination reactions, the interconversion between the two components must be very slow. These conclusions, reached qualitatively, can be tested quantitatively from data in Figure 1 in which the amplitude of the observed slow phase (□) in CO combination reactions [$\Delta A_s/\Delta A_{(total)}$] is plotted against the aging time (A_t in seconds). In calculations described below we make no assumption regarding the nature of the fast or slow CO-reacting species, their initial concentrations, or their tetramer–dimer equilibrium constants. The starting point in these calculations is the distribution of ferric species into dimers and tetramers: $\alpha_2^{CO}\beta_2^{+H_2O} \rightleftarrows 2\alpha^{CO}\beta^{+H_2O}$, $k_{4,2} = k_2/k_1$ (reaction scheme I). In our earlier studies we had assumed $k_{4,2}$ = 1 s^{-1}, on the basis of values reported for ferric hybrids (Smith & Ackers, 1985). This implied significant dissociation of ferro hybrids into dimers. This is not supported by the kinetic data obtained in the present study as well as in studies described by Cassoly and Gibson (1972) and the NMR data of Ogawa and Shulman (1971). From the tetramer–dimer dissociation equilibrium constant (1 × 10^{-6} M) one can calculate the concentrations of $\alpha^{CO}\beta^{+H_2O}$ prior to reduction with dithionite. All unliganded subunits are in the ferric state and coordinated predominantly to water and, therefore, presumed to be in the R state; the T-state population at this stage is zero as indicated by observation C (Results). The initial concentrations of the reduced species ($\alpha_2^{CO}\beta_2$ and $\alpha^{CO}\beta$) are zero and, therefore, in dilute solutions the rates of the reduction reaction with dithionite can be assumed to be much faster than the rates of dimer ⇄ tetramer relaxation of the reduced species. The data are fitted to the reaction (model A) shown in Scheme I (in which D is dimer, R is the fast reacting species, and T is the slow reacting species). In the case of reactions involving CO, k_i (i are numerals given to various rate constants in Scheme I) and k'_i are for CO dissociation and combination reactions, respectively. The values of fixed constants k_3 and k_5 (=5 × 10^5 M^{-1} s^{-1}) were taken from the values reported in the literature for similar reactions (Benjamin et al., 1981). Constants k_{10} and k_{11} were fixed at 0.015 and 0.09 s^{-1}, respectively (Sharma et al., 1976). The lack of concentration dependence of ΔA_s suggests that k_4 and k_6 are either very small or zero. Rate equations for numerical integration were written

Table I: Kinetic Data for the Reactions of $\alpha_2^{CO}\beta_2$ and $\alpha_2\beta_2^{CO}$ at 20 °C in 0.1 M Bistris, pH 7.0[a]

reaction	rate constant
(1) $\alpha_2^{CO}\beta_2$ + CO	k'_9, (3 ± 0.4) × 10^6 M^{-1} s^{-1}
	k'_{10}, (9 ± 0.8) × 10^6 M^{-1} s^{-1}
	k'_{11}, (2 ± 0.1) × 10^5 M^{-1} s^{-1}
$\alpha_2^{CO}\beta_2$ − CO	k_{11}, 0.07 ± 0.002 s^{-1} [0.08 s^{-1}, 27%]
	k_{10}, 0.017 ± 0.002 s^{-1} [0.02 s^{-1}]
$\alpha_2^{CO}\beta_2$ + Me	k_1, (5.8 ± 0.2) × 10^5 M^{-1} s^{-1} [5.7 × 10^5]
	k_5, (6.0 ± 0.7) × 10^4 M^{-1} s^{-1} [6 × 10^4]
$\alpha_2^{CO}\beta_2$ − Me	k_6, (83 s^{-1}) fixed constant
	k_2, 6.8 ± 1 s^{-1} [7]
(2) $\alpha_2\beta_2^{CO}$ + CO	k'_9, (1.5 ± 0.4) × 10^6 M^{-1} s^{-1}
	k'_{10}, (7 ± 0.3) × 10^6 M^{-1} s^{-1}
	k'_{11}, (2.3 ± 0.02) × 10^5 M^{-1} s^{-1}
$\alpha_2\beta_2^{CO}$ − CO	k_{11}, 0.06 ± 0.008 s^{-1} [0.06 s^{-1}, 32%]
	k_{10}, 0.012 ± 0.004 s^{-1} [0.012 s^{-1}]
$\alpha_2\beta_2^{CO}$ + Me	k_1, (3.5 ± 0.2) × 10^5 M^{-1} s^{-1} [1.4 × 10^5]
	k_5, (6.9 ± 0.9) × 10^4 M^{-1} s^{-1} [3.3 × 10^4]
$\alpha_2\beta_2^{CO}$ − Me	k_6, (140 s^{-1}) fixed constant
	k_2, 5.4 ± 0.7 s^{-1} [3.6]
(3) $\alpha_2^{CO}\beta_2^{+H_2O}$ + CO	l'_4, 2.3 × 10^7 M^{-1} s^{-1}
(4) $\alpha_2^{+H_2O}\beta_2^{CO}$ + CO	l'_4, 3 × 10^7 M^{-1} s^{-1}
(5) Hb_4 + Me	i', 7.7 × 10^4 M^{-1} s^{-1}
(6) $Hb_4(Me)_3$ + Me	i'_4, 1 × 10^6 M^{-1} s^{-1}

[a] For the reactions of methyl isocyanide, values in square brackets are for Hb A as reported by Reisberg and Olson (1980b). For CO dissociation reaction, values in square brackets were obtained by nonlinear least-squares fit to eq 2. Rate constants k'_9, k'_{10}, k'_{11}, k_{10} and k_{11} are based on reaction scheme I and k_1, k_2, k_5 and k_6 on reaction scheme II.

on the basis of the reaction in Scheme I, and the variable rate constants (k_7 and k_8 in the present case) were estimated by minimizing the sum-square of residuals between the calculated and observed values of the fraction of the total free Hb present in the form of slow phase:

$$\Delta A_s/\Delta A_{tot} = (2[T] + 4[\alpha_2\beta_2])/[Hb_{tot}] \quad (1)$$

For CO combination or dissociation reactions the function used for least-squares analysis was

$$F = ([Hb_{tot}] - ([D] + 2[T] + 2[R]))/[Hb_{tot}]$$

where F represents the fraction of the reaction completed.

The species $\alpha_2\beta_2$ in eq 1 is formed by the disproportionation reactions of the partially liganded species: $\alpha_2^{CO}\beta_2^{T} \rightleftarrows \alpha_2\beta_2 + 2CO$, $\alpha_2^{CO}\beta_2^{R} + 2CO \rightleftarrows \alpha_2^{CO}\beta_2^{CO}$. In eq 1, Hb_{tot} represents free Hb on heme basis (or liganded Hb in CO dissociation reactions) and the concentrations of D, R, T, and $\alpha_2\beta_2$ are on dimer (d) or tetramer (R, T, $\alpha_2\beta_2$) basis. The estimated values of the constants were k_7 = 0.04 s^{-1} and k_8 = 0.002 s^{-1}. We emphasize that the number of data points in Figure 1 is small, the correlation coefficients between various constants were high, and analysis based on zero-time amplitudes is inherently not as accurate as analysis of continuous reaction time course. Therefore, the numerical values of k_7 and k_8 given above should be considered only as the least-squares fit parameters. A more rigorous estimation of these parameters will be attempted later in the analysis of CO dissociation reaction time course, but it is safe to conclude on the basis of analysis given above that dimers can account for only a small portion of the fast phase (≈16%; see Figure 1).

Analysis of CO Combination and Dissociation Reactions. In view of the preceding calculations it is clear that the reaction time course of CO combination and dissociation can also be analyzed in terms of model-independent parameters by making a least-squares fit to an equation of the type

$$Abs_t = \sum_{i=1}^{n} A_i e^{-k_i t} + Abs_\infty \quad (2)$$

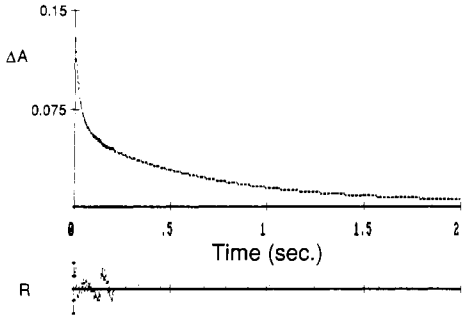


FIGURE 2: Calculated and observed absorbance changes versus time for the reaction of $\alpha_2^{CO}\beta_2$ with CO at A_t = 2 s. [$\alpha_2^{CO}\beta_2$] = 10 μM; [CO] = 15.5 μM in 0.1 M Bistris, pH 7.0. R, magnified residuals for data in ΔA plot; line R = 0. Each small division represents 1% deviation from zero residual.

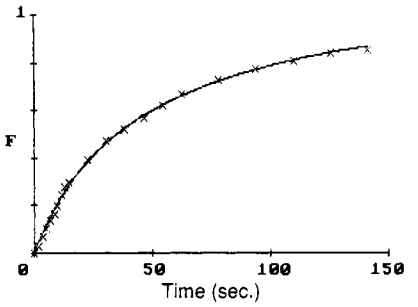


FIGURE 3: Fractional saturation versus time for the reaction of $\alpha_2\beta_2^{CO}$ with microperoxidase at A_t = 2 s. [Mp] = 500 μM; [$\alpha_2\beta_2^{CO}$] = 70 μM in 0.1 M Bistris, pH 7.0. The best-fit was calculated by using the constants listed in Table I (k_{10} and k_{11}).

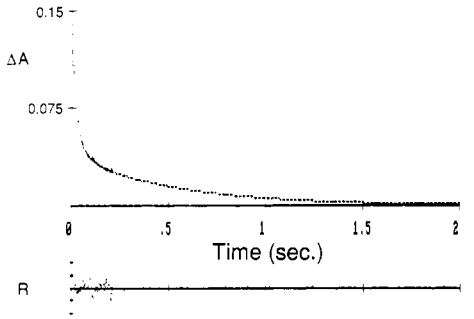


FIGURE 4: Calculated and observed absorbance changes versus time for the reaction of $\alpha_2\beta_2^{CO}$ with CO at A_t = 2 s. [$\alpha_2\beta_2^{CO}$] = 10 μM; [CO] = 15.5 μM in 0.1 M Bistris, pH 7.0. R, magnified residuals for data in ΔA plot; line R = 0. Each small division represents 1% deviation from zero residual.

where Abs_t is the absorbance at time t, n is the number of discrete exponentials, and Abs_∞ is the absorbance at infinite time.

Least-squares analyses of CO combination reaction time course (eq 2, i = 3) yielded rate constants listed in Table I. The zero-time amplitudes yielded [D] = 19%, which is in agreement with the value of dimer concentration (≈16%) predicted by the analysis shown in Figure 1. This was also the percentage by which ΔA_s was increased in CO combination reactions observed in the presence of 0.2 mM IHP in CO solution (Results, observation V).

The calculated and observed reaction time course for CO combination for $\alpha_2^{CO}\beta_2$ is shown in Figure 2. Figures 3 and 4 show the CO dissociation and combination reaction time course for $\alpha_2\beta_2^{CO}$, respectively. Table I lists various rate constants obtained by the method of least-squares analysis as described earlier.

Since CO combination reactions are fast, they could yield no information regarding the slow reactions corresponding to

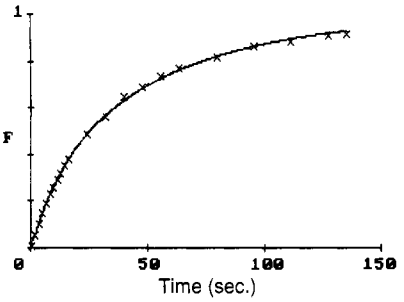


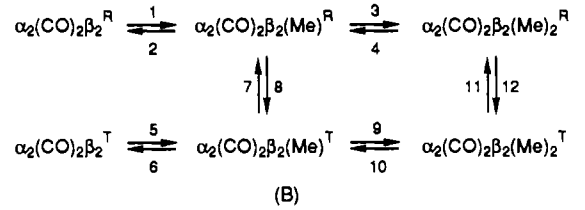
FIGURE 5: Fractional saturation versus time for the reaction of $\alpha_2^{CO}\beta_2$ with microperoxidase at A_t = 2 s. [Mp] = 500 μM; $[\alpha_2^{CO}\beta_2]$ = 70 μM in 0.1 M Bistris, pH 7.0.

k_4, k_6, k_7, and k_8. The reaction time course for CO dissociation provided data suitable for estimating the rate constants for these slower reactions. Details of the numerical analysis of the CO dissociation reaction time course of $\alpha_2^{CO}\beta_2$ hybrid are described below. The results are very instructive and also apply to $\alpha_2\beta_2^{CO}$ hybrid. The calculations were started on the basis of the distribution of dimers ($\alpha^{CO}\beta^{+H_2O}$) and tetramers ($\alpha_2^{CO}\beta_2^{+H_2O}$) before reduction with dithionite. The T-state population was assumed zero at this stage. Assuming fast reduction (reaction of Scheme I), the concentrations of $\alpha^{CO}\beta$, $\alpha_2^{CO}\beta_2^{T}$, and $\alpha_2^{CO}\beta_2^{R}$ were calculated at A_t = 2 s. These calculations indicated that, at A_t = 2s, [T] = 30% and that about 5% of the fast phase could be due to the presence of dimers because of incomplete association of dimers subsequent to the reduction reaction. Six rate constants, k_4, k_6, k_7, k_8, k_{10}, and k_{11}, were treated as variable constants to be estimated by least-squares analysis of the reaction time course. At the final convergence of the residuals, k_4, k_6, k_7, and k_8 were reduced to zero or less than 10^{-5} and the values of the two remaining constants were k_{10} = 0.017 ± 0.002 s^{-1} and k_{11} = 0.07 ± 0.002 s^{-1}. The calculated and observed reaction time course is shown in Figure 5. We should note that without making any assumption regarding the initial concentration of the species, the analysis given above leads to the conclusion that the dissociation rate constant for the tetramer of the reduced hybrid ($\alpha_2^{CO}\beta_2$) is very small and that the interconversion between the fast and slow CO reacting species is very slow. The numerical values of k_{10} and k_{11} are also in agreement with the model-independent parameters estimated from eq 2; k_f = 0.08 s^{-1}, k_s = 0.02 s^{-1}. In our earlier study (Sharma, 1988) CO dissociation from $\alpha_2^{+}\beta_2^{CO}$ was obtained at 10 μM (as compared to 80 μM in the present study) heme concentration, and the data were analyzed graphically. Since the *rates* of the two phases differ by only a factor 2, smaller absorbance changes and the graphical analysis in that study could not resolve the two components of the reaction time course.

Further Experiments Suggesting That Diliganded Species (Hb_4L_2) Exist at Least in Two States Not in Rapid Equilibrium. It is difficult to accept that two diliganded (Hb_4L_2) tetrameric species with CO combination rate constants close to the values expected for the R and T states are not in rapid equilibrium with each other. We describe below experiments which indicate that diliganded species Hb_4L_2 exist at least in two states which are not in rapid equilibrium with each other.

(*a*) Stripped carboxyHb solution at about 350 μM (heme basis, 0.1 M Bistris, pH 7.0) was partially photolyzed to yield 25% free hemes. The reaction time course of CO combination after partial photolysis was analyzed by using eq 2. The zero-time amplitude of the slow phase was ≈17% of the total absorbance change. Assuming random photodissociation (free heme as Hb_4L_3 = 42.5%, Hb_4L_2 = 42.5%, Hb_4L_1 = 13.9%), at 25% photolysis statistical distribution of species would require at least 43% slow phase ($l'_3 \approx 1$–3×10^5 M^{-1} s^{-1}) due to the reaction of Hb_4L_2 and 57% fast phase ($l'_4 \approx 3$–6×10^6 M^{-1} s^{-1}). The observed value is less than half of the expected value, indicating that more than half of the diliganded species are in fast CO reacting state. This is consistent with the results described earlier for hybrids isolated by HPLC. It should be pointed out that at this concentration of hemoglobin dimers are insignificant.

Scheme II



(B)

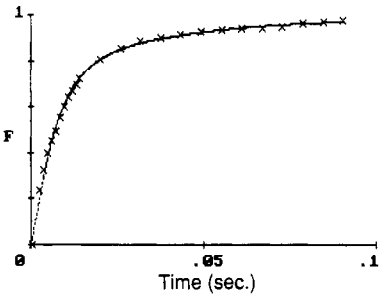


FIGURE 6: Fractional saturation versus time for the reaction of $\alpha_2^{CO}\beta_2$ with methyl isocyanide (Me). $[\alpha_2^{CO}\beta_2]$ = 20 μM; [Me] = 1113 μM in 0.1 M Bistris, pH 7.0. Wavelength of observation was 440 nm.

(*b*) Tomoda et al. (1978) have reported that in the presence of IHP β chains in ferric Hb are reduced by ascorbic acid much faster than α chains and that only one partially reduced intermediate $\alpha_2^{3+}\beta_2^{2+}$ is formed. CO combination reaction of $\alpha_2\beta_2^{CO}$ produced by this method yielded a biphasic reaction time course for CO combination, and the zero-time amplitudes of the two phases in the absence of IHP were approximately equal to those observed in hybrids prepared by the HPLC method.

Reaction of Methyl Isocyanide with $\alpha_2^{CO}\beta_2$ and $\alpha_2\beta_2^{CO}$. The reaction time course of the deoxyHb with isocyanides is complex and subject to varying interpretations. Recently, Reisberg and Olson (1980b) studied the reactions of several isocyanides with deoxyHb and interpreted the data on the basis of the two-state model with remarkable consistency. We have used a similar model for analyzing the reaction time course of methyl isocyanide with $\alpha_2^{CO}\beta_2$ and $\alpha_2\beta_2^{CO}$ (Scheme II). The model assumes the presence of two starting species as indicated by the biphasic nature of the reaction time courses. The initial concentrations of the two species were estimated by making a least-squares fit to the reaction time course on the basis of eq 2. The initial relative amounts of the two species were similar to those estimated in the reactions of CO with the two hybrids. The values of k_7 and k_8 were kept fixed at the values reported by Ferrone et al. (1985) for the rates of R → T and T → R transitions in the reaction $Hb_4(CO)_3^{R} \rightleftarrows Hb_4(CO)_3^{T}$: $k_7 = 1 \times 10^3$ s^{-1}, $k_8 = 3 \times 10^3$ s^{-1}. It was assumed that the *tetra*liganded species ($\alpha_2^{CO}\beta_2^{(Me)}$ or $\alpha_2^{(Me)}\beta_2^{CO}$) is predominantly in the R state (i.e., $k_{12} \ll k_{11}$). Since $k_7 \gg k_5$, the species $\alpha_2^{(CO)}\beta_2Me_1$ is never populated sufficiently to allow accurate estimation of k_6. Therefore, this constant was kept fixed at the values reported by Reisberg and Olson (1980b): k^{β}_6 = 83 s^{-1}; k^{α}_6 = 140 s^{-1}. Constants 1 and 3 and constants 4 and 2 are for the same quaternary structure

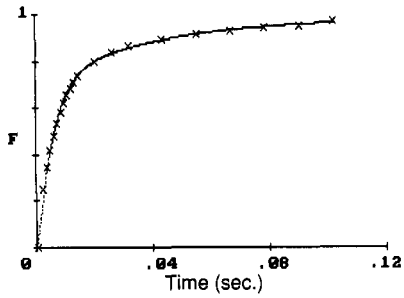


FIGURE 7: Fractional saturation versus time for the reaction of $\alpha_2\beta_2^{CO}$ with methyl isocyanide. $[\alpha_2\beta_2^{CO}] = 20\ \mu M$, [Me] = 1484 μM in 0.1 M Bistris, pH 7.0. Wavelength of observation was 440 nm.

and, therefore, were assumed to be statistically related: $k_1 = 2k_3$; $k_4 = 2k_2$. Figures 6 and 7 show the calculated and observed reaction time courses for the reaction of the two hybrids with methyl isocyanide, and the estimated rate constants are listed in Table I. The constants reported by Reisberg and Olson (1980b) have a standard deviation of ±10–150%. According to Reisberg and Olson (1980b), the fixed constant k^{α}_6 (140 ± 210 s^{-1}) is least reliable. The starting species in the reaction of deoxyHb is unliganded, while in the present case it is diliganded. Considering this and the differences in the experimental conditions, the agreement between values obtained by us and those reported by Reisberg and Olson (1980b) for $\alpha_2^{CO}\beta_2$ is remarkably good. The values of various rate constants for $\alpha_2\beta_2^{CO}$ obtained by us are about twice the values reported by Reisberg and Olson (1980b). This difference is mainly due to a much smaller difference in the reactivities of α and β chains in the experimental conditions of the present investigation. Alternatively, the chain differences probably decrease as the degree of ligation increases. These results indicate that while in the diliganded species there is slow (or no) relaxation between the fast and slow reacting species, after the ligation of the third ligand, there is a fast T ⇄ R relaxation.

Discussion

The most consistent feature of the kinetic data obtained in this study, and in studies by others, is the absence of a distinct effect of protein concentration on the relative amplitudes of the fast and slow phases in the reactions of the two hybrids with CO and methyl isocyanide. Model calculations based on the reaction of Scheme I show that at 10 μM heme concentration ΔA_s at A_t = 2 s is 28% and at 1 μM 23%; the experimental values are 29 and 26%, respectively. These small variations in ΔA_s over a 10-fold variation in protein concentration are less than the accuracy with which we were able to determine the zero-time amplitudes. Kinetic studies in 2 M urea solutions indicate that if one artificially increases the dissociation of reduced hybrids into dimers, the expected concentration dependence of the relative amplitudes is exhibited.

We should also point out that the analysis of data in Figure 1 was carried out without making any assumption regarding the nature of the fast or slow phases. The initial concentrations were calculated on the basis of the equilibrium constant for the reaction $2\alpha^{CO}\beta^{+H_2O} \rightleftarrows \alpha_2^{CO}\beta_2^{+H_2O}$. Analysis for the CO dissociation reaction was also made on this basis. We consider the data for CO dissociation more accurate, as it covers a very wide time domain and the data points are from a continuous reaction time course. These data suggest that k_4, k_6, k_7, and k_8 are very small. In our earlier study we made two main mistakes: (a) The analysis of the data was carried out by assuming k_4 = 1 s^{-1} as suggested by the earlier studies of fully liganded Hb and valency hybrids (Smith & Ackers, 1985). The kinetic data do not support this (Cassoly & Gibson, 1972). (b) The zero-time amplitude of the slow phase (ΔA_s) at 10 μM was studied at 437.8 nm. This was compared with ΔA_s at 418 nm for 1 μM heme concentration. The comparison should have been made between the data obtained at the same wavelength, as is the case in the present study. In addition, in analyzing the aging time (A_t) versus ΔA_s plot we have taken into consideration the disproportionation reaction ($\alpha_2^{CO}\beta_2^{T} \rightleftarrows \alpha_2\beta_2 + 2CO$, $2CO + \alpha_2^{CO}\beta_2^{R} \rightleftarrows \alpha_2^{CO}\beta_2^{CO}$), which can also contribute in the evolution of the slow phase.

Analysis of the kinetic data for the reactions of methyl isocyanide on the basis of the reaction in Scheme II (model B) implies fast T ⇄ R equilibria after the binding of the third ligand and indicates that the slow equilibrium between the fast and slow reacting species responsible for the biphasic ligand combination kinetics is present only in the diliganded species. A remarkably good agreement between the values of rate constants obtained in this study and those obtained by Reisberg and Olson (1980a,b) for HbA on the basis of the two-state model and for conditions in which there was insignificant dissociation into dimers suggests that the fast and slow reacting components are two tetrameric species.

It is difficult to explain slow interconversion between the fast (R) and slow (T) reacting species. Liddington et al. (1988) and Brzozowski et al. (1984) succeeded in studying the crystal structure of the diliganded species $\alpha^{02}{}_2\beta_2$. They observed that, in the diliganded T state α subunits, both the tight packing of heme and the intersubunit contacts inhibit a conformational change betwen the F helix and FG corner which would allow the heme to become planar and the iron to assume symmetrical R-like coordination. In β subunits, by contrast, they found no strain on the proximal sides, but the intersubunit contacts prevented the heme from tilting, which would open up the binding site to ligand binding. The crystals for these studies could be grown only in poly(ethylene glycol), and the results obtained may have no bearing on the behavior of diliganded species in aqueous environment. Somewhat similar findings have been made in a recent theoretical study of hemoglobin intermediates by Arata et al (1988), who pointed out that when either an α or a β subunit (in deoxyHb) is substituted with a corresponding carboxy subunit, serious steric hindrance is produced between α_1 FG4 (92) Arg and β_2 C3(37) Trp [or between α_1C6(41) Thr and β_2FG4(97) His], all of which belong to the allosteric core affected directly by ligand binding. These steric hindrances become more serious when both α_1 (α_2) and β_2 (β_1) subunits are substituted. The common observation in both of these studies (Liddington et al., 1988; Arata et al., 1988) is the very tight or constrained α_1–β_2 (or α_2–β_1) subunit contacts in the T state of the diliganded species, making structural changes in the allosteric core (which includes the ligand binding site) very difficult. If these findings are to be used to explain the kinetic behavior of the two hybrids studied, it would mean that both the fast and slow ligand reacting species are in quaternary T state in which the subunit contacts are so constrained that they do not switch from the fast reacting to the slow reacting form unless a strong allosteric effector such as IHP is added to the system or from the slow to the fast reacting form unless the degree of ligation is increased to three. The suggestion of two T states not in rapid equilibrium was first made by Nagel and Gibson (1972). Very low values of tetramer to dimer dissociation rate constants (k_4 and k_6) are also consistent with this postulate. Since both the rates of quaternary structural changes and dissociation of tetramer into dimers

depend on the nature of α_1–β_2 (or α_2–β_1) subunit contacts, it makes some logic to observe slow rates of structural changes linked to slow rates of dissociation of tetramer into dimers.

Reaction Mechanism. The reaction of Scheme I (reaction model A) implies that dimers have two paths for associating into tetramers: reaction 3 leads to the formation of R species and reaction 5 to T species. How does the dimer discriminate between the two paths? In fact, there are reasons to believe that for normal HbA the R-state tetramer is an intermediate in the formation of the T-state tetramer. This conclusion is based on the following calculations:

$$2D \underset{4}{\overset{3}{\rightleftharpoons}} R \underset{8}{\overset{7}{\rightleftharpoons}} T \qquad (C)$$

$k_3 = 5 \times 10^5$, $k_4 = 1$, $k_7/k_8 = 3 \times 10^5$ (Edelstein, 1971)

$$K_{D,T} = [T]/[D]^2 = 15 \times 10^{10}\ M^{-1}$$

A value of $1.2 \times 10^{10}\ M^{-1}$ is obtained if, instead of using the value of $L = 3 \times 10^5$ (Edelstein, 1971), we use the values of rate constants of k_7 and k_8 reported by Ferrone et al. (1985) for triliganded species and make allowance for the increase of k_7 and decrease of k_8 by a factor of ≈ 3 for each ligand removed. Both values are in reasonable agreement with the experimentally determined value of $4.2 \times 10^{10}\ M^{-1}$ of $K_{D,T}$.

Therefore, if dimers cannot associate directly into the T-state tetramer, reactions 5 and 6 get eliminated from model A and we obtain reaction model C. A similar conclusion is reached from completely different considerations as well: using the constants due to Smith and Ackers (1985) and the model 2D $\rightleftarrows$ R (or T), the tetramer population should be $\approx 75\%$ of the total heme. Since the observed ΔA_s is $30 \pm 5\%$, approximately half of the hemes must be in the form of fast reacting tetramer, suggesting that model is more accurately represented by model C. It also implies that $K_{T/R} = [T]/[R] = Lc^2 \approx 1$. As the T state (or species) is not formed by the association of dimers and the R state (or species) is not in rapid equilibrium with the T species (both assumptions implied in model C), the question arises as to how the T species is formed at $A_t = 2$ s. One possibility is that a rapid equilibrium exists initially which is followed by changes in the structures of the R and T species which render them slow equilibrium species. With slightly different (compared to those obtained on the basis of model A) rate constants, this model fits the kinetic data obtained in this study: for $\alpha_2^{CO}\beta_2$ $k_{11} = 0.066\ s^{-1}$, $k_{10} = 0.019\ s^{-1}$, $k_7 = 0.02\ s^{-1}$, and $k_8 = 0.008\ s^{-1}$; for $\alpha_2\beta_2^{CO}$ $k_{11} = 0.044\ s^{-1}$, $k_{10} = 0.01\ s^{-1}$, and k_7, $k_8 = 0$. We do not put much reliance on the values of k_7 and k_8 and consider them only as the least-squares fit parameters. The values of CO dissociation rate constants are not very different from those listed in Table I and calculated on the basis of reaction model A.

Model C has two additional features in its favor: (1) It requires much reduced dependence of ΔA_s on protein concentration than if the model were 2D $\rightleftarrows$ R (or T). Such small variations in the fraction of the slow phase cannot be detected easily with absolute certainty, unless the protein concentration is varied over a very wide range. This is generally not possible to achieve in stopped-flow studies of CO combination reactions. As a result, the observed proportions of the fast and slow phases may appear to be concentration independent. (2) It also explains the observation that in very dilute solutions (1 μM after mixing) the reaction time course of CO dissociation, which was biphasic at 80 μM heme concentration, becomes almost monophasic with $k_{obsd} \approx 0.08\ s^{-1}$ (Sharma, 1988). This is expected from model C, if in dilute solutions the rate of change of equilibria involving k_3/k_4 and k_7/k_8 becomes faster than the rate of CO dissociation from the T state:

$$2D \underset{4}{\overset{3}{\rightleftharpoons}} R \underset{8}{\overset{7}{\rightleftharpoons}} T \xrightarrow[k_{11}]{-CO}$$

$k_{obsd} = (K/(K+1))\ k_{11}$, where $K = (k_3/k_4)(k_7/k_8)$

if $K \gg 1$, $k_{obsd} = k_{11}$

The observed value of $k_{obsd} \approx 0.08\ s^{-1}$ is in good agreement with the value calculated on the basis of model A ($=0.07\ s^{-1}$) or the model-independent value ($0.08\ s^{-1}$) calculated on the basis of aq 2 or with the value of $0.085\ s^{-1}$ reported by Samaja et al. (1987) or model C ($0.066\ s^{-1}$).

In summary, the reaction time course of CO and methyl isocyanide combination with $\alpha_2^{CO}\beta_2$ and $\alpha_2\beta_2^{CO}$ is biphasic and independent of protein concentration, indicating the presence of two species not in rapid equilibrium. Data anlysis on the basis of the two-state model for the reactions of methyl isocyanide indicates that after the binding of the third ligand, equilibria between the fast and slow reacting species become rapid. Although the kinetic data for CO best fit a model consisting of two tetrameric species not in rapid equilibrium (R $\rightleftarrows$ T), detailed kinetic considerations suggest that the model is more appropriately represented as 2D $\rightleftarrows$ R $\rightleftarrows$ T.

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Registry No. CO, 630-08-0; Me, 593-75-9; Fe, 7439-89-6.

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Peptide Substrate Specificity of the Membrane-Bound Metalloprotease of *Leishmania*†

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ABSTRACT: The promastigote surface protease (PSP) of *Leishmania* is a neutral membrane-bound zinc enzyme. The protease has no exopeptidase activity and does not cleave a large selection of substrates with chromogenic and fluorogenic leaving groups at the P_1' site. The substrate specificity of the enzyme was studied by using natural and synthetic peptides of known amino acid sequence. The identification of 11 cleavage sites indicates that the enzyme preferentially cleaves peptides at the amino side when hydrophobic residues are in the P_1' site and basic amino acid residues in the P_2' and P_3' sites. In addition, tyrosine residues are commonly found at the P_1 site. Hydrolysis is not, however, restricted to these residues. These results have allowed the synthesis of a model peptide, H_2N-L-I-A-Y-L-K-K-A-T-COOH, which is cleaved by PSP between the tyrosine and leucine residues with a k_{cat}/K_m ratio of 1.8×10^6 M^{-1} s^{-1}. Furthermore, a synthetic nonapeptide overlapping the last four amino acids of the prosequence and the first five residues of mature PSP was found to be cleaved by the protease at the expected site to release the mature enzyme. This result suggests a possible autocatalytic mechanism for the activation of the protease. Finally, the hydroxamate-derivatized dipeptide Cbz-Tyr-Leu-NHOH was shown to inhibit PSP competitively with a K_I of 17 μM.

Proteolytic enzymes are involved in numerous pathogenic processes, including parasitic disease (Mignatti et al., 1986; Chen & Chen, 1987; McKerrow, 1989). There has been increasing interest in the roles that parasite proteases play in the invasion of host tissues (McKerrow et al., 1989), evasion of the host immune response (Verwaerde et al., 1988), and degradation of host proteins (Rosenthal et al., 1988). These enzymes have therefore been proposed as targets for the rational design of new drugs for chemotherapy, offering an alternative to vaccination (Wang, 1984; Schnebli & Braun, 1986). In this respect, the promastigote surface protease (PSP)[1] of *Leishmania* could be such a target. This enzyme, also known as "gp63", is a glycoprotein expressed at high density (5×10^5 molecules/cell) at the surface of the parasite (Bouvier et al., 1985; Etges et al., 1986a; Bordier, 1987; Chaudhuri & Chang, 1988). It is bound to the membrane by a glycosylphosphatidylinositol (GPI) anchor (Etges et al., 1986b; Bordier et al., 1986) that attaches a wide variety of proteins to membranes (Ferguson & Williams, 1988; Low & Saltiel, 1988). The enzyme is present on the promastigotes residing in the midgut of the phlebotomine sandfly vector (Grimm et al., 1987) and has been detected at the surface of all *Leishmania* species examined so far (Bouvier et al., 1987). Surface metalloprotease activity not only is a highly conserved feature of the genus *Leishmania* but also occurs at the surface of the monogenetic trypanosomatids *Crithidia* and *Herpetomonas* (R. Etges, personal communication). The complete nucleotide sequence for the protease has been deduced (Button & McMaster, 1988, 1990; Miller et al., 1990), and a recent report has shown that the enzyme is encoded by a family of tandemly linked genes, all of which map to a single chromosome (Button et al., 1989). The synthesis and expression of the protease by *Leishmania* amastigotes, the intracellular form infecting the host macrophages, indicate that the enzyme is not stage-specific (Colomer-Gould et al., 1985; Chaudhuri et al., 1989; Medina-Acosta et al., 1989; Frommel et al., 1990). Its involvement in the early phases of infection as a ligand for the mannosylfucosyl receptor, as an acceptor for C3b deposition, or as the major surface antigen and vaccine candidate has been discussed (Russell & Wilhelm, 1986; Mosser & Edelson, 1987; Russell & Alexander, 1988; Puentes et al., 1989). Recently, PSP was shown to be a zinc enzyme, the active site of which has strong similarities to those of other

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[1] Abbreviations: PSP, promastigote surface protease; Cbz (Z), benzyloxycarbonyl; Suc, *N*-succinyl; AMC, 7-amido-4-methylcoumarin; pNa, *p*-nitroanilide; TBS, Tris-buffered saline; HPLC, high-pressure liquid chromatography.

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