Viscosity Dependence of the Kinetics of the Diffusion-Controlled Reaction of Carbon Monoxide with the Separated α and β Chains of Hemoglobin†

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ABSTRACT: The kinetics of the recombination reaction of carbon monoxide with the isolated α and β chains of human hemoglobin have been examined by laser flash photolysis in glycerol–water as a function of temperature and solvent viscosity. The second-order recombination rate constant is inversely proportional to viscosity raised to the 0.5 power—paralleling that predicted for the CO diffusion coefficient. This viscosity exponent is independent of the protein. These results are consistent with the reaction kinetics being essentially diffusion controlled in the high viscosity glycerol–water. For the α and β chains, respectively, the diffusion-controlled rate constant is 0.003 and 0.002 of that predicted from the simple von Smoluchowski model based on the diffusion coefficients and molecular sizes of uniformly reactive spherical molecules. Several models incorporating steric requirements are used to rationalize the results. These models indicate that steric requirements for reaction in the diffusion-controlled limits are not greatly different in the α and β chains and are only slightly less stringent than for myoglobin.

In the absence of chemical activation effects (e.g., bond making and breaking) the measurement of the diffusion-controlled rate limit provides a measure of the steric requirements for reaction. When the solvent viscosity is increased, a reaction may become largely diffusion controlled. The influence of diffusion has been considered for ligand binding to Hb[1] (Hasinoff, 1978; Szabo, 1978; Morris & Gibson, 1980) and Mb (Hasinoff, 1977; Hasinoff & Chishti, 1982). A reanalysis of published data (Peak, 1982) has also pointed to the importance of diffusion in these systems. In glycerol–water solvents nonlinear Arrhenius plots of second-order ligand recombination rate constants for ligand binding to hemes and heme proteins are observed (Hasinoff, 1977, 1978, 1981; Austin et al., 1975; Alberding et al., 1978). In more recent studies on Mb (Beece et al., 1980; Hasinoff & Chishti, 1982) the CO recombination rate constant was observed to vary inversely as the solvent viscosity raised to a fractional power.

A common interpretation of the effect of solvent viscosity is through the use of the Stokes–Einstein expression

$$D = \mathbf{k}T/(6\pi r\eta) \tag{1}$$

($\mathbf{k}$ is Boltzmann's constant, r is the molecular radius, and η is the solvent viscosity) in the simple von Smoluchowski (1917) expression

$$k_D = 4\pi RDN/1000 \tag{2}$$

(k_D is the diffusion-controlled rate constant in M^{-1} s^{-1}, R is the encounter distance in cm, D is the sum of the translational diffusion coefficients of the reacting species in cm^2 s^{-1}, and N is Avogadro's number). The encounter distance R is often taken as the sum of the radii of the two reacting species. The failure of eq 1 for small molecular solutes is well-known (Jordan et al., 1956; Lamm & Sjostedt, 1938) and has been reconfirmed in recent extensive studies of the effect of solvent viscosity on small solute diffusion coefficients (Evans et al., 1979, 1981; Davis et al., 1980). The striking empirical correlation between the small molecule diffusion coefficient and solvent viscosity that was found is contained in

$$D = AT/\eta^p \tag{3}$$

where D is the diffusion coefficient in cm^2 s^{-1}, A an empirical constant, and η the solvent viscosity in Pa s (1 mPa s = 1 cP). The viscosity exponent p is a function of solute size given by

$$p = -1.296\ \text{Å}/r + 1.666 \tag{4}$$

where the solute radius is estimated simply from

$$r = (V/N)^{1/3}/2 \tag{5}$$

where V is the molar volume of the pure solute in the liquid state at low pressure.

Combining eq 3 with eq 2 and including a factor f which might include steric, interaction, and probability factors give with $D_{CO} \gg D_\alpha, D_\beta$

$$k_D = f(4\pi RATN)/(1000\eta^p) \tag{6}$$

The viscosity dependence of eq 6 can be verified by measurement of the diffusion-controlled rate constant and correlated both with a p determined experimentally from eq 3 and with the more general empirical eq 4.

In general, any reaction is expected to be simultaneously influenced by chemical activation and diffusion control (Noyes, 1961) through

$$k^{-1} = k_D^{-1} + k_0^{-1} \tag{7}$$

where k is the observed second-order rate constant and the chemical activation k_0 has the usual transition state temperature dependence:

$$k_0 = (\mathbf{k}T/h)\exp[-\Delta H^\ddagger/(\mathbf{R}T)]\exp(\Delta S^\ddagger/\mathbf{R}) \tag{8}$$

where $\Delta H^\ddagger$ and $\Delta S^\ddagger$ are the activation enthalpy and entropy, respectively.

Experimental Procedures

Materials. The isolated α and β chains were prepared from freshly collected human blood that had been washed 3 times with 0.9% NaCl, lysed with distilled water, and freed from organic phosphates on a Sephadex G-25 column. The chains

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[1] Abbreviations: α and β, the ferrous α and β chains of human hemoglobin; Hb, hemoglobin; Mb, myoglobin; β_4, β chain tetramer.

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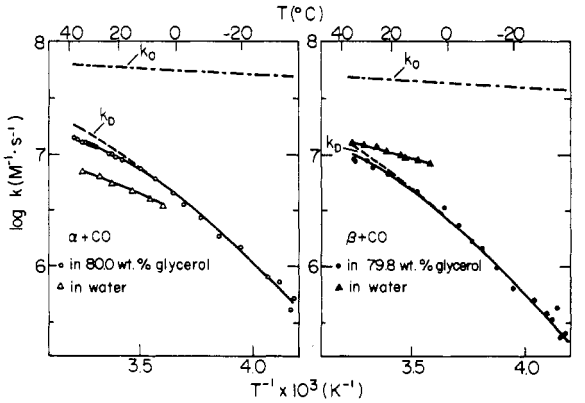

FIGURE 1: Arrhenius plots for the reaction of CO with isolated α and β chains of Hb in glycerol–water and water. (△, ▲) Water; (○, ●) glycerol–water. $[CO]_0$ = 450 μM. (—) Calculated from best fit parameters (Table I) to eq 7. The diffusion-controlled k_D (- - -) and chemical activation k_0 (— - —) components are indicated.

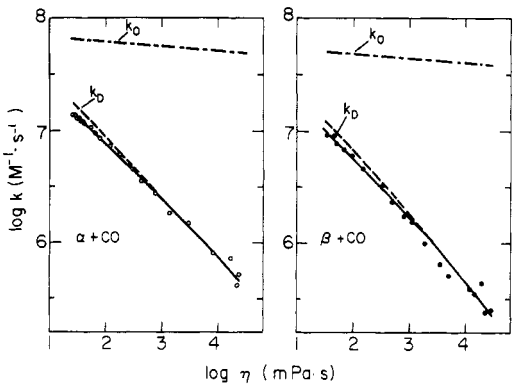

FIGURE 2: log k vs. log η plots for the reaction of CO with isolated α and β chains of Hb in glycerol–water. Other comments as in Figure 1.

were separated with *p*-(chloromercuri)benzoate according to the procedure of Bucci & Fronticelli (1965) and isolated on a DEAE-cellulose column (Geraci et al., 1969). Organic mercury bound to the α chain was removed with mercaptoethanol (Geraci et al., 1969) on a CM-cellulose column. Mercury bound to β chain was also removed with mercaptoethanol on a Sephadex G-10 column (Sugita et al., 1972). The absorption spectrum of the deoxy and CO derivatives was in agreement with Antonini et al. (1965). CO-saturated glycerol–water solutions were prepared by weight and contained 0.1 M pH 7.0 (aqueous) phosphate buffer. The total $[CO]_0$ was determined from solubility data (Ackerman & Berger, 1963).

Apparatus and Methods. The dye laser flash photolysis apparatus with its spectrophotometric detection has been described (Hasinoff, 1981) as has the data acquisition and analysis (Hasinoff, 1978). The reaction was followed at 435 nm with a large excess of $[CO]_0$ over the heme protein. The pseudo-first-order rate constant k_{obsd} was determined by a three-parameter nonlinear least-squares exponential fit. Averaged experimental second-order rate constants k were calculated from $k = k_{obsd}/[CO]_0$. The viscosities of the solutions were measured on a Wells-Brookfield viscometer. The viscosity varied about 1000-fold (27 to 28 000 mPa s) over the temperature range. The viscosity data were fit to a polynomial in T, and interpolated values were obtained at each temperature kinetic measurements were made.

Results and Discussion

Effect of Viscosity and Temperature on the Kinetics of the Isolated α and β Chain CO Recombination. The recombination of the isolated α and β chains with CO at long times after laser flash photolysis is, for example, in the case of αCO

$$\alpha CO \xrightarrow{h\nu} \alpha + CO \text{ (photodissociation)} \quad (9)$$

$$\alpha + CO \xrightarrow{k} \alpha CO \text{ (recombination)} \quad (10)$$

The Arrhenius plots are given in Figure 1, and log k is plotted as a function of log η in Figure 2. The accuracy of k is less at low temperatures since the fraction of CO that photodissociates into the bulk solvent is substantially decreased. The k values were analyzed by a three-parameter two-independent variable (η and T) weighted nonlinear least-squares analysis of eq 6 and 8 in eq 7 to obtain f, p, and $\Delta S^{\ddagger}$ (Table I), with the constraint $\Delta H^{\ddagger} = 0$. This was done as the four-parameter analyses that included $\Delta H^{\ddagger}$ (with the reasonable constraint $\Delta H^{\ddagger} > 0$) consistently converged on values of $\Delta H^{\ddagger}$ that were close to zero. This arises because the degree of diffusion control ($k_D^{-1} > k_0^{-1}$) over the whole temperature range is large. Hence k_0 and $\Delta S^{\ddagger}$ can be considered approximate only. The empirical constant A in eq 3 and 6 was estimated from D_{CO} = 2.0 × 10^{-5} cm^2 s^{-1} at 20 °C in water (Roughton, 1959) and the experimentally determined values of p of Table I. The encounter distance R in eq 2 was estimated from $R = r_\alpha + r_{CO}$ and $R = r_{\beta_4} + r_{CO}$ with r_{CO} = 1.9 Å from eq 5. Both isolated α and β chains by themselves associate to form dimers or tetramers, respectively (Valdes & Ackers, 1977, 1978). At the concentration of αCO (3.4 μM) used in this study the α chains in water can be expected to be present mainly as monomer. This is also likely to be the case in glycerol–water since the solvent properties are similar. However, in aqueous buffer solution at the concentration of βCO used (4.7 μM) the large majority of the hemes are present as the β_4 tetramer. Again glycerol–water should not be too different. From the molecular weight of the α monomer and β_4 tetramer r_α = 18.1 Å and r_{β_4} = 28.8 Å by using the method of Wherland & Gray (1976).

Table I: Diffusion and Chemical Activation Control Parameters for the Reaction of Isolated α and β Chains of Hb with CO in Glycerol–Water and Water[a]

reactant	steric factor f	$\Delta H^{\ddagger}$ (kcal mol^{-1})	$\Delta S^{\ddagger}$ (cal K^{-1} mol^{-1})	viscosity exponent p	k_0[b] (μM^{-1} s^{-1})
			Glycerol–Water		
α[c]	(3.0 ± 0.1) × 10^{-3}	0	−23 ± 1	0.50 ± 0.02	45
β_4[d]	(1.7 ± 0.1) × 10^{-3}	0	−23 ± 2	0.55 ± 0.03	70
			Water		
α		3.5 ± 0.7	−16 ± 2		5.3[e]
β_4		1.9 ± 0.8	−20 ± 3		11[e]

[a] In pH 7.0 (aqueous) 0.1 M phosphate buffer. Error values are fitting errors only from least-squares analyses. [b] Chemical activation controlled rate constant at 20 °C from eq 8. [c] In 80.0 wt % glycerol. [d] In 79.8 wt % glycerol. [e] Compares to k_0 (20 °C, pH 7, 0.05 M phosphate) average of flow and flash measurements for α chain of 5.2 μM^{-1} s^{-1} and β chain 12 μM^{-1} s^{-1} (Geraci et al., 1969).

An independent check of eq 3 and hence eq 6 can be made from polarographically measured diffusion coefficients for the similarly sized O_2 in glycerol–water (Jordan et al., 1956; Ackerman et al., 1963). Linear least-squares fits of the logarithmic form of eq 3 give the viscosity exponent p values of Table II. A second independent check is the viscosity exponent p predicted from the molecular size of CO and O_2 in eq 4. The viscosity exponent p values for the α and β chains

Table II: Comparison of Viscosity Exponent p Determinations[a]

ligand	viscosity exponent p	determination
CO	0.49	molecular size[b]
CO	0.50 ± 0.02	α chain[c]
CO	0.55 ± 0.03	β chain[c]
CO	0.55 ± 0.03	Mb[d]
O_2	0.44	molecular size[e]
O_2	0.48 ± 0.05	D_{O_2} in glycerol-water[f]
O_2	0.53 ± 0.08	D_{O_2} in glycerol-water[g]
O_2	0.37 ± 0.07	D_{O_2} in sucrose-water[f]

[a] Error values are from least-squares fits only. [b] From eq 4 with r_{CO} = 1.93 Å from eq 5. [c] This study. [d] In 80 wt % glycerol-water (Hasinoff & Chishti, 1982). [e] From eq 4 with r_{O_2} = 1.80 Å from eq 5. [f] From data from Jordan et al. (1956) in eq 3. [g] From data from Ackerman et al. (1963) in eq 3.

Table III: Diffusion Control Steric Factors for Models of the Reaction of CO with α and β Hb Chains and Mb[a]

model	steric parameter	reaction		
		α + CO	β_4 + CO	Mb + CO[b]
surface reactivity[c]	f(exp)	3.0×10^{-3}	1.7×10^{-3}	1.4×10^{-3}
	f(calcd)	1.4×10^{-3}	0.54×10^{-3}	1.1×10^{-3}
	A_b (Å^2)	25	35	14
solid angle[d]	θ (deg)	23	22	18
	r_T (Å)	0.8	0.7	0.6
reactive site[e]	a (Å)	0.20	0.18	0.1
buried active site[f]	θ' (deg)	7	5	4

[a] From 79.6 to 80.0 wt % glycerol in pH 7 (aqueous) 0.1 M phosphate buffer. [b] Hasinoff & Chishti (1982). [c] Weller (1961). [d] Schurr & Schmitz (1976). [e] Shoup et al. (1981). [f] Buried to a depth of $^1/_2 r_p$ (Samson & Deutch, 1978).

are very close to values determined for the reaction of Mb and CO in glycerol–water (Hasinoff & Chishti, 1982), indicating that p is independent of the protein. This experimental result is inconsistent with the mechanism of Beece et al. (1980) in which the viscosity exponent is said to be due to the solvent viscosity being "shielded and attenuated" by the protein and thus, it follows, protein dependent.

It has been noted that k for Mb at first increases upon the addition of glycerol to water (McKinnie & Olson, 1981; Hasinoff, 1977) but then decreases as the viscosity affects the reaction through diffusion control. Similarly here the chemical activation k_0 values for α and β chains are approximately some 6 and 8 times greater than in water. This increase in rate comes about from the decrease in $\Delta H^{\ddagger}$ in glycerol–water. Free energy changes in either the reactants or the transition state with changing solvent composition are well-known.

Diffusion Models with Steric Constraints. The diffusion of reactant to a sterically hindered site on a protein is predicted to result in a significant reduction in the diffusion-controlled rate constant (Weller, 1961; Hill, 1975; Schurr & Schmitz, 1976; Samson & Deutch, 1978; Shoup et al., 1981; Chou & Zhou, 1982). The Weller (1961) surface area reactivity model uses the fraction of the surface area of each spherical molecule that is reactive and gives $f = \sigma_P \sigma_{CO}$ where σ_P is the fraction of the surface area of the heme protein that is reactive. Since only half of CO is reactive $\sigma_{CO} = {}^1/_2$. The value of σ_P calculated from the experimental f can be compared with σ_P, obtained from a simple model based on the ratio of the cross-sectional area of CO to the surface area of the protein: $\sigma_P = \pi r_{CO}^2/(4\pi r_P^2)$. While this model is perhaps overly simplistic, it is useful because it has no adjustable parameters and thus allows a direct comparison with the experimental f's. If the reactive site is considered to be a hole rather than a patch on the surface of a sphere through which CO must enter (Hill, 1975), then the σ_P above is the special case of $r_h = 2r_{CO}$ in $\sigma_P = \pi(r_h - r_{CO})^2/(4\pi r_P^2)$ where r_h is the radius of the "hole". The observed f for the α and β_4 chains is respectively 2 and 3 times larger than the simple Weller model predicts (Table III). By contrast the f found for Mb was close to that predicted. The values of the steric parameters of Table III for the β_4 tetramer are based on the assumption that on the large majority of the β_4 molecules only a single CO is photodissociated because the fraction of CO that is photodissociated is small over most of the temperature range. On this basis the area of the binding site, A_b, of a single reactive uncomplexed heme on the β_4 tetramer is some 40% larger than on an α chain.

The solid angle model of Schurr & Schmitz (1976) in which both translational diffusion and rotational diffusion were considered gives $f = (1 - \cos\theta)\theta r_{CO}/(r_P + r_{CO})$ where θ is the minimum half-cone solid angle (in rad) over which reaction can take place and $r_{CO}\theta = r_T$ is the hemispherical target radius. In this model α and β_4 chains are equally reactive but are both more reactive than Mb.

The Shoup et al. (1981) model, using new boundary conditions to yield analytical solutions, considers the encounter of a uniformly reactive ligand to a small reactive site of radius a. If rotational diffusion of the protein is unimportant, $f = 3\pi a/[32(r_P + r_{CO})]$. In this model the α and β_4 binding sites are of about the same size and both are larger than Mb.

The buried active site model of Samson & Deutch (1978) considers the diffusion of a unrealistic pointlike substrate molecule to a reactive spherical buried cap. The experimental f yields the half-cone angles θ' formed by the cap at $^1/_2 r_P$. In this model the opening angle of the α chain is larger than for β_4 and both are larger than for Mb.

The consistent picture that is obtained by using these different models is one in which α and β_4 chains in the diffusion-controlled limit are of approximately equal reactivity and both are significantly more reactive than Mb. In water as in glycerol–water α and β_4 are both considerably more reactive (~10-fold) than Mb (k = 0.5 μM^{-1} s^{-1}) (Parkhurst, 1979; Moffat et al., 1979), consistent with a more hindered heme in Mb, though this difference is reduced in the diffusion-controlled limit. The isolated chains are probably similar in conformation to the fast-reacting R state of "oxy" Hb. It was also concluded in a review of the literature (Parkhurst, 1979) on the question of α–β chain inequivalence in R-state human Hb tetramer that at least for CO there is not good evidence for significant kinetic heterogeneity. It is clear that the heme pocket of Mb and Hb is quite crowded. Case & Karplus (1978) estimated that in Mb for an assumed linear perpendicular CO the oxygen of the CO would be but 2.1 Å away from the N_ϵ of the distal His-E7, which is less than the minimum van der Waals' distance of 3.2 Å. In human HbCO (Baldwin, 1980) this same distance would be 3.6 and 3.1 Å for the α and β subunits. (Actual distances for the bent CO are 4.1 and 3.4 Å.) These results are consistent with the diffusion-controlled kinetics in which the Hb chains have a more accessible heme pocket than in Mb.

Registry No. Carbon monoxide, 630-08-0.

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Human Skin Fibroblast Procollagenase: Mechanisms of Activation by Organomercurials and Trypsin†

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ABSTRACT: Pure human skin fibroblast procollagenase has been utilized in this study as a model system in which to examine the pathways of organomercurial and trypsin activation. Three organomercurials, *p*-(hydroxymercuri)benzoate, mersalyl, and *p*-aminophenylmercuric acetate, were able to fully activate human skin procollagenase with no accompanying loss of molecular weight. Lower molecular weight species were subsequently produced, particularly with a fourth organomercurial, phenylmercuric chloride. The activation process was dependent upon the concentration of the organomercurial compound and the time of incubation, but not on enzyme protein concentration. No evidence of a role for free sulfhydryls was found. Trypsin produced an initial cleavage product of procollagenase which was collagenolytically inactive yet underwent a concentration independent autocatalysis. Thus, procollagenase appeared to have an autocatalytic property which was enhanced by treatment with a variety of agents, all of which may function by perturbation of the zymogen conformation.

The degradation of collagen is initiated by a specific class of proteases, the collagenases. In general, these enzymes, at least in mammalian species, are found in an inactive state, and, consequently, the nature of this latency and its modulation are factors crucial to the regulation of the connective tissue matrix [for recent reviews, see Murphy & Sellers (1980) and Bauer et al. (1982)]. This latency has been variously attributed to the secretion of the enzyme as a zymogen (Vaes, 1971, 1972a,b; Harper et al., 1971; Stricklin et al., 1977), or as an enzyme–inhibitor complex (Abe & Nagai, 1972; Abe et al., 1973; Sellers et al., 1977). Much uncertainty concerning the nature of latent collagenase has been engendered by the multitude of pathways by which activation occurs such as proteolysis by trypsin (Vaes, 1972a; Harper et al., 1971; Bauer et al., 1975) or tissue proteases (Vaes, 1972a; Harper et al., 1971), incubation with chaotropic ions such as I^- or SCN^- (Abe & Nagai, 1972; Abe et al., 1973), the action of nonenzymatic tissue activators (Tyree et al., 1981), autoactivation

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