sulfur atoms in biotin, thiamin, and iron–sulfur proteins.

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Viscosity Dependence of the Kinetics of the Diffusion-Controlled Reaction of Carbon Monoxide and Myoglobin†

Brian B. Hasinoff* and S. Bano Chishti

ABSTRACT: The kinetics of the reaction of CO with myoglobin have been studied by laser flash photolysis in glycerol–water as a function of solvent viscosity and temperature. At high viscosities and low temperatures the second-order rate constant is inversely proportional to the viscosity raised to approximately the 0.5 power. This result parallels the behavior of the oxygen diffusion coefficient in glycerol–water. It is concluded that the reaction kinetics in high viscosity glycerol–water are largely diffusion controlled. At higher temperatures, though, the effect of simultaneous chemical activation control of the reaction is observed. The diffusion-controlled rate constant is 1.4×10^{-3} of that predicted from simple von Smoluchowski theory based on diffusion coefficients and molecular radii. Several models with steric requirements for diffusion-controlled reactions are examined.

The effect of solvent viscosity on the binding of CO or O_2 to Mb[1] in glycerol–water has been the object of several studies (Strother et al., 1959; Fesenko et al., 1972; Austin et al., 1975; Hasinoff, 1977; Beece et al., 1980; McKinnie & Olson, 1981). Nonlinear Arrhenius plots of the second-order ligand recombination rate constant were interpreted in a previous study as due to simultaneous chemical activation and diffusion control of the reaction (Hasinoff, 1977). At temperatures below ~ -25 °C, an additional fast phase is seen exhibiting kinetics indicative of a caged geminate transient diffusion process (Hasinoff, 1981). In a recent study (Beece et al., 1980) the ligand combination rate constant was observed to vary inversely as the solvent viscosity raised to a fractional power.

Simple von Smoluchowski (1917) theory predicts that for a diffusion-controlled reaction

$$k_D = 4\pi RDN/1000 \quad (1)$$

where 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

† From the Department of Chemistry and Faculty of Medicine, Memorial University of Newfoundland, St. John's, Newfoundland, Canada A1B 3X7. *Received March 25, 1982.* This work was supported in part by Natural Sciences and Engineering Research Council (Canada) Grant A9430.

* Address correspondence to this author at the Department of Chemistry.

[1] Abbreviation: Mb, ferrous myoglobin.

0006-2960/82/0421-4275$01.25/0

translational diffusion coefficients of the two reacting species in $cm^2\ s^{-1}$, and N is Avogadro's number. The Stokes–Einstein expression

$$D = \mathbf{k}T/(6\pi r\eta) \quad (2)$$

(where $\mathbf{k}$ is Boltzmann's constant, η is the solvent viscosity, and r is the molecular radius) has often been used with eq 1 to yield a result that predicts that $k_D \propto 1/\eta$. The failure of eq 2 to predict physically reasonable radii for small molecules is well-known. This has also been noted for O_2 (Jordan et al., 1956) and H_2O (Lamm & Sjostedt, 1938) in glycerol–water. Thus in the previous study (Hasinoff, 1977) effective Stokes' radii from eq 2 were used. However, more recently (Evans et al., 1981; Davis et al., 1980; Evans et al., 1979) extensive studies have been made of the variation of solute diffusion coefficients with viscosity for a range of small (monoatomic) to large solutes in a number of solvents covering a wide range of temperatures and solvent viscosities. The failure of eq 2 has been reconfirmed for small solutes, and some striking empirical correlations have been presented for the relationship between the diffusion coefficient and the viscosity that involve the molecular size of the small solute:

$$D = AT/\eta^p \quad (3)$$

where D is the diffusion coefficient in $cm^2\ s^{-1}$, A is an empirical constant, T is the absolute temperature, η is the solvent viscosity in Pa s, and the viscosity exponent p is a function of solute size:

$$p = -1.296\ \text{Å}/r + 1.666 \quad (4)$$

The solute radius is estimated from

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

where V is the molar volume in the liquid state at a low pressure.

Inclusion of a factor f in eq 1 combining steric, interaction, and probability factors yields

$$k_D = f(4\pi RDN)/1000 \quad (6)$$

The diffusion of reactant to a sterically restricted site on a protein is predicted to result in a significant reduction in k_D (Hill, 1975; Schurr & Schmitz, 1976; Samson & Deutch, 1978; Shoup et al., 1981). With the empirical eq 3 in eq 6 where D is that of the small ligand ($D_{CO} \gg D_{Mb}$)

$$k_D = f(4\pi RATN)/(1000\eta^p) \quad (7)$$

Equation 7 provides a rational basis for the effect of viscosity on a diffusion-controlled reaction. For verification of eq 7, the exponent p can be determined three ways: (i) by kinetic means through eq 7, (ii) from the empirical correlation involving molecular size in eq 4, and (iii) through the viscosity dependence of the ligand diffusion coefficient in eq 3. A correspondence between these would be strong evidence in favor of the influence of diffusion on the reaction. In general a reaction is expected to be simultaneously influenced by chemical activation and diffusion control (Noyes, 1961) with

$$k^{-1} = k_D^{-1} + k_0^{-1} \quad (8)$$

where the chemical activation rate constant k_0 has the usual transition state temperature dependence:

$$k_0 = (\mathbf{k}T/h)\exp[-\Delta H^{\ddagger}/(RT)]\exp(\Delta S^{\ddagger}/R) \quad (9)$$

where $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are the activation enthalpy and entropy, respectively. A determination of the variation of k over a much greater range of η and T than previously studied (Hasinoff, 1977) is necessary in order to verify eq 7 and the influence of diffusion control on the reaction of Mb and CO.

Table I: Diffusion and Chemical Activation Control Parameters for the Reaction of Mb and CO in Glycerol-Water[a]

[CO] (μM)	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})
16[c]	$(1.6 \pm 0.2) \times 10^{-3}$	0.6 ± 1.0	-26 ± 3	0.56 ± 0.02	4.6
440[d]	$(1.1 \pm 0.1) \times 10^{-3}$	0.0 ± 2.5	-27 ± 8	0.54 ± 0.03	6.5

[a] In pH 7.0 (aqueous) 0.1 M phosphate buffer. Error values are fitting errors only from nonlinear least-squares analysis. [b] Chemical activation controlled rate constant at 20 °C from eq 9. [c] In 80.0 wt % glycerol. [d] In 79.6 wt % glycerol.

Experimental Procedures

Materials. Myoglobin (sperm whale), dissolved in 0.1 M pH 7.0 KH_2PO_4–NaOH buffer, reduced with a few grains of dithionite and saturated with CO, was added to glycerol–water solutions prepared by weight also containing 0.1 M pH 7.0 (aqueous) phosphate buffer. The initial CO concentrations ($[CO]_0$) were determined from data for the solubility of CO in glycerol–water mixtures (Ackerman & Berger, 1963).

Apparatus and Methods. The laser low-temperature flash photolysis apparatus (maximum 3 J output at 578 nm with Rhodamine 590 dye, 300 ns pulse width) and spectrophotometric detection system have been described (Hasinoff, 1981) as has the analogue to digital data aquisition system and data analysis (Hasinoff, 1977). Reactions were followed at 440 nm under pseudo-first-order conditions. Averaged pseudo-first-order rate constants, k_{obsd}, obtained from three-parameter nonlinear least-squares exponential fits, were used to calculate the experimental second-order rate constant from $k = k_{obsd}/[CO]_0$. The viscosities of the reaction solutions were measured from 35 to −15 °C (~1600 mPa s; 1 mPa s = 1 cP) on a calibrated cone-plate Wells-Brookfield microviscometer. Overlapping (by some 35 °C) measurements were made on larger volumes of matched composition with the accessory spindle apparatus down to −37 °C ($\eta \sim 46000$ mPa s). The viscosity data were fit to the empirical three-parameter (B, C, and T_0) equation $\eta = C\exp[-B/(T - T_0)]$ (Buxton et al., 1975) which provides an accurate fit to mass transport processes in glass-forming liquids. From this empirical equation, interpolated and extrapolated (a maximum of 10 °C at the lowest temperatures) values of η were calculated at each temperature kinetic measurements were made.

Results and Discussion

Effect of Viscosity and Temperature on Mb plus CO Recombination. The recombination of Mb with CO at long times after laser flash photolysis is

$$\text{MbCO} \xrightarrow{h\nu\ \sim} \text{Mb} + \text{CO (photodissociation)} \quad (10)$$

$$\text{Mb} + \text{CO} \xrightarrow{k} \text{Mb} + \text{CO (recombination)} \quad (11)$$

Arrhenius plots of the data are given in Figure 1. The values of k (Figures 1 and 2) as a function of both η and T were analyzed by four-parameter weighted nonlinear least-squares analysis of eq 7 and 9 in eq 8 to obtain f, p, $\Delta H^{\ddagger}$, and $\Delta S^{\ddagger}$ (Table I). The chemically reasonable constraint that $\Delta H^{\ddagger} > 0$ was used in the analysis. The encounter distance R in eq 7 was estimated from $R = r_{Mb} + r_{CO}$ with $r_{Mb} = 20$ Å (Hasinoff, 1977) and $r_{CO} = 1.9$ Å from eq 5. The value of the empirical constant A in eq 7 was estimated from $D_{CO} = 2 \times 10^{-5}\ cm^2\ s^{-1}$ at 20 °C in water (Roughton, 1959) and the

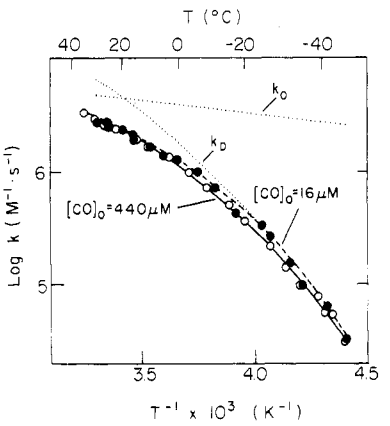

FIGURE 1: Arrhenius plots for the reaction of CO with Mb in glycerol–water. (●) $[CO]_0$ = 16 μM; (○) $[CO]_0$ = 440 μM. Calculated from best-fit parameters (Table I) to eq 8 for the (---) 16 and (—) 440 μM $[CO]_0$ data, respectively. For the $[CO]_0$ 16 μM data, the diffusion-controlled k_D and chemical activation k_0 components (···) are also indicated.

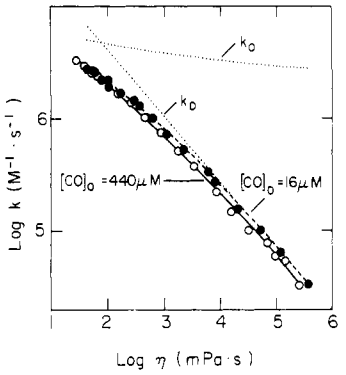

FIGURE 2: log k vs. log η plots for the reaction of CO with Mb in glycerol–water. Other comments as in Figure 1.

experimentally determined values of p of Table I in eq 3. The chemical activation rate constants, k_0, are not well determined as the degree of diffusion control over most of the temperature range is large.

Diffusion coefficients for O_2 have been measured polarographically in glycerol–water solutions of varying composition (Jordan et al., 1956; Ackerman et al., 1963), thus providing an experimental test of eq 3 and a comparison with the exponent p predicted from molecular size in eq 4. These values can also be compared with the kinetically measured values of p in Table I, since CO and O_2 are very similar in size and their p values should show a correspondence. Linear least-squares analysis of a logarithmic form of eq 3 gave the viscosity exponent p values of Table II. The good agreement between p values determined these different ways is strong evidence for the effect of diffusion control on reaction 11. Using a different kinetic model, Beece et al. (1980) found that ligand binding to Mb gave for CO a second-order rate constant proportional to $1/\eta^{0.6}$ and for O_2 $1/\eta^{0.5}$ which is in good agreement with the p values of Table II.

Diffusion Models with Steric Constraints. The binding of ligand to the Fe^{2+} in the restricted heme pocket of Mb might be expected to result in a significant rate reduction. Several models are available on which to rationalize the average experimental steric factor f of 1.35×10^{-3}.

The Weller (1961) surface reactivity model uses the fraction of surface area of each spherical molecule that is reactive and gives $f = \sigma_{Mb}\sigma_{CO}$. With $\sigma_{CO} = {}^1/_2$ and σ_{Mb} given by the ratio of the cross-sectional area of CO to the surface area of Mb, $\sigma_{Mb} = \pi r_{CO}^2/(4\pi r_{Mb}^2) = 2.2 \times 10^{-3}$, the predicted f is 1.1×10^{-3} which is in excellent agreement with the experimental value.

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

ligand	viscosity exponent p	determination
CO	0.49	molecular size[b]
CO	0.56 ± 0.02	kinetic[c]
CO	0.54 ± 0.03	kinetic[c]
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 SE's from least-squares fits. [b] From eq 4 with r_{CO} = 1.93 Å from eq 5. [c] This study, at [CO] = 16 μM. [d] This study, at [CO] = 440 μM. [e] From eq 4 with r_{O_2} = 1.80 Å from eq 5. [f] Data from Jordan et al. (1956) in eq 3. [g] Data from Ackerman et al. (1963) in eq 3.

The Schurr & Schmitz (1976) solid angle model considered both translational diffusion and rotational diffusion and gives $f = (1 - \cos\theta)\theta r_{CO}/(r_{Mb} + r_{CO})$ where θ is the minimum half-cone solid angle (in rad) necessary for reaction and $r_{CO}\theta = r_T$ is the hemispherical target radius. The experimental f yields $\theta = 18°$ and $r_T = 0.6$ Å.

The Hill (1975) planar capture window model in which a "hole" on the surface of Mb captures CO once it has diffused through yields $f = (R_w - r_{CO})/[\pi(r_{Mb} + r_{CO})]$ in terms of the window radius R_w. Thus with $R_w - r_{CO}$ of 0.09 Å, the window is but 5% larger than CO, a result that is again consistent with a relatively restricted binding site on Mb.

The application of new boundary conditions (Shoup et al., 1981) has yielded analytical solutions for previously intractable problems. For reaction of a uniformly reactive ligand with a small reactive site of radius a on a macromolecule where rotational diffusion is unimportant, $f = 3\pi a/[32(r_{Mb} + r_{CO})]$. The radius of the reactive site is 0.1 Å which is much less than the simple surface reactivity model predicts.

In a model that is perhaps more realistic for Mb, with its binding site in the heme pocket, the effect of burying the active site inside an inert sphere was considered (Samson & Deutch, 1978). In the absence of rotational diffusion for a pointlike (unrealistic) substrate molecule for an active site at a depth of ${}^1/_2 r_{Mb}$ (10 Å) the half-cone angle formed by the spherical buried cap is 4°, indicating once more a restricted binding site.

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Kinetics of Reduction of the Intersubunit Disulfides of the Carboxyl Propeptide of Type I Procollagen†

Phyllis Anne Kosen‡ and Carl Franzblau*

ABSTRACT: The carboxyl propeptide produced by proteolysis during maturation of type I procollagen to collagen was purified to homogeneity from the medium of cultured chick embryo calvaria by a new method. The propeptide was identified as such by its amino acid composition and migration pattern through sodium dodecyl sulfate-polyacrylamide gels in the absence and presence of dithiothreitol. Reduction of the intersubunit disulfides, which covalently join the two C1 and one C2 polypeptides of the carboxyl propeptide, was studied by incubating the propeptide in the presence of dithiothreitol for various times under nondenaturing conditions at pH 8.2. The reduction process was characterized by the appearance of disulfide-linked dimers. The appearance of dimers correlated with the disappearance of the carboxyl propeptide. Monomers, retaining intrasubunit disulfides, appeared concomitant with dimer formation. Reduction of the intersubunit disulfides of the dimers followed; intrasubunit disulfides were retained. The rate of the first process, trimer to dimer plus monomer, was an order of magnitude larger than the rate for the second process, dimer to monomers. The dimeric intermediates were composed of approximately equivalent amounts of $(C1)_2$ and (C1, C2). The kinetics of formation and reduction of $(C1)_2$ and (C1, C2) could not be differentiated by the techniques used. The relative amounts of intermediates found were not those expected if quasi-equivalent intersubunit disulfides were reduced in a random fashion. A possible model for reduction of the intersubunit disulfides of the propeptide has been proposed, and implications for the intersubunit polypeptide surface contacts have been discussed.

The carboxy-terminal extension of the several pro α[1] chains of the procollagens has been strongly implicated as the site for pro α chain association during collagen maturation (Schofield et al., 1974; Harwood et al., 1976, 1977; Rosenbloom et al., 1976; Kao et al., 1979; Bächinger et al., 1980; Gerard et al., 1981). Little is known concerning the conformation and other possible functions of the carboxy-terminal extension; however, the amino acid sequences of the type I extensions have been deduced from nucleotide sequences of cDNA clones (Fuller & Boedtker, 1981). Unlike the amino-terminal extension which can be obtained in large yield from skins of dermatosporatic animals (Becker et al., 1976; Engel et al., 1977), carboxy-terminal extensions are not readily available; only the carboxyl propeptide derived from type I procollagen has been obtained in reasonable quantities (Olsen et al., 1977). This carboxyl propeptide, formed by proteolysis during the maturation of type I procollagen to collagen, appears to be potentially useful for studying processes involved in the folding of polypeptides and in polypeptide chain association for reasons outlined below.

Both type I collagen and its biosynthetic precursor, type I procollagen, are composed of two genetically distinct, but related, chains which associate in a 2:1 ratio as $[\alpha1(I)]_2\alpha2$ and [pro $\alpha1(I)]_2$ pro $\alpha2$, respectively. This ratio predominates in vivo, but a procollagen and a collagen composed of only a single polypeptide ([pro $\alpha1(I)]_3$) and $[\alpha1(I)]_3$ have also been identified (Mayne et al., 1976; Jimenez et al., 1977; Little et al., 1977; Wohllebe & Carmichael, 1978; Crouch & Bornstein, 1978; Smith & Niles, 1980). On the other hand, neither [pro $\alpha2]_3$ nor $[\alpha2]_3$ has ever been identified in vivo. Further, in tissues or cell cultures producing more than one type of collagen, heterogeneous mixtures of genetically distinct collagen

† From the Department of Biochemistry, Boston University School of Medicine, Boston, Massachusetts 02118. *Received January 19, 1982.* This work was supported by Grants HL13262 and HL19717 from the National Institutes of Health and Postdoctoral Fellowship Grant 5F32HL05874 to P.A.K. from the National Institutes of Health.

‡ Present address: Department of Chemistry, Reed College, Portland, OR 97202.

[1] Abbreviations: pro α, intact polypeptide chain of procollagen; pC-collagen, procollagen lacking the amino-terminal extensions; carboxyl propeptide, disulfide-linked carboxy-terminal extensions derived from proteolysis during maturation of type I procollagen to collagen; C1 and C2, individual polypeptide chains of the carboxyl propeptide without reference to the redox state of the chains' cysteines; T and T′, trimeric states of the carboxyl propeptide visualized on polyacrylamide gels (T′ is an open form of T); D and M, dimeric and monomeric states, respectively, of the carboxyl propeptide visualized on polyacrylamide gels (both species retain intrasubunit disulfides, and D retains intersubunit disulfides also); Con A, concanavalin A; DEAE, diethylaminoethyl; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; TPE buffer, 0.1 M Tris-PO_4, pH 8.2, 1.0 M K_2HPO_4, and 5 mM EDTA; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/82/0421-4278$01.25/0