THE RATE OF ENTRY OF DIOXYGEN AND CARBON MONOXIDE INTO MYOGLOBIN

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ABSTRACT The model for carbon monoxide or dioxygen recombination with heme proteins developed by the group at the University of Illinois is reexamined. We propose that the carbon monoxide or dioxygen molecule enters the protein at essentially a diffusion-limited rate determined by the solvent viscosity and that the protein offers no important barriers to this entry. The viscosity dependence of the entry rate k_{ED}, its magnitude (1×10^{10} $M^{-1}s^{-1}$), and the rate of quenching of triplet states of protoprophyrin IX in apomyoglobin by dioxygen are used as supporting evidence. Comparison is made to the model of a fluctuating protein developed by G. Weber (1).

INTRODUCTION

The classic picture of a protein molecule as presented by X-ray crystallography is a static one, yet most protein molecules spend their working lives in fluid environments. How rigid is a globular protein in solution? How readily can small molecules penetrate the interior of a globular protein? Basically, there are two opposing models of protein permeability in solution, with supporting experiments. (*a*) The fluorescence quenching experiments of Lakowicz and Weber (1) showed that tryptophan groups buried within protein molecules were quenched by dioxygen at nearly the rate they were quenched free in solution. The model that comes from these carefully performed experiments is one of a floppy protein molecule, with large fluctuations in conformation on the nanosecond time scale. The recent calculations of Karplus (2) also seem to support this view. (*b*) An opposite view comes from the phosphorescence work of Saviotti and Galley (3). In their experiments, the phosphorescence of tryptophan groups in the interiors of some proteins can have lifetimes in the tenths of seconds range at room temperature in air-saturated water. This implies an access rate of dioxygen to the interior of the protein very much less than the rates seen by Lakowicz and Weber.

The work of the group at Illinois (4–6) using the recombination kinetics of carbonmonoxide or dioxygen with heme proteins also provides information on the access of a small ligand with the interior of a protein. In this communication we would like to examine the Illinois model and see whether it can be interpreted in terms of either of the two opposing models just stated.

The particular question we would like answered by the carbon monoxide recombination work is: What is the rate at which the carbon monoxide molecules enter the heme pocket of the protein? In the Illinois experiment, it was possible to separate internal recombination from external, concentration-dependent recombination. This

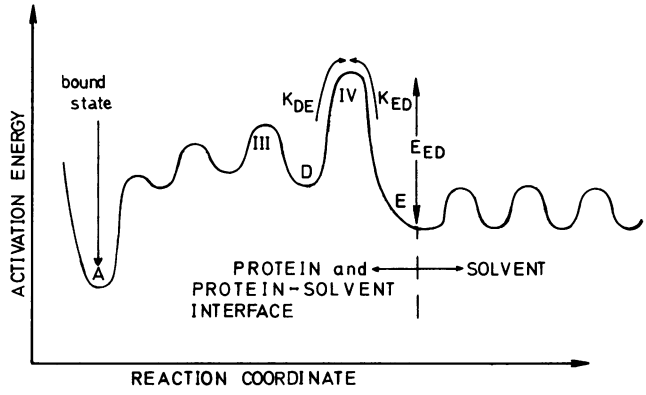

FIGURE 1 The postulated energy barrier scheme for CO recombination with Mb, taken from Austin et al. (4). Barrier IV represents the solvent-protein interface that the CO must surmount to enter the heme crevice. The solvent is represented by the smaller activation energy barriers to the right. To be consistent with Alberding et al. (5,6), where barrier III is lacking, the reader should rename barrier III barrier II and well D well C for those cases where no evidence for barrier III exists.

allowed the construction of a set of differential equations with one of the rate parameters interpreted as the actual rate of entry of the carbon monoxide molecule into the binding area near the heme. Fig. 1 shows the scheme of activation energy barriers and their associated rates as postulated by the Illinois group. The barrier of most interest to us here is barrier IV, and the rate k_{ED} for passage over it. Ideally, k_{ED} should represent the rate of entry of the carbon monoxide into the protein (as distinguished from binding at the iron site) and k_{DE} the rate of exit. This means that the rate k_{ED} should be roughly the same as the dioxygen quenching rate measured in the fluorescence quenching experiments. We have used the techniques of transient optical anisotropy decay and triplet lifetime quenching in an attempt to test this proposal.

METHODS AND MATERIALS

The myoglobin (Mb) was purchased from Sigma Chemical Co. (St. Louis, Mo.). The protein was freshly dissolved in distilled water and centrifuged to remove any precipitates.

Glycerol, reagent grade, was purchased from the J. T. Baker Chemical Co. (Phillipsburg, N.J.). Glycerol-water mixture ratios were determined by measurement of the viscosity of the sample with a Schott Capillary Viscometer (Schott, Inc., New York) and compared to viscosities of known mixtures (Chemical Rubber Company Handbook of Chemistry and Physics, 1962). The protein sample was dialyzed for 2 days at 4°C against the desired glycerol-water mixture.

The carbon monoxide (CO) and argon gases used are 99.995% pure. Equilibration of the gas was via slow bubbling (CO) or by stirring under an atmosphere (Argon) for $\frac{1}{2}$ h. Dioxygen (O_2) was similarly introduced by slowly stirring the sample with exposure to air. Thus, the external O_2 pressure in these experiments was 0.21 bar, while the CO pressure was 1.0 bar. The solubilities of CO and O_2 in our solvents were taken from the Landolt-Boernstein tables (7).

The disodium salt of protoporphyrin IX was purchased from ICN Nutritional Biochemicals Div., International Chemical & Nuclear Corp. (Cleveland, Ohio). It was freshly dissolved in

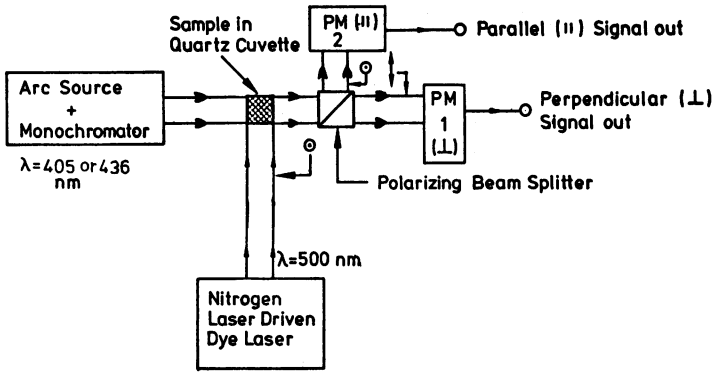

FIGURE 2 Block diagram of the apparatus. The polarization of the exciting laser pulse was out of the plane of the paper (⊙). The monochromator was set to 405 nm for PP9-Mb triplet state measurements (singlet depletion signal measured) or 436 nm for Mb-CO photolysis (creation of Fe[+2] state with no sixth ligand). The repetition rate of the laser was typically 10 Hz, and 1,000 shots were averaged at each temperature.

reagent grade dimethylformamide (DMF) purchased from Baker Chemical Co. to which 4 ml/1,000 ml of 1 N HC had been added. The apomyoglobin was prepared via the procedure of Rossi Fanelli et al. (8) and stored at −20°C in 50% glycerol. A Bio-Gel P-4 column (Bio-Rad Laboratories, Richmond, Calif.) equilibrated with a 50% glycerol-10 mM phosphate buffer system, pH 7.5, was used to separate unbound protoporphyrin IX (PP9) from the bound PP9-Mb complex.

The photolysis system is shown in Fig. 2. A nitrogen laser (Lambda Physik GmbH, Göttingen, W. Germany) pumped a Coumarin 307 dye laser, emission at 500 nm and pulse duration 5 ns. The polarization of the laser output was approximately 90%, vertical. The unpolarized emission of a mercury-xenon arc lamp was passed through a monochromator and through the sample. The transmitted beam passed through a polarizing beam splitter into two 1P28A/V1 photomultipliers. The anode had a 1-kohm load resistor followed by a LH0033 high-speed buffer amplifier (National Semiconductor Corp., Santa Clara, Calif.) and a LeCroy VV100 amplifier (LeCroy Research Systems Corp., Spring Valley, N.Y.). The measured rise time of the entire system was 20 ns for a 5-ns light pulse. The outputs of the two channels were fed to a Tektronix 7A13 differential amplifier (Tektronix, Inc., Beaverton, Oreg.) and then to a Data Lab model DL920 transient recorder (Data Laboratories Ltd., Mitcham, Surrey, U.K.), maximum sample rate 20 MHz. The final rise time measured on the transient recorder was 100 ns, determined by the transient recorder amplifiers. The digital output of the transient recorder was averaged in a Fabritek averager (Fabri-Tek Instruments Inc., Madison, Wis.) and fed to a Univac computer for analysis.

The anisotropy was determined from the expression,

$$\alpha(t) = (\Delta A_{\parallel} - \Delta A_{\perp})/(\Delta A_{\parallel} + 2\Delta A_{\perp}), \quad (1)$$

where $\Delta A_{\parallel}$ and $\Delta A_{\perp}$ are the transient absorbance changes parallel and perpendicular, respectively, to the exciting laser polarization. The absorbance changes were measured at 436 nm for CO-Mb and 405 nm for protoporphyrin IX. The anisotropy corrected absorbance change was determined from the expression,

$$\Delta A = \Delta A_{\parallel} + 2\Delta A_{\perp}. \quad (2)$$

RESULTS AND DISCUSSION

In the anisotropy decay experiments, the excitation laser pulse photolyzed the CO-Fe bond, creating an absorbance change. The photolyzing beam was polarized in the vertical direction and thus created a population of photolyzed Mb molecules whose heme planes were preferentially aligned along the exciting laser beam polarization vector. The unpolarized monitoring beam was at a right angle to the exciting beam and becomes polarized after passage through the photolyzed sample because of the induced optical anisotropy. A complete calculation for a chromophore of planar symmetry shows that the maximum anisotropy expected is 0.14 (9). In reality, our anisotropy 100 ns after the flash varied from 0.045 to 0.07, the higher initial anisotropy occurred in the most viscous solvents. The subsequent anisotropy decay was always a good fit to a single exponential even when the absorbance changes with time were complex, as is true for Mb-CO at low temperatures. This also implies that our corrected absorbance changes had no polarization effects in them. Corrections for polarization effects in optical photolysis or other techniques (such as T-jump) in viscous solvents or for large linear molecules like DNA are not generally done, although it is possible to get very large, apparent "absorbance changes" which are nothing more than induced dichroism changes due to alignment of an asymmetric chromophore.

The anisotropy decay rate is the rotational rate of the Mb molecule, k_{rot}. From the Stokes-Einstein relation, we can determine either the hydrodynamic radius of the protein molecule (r) or the viscosity of the medium η:

$$k_{rot} = 3k_B T/4\pi r^3 \eta. \tag{3}$$

Thus, if the viscosity of the medium is measured at some temperature and the hydrodynamic radius of the protein calculated, it is then possible to determine the viscosity of the medium at any other temperature if it is assumed that the radius of the protein does not change. In Fig. 3 we have plotted log k_{rot} vs. $1/T$ over the range of temperatures of interest. In this manner we obtain viscosities difficult to measure by normal means. We calculate the hydrodynamic radius of Mb to be 2.4 nm at 293 K, in good agreement with the approximate value of 2.0 nm as determined from X-ray crystallography.

The slope of $\log(k_{rot}/T)$ vs. $1/T$ is a measure of the activation energy for viscosity changes, E_η. Although we have plotted log k_{rot} vs. $1/T$, over our temperature range and with our error bars the curvature induced by the correct formulation is slight and the slope given remains valid. Because we determined the glycerol-water content of our sample as 84% by volume, we denote this activation energy as E_η (84%). In our preparation of the sample, we tried to duplicate the Illinois procedure as closely as possible. The percentage of glycerol is slightly different from the value quoted in refs. 4–6, although no absolute determination of the glycerol-water content was made at Illinois. This small difference should not affect the main conclusions.

Let us now look at barrier IV in Fig. 1 and its associated rates, k_{ED} and k_{DE}. We have plotted in column 1 in Table I the energy barrier height of IV for various heme

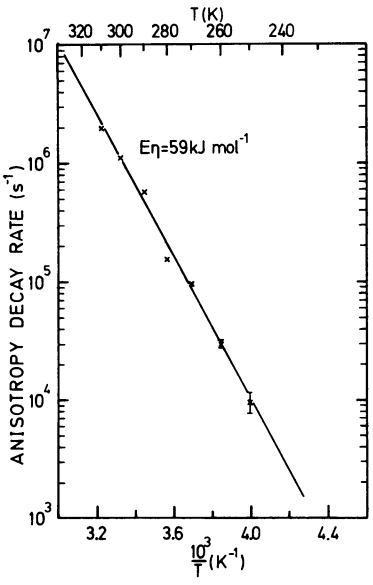

FIGURE 3

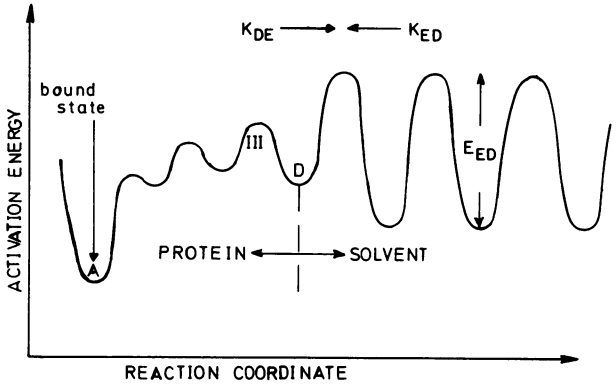

FIGURE 4

FIGURE 3 The log of the anisotropy decay rate of Mb, k_{rot}, vs. $1/T$. The solvent was 84% glycerol-water by volume, as determined by a Schott Capillary Viscometer. Although tables of glycerol-water viscosity do not extend down into the temperatures measured, extrapolations of existing tables gave reasonable agreement with our measured values using the radius of Mb (2.4 nm) determined at 293 K.

FIGURE 4 Revised model of the entry of CO or O_2 into the Mb molecule. Barrier IV no longer exists as a single barrier but instead is the activation energy for the viscosity of glycerol-water. The small gas molecule is postulated to enter the region near the binding site (barrier III) at a solvent diffusion-limited rate. The rate k_{DE} is the diffusion-limited dissociation rate. This picture may be modified at lower ratios of glycerol-water, where the small protection barrier provided by the protein may become larger than the activation energy for the viscosity of water (12.6 kJ/mol).

TABLE I

ROTATIONAL RATE AND LIGAND ENTRY RATES FOR VARIOUS PROTEINS

Substance	E_η or E_{ED}	k_{rot} or k_{DE} at 280K	k_{ED} at 280K	$\frac{k_{ED}}{[C]} \times \frac{\eta}{\eta_{H_2O}}$	Reference
	kJ/mol	(s^{-1})	(s^{-1})	($M^{-1}s^{-1}$)	
Viscosity of 84% glycerol-water	59	1.56×10^5 (Mb)	—	—	This work
Mb-O_2	53	2×10^5	2×10^3	1×10^{10}	4
Mb-CO	79	9×10^5	7×10^3	7×10^{10}	4
β_{SH}-CO	44	5×10^5	5×10^3	5×10^9	6
α_{SH}-CO	37	2×10^5	5×10^3	5×10^9	6
Heme-CO	74	7×10^8	2×10^4	2×10^{10}	5

proteins as determined by Austin et al. (4) and Alberding et al. (5,6), and in columns 2 and 3 the rate parameters k_{ED} and k_{DE} at 280 K. With the results of Lackowicz and Weber (1) in mind, one is immediately struck by the high value (34–75 kJ/mol) that barrier IV presents to the diffusion of a small ligand into the protein. However, it must be kept in mind that the measurements at Illinois were done in 75% glycerol. The well-known Smoluchowski equation (10) gives a reliable prediction of the maximum value that a bimolecular rate parameter can have (the diffusion-limited rate),

$$k_{diff} = (4\pi N_{AV}/1{,}000)D \cdot a, \tag{4}$$

where a is the sum of the molecular radii and D is the sum of the diffusion coefficients. Because the diffusion constant is inversely proportional to viscosity,

$$D = k_B T/6\pi a\eta, \tag{5}$$

where η is the viscosity of the solvent in poise (cgs unit), to compare rates in different viscosities we will multiply the observed rates by the ratio of the measured viscosity to the viscosity of water at 280 K, and divide by the concentration of the gas in solution. The fourth column in Table I contains the raw and corrected k_{ED} for various proteins that have been measured. The final entry in Table I is our measured rate at which oxygen quenches the triplet state of PP9 in DMF, corrected for the measured viscosity and the known concentration of atmospheric O_2 in DMF. One can also calculate roughly the expected rate at which O_2 should quench the triplet PP9 from Eqs. 4 and 5. This value is given in Table II.

Several relationships can be seen in Table I. First, the activation energies of the k_{ED}s (34–75 kJ/mol) are similar to $E\eta$ (84%), 59 kJ/mol. Some of the activation energies, it must be confessed, are closer to Eη (84%) than others. The activation energy for Mb-CO is clearly too large (75 kJ/mol), whereas the value for Mb-O_2 is quite close

TABLE II
TRIPLET QUENCHING RATES

Parameter at 280K	Raw value	$\frac{\text{Rate}}{[O_2]} \times \frac{\eta}{\eta_{H_2O}}$	Reference
	(s^{-1})	$(M^{-1}s^{-1})$	
Quenching rate of PP9 triplet by O_2 in DMF	1.8×10^6	1.4×10^9	This work
Quenching rate of PP9-Mb triplet in air-saturated water	3.1×10^4	2.0×10^8	This work
Quenching rate of PP9-Mb triplet in air-saturated 75% glycerol-water	2.7×10^2	2.8×10^8	This work
Calculated rate of O_2 quenching of PP9 triplet in medium of 0.01 poise	—	9.2×10^9	
k_{ED} for Mb-O_2	2×10^3	1×10^{10}	4

(54 kJ/mol). It should be stressed that k_{ED} is a computer-derived fit, not directly measured. Second, the corrected bimolecular rates at 280 K are actually *greater* than the expected diffusion-limited rate, although it is not totally impossible for the diffusion-limited rate for arrival at the area near the heme group to be different from the diffusion-limited quenching rate of O_2 with PP9. Granting the imperfections, we feel that the evidence is sufficiently encouraging to propose the following: Barrier IV has little to do with the protein-solvent interface; it is simply the activation energy E_η for the diffusion of CO or O_2 in 75% glycerol-water. The O_2 or CO arrives at barrier III at the diffusion-limited rate.

There is a further test we can make, quite similar to a fluorescence quenching experiment. Because the lifetime of the triplet state of PP9 in water at 280 K is 1.3 ms, it is easy to measure the quenching of the PP9 triplet in a protein by O_2 at atmospheric pressure if the quench rate is in the diffusion-limited range. The unprotected quench rate of PP9 by O_2, corrected again for viscosity, is given in Table II. We have also measured the quenching rate of O_2 for PP9 bound to apomyoglobin in water and in 75% glycerol. From Table II we see that the reduction of the quenching rate by the protein (0.15) is in the range of values found in Lackowicz and Weber (1), 0.17–0.60. Because the O_2 must penetrate the protein to some extent to quench, it would not even be unreasonable to expect that $k_{ED} = k_{quench}$. They are not, but $k_{ED} > k_{quench}$, once again supporting the picture that k_{ED} is a diffusion-limited rate. There certainly is a barrier presented by the protein—the PP9 is protected to some extent—but not the 50- to 84-kJ/mol barrier originally associated with the protein-solvent interface. Our modified view of the potential barriers is given in Fig. 4.

The above statement is our main conclusion. There are further puzzles which need much more careful work. Why does k_{DE} have the same activation energy as k_{ED}? We would like to argue that k_{DE} represents the diffusion-limited dissociation of CO or O_2 from the protein, with its correspondingly greater "effective radius" and thus higher rate, but this remains speculative. We originally were struck by the equivalence of k_{DE} and k_{rot} and had thought that the solvent-cage barrier originally postulated for barrier IV had a lifetime given by the rotational rate of the Mb, but our further experiments have convinced us that barrier IV in effect is the solvent alone. Only further experiments, in particular determination of the barriers at other ratios of glycerol-water, can judge the correctness of our proposal. For now we would say that the fluctuating protein model of G. Weber seems to give the best explanation of our data, at least for access of small molecules to groups near the surface of a protein.

We would like to acknowledge helpful comments and discussions from Prof. H. Frauenfelder. We are indebted to G. Striker for his expert computer data analysis.

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NOTE ADDED IN PROOF

It has been brought to our attention that the quenching of triplet protoporphyrin IX in proteins by oxygen has previously been done by B. Alpert and L. Lindqvist [1975. Porphyrin

triplet state probing the diffusion of oxygen in hemoglobin. *Science (Wash. D.C.).* **187**:836–837; and 1976. Laser study of triplet porphyrin quenching by oxygen in porphyrins globins. *In* Excited States of Biological Molecules. J. B. Birks, editor. John Wiley & Sons, Inc., New York]. Their results for the triplet quenching are similar to ours and we apologize to them for our oversight.

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DISCUSSION

VANDERKOOI: Oxygen is a small lipophilic molecule. Its partition coefficient for organic solvents is about 10 times greater than that for aqueous solution. Would you not expect oxygen to partition into the hydrophobic interior of proteins so that the effective concentration of oxygen in the interior is higher than in the aqueous phase? How would this affect your calculations?

AUSTIN: That is an excellent question. (*a*) Let us state the obvious and note that since our protein concentration was only about 10 μM, a net increase in solubility of the CO in the protein would not have affected our bulk overall solubility. (*b*) Let us try to calculate the probability of finding a CO molecule "dissolved" in a myoglobin molecule. The volume of a myoglobin molecule is about 6×10^{-20} cm^3. The volume for each O_2 molecule at 0.2 bar pressure in 75% glycerol-water is about 2.5×10^{-17} cm^3. If we assume that the actual local concentration of the O_2 in the protein is 10 times that in water (or 30 times that in 75% glycerol-water), then the probability of finding an O_2 molecule in the protein molecule is $[(6 \times 10^{-20})/(2.5 \times 10^{-17})] \times 30 = 0.08$. This implies that the triplet quenching of the heme should show two phases: an oxygen concentration-independent quenching of 0.08 of the molecules and an oxygen concentration-dependent quenching of the remaining 0.92 molecules. What we see is the following: the single exponential triplet lifetime of the argon-saturated sample of several milliseconds changes to a single exponential with a lifetime of several microseconds