Diffusion of Small Molecules through the Structure of Myoglobin. Environmental Effects†

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ABSTRACT: The effect of the ambient solvent viscosity on the mobility of small molecules within myoglobin was studied by substituting Zn–protoporphyrin (ZnPP) for the native Fe–protoporphyrin and using it as an optical probe in the protein (ZnPPMb). The quenching of the ZnPPMb triplet state by oxygen, by anthraquinonesulfonate, and by methyl viologen was followed by exciting it with a laser flash and measuring its decay rate as a function of quencher concentration. The quenching rate constants were taken to measure the diffusion rate of the quencher within the protein. At room temperature, these constants were determined in aqueous and in 37% and 55% (by weight) glycerol–water solutions by measuring the ZnPPMb-delayed fluorescence at 606 nm. It was found that although the quenching rate constants varied the activation energies in the protein were very similar for the different quenchers. In aqueous solution, E_a = 6.0–7.4 kcal/mol; in 37% glycerol, E_a = 6.8–7.5 kcal/mol; and in 55% glycerol, E_a = 8.5–9.2 kcal/mol. The quenching rate of ZnPPMb by oxygen was also measured between 190K and 293K in 80% glycerol, and its triplet decay in the absence of oxygen was determined down to 120K in 88% glycerol. In all experiments, the quenching rates in the protein were compared to those of Zn–hematoporphyrin in the same solvent. The results are discussed in terms of Northrup and McCammon's gated reaction theory. It is concluded that (a) the mobility within the protein persists at least down to the temperature where the solvent solidifies and (b) the activation energies obtained measure the energetics of the conformational changes in the protein which enable large and small quencher molecules to penetrate the protein with equal ease.

The reaction between a protein and its substrate includes both the reaction step itself and the movement of the reactant molecule through the protein matrix toward the reation site. We concern ourselves here with the latter part of the process, i.e., the diffusion of small molecules through the protein. Various experiments (Lumry & Rosenberg, 1976; Lakowicz & Weber, 1973), most notably the determination of the mean square displacements of atomic positions (Frauenfelder et al., 1979; Artymiuk et al., 1979), from X-ray crystallography as well as calculations (Karplus & McCammon, 1981), indicate that proteins are dynamic entities which undergo structural fluctuations. It is these movements of amino acid side chains, or even sometimes of the polypeptide backbone, which are thought to facilitate the diffusion of oxygen through the otherwise compact protein structure (Lakowicz & Weber, 1973). Therefore, the mobility of small molecules within the protein and its temperature dependence might serve as an indicator for the energetics of the above conformational dynamics.

Following the above pioneering work of Lakowicz and Weber, the fluorescence and triplet-state quenching of tryptophan residues in proteins by different quenchers have been extensively studied. There is general agreement on the efficient fluorescence quenching by oxygen (Lakowicz & Weber, 1973; Calhoun et al., 1983; Eftink & Jameson, 1982) which indicates that O_2 penetrates the structure of most proteins. Of interest are the quenching rate constants for medium-sized quenchers, notably acrylamide, with proteins whose tryptophan residues are not directly accessible to the solvent as evidenced by the spectral distribution of their fluorescence (Calhoun et al., 1987). These rate constants lie in the vicinity of 10^8 M^{-1} s^{-1} for RNase T1 and parvalbumin (Eftink & Ghiron, 1984) and 10^7 M^{-1} s^{-1} for LADH (Eftink & Selvidge, 1982) and for aldolase (Calhoun et al., 1986). Different mechanisms have been proposed by the authors to account for the experimental data. The quenching rate constants of the tryptophan triplet state in proteins by oxygen are an order of magnitude smaller than those for the singlet state (Strambini, 1987; Barboy & Feitelson, 1978; Ghiron et al., 1988). For other quenchers, even smaller values are often obtained. The phosphorescence quenching rate constants for a number of proteins including the above-mentioned proteins by different size quenchers have recently been reported (Calhoun et al., 1988). The authors propose criteria for the mechanisms of quenching which include protein penetration, gate opening, and long-range energy and electron transfer. It appears that at present no single mechanism describes adequately the diverse fluorescence and triplet-state quenching data.

The binding of carbon monoxide and of oxygen to myoglobin and to hemoglobin has been studied by following the photodissociation of the bound ligand and its subsequent rebinding at the heme site (Debrunner & Frauenfelder, 1982; Hofricher et al., 1985; Friedman, 1985). It was found that preceding the actual binding step with its specific potential barrier the substrate must overcome a number of (Austin et al., 1975) or at least one (Marden et al., 1986) potential barrier on its way through the protein. In a previous paper, we studied the diffusional movement of small molecules into the myoglobin binding site (Barboy & Feitelson, 1987). Zn–protoporphyrin (ZnPP) was substituted for the native iron–protoporphyrin. This substitution did not, for all practical purposes, affect the protein structure (Feitelson & Spiro, 1986). The quenching of the long-lived ZnPP-delayed fluorescence which stems from the triplet state served as a measure of the above diffusion process. We obtained a rate constant for oxygen quenching of ZnPP within myoglobin of $k_q = 10^8$ M^{-1} s^{-1}. Similar values

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between 1×10^8 and 2×10^8 M^{-1} s^{-1} have been obtained for Fe-free hemoglobin (Alpert & Lindquist, 1975) for Zn-protoporphyrin-substituted myoglobin (Zemel & Hoffman, 1981) and for Fe-free myoglobin (Jameson et al., 1984). It was concluded that in aqueous solution conformational fluctuations in the protein act as a gating mechanism (Northrup & McCammon, 1984) which allows small molecules of various sizes to penetrate the protein structure with similar rates and similar energetics. We shall now inquire whether and to what extent the above structural fluctuations are influenced by the protein environment.

It has been suggested that the solution viscosity is the predominant property likely to influence the conformational dynamics within the protein structure, and a relation has been proposed between the measurable solution viscosity and the viscosity within the protein (Gavish, 1980) which determines the rate of diffusion of small molecules through the protein matrix. The effects of viscosity on ligand binding to myoglobin have been studied (Beece et al., 1980), and the subject has recently been reviewed (Gavish, 1986). In these studies, the temperature dependence of the binding rate in the protein was measured in a series of hydroxylic solvents under isoviscous conditions. Specific solvent–protein and solvent–ligand interactions are not taken into account by this approach. The results showed that the solvent viscosity does affect the mobility of the ligand in the protein. The older binding data (Austin et al., 1975) were reexamined (Austin & Chan, 1978), and it was concluded that the entry rate of the ligand into the protein is determined by the solvent viscosity and that the protein offers no potential barrier to this entry. Here we wish to study the influence of the solvent viscosity, including its temperature dependence, on the mobility of the quencher within the protein and to compare it to the viscosity effect in solution. As before, the inward movement of the quencher through the protein matrix is monitored by the loss of the ZnPP excitation energy upon colliding with it. The quenching step itself, which is probably of an electron-transfer nature, is very rapid and is thought not to depend on the temperature. We assume that the potential barriers encountered by the quenchers within the proteins are lowered through conformational changes in the latter. We describe, therefore, the migration of the quencher through the protein by a diffusional process facilitated by the above changes in the protein structure. These changes might be coupled to the movement of the solvent molecules surrounding the protein and thus to the macroscopic viscosity of the solvent (Northrup & McCammon, 1984).

We shall compare the rates and the energetics derived from the Zn–hematoporphyrin (ZnHP) quenching by quenchers of various sizes in aqueous and in glycerol-containing solutions to those of ZnPP incorporated in the myoglobin molecule (ZnPPMb) in a similar solvent. From the results obtained, we shall try to draw conclusions on the solvent dependence of the protein structural fluctuations as a function of temperature.

Experimental Procedures

The ZnPP was incorporated into myoglobin as described previously (Barboy & Feitelson, 1987). Briefly, the iron–protoporphyrin was extracted with acidified (pH 3) ethyl methyl ketone. The apomyoglobin was reconstituted at pH 8.7 with ZnPP (Porphyrin Products, Logan, UT). After standing for about half an hour, the ZnPP-myoglobin (ZnPPMb) at pH 7.0 was loaded onto a CM-52 column, which was preequilibated at pH 7.0 with 0.01 M phosphate buffer. It was subsequently eluted with 0.15 M phosphate buffer, pH 7.0, and stored as 0.5-mL aliquots at −28 °C. The whole procedure was carried out at 3–4 °C.

The ~0.5-mL portions of ZnPPMb were diluted with 0.01 M phosphate buffer, pH 7.8, or with buffer–glycerol mixtures to about 4 mL, i.e., to a final buffer concentration of 0.025 M. They were deoxygenated by flushing with >99.999% nitrogen (further purified by passing through an Alltech Oxy-Trap) and stirring for 2 h in the dark. High-viscosity solutions had to be left overnight to equilibrate under nitrogen followed by additional flushing for 1 h at room temperature prior to the actual measurements. A deoxygenation vessel with an optical cell as a side arm was used. This vessel was designed so that measured amounts of air or, alternatively, two aliquots of quencher solution could be added sequentially to the ZnPPMb solution for kinetic runs. For temperature dependencies in the 273–305 K range, the cell was thermostated in a brass cell holder. It was illuminated at 545 nm by a 10-ns Molectron DL 220 nitrogen laser pumped dye laser, and the decay of the E-type delayed fluorescence was monitored by a R928 photomultiplier (Hamamatsu Photonics K.K., Hamamatsu City, Japan) at 606 nm. This kind of delayed fluorescence (DF) derives from the ZnPP triplet state (see below) and hence enables us to follow the slow decay of the latter which falls into the microsecond to millisecond range. To prevent saturation of the photomultiplier, the high voltage was switched off by a gating circuit for about 2 μs during the laser flash. For the low-temperature measurements in the 120–293 K range, the cell was contained in a cryostat (Oxford Instruments, England) with three-way windows fitted with a temperature regulator. At the low temperatures, no DF is observed, and therefore the time dependence of the triplet–triplet absorption at 460 nm was measured. The cell in the cryostat was illuminated by the second harmonic (532 nm) of a Nd-YAG (Quantaray DCR1, Spectra Physics) laser flash. A pulsed, shuttered Xe arc was used as monitoring light source. The solution was exposed for at most 0.5 s to the monitoring light.

The solvents were distilled water purified by passing through a MilliQ absorbent train (Millipore Corp.) and either Matheson Coleman and Bell (Norwood, OH) or Analar (BDH) glycerol. Other reagents used were of analytical grade.

Glycerol–water mixtures of 37%, 55%, and 80% (by weight) were used as solvents. These solutions have relative viscosities of 1, 3.6, and 8 cP, respectively (CRC Handbook, 1979).

The solubility of oxygen in glycerol–water was obtained by assuming a linear dependence of the Bunsen coefficient (α) between 13 and 37 °C for which data are available (IUPAC Solubility Data, 1981) and extropolating the data to 1 °C. The oxygen concentration in solution was calculated from the α values. The solutions were equilibrated with an atmosphere of a given partial oxygen pressure at the temperature of the experiment. We estimate that the above procedure of linearizing the α versus T dependence might lead to an error in the oxygen concentrations of 10–15%. For the low-temperature quenching experiments (190–290 K), where the solvent was glycerol–water 80% by weight, the exact concentration of oxygen in equilibrium with the solution is not known since to our knowledge no data are available for glycerol–water mixtures at low temperatures. The solutions were deoxygenated and equilibrated with air or with oxygen at room temperature. They were then rapidly cooled to the appropriate temperatures. Since the equilibration of oxygen with the highly viscous 80% glycerol solution is a very slow process, it was assumed that at low temperatures and without stirring the oxygen concentration in this solution did not change from that at room temperature over the duration of the experiment.

Table I: ZnHP and ZnPPMb Triplet Lifetimes, Quenching Rate Constants, and Activation Energies in H_2O and in 37% and 55% Glycerol–H_2O (by Weight)[a]

	quencher	ZnHP			ZnPPMb		
		H_2O	37% glyc	55% glyc	H_2O	37% glyc	55% glyc
τ^0 (ms)		1.2*	1.5*	3.0	13.7*	13.0	13.5
$k_q \times 10^{-8}$ (M^{-1} s^{-1})	O_2	11*		8	1.0*	1.1	1.1
	AQS	22*	5.5	2.7	2.9*	1.6	0.63
	MV	36	13.0	10.0	0.45	0.32	0.3
E_a (kcal/mol)	O_2	3.2*		4.1	6.0*	6.8	8.5
	AQS	3.1*	6.6	8.5	5.8*	7.0	8.7
	MV	3.9	5.4	8.6	7.4	7.5	9.2

[a] Some of the data for aqueous solutions indicated by an asterisk have been reported before (Barboy & Feitelson, 1987) and are presented for comparison. The quenchers were oxygen (O_2), anthraquinonesulfonate (AQS), and methyl viologen (MV). The estimated errors in lifetimes and rate constants are ±12% and in the activation energies ±0.5 kcal/mol. τ^0, lifetime in the absence of quencher at 20 ± 1 °C; k_q, bimolecular quenching rate constant at 20 ± 1 °C; E_a, activation energy.

This was verified by an experiment in which oxygen was added to the measuring vessel containing the N_2-purged ZnPPMb solution in 80% glycerol at 1 °C without stirring. Over a period of 2 h, no quenching of the triplet ZnPPMb was observed, meaning that no appreciable dissolution of oxygen had taken place.

For measurements of the ZnPPMb lifetime below 190 K, 88% (by weight) glycerol–water was used as solvent.

The viscosity of the 80% (by weight) glycerol–water solution as a function of temperature was measured between 292 and 250 K with an Ostwald viscosimeter immersed in a low-temperature bath.

Results

When either ZnHP or ZnPPMb in the absence of air is flash-illuminated into one of their absorption bands, an emission with a spectral distribution of its fluorescence but with a lifetime resembling that of its triplet state is observed (Barboy & Feitelson, 1987). Illuminating with a low-power laser produces an emission whose intensity is proportional to that of the laser flash and which decays exponentially. This, so-called, E-type delayed fluorescence (DF) derives from the porphyrin triplet state by thermal excitation to the singlet. The properties of the porphyrin DF have been described previously (Parker & Joyce, 1967; Feitelson & Mauzerall, 1982). Where possible, i.e., in the range near room temperature, we preferred measurements of the delayed fluorescence over triplet absorption to monitor the triplet state. In the presence of oxygen, porphyrins undergo a photochemical change. The absorbance near 460 nm measures both the decay of the triplet and the time dependence of the photochemical product produced by the illumination, while the DF derives from the triplet is not influenced by the above photochemistry (Barboy & Feitelson, 1987). At low temperatures, the intensity of the E-type DF decreases drastically, and we had to use triplet absorption measurements.

Since the actual quenching of the excited ZnHP triplet in solution and of the ZnPP in myoglobin (ZnPPMb) is an instantaneous process, the decrease in the DF or triplet absorption decay times measures the diffusion rate of the quencher toward the excited porphyrin and thus directly the diffusion constant in the solution or in the protein phases, respectively, as will be shown under Discussion.

Quenching of the ZnHP- and ZnPPMb-Delayed Fluorescences in the 273–305 K Range. Solutions of ZnHP and of ZnPPMb ($\sim 10^{-5}$ M) in water and in 37% and 55% glycerol–water were thoroughly deoxygenated by flushing with prepurified nitrogen. The triplet lifetimes of the porphyrins were measured by illuminating them at 545 nm and following the DF decay at 606 nm as a function of temperature. The quenchers oxygen (O_2), the negatively charged anthraquinonesulfonate (AQS), and the positively charged methyl viologen (MV) were added to determine the quenching rates for molecules of different size and electric charge. At the low porphyrin (10^{-5} M) and quencher concentrations used (5×10^{-6} to 4×10^{-5} M), the decay rates followed the Stern–Volmer relation $1/\tau - 1/\tau^0 = k_q[Q]$, where τ and τ^0 are the DF lifetimes in presence and in absence of the quencher Q, respectively.

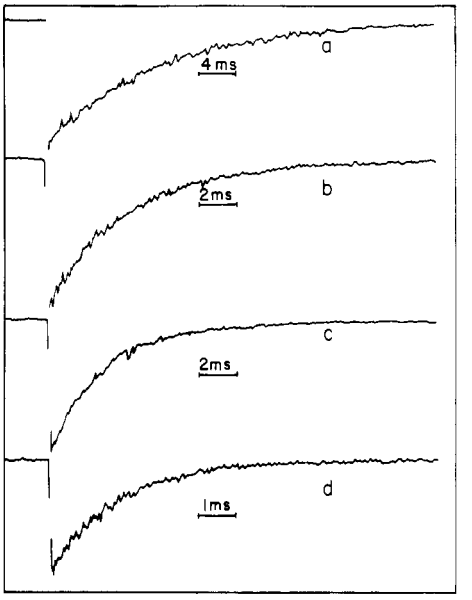

FIGURE 1: ZnPPMb quenching by MV in 55% (by weight) glycerol–water at 20 °C. From top to bottom, [MV] = 0.0, 5×10^{-6}, 10^{-5}, and 2×10^{-5} M.

Our data (Barby & Feitelson, 1987) for O_2 and the negatively charged AQS were supplemented by quenching data for the positively charged methyl viologen. In Table I, the results in aqueous solution are compared to those in the glycerol–water mixtures. Since, as noted under Experimental Procedures, the solubilities of oxygen in 37% and in 55% glycerol as a function of temperature are not exactly known, the rate constants might be in error by as much as 10–15%. However, the general trends of the data are clearly discernible.

Quenching of the ZnHP and ZnPPMb Triplets in the 120–293 K Range. Solutions of ZnHP and of ZnPPMb ($\sim 10^{-5}$ M) in 80% and 88% (by weight) glycerol–H_2O were thoroughly deoxygenated as described under Experimental Procedures. More extensive deoxygenation did not lead to an increase in the triplet lifetimes. These lifetimes were measured by illuminating the solutions in a cryostat at the appropriate temperature with a 532-nm laser flash and measuring the triplet absorption at 460 nm. Precautions were taken to minimize the illumination time of the solution to avoid pho-

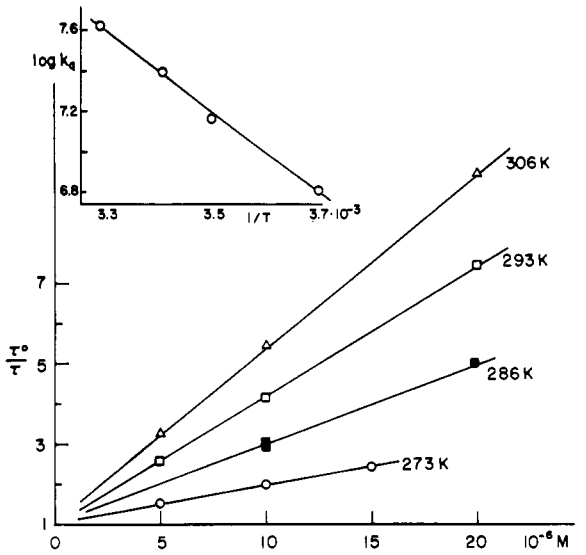

FIGURE 2: ZnPPMb quenching by MV. Temperature dependence. Insert: Activation energy for quenching process. E_a = 9.2 kcal/mol.

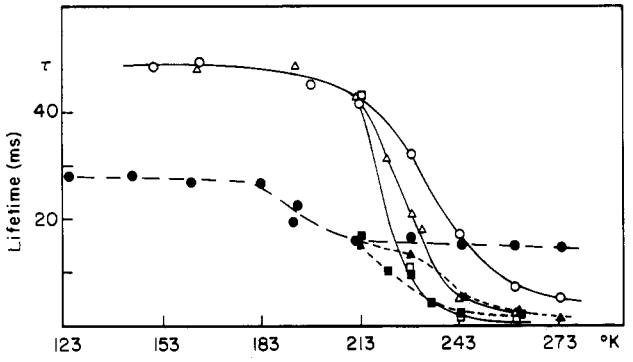

FIGURE 3: Lifetimes of ZnHP and ZnPPMb in 80% (by weight) glycerol–water in the absence of oxygen (circles) and in the presence of 8×10^{-5} M oxygen (triangles) and 4×10^{-4} M oxygen (squares). Open symbols denote ZnHP quenching and closed symbols ZnPPMb quenching.

tochemical damage. The decay times were measured in the absence of oxygen and after equilibrating the solutions at room temperature with air and with oxygen, respectively. The oxygen concentrations were 8×10^{-5} and 4×10^{-4} M for air and for oxygen-saturated solutions, respectively, as obtained from the Bunsen coefficients α, and these concentrations were used when evaluating the quenching rate constants at low temperatures (see Experimental Procedures). The lifetimes of ZnHP and of ZnPPMb as a function of temperature and of oxygen concentration are shown in Figure 3. The corresponding rate constants together with the solvent viscosity are presented in Figure 4. It is noteworthy that the lifetimes of ZnHP in the absence of oxygen increase from 5 ms at 273 K to 46 ms at 203 K. In ZnPPMb, on the other hand, the triplet lifetime in the absence of oxygen changes only slightly from 13 to 16 ms in the same temperature range. It does, however, increase at about 185 K to 28 ms, where it remains down to 120 K. No unique activation energy can be assigned to the quenching process over the whole temperature range. The log k vs $1/T$ slope, however, does increase with decrease in temperature, yielding an apparent activation energy of ~15 kcal/mol at 230 K. The quenching rate in ZnPPMb follows the temperature dependence of the solvent viscosity.

Discussion

In view of the quenching data of ZnHP in aqueous solution, which show that the rate of the process is diffusion controlled,

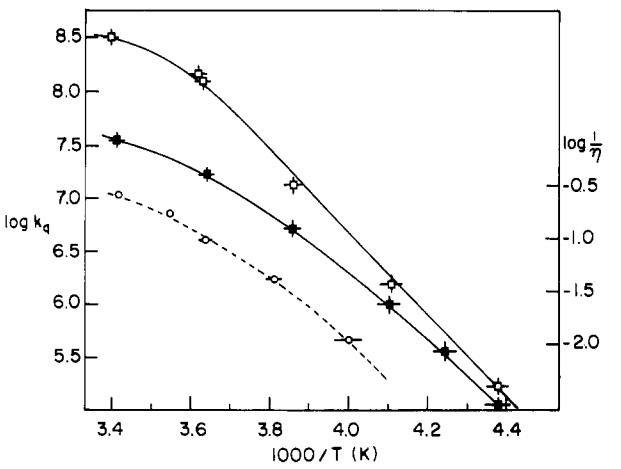

FIGURE 4: Temperature dependence of the oxygen quenching rate constants. The temperature range is 273–220 K, and the solvent is glycerol–water, 80% (by weight). Open squares refer to ZnHP quenching and closed squares to ZnPPMb quenching.

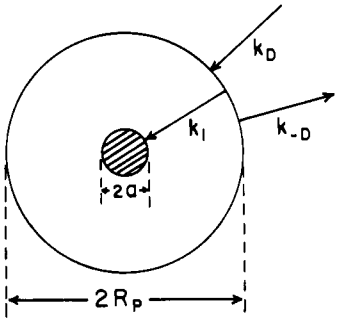

FIGURE 5: Diffusion of quencher into the protein. R_P is the radius of the protein, a is the distance of closest approach of quencher to excited chromophore, k_D and k_{-D} are the diffusional rate constants in solution, and k_1 is its value within the protein.

we assume that in all the media here considered, the high-viscosity solutions and the protein interior, the quenching rate is determined by the diffusion of the quencher.

Diffusion in Heterogeneous Media. In the reaction

$$P_P^* + Q_S \underset{k_{-D}}{\overset{k_D}{\rightleftharpoons}} (P^* + Q)_P \xrightarrow{k_1} (P^*Q)_P \xrightarrow{\kappa} P_P + Q_P \qquad (1)$$

a quencher molecule (Q) in solution encounters a protein (containing an excited ZnPP at the active site) where it reaches, by diffusion, the excited porphyrin (P_P^*) and quenches it. The quencher is not bound at the porphyrin site as has been shown previously (Barboy & Feitelson, 1987). S and P denote the solution and protein phases, respectively. $(PQ^*)_P$ is the encounter complex in the protein, and κ represents the instantaneous quenching step. Such a system assumes a homogeneous protein interior and therefore can serve only as a much simplified model for a real protein. At low protein and quencher concentrations (in the 10^{-5} M range), practically all Q molecules reside in the solution. Because of the ultrafast quenching step, the excited porphyrin in the protein acts as a sink so that no back-dissociation into P_P^* + Q takes place. We can evaluate the diffusion constant within the protein for such a simple model of a protein molecule of radius R_P which is permeable to the quencher Q. After entering the protein, the quencher encounters the excited porphyrin P_P^* at a distance of closet approach, a, by a diffusional process (Figure 5). We adopted the treatment of Agmon (Agmon et al., 1988) for the solution of the diffusion equation of the mathematically similar problem of geminate recombination in proton transfer. The difference from that approach is that the authors start with a species AB which dissociates first into a complex A···B

before separating into A + B, while in our case Q is initially located in the surrounding solution from which it diffuses towards P*, the excited porphyrin, yielding the encounter complex. In the absence of a potential field, the rate constants obtained by use of the appropriate boundary conditions are

$$k_D = 4\pi D_S R_P \quad \text{and} \quad k_{-D} = 4\pi D_S R_P \tag{2}$$

with D_S the sum of the diffusion constants for the protein and quencher in solution and

$$k_1 = 4\pi D_P/(1/a - 1/R_P) \tag{3}$$

which is obtained by applying the completely absorbing boundary condition. D_P is the diffusion constant in the protein. The measured quenching rate constant, k_q, obtained from eq 1 in the steady-state approximation (Shoup & Szabo, 1982) is

$$k_q(\text{obsd}) = k_1 k_D/(k_{-D} + k_1) \tag{4}$$

By substitution of eq 2 and 3 into eq 4, the measured rate constant for a solution containing the protein-bound porphyrin becomes

$$k_q(\text{obsd}) = 4\pi D_P R_P/[(R_P/a) - 1 + D_P/D_S]$$

For a protein whose dimensions are large compared to those of the excited chromophore, i.e., $R_P \gg a$, and for $0 < D_P/D_S < 1$ (the diffusion rate in the protein does not exceed that in solution), this equation reduces to

$$k_q(\text{obsd}) = 4\pi D_P a \tag{5}$$

i.e., the measurement reflects the diffusion rate within the protein as can be expected under these conditions. The corresponding quantity for the quenching of a porphyrin molecule, P_S*, in solution is given by the Smoluchovsky equation (Shoup & Szabo, 1982):

$$k_q^S = 4\pi D_S a \tag{6}$$

The time-dependent term in the Smoluchovsky equation can be omitted for long-time phenomena such as the decay of the triplet.

Measurements in the 273–305 K Temperature Range. In aqueous solution, the quenching of ZnHP by all three quenchers is, or is almost, diffusion-controlled, and the activation energy for the process reflects roughly the temperature dependence of the viscosity. A slight increase in the rate of quenching by the positively charged methyl viologen (MV) over that of anthraquinonesulfonate is probably due to an electric charge effect on the quenching of the negatively charged ZnHP. The data for O_2 ought to be corrected by the statistical factor for triplet–triplet quenching which for aromatic systems in polar solvents has a value between 9 and $^9/_4$ (Saltiel & Atwater, 1988). This makes oxygen a slightly better quencher than AQS.

The connection between the mobility of small molecules in solution and the viscosity has recently been reviewed (Gavish, 1986). Given the reciprocal Stokes–Einstein relation between the diffusion coefficient, D, and the viscosity, η, it might be expected that the quenching rate constants in solution will behave in a similar manner. This was found to be approximately the case for the ZnHP quenching by AQS and by MV where the increase in solvent viscosity from 1 cP in aqueous solution to 8 cP in 55% glycerol–water at 20 °C (Handbook of Chemistry, 1979) can be compared with the corresponding quenching rate constants in Table I. The effect for the doubly charged MV was only half that expected. Similarly, the increase in activation energy for the quenching process by AQS and by MV from 3.1 and 3.9 kcal/mol to 8.5 and 8.6 kcal/mol, respectively, is comparable to the corresponding values for the temperature dependence of the reciprocal viscosity, $1/\eta$, E_a = 3.8 and 7.1 kcal/mol (Landolt-Bornstein, 1969). On the other hand, the quenching rate by oxygen and its activation energy are very little affected by the above increase in solvent viscosity. It is, however, known that the mobility of oxygen does not follow the Stokes–Einstein relation. Especially in the low-viscosity range of 1–10 cP, the mobility of O_2 does not markedly decrease with increase in solvent viscosity (Ware, 1962; Alvattar & Birks, 1973).

If the protein were a semirigid structure independent of the solvent environment, we would expect that the latter would not affect the rates of processes taking place within the protein. Any change in protein reactivity or in the quenching parameters due to changes in the environment can therefore be ascribed to the influence that the environment exerts on the protein structure and/or its dynamics.

The quenching rate of the porphyrin within myoglobin, ZnPPMb, in all solutions is lower than that of ZnHP in free solution. Equation 5 shows that in this case the measured quenching rate constant reflects the diffusion rate in the protein. Differences in the room temperature quenching constants for the various quenchers might be due to differences in solute–protein interactions. Thus, the marked decrease in the quenching rate by the positively charged MV with respect to AQS in ZnPPMb could indicate the location of a positively charged group along the passageway of the quencher in the protein.

For all three quenchers, it is found that the activation energy for the quenching process increases with solvent viscosity. This increase, however, is less pronounced in the protein than in aqueous solution so that for myoglobin in 55% glycerol–water it exceeds only marginally the E_a value in water. This can be taken to mean that although the solution viscosity does affect the quencher mobility in the protein, the effect is only partially transmitted from the solution to the protein interior. It must be noted that within myoglobin all three quenchers, O_2, AQS, and MV, exhibit a similar activation energy in water and an increasingly larger but similar E_a value in the glycerol–water mixtures. This indicates that contrary to the ambient solution the protein medium does not distinguish between quenchers of different sizes. We shall try to account for this behavior in terms of so-called gated reactions (Szabo & Shoup, 1982; Northrup & McCammon, 1984; Agmon & Kosloff, 1988).

When Northrup and McCammon's approach is adopted, a system, where steric factors obstruct the reaction, should be described by a reaction coordinate and an auxiliary coordinate. In our case, where the process under consideration is the passage of the quencher through constrictions in the protein structure, the auxiliary coordinate describes the conformational changes which act as a gate for the quencher on its way toward the excited ^{3}ZnPP. The diffusion of a quencher molecule from location A, before the constriction, to location B, after the constriction, takes place via the passageway $A_1 \rightarrow A_2 \rightarrow B_2 \rightarrow B_1$ where subscripts 1 and 2 denote protein conformations for which the free energy of activation $\Delta G(A_2 \rightarrow B_2) < \Delta G(A_1 \rightarrow B_1)$. Two limiting cases are discussed by the authors which, for the diffusion of the quencher, take the following form. If the conformational changes which allow the quencher to pass are rapid with respect to the mobility of the latter, the protein conformation adapts to the quencher movement, and the (Gibbs) activation energy of the process represents the lowered energy barrier $\Delta G(A_2 \rightarrow B_2)$ in the new protein conformation. The picosecond movements of amino acid side chains would constitute such conformational fluctuations. These movements,

however, would not facilitate the passage of small and of large quencher molecules to the same extent, and, hence, different activation energies should be obtained for different quenchers. In the second case, the gating process is considered to be slow with respect to the passage of the quencher. The theory shows that in this case the diffusion rate is determined by the rate of the gating and the activation energy represents the energy needed for the conformational change, $A_1 \rightarrow A_2$ which acts as a gate, to take place. These large dynamic structural changes in proteins are expected to be influenced by the ambient viscosity (Northrup & McCammon, 1984). On the other hand, the above rapid small fluctuations will probably not influence the gross protein structure and thus will not experience any influence of the ambient solvent viscosity. The similarity of the measured activation energies for all three quenchers in ZnPPMb and their increase with the solvent viscosity indicate that the second, slow gating, case applies here. It appears that the slow configurational changes are of sufficient amplitude to let small and large quenchers to pass with equal ease, and we therefore propose that in this case the activation energies measure the energetics of the configurational changes in the protein. A gating mechanism has also been suggested for the tryptophan phosphorescence quenching in LADH (Calhoun et al., 1988).

Measurements in the 120–275 K Temperature Range. In the low-temperature range, two interesting features are observed (Figure 3): (a) The lifetime of the ZnHP-delayed fluorescence in the absence of oxygen increases greatly with decreasing temperature, leveling off at a value of ~46 ms below 210 K. The short lifetime of the triplet state in deoxygenated solutions at higher temperatures might be due either to a residual impurity or to a, rather inefficient, interaction with the solvent. Both processes would cease when the solvent solidifies. It is not very likely that an impurity is responsible for the effect since even for a very efficient quencher (of e.g., $k_q = 10^8$ M^{-1} s^{-1} in a viscous low-temperature solution) a concentration of 5×10^{-7} M would be required to account for the process. A qualitatively similar picture emerges for the protein. However, here the lifetime of 13–16 ms persists at lower temperatures till 185 K. Only at 180 K does the lifetime increase to 28 ms, where it remains down to 120 K. This indicates that the porphyrin is well protected from the ambient solution and that its lifetime is determined by its spectroscopic properties and by the immediate neighborhood only. A glass transition in the protein has been predicted at 175 K (Austin, private communication). The increase in the ZnPPMb lifetime at 180 K might indicate such a transition at which the conformational fluctuations in the proteins cease. (b) The quenching data (triangles and squares in Figure 3) show that at about 230 K the solvent becomes very viscous so that the mobility of the quencher in solution is drastically decreased and no quenching of the ZnHP in solution takes place. Quenching within the protein stops at the same temperature. However, this does not mean that all mobility in the protein ceases but only that the ambient solution cannot any more supply quencher molecules to the protein at these low temperatures within the ^{3}ZnPPMb lifetime. A comparison between the quenching of ZnHP in solution and of ZnPP in the protein with the solution viscosity shows that below 265 K both processes run in parallel with the viscosity. Although no well-defined activation energy can be assigned to the quenching and to the viscosity, the similarity between the log k_q and log η^{-1} vs $1/T$ values points toward a similar mechanism for the molecular mobility in the two systems, i.e., molecular or group displacements in the near vicinity which enable small molecules to thread their way through the environment.

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References

Agmon, N., & Kosloff, R. (1987) *J. Phys. Chem. 91*, 1988–1996.

Agmon, N., Hupert, D., & Pines, E. (1988) *J. Chem. Phys.*, 5631–5638.

Alpert, B., & Lindquist, L. (1975) *Science 187*, 836–837.

Altwattar, A. H., Lumb, M. D., & Birks, J. B. (1973) in *Organic Molecular Photophysics* (Birks, J. B., Ed.) Vol. 1, Chapter 8, Wiley, New York.

Artymink, P. J., Blake, C. C. F., Grace, D. E. P., Oatley, S. J., Phillips, D. C., & Sternberg, M. J. E. (1979) *Nature 280*, 563–568.

Austin, R. H., & Chan, Sh. S. (1978) *Biophys. J. 34*, 175–182.

Austin, R. H., Beeson, K., Eisenstein, L., Frauenfelder, H., & Gusalus, I. C. (1975) *Biochemistry 14*, 5355–5375.

Barboy, N., & Feitelson, J. (1987) *Biochemistry 26*, 3240–3244.

Beece, D., Eisenstein, L., Frauenfelder, H., Good, D., Marden, M. C., Reinisch, L., Reynolds, A. H., Sorensen, L. B., & Yue, K. T. (1980) *Biochemistry 19*, 5147–5157.

Calhoun, D. B., Vanderkooi, J. M., & Englander, S. W. (1983) *Biochemistry 22*, 1526–1539.

Calhoun, D. B., Vanderkooi, J. M., Holtom, G. R., & Englander, S. W. (1986) *Proteins: Struct., Funct., Genet. 1*, 109–115.

Calhoun, D. B., Englander, S. W., Wright, W. W., & Vanderkooi, J. M. (1988) *Biochemistry 27*, 8466–8474.

CRC Handbook of Chemistry and Physics (1979) p D–240, Chemical Rubber Publishing Co., Cleveland, OH.

Debrunner, P. G., & Frauenfelder, H. (1982) *Annu. Rev. Phys. Chem. 33*, 283–290.

Eftink, M. R., & Jameson, D. M. (1982) *Biochemistry 21*, 4443–4449.

Eftink, M. R., & Selvidge, L. A. (1982) *Biochemistry 21*, 117–125.

Eftink, M. R., & Ghiron, C. (1984) *Biochemistry 23*, 3891–3899.

Feitelson, J., & Mauzerall, D. (1982) *J. Phys. Chem. 86*, 1623–1628.

Feitelson, J., & Spiro, T. G. (1986) *Inorg. Chem. 25*, 861–865.

Frauenfelder, H., Petsko, G. A., & Tsernoglou, D. (1979) *Nature 280*, 558–563.

Friedman, J. M. (1985) *Science 228*, 1273–1280.

Gavish, B. (1980) *Phys. Rev. Lett. 44*, 1160–1163.

Gavish, B. (1986) in *The Fluctuating Enzyme* (Welch, G. R., Ed.) Wiley-Interscience, New York.

Ghiron, C., Bazin, M., & Santus, R. (1988) *Photochem. Photobiol. 48*, 539–543.

Hofricher, J., Henry, E. R., Sommer, J. H., Deutsch, R., Ikeda-Saito, M., Yonetani, T., & Eaton, W. A. (1985) *Biochemistry 24*, 2667–2679.

IUPAC Solubility Data Series (1981) Vol. 7, p 207, 409, Pergamon Press, New York.

Jameson, D. M., Gratton, E., Alpert, B., & Weber, G. (1984) *Biophys. J. 45*, 795–803.

Karplus, M., & McCammon, J. A. (1981) *CRC Crit. Rev. Biochem. 9*, 293–349.

Lakowicz, J. R., & Weber, G. (1973) *Biochemistry 12*, 4171–4179.

Lumry, R., & Rosenberg, A. (1976) *Colloq. Int. C.N.R.S. No. 246*, 53–62.

Marden, M. C., Hazard, E. S., III, & Gibson, Q. H. (1986) *Biochemistry 25*, 2786–2792.

Northrup, S. H., & McCammon, J. A. (1984) J. Am. Chem. Soc. 106, 930-934.
Parker, C. A., & Joyce, T. A. (1967) Photochem. Photobiol. 6, 395-406.
Saltiel, J., & Atwater, W. (1988) Adv. Photochem. 14, 1-90.
Shoup, D., & Szabo, A. J. (1982) J. Biophys. Soc. 40, 33-39.
Strambini, G. B. (1987) Biophys. J. 52, 23-28.
Szabo, A. J., Shoup, D., Northrup, S. H., & McCammon, A. (1982) J. Chem. Phys. 77, 4484-4493.
Vanderkooi, J. M., Calhoun, D. B., & Englander, S. W. (1987) Science 236, 568-569.
Ware, R. W. (1962) J. Phys. Chem. 66, 455-458.
Zemel, H., & Hoffman, B. M. (1981) J. Am. Chem. Soc. 103, 1192-1201.

Semisynthetic Hemoglobin A: Reconstitution of Functional Tetramer from Semisynthetic α-Globin†

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ABSTRACT: The optimal conditions for the semisynthesis of α-globin through *Staphylococcus aureus* V8 protease condensation of a synthetic fragment (α_{1-30}) with the complementary apo fragment (α_{31-141}) in the presence of structure-inducing organic cosolvents and the reconstitution of the functional tetramer from semisynthetic α-globin have been investigated. The protease-catalyzed ligation of the complementary apo fragments α_{1-30} and α_{31-141} proceeds with very high selectivity at pH 6.0 and 4 °C in the presence of 1-propanol as the organic cosolvent. A 30% 1-propanol solution was optimal for the semisynthetic reaction, and the synthetic reaction attained an equilibrium (approximately 50%) in 72 h. The synthetic reaction proceeds smoothly over a wide pH range (pH 5-8). Besides, the semisynthetic system is flexible, and it also proceeded well if trifluoroethanol or 2-propanol was used instead of 1-propanol. However, glycerol, a versatile organic cosolvent used in all other proteosynthetic reactions reported in the literature, was not very efficient as an organic cosolvent in the present synthetic reaction. The semisynthetic α-globin prepared with 1-propanol as the organic cosolvent has been reconstituted into HbA. The semisynthetic HbA was then purified by CM-cellulose chromatography. The semisynthetic HbA is indistinguishable from native HbA, in terms of its structural and functional properties. The semisynthetic approach provides the flexibility in protein engineering studies for the incorporation of spectroscopic labels (^{13}C- and/or ^{15}N-labeled amino acids), noncoded amino acids, or unnatural bond functionalities, which at present is not possible with genetic approaches.

Preparation of structural variants of a protein is a direct and powerful method that permits the delineation of the role(s) of specific amino acid residues in the structure/function of a protein. Recent advances in oligonucleotide-directed site-specific mutagenesis have made the generation of molecular variants much simpler (Gerlt, 1987; Ward & Fersht, 1988; Knowles, 1987) compared to the total chemical synthesis of proteins (Gutte & Merrifield, 1969; Clark-Lewis et al., 1986). Nonetheless, the semisynthesis of proteins still remains a potentially powerful alternative to site-directed mutagenesis in protein chemistry, especially since it provides a flexibility to introduce ^{13}C- or ^{15}N-labeled amino acids as well as noncoded amino acids at selected sites (Offord, 1985, 1987; Dimarchi et al., 1986). In these semisynthetic approaches, a synthetic polypeptide segment of a protein is ligated with the complementary fragment derived from the native protein either by enzymic or by nonenzymic methods (Chaiken, 1981).

The protease-catalyzed peptide bond formation (reverse proteolysis) has gained considerable interest in synthetic peptide chemistry in recent years (Bodansky, 1985; Kullman, 1985; Fruton, 1983). At first sight, the potential of proteases to hydrolyze preexisting susceptible peptide bonds in the fragments during condensation would seem to limit the general applicability of proteases in semisynthesis of covalent analogues of proteins (Kullman, 1985). However, the protease-catalyzed re-formation of the peptide bond at the discontinuity regions of "fragment complementing systems" is an exception in that respect. The specific noncovalent interactions of the complementary fragments of the complex maintaining a "nativelike" conformation generally limit the nonspecific digestion of the fragment complement systems by the protease (Chaiken, 1981; Kullman, 1985; Homandberg & Laskowski, 1979; Homandberg & Chaiken, 1980; Komariya et al., 1980).

The region of α-chain of hemoglobin A (HbA)[1] corresponding to the junction of the translation products of exon 1 and exon 2 of the α-globin gene has been recently identified as a "permissible discontinuity region" of the polypeptide chain

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[1] Abbreviations: Hb, hemoglobin; DPG, 2,3-diphosphoglycerate; HMB, *p*-(hydroxymercuri)benzoate.

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