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# Quenching of the Zinc–Protoporphyrin Triplet State as a Measure of Small-Molecule Diffusion through the Structure of Myoglobin[†]

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*Received August 18, 1986; Revised Manuscript Received November 24, 1986*

ABSTRACT: The diffusion of small molecules through the myoglobin structure was studied. It has been shown that the fluorescent Zn–protoporphyrin substitutes easily for the native nonfluorescent Fe–protoporphyrin in myoglobin. The quenching rate of the E-type delayed fluorescence of Zn–protoporphyrin in a substituted myoglobin by the quenchers oxygen and anthraquinonesulfonate was used to measure their diffusion from the ambient solution through the protein to the ligand binding site. The quenching rate constant (at 21 °C) for oxygen is $k_q = (9.6 \pm 0.9) \times 10^7$ $M^{-1}$ $s^{-1}$, only 1 order of magnitude less than that for Zn–hematoporphyrin quenching in aqueous solution. The activation energy in the range between 2 and 40 °C is $E_a = 6.0 \pm 0.6$ kcal/mol. The corresponding data for anthraquinonesulfonate are $k_q = (2.1 \pm 0.3) \times 10^8$ $M^{-1}$ $s^{-1}$ and $E_a = 5.8 \pm 0.6$ kcal/mol. Taking into account the statistical factor involved in the oxygen quenching of the Zn–porphyrin triplet, the quenching rates are very similar. The data are discussed in terms of the "gated reaction" theory of Northrup and McCammon. The similar rate constants and activation energies indicate that the diffusion rate in the protein is determined by the frequency of the conformational changes that open "gates" for the passage of the quencher through the protein.

Myoglobin (Mb) is one of the most intensively studied proteins with respect to structural implications for its biological functions. Most notable are the studies on ligand binding following photodissociation of the Mb–CO and the Mb–$O_2$ complexes over a wide range of temperatures (Austin et al., 1975; Debrunner & Frauenfelder, 1982) and the resonance Raman studies on Mb and on hemoglobin that describe in detail the conformational changes at the ligand binding site which accompany the above binding process (Spiro, 1985; Friedman, 1985). The most important conclusions drawn from these binding studies are the following: The protein exists in a number of conformational states, each of which binds the ligand at its specific rate. At room temperature these substrates are in rapid equilibrium with each other via conformational fluctuations while at low temperatures ($T < 180$ K) each protein molecule remains in its given substate. Similar conclusions about structural fluctuations have been drawn from X-ray diffraction studies (Artymiuk et al., 1979; Frauenfelder et al., 1979) and from molecular dynamics calculations (Karplus & McCammon, 1981). Moreover, the above binding studies indicate that, in order for a ligand molecule in the ambient solution to be bound to the myoglobin porphyrin, it must overcome a number of potential barriers on its way through the protein structure to the binding site.

The question being addressed here concerns one part of the above overall ligand binding process, namely, the diffusional movement of the small molecule from the solution through the protein. This movement has not yet been studied extensively in myoglobin. A rate constant of $k = 2 \times 10^8$ $M^{-1}$ $S^{-1}$ has been assigned to the diffusion in de-Fe myoglobin (Jameson et al., 1984). Here we describe the measurements of small-molecule diffusion through Mb without the process being influenced by the actual binding step. To this end, we use Mb whose Fe–protoporphyrin has been exchanged for Zn–protoporphyrin (ZnPP). The spectral properties of the latter are easy to follow, and the quenching rate of its long-lived excited triplet state yields a direct measure for the rate of diffusion of the quencher molecule from the external solution to the (ZnPP-substituted) binding site.

## EXPERIMENTAL PROCEDURES

The detailed procedure used for the preparation of ZnPP-substituted myoglobin (ZnPP-Mb) has been described previously (Feitelson & Spiro, 1986). Since apomyoglobin is very heat sensitive, its preparation and subsequent reconstitution were carried out in the cold room at approximately 3 °C. All solutions were precooled in an ice bath. Briefly, about 200 mg of sperm whale myoglobin (Sigma) were dissolved in 10 mL of water, acidified to pH 4, and shaken in a separating funnel with 15 mL of cold 2-butanone (Teale, 1959). The light-colored aqueous apomyoglobin phase was dialyzed overnight against water at 3 °C. The protein was reconstituted

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[†] The financial support of the Israel Academy of Sciences and Humanities is gratefully acknowledged.

with ZnPP obtained from Porphyrin Products (Logan, UT) by a procedure similar to that of Scholler (1981). For the above amount of apomyoglobin, about 12 μmol of ZnPP was wetted with 2 drops of pyridine, dissolved in 0.5 mL of 0.1 M $NH_4OH$, and diluted to 2 mL with water. The apomyoglobin solution was adjusted to pH 8.6 and the ZnPP solution added dropwise with shaking. The solution pH was readjusted to pH 8.6 and left in the ice bath for about 30 min. The pH was lowered to pH 7 and the solution loaded onto a CM-52 column that had been preequilibrated with 0.01 M phosphate buffer, pH 7.0. After elution was begun with about 50 mL of this buffer, the ZnPP-Mb was eluted as a sharp band with 0.15 M phosphate buffer, pH 7.0, and was stored in 0.6-mL aliquots at −70 °C. All work with the light-sensitive ZnPP was carried out in the dark or, when necessary, under very dim illumination.

To measure the delayed fluorescence of the ZnPP incorporated in myoglobin, the solution had first to be thoroughly deoxygenated. Four milliliters of about $10^{-5}$ M ZnPP-Mb contained in a 50-mL flask with a 1-$cm^2$ optical cell as side arm was stoppered with a rubber septum. It was freed of oxygen by flushing the magnetically stirred solution with 99.999% nitrogen (further purified by passing through an Alltech Associates Oxy-Trap). Alternatively, the ZnPP-Mb solution was freed from air on the vacuum line without freezing it in a specially designed cell assembly (Barboy & Feitelson, 1985). The temperature between 2 and 40 °C was controlled by circulating water from a thermostat through a brass cell holder. The ZnPP-Mb solution was illuminated by a 10-ns dye laser (Molectron 400) flash at 550 nm, and the delayed fluorescence decay was observed at right angles through a Jarrell-Ash 25-cm monochromator, set at 606 nm, by a Hamamatsu R-928 photomultiplier. In order not to saturate the photomultiplier by the strong prompt fluorescence, the latter was gated so as to develop the full high voltage across the dynode chain about 6 μs after the laser flash (Ballard, 1983). The signal was recorded on a Biomation 8100 and averaged on a Nicolet averager. Triplet–triplet absorption was measured on the same instrument in a slightly different configuration with a shuttered, pulsed Xe arc as analyzing light source. In the quenching experiments the required amount of oxygen was dissolved in the solution by adding a measured volume of air with a syringe to the nitrogen atmosphere in the cell assembly and equilibrating the stirred solution with it. When the solution was deoxygenated by evacuation, the measured amount of air was admitted to the cell assembly on the vacuum line. In the quenching experiments with anthraquinonesulfonate (AQS) an aliquot of $10^{-4}$ M AQS (Fluka, purissimum) in water was added to the ZnPP-Mb solution and the cell assembly kept under a stream of nitrogen throughout the experiment.

Triply distilled water was used for all solutions. The 2-butanone was a Riedel de Haen product. All other chemicals were of reagent grade. Absorption spectra of the solutions before and after illumination by the laser flashes were measured on a Cary 219 spectrophotometer.

## RESULTS

The diffusion of small molecules through myoglobin was measured by following the quenching of its porphyrin delayed fluorescence by oxygen and by anthraquinonesulfonate. Since the native Mb is nonfluorescent, myoglobin in which the Fe–protoporphyrin had been substituted by the fluorescent Zn–protoporphyrin (ZnPP) was used. Both the resonance Raman spectrum and the Q-band absorption spectrum of ZnPP-Mb show clearly that the ZnPP is bound in Mb to the proximal histidine in the Mb pocket (Feitelson & Spiro, 1986). The Zn–histidine stretching frequency lies, however, at a lower frequency than that of the Fe–histidine in the native protein, indicating a weaker bond in the ZnPP-substituted protein.

*ZnPP-Mb Delayed Fluorescence* (*DF*). When ZnPP-Mb in a deaerated aqueous solution is illuminated into one of its absorption bands, a long-lived emission in addition to the short-lived prompt fluorescence is observed. The spectral distribution of this emission is identical with that of the prompt fluorescence, it decays by a first-order process, and its intensity is proportional to that of the exciting laser flash. The lifetime of this emission, $\tau_T = 13.7 \pm 0.6$ ms at room temperature in a $10^{-5}$ M ZnPP-Mb solution, equals that of the ZnPP-Mb triplet state as measured by triplet–triplet absorption. Similarly to the emission previously described for ZnPP in solution (Feitelson & Barboy, 1986), it is therefore attributed to the E-type delayed fluorescence of ZnPP-Mb. Unlike the above data obtained for ZnPP and for Zn–octaethylporphyrin (ZnOEP) (Feitelson & Mauzerall, 1982) in solution, where both the triplet lifetime, $\tau_T$, and the fluorescence yield $\phi_{PF}$ increased somewhat with decrease in temperature, Figure 2 shows that $\tau_T$ in ZnPP-Mb is independent of the temperature between 2 and 40 °C. It was also found that its prompt fluorescence yield did not change in that temperature range. It has previously been shown (Feitelson & Barboy, 1986) that the intersystem crossing yield of ZnPP is very similar to that of ZnOEP (Gradyushko & Tsvirko, 1971), i.e., $\phi_{ISC} > 0.9$, and the same value is used here for ZnPP-Mb. Parker (1967) has proposed a relation for the population of the singlet state obtained by thermal excitation from the triplet: $\phi_{DF}/\phi_{PF}\tau_T = \phi_{ISC}Ae^{-E/RT}$, where $E$ is the singlet–triplet energy gap and the subscripts of the yields, $\phi$, denote the delayed fluorescence (DF), the prompt fluorescence (PF), and the intersystem crossing (ISC), respectively. $\tau_T$ is the triplet lifetime as measured by the delayed fluorescence decay in the absence of quencher. $\phi_{DF}$ is proportional to the integrated intensity of the emission, and $\phi_{DF}/\tau_T$ is proportional to the intensity of what is practically time zero $I^0_{DF}$. Since in our case $\tau_T$ and $\phi_{PF}$ are constant over the whole temperature range, a plot of $\phi_{DF}$ against $1/T$ yields the activation energy. A value for $E$ and hence a singlet–triplet gap of 7.25 ± 0.7 kcal/mol or 2580 ± 260 $cm^{-1}$ was obtained. This value should be comparable to the wavenumber difference between the fluorescence and phosphorescence of ZnPP-Mb. We were unable to measure the low-temperature phosphorescence of ZnPP-Mb, but the above value compares well with the $\Delta(S - T)$ value for ZnPP, 2750 $cm^{-1}$, obtained in an ethanol glass at 77 K.

*Quenching of the ZnPP-Mb Delayed Fluorescence.* The E-type delayed fluorescence, DF, is a convenient tool to study the porphyrin triplet state since it derives from that state and is not influenced by the presence of nonemitting transients that might be formed photochemically. Figure 1 shows that in the absence of oxygen the lifetimes measured by DF and by triplet–triplet absorption are identical. In the presence of low concentrations of oxygen, however, the triplet decay as measured by triplet–triplet absorption is accompanied by the appearance of a long-lived species. The absorption curve does not decay to zero, an effect that increases with subsequent flashes (see Figure 1-IIb). A new species seems to underlie the decay curve, a species that eventually disappears on a time scale of seconds. This photochemistry is probably brought about by the exposure of the solution to both the laser flash and the strong pulsed (1-ms) Xe arc used as analyzing light.

(*A*) *ZnPP-Mb Quenching by Oxygen.* A measured amount of air was added to the oxygen-free cell (see Experimental

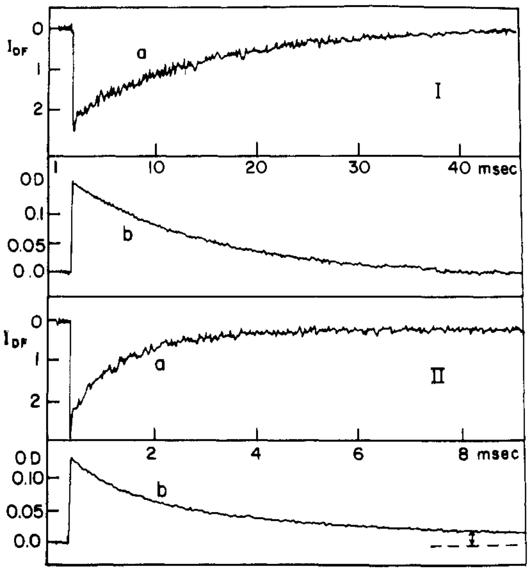

FIGURE 1: Decay of ZnPP-Mb triplet state (I) in the absence and (II) in the presence of $1.0 \times 10^{-5}$ M oxygen: (a) delayed fluorescence; (b) triplet–triplet absorption. The temperature was 21 °C; [ZnPP-Mb] = $10^{-5}$ M.

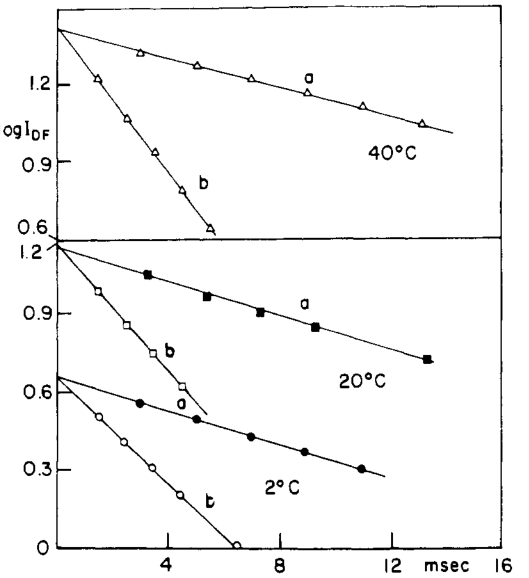

FIGURE 2: First-order decay of ZnPP-Mb delayed fluorescence. The intensities, $I_{DF}$, are in arbitrary units. The relative values of log $I_{DF}$ are given at three temperatures in the absence (a) and in the presence (b) of oxygen. [$O_2$] equals $3.3 \times 10^{-6}$ M at 2 °C, $2.0 \times 10^{-6}$ M at 20 °C, and $1.5 \times 10^{-6}$ M at 40 °C. [ZnPP-Mb] = $10^{-5}$ M.

Table I: ZnPP-Mb and ZnHP Triplet Lifetimes, Quenching Rate Constants, and Activation Energies in Aqueous Solution at 21 ± 1 °C[a]

| | quencher | ZnHP | ZnPP-Mb |
|---|---|---|---|
| $\tau^0$ (ms) | | 1.2 ± 0.2 | 13.7 ± 0.6 |
| $k_q \times 10^{-8}$ ($M^{-1}$ $s^{-1}$) | $O_2$ | 11.2 ± 2 | 0.96 ± 0.1 |
| $E_a$ (kcal/mol) | $O_2$ | 3.0 ± 0.3 | 6.0 ± 0.6 |
| $k_q \times 10^{-8}$ ($M^{-1}$ $s^{-1}$) | AQS | 22.0 ± 2 | 2.9 ± 0.3 |
| $E_a$ (kcal/mol) | AQS | 3.1 ± 0.3 | 5.8 ± 0.6 |

[a] [ZnHP] = $10^{-5}$ M. [ZnPP-Mb] = $10^{-5}$ M.

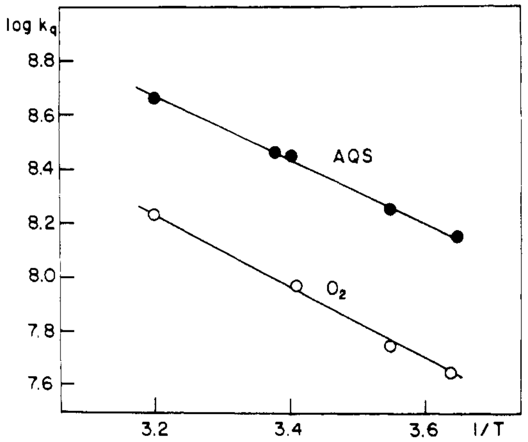

FIGURE 3: Activation energies for the quenching of ZnPP-Mb by oxygen and by anthraquinonesulfonate. [ZnPP-Mb] = $10^{-5}$ M.

Procedures) assembly and equilibrated thoroughly, by stirring, with the ZnPP-Mb solution. The oxygen concentration in solution was calculated from its partial pressure over the solution and its solubility at the given temperature in water. A value of $2.8 \times 10^{-4}$ M was used for the oxygen concentration in aqueous solution in equilibrium with air at 21 °C. The quenching of the ZnPP-Mb triplet was measured by the decrease in its lifetime in the presence of oxygen. Representative data at three temperatures are shown in Figure 2. The ZnPP-Mb concentration is $10^{-5}$ M. It is seen that an exponential decay with the same intensity at time zero, i.e., the same intercept on the log $I_{DF}$ axis, was obtained in the presence and in the absence of oxygen at any given temperature. This means that no significant static quenching takes place, i.e., no ground-state porphyrin–oxygen complex is present prior to excitation. The Stern–Volmer relation $\phi^0/\phi - 1 = k_q\tau_T{}^0[O_2]$ was obeyed at all temperatures. The value obtained at 21 ± 1 °C for the quenching rate constant $k_q = (9.6 \pm 0.9) \times 10^7$ $M^{-1}$ $s^{-1}$ compares well with that measured by Zemel (1981), $k_q = 1.2 \times 10^8$ $M^{-1}$ $s^{-1}$, and with the data of Alpert and Lindquist (1975) on the quenching of Fe-free hemoglobin, $k_q = 1.8 \times 10^8$ $M^{-1}$ $s^{-1}$. Quenching constants for ZnPP-Mb and for the water-soluble Zn–hematoporphyrin (ZnHP) are presented in Table I. The temperature dependence of the quenching rate constant between 2 and 40 °C yields an activation energy of $E_a = 6.0 \pm 0.6$ kcal/mol with a preexponential factor of $A = 2.5 \times 10^{12}$ $M^{-1}$ $s^{-1}$ (see Figure 3). The data show that the quenching rate of ZnHP by $O_2$ in solution is diffusion controlled.

(*B*) *ZnPP-Mb Quenching by Anthraquinonesulfonate* (*AQS*). Anthraquinone as well as other aromatic quinones is a very efficient quencher of porphyrin triplets (Feitelson & Mauzerall, 1982; Feitelson & Barboy, 1986). Aliquots of $10^{-4}$ M anthraquinonesulfonate (AQS) were added with a syringe to the ZnPP-Mb solution in the above-mentioned vessel with an optical cell as a side arm. The solutions were freed from oxygen by flushing the stirred solutions with purified nitrogen (see Experimental Procedures), and measurements were carried out with the solution under a stream of nitrogen. The DF was determined as a function of AQS concentration between 2 and 40 °C. Again, as in the oxygen quenching experiments the exponential decay rates obeyed the linear Stern–Volmer relation from which the quenching rate constants $k_q$ were calculated. From the temperature dependence of the quenching rate constants an Arrhenius activation energy of $E_a = 5.8 \pm 0.6$ kcal/mol, with $A = 5 \times 10^{12}$ $M^{-1}$ $s^{-1}$, was obtained (see Figure 3). The room temperature rate constants and activation energy for the AQS quenching of ZnPP-Mb and of Zn–hematoporphyrin (ZnHP) in aqueous solution are presented in Table I. It is seen that the AQS quenching of ZnHP in solution is diffusion controlled.

A few experiments of ZnPP-Mb quenching by naphthoquinonesulfonate were also carried out. They gave quenching rate constants very similar to those for AQS. However, the naphthoquinonesulfonate solutions deteriorated in the course of the experiments, and these data are therefore of qualitative value only.

## DISCUSSION

The long triplet lifetime of ZnPP-Mb, 13.7 ms as against 0.7–1.2 ms for ZnPP and Zn–hematoporphyrin in solution, as well as the constancy of the lifetime between 2 and 40 °C (see Figure 2) indicates that the ZnPP in the myoglobin pocket is well protected from impurity or solvent quenching.

We feel that the use of the long-lived triplet state to follow the ZnPP-Mb quenching process is preferable to the singlet-state fluorescence quenching. The long lifetime of the former makes it possible to use very low quencher concentrations. At our ZnPP-Mb concentration of $10^{-5}$ M the volume fraction occupied by the protein in solution is about $2 \times 10^{-4}$. If we assume that the distribution coefficient for the quencher between the protein and solution phases is not very different from unity, say $1 < [\mathrm{Q}]_{\mathrm{prot}}/[\mathrm{Q}]_{\mathrm{soln}} < 10$, then for a $10^{-5}$ M quencher concentration the latter resides practically wholly in the solution. Even for a quencher concentration of $10^{-4}$ M only about 1% of the myoglobin molecules will, on the average, contain one quencher molecule. Also, the similar intensities of the delayed fluorescence at time zero in the presence and in the absence of $O_2$ (Figure 2) show that no oxygen is bound in the ZnPP-Mb pocket. We describe the quenching process by

$$\mathrm{Q}_{\mathrm{soln}} \rightleftharpoons \mathrm{Q}_{\mathrm{prot}} \rightarrow \mathrm{QP}_{\mathrm{prot}} \rightarrow \mathrm{QP}$$

where $\mathrm{QP}_{\mathrm{prot}}$ is the quencher–triplet ZnPP encounter complex in the protein and the actual quenching step can be assumed to be instantaneous. As long as the quencher diffusion through the solution is rapid compared to that in the protein (see Table I), i.e., the solution–protein equilibrium with regard to the quencher is quickly established, the diffusion of the latter in the protein is the rate-determining step and the quenching rate becomes a direct measure of this diffusion through the protein.

The quenching data show two interesting features: (a) the rate constants for the ZnPP quenching in myoglobin are quite large, their value being only 1 order of magnitude smaller than those for ZnHP in aqueous solution; (b) the rate constants for the ZnPP-Mb triplet quenching by the small oxygen molecule and by the much larger anthraquinonesulfonate are similar. Taking into account that the ZnPP-Mb triplet quenching rate by the triplet oxygen is decreased by a statistical factor of 9 (Porter & Wright, 1959) and that the quenching by AQS, which presumably takes place by electron transfer, does not involve such a factor, the diffusion rates of the two quenchers differ only by a factor of 3—less than in aqueous solution. There seems to be no specific effect of the solute on the diffusion rate through the protein structure.

Lately, reactions in which the structure of a dense medium might obstruct a given process have been treated by introducing auxiliary coordinates in addition to the ordinary reaction coordinate (Northrup & McCammon, 1984). These coordinates describe atom or group displacements in the medium that relieve local obstructions so as to allow the reaction to proceed. It is generally accepted that processes like the diffusion of small molecules within a protein, with which we are concerned here, are made possible by conformational fluctuations of the protein that involve the movements of amino acid side chains or larger parts of the protein structure (Karplus & McCammon, 1981). These movements can relieve local steric hindrances in the diffusion path and thus facilitate the small molecule passage through temporarily opened gates (Szabo & Shoup, 1982). The conformational changes are expressed as variations along the above auxiliary gating coordinate.

Adopting Northrup and McCammon's treatment, we denote by $A_1$ and by $B_1$ the state of a quencher molecule in a protein of conformation 1 before and after a constriction along the diffusion path. The direct transition from $A_1$ to $B_1$ is effectively blocked by the protein structure; i.e., the free energy of activation for the transition, $\Delta G^{\ddagger}_{A_1-B_1}$, is very high. $A_2$ and $B_2$ are the corresponding states when the protein assumes a different conformation (2) in which the above constriction is removed. The conformational change (1) to (2), i.e., the opening of a gate in the diffusion path, which is accompanied by a decrease in the free energy of activation for the process ($\Delta G^{\ddagger}_{A_2-B_2} < \Delta G^{\ddagger}_{A_1-B_1}$), is described by a variation along the gating coordinate with its own free energy of activation $\Delta G^{\ddagger}_{A_1-A_2}$. The actual diffusion path is thus $A_1$–$A_2$–$B_2$–$B_1$. It has been shown by the above authors that, in the limiting case of very rapid conformational fluctuations, where the gating rate is much larger than the translational movement of the quencher molecules from $A_1$ to $B_1$, the protein structure seems to adapt to that movement and the diffusion rate is determined by the free energy of activation $\Delta G^{\ddagger}_{A_2-B_2}$. In the other limiting case, where the gating process is much slower than the transition along the reaction coordinate, it is shown that the gating rate determines the rate of diffusion. In this case the free energy of activation for the process is the energy needed for the gating, i.e., the conformational change, to take place, $\Delta G^{\ddagger}_{A_1-A_2}$, and not the energy to overcome the barrier along the diffusion path $\Delta G^{\ddagger}_{A-B}$.

Within the framework of the above theory our data cannot be explained by rapid subpicosecond or picosecond bond rotations or amino acid side chain displacements. These small amplitude fluctuations will not assist the passage of small as well as large molecules through the protein structure to the same extent; i.e., even the lowered free energy of activation along the diffusion path $\Delta G^{\ddagger}_{A_2-B_2}$ can be expected to depend on the size of the quencher. Rather, the second of the above limiting cases seems to apply here, where larger conformational changes open channels in the protein structure that enable both the small $O_2$ and the large AQS molecules to pass with equal ease. The similar activation energies obtained with the two quenchers ought not, in this case, be attributed to the frictional forces opposing diffusional movement but rather to the protein conformational changes, which make the diffusion of both oxygen and AQS possible. Further, if the gating mechanism applies to the diffusion of small molecules through myoglobin, one can, in principle, estimate a lower limit for the amplitude of the conformational change necessary for their passage. We have at present no reliable data for the dimensions of $O_2$ and of AQS to be used in the diffusion process through a protein. However, if we represent quite arbitrarily the dumbbell-shaped $O_2$ molecule by a cylinder with effective diameter $d = 2.8$ Å and the AQS molecule by an effective ellipsoid with short axis of 4.8 Å, the motions of the gate must lie between $X < 2.8$ Å, the distance between the blocking atoms in the closed gate position, and $X < 4.8$ Å for the open gate.

Finally, we note that this study deals with the diffusion in myoglobin at and near room temperature only. At lower temperatures the diffusion will presumably be influenced by the temperature dependence of the protein conformational changes.

**Registry No.** $O_2$, 7782-44-7; Zn–protoporphyrin, 15442-64-5; anthraquinonesulfonate, 60081-98-3.

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