Suva, L. J., Winslow. R. E. H., Wettenhall, R. G., Hammonds, G. M., Moseley, H., Diefenbach-Jagger, H., Rodda, C. P., Kemp, B. E., Rodriguez, H., Chen, E. Y., Hudson, P. J., Martin, T. J., & Wood, W. I. (1987) *Science 237*, 893–896.
Thrift, R., Forte, T. M., Cahoon, B. E., & Shore, V. G. (1986) *J. Lipid Res. 27*, 236–250.
Tomlinson, J. E., McLean, J. W., & Lawn, R. M. (1989) *J. Biol. Chem. 264*, 5957–5965.
Trieu, V. N., & McConathy, W. J. (1990) *Biochemistry 29*, 5919–5924.
Utermann, G., & Weber, W. (1983) *FEBS Lett. 154*, 357–362.
Utermann, G., Menzel, H. J., Kraft, H. G., Duba, H. C., Kemmler, H. G., & Seitz, C. (1987) *J. Clin. Invest. 80*, 458–465.
Vehar, G. A., Kohr, W., Bennett, W. F., Pennica, D., Ward, C. A., Harkins, R. N., & Collen, D. (1984) *Biotechnology 2*, 1015–1057.
Wong, W. L. T., Eaton, D. L., Berloui, A., Fendly, B., & Hass, P. E. (1990) *Clin. Chem. 36*, 192–197.

Migration of Small Molecules through the Structure of Hemoglobin: Evidence for Gating in a Protein Electron-Transfer Reaction†

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ABSTRACT: It has previously been shown that the rates and activation energies for migration molecules of different sizes through myoglobin are very similar. The results were interpreted in terms of conformational changes in the protein structure that facilitate the passage of the different molecules to a similar extent. Here we ask whether the quaternary structural changes that accompany the binding of ligands (O_2 or CO) to hemoglobin might influence the migration rate from the solution into the protein's binding site. As a model for the R state of hemoglobin, we used the protein in which the Fe protoporphyrin (FePP) in the α subunit was substituted by Zn protoporphyrin (ZnPP) and the oxidized heme was ligated by CN^-. The T state of hemoglobin was represented by the protein in which all four FePP groups were substituted by ZnPP. The quenching rate of the excited ZnPP triplet state within the hemoglobin by oxygen, methyl viologen, and anthraquinonesulfonate served as a measure of the migration rate through the protein into the binding site. It was found that the activation energies for all three quenchers were very similar and closely resembled those in myoglobin, suggesting that the migration rates are determined by the subunit structure only and that the quaternary configurational changes do not influence the quenching rates. The implications of the results for electron transfer in proteins are briefly discussed.

The reaction between a protein and small molecules, for example, a substrate, depends not only on the specific reaction rate constant(s) but also on the diffusional migration of the small molecule through the protein matrix toward the reaction site. What is usually measured is the overall process. Therefore, in order to understand fully the reaction within a biological macromolecule, it is necessary to individually determine the two components of the above complete reaction.

The binding of ligands to myoglobin and to hemoglobin has been studied extensively by following the photodissociation of the bound ligand and its subsequent rebinding at the heme site (Frauenfelder & Debrunner, 1982; Ansari et al., 1986; Henry et al., 1984; Murray et al., 1988; Marden et al., 1986; Friedman, 1985). In myoglobin, it was found that the ligand must overcome one or more potential barriers on its way toward the binding site (Austin et al., 1978; Marden, 1986) before the actual binding step occurs with its own specific activation energy. In hemoglobin, the question has been raised whether the quaternary structural changes upon ligand binding affect the reentry rate of the latter into the heme pocket. It was suggested by a group at NIH (Henry et al., 1984; Murray et al., 1988) that the diffusion rate into the protein is not responsible for the differences in the ligand association rates of the hemoglobin R and T states.

We shall concern ourselves here with the migrational movement of the potential ligand O_2 and other, somewhat larger, molecules through the structure of hemoglobin.

Various experiments, such as hydrogen exchange (Lumry & Rosenberg, 1976), fluorescence quenching (Lakowicz & Weber, 1973), and X-ray diffraction (Frauenfelder et al., 1979; Artymiuk et al., 1979) and calculations (Karplus & McCammon, 1981) indicate that proteins are dynamic entities that undergo structural fluctuations. It is these movements within the protein that are thought to enable molecules like oxygen to migrate through the otherwise compact protein structure. Following the above seminal work by Weber and Lakowicz, the fluorescence and also the triplet-state quenching of tryptophan residues in proteins by different quenchers have been extensively studied (Calhoun et al., 1988; Ghiron, 1988; Papp & Vanderkooi, 1989). While it is generally agreed that oxygen efficiently penetrates most proteins and thus quenches easily the excited tryptophans, the mechanism for larger quenchers is less well understood. The phosphorescence-quenching rate constants for a number of proteins by various

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quenchers have been reported (Calhoun et al., 1988). Criteria for quenching mechanisms of internal tryptophans, which include gate opening as well as energy and electron transfer, are proposed here.

One useful model system for study involves the triplet-state quenching of Zn protoporphyrin (ZnPP) that is substituted for the native Fe porphyrin in myoglobin (Barboy & Feitelson, 1987, 1989). It was shown that as long as the quenching rate of ZnPP by small molecules in solution is considerably larger than the quenching rate in the protein, the protein rate is determined by the migration rate of the quencher through the protein matrix. Further, it was found that the room temperature activation energy for a small neutral molecule like O_2 is surprisingly similar to that of the large charged anthraquinonesulfonate and methyl viologen molecules. It was concluded that in this case the migrational movement through the protein is apparently facilitated by conformational changes, a kind of "gating" process, by which temporary channels are formed in the protein structure. In a like manner, the quenching kinetics of Zn porphyrins substituted into hemoglobin could probe whether the structure of hemoglobin (with its myoglobin-like subunits) affects the above migration rate. It might be imagined that the quaternary structural changes that take place during the binding process of ligands to hemoglobin might either restrict or facilitate the migration of the small molecules. Thus, these experiments provide a direct test of the suggestions of Henry et al. (1984).

To prove such tests, two types of Zn-substituted hemoglobins were used. Hemoglobin, in which the native Fe protoporphyrin (FePP) in its α subunits was substituted by Zn protoporphyrin (ZnPP) while the β subunits were oxidized to the Fe^{3+} state and ligated with –CN, ($\alpha Zn\beta Fe^{3+}CNHb$), served as a model for the R state hemoglobin. The protein in which all four iron porphyrins were substituted by Zn protoporphyrin (Zn_4Hb) represents the T state of hemoglobin (Simolo et al., 1985). It has been shown that these substitutions do not significantly affect the secondary or tertiary structures of hemoglobin (Scholler et al., 1978; Simolo et al., 1986) or myoglobin (Feitelson & Spiro, 1985). The Zn protoporphyrins in the hemoglobin were flash excited, and the subsequent quenching reactions of their triplet state by anthraquinonesulfonate (AQS), by methyl viologen (MV), and by oxygen were followed as a function of both quencher concentration and temperature. As in myoglobin, this quenching represents the rate of small molecule movement from the ambient solution through the protein structure toward the quenching site. The quenching rate constants and the activation energies obtained can yield information about the protein structural dynamics that enables the quencher molecules to penetrate the protein matrix on their way to the excited triplet state.

Experimental Procedures

$\alpha Zn\beta Fe^{3+}CNHb$ was prepared as described previously (Simolo et al., 1985). It was fully oxidized by reacting it with $K_3Fe(CN)_6$ and removing the surplus ferricyanide by chromatography on a Sephadex G25 column. Zn_4Hb was prepared as previously described by Hoffman (Scholler et al., 1978). The proteins were stored in the form of frozen pellets in liquid nitrogen. For each experiment, a small amount of the hemoglobins was dissolved in pH 7.2, 0.02 M phosphate buffer to a concentration of about 8×10^{-5} M. The $\alpha Zn\beta FeHb$ was cyanide ligated by addition of an equimolar amount of aqueous KCN to the solution. The ligand exchange was followed by the disappearance of the 408-nm shoulder in the Soret absorption band. Subsequently, the protein and the quencher solutions were deoxygenated by flushing with high-purity nitrogen in septum-stoppered Erlenmeyer flasks and transferred to a glove box.

Table I: Zinc Hemoglobin, Zinc Myoglobin, and Zinc Hematoporphyrin Quenching by O_2, MV, and AQS in Aqueous Solution[a]

	$\alpha Zn\beta FeCNHb$	Zn_4Hb	ZnMb	ZnHP
	quenching rate constants (M^{-1} s^{-1})			
k_q(AQS)	2.1×10^8	1.7×10^8	2.9×10^8	22×10^8
k_q(MV)	8.2×10^7	8.3×10^7	4.5×10^7	36×10^8
$k_q(O_2)$	9×10^7	1.5×10^8	1×10^8	11×10^8
	activation energies (kcal/mol)			
E_a(AQS)	5.4	6.0	5.8	3.1
E_a(MV)	5.6	6.2	7.4	3.9
$E_a(O_2)$	6.3	6.9	6.0	3.2

[a] k_q values are the quenching rate constants at 25 °C for the hemoglobins and at 20 °C for myoglobin. The estimated errors in the quenching rate constants are ±12% and in activation energies are ±0.5 kcal/mol.

For quenching with AQS and MV, a 1-mL portion of protein solution and 3 mL of deoxygenated buffer and the appropriate volume of quencher solution were anaerobically transferred to an optical cell and stoppered with subaseal septa. The cells containing the solution were illuminated in a water-jacketed cell holder by a DCR2 NdYAG laser at 532 nm, and the transient absorption was followed by an R-928 (Hamamatsu) photomultiplier. The signal was digitized by a Tektronix 7912 digitizer and transferred to an IBM PC for data analysis.

For quenching with oxygen, the solution contained in a deoxygenation vessel with an optical cell as a side arm (Barboy & Feitelson, 1989b) was freed of oxygen by flushing with prepurified nitrogen. A measured amount of air was injected with a syringe into the apparatus and the solution equilibrated with the atmosphere in the vessel for at least $^1/_2$ h by stirring at the temperature of the experiment. The oxygen concentration in solution was determined from the solubility of oxygen at the given temperature (Critical Tables, 1928).

Oxygen-containing porphyrin solutions readily undergo irreversible photochemical changes producing products that absorb light in the absorption range of the triplet and thus interfere with the determination of the decay constants. Therefore, the quenching rate by oxygen was determined by illumination of the solution with a weak laser flash from a nitrogen-laser-pumped dye laser (Molectron DL220) at 550 nm. Both the decay of the E-type delayed fluorescence from the Zn protoporphyrin in hemoglobin and its triplet absorption were determined as a function of temperature.

Results and Discussion

The absorbance of the ZnPP-substituted hemoglobins in the presence of AQS or MV does not decay entirely to zero. Therefore, the raw decay curves obtained from transient absorption had to be corrected for the slight absorbance of the photoproduct formed.[1] The decay constants were determined as a function of quencher concentration to obtain the actual second-order rate constants. These rate constants were measured at several temperatures between 5 and 40 °C to

[1] If the absorbance of a short-lived excited species, A, does not decay to zero due to the simultaneous formation of a photoproduct, B, the decay is analyzed as follows: For a first-order process, the true decay of A is given by A_0e^{-kt}, where A_0 is the initial concentration of A. The formation of B is described by $B_\infty - B_\infty e^{-kt}$, with B∞ denoting the final concentration of B. The measured decay is therefore given by $(A_0 - B_\infty)e^{-kt} + Ba_\infty a$. Hence, to obtain the decay constant k, the baseline to be used is raised to the value B_∞ and k is evaluated from a logarithmic plot of $(A_0 - B_\infty)e^{-kt}$ against t.

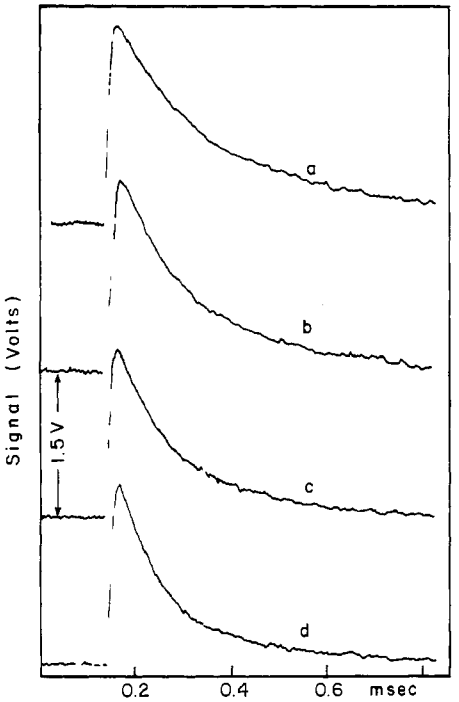

FIGURE 1: Transient absorption signal for the first-order decay of the Zn_4Hb triplet in prescence of 385 μM AQS at (a) 7 °C, (b) 17.5 °C, (c) 25 °C, and (d) 36.8 °C. The ordinate shows the decrease in light transmittance in volts as measured by the digitizer.

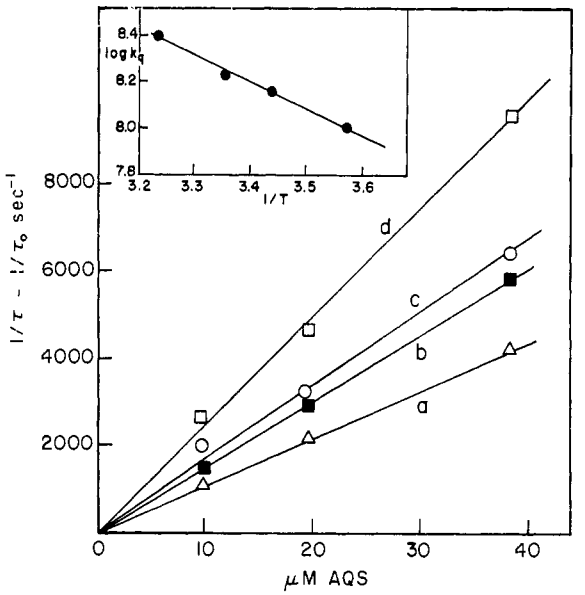

FIGURE 2: Stern–Volmer plots for the quenching of Zn_4Hb by 38.5 μM AQS at (a) 7 °C, (b) 17.5 °C, (c) 25 °C, and (d) 36.8 °C. Insert: activation energy for Zn_4Hb quenching by AQS.

determine the activation parameters for the reaction. The second-order rate constants, k_q, were calculated from the Stern–Volmer relation, $1/\tau - 1/\tau_o = k_q[Q]$. The results are presented and compared to those obtained previously for myoglobin and for hematoporphyrin in Table I. Representative decay curves, Stern–Volmer plots, and activation energies are shown in Figures 1 and 2.

It is seen that, to a first approximation, both the quenching rate constants, k_q, and the activation energies, E_a, are very similar for all three quenchers. The k_q values for the positively charged MV^{2+} are slightly lower than those for AQS^-, which carries a negative charge. This difference might be attributed to a charge effect along the migratory pathway toward the ZnPP in the protein, or perhaps to greater solvation of the MV^{2+} cation.

The activation energies at 25 °C for the tetrazinc hemoglobin are somewhat higher than those for the $\alpha Zn\beta Fe$ hemoglobin. Although the effect falls within the experimental error range, the consistently higher values for all three quenchers suggest there is a small quaternary effect. This apparent change in activation energy is offset by a slightly larger preexponential factor (hence a larger activation entropy) for (T-state) Zn_4Hb. The difference in activation entropies thus derived between the high-affinity T state and the low-affinity R states of hemoglobin amounts to about 2 eu.

The heme site in hemoglobin is where ligand binding occurs and also where the observed small molecule mediated quenching occurs in the present experiments. The data show that migration of the quencher (or ligand) to the heme site is primarily determined by the tertiary structure of the subunits and not by changes in the quaternary structure of hemoglobin. This agrees with the suggestion of a group at NIH (Murray et al., 1988) that the energetics of the ligand binding reflects mainly the heme binding rates upon changing from the R to the T state of hemoglobin and not earlier processes along the reaction coordinate, such as internal diffusion. Therefore, the diffusive processes measured for Mb, Hb(R), and Hb(T) are remarkably similar. In the hemoglobins studied here, the activation energies for the quenching process by the small O_2 molecule and the much larger MV^{2+} and AQS– resemble each other even more than in Zn myoglobin. The specific quenching step is likely to occur by electron transfer

$$[\text{Zn porphyrin}]^* + Q \rightarrow Q^- + [\text{Zn porphyrin}]^+$$

This mechanism has been clearly established by transient absorbance measurements for the reaction of hemoglobin with methyl viologen (Magner & McLendon, 1989) and simple quinones (Simmons and McLendon, unpublished data). Oxygen might react by either electron transfer or triplet–triplet annihilation. All these mechanisms have strong dependence on distance and thus are favored by close approach (although not necessarily direct collisions) between the porphyrin and quencher.

The rates and activation parameters for diffusion of the reactants in the solution phase can be modeled by the quenching of the protein-free Zn hematoporphyrin (Table I). ZnHP + Q reactions give small activation energies ($\sim 2 \times 10^9$ M^{-1} s^{-1}). Clearly, the protein presents a barrier to the diffusion of the reactants toward close contact. As previously noted, diffusion is facilitated into the closely packed protein interior by fluctuations in the protein structure (Frauenfelder et al., 1979; Frauenfelder & Debrunner, 1982).

It might be expected, however, that MV^{2+}, with an effective radius > 6 Å, would require quite different fluctuations than the small, neutral O_2 molecule. Why, then, do these three quenchers of different size, structure exothermicity, and charge show such similar rates and activation energies? One possible quenching mechanism that should be considered involves direct collisional quenching by the acceptors at an exposed heme edge. This direct quenching can probably be excluded for several reasons. For such a direct collisional quenching process, the protein would primarily provide a steric effect, which limits the accessible quenching area (Sutin, 1974): $k_{obs} = k_q(f)$, where k_q is the rate constant for quenching of a fully exposed porphyrin ($\sim 2 \times 10^9$ M^{-1} s^{-1}) and f is the fractional area exposed. Thus, in comparing the direct collisional mechanism for the protein with that for hematoporphyrin, it is expected that while the protein should show a smaller rate constant (due to the steric factor), only the prefactor should change but the activation energy, which would be controlled by small molecule

diffusion, should not change. This prediction does not agree with the observed data. Furthermore, if reaction were to occur at the edge, the steric protection factor (f) would in fact differ substantially for different quenchers with $f(O_2) \gg f(MV^{2+})$, thus predicting $k_{obs}(O_2) \gg k_{obs}(MV^{2+})$, again in contrast with experiment. Finally, for electron-transfer quenching at the heme edge, one would predict that the observed rate (and activation energy) should depend on the free energy of the electron-transfer reaction, in contrast with observation. These results for these heme proteins are indeed in contrast with results of Vanderkooi et al. (Papp & Vanderkooi, 1989) on quenching of buried tryptophans, where large steric effects *are* seen. These differences would be further exacerbated by local charges at the reaction site (e.g., the heme propionates). For these reasons, it is unlikely that the quenching mechanism involves direct attack at the partially exposed heme edge without some associated fluctuation of the protein, which gives rise to a higher activation energy. However, the conformational fluctuations that prevent quenching might indeed occur near the heme edge, so that complete diffusion to the Zn^{2+} center may be unnecessary.

However, the data *might* be explained by invoking a gating mechanism (Shoup & Szabo, 1982; Northrup & McCammon, 1984; Agmon & Kosloff, 1987), as previously suggested for ZnPPIX-substituted myoglobin (Barboy & Feitelson, 1989a). Adopting Northrup and McCammon's theoretical approach, a process in a restricted medium, such as a protein, is described by the reaction coordinate for the electron transfer and by an additional auxiliary coordinate that is related to changes in the protein conformation. The potential energy surface is depicted as a topographic map where the x axis is the reaction coordinate and the y axis is the (conformational) auxiliary coordinate. If the direct passage from location A_1 to location B_1 in the protein is blocked by a higher potential barrier, $\Delta G(A_1 \rightarrow B_1)$, i.e., a bottleneck in the passageway, there might exist a different protein conformation where the free energy of activation for the passage from A to B is lower, $\Delta G(A_2 \rightarrow B_2)$. The subscripts 1 and 2 denote protein conformation such that $\Delta G(A_2 \rightarrow B_2) < \Delta G(A_1 \rightarrow B_1)$. The theory describes two limiting cases. If the conformational fluctuations in the protein occur on a time scale that is short in comparison to the migration of the quencher, the protein conformation adapts to the movement of the latter and the activation energy of the process represents the lowered energy barrier $\Delta G(A_2 \rightarrow B_2)$. The picosecond fluctuations of the amino acid side chains described by molecular dynamics calculations would constitute such fluctuations. These small, local fluctuations cannot be expected to facilitate the passage of the small O_2 molecule and the much larger AQS^- and MV^{2+} molecules to the same extent. Therefore, the energy barrier experienced by these different size molecules would not be the same, in contrast with the observed similarities in rate constants and activations energies. In the other limiting case, the conformational changes are slow with respect to the time it takes the quencher to pass the constriction. In this case, the activation energy measures the energetics of the protein dynamics, i.e., the opening of a "gate". This is denoted on the above two-dimensional energy surface as a change along the auxiliary coordinate from point A_1 to point A_2. If we approximate the free energy of activation by the enthalpy of activation, then the similarity of the activation energies for all three quenchers indicates that the gating mechanism operates in hemoglobin and that the measured E_a values represent the energy needed to effect the above conformational changes. Once this conformation change, the "opening of the gate", has taken place, small and large quencher molecules alike can pass through the above constricted region with equal ease on their way from location A to location B. This suggested mechanism necessarily implies that a rather large gate, i.e., a passage of >6-Å radius is required to accommodate the large AQS^- or MV^{2+} quenchers. These data are particularly germane in the context of recent interest in electron transfer in proteins. Conformational gating has been proposed to play an important role in protein redox reactions (Hoffman & Ratner, 1987), but few examples exist of such gating (McLendon et al., 1987). The present data provide a possible example of one type of conformational gating in electron transfer in which relatively large structural rearrangements allow a close approach between the reactants. These data also point out a possible difficulty in other studies in interpreting the activation parameters observed for protein electron transfer. In the present case, the observed activation energy bears no relationship to the fundamental electron-transfer step, but rather to a preceding conformational rearrangement. Similar results can be predicted for other proteins for which gating is involved in the rate-determining step.

Registry No. O_2, 7782-44-7; MV, 1910-42-5; AQS, 30637-95-7.

REFERENCES

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

Ansari, A., Dolorio, E. E., Frauenfelder, H., Iben, I. E. T., Langer, P., Roder, H., Sauke, T. B., & Shyamsundu, E. (1986) *Biochemistry 25*, 3189–3146.

Artymiuk, 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.

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

Barboy, N., & Feitelson, J. (1989a) *Biochemistry 28*, 5450–5456.

Barboy, N., & Feitelson, J. (1989b) *Anal. Biochem. 180*, 384–386.

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

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

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

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

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

Henry, E. R., Hofrichter, J., Sommer, J. H., & Eaton, W. A. (1984) in *Photochemistry and Photobiology* (Zewail, A. W., Ed.) Vol. 2, pp 791–880, Harwood Academic Publishing, New York.

Hoffman, B., & Ratner, A. (1987) *J. Am. Chem. Soc. 109*, 6237–6243.

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. 246*, 53–62.

Magner, E., & McLendon, G. (1989) *Biochem. Biophys. Res. Commun. 159*, 472–476.

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

McLendon, G., Pardue, K., & Bak, P. (1987) *J. Am. Chem. Soc. 109*, 7540–7541.

Murray, L. P., Hofrichter, J., Henry, E. R., & Eaton, W. A.

(1988) *Biophys. Chem. 29*, 63-76.
Northrup, S. H., & McCammon, J. A. (1984) *J. Am. Chem. Soc. 106*, 930-934.
Papp, S., & Vanderkooi, J. M. (1989) *Photochem. Photobiol. 49*, 775-784.
Scholer, D. M., Warig, M. R., & Hoffman, B. (1978) *Methods Enzymol. 52C*, 487-493.
Shoup, D., & Szabo, A. J. (1982) *Biophys. J. 40*, 33-39.
Simolo, K., Stucky, G., Chen, B., Bailey, M., Scholes, C., & McLendon, G. (1985) *J. Am. Chem. Soc. 107*, 2865-2872.
Simolo, K., Korszun, Z. R., Stucky, G., Moffat, K., & McLendon, G. (1986) *Biochemistry 25*, 3773-3778.
Sutta, N. (1974) in *Metals in Biology* (Eichhorn, G., Ed.) Elsevier, New York.

Thermodynamic Identification of Stable Folding Intermediates in the B-Subunit of Cholera Toxin†

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ABSTRACT: The structural stability and domain structure of the pentameric B-subunit of cholera toxin have been measured as a function of different perturbants in order to assess the magnitude of the interactions within the B-subunits. For these studies, temperature, guanidine hydrochloride (GuHCl), and pH were used as perturbants, and the effects were measured by high-sensitivity differential scanning calorimetry, isothermal reaction calorimetry, fluorescence spectroscopy, and partial protease digestion. At pH 7.5 and in the absence of any additional perturbants, the thermal unfolding of the B-subunit pentamer is characterized by a single peak in the heat capacity function centered at 77 °C and characterized by a ΔH_{cal} of 328 kcal/mol of B-subunit pentamer and $\Delta H_{vh}/\Delta H_{cal}$ of 0.3. Lowering the pH down to 4 or adding GuHCl up to 2 M results in a decrease of the calorimetric enthalpy with no significant effect on the van't Hoff enthalpy. The transition enthalpy decreases in a sigmoidal fashion with pH, with an inflection point centered at pH 5.3. Isothermal titration calorimetric studies as a function of pH also report a transition centered at pH 5.3 and characterized by an enthalpy change of 27 kcal/mol of B-subunit pentamer at 27 °C. Below this pH, the enthalpy change for the unfolding transition is reduced to approximately 100 kcal/mol of B-subunit pentamer. Similar behavior is obtained with GuHCl. In this case, a first transition is observed at 0.5 M GuHCl and a second one at 3 M GuHCl. Trypsin digestion studies show that at pH 5.0 the B-subunit is 4 times more susceptible to digestion than at pH 7.0 and that at pH 5.0 limited proteolysis results in two fragments of ~7 and ~5 kDa. These studies provide strong evidence that the B-subunits of cholera toxin are composed of two folding/unfolding domains and that the interactions between the two domains within the same subunit and between subunits are able to account for the cooperative behavior of the entire pentameric ring.

Cholera toxin, the enterotoxin of *Vibrio cholera*, is a globular protein (M_r 85000) composed of two protomeric species, A (M_r 27000) and B (M_r 58000) (Finkelstein, 1973; Sattler et al., 1975; Lai, 1980). The A-promoter contains two nonidentical subunits: an ADP-ribosylating protein, A1 (M_r 21000), which activates adenylate cyclase, and a small subunit, A2 (M_r 6000), which plays a structural role in holding the A- and B-subunits together. The two components of the A-subunit are linked by a single disulfide bond (Gill & King, 1975). The B-promoter contains five identical polypeptide chains (each of M_r 11500) arranged in a noncovalently associated, ringlike pentameric configuration surrounding the dimeric A-subunit (Gill, 1976; Ribi et al., 1988; Spangler & Westbrook, 1989). Cholera toxin binds specifically through the B-subunit to ganglioside GM1 present on the plasma membrane of most eukaryotic cells. This association is believed to cause a conformational change in the protein (Sillerud et al., 1981; Fishman et al., 1978; Goins & Freire, 1985, 1988a; Schon & Freire, 1989; Surewicz et al., 1990) that facilitates the exposure and subsequent penetration of the A-subunit into the membrane.

Previous studies on the thermal stability of intact cholera toxin and cholera toxin subunits from this laboratory (Goins & Freire, 1985, 1988a,b) have shown that cholera toxin undergoes two distinct thermally induced transitions; a low-temperature transition (51 °C) due to the irreversible denaturation of the A-subunit and a high-temperature, reversible transition centered at approximately 75 °C due to the thermal unfolding of the B-subunit. The characteristics of the B-subunit transition are not affected by the presence or absence of the A subunit. In the absence of ganglioside GM1, the B-subunit pentamer exhibits a single transition in aqueous solution and is characterized by little or no intersubunit cooperative interactions. Upon binding to ganglioside GM1, the unfolding process becomes highly cooperative, and the pentameric B-subunit ring effectively behaves as a single cooperative unit (Goins & Freire, 1988; Schon & Freire, 1989).

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