Gaugement of the inner space of the Apomyoglobin's heme binding site by a single free diffusing proton

II. Interaction with a bulk proton

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ABSTRACT The reaction mechanism and the dynamic aspects of protonation of a defined moiety located inside an aqueous cavity in a protein were monitored by time resolved spectroscopy using the pyranine apomyoglobin complex as a model (Shimoni, Tsfadia, Nachliel, and Gutman, 1993, *Biophys. J.* 64:472–479).

The reaction was synchronized by a short laser pulse and the reprotonation of the ground state pyranine anion (ΦO^-) was monitored, in the microsecond time scale, by its transient absorption at 457 nm.

The observed signal was reconstructed by a numeric solution of nonlinear, coupled differential equations which account for the direct reaction of ΦO^- with bulk proton and by proton transfer from the nearby amino acids: His 64, Asp 44, Asp 60, and Glu 59. A unique combination of rate constant was obtained which quantitates the contribution of each pathway to the overall relaxation process.

In the first phase of the dynamics ΦO^- abstracts a proton from the nearby protonated histidine. The bulk proton interacts preferentially with the cluster of three carboxylates and immediately shuttled to the deprotonated histidine.

The high proximity of the reactive groups and the strong electrostatic forces operating inside the heme binding cavity render the rate of proton transfer in the site ultrafast.

INTRODUCTION

With respect to proton transfer reactions, a protein represents a scaffold supporting a multitude of protonable groups (Cao et al., 1992; Henderson et al., 1990; Gerwert et al., 1990; Cybulski and Scheiner, 1989). The low dielectric constant of the protein intensifies the electrostatic forces at the interface with subsequent expansion of the Coulomb cage of the surface bound charges (Matthew, 1985). The overlap of the Coulomb cages increases the probability of proton passage between surface groups (Gutman and Nachliel, 1990), while clustering of negatively charged moieties forms proton attracting regions with high probability for capturing a bulk proton (Yam et al., 1991). The combination of a preferential proton binding region with adjacent proton acceptors can provide an alternative mode for protonation of a specific site, which may be even faster than its direct protonation by a diffusion controlled reaction. In this publication we demonstrate the validity of this scenario by measuring the time resolved reaction of a bulk proton with a chromophore, pyranine anion (8 hydroxy pyrene 1,3,6 trisulfonate) located in the heme binding site of apomyoglobin (Gutman and Nachliel, 1982; Shimoni et al., 1993).

The three-dimensional structure of the protein dye complex, visualized by the FRODO program (Jones, 1985) and the coordinates of oxymyoglobin (Phillips, 1980), reveals that the dye occupies only a fraction of the space taken up by the heme group. It is anchored by salt bridges with two histidines (His 93, His 97), which must be in their protonated state to form a tight complex, and Arg 45 (Gutman and Nachliel, 1982). The nearby His 64, too far to serve as a ligand, is located between the dye and a cluster of three carboxylic acids Asp 60, Asp 44, and Glu 59.

To investigate how and to what extent these amino residues act as a proton antenna that shuttles protons to the dye, we monitored the dynamics of proton passage from bulk to the dye by a time resolved spectroscopy. The proton was evicted from the site by exciting the pyranine (ΦOH) to its first electronic singlet state (ΦOH^*) which rapidly dissociates to ΦO^{*-} and H^+. In sites from which H^+ leaked out, the dye relaxed to ground state anion that is characterized by a strong absorption band at 450 nm. The monitoring of the reprotonation of the dye in the microsecond time scale produces an experimental curve which was analyzed by numerical integration of a set of coupled, nonlinear differential equations, where the rate constants are adjustable parameters (Yam et al., 1988). As will be shown below, the reconstructed dynamics has a unique solution, indicating that the most probable route of proton involves both the carboxylates and His 64. In the initial phase H^+ His serves as immediate proton donor to ΦO^-, while the carboxylates provide an electrostatic trap for bulk protons, yet as soon as the protons react with the carboxylates, it is shuttled to the proton deprived histidine. The rate constants of these steps were determined and their magnitude is comparable with that of solvent mediated intramolecular proton transfer reactions (McMorrow and Kasha, 1984).

Based on these observations, the mechanistic role of charged moieties surrounding an active site should be incorporated in the structure function relationship of the protein.

MATERIALS AND METHODS

Apomyoglobin was prepared as described in the preceding manuscript (Shimoni et al., 1993). The reprotonation dynamics of the pyranine in

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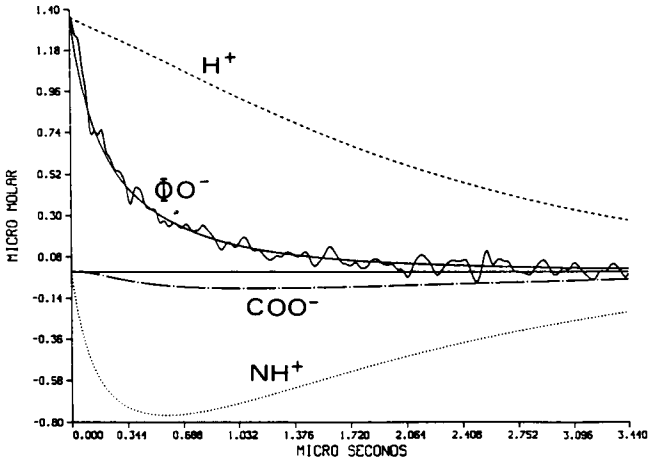


FIGURE 1 Time resolved dynamics of the reprotonation of pyranine in the heme binding site following its photo dissociation. The reaction was carried at pH 5 using 176 μM pyranine and 400 μM Apomyoglobin. The excitation was at the third harmonic frequency of Nd-Yag laser (λ = 355 nm) at energy density of 1 MW/cm^2. The absorbance of ΦO^- was monitored at λ = 457 nm.

The line marked ΦO^- depicts the measured absorbance of the sample and a reconstructed dynamics using the rate constants listed in Table 1. The other curve denotes the free proton concentrations, protonation of carboxylates, and deprotonation of the Histidin 64 marked: H^+, COO^-, and NH^+, respectively.

the heme binding site was measured with dye protein complex (176 μM dye and 400 μM protein) at pH = 5 in absence of any buffer. Under these conditions the fraction of the free dye is less than 0.05%.

The sample under study was irradiated by the third harmonic frequency (λ = 355 nm) of a Nd.Yag laser (2 ns full width at half maximum, 1.5 mj/pulse, reaching energy density of 1 MW/cm^2). The anion (ΦO^-) was monitored by a probing beam of an Argon laser (λ = 457 nm) where it has a strong absorption (ϵ_{457} = 24,000 M^{-1} cm^{-1}). The transient absorbancies were digitized and averaged by Tektronix 2430A oscilloscope. The analysis of the dynamics was carried out using the procedure and program detailed by Yam, Nachliel, and Gutman (1988) and Yam et al. (1991).

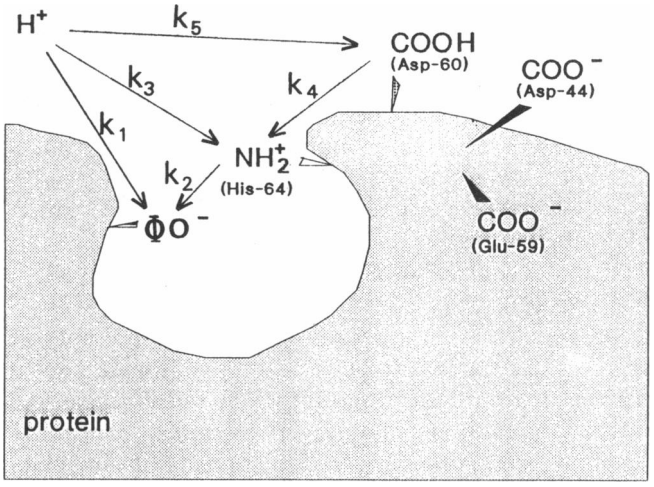
RESULTS

Fig. 1 depicts the transient dynamics of reprotonation of the pyranine anion, measured under conditions where 99.95% of it is bound to the protein. The ΦO^- absorption, at t = 0, corresponds with 1.4 μM of the dye. This is a rather low yield (0.8%) compared with the ~12% of ΦO^- measured, under comparable conditions, with dye dissolved in water. This value is consistent with our observation (see preceding publication; Shimoni et al., 1993) of a reduced efficiency of proton dissociation within the site.

The analysis of the experimental curve was attained by integrating a set of differential, nonlinear, rate equations describing the events presented in scheme 1.

The reprotonation of ΦO^-, located in the cavity, proceeds by more than one reaction. It can be directly protonated by a proton coming from the bulk (k_1). It can also be protonated by abstracting a proton from H^+His (k_2), with a back reaction k_{-2} (where log (k_2/k_{-2}) = ΔpK). The Histidin is reprotonated by free proton (k_3) or by a protonated carboxylate (k_4). The carboxylates which at the initial state are mostly deprotonated (pK = 4) (Matthew, 1985) react with the discharged protons with rate constant k_5. This setup was converted into a set of coupled nonlinear differential equations which maintain a detailed balance (for detailed equations see Yam et al., 1988). Their integration over time reconstructs the dynamics of the whole system. In the integration procedure each rate constant was varied independently of all others. The iteration was repeated till the calculated dynamics of ΦO^- was identical with the observed signal (see *continuous line* in Fig. 1). Any combination of rate constant which reconstructs the observed dynamics is a legitimate solution, but in order to comply with the present knowledge of the structure of the site, some restriction with respect to the magnitude of rate constants and concentration of reactants was implemented. The rate constants k_1, k_3, and k_5 were set to be compatible with those measured for similar reactions (see Gutman, 1986). This restriction is not applicable to the rates of proton exchange between the fixed components (k_2, k_4) (Nagoaka et al., 1991).

The reactant concentrations used for the integration were proportioned with the measured amount of ΦO^- (1.4 μM). The relative concentrations of ΦO^-, His, and carboxylates were based on a 1:1:3 stoichiometry and adjusted for the initial state of protonation using the experimental pH and the pK values given in Table 1. All



SCHEME I Mechanistic description of the protonation of the pyranine anion in the heme binding site of Apomyoglobin.

The dye is located within the cavity, and reacts with bulk protons with rate constant k_1, the Histidin 64 at the opening of the cavity protonates the pyranine anion with rate constant k_2, and reacts with free protons with rate constant k_3. The carboxylates Asp 44, Asp 60, and Glu 59 are protonated with the rate constant k_5 (common to all three) and protonate the Histidin with the rate constant k_4. The distance between the oxy anion of pyranine and the imidazolium ring of His 64 is ~5 Å. The distance between His 64 and Asp 60 is ~8 Å.

TABLE 1 **Kinetic and thermodynamic parameters characterizing the reactants involved in reprotonation of pyranine anion in the heme binding site of apomyoglobin**

Constant	Reaction	Rate	pK
		$M^{-1}\,s^{-1}$	
k_1	$\Phi 0^- + H^+$	$2.5 \cdot 10^{10}$	7.7*
k_3	$His + H^+$	$5.0 \cdot 10^9$	6.4‡
k_2	$His \cdot H^+ + \Phi 0^-$	$3.8 \pm 0.7 \cdot 10^{12}$	
k_5	$COO^- + H^+$	$2.5 \cdot 10^{10}$	4‡
k_4	$COOH + His$	$8 \pm 1.1 \cdot 10^{12}$	

* Gutman and Nachliel (1982). ‡ Matthew (1985).

other protein molecules are experimentally unobserved and were treated as a low pK buffer (Shimoni, 1991). Within these limiting restrictions only one combination of rate constant, that given in Table 1, yielded a good reconstruction of the observed dynamics. This is a unique solution for the experiment. Any attempt to omit one of the reactants, either His H^+ or the carboxylates, failed to reproduce dynamics, whatever the other rate constants were.

DISCUSSION

Out of the five rate constants controlling the reaction three are diffusion controlled ones, limited by the mutual search of the reactants. When an encounter between them takes place, the ensuing formation of the covalent bond is practically instantaneous. The other two reactions, proton transfer from COOH to His and from His H^+ to the dye, appear to be much faster. These extremely fast reactions are not limited by a diffusional step. The reactants, already at proton transfer range, exchange the proton at a rate controlled by the energetic parameters of the transfer (McMorrow and Kasha, 1984; Kirshman et al., 1990), which is very highly dependent on the distance and relative orientation of the reactants (Cybulski and Scheiner, 1989).

The rate constants for proton transfer between ΦO^-, His, and the carboxylates are given in Table 1 by $M^{-1}\,s^{-1}$ units, which are improper for reactants that are not free species. Yet this is the penalty we pay for the convenience of using the formalism of chemical relaxation, where reactants are quantitated by molar concentrations. Indeed the magnitude of these reactions indicates that the rate is unrestricted by diffusive processes. Actually the transition frequencies of proton exchange between the reactants should be deduced by the formalism used in the previous paper (Shimoni et al., 1993). But because of the long observation period (microseconds) the computation expenses for such simulations are prohibitive.

The reconstructed dynamics shown in Fig. 1 depict not only the reprotonation of ΦO^-. It also draws the dynamics of the free proton, and the protonation states of the Histidin and carboxylates. A striking feature in this figure is that reprotonation of ΦO^- is faster than the recapturing of the free protons from the solution. During the initial phase of the reaction most of ΦOH is formed due to proton abstraction from the histidinum. A little later, when the reaction with H^+ is accelerated by the appearance of the deprotonated His 64, the protonation state of His assumes a steady-state that relaxes to the equilibrium level. The carboxylates which initially are mostly deprotonated, exercise a transient protonation, but due to the fast rate of exchange with the imidazol ring, their dynamics follow a prolonged phase of steady-state where they channel protons from the bulk to the deprotonated Histidin. Thus, we observe that the state of protonation of the reactants varies rapidly due to the direct proton transfer while the overall protonation state of the system (dye, Histidin and carboxylates) relaxes more slowly, as determined by the diffusion controlled reaction with the proton.

The multiple relaxation process indicates that proton transfer between a site in protein and bulk should not be regarded as a monotonous process. It is better to consider the process as the sum of parallel reactions consisting of both direct, diffusion controlled reactions, and free energy controlled proton transfer between adjacent components whose relative position and pK difference provide a rigid path for enhanced proton transfer.

The contribution of each process is to be scaled by hierarchy of dominance, determined by the rate constants, probability of transition and the progression of the reaction.

CONCLUDING REMARKS

The multiple pathway for proton re-entry to the heme binding site of Apomyoglobin demonstrates that the surface of a protein is an integral component of the catalytic mechanism. The surface provides a large area exposed to bulk which can trap the substrate and channel it to the active site. In this study our observation was limited to proton as a convenient ion, yet our conclusions are applicable to other charged moieties with which a protein interacts. Thus in evaluating a structure function relationship for a protein, the consideration must be expanded beyond the boundaries of the active site per se.

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