# Equilibrium and Kinetics of the Reaction of Aplysia Myoglobin with Azide†

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ABSTRACT: The present paper reports a study on the equilibria and kinetics of the acid–alkaline transition and the azide binding reaction by ferric Aplysia myoglobin. A single completely reversible spectrophotometric titration curve is found over the pH range from ~5 to ~9, with an apparent p$K$ = 7.5 for the acid–alkaline transition. The kinetics of the process, followed by the temperature-jump method, gives, at pH values close to the p$K$ of the transition, one single, well-resolved, relaxation independent of protein concentration and of type of buffer used. The pattern accords to a simple pH dependent reaction, in buffered medium, between the two forms of the protein. The results of the azide binding reaction show that the process conforms to simple equilibrium as expected for a single site protein. The measured association constant is reported as a function of pH. The kinetics of the reaction of Aplysia metMb with $N_3^-$ shows, on the other hand, a complex behavior. The relaxation pattern is found to strongly depend on pH and ligand concentration in such a way to suggest a linkage between ligand binding and acid–alkaline transition. The system is discussed on the basis of two simplifying conditions, i.e., at low and higher pH with respect to the p$K$ of the acid–alkaline transition. At acid pH the reaction corresponds to a single bimolecular process as expected for a simple binding reaction; at alkaline pH, the dependence of relaxation time on ligand concentration implies the existence of a rate-limiting monomolecular step. On the basis of a reaction scheme implying that binding of the ligand can only occur through the acid (aquomet) form of the protein via the displacement of the water molecule, the experimental data are quantitatively accounted for.

The myoglobin of Aplysia, a mediterranean mollusk, has been the object of several investigations because its special properties make it a suitable system for studying function–structure relationships in hemoproteins. Aplysia myoglobin has also proved to be a good model to study the conformational transitions involved in protein denaturation, since it behaves as a completely reversible system under a wide range of experimental conditions (Brunori et al., 1968a, 1972).

This paper reports a detailed investigation of the equilibria and kinetics of ligand binding (azide) to ferric Aplysia myoglobin. This derivative of Aplysia myoglobin, like other ferric hemoproteins, undergoes an acid–alkaline transition which involves a high- to low-spin change (Antonini and Brunori, 1971). In the course of the study it soon became clear that the kinetics of reaction with $N_3^-$ does not conform to a simple one-step process, and that it may reflect a linkage between ligand binding and the acid–alkaline transition. The present paper deals with the equilibrium and kinetics analysis of the pH dependent transition of ferric Aplysia Mb, with the binding of azide, and with the linkage between the two phenomena. A formal scheme which accounts for the observations is proposed to describe the behavior of this system. Some of the results given here have already been reported in preliminary form (Giacometti et al., 1973).

## Experimental Section

Aplysia metmyoglobin was prepared from the frozen buccal muscles of the mollusc following the procedure of Rossi Fanelli and Antonini (1957), with minor modifications. The protein was stored in the ferric form under ammonium sulfate at 95% saturation or, for a limited time, in water at −20°. The concentration was determined spectrophotometrically using published extinction coefficients (Rossi Fanelli and Antonini, 1957).

Fresh solutions of azide were prepared for each experiment from Merck reagent grade sodium azide dissolved in the buffer used for the titration. The following buffer systems, 0.2 $M$ ionic strength, were used: sodium acetate buffers for pH 4–6, potassium phosphate buffers for pH 6–8, sodium borate buffers for pH 8–11. All the reagents employed were of analytical grade and were used without further purification. Spectroscopic measurements were performed with a Cary Model 14 or Beckman DB-GT recording spectrophotometer, equipped with thermostated cell holders. The pH values were determined before and immediately after each experiment with a Radiometer Model A pH meter.

In the equilibrium experiments, the spectrum of the protein was recorded from 360 to 440 nm in a 1-cm light path. Known volumes of azide solution at various concentration were added with an Agla microsyringe. Protein concentrations of about 6 $\mu$M were used throughout. At this protein concentration (because of the low affinity) the fraction of azide bound to myoglobin is small compared to the total azide concentration which minimizes errors involved in the determination of the free ligand concentration.

Kinetic measurements were performed with a single beam temperature-jump apparatus manufactured by Messanlagen (Gottingen, Germany). A temperature-jump cell of 7-ml capacity and 1-cm light path was used; changes in absorbancy in the Soret region at suitable wavelengths were used to follow the kinetics. Generally a temperature change of about 5° was imposed by a discharge of 30 kV.

The kinetic difference spectra were obtained from the de-

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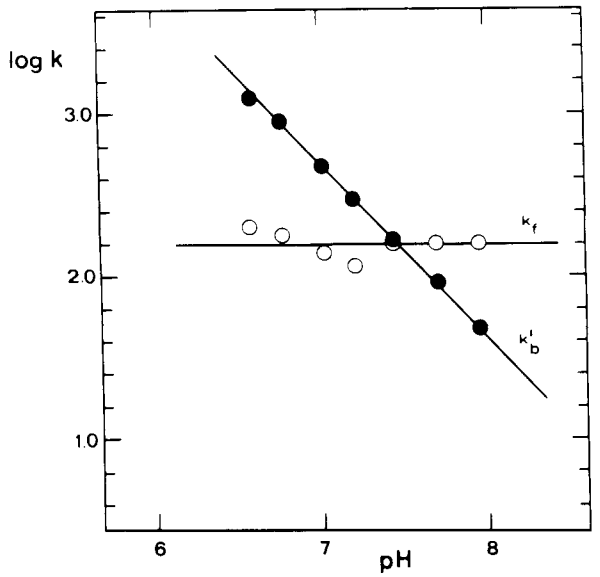


FIGURE 1: Dependence of the apparent kinetic constants ($k_f$, $k_b'$) on pH for the transition between the alkaline and the acid form of Aplysia metmyoglobin, calculated according to eq 3. Temperature, 20°; myoglobin concentration, $6 \times 10^{-6}$ *M*; phosphate buffers 0.2 *M* ionic strength.

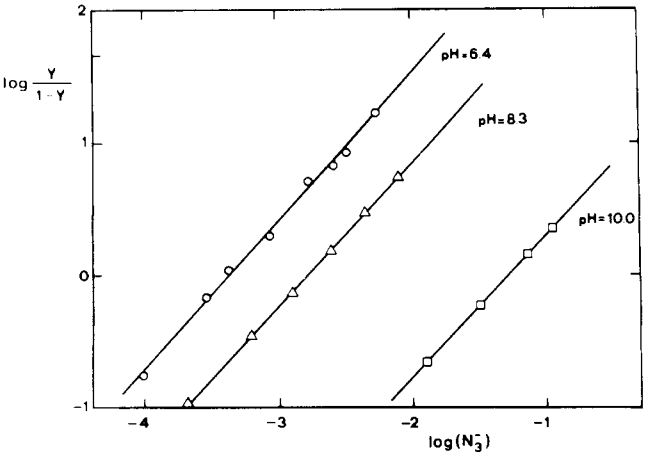


FIGURE 2: Hill plot of equilibrium curves for binding of azide to Aplysia metmyoglobin. Temperature, 21°; myoglobin concentration, $6 \times 10^{-6}$ *M*. (○) Phosphate buffer 0.2 *M*; (△) 0.2 *M* Tris-HCl buffer; (□) 2% borate-NaOH buffer.

pendence of the optical density change on the observation wavelength at time zero after the temperature jump or after mixing. The comparison with the same spectrum obtained statically (in a Cary 14 spectrophotometer) has been performed by normalizing the curves at one wavelength (see also caption of Figure 7).

## Results

(*a*) *Acid–Alkaline Transition.* As mentioned above, the acid–alkaline transition involves a variation in the total spin of the heme iron which is reflected in large changes of the absorption bands in the Soret and visible regions. The reaction is believed to involve primarily a deprotonation of the water molecule at the sixth coordination position of the iron according to (Antonini and Brunori, 1971):

$$\text{protein–Fe}^{+}\text{H}_2\text{O} \rightleftharpoons \text{protein–Fe}^{+}\text{OH}^{-} + \text{H}^{+} \qquad (1)$$

For Aplysia myoglobin the titration, monitored in the Soret region, yielded a simple, completely reversible, transition over the pH range 5–9 with an apparent p$K$ (p$K'$) of 7.5 at 25° (Giacometti et al., 1973). Such a result is in accord with the previously reported value (Brunori et al., 1968b).

The kinetics of the transition was followed by the temperature-jump method. In buffered solution, at pH values close to the p$K'$, a single well-resolved relaxation effect was observed in the millisecond time range. The measured relaxation time ($\tau$) was found to be independent of protein concentration (over the range from 3 to 15 $\mu M$), and of the type of buffer used.

As previously reported for Aplysia Mb (Giacometti et al., 1973) the amplitude of the relaxation was found to vary with pH, and, as expected, a plot of the amplitude vs. pH gives a bell-shaped curve with the maximum at pH 7.5, approximately equal to the p$K'$ of the transition. This fact, together with the identity of the static and kinetic difference spectra, is to be expected if the kinetic and static measurements reflect the same process.

The relaxation time was found to be pH dependent increasing with increase in pH. If a simple equilibrium between two forms of the protein is assumed

$$\text{MbOH}_2 \underset{k_b}{\overset{k_f}{\rightleftharpoons}} \text{MbOH}^{-} + \text{H}^{+} \qquad (2)$$

it is possible to derive, for any experimental condition, the relevant rate constants ($k_f$ and $k_b$) from a knowledge of the rate of approach to equilibrium ($\tau^{-1} = k_f + k_b\,(\text{H}^+)$) and of the equilibrium constant between the two forms

$$K = \frac{k_f}{k_b} = \frac{(\text{MbOH}^{-})(\text{H}^{+})}{(\text{MbH}_2\text{O})} \qquad (3)$$

As shown in Figure 1, log $k_b'$ depends linearly on pH, whereas log $k_f$ is practically pH independent. At the p$K'$ value, $k_f = k_b\,(\text{H}^+) = 160\ \text{sec}^{-1}$ at 20°.

Table I: Values of Log of the Apparent Association Constant and Intrinsic Association Constant for the Equilibrium between Azide and Aplysia Metmyoglobin as a Function of pH and Ionic Strength at 21 ± 1°.

| pH | Molarity | Log $K_{ass}^{obsd}$ | Log $K_i$ |
|---|---|---|---|
| 4.50 | 0.2 | 4.75 | 5.27 |
| 5.45 | 0.2 | 3.93 | 4.02 |
| 6.00 | 0.2 | 3.67 | 3.70 |
| 6.39 | 0.05 | 3.39 | 3.42 |
| 7.00 | 0.2 | 3.40 | 3.50 |
| 7.05 | 0.05 | 3.27 | 3.38 |
| 8.28 | 0.05 | 2.80 | 3.57 |
| 8.37 | 0.2 | 2.69 | 3.53 |
| 8.68 | 0.2 | 2.60 | 3.71 |
| 9.00 | 0.05 | 2.10 | 3.51 |
| 9.10 | 0.2 | 2.05 | 3.58 |
| 9.90 | 0.2 | 0.75 | 3.95 |
| 10.0 | 0.05 | 1.16 | 3.56 |
| | 0.2 | 0.8 | 3.25 |

(*b*) *Reaction with Azide.* (i) EQUILIBRIA. Titration of ferric Aplysia myoglobin with a freshly prepared solution of sodium azide was performed spectrophotometrically as a function of temperature and pH. Under all the experimental conditions, the titrations conform to simple expectations, with a value of $n$ equal to 1 (Figure 2). The apparent association constants obtained under different conditions are summarized in Table I. The apparent affinity constant for the binding of azide to Aplysia Mb is pH dependent (Figure

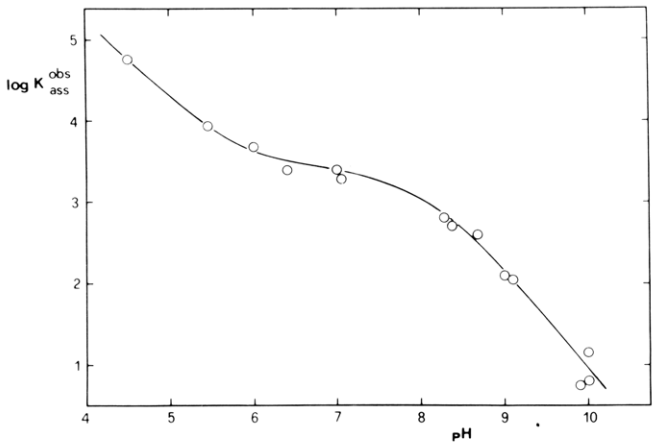


FIGURE 3: Dependence on pH of the logarithm of apparent association constant for binding of azide to Aplysia metmyoglobin at 21 ± 1°.

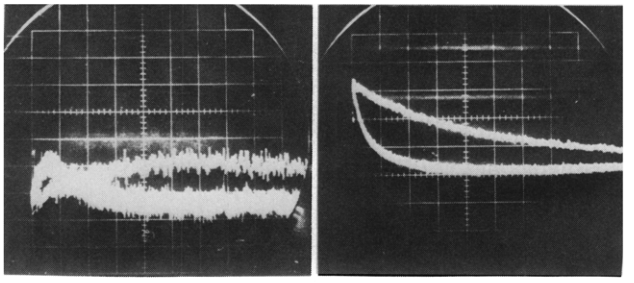


FIGURE 4: Oscilloscope traces of temperature jump experiments on Aplysia metmyoglobin close to the p$K'$ of the acid to alkaline transition of the protein (pH 7.45, 0.2 $M$ phosphate buffer) and 20°. Observation wavelength $\lambda$ 425 nm; (A) at low saturation of azide ($Y$ = 2.2%); sweep = 1 and 10 msec/grid division; (B) at higher saturation of azide ($Y$ = 77%); sweep = 0.5 and 5 msec/grid division.

3) similarly to what was reported for other hemoproteins (Anusiem et al., 1966, 1968). At alkaline pH the reaction with $N_3^-$ is in competition with the ionization of myoglobin to its alkaline form; following Anusiem et al. (1966), an intrinsic affinity constant ($K_i$) for the binding of $N_3^-$ may be evaluated, as follows

$$K_i = K_{ass}^{obsd}[(K + (H^+))/(H^+)] \quad (4)$$

where $K$ is the equilibrium constant for the acid–alkaline transition of the protein as defined in eq 3. The calculated values of $K_i$ were found to be essentially pH independent between ~6 and ~10, with an average value of $3.2 \times 10^3$ $M^{-1}$, which closely corresponds to the value of the observed equilibrium constant in the pH region between 6 and 7 (see Table I). Below pH 5, in a region where the ligand begins to be protonated ($K_{HN_3} = 1.4 \times 10^{-5}$ $M$, Burns and Chang, 1959), the additional increase in $K_{ass}^{obsd}$ may be taken as an indication that the protonated form of azide is preferentially bound. However, since it cannot be excluded that the protein undergoes a pH dependent structural change in this pH range (which is close to the isoelectric point), the matter was not examined in any further detail. The temperature dependence of the association constant was estimated in the range from 10 to 40°. At each pH explored, negative $\Delta H°$ values were found ranging from about −3 kcal/mol at alkaline pH to about −9 kcal/mol at acid pH.

(ii) KINETICS. The relaxation kinetics for the reaction of a macromolecule having a single binding site with a ligand

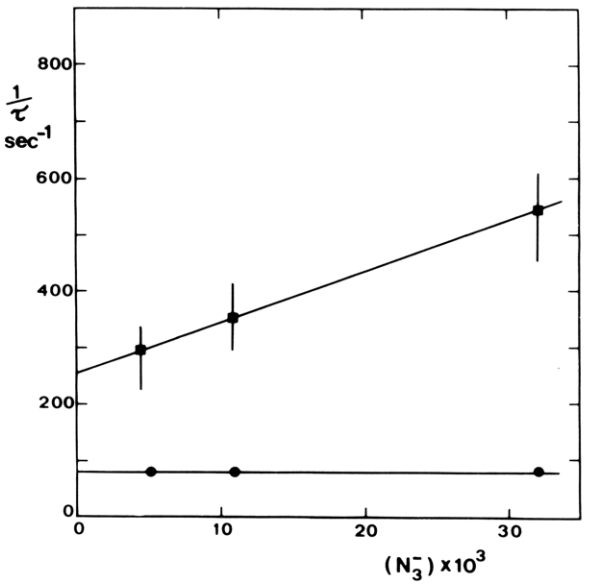


FIGURE 5: Dependence of $1/\tau$ for the slow and fast relaxation processes observed in temperature-jump experiments, on the concentration of $N_3^-$. Temperature, 20°; borate–boric acid buffer 0.2 $M$, pH 8.0.

(L) should conform to a simple scheme, e.g.

$$Mb + L \underset{k_2}{\overset{k_1}{\rightleftharpoons}} MbL$$

$$K = \frac{k_1}{k_2} = \frac{(MbL)}{(Mb)(L)} \quad (5)$$

with a relaxation time ($\tau$) given by

$$\tau^{-1} = k_2 + k_1(\bar{Mb} + \bar{L}) \quad (6)$$

where $\bar{L}$ represent the equilibrium concentration of free ligand and $\bar{Mb}$ that of free sites (Eigen and De Mayer, 1963). The kinetics of the reaction of $N_3^-$ with Aplysia Mb, however, shows a more complex behavior than indicated by eq 5, the relaxation pattern being pH dependent.

At pH values close to the p$K'$ of the acid–alkaline transition of the protein (≃7.5), the relaxation pattern is complex, and depends on the fractional saturation with the ligand (i.e., on the absolute $N_3^-$ concentration) (see Figure 4). At low saturations, two processes, well separated on the time axis, are easily observed since they involve changes in optical density of opposite sign (Figure 4a). The faster process, which at $\lambda$ 425 nm corresponds to an increase in the optical density, reflects a perturbation of the acid–alkaline equilibrium in the free protein (see above); the slower one, involving a decrease in the absorbance at the same wavelength, is due to the reaction with azide. These assignments were obtained from the spectral properties of the two events and from the direction of the OD changes in relation to the known enthalpy of the processes (Giacometti et al., 1973). At higher ligand concentration (i.e., higher saturation) the optical density at $\lambda$ 425 nm decreases monotonically during the relaxation (Figure 4b); however, the process does not correspond to a single event, and the analysis requires at least two exponential processes.

In so far as the analysis is feasible, it is found that the inverse relaxation time for one process depends linearly on concentration of the ligand (since $\bar{L} \gg \bar{Mb}$), whereas the other relaxation time is independent of ligand concentration (Figure 5). These findings suggest that at fractional saturations above 10% the faster relaxation phase reflects a bi-

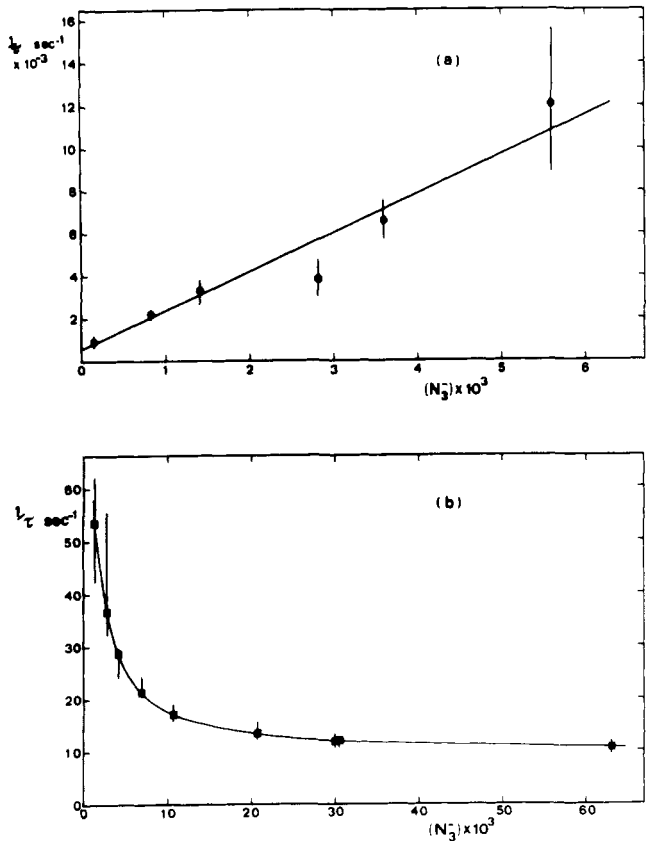


FIGURE 6: Dependence of $1/\tau$ on free azide concentration as obtained in temperature-jump experiments, at 20° (after a jump of 5°). (a) pH 6.15, 0.2 $M$ phosphate buffer. Straight line is a linear regression yielding the following values of rate constants: $k_1 = (1.8 \pm 0.2) \times 10^6\ M^{-1}$ $sec^{-1}$; $k_2 = (7.5 \pm 1) \times 10^2\ sec^{-1}$. (b) pH 9.37, borate-2% NaOH. Continuous line is the theoretical curve calculated according to eq 9 with the following values of the rate constants: $k_f = 160\ sec^{-1}$; $k_b(H^+) = 10\ sec^{-1}$; $K_1 = 1.7 \times 10^6\ M^{-1}\ sec^{-1}$; $k_2 = 8.5 \times 10^2\ sec^{-1}$.

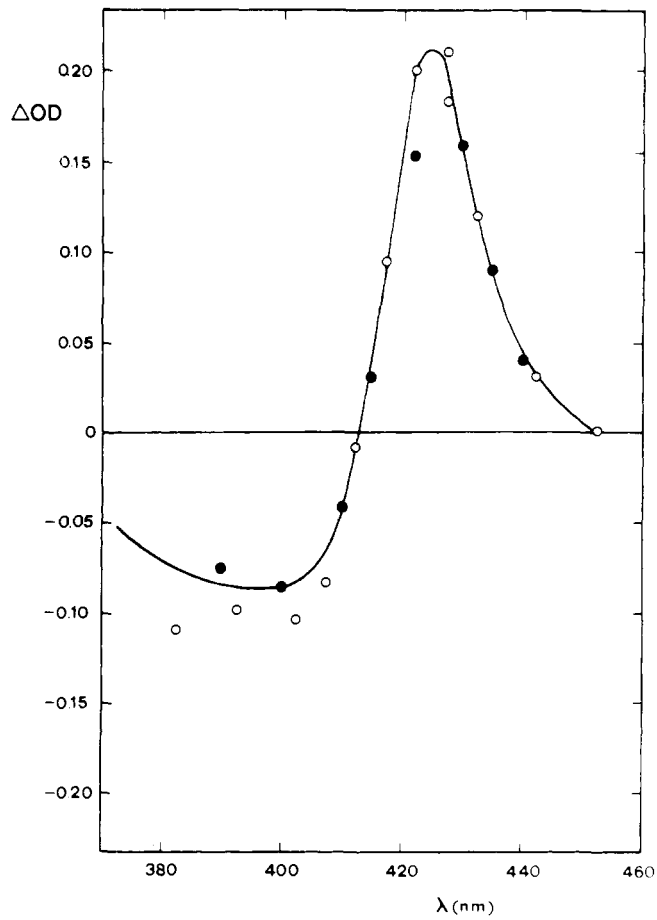


FIGURE 7: Difference spectrum of $MbN_3^-$ minus $MbOH^-$ (—) obtained statically; (●) amplitude of the relaxation process as a function of wavelength from flow experiments; (○) the same from temperature-jump experiments. The kinetic difference spectrum has been normalized to the static one at λ 430 nm. Temperature, 20°; protein concentration, $6 \times 10^{-6}\ M$; azide equilibrium concentration, $3 \times 10^{-3}\ M$; borate buffer, pH 9.37.

molecular step, whereas the slower one reflects a monomolecular event.

At pH values considerably removed from the p$K'$ of the acid–alkaline transition a single relaxation time is observed. However, at pH 6 the overall process is much faster than at pH 9. In addition a very different dependence of the reciprocal relaxation time on ligand concentration is observed under the two conditions, as shown in Figure 6. At pH 7.5, where the acid form of the protein dominates (Figure 6a), the dependence of the relaxation time on reagent concentration is consistent with a simple bimolecular process; as depicted in eq 6. Therefore under these conditions no evidence for a deviation from a simple behavior is observed. At pH $\gg$7.5 (Figure 6b), where the alkaline form of the protein dominates, the reciprocal relaxation time decreases with increasing the ligand concentration and tends toward an asymptotic value of about 10 $sec^{-1}$ (at pH 9.4 and 20°). The analysis of the amplitude vs. the observation wavelengths is reported in Figure 7 where the experimental points are superimposed, after normalization at one wavelength, to the static difference spectrum between the ligand free and ligand bound forms of the protein at the same pH. This shows that the changes in optical density following the temperature increase are due to a perturbation of the equilibrium with azide. It might be noted that the spectrum of the complex with azide is pH independent over the region where the acid–alkaline transition occurs.

## Discussion

The kinetics of the acid–alkaline transition in sperm whale myoglobin have been investigated employing the electric field-jump relaxation method (Ilgenfritz and Schuster, 1971). This process in whale myoglobin is much too fast to be followed by the temperature-jump technique, as shown by earlier unsuccessful attempts (T. M. Schuster and M. Brunori, unpublished results). On the other hand, in Aplysia myoglobin the transition occurs in the millisecond time range, and therefore may be investigated using the temperature-jump method.

At 412 nm the observed effect corresponds to an increase in optical density implying that the alkaline form of the protein is favored by an increase in temperature. This result is consistent with the known enthalpy of the acid–alkaline transition which, in the case of Aplysia Mb, has a value of $\Delta H^\circ \simeq +3$ kcal/mol (Brunori et al., 1968b).

The kinetic results reported in Figure 1 are consistent with a simple protonation process in buffered medium, as previously reported (Giacometti et al., 1973). However, two points deserve comment: (i) the apparent p$K'$ for Aplysia myoglobin is lower than that reported for sperm whale myoglobin or human hemoglobin, and (ii) the relaxation occurs in a time range which is unusual for simple ionization processes (Eigen and De Mayer, 1963). Although an exact interpretation of these findings is not yet possible, they point

out the importance of the distal residue (which is not a histidine in Aplysia) (Tentori et al., 1968) and/or of neighboring groups on the reactivity of the heme, and suggest the possibility of a conformational change of the protein associated to the acid–alkaline transition.

Also in mammalian myoglobin and hemoglobin it has been suspected that the acid–alkaline transition is not a simple ionization process, but that the change involves other protein groups in the proximity of the heme. The "slow" kinetics of the acid–alkaline transition in Aplysia myoglobin indicates, more directly that, at least in this protein, the ionization process, with the accompanying change in spin of the iron, is associated with structural changes in the protein.

The static results on the reaction with azide show that the process conforms to a simple equilibrium ($n = 1$) as expected for a protein containing a single site. The intrinsic affinity constant ($K_i$), computed as indicated in eq 4, is independent of pH, and its absolute value is between one and two orders of magnitude smaller than that of sperm whale myoglobin.

As documented above, the kinetics of the reaction of Aplysia metmyoglobin with azide are complex. In the intermediate pH region (e.g., pH 8.0), where two relaxation processes are directly observed, a complete analysis of the results has not been carried out. However, two simplifying conditions have been fully characterized, i.e., at low and high pH in respect to the p$K'$ of the acid–alkaline transition. At acid pH, where only one relaxation process is observed, the reaction corresponds to a simple bimolecular process (Figure 6), and can be described by

$$MbOH_2 + N_3^- \underset{k_2}{\overset{k_1}{\rightleftharpoons}} MbN_3^- + H_2O \qquad (7)$$

The measured rate constants, $k_1 = 1.8 \times 10^6\ M^{-1}\ sec^{-1}$ and $k_2 = 7.5 \times 10^2\ sec^{-1}$, are in good accord with the intrinsic affinity constant calculated from equilibrium experiment ($K_{Eq} = 2.4 \times 10^3\ M^{-1}$).

At alkaline pH, the dependence of the relaxation time on ligand concentration implies that a rate-limiting monomolecular step is involved in the reaction of the Aplysia metmyoglobin with azide, since the reciprocal relaxation time levels off at high ligand concentration (Figure 6b).

On the assumption that binding of azide can take place, even at alkaline pH, only through the acid (or aquomet) form of the protein, the following reaction scheme may be proposed:

$$\begin{aligned} MbOH^- + H^+ &\underset{k_f}{\overset{k_b}{\rightleftharpoons}} MbOH_2 \quad \text{(step I)} \\ MbOH_2 + N_3^- &\underset{k_2}{\overset{k_1}{\rightleftharpoons}} MbN_3^- + H_2O \quad \text{(step II)} \end{aligned} \qquad (8)$$

According to this scheme, because of the negligible concentration of $MbOH_2$ at pH ~9, only one relaxation time is expected, in agreement with the observations. The dependence of the observed relaxation time on ligand concentration, which may be obtained on the basis of the steady-state approximation for $MbOH_2$ (Eigen and De Maeyer, 1963; Czerlinski, 1966), is

$$1/\tau = \frac{k_1 k_b \bar{C}_{N_3^-} \bar{C}_{H^+} + k_f k_2}{k_1 \bar{C}_{N_3^-} + k_f} \qquad (9)$$

where $\bar{C}_{N_3^-}$ and $\bar{C}_{H^+}$ represent respectively the equilibrium concentration of $N_3^-$ and $H^+$. The equation has been obtained by taking into account that both the concentration of azide and that of protons are buffered. Choosing values of $k_f$, $k_b$, $k_2$, and $k_1$ very close to those independently derived from Figures 1 and 6a, eq 9 gives a plot of $1/\tau$ as a function of $\bar{C}_{N_3^-}$ in good agreement with the experimental values, as shown in Figure 6b. In addition, the difference spectrum corresponding to the process agrees, as expected, with the static difference spectrum: ($MbN_3^- - MbOH^-$).

Therefore it is possible to quantitatively account for the experimental data on the basis of the scheme represented in eq 8, which essentially implies that binding of anionic ligands in ferric Aplysia Mb can only occur via the displacement of the water molecule. It may be tempting to speculate on the structural basis for this behavior. Thus it is possible that the acid–alkaline transition does not involve the ionization of the bound water molecule, but that of an amino acid side chain of the protein in close proximity with the iron. Binding of azide would therefore only occur when this group exists in the protonated form. Although no proposal for the nature of this group can be offered at present, this possibility appears consistent with the analysis of the data along the lines shown above.

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