Temperature Jump Studies of Sperm Whale Metmyoglobin

II. AZIDE AND CYANATE BINDING BY METMYOGLOBIN*

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The determination of the crystal structure of sperm whale metmyoglobin and several of its complexes by Stryer, Kendrew, and Watson (1) has established the chemical environment around the iron atom in this molecule. It has been shown that a histidine group (called the "proximal heme-linked" group) is coordinated to the iron atom above the plane of the porphyrin system and that a second histidine group (called the "distal heme-linked" group) lies close to the sixth coordination position of the iron atom to which a water molecule or a variety of ligands binds.

In the last decade, extensive spectrophotometric and magnetic susceptibility studies on the binding of a variety of ligands by horse metmyoglobin have been carried out (2-4) in order to investigate the influence of these heme-linked groups on the mode of binding. Although such equilibrium studies are necessary for a determination of thermodynamic properties of the system, it is desirable to obtain kinetic data for both the forward and reverse reactions in order to obtain a detailed understanding of the individual steps of the binding mechanism.

In our previous paper (5) we reported the kinetics of the binding of imidazole to sperm whale metmyoglobin over the pH range from 6.0 to 9.0. In this case, the basic form of the ligand was neutral and the acid form positively charged. The pH variation of the forward rate constant could be accounted for by a simple electrostatic interaction between the active site of the protein and the two forms of the ligand.

As an extension of these studies, temperature jump measurements on the binding of azide and cyanate by sperm whale metmyoglobin have been carried out in order to determine the kinetics of binding of a negative ion and to obtain information about the number and nature of heme-linked ionizing groups in the pH region from 6.0 to 7.5 for sperm whale metmyoglobin.

EXPERIMENTAL PROCEDURE

The kinetic experiments were performed on the temperature jump apparatus described previously (5). Sperm whale metmyoglobin was obtained from the Mann Research Company and was indistinguishable kinetically from that kindly given to us by Professor F. R. N. Gurd of Indiana University Medical School. Maleate buffer was used in the pH range from 5.0 to 6.5, and phosphate buffer was used in the range from 6.0 to 7.5. In the overlapping region of pH between 6.0 and 6.5, all experiments were carried out with both buffers. The relaxation times

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photometrically determined dissociation constant. Since the dissociation constants were only known approximately, an iteration procedure was adopted to obtain the final straight line with a ratio of intercept to slope which agreed within experimental error with the dissociation constant used to calculate the equilibrium concentrations.

It has been shown that sperm whale metmyoglobin has an ionizing group with a pK of 8.9 (6). Since our experiments were restricted to the region between pH 5.0 and pH 7.5, this ionization would not be expected to influence significantly the observed rate and hence will not be considered further. However, if the investigations were to be extended to higher pH values, more steps would have to be added to the mechanism, as shown by the imidazole binding kinetics.

In the acid region, there is a question as to whether there are one or two heme-linked ionizing groups in horse metmyoglobin (2-4). For the analysis of our data, we will therefore consider the following two mechanisms. We will also assume that all ligand species are bound by metmyoglobin.

Mechanism 1 has one heme-linked acidic group,

\[
\begin{align*}
HMB^+ + HL & \quad k_{+1} \\ 
K_B & \quad \frac{HMBHL^+}{K_{BL}} \\
M^+ + HL & \quad k_{-2} \\
K_L & \quad \frac{ML}{K_{BL}}
\end{align*}
\]

and Mechanism 2, two heme-linked acidic groups.

\[
\begin{align*}
HMB^+ + HL & \quad k_{+1} \\ 
K_A & \quad \frac{HMBHL^+}{K_{AL}} \\
M^+ + HL & \quad k_{-2} \quad \frac{ML}{K_{AL}}
\end{align*}
\]

Here \(HMB^+, HMBL^+, M^+\) represent the different protonic forms of metmyoglobin and \(HL\) and \(L\), those of the ligand. \(K_A\) and \(K_B\) are the acid dissociation constants of the two heme-linked groups, and \(K_L\) is that of the ligand. Similar notation applies to the species of the reverse reaction. The capital \(K\)'s in all cases represent dissociation constants.

To calculate the relaxation time, we have

\[
\frac{1}{\tau} = k_{-1(app)} + k_{+1(app)} \left[ \sum_i \langle M_i \rangle + \sum_j \langle L_j \rangle \right]
\]

where \(\sum_i \langle M_i \rangle\) is the sum of the equilibrium concentrations of all the forms of metmyoglobin, \(\sum_j \langle L_j \rangle\) is the sum of the equilibrium concentrations of all the forms of the ligand, and \(k_{-1(app)}\) and \(k_{+1(app)}\) represent the apparent backward and forward rate constants, respectively.

For Mechanism 1

\[
k_{-1(app)}(1) = \left( k_{+1} + k_{-2} \right) \frac{K_B}{K_L} + k_{+1} \frac{H}{K_L} + k_{+4} \frac{K_B}{K_L}
\]

For Mechanism 2

\[
k_{-1(app)}(2) = \left( k_{+1} + k_{-2} \right) \frac{K_B}{K_L} + k_{+1} \frac{H}{K_L} + k_{+4} \frac{K_B}{K_L}
\]
TABLE II
Rate constants obtained assuming one and two heme-linked dissociating groups

<table>
<thead>
<tr>
<th></th>
<th>One pK mechanism</th>
<th>Two pK mechanisms</th>
<th>One pK mechanism</th>
<th>Two pK mechanisms</th>
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</thead>
<tbody>
<tr>
<td><strong>Azide</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>$K_L$</td>
<td>$2.9 \times 10^{-4}$</td>
<td></td>
<td>$K_A$</td>
<td>$3.0 \times 10^{-4}$</td>
</tr>
<tr>
<td>$K_B$</td>
<td>$2.0 \times 10^{-4}$</td>
<td></td>
<td>$K_B$</td>
<td>$2.0 \times 10^{-4}$</td>
</tr>
</tbody>
</table>
| $k_{+1}$           | $9.1 \times 10^4$  | $k_{+1}$          | $8.5 \times 10^4$ | *
| $k_{+2}$           | $2.2 \times 10^5$  | $k_{+2}$          | $3.6 \times 10^4$ | *
| $k_{-1}$ + 0.007   | $4.0 \times 10^4$  | $k_{-1}$          | $2.5 \times 10^3$ | *
| **Cyanate**        |                  |                   |                  |                   |
| $K_L$              | $1.95 \times 10^{-4}$ |                  | $K_A$            | $2.0 \times 10^{-4}$ |
| $K_B$              | $2.0 \times 10^{-4}$ |                  | $K_B$            | $1.8 \times 10^{-4}$ |
| $k_{+1}$           | $1.8 \times 10^4$  | $k_{+1}$          | $1.1 \times 10^4$ | *
| $k_{+2}$           | $4.2 \times 10^3$  | $k_{+2}$          | $1.5 \times 10^4$ | *
| $k_{-1}$ + 0.01    | $6.2 \times 10^3$  | $k_{-1}$          | $2.7 \times 10^3$ | *

For Mechanism 2

$$k_{+1(\text{app})} = \left[ \frac{k_{+1} + k_{+1} (\frac{H}{K_A}) + k_{+1} (\frac{H}{K_B})}{1 + (\frac{H}{K_A}) + (\frac{H}{K_B})} \right] \left[ \frac{1 + (\frac{H}{K_A}) + (\frac{H}{K_B})}{1 + (\frac{H}{K_A})} \right]$$

or

$$k_{-1(\text{app})} = \left[ \frac{k_{-1} + k_{-1} (\frac{H}{K_A}) + k_{-1} (\frac{H}{K_B})}{1 + (\frac{H}{K_A}) + (\frac{H}{K_B})} \right] \left[ \frac{1 + (\frac{H}{K_A}) + (\frac{H}{K_B})}{1 + (\frac{H}{K_A})} \right]$$

where $k_{+1(\text{app})}$ represents the apparent forward rate constant corrected for the concentration of ligand present as the acidic form.

The data were analyzed by evaluation of bell-shaped curves obtained from Equations 3 and 6, as has been previously described (5), and the rate constants derived from these parameters are given in Table II. Since a bell-shaped curve yields only three parameters, only three rate constants or combinations of rate constants may be obtained. For Mechanism 1 only $k_1$ and $k_2$ can be obtained explicitly, and $k_3$ and $k_4$ cannot be separated.

Since, for Mechanism 1, $K_L$ is known for each ligand ($K_{\text{NN}} = 2.0 \times 10^{-5}$ and $K_{\text{HUCN}} = 1.05 \times 10^{-4}$ at 25°C and 0.1 M) but $K_B$ is unknown, the value of $K_B$ was found by varying $K_B$ until a symmetrical bell was obtained; the parameters of this bell then gave a good fit to the original data for each ligand. For Mechanism 1 only $k_1$ and $k_2$ can be obtained explicitly, and $k_3$ and $k_4$ cannot be separated.

For Mechanism 2, on the other hand, both $K_A$ and $K_B$ are unknown. A computer program was written for the General Electric model 615 computer which calculated a series of bell-shaped curves with values of $pK_A$ ranging from 4 to 6 and of $pK_B$ ranging from 5.5 to 7.5. The most symmetrical bell-shaped curves were obtained in the region of $pK_A = 4.5$ to 5.0 and $pK_B = 5.5$ to 6.0. The values for $K_A$ and $K_B$ in this region were then selected to obtain the best fit of the original data for both ligands. Fig. 2 shows the agreement between the observed values of $k_{+1(\text{app})}$ and those calculated from the parameters given in Table II for Mechanism 2. Both Mechanisms 1 and 2 fit the data within experimental error with the parameters given in Table II for the azide ligand, but the parameters for the cyanate case for Mechanism 1 did not give as good a fit in the low pH region as did those of Mechanism 2.

The values for the rate constants obtained for Mechanism 1 suggest that the neutral form of the ligand binds much faster than the ionized form and that the binding is not electrostatically controlled. George and Hanania have found a similar relation for the binding of fluoride by horse metmyoglobin (7). On the other hand, the rate constants for Mechanism 2 do show an electrostatic dependence since they increase for both ligands with decreasing pH.

The data for the reverse reaction for both ligands are given in Table I. It is readily evident that these data show the same dependence on pH as the apparent forward rate constants.

For Mechanism 1

$$k_{-1(\text{app})} = \frac{k_{-1} K_{BL}}{1 + (\frac{H}{K_D}) + (\frac{H}{K_B}) + (\frac{H}{K_B} K_{BL}) K_D}$$

and $K_B K_{BL} = K_D K_{BL}$. For Mechanism 2

$$k_{+1(\text{app})} = \frac{k_{+1} K_{AL}}{1 + (\frac{H}{K_D}) + (\frac{H}{K_A}) + (\frac{H}{K_A} K_{AL}) K_D}$$

where $K_{AL} = K_A K_{BL}$.
and $K_{c}K_{aL} = K_{b}K_{aHL}$ and $K_{b}K_{BL} = K_{b}K_{bHL}$.

For both cases, an explicit analysis of these data is at present too involved because of the large number of unknown parameters.

As has been shown, there are two possible interpretations of these data; one assuming one heme-linked ionizing group with a pK of 5.7 and the other assuming two heme-linked ionizing groups with pK values of approximately 5.7 and 4.7.

According to the first mechanism the HL species binds 100 times faster than the L- species. These results would require some explanation other than a simple electrostatic picture of the binding. On the other hand, according to the second mechanism (this involves an additional heme-linked ionizing group), the pH dependence of the binding rate constants can be explained in terms of a simple electrostatic picture since $k_{4}k_{L}k_{G} = 400:20:1$, as would be expected for a binding site which has singly, doubly, and triply positive charged forms with decreasing pH. This increasing charge could be accounted for by the proximal and distal histidine groups around the iron-porphyrin group.

The pH dependence of the dissociation rate constant can be explained in terms of a simple electrostatic dependence.

**SUMMARY**

1. The kinetics of binding of azide and cyanate ligands by sperm whale metmyoglobin have been studied over the pH range from 5.0 to 7.5 by the temperature jump method. The forward rate constant for both ligands varies in the same manner over two orders of magnitude, increasing with decreasing pH.

2. The forward rate data can be interpreted in terms of two mechanisms. The first mechanism has only one heme-linked group with a pK around 5.7, but requires that the neutral acidic form of the ligand binds much faster than the negative basic form. The second mechanism includes two heme-linked ionizing groups with pK values of approximately 4.7 and 5.7. The relative orders of magnitude of the rate constants in this case are such as would be expected from a simple electrostatic dependence.

3. The pH dependence of the dissociation rate constant can be explained in terms of a simple electrostatic model.

**REFERENCES**

Temperature Jump Studies of Sperm Whale Metmyoglobin: II. AZIDE AND CYANATE BINDING BY METMYOGLOBIN
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