Methemoglobin

Electron Paramagnetic Resonance Study of Carp Methemoglobin

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The $g$ anisotropy of the EPR spectra of carp azidomethemoglobin is found to be pH-dependent, whereas, the spectra of human azidomethemoglobin are not. The two hemoglobins have the same $g$ values at alkaline pH values. Crystal field analysis yielded values of 2.25 and 3.31, respectively, for the rhombic distortion, $V/A$, and the tetragonal distortion, $\Delta A$. The spin orbit coupling constant is $\lambda$. At pH 4.0, the values of $V/A$ and $\Delta A$ for carp azidomethemoglobin became 1.95 and 4.76, respectively, whereas those for the human hemoglobin are virtually unchanged. The results are interpreted to mean an increase of out-of-plane displacement of the iron atom and stabilization of the T form of carp azidomethemoglobin by high proton concentration. At pH 6.0 and lower, the EPR spectra of carp azidomethemoglobin showed the presence of about 1.5% of high spin species, the amount is not affected by excess of either inositol hexaphosphate or sodium azide. The EPR spectra of aquo- and fluoro-derivatives of carp methemoglobin were not affected by pH changes.

The possibility that the hemes in some derivatives of methemoglobins may exist in thermal equilibrium of different electronic states was first suggested by Taube (1). This was verified experimentally (2-4). Ultraviolet absorption, circular dichroism, sulfhydryl reactivities, and other spectroscopic evidences (5) showed that solutions of high spin methemoglobin derivatives normally contain the R and T quaternary forms in dynamic equilibrium, the R form being favored by high pH and the T form by low pH and by organic phosphates. The effect of IHP on low spin hemoglobins is much weaker. The spin state equilibria are temperature-dependent. Lowering of temperature decreases the amount of low spin species in fluoromethemoglobin and reduces the concentration of high spin species in azidomethemoglobin (2, 3, 6). Different methods for the determination of spin state concentrations sometimes gave very different results. For instance, IHP was found to increase the high spin concentration in azidomethemoglobin from 2 to 8% by Gouy balance; the increase was 40% by NMR (6).

Carp hemoglobin exhibits an interesting pH-dependent ligand binding affinity and cooperativity (7, 9), which have been explained by the existence of two and only two quaternary conformational states. The same interpretation was given (8) for the effects of pH and IHP on the visible spectra of carp methemoglobin. Difference peaks were observed even in the case of carp cyanomethemoglobin. Difference spectrophotometry has the advantages of high sensitivity and room temperature measurements. However, the changes cannot be given molecular interpretations because of contributions from the heme, its axial ligands, and other residues. In this work, EPR was used to study the effects of pH and IHP on the electronic configuration of carp methemoglobin derivatives.

Materials and Methods

Carp Hemoglobin—Carp were anesthetized with 0.5 g liter$^{-1}$ of ethyl-$m$-aminobenzoate methanesulfonic acid (Tricaine from Sigma). The blood was collected by either peduncle severance or extraction from the peduncular artery with a heparinized syringe. The blood was washed four times in Alsevers solution and frozen in liquid nitrogen. Cell membranes were lysed with an equal volume of water during the thaw process. The debris were removed by ultracentrifugation at 20,000 x $g$ for 20 min. The hemolysate was stripped of endogenous polyphosphates on a Sephadex G-25 column (30 x 2 cm) equilibrated with 0.1 M NaCl. Oxygenation curves at pH 7.45 with or without IHP agree within experimental error with the half-saturation pressure and Hill coefficient values reported by Noble et al. (7). The hemoglobin was oxidized with a 50% excess of K$_2$Fe(CN)$_6$, and purified on a Sephadex G-25 column.

Human hemoglobin A was prepared similarly except that the erythrocytes lyse readily without a freeze-thaw treatment. This step is therefore omitted.

Electron Paramagnetic Resonance—EPR spectra were obtained with a Varian E-9 spectrometer at 77 K. The samples are 1.9 mm in height. A given methemoglobin derivative was prepared by introduction of a 10-fold excess of the appropriate sodium salt.

Results

A typical low spin EPR spectrum of stripped human azidomethemoglobin is shown at the bottom of Fig. 1. At the top of this figure is also shown the spectrum for the carp derivative at the same low pH. The reduced signal to noise ratio for this spectrum arises because carp hemoglobin is a fragile protein which begins to precipitate at pH 4.0. Comparison of the two spectra clearly shows the difference in $g$ values for the two hemoglobins.

EPR spectra for stripped human and carp azidomethemoglobins were recorded from pH 4 to 9 and the pH dependence of the $g$ values are summarized in Fig. 2. In the alkaline region of pH 8 to 9, the two hemoglobins have nearly identical $g$ values. As the pH is lowered in steps, there is no significant change in the spectra for human azidomethemoglobin. In con-

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The abbreviations used: IHP, inositol hexaphosphate; $V$, rhombic crystal field distortion; $\Delta$, tetragonal crystal field distortion.
EPR Study of Carp Methemoglobin

FIG. 1. EPR spectra of human and carp azidomethemoglobin at 77 K and pH 4.0.

FIG. 2. Variation of g values for carp and human azidomethemoglobin as a function of pH at 77 K.

Tradistinction, there is a monotonic increase of g anisotropy with the lowering of pH for carp azidomethemoglobin samples. The effect of IHP on the EPR spectra of carp azidomethemoglobin is small showing a slight increase in the g anisotropy which is just outside of the experimental accuracy of ±0.005. The results are given in Table I. There was no detectable effect of IHP in parallel measurements on human hemoglobin.

The presence of high spin species in azidomethemoglobin was examined. For the carp derivative, none was found at pH 8.0. At pH 6.0 to 4.0, a high spin signal was seen at g = 6 which corresponds to about 1.5% of the total heme concentration. Addition of IHP to the low pH samples made no difference in the intensity of this signal; it was also not affected by 100-fold excess of sodium azide. No high spin signal was observed for human azidomethemoglobin from pH 4 to 8.

EPR spectra of aquo- and fluoromethemoglobins of carp were recorded as a function of pH. Neither the g values nor the signal intensities were found to depend on pH or IHP.

DISCUSSION

The g anisotropy of human azidomethemoglobin is pH-invariant whereas that of carp is pH-dependent. The crystal field of the ligands determines the splitting of the 3d orbitals of iron and in turn the g anisotropy. Conversely, from the changes in g anisotropy it is possible to elucidate the influence of heterotropic effector molecules, such as proton and IHP, on the binding of ligands to the iron ion.

The g anisotropy of azidomethemoglobin has been explained by Griffith (10) as arising from a combination of tetragonal and rhombic distortions of the ligand field of the iron ion. A more detailed expansion of this analysis has also been given by Weissbluth (11). In a weak octahedral field the t_{2g} orbitals are triply degenerate with the doubly degenerate e_{g} orbitals. Rhombic distortion completely removes these degeneracies. The t_{2g} orbitals give rise to three Kramers doublets; only the lowest of these is involved in the EPR transition observed under our experimental conditions. The wave function of the lowest Kramers doublet is

\[
\psi_{\pm} = \begin{pmatrix} A |+1a> + B |\zeta \beta> + C|-1a> \\ A |+1a> - B |\zeta \beta> + C|+1\beta> \end{pmatrix}
\]

where \(a\) and \(b\) are the spin functions. The orbital functions \(|+1a>\), \(|-1a>\), and \(|\zeta \beta>\) are eigenfunctions of \(L_z\) and correspond, respectively, to the \(d_{x^2-y^2}\) and \(d_{xy}\) and a linear combination of atomic orbitals given by

\[
|\zeta \beta> = \frac{1}{\sqrt{2}} \left( |+2> - |-2> \right)
\]

with

\[
|+2> = \frac{1}{\sqrt{2}} \left( |d_{x^2-y^2}> + |d_{xy}> \right)
\]

and

\[
|-2> = \frac{1}{\sqrt{2}} \left( |d_{x^2-y^2}> - |d_{xy}> \right)
\]

The notations used above are in the "hole" formalism which is particularly convenient for low spin 3d5 configuration. That is,

\[
|+1a> = |-1\beta, +1a, +1\beta, \zeta \alpha, \zeta \beta>
\]

The coefficients \(A\), \(B\), and \(C\) in Equation 1 are real (11) and normalized i.e.

\[
A^2 + B^2 + C^2 = 1
\]

In order to interpret the g values of an EPR spectrum, it is necessary to obtain the eigenvalues of \(\psi_{\pm}\) of the magnetic interaction Hamiltonian i.e.

\[
E^{\pm}(\psi_{\pm}) = \mathbf{S} \cdot \mathbf{H}_{\pm} \cdot \psi_{\pm} = L_{\pm} + \mathbf{g} \cdot \mathbf{S} \cdot \psi_{\pm}
\]

where \(i = x, y, z\). The difference in the energy of each Kramers doublet is related to each principal g value by

\[
\Delta g = \mathbf{g} \cdot \mathbf{S} \cdot \mathbf{H}_{\pm} = E^{+}(\psi_{+}) - E^{-}(\psi_{-})
\]

The results are:

\[
\begin{array}{l}
g_x = 2 \left( 2A^2 - B^2 \right) \\
g_y = 2 \left( \sqrt{2} A + B \right) \left( \sqrt{2} C - B \right) \\
g_z = 2 \left( \sqrt{2} A + B \right) \left( \sqrt{2} C + B \right)
\end{array}
\]

Using the g values observed at pH 4.0, and Equation 6, we

<table>
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<th>pH</th>
<th>IHP</th>
<th>(g_x)</th>
<th>(g_y)</th>
<th>(g_z)</th>
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</thead>
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<tr>
<td>4</td>
<td>+</td>
<td>1.60</td>
<td>2.19</td>
<td>2.88</td>
</tr>
<tr>
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<td>-</td>
<td>1.61</td>
<td>2.18</td>
<td>2.87</td>
</tr>
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<td>1.61</td>
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<td>2.87</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>1.82</td>
<td>2.18</td>
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<td>+</td>
<td>1.86</td>
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<td>-</td>
<td>1.87</td>
<td>2.19</td>
<td>2.81</td>
</tr>
</tbody>
</table>
calculate the coefficients $A$, $B$, and $C$. We exclude imaginary and physically unrealistic solutions. The following functions result for carp azidomethemoglobin:

$$
\psi_+ = 0.857 | +1a > + 0.123 | -1a > + 0.500 | -1a > 
$$

$$
\psi_- = 0.657 | +1a > - 0.123 | -1a > + 0.500 | +1a > 
$$

and for human hemoglobin:

$$
\psi_+ = 0.842 | +1a > + 0.128 | -1a > + 0.525 | -1a >
$$

$$
\psi_- = 0.842 | +1a > - 0.128 | -1a > + 0.525 | +1a >
$$

One can now calculate the energy of the orbitals $| +1a >$, $| -1a >$, and $| -1a >$ by using the combined Hamiltonian of the spin orbit coupling and the crystal field, $-\lambda L \cdot S + V$, where $V$ is the crystal field and $\lambda$ is the spin orbit coupling coefficient.

$$
(- \lambda L \cdot S + V) \psi_n = \lambda_n \psi_n
$$

From the solutions of the secular determinants we obtain the relative orbital energies as given in Fig. 3, where the value of $\lambda$ has taken to be 345 cm$^{-1}$ which is the value for free ferric ion. Spin orbit coupling is undoubtedly a lower but indeterminant value in heme complexes (12). Note that the energies given in Fig. 3 are in terms of one-electron orbital energies and not $\lambda$s.

The $g$ values can be calculated from these orbital energies and are given in Table II. The values $g_z$ are taken to be the same as the observed values in order to solve Equation 6. The small discrepancies between the calculated and observed values of $g_z$ and $g_x$ probably arise from oversimplicity of the model as it excludes higher excited states and orbital reduction factors. The latter is equivalent to setting $h = 1$ in Griffith's treatment.

At alkaline pH values, the azidomethemoglobins of carp and human have the same crystal field distortions. This implies that the positions of iron with respect to the porphyrin ring and the axial ligands are the same in the two proteins.

The crystal field of carp azidomethemoglobin changes with acidic pH but that of human azidomethemoglobin does not. The changes are a decrease in the rhombic distortion but an increase in the tetragonal distortion. The $| -1a >$ orbital is largely $d_{xz}$ in character and lies parallel to the porphyrin plane. A lowering of its energy may be interpreted as an out-of-plane displacement of the iron atom. The energy separation between the $| +1a >$ or $d_{yz}$ orbitals and the $| -1a >$ or $d_{xz}$ orbital has been attributed by Griffith (10) to the $p$ interaction of the imidazole group of the F8 histidine, which is taken to be in the xz plane, with the $d_{yz}$ orbital of the iron. In this model, a smaller $V$ in carp azidomethemoglobin at low pH suggests a weaker longer iron-imidazole bond. On the other hand, Kotani (13, 14) estimated this contribution to be less than 60 cm$^{-1}$ and concluded that the contribution to $V$ from the $p$ interaction between the N$_3$ ligand and iron atom to be more important. Thus, a smaller $V$ implies a weaker Fe-N$_3^-$ bonding.

The orientations and interatomic distances in azidometoglobin have been determined by three-dimensional Fourier difference synthesis (15). The three nitrogen atoms (N$_3$N$_3$N$_3$) of the azida ion are linear, making an angle of 21° with the heme plane. The Fe-N$_3$ (2.05 Å) and N-N$_3$ (1.15 Å) bond axes lie in a plane which intersects the heme plane along the line joining two opposite methine carbons, the N$_3$, atom being situated 3.2 Å above CH$_3$. The N$_3$ of azide is only 2.5 Å above the heme plane and 3.1 Å from the nitrogens of pyrroles 2 and 3. There is probably a hydrogen bond between N$_3$ of azide and N$_6$ of the E6 histidine. Overall, the position of the azide ligand minimizes nonbonding repulsive interactions with other residues such as the imidazole of E7 histidine, phenyl of CD1 phenylalanine, and methyl of E11 valine. If one assumes that this arrangement is also the one favored by carp azidomethemoglobin at alkaline and neutral pH, and if low pH causes an out-of-plane displacement of the iron atom toward the F8 histidine, the azide ligand may be prevented from a similar movement by virtue of the fact that the N$_3$ and N$_6$ atoms would come to less than the minimum van der Waals contact distances with the other atom mentioned above. Furthermore, the possible hydrogen bond with the E7 histidine may also weaken as a result.

The above considerations favor the interpretation that at low pH there is an out-of-plane movement of the iron atom but not for the azide ligand in carp azidomethemoglobin.

There exists a considerable body of evidence (5-8) which points to the stabilization of the T quaternary form of methemoglobin at low pH. The EPR results presented and discussed above suggest that this also is the case for carp azidomethemoglobin. In the case of human azidomethemoglobin, the hysteresis effect of the protons is absent; the iron atom does not feel a significant modification of crystal field. This means that it is highly unlikely that the pH effect on the carp derivative is due to protonation of a local residue such as F8 or E1 histidine.

At low pH, about 1.5% of the carp azidomethemoglobin was found to be in the high spin state at 77 K. The amount of high spin species may, however, be considerably greater at room temperature. It lies higher in energy than the low spin species and its population should increase with an increase of temperature. The observed population difference would of course depend upon the rate of sample cooling and approach to equilibrium.

The near absence of IHP effect (Table I) on the EPR spectra of carp azidomethemoglobin is very interesting. Tan and Nobel (16) reported that the addition of 0.7 and 7 mM IHP to stripped carp hemoglobin has nearly the same effect on all aspects of ligand binding, affinity, kinetics, and cooperativity,
as decreasing the pH of stripped hemoglobin by 1.6 and 1.8 pH units, respectively. In spite of this, the kinetics of CO dissociation (10) showed that IHP actually causes some changes in the properties of the high and low affinity states of carp hemoglobin. Therefore, pH and IHP do not affect the protein conformation in the same manner. The results described here show that the two heterotropic effector molecules change the conformation of carp azidomethemoglobin differently. The protons cause a change in the crystal field distortion of the prosthetic group whereas IHP does not. In the future paper, circular dichroism results will be given which show that protons change the quaternary structure of carp hemoglobin whereas the effect of IHP is confined more to the tertiary level.

REFERENCES

Electron paramagnetic resonance study of carp methemoglobin.
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