Magnetic Susceptibility Measurements of Cytochrome c Peroxidase and Its Complexes*

T. IIZUKA, M. KOTANI, AND T. YONETANI+

From the Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pennsylvania 19104 and the Department of Biophysics, Faculty of Engineering Science, Osaka University, Osaka, Japan

SUMMARY

A highly purified preparation of cytochrome c peroxidase was prepared by reconstituting the enzyme from the apoprotein and protohemin. Paramagnetic susceptibilities of the enzyme and of its complexes with cyanide, azide, cyanate, and fluoride were measured at temperatures from 77° K to 273° K. The susceptibilities of the cyanide and fluoride complexes obeyed Curie's law over the entire temperature range and it was confirmed that these complexes are in purely low spin and high spin states. All of the other complexes, in addition to the free enzyme, were found to deviate from Curie's law above certain temperatures. The effective Bohr magneton numbers \( n_{\text{eff}} \) of the enzyme at different pH values were explained by assuming that cytochrome c peroxidase is a mixture of acidic and alkaline forms and that both forms have low spin and high spin states in thermal equilibria. The temperature dependence of the equilibria was also characterized spectrophotometrically for the enzyme at pH 7 and its cyanate complex. The optical measurements were in good agreement with the susceptibility measurements of the corresponding samples.

The temperature-dependent changes of \( n_{\text{eff}} \) were analyzed on the basis of a thermal equilibrium between high spin and low spin states. Several thermodynamic parameters such as the energy difference \( \epsilon \), the enthalpy difference \( \Delta H^0 \), and the entropy difference \( \Delta S^0 \) between the two spin states, the entropy factor \( \gamma \), and the compensation temperature \( T_c \) were estimated from the temperature dependence of the equilibrium constants of the mixed spin states. The relation between \( \Delta H^0 \) and \( \Delta S^0 \) is discussed and compared with myoglobin and hemoglobin.

Cytochrome c peroxidase is a ferric protohemoprotein obtained from baker's yeast. Previous optical (1), electron paramagnetic resonance (2), and magnetic (2, 3) studies indicate that the enzyme in neutral solution is a mixture of acidic and alkaline forms. Because of the thermal excitation of spin states, the enzyme is predominantly in a high spin state at room temperature, whereas it is a low spin-rich mixture of high and low spin components in the ground state below 173° K (3, 4).

In order to determine the effect of heme substitution on the thermal excitation characteristics of this enzyme, a highly purified cytochrome c peroxidase has been reconstituted from the apoenzyme and protohemin as a control preparation and examined by magnetic susceptibility measurements. The paramagnetic susceptibilities of the reconstituted cytochrome c peroxidase at different pH values and its complexes with fluoride, cyanate, azide, and cyanide have been measured in a temperature range from 77° K to 273° K and analyzed to determine the thermodynamic parameters associated with the thermal excitation of the spin states of the enzyme. The results of these investigations are described and discussed in this paper.

A summary of the basic equations required in the analysis of experimental data (5) is given below. In a mixture of two paramagnetic isomers, high spin and low spin, the effective Bohr magneton number \( n_{\text{eff}} \) is given by Equation 1

\[
n_{\text{eff}} = \alpha n_L + (1 - \alpha)n_H
\]

where \( \alpha \) and \( 1 - \alpha \) are the relative concentrations and \( n_L \) and \( n_H \) are the effective Bohr magneton numbers of the low spin and high spin states, respectively. The equilibrium constant, \( K \), is given by Equation 2

\[
K = \frac{\alpha}{1 - \alpha} = \frac{(3\gamma)^{-1}}{\exp(\epsilon/kT)}
\]

where \( \epsilon \) is the energy difference between high spin and low spin states, \( \gamma \) is the ratio of the statistical weights of the high spin and low spin states excluding the spin weight, \( k \) is the Boltzmann constant, and \( T \) is the absolute temperature (see References 5 and 6). Substituting Equation 2 in Equation 1, we obtain Equation 3, which represents the temperature dependence of \( n_{\text{eff}} \)

\[
n_{\text{eff}}^2 = 5\frac{\exp(\epsilon/kT) + 2\gamma}{\exp(\epsilon/kT) + 3\gamma}
\]

When there exist two components, I and II, the apparent \( n_{\text{eff}}^2 \) is given by Equation 4

\[
n_{\text{app}}^2 = \Delta n_L^2 + (1 - \Lambda)n_H^2
\]

where \( \Lambda \) is the relative concentration of Component I and \( n_L \) and \( n_H \) are the effective Bohr magneton numbers of the low spin and high spin states, respectively.
Magnetic Susceptibility of Cytochrome c Peroxidase

Vol. 246, No. 15

High Spin

Low Spin

Fig. 1. The temperature dependence of $n_{\text{eff}}$ of several complexes of reconstituted protoheme-cytochrome c peroxidase (R-Proto-CCP). The data for the free enzyme at pH 5.55, 6.50, 7.05, and 7.50 are identified by pH value. The data of fluoride, cyanate, azide, and cyanide complexes are specified by F−, OCN−, N3−, and CN−, respectively.

and $n_{\text{eff}}$ are effective Bohr magneton numbers of Components I and II (8, 5).

EXPERIMENTAL PROCEDURE

Cytochrome c peroxidase was purified from bakers' yeast and twice recrystallized (7, 8). The apoenzyme was prepared by the acid-butanol method (9). The reconstituted holoenzyme was prepared from the apoenzyme and protohemin (Sigma), purified by column chromatography on DEAE-cellulose, and crystallized as described previously (9). The crystals of the reconstituted enzyme were dissolved in a small volume of 1 M potassium phosphate buffer, pH 6, to give a concentration of about 10 mM. The pH of the concentrated solution was adjusted with 1 M KH2PO4. The heme concentration was determined spectrophotometrically by the pyridine hemochromogen procedure.

Paramagnetic susceptibilities were measured with a magnetic torsion balance. The block diagram and operational principle of the balance has been described elsewhere (5). Higher stability and sensitivity were obtained with the balance used in the present study at the Johnson Foundation than with the original balance by the use of a higher magnetic field (12 koersted) and a vibration-free stone table. The minimal measurable amounts of high spin and low spin ferric hemoproteins were approximately 0.6 and 3 μmoles, respectively. Low temperature optical absorption spectra were measured with a dual-beam spectrophotometer (10, 11). The temperature was measured with a thermometer of gold-cobalt alloy versus copper (3).

RESULTS

Fig. 1 shows the temperature dependence of the square of the effective Bohr magneton number ($n_{\text{eff}}^2$) of frozen solutions of reconstituted cytochrome c peroxidase at pH 5.55, 6.50, 7.05, and 7.50 and its complexes with fluoride, cyanate, azide, and cyanide. The values of $n_{\text{eff}}^2$ for the fluoride and cyanide complexes were constant at 35 and 4.5, respectively, in the temperature range from 77-273° K, indicating that these complexes obey Curie's law over this temperature range. The values of $n_{\text{eff}}^2$ for the other complexes except cyanate were constant only below 153° K. The $n_{\text{eff}}^2$ value of the enzyme at pH 5.55 decreased above 173° K, whereas those of the enzyme at other pH values and of its azide complex increased sharply above 173° K. The $n_{\text{eff}}^2$ value of the cyanate complex showed a gradual and continuous increase with increasing temperature above 77° K. The curves for the enzyme at pH 5.55, 6.50, and 7.05 have a common crossover point near 263° K. Values of $n_{\text{eff}}^2$ and $n_{\text{eff}}$ at 77° K and 250° K for each of these complexes are listed in Table I.

Absorption spectra of the enzyme and its complexes were measured as a function of temperature. Fig. 2 illustrates the absorption spectra of the enzyme (pH 7) at 91°, 221°, 235°, and 242° K. As the temperature increased, the intensities of the α and β bands at 575 and 542 nm, characteristic of a low spin ferric hemoprotein, decreased and those of the 505- and 640-nm bands, characteristic of a high spin state, increased correspondingly. Fairly good isosbestic points were observed at 482, 525, and 597 nm, indicating that the transition involved essentially two components. Fig. 2B shows the absorption spectra of the cyanate complex of the enzyme at 91°, 183°, 200°, and 217° K. The intensities of the α and β bands at 570 and 540 nm decreased with increasing temperature. Absorption bands at 506 and 628 nm, characteristic of a high spin state, were observed to increase gradually at higher temperatures. Well defined isosbestic points were observed at 492, 514, and 598 nm. In Fig. 3, the intensities of these bands are plotted against the inverse of the
Fig. 2. The temperature dependences of the absorption spectra in the visible region. A, reconstituted protoheme-cytochrome c peroxidase (R-Proto-CCP) at pH 7.0; B, the cyanate complex of reconstituted protoheme-cytochrome c peroxidase at pH 7.0.

FIG. 3. The temperature dependence of absorbance for the reconstituted protoheme-cytochrome c peroxidase (R-Proto-CCP). A, 542, 575, 505, and 640 nm for the enzyme; B, 540, 570, 505, and 628 nm for the cyanate complex.

absolute temperature in order to compare the temperature dependence of the absorption bands with that of $\gamma$ shown in Fig. 1. Optical data of the enzyme at pH 7 (Fig. 3A) and its cyanate complex (Fig. 3B) are consistent with the magnetic data of these samples (Fig. 1).

Discussion

Fluoride and Cyanide Complexes—As shown in Fig. 1 and Table I, fluoride and cyanide complexes of cytochrome c peroxidase have essentially temperature-independent values of $n_{\text{eff}}^2$ of 35 and 4.5, respectively. The value 35 corresponds to the spin only value of the high spin configuration $(d^6)^2$ $(dy^2)^2$. The fluoride complex of the enzyme is, therefore, a purely high spin compound as are complexes of ferric myoglobin, hemoglobin, horseradish peroxidase, and catalase (11-15). On the other hand, the value of 4.5 is much larger than the expected spin only value of 3 for one unpaired electron $(S = 4)$. This large value of $n_{\text{eff}}^2$ is considered to arise from the contribution of residual orbital degeneracy in the low spin configuration $(d^6)^5$. Since low spin compounds of the other ferrihemoproteins have $n_{\text{eff}}^2 \approx 4.5$ to 5.5 (1-5, 12-16), the cyanide complex of cytochrome c peroxidase is also considered to be in the low spin state.

Azide Complex—Fig. 1 and Table I show that the $n_{\text{eff}}^2$ value of the azide complex below 173°K is 5.4, indicating that the azide complex is a low spin compound in this temperature range.

However, $n_{\text{eff}}^2$ increases sharply above 173°K. The complex is, therefore, considered to be a mixture of high spin and low spin states above this temperature. We can analyze this temperature-dependent change of $n_{\text{eff}}^2$ on the basis of an equilibrium between two spin states. A similar treatment has been applied to myoglobin, hemoglobin, and other ferric hemoproteins successfully (5, 12, 13, 16). Adopting 35 and 5.4 as values of $n_{\text{eff}}^2$ and $n_{\text{eff}}^2$ and using Equations 1 and 2, we calculated the value of the equilibrium constant $K$ at each temperature. These values were plotted against the inverse of the absolute temperature in Fig. 4. The energy difference between two spin states, $\epsilon$, and the entropy factor, $\gamma$, were estimated from the slope of the straight line and the extrapolated value of $K$ at $1/T = 0$ in Fig. 4. Values of $\epsilon$ and $\gamma$ are summarized in Table II.

Cyanate Complex—As shown in Fig. 1 and Fig. 2B, the cyanate complex seems to change from a predominantly low spin state to a high spin-rich state with increasing temperature. Using $n_{\text{eff}}^2 = 9.3$ at the low temperature limit and $n_{\text{eff}}^2 = 5$ and $n_{\text{eff}}^2 = 35$ in Equation 4, we obtain $A = 0.86$. This means that this complex consists of 86% low spin and 14% high spin in the ground state. Since the relative contents of major and minor components are considered to be temperature independent in a frozen solution, we can estimate the temperature dependence of $n_{\text{eff}}^2$ of the major component by using Equation 4 with the additional assumption that the spin state of the minor component is temperature independent. The same method of analysis was used for the cyanate complex as for the azide complex, and the relation of log $K$ to $1/T$ is shown in Fig. 4. Values of $\epsilon$ and $\gamma$ were estimated in the same way as for the azide complex and are listed in Table II.

pH Dependence—The paramagnetic susceptibilities of native cytochrome c peroxidase at pH 5.0 and 7.0 are measured and reported in a previous paper (3). In the present experiment, we measured the susceptibility of the reconstituted and purified enzyme three times. The $n_{\text{eff}}^2$ value of the reconstituted enzyme was found to have a wider pH variation than native enzyme, while the temperature-dependent change was similar in both cases.

$n_{\text{eff}}^2$ in Ground State—The temperature-independent values of $n_{\text{eff}}^2$ of the enzyme at pH 5.55, 6.50, and 7.05 below 173°K are discussed first. In this temperature range, no change was observed in the broad high spin bands near 640 and 505 nm or in the low spin bands near 542 and 575 nm (See Fig. 2A and Fig. 3A). Detailed measurements of electron paramagnetic...
resonance and optical absorption spectra showed the increase of high spin state and decrease of low spin state with lowering pH. These results indicate that the electronic state of the enzyme changes from an "acidic" form of high spin to an "alkaline" form of low spin with increasing pH and that both forms are in the ground state below 173° K. Since there exist only two forms in the reconstituted and purified enzyme, 1) n_eff at a certain pH value in the ground state may be described by the combination of two components, I and II, by Equation 4, where the components I and II are the alkaline and acidic forms, respectively. n_I and n_II are considered to be 5 (low spin) and 35 (high spin) in the ground state. Values of A (the relative content of the alkaline form) calculated on this basis are given in Table III, together with the percentage of each form.

Values of ε and γ are listed in Table II. ε > 0 and ε < 0 in Table II correspond to positive and negative slopes in Fig. 4, indicating that the ground states are low spin and high spin states, respectively.

Curves a to e in Fig. 5 are theoretical curves derived by inserting the values of ε and γ from Table II into Equations 3 to 6. The calculated curves agree very well with the experimental data, including those for the experiment at pH 6.5 which were not used in deriving the values of ε and γ.

Since there is only one alkaline and one acidic form of the enzyme and since both show fairly sharp changes of n_eff near 263° K, it is reasonable that the curves at pH 5.5, 6.5, and 7.05 have a single crossover point at 263° K. This is analogous to the isosbestic points observed in optical spectra. This kind of crossover point (or region) appears near 260-280° K not only in cytochrome c peroxidase but also in horseradish peroxidase (15), and catalase (17), whereas it is not observable and is expected to be located near 400° K in the case of myoglobin or hemoglobin (5, 14). If more than two components are present because of partial denaturation or some other reason, the curve of n_eff against 1/T will deviate from the crossover point. The enzyme at pH 7.5 seems to show this behavior as seen in Fig. 1.

Finally we discuss another possible cause of the temperature-dependent change of n_eff, namely a shift of effective pH in frozen solutions with increasing temperature above 173° K, which would change the equilibrium

$$
\text{Acidic form (high spin) \rightarrow alkaline form (low spin)}
$$

and cause a transition between low spin and high spin states. As shown in Fig. 1, n_eff at pH 5.5 decreases with increasing temperature, indicating the shift of effective pH to the alkaline side in Equation 7. On the contrary, n_eff at pH 7.05 increases with increasing temperature, suggesting a shift to the acidic

---

1. T. Yonetani and T. Iizuka, unpublished data.
side in Equation 7. It seems unlikely that the effective pH changes with increasing temperatures are inverted within such a narrow range of pH. For this reason and since the azide and cyanate complexes show temperature-dependent transitions caused by a thermal mixing of two magnetic isomers (high spin and low spin), we can assume that there is a thermal equilibrium between high spin and low spin states in both the alkaline and acidic forms and that the relative contents of these forms remain constant in frozen solutions.

Thermodynamic Parameters—The enthalpy and entropy changes associated with spin change were estimated from values of $\epsilon$ and $\gamma$ for each sample by use of the relations, $\Delta H = -N\epsilon$ and $\Delta S = -R \ln 3\gamma$ (6, 16), where $N$ is Avogadro’s number and $R$ is the molar gas constant. The results are listed in Table II together with the compensation temperature, $T_c$ ($=\Delta H^0/\Delta S^0$) at which the concentrations of the high spin and low spin states are expected to be equal. The energy differences ($\epsilon$) of various derivatives of cytochrome c peroxidase increase in the following order:

$$\epsilon(F^-) < \epsilon(\text{acidic form}) < \epsilon(\text{OCN}^-) < \epsilon(\text{N}_2^-) \approx \epsilon(\text{alkaline form}) < \epsilon(\text{CN}^-)$$

A similar order has been deduced before for hemoglobin and myoglobin complexes (5, 14):

$$\epsilon(F^-) < \epsilon(\text{acidic form}) < \epsilon(\text{OCN}^-) < \epsilon(\text{N}_2^-) \approx \epsilon(\text{alkaline form}) < \epsilon(\text{CN}^-)$$

For these proteins, it was concluded that this order corresponds to the order of ligand field strength or covalency of the ligand (5, 14). Similarly, in the case of cytochrome c peroxidase, the ligands CN$^-$ and F$^-$ may be considered to be highly covalent (strong ligand field) and ionic (weak ligand field), respectively. The alkaline form of cytochrome c peroxidase occupies almost the same position as the acid form of the complexes. This suggests that the sixth ligand of the alkaline form may not be the OH$^-$ ligand from the medium, but a ligand from the apoprotein such as an side chain of an amino acid residue. This would be in contrast to the situation obtained with the alkaline form of myoglobin and hemoglobin. Since the direct coordination of an amino acid residue of the apoprotein to the 6th position of the iron would be expected to cause a large change in protein conformation and thus a larger effect on the entropy factor ($\gamma$) than the axial ion added externally, it might be reasonable that the absolute value of $\Delta S^0$ is 3 entropy units (calories per mole per degree) higher in the alkaline form than in the acid form, in spite of the fact that the value of $\Delta H^0$ is almost the same.

Fig. 6 illustrates a plot of $\Delta H^0$ versus $\Delta S^0$ for various complexes of cytochrome c peroxidase. We can draw a straight line to fit four points, and the line will go through the origin. This straight line means that the enthalpy changes are compensated by the entropy changes. The slope of the line corresponds to the temperature $T_c$. Similar plots for myoglobin and hemoglobin complexes are indicated by $\cdots$ and $\cdot\cdot\cdot\cdot$, which has a different slope from that of the peroxidase (5, 14). The values of $T_c$ given in Table II are relatively independent of the 6th ligand for four complexes, whereas the individual values of $\Delta H^0$ and $\Delta S^0$ are highly dependent on the nature of the 6th ligand. The mean value of $T_c$ for these four cases is 272$^\circ$ K, which is about 100$^\circ$ K lower than that of hemoglobin (372$^\circ$ K) and myoglobin (368$^\circ$ K) (5, 14). Otsuka (18) theoretically calculated that $T_c$ may be determined not by the nature of the 6th ligand, but by environmental factors such as the nature and extent of van der Waals contacts between heme and apoprotein and thus by the type of the apoprotein.

Since the reconstituted cytochrome c peroxidase exhibited spectral, electron paramagnetic resonance, and enzyme properties indistinguishable from those of the freshly prepared native enzyme, it was concluded that the original mode of the heme-apoprotein interaction is fully restored in the reconstituted enzyme (9). This conclusion has been further supported by the present susceptibility study. The thermodynamic parameters of the spin transition of the reconstituted enzyme are in good agreement with those of the native enzyme reported previously (5). The compensation temperature, $T_c$, which we consider a particularly sensitive measure of the heme-apoprotein interaction, was found to be essentially identical in the native and reconstituted enzymes. The presence of a definite crossover point at 263$^\circ$ K, which was previously assumed from the measurements at two pH values (3), has been unequivocally established by the measurements at three pH values, pH 5.55, 6.50, and 7.05 (Fig. 1). The slight deviation of the plot at pH 7.50 from the crossover point in Fig. 1 indicates that the enzyme can no longer be regarded as a simple mixture of acidic and alkaline forms at pH 7.50. It appears that another "alkaline" form was formed at higher pH values. This was not a property inherent to the reconstituted enzyme, because the native enzyme was found to be unstable above pH 7.50 (1). The formation of another "alkaline" form of the enzyme above pH 8.0 could be readily detected by low temperature optical and electron paramagnetic resonance measurements.1 The instability of the enzyme at extreme pH values may provide an explanation for the fact that the pH dependence of the $A$ value of the enzyme (Table III) does not conform to the conventional pH curve of

<table>
<thead>
<tr>
<th><strong>Table II</strong></th>
<th>Various thermodynamic parameters in temperature-dependent transition of spin state of cytochrome c peroxidase</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CCP</strong> (full form)</td>
<td><strong>Ground state</strong></td>
</tr>
<tr>
<td><strong>Acidic form</strong></td>
<td>High spin</td>
</tr>
<tr>
<td>OCN$^-$</td>
<td>Low spin</td>
</tr>
<tr>
<td>N$_2^-$</td>
<td>Low spin</td>
</tr>
<tr>
<td><strong>Alkaline form</strong></td>
<td>Low spin</td>
</tr>
</tbody>
</table>

1 CCP, cytochrome c peroxidase.

2 $T_c$ is defined as the temperature, where high spin amounts are equal to low spin amounts or $T_c = \Delta H^0/\Delta S^0$ (5).

<table>
<thead>
<tr>
<th><strong>Table III</strong></th>
<th>Relative concentration of alkaline and acidic forms of cytochrome c peroxidase at various values of pH</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>pH</strong></td>
<td><strong>$n_{eff}$</strong></td>
</tr>
<tr>
<td><strong>%</strong></td>
<td><strong>%</strong></td>
</tr>
<tr>
<td>5.55</td>
<td>29.0</td>
</tr>
<tr>
<td>6.50</td>
<td>67.5</td>
</tr>
<tr>
<td>7.05</td>
<td>71.8</td>
</tr>
</tbody>
</table>
acid-alkaline transition. Cytochrome c peroxidase could not be converted fully to either high spin acidic or low spin alkaline form without some modification of the enzyme.

On the basis of the present results, it is feasible now to determine the effect of heme modifications on the thermodynamic parameters and the heme-apoprotein interaction through magnetic susceptibility measurements of artificial hemoproteins with substituted hemes.

REFERENCES

Magnetic Susceptibility Measurements of Cytochrome c Peroxidase and Its Complexes
T. Iizuka, M. Kotani and T. Yonetani


Access the most updated version of this article at http://www.jbc.org/content/246/15/4731

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/246/15/4731.full.html#ref-list-1