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A THERMAL EQUILIBRIUM BETWEEN HIGH- AND LOW-SPIN STATES IN FERRIC CYTOCHROME *c* PEROXIDASE AND SOME DISCUSSION ON THE ENZYME–SUBSTRATE COMPLEX

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SUMMARY

Paramagnetic susceptibilities of cytochrome *c* peroxidase (ferriperoxidase) and its peroxide compound (*E–S* complex) were measured in a range of temperature from liquid nitrogen to room temperatures. The susceptibilities of this enzyme in dissolved and polycrystalline states were found to deviate from the Curie law above −100°, whereas the susceptibility of the *E–S* complex was found to obey the Curie law rather closely in the whole range of temperature tested. The abnormal temperature- and pH dependences of paramagnetic susceptibility of ferriperoxidase are explained on the assumptions that these samples are pH-dependent mixtures of two kinds of molecules, probably $(Fe^{3+}) \cdot OH^-$ and $(Fe^{3+}) \cdot H_2O$ and that both of these molecules have high- and low-spin states populated in thermal equilibria.

From the analysis of temperature dependence of the equilibrium constants, energy and entropy differences between high- and low-spin states were estimated, as shown below:

	$(Fe^{3+}) \cdot OH^-$	$(Fe^{3+}) \cdot H_2O$
Excited state	high-spin state	low-spin state
Lowest state	low-spin state	high-spin state
Excitation energy	1830 cm^{-1}	1230 cm^{-1}

The entropy differences between these states deviated considerably from $R \cdot \ln 3$ expected from weight differences of these spin states. Thermodynamical quantities, ΔH° and ΔS°, were calculated from energy difference (ε) and entropy factor (γ).

The electronic structure of the peroxide compound (*E–S* complex) was discussed in terms of a compound containing a free radical and Fe^{4+}.

INTRODUCTION

Some ferric hemoproteins such as the hydroxide complexes of ferric hemoglobin[1]

and myoglobin[2], $Hb(Fe^{3+})\cdot OH^-$ and $Mb(Fe^{3+})\cdot OH^-$, respectively, have magnetic susceptibilities between the values characteristic of 5 and 1 unpaired electrons. It was suggested by THEORELL AND EHRENBERG[2] and TAUBE[3] that these compounds might be a mixture of two magnetic isomers, one high-spin and the other low-spin. GRIFFITH[4] extended this concept further on the basis of theoretical calculation and concluded that ferric hemoproteins with intermediate magnetic susceptibilities may be in thermal equilibria between high-spin and low-spin states based on the Boltzmann distribution. This concept was experimentally verified by GEORGE, BEETLESTONE AND GRIFFITH[5], who determined the temperature dependences of magnetic susceptibility and light absorption spectra in a range of ambient temperatures from 0 to 30°.

Recent measurements of paramagnetic susceptibilities and light absorption spectra of some ferric hemoproteins at cryogenic temperatures[6,7] indicate that the temperature-dependent transition between high-spin and low-spin states does occur even below 0°. IIZUKA AND KOTANI[6] recently carried out the accurate measurement of paramagnetic susceptibility of ferrimyoglobin azide ($Mb(Fe^{3+})\cdot N_3^-$) between liquid nitrogen temperature and room temperature. They found that $Mb(Fe^{3+})\cdot N_3^-$ is a purely low-spin compound with an effective Bohr magneton number (n_{eff}) of 2.24 between −130° and −196° and obeys the Curie law in this range of temperature. However, $Mb(Fe^{3+})\cdot N_3^-$ becomes a thermally equilibrated mixture of high- and low-spin states above −120° and its susceptibility deviates considerably from the Curie law between −120° and room temperature.

Cytochrome *c* peroxidase (ferriperoxidase) is a ferric protohemoprotein purified from baker's yeast[8]. The magnetic susceptibility ($n_{eff} = 5.6$ at 20° (ref. 9)) and light absorption spectrum of this enzyme at pH 7 (ref. 7) indicate that ferriperoxidase is a predominantly high-spin compound at ambient temperatures. However, its EPR spectrum at liquid nitrogen temperature indicates that this enzyme becomes a low-spin rich mixture of high- and low-spin compounds at −196° (ref. 10). The quantitative EPR examination of the spin transition of this enzyme is rather difficult above −100° because of considerable broadening of EPR signals derived from the heme iron at higher temperatures. Thus, YONETANI, WILSON AND SEAMONDS[7] attempted to investigate the temperature-dependent transition of the spin states of this enzyme by low-temperature spectrophotometry. They showed that the light absorption spectrum of this enzyme is temperature independent below −100°, whereas it becomes highly temperature dependent above −100°. The low-spin bands at 540 and 574 mμ were shown to disappear above −100°, whereas the high-spin bands at 500 and 640 mμ appeared above −60°. Therefore, it was concluded that the temperature-dependent transition of the spin states of this enzyme takes places in a relatively narrow range of cryogenic temperature from −100 to 0°. The non-synchronous appearance and disappearance of these absorption bands and the apparent absence of isosbestic points in absorption spectra at different temperatures indicate the involvement of more than two components in these absorption changes. Since the absorption spectrum of each component was not known, the detailed quantitative analysis of these optical data was not performed.

Since a highly sensitive magnetic balance for measurements at ambient and cyogenic temperatures has become available[6], it is now possible to investigate in detail the temperature-dependent transition of the spin state of cytochrome *c* peroxidase by magnetic susceptometry in a wide range of temperature.

Cytochrome *c* peroxidase forms a relatively stable intermediate, the *E–S* complex, in the reaction with H_2O_2[11]. The *E–S* complex was demonstrated to have an oxidation state 2-equivalents higher than the original ferric enzyme[12] and an effective Bohr magneton number of 4.1 at 20°[10]. The EPR spectrum of the *E–S* complex at −196° (ref. 10) exhibited an intense EPR signal of a free radical type at $g = 2.004$. On the basis of these findings, it was speculated that the *E–S* complex might retain one of the two oxidizing equivalents as a free radical and the other in the heme group as Fe^{4+} (ref. 10). In order to understand further the chemical nature of the *E–S* complex, it is highly desirable to measure the paramagnetic susceptibility of the *E–S* complex in a wide range of temperature.

In this paper, we shall describe paramagnetic susceptibilities of cytochrome *c* peroxidase and the *E–S* complex measured between −196° and room temperature. These results will be discussed in terms of thermal equilibria between high- and low-spin states of $(Fe^{3+}) \cdot OH^-$ and $(Fe^{3+}) \cdot H_2O$. Some discussions of the electronic structure of the *E–S* complex will be also given.

EXPERIMENTAL PROCEDURES

Preparation

Cytochrome *c* peroxidase was purified from baker's yeast[8] and recrystallized twice by dialysis against distilled water, as described elsewhere[13]. Crystals of the enzyme were collected by centrifugation and used as a polycrystalline preparation. The pH value of the polycrystalline preparation could not be determined, since the enzyme was crystallized from distilled water. Since the enzyme is an acidic protein with an isoelectric point of pH 5.1 (ref. 14), it is expected that the internal pH value of the enzyme crystals is close to this pH value. The enzyme was dissolved in 0.2 M K_2HPO_4 and adjusted to pH 7.0 and pH 5.0 with 0.5 M KH_2PO_4 and 0.5 M H_3PO_4, respectively. The pH values indicated are determined at 23°. The quantity of the enzyme was determined spectrophotometrically by the use of an absorbance coefficient of 93 $mM^{-1} \cdot cm^{-1}$ at 408 mμ (pH 6.0 and 23°) (ref. 11). The *E–S* complex was prepared by mixing approx. 0.9 ml of 6 mM ferriperoxidase with 0.1 ml of 60 mM ethylperoxide. The thus prepared *E–S* complex was immediately transferred to the sample capsule of the magnetic balance and rapidly frozen by immersing in liquid nitrogen within 40 sec after mixing. The preparation of the *E–S* complex was kept frozen below −5° during the magnetic measurements. Aliquots of the *E–S* complex were spectrophotometrically examined before and after the magnetic measurements to ensure the full formation of the *E–S* complex.

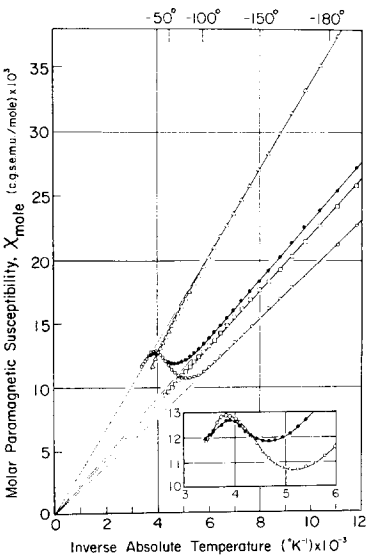
Method

The measurements of magnetic susceptibility were carried out with a magnetic torsion balance[6]. This magnetic balance automatically measures the susceptibility by means of a twin photocell and feedback mechanism and is operated at a field strength of 4000 Oe and in a range from liquid nitrogen to room temperatures, as described in detail elsewhere[6]. Pure water ($0.720 \cdot 10^{-6}$ c.g.s. e.m.u. at room temperature) was used for the calibration of the susceptibility. The measurements of temperature was made by the use of a thermocouple of Au–Co alloy and Pt. The output voltage of this thermocouple was an approximately linear function of temperature

between $-200°$ and $+100°$. The linearity was checked by a chrome alum standard which is known to obey the Curie law in this range of temperature. The magnetic balance plotted automatically and continuously the susceptibility of a sample as a function of temperature on an X–Y recorder. A minimal quantity of a sample required was of the order of $3 \cdot 10^{18}$ spins, which corresponds approximately to $1 \cdot 10^{-6}$ and $7 \cdot 10^{-6}$ gatom of iron for high- and low-spin ferric hemoproteins, respectively. A sample capsule, which holds a sample of approx. 0.7 ml was made from a purely diamagnetic plastic which had enough flexibility to withstand the stress caused by the volume change of a sample at different temperatures.

RESULTS

Fig. 1 illustrates the temperature dependence of χ_{mole}, the molar paramagnetic susceptibility, of ferriperoxidase in dissolved and polycrystalline states and the *E–S* complex in a dissolved state. It is expected that χ_{mole} will be a linear function of inverse absolute temperature, if it obeys the Curie law. As seen in Fig. 1, observed values of χ_{mole} of ferriperoxidase in both dissolved and polycrystalline states obeyed the Curie law rather well below $-100°$. However, they deviated from the law above $-100°$. The temperature dependence of χ_{mole} of ferriperoxidase in solution at pH 5 deviated from the Curie law differently from that of ferriperoxidase in solution at pH 7 or in a polycrystalline state. The former deviated downwards above $-100°$



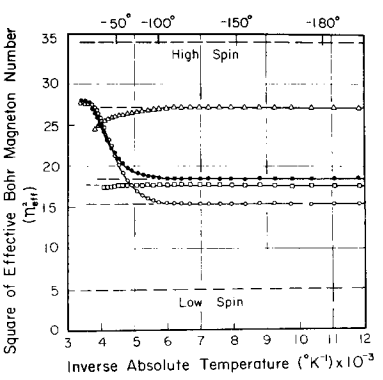


Fig. 1. The temperature dependence of molar paramagnetic susceptibilities of cytochrome *c* peroxidase and the *E–S* complex. The higher temperature region is shown expanded in the insert. Cytochrome *c* peroxidase solution: at pH 5 (△—△); at pH 7 (○—○). Cytochrome *c* peroxidase polycristalline, (●—●). *E–S* complex solution at pH 7 (□—□).

Fig. 2. The temperature dependence of n_{eff}^2 values (squares of effective Bohr magneton number). High- and low-spin states are indicated by the broken horizontal lines at n_{eff}^2 values of 35 and 5, respectively. For symbols see Fig. 1.

from the straight line representing the Curie law, whereas the latter two deviated upwards. However, the χ_{mole} value of the *E–S* complex was found to be almost proportional to inverse absolute temperature over a wide range of temperature.

The χ_{mole} values were converted to the n_{eff}^2 values, by the use of Langevin equation:

$$n_{eff}^2 = \frac{3kT}{N\beta^2} \chi_{mole}$$

where n_{eff} is the effective Bohr magneton number, k is Boltzmann's constant, T is absolute temperature, β is Bohr magneton, and N is Avogadro number. The temperature dependences of n_{eff}^2 values of different samples are illustrated in Fig. 2. All the n_{eff}^2 values of each sample obtained at different temperatures were found to fall in a range between 35 and 5, which are the values characteristic of high- and low-spin compounds with 5 and 1 unpaired electrons, respectively. The temperature-dependent changes of the n_{eff}^2 values of ferriperoxidase in dissolved and polycrystalline states above $-100°$ are more clearly seen in Fig. 2. The n_{eff}^2 values of three different samples of ferriperoxidase were found to be strictly temperature independent below $-100°$. However, they were pH dependent. The n_{eff} values of ferriperoxidase calculated for dissolved preparations at pH 5 and 7 and for a polycrystalline preparation were 3.91, 5.20, and 4.29 between $-100°$ and $-196°$, respectively. The n_{eff} value of the *E–S* complex was found to be 4.17, which was almost temperature independent between $-5°$ and $-196°$.

Fig. 3 shows the temperature dependence of the absorbance at 574 mμ of ferriperoxidase in a frozen solution at pH 7. This figure was reconstructed from the data of YONETANI, WILSON AND SEAMONDS[7] by replotting the 574 mμ absorbance against inverse absolute temperature, in order to compare it with the present magnetic data. The absorbance at 574 mμ of ferriperoxidase may be considered to be derived mainly from a low-spin molecule, since it is far distant from the absorption bands at 500 and 640 mμ associated with a high-spin compound. The temperature dependence of the absorbance at 574 mμ shows two regions, namely, a temperature-independent region below $-100°$ and a temperature-dependent region above $-100°$. The general features of the temperature dependences of the n_{eff}^2 value (*cf.* Fig. 2) and the 574 mμ absorbance (*cf.* Fig. 3) of ferriperoxidase in solution at pH 7 are similar.

ANALYSES AND DISCUSSIONS

First let us discuss the intermediate values of n_{eff}^2 of ferriperoxidase obtained

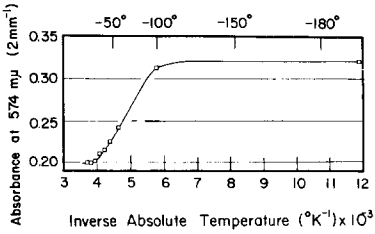


Fig. 3. The temperature dependence of the absorbance at 574 mμ of cytochrome *c* peroxidase in solution (70 μM, pH 7). The data of YONETANI, WILSON AND SEAMONDS[7] are replotted here against inverse absolute temperature in order to compare with Fig. 2.

between −100° and −196°. In this range of temperature, not only n_{eff}^2 but also light absorption and EPR spectra are strongly influenced by the pH value of a sample. The pH dependences of n_{eff}^2, light absorption and EPR spectra suggest that the numbers of molecules of ferriperoxidase in a low-spin state increase at higher pH values. Therefore, it is reasonable to consider that H_2O and OH^- are bound to the sixth coordination position of the heme iron (Fe^{3+}), as assumed in other ferric hemoproteins such as Hb(Fe^{3+}) and Mb(Fe^{3+}), and that (Fe^{3+})·OH^- is in a purely low-spin state and (Fe^{3+})·H_2O is in a purely high-spin state between −100° and −196°. Since n_{eff}^2 has additivity, n_{eff}^2 at a certain pH value may be described by Eqn. 1:

$$n_{eff}^2 = A\, n_{OH^-}^{-2} + (1 - A)\, n_{H_2O}^2 \qquad (1)$$

where A and 1–A are the pH-dependent terms representing the relative concentrations of (Fe^{3+})·OH^- and (Fe^{3+})·H_2O, respectively, and n_{OH^-} and n_{H_2O} are the Bohr magneton numbers of (Fe^{3+})·OH^- and (Fe^{3+})·H_2O, respectively. The values of $n_{OH^-}^{-2}$ and $n_{H_2O}^2$ may be put as 5 and 35, respectively, below −100°, since (Fe^{3+})·OH^- and (Fe^{3+})·H_2O are considered to be in purely low- and high-spin states in this range of temperature, respectively. This assumption is reasonable, since n_{eff}^2 values of Mb(Fe^{3+})·H_2O, Mb(Fe^{3+})·N_3^-, and Mb(Fe^{3+})·imidazole are 34.8, 4.9, and 5.3, respectively, between −120° and −196°, although they are intermediate values above −120°.

Using the n_{eff}^2 value at each pH, the A value is calculated from Eqn. 2:

$$n_{eff}^2 = 5A + 35(1 - A) \qquad (2)$$

Since the n_{eff}^2 value of ferriperoxidase in solution at pH 7 was 15.32, the A value was calculated to be 0.656, indicating that ferriperoxidase in solution at pH 7 consists of 65.6% (Fe^{3+})·OH^- and 34.4% (Fe^{3+})·H_2O. The A value of ferriperoxidase in solution at pH 5 was determined to be 0.267 in the same way.

We discuss next the temperature-dependent changes of n_{eff}^2 values of ferriperoxidase above −100° (*cf.* Fig. 2). Here we analyze our data in terms of thermally equilibrated mixture of high- and low-spin states, according to the method of GEORGE, BEETLESTONE AND GRIFFITH[5]. However, the analysis of the present data on n_{eff}^2 of ferriperoxidase is more complex, since there exist two kinds of molecules, (Fe^{3+})·OH^- and (Fe^{3+})·H_2O, which are purely low- and high-spin states, respectively, below −100°. However, the spin state of each of these molecules is changeable above −100°. The change from low- to high-spin states appears mainly at pH 7 (65.6% (Fe^{3+})·OH^- and 34.4% (Fe^{3+})·H_2O) and change from high- to low-spin states appears mainly at pH 5 (26.7% (Fe^{3+})·OH^- and 73.3% (Fe^{3+})·H_2O), for n_{eff}^2 values increased at pH 7 and decreased at pH 5 as temperature was increased above −100°, as shown in Fig. 2. If we assume that the relative amounts of (Fe^{3+})·OH^- and (Fe^{3+})·H_2O are fixed throughout the temperature range below 0°, parameter A in Eqn. 1 becomes a temperature-independent term. The values of A are already determined from the data below −100°. Therefore, $n_{OH^-}^{-2}$ and $n_{H_2O}^2$ values at each temperature can be calculated by solving two simultaneous equations for pH 5 and 7 based on Eqn. 1. The values of $n_{OH^-}^{-2}$ and n_{H_2O} calculated are indicated by filled circles on Curves a and b in Fig. 4. These points indicate clearly that (Fe^{3+})·OH^- has a thermally excited high-spin state and (Fe^{3+})·H_2O has a thermally excited low-spin state.

The subsequent analyses are quite similar to those reported in a previous

paper[6]. The α value, the relative concentration of the low-spin state in $(Fe^{3+})\cdot OH^-$, is determined as a function of temperature by the use of Eqn. 3:

$$n_{OH^-}{}^2 = \alpha\, n_L{}^2 + (1-\alpha)\, n_H{}^2 = 5\,\alpha + 35(1-\alpha) \tag{3}$$

where n_L and n_H are effective Bohr magneton numbers of low- and high-spin states of $(Fe^{3+})\cdot OH^-$, respectively. Then the equilibrium constant between high- and low-spin states, K, is expressed by Eqn. 4:

$$K_{OH^-} = \frac{\alpha}{1-\alpha} = (3\gamma)^{-1}\, e^{\varepsilon/kT} \tag{4}$$

where ε is the energy difference between two spin states and γ is an entropy factor[6]. The K_{OH^-} values determined are plotted as a function of inverse absolute temperature in Fig. 5. The ε and γ are graphically determined as the slope and the intercept at the $\log K_{OH^-}$ axis of the $\log K_{OH^-}$ *versus* $1/T$ plot, respectively. The ε and γ values obtained for $(Fe^{3+})\cdot OH^-$ are listed in Table I.

TABLE I

THERMODYNAMICAL QUANTITIES OF THE TRANSITION OF SPIN STATES OF CYTOCHROME *c* PEROXIDASE BETWEEN LIQUID-NITROGEN TEMPERATURE AND ROOM TEMPERATURE

The data for $Mb(Fe^{3+})\cdot N_3^-$ were taken from IIZUKA AND KOTANI[6].

Compound	*Excitation energy* (ε, ε' *in* cm^{-1})	*Entropy factor* (γ, γ')	ΔH^0 *(cal/mole)*	ΔS^0 *(e.u.)*	ΔF^0 *(cal/mole)*	T *at* $\Delta F^0 = 0$		*Excitation state*
						°K	°C	
$(Fe^{3+})\cdot OH^-$	+1830	$2.58\cdot 10^4$	−5220	−22.5	$-5220 + 22.5T$	232	−41	High spin
$(Fe^{3+})\cdot H_2O$	−1230	$5.59\cdot 10^{-4}$	+3510	+12.8	$+3510 - 12.8T$	274	+1	Low spin
$Mb(Fe^{3+})\cdot N_3^-$	+1280	$5.35\cdot 10^1$	−3660	−10.15	$-3660 + 10.15T$	360	+87	High spin

Similarly we adopt Eqns. 5 and 6 for $(Fe^{3+})\cdot H_2O$:

$$n_{H_2O}{}^2 = \alpha' n_L{}^2 + (1-\alpha')\, n_H{}^2 \tag{5}$$

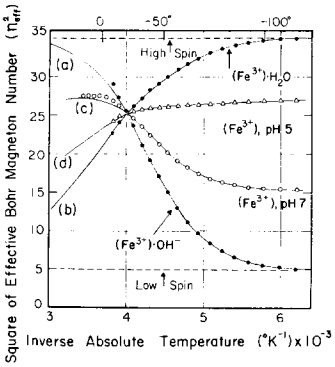
$$K_{H_2O} = \frac{\alpha'}{1-\alpha'} = (3\gamma')^{-1}\, e^{\varepsilon'/kT} \tag{6}$$

where α' is the relative concentration of the low-spin state in $(Fe^{3+})\cdot H_2O$, K_{H_2O} is the equilibrium constant between two spin states of $(Fe^{3+})\cdot H_2O$, ε' is the energy difference, and γ' is an entropy factor. The calculated values of ε' and γ' are also listed in Table I. The conditions, $\varepsilon > 0$ and $\varepsilon < 0$, indicate that low-spin and high-spin states are the ground states, respectively.

From the values of ε, ε', γ, and γ', the temperature dependence of $n_{eff}{}^2$ at pH 7 and 5 can be simulated. From Eqns. 3–6 we obtain Eqns. 7 and 8:

$$n^2{}_{OH^-} = 5\cdot\frac{e^{\varepsilon/kT} + 21\gamma}{e^{\varepsilon/kT} + 3\gamma} \tag{7}$$

$$n^2{}_{H_2O} = 5\cdot\frac{e^{\varepsilon'/kT} + 21\gamma'}{e^{\varepsilon'/kT} + 3\gamma'} \tag{8}$$



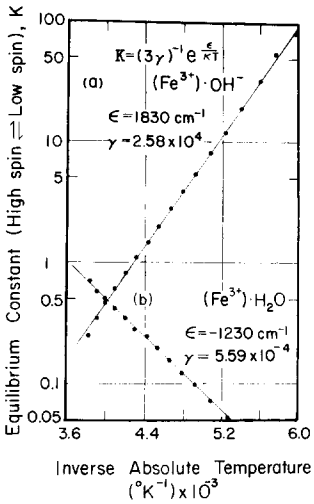


Fig. 4. The temperature dependence of n_{eff}^2 values determined for cytochrome *c* peroxidase at pH 5 (△—△) and pH 7 (○—○) and $n_{OH^-}{}^{-2}$ and $n_{H_2O}{}^2$ (●—●), which are n_{eff}^2 values for $(Fe^{3+})\cdot OH^-$ and $(Fe^{3+})\cdot H_2O$ estimated from two simultaneous equations (Eqn. 1) for pH 5 and pH 7, respectively. Solid Curves a and b are constructed from Eqns. 8 and 9 by applying the determined values of ε and γ, respectively. Solid Curves c and d are made from Eqn. 10 for pH 7 and pH 5, respectively.

Fig. 5. The temperature dependence of logarithm of K (equilibrium constants between high- and low-spin states). These results were calculated from the temperature dependence of $n^2{}_{OH^-}$ and $n_{H_2O}{}^2$ in Fig. 4.

Solid Curves a and b in Fig. 4 are simulated by Eqns. 7 and 8 with the obtained values of ε, ε', γ, and γ'.

Putting Eqns. 7 and 8 into Eqn. 1, n_{eff}^2 can be expressed by Eqn. 9:

$$n^2_{eff} = 5A \cdot \frac{e^{\varepsilon/kT} + 21\gamma}{e^{\varepsilon/kT} + 3\gamma} + 5(1 - A) \cdot \frac{e^{\varepsilon'/kT} + 21\gamma'}{e^{\varepsilon'/kT} + 3\gamma'} \qquad (9)$$

Solid Curves c and d in Fig. 4 represent $n^2{}_{eff}$ calculated from Eqn. 9 with A = 0.656 (pH 7) and A = 0.264 (pH 5), respectively. The observed values at pH 7 above −20° deviate slightly from the simulated Curve c, but their temperature dependence above 0° agree with Curve c qualitatively.

The corresponding values of thermodynamical quantities, ΔH° (= $-N\varepsilon$), ΔS° (= $-R\ln 3\gamma$), ΔF°, and T at ΔF° = 0 are summarized in Table I.

Since the values of γ and γ' are far different from unity, the values of $|\Delta S^\circ|$ become very large. Therefore, the condition, ΔF° = 0, is realized near room temperature (−41° and +1° for $(Fe^{3+})\cdot OH^-$ and $(Fe^{3+})\cdot H_2O$, respectively) inspite of very large $|\Delta H^\circ|$ values. The temperature at which ΔF° = 0 gives the inflection point of the n_{eff}^2 *versus* $1/T$ curve. The concentrations of high- and low-spin states are equal at this temperature. These results on ferriperoxidase are very suggestive that thermal equilibria may be uniquely characteristic of ferric hemoproteins, because hemin and its solution at high pH values were found to obey the Curie law strictly and they are not considered to be thermally equilibrated mixtures[15]. Large values of γ and $|\Delta S^\circ|$ are considered to originate mainly from the protein structure and its interaction with solvent.

As to the crystalline sample of ferriperoxidase, the percentage content of $(Fe^{3+})\cdot OH^-$ and $(Fe^{3+})\cdot H_2O$ can be determined from n_{eff}^2 value of 18.40 below $-100°$, on the same assumptions employed in the analysis of dissolved samples. The polycrystalline ferriperoxidase was found to contain 55.2% $(Fe^{3+})\cdot OH^-$ and 44.8% $(Fe^{3+})\cdot H_2O$. However, the temperature dependence of n_{eff}^2 above $-100°$ for the polycrystalline ferriperoxidase, which was reconstructed by use of the values of ε and γ for $(Fe^{3+})\cdot OH^-$ and $(Fe^{3+})\cdot H_2O$ in dissolved states did not agree well with the observed curve. It may be considered that this discrepancy is caused by the difference in γ and γ' values between dissolved and polycrystalline states. In this connection it is to be remarked that $Mb(Fe^{3+})\cdot N_3^-$ was found to have different γ values between polycrystalline and dissolved states, but to have almost the same values of ε for the two states[15].

Finally let us discuss the *E–S* complex. The peroxide compounds of hydroperoxidases, such as peroxidases and catalases, have been investigated by various physical and chemical techniques, since the chemical nature of these compounds bears importance in the elucidation of the mechanism of action of these enzymes. Since the majority of these peroxide compounds are highly unstable, the accurate measurements of physical and chemical parameters of these compounds have been rather difficult.

Nevertheless, GEORGE[16] demonstrated that the red peroxide compound of horseradish peroxidase (Compound II) has an oxidation state 1 equivalent higher than the original ferric enzyme. He proposed a ferryl state (Fe^{4+}) as one of the most likely states of the heme prosthetic group in Compound II. On the reaction with a stoichiometric amount of H_2O_2, ferriperoxidase is rapidly converted to a stable *E–S* complex, the light absorption spectrum of which is very similar to that of Compound II (refs. 11, 12). Moreover, EPR spectrum of the *E–S* complex exhibited a signal at $g = 2.004$, which was interpreted to be derived from a free radical. This result seems to indicate that iron in the *E–S* complex has an even number of electrons, and it may be reasonable to assume that the *E–S* complex has an oxidation state 2 equivalents higher than the ferric state. Thus, it was speculated that one of the two equivalents of the *E–S* complex is retained as a free radical and the other as Fe^{4+} (ref. 10). Since the contribution of the radical to the n_{eff}^2 value is considered to be $3(S = {}^1/_2)$, the contribution of the iron to the n_{eff}^2 value, n_{Fe}, can be expressed by Eqn. 10:

$$n_{Fe} = \sqrt{n_{eff}^2 - 3} \qquad (10)$$

where no interaction between the free radical and iron ion is assumed at present and the additivity of n_{eff}^2 is considered to be allowed. The temperature dependence of n_{Fe} is shown expanded in Fig. 6, from which the average value of n_{Fe}^2 in this range of temperature is found to be 14.60.

Although the n_{Fe} value of 14.60 is very close to the spin-only value of 15 for $S = {}^3/_2$, this coincidence seems to be fortuitous, because in the aforementioned interpretation the iron is to be in the Fe^{4+} state. Now, as a paramagnetic Fe^{4+}, two possibilities may be considered: $(d\varepsilon)^3(d\gamma)^1$ $S = 2$ or $(d\varepsilon)^4$ $S = 1$. However, the first possibility is difficult to accept, because $S = 2$ gives an n_{eff}^2 value of 24 and this value is too large. This situation will not change, even if there exists a small contribution of the orbital angular momentum originating from the degeneracy in $(d\varepsilon)^3(d\gamma)^1$ state.

The second possibility seems to be more reasonable to us. The iron in the *E–S*

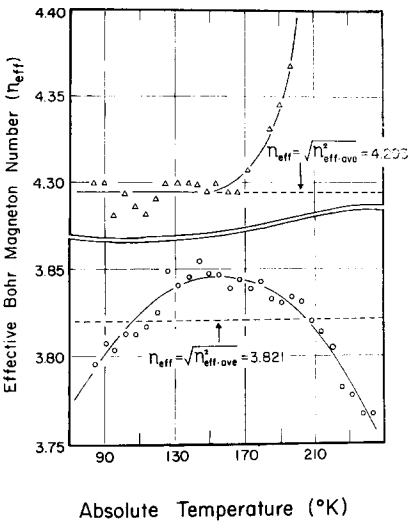


Fig. 6. The temperature dependence of n_{eff} values of cytochrome *c* peroxidase in a polycrystalline state (△—△) the *E–S* complex in solution at pH 7 (○—○).

complex is considered to be in the lowest state of $(d\varepsilon)^4$ S = 1. In this state the contribution of the orbital angular momentum may become fairly large. In fact, in a cubic field the theoretical curve of n_{Fe} against kT/A has a peak value of $n_{Fe} = 3.7$ at $kT/A = 0.5$, where A is the parameter of spin-orbit interaction (*cf.* KOTANI[17]). This peak value may be lower in a tetragonal field, but as long as the deviation from a cubic field is not large, n_{eff} is expected to show a qualitatively similar behavior. Since the experimental curve of the *E–S* complex in Fig. 6 has a peak value of $n_{Fe} = 3.85$ at a temperature of 150 °K, the A value ($A = kT/0.5$) is calculated to be approx. 200 cm^{-1}. If the peak value for the cubic case is tentatively adopted, the agreement of theoretical and observed peak values of n_{Fe} (3.7 and 3.85, respectively) and the reasonable value of A = 200 cm^{-1} derived from this analysis seem to favor this interpretation. Now according to the theory, the lowest sublevel of Fe^{4+} in a $(d\varepsilon)^4$ S = 1 state is non-degenerate both in cubic and lower symmetry fields, so that n_{eff} must fall to zero as the temperature approaches to 0 °K. The values of n_{eff} at the lowest temperature are not known, but the recent measurement of Mössbauer effect of the *E–S* complex by LANG, ASAKURA AND YONETANI[18] seems to show that the iron in the *E–S* complex behaves as diamagnetic in the lower temperature range. This may be considered as a support to our interpretations. Anyway the accurate measurements of the paramagnetic susceptibility of the *E–S* complex below −196° is needed to reach a more definite conclusion concerning the electronic structure of the *E–S* complex. Such a measurement will be carried out in the near future.

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