Nuclear Magnetic Resonance Relaxation Time Studies on the Manganese(II) Ion Complex with Succinyl Coenzyme A Synthetase from *Escherichia coli*†

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ABSTRACT: Nuclear magnetic resonance, as well as electron paramagnetic resonance, experiments were carried out in a study of the Mn(II) ion complex with phosphorylated succinyl-CoA synthetase. For high specific activity enzyme samples, there are 3.5 ± 0.7 metal ion binding sites per enzyme molecule with indistinguishable dissociation constants (K_D = 6.9 × 10^{-4} M). However, for enzyme samples with a lower specific activity yet equivalent purity, there are 1.6 strong metal ion binding sites (K_D = 6.6 × 10^{-4} M) and 2.0 weak metal ion binding sites (K_D = 4.0 × 10^{-3} M), a result that is easily reconciled with the $\alpha_2\beta_2$ subunit structure of the enzyme. Water proton relaxation rate measurements indicate that each strongly bound Mn(II) ion is coordinated to three water molecules. The present results strongly suggest that the existence of nearly 4 high-affinity metal binding sites per enzyme molecule is related to the integrity or configuration of that portion of the molecule which interacts with substrates.

Succinyl-CoA synthetase catalyzes the "substrate-level" phosphorylation step of the tricarboxylic acid cycle, the formation of nucleoside triphosphate at the expense of succinyl-CoA. Recently, attention has been directed toward its interesting subunit structure and toward the number of substrate binding sites which are present in the molecule. Summarizing the properties of the enzyme from *Escherichia coli*, the subject of this investigation, the enzyme has an $\alpha_2\beta_2$ subunit structure with the active site phosphohistidyl residue contained in the smaller α subunit (Bridger, 1971; for a review see Bridger, 1974). The enzyme has been dissociated into its constituent subunits and reassembled (Pearson and Bridger, 1975a), and the α subunit has been shown to catalyze a partial reaction, namely its own phosphorylation by ATP[1] (Pearson and Bridger 1975b). However, there has been considerable controversy concerning the number of available active sites in the tetrameric enzyme molecule. For example, it has been reported that the enzyme shows half-sites reactivity with regard to phosphorylation by ATP (Ramaley et al., 1967; Moffet et al., 1972), but Bowman and Nishimura (1975) have observed that both the degree of phosphorylation and capacity for binding CoA may approach the anticipated value of 2 sites/molecule with enzyme preparations of highest specific activity. In this communication, we report the results of a magnetic resonance study of the binding of Mn(II) to the phosphorylated form of succinyl-CoA synthetase. The data indicate that the integrity of certain metal binding sites is related to the specific activity of the enzyme.

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[1] Abbreviations used are: PRR, proton spin-lattice relaxation rate; uv, ultraviolet; NMR, nuclear magnetic resonance; EPR, electron paramagnetic resonance; T_1, spin-lattice relaxation time; ATP, adenosine triphosphate; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid.

Experimental Section

Materials. Phosphorylated succinyl-CoA synthetase from *E. coli* (Crooke's strain) was purified using the standard purification procedure (Leitzmann et al., 1970; Moffet and Bridger, 1973) and then the enzyme extract was stored as a precipitate in a 75% saturated ammonium sulfate solution at 4 °C. For each set of experiments, a small quantity of the precipitate was dissolved in 0.05 M Tris-HCl, 0.1 M KCl, 0.1 mM EDTA, pH 7.2. The buffered enzyme solution was then introduced into a column packed with Sephadex G-25 (fine) resin at the bottom (1.0 × 30 cm), and AG-1 anion-exchange resin at the top (1.0 × 4.0 cm) to eliminate the EDTA and any EDTA–metal ion complexes. Before use, the column was equilibrated with EDTA-free Tris buffer, pH 7.2. The effluent enzyme solution was then concentrated using an 8MC Diaflo cell (Amicon). These preparations were carried out at 4 °C and the purified enzyme solutions were always kept at 4 °C between measurements to prevent denaturation.

Enzyme Assay. The molar concentration of the enzyme was determined by measuring the uv absorption at 280 nm using the extinction coefficient $E_{1cm}^{1\%}$ = 4.9 ± 0.2 (Krebs and Bridger, 1974). The enzyme activity was measured spectrophotometrically as described by Bridger et al. (1969). As has been noted elsewhere (Ramaley et al., 1967; Moffet et al., 1972; Bowman and Nishimura, 1975), preparations of enzyme of apparently equivalent high purity may differ in specific activity over the range 20–40 units/mg. The origins of this unusual property of the enzyme are poorly understood.

EPR and NMR Measurements. All samples for the EPR and NMR measurements were prepared in Tris buffer. The buffer was made up from 0.05 M Tris and 0.1 M KCl in triply distilled water. The pH was 7.2. NMR and EPR experiments were immediately carried out and completed within 1 week after the samples were prepared. The specific activity was then rechecked and some of the NMR and EPR measurements were repeated. The results were reproducible. Samples which experienced a large change in activity (>25%) were discarded.

For the EPR experiments, all the samples were prepared with the same enzyme concentration of 0.074 mM, but dif-

ferent Mn(II) ion concentrations that ranged from 0.08 to 1.0 mM. A series of reference samples with the same Mn(II) ion concentrations, but no enzyme, were also prepared. All samples were carefully sealed into small capillary tubes (1-mm i.d.) and all had the same volume (30 μl).

The EPR measurements were carried out on a Varian X-4502 EPR spectrometer (9.2 GHz). The temperature was controlled using the Varian V-4557 temperature control unit and was measured using a copper-constantan thermocouple. The EPR spectra of the enzyme sample and of the reference sample (enzyme free) containing the same total Mn(II) ion concentration were obtained under controlled instrumental settings. The experimental uncertainty in these measurements was ±5%. The total amplitude of each spectrum was measured by adding the six peak to peak heights of the EPR signal. The free Mn(II) ion concentration for each enzyme sample was calculated from the relative signal amplitude with respect to the reference sample (Cohn and Townsend, 1954).

The dissociation constant, K_D, for the enzyme-manganese(II) ion complex can be determined using this EPR technique by titrating the enzyme solution with an Mn(II) ion solution (Cohn and Townsend, 1954; Cohn and Leigh, 1962; Mildvan and Cohn, 1963; Mildvan and Engle, 1972). The results of this EPR-M titration experiment are then analyzed in terms of the Scatchard equation (Scatchard, 1949):

$$\frac{\bar{\nu}}{[M_f]} = \frac{n}{K_D} - \frac{\bar{\nu}}{K_D}, \text{ where } \bar{\nu} = \frac{[M_b]}{[E_t]} \qquad (1)$$

The concentrations of the enzyme-free (hexaaquo) and the enzyme-bound Mn(II) ion complexes are $[M_f]$ and $[M_b]$, respectively, $[E_t]$ is the total enzyme concentration, and n is the number of metal ions that are bound per mol of enzyme. In general, a plot of $\bar{\nu}/[M_f]$ vs. $\bar{\nu}$ yields a straight line from which the values of n and K_D can be easily obtained graphically. However, when there exist several groups of equivalent metal ion binding sites with different dissociation constants, the observed data represents the overall summation of several straight lines. Consequently, nonlinearity in the Scatchard plot is observed.

The NMR water proton spin-lattice relaxation rate (PRR) measurements were carried out as a function of frequency and temperature. With the knowledge of the dissociation constant obtained from the above EPR experiments, the samples were prepared in such a way that the ratio of the enzyme-bound Mn(II) ions to the enzyme-free Mn(II) ions was maximized. The concentrations of the Mn(II) ions and the enzyme used were 0.14 mM and 0.078 mM, respectively. A reference sample containing only 0.14 mM Mn(II) ion in the same buffer was also prepared. All samples were prepared under the same conditions, and small aliquots were taken for each temperature measurement. All samples were carefully sealed in NMR tubes (5 mm × 4 cm), and each had a volume of 75 μl. The free Mn(II) ion concentration of each NMR sample was determined using EPR.

Water proton spin-lattice relaxation times (T_1) were measured as a function of temperature (4–38 °C), the range over which the enzyme is stable. Measurements in the 6.0–60.0 MHz range were carried out with a Bruker SXP NMR spectrometer, while the 100.0 MHz experiments were carried out with a Varian-HA-100-15 spectrometer interfaced with a Digilab Fourier transform system. The relaxation times were measured using the 180°–τ–90° two pulse sequence. (A review of this technique has been presented by Farrar and Becker (1971).) The T_1 was calculated using a linear least-squares technique on at least 20 data points/sample. On each sample, four experiments were carried out, thus giving T_1 values with a standard deviation of 2–4%.

The water PRR for the enzyme-bound Mn(II) ions, $1/T_{1p_{bk}}$, can be evaluated from the experimentally observed water PRR, $T_{1p_{obsd}}{}^{*-1}$, using the following equation (Eisinger et al., 1962; Mildvan and Cohn, 1963):

$$T_{1p_{obsd}}{}^{*-1} = \frac{[M_f]}{[M_t]} T_{1p_f}{}^{-1} + \sum_{bk=1}^{j} \left(\frac{[M_{bk}]}{[M_t]} T_{1p_{bk}}{}^{-1} \right) \qquad (2)$$

The mole fractions $[M_i]/[M_t]$ are determined from the stoichiometric constants. T_{1p_f} as well as $T_{1p_{obsd}}{}^*$ are measured experimentally. The theoretical basis for this study has been well reviewed (Mildvan and Engle, 1972; Dwek et al., 1974, and references therein).

The paramagnetic water proton relaxation rate, $T_{1p}{}^{-1}$, is given by the following equation (Swift and Connick, 1962; O'Reilly and Poole, 1963; Luz and Meiboom, 1964):

$$(P_m T_{1p_i})^{-1} = \frac{q_i}{T_{1m_i} + \tau_{m_i}} \qquad (i = f, bk) \qquad (3)$$

When the subscript i = f, the equation refers to the enzyme-free complex and the subscript bk indicates that the terms in this equation now refer to the kth group of enzyme-metal ion binding sites in the enzyme-bound complex. $P_m = [M_t]/[H_2O] = [M_t]/55.5$, τ_m is the exchange lifetime, T_{1m} is the water proton spin-lattice relaxation time when the water molecule is in the coordination sphere of the metal ion, and q is the number of water molecules in the first coordination sphere of the metal ion (six for the hexaquo manganese(II) complex).

For aqueous Mn(II) ion solutions, T_{1m} is given by the Solomon-Bloembergen equation (Solomon, 1955; Solomon and Bloembergen, 1956; Bloembergen, 1957; Bernheim et al., 1959):

$$T_{1M_i}{}^{-1} = \frac{C_D}{R_i{}^6} \left[\frac{3\tau_c}{1 + \omega_I{}^2\tau_c{}^2} + \frac{7\tau_c}{1 + \omega_S{}^2\tau_c{}^2} \right]_i \qquad (4)$$

$$(i = f, bk)$$

where $C_D = 2S(S+1)\gamma_I{}^2 g^2\beta^2/15$, R is the distance between the water protons and the Mn(II) ion, and τ_c is the dipolar correlation time. The other symbols have their usual meaning. The distinction between τ_{c1} and τ_{c2} (Reuben et al., 1970) is neglected in the present analysis (Koenig, 1972). The dipolar correlation time is composed of three independent correlation times as follows:

$$\tau_{c_i}{}^{-1} = \tau_{m_i}{}^{-1} + \tau_{R_i}{}^{-1} + \tau_{1S_i}{}^{-1} \qquad (5)$$

where τ_R is the rotational correlation time. The electronic relaxation time, τ_{1S}, is best approximated by the following expression (Bloembergen and Morgan, 1961; Rubinstein et al., 1971):

$$\tau_{1S}{}^{-1} = B \left[\frac{\tau_v}{1 + \omega_S{}^2\tau_v{}^2} + \frac{4\tau_v}{1 + 4\,\omega_S{}^2\tau_v{}^2} \right] \qquad (6)$$

where B is a constant that depends on the zero-field splitting and τ_v is the correlation time of the modulation. This equation governs the frequency dependence of τ_{1S}.

The temperature dependence of the water PRR arises from the temperature dependence of the correlation times. For τ_R and τ_v, the temperature dependence is given by

$$\tau_j = \tau_j{}^\circ \exp(E_j/RT) \qquad (7)$$

$$(j = R, v)$$

where E_j is the activation energy for the relaxation process. The

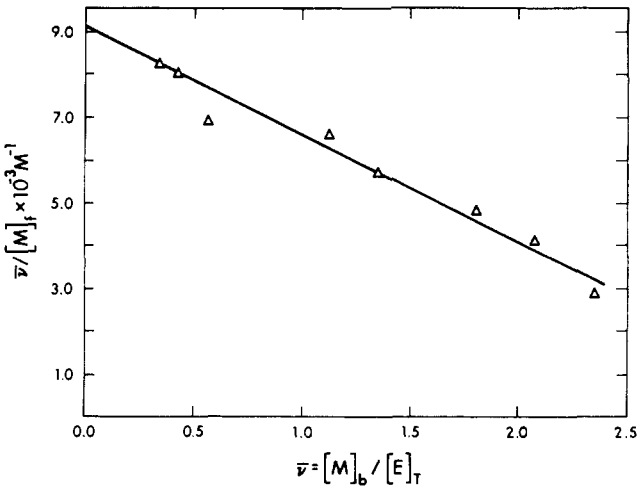

FIGURE 1: The Scatchard plot of the data obtained from an EPR M-titration experiment. The experiments were carried out at 25 °C and at a frequency of 9.2 GHz. All samples were prepared in 0.05 M Tris-HCl buffer (pH 7.2) and 0.1 M KCl. The enzyme concentration, $[E_t]$, was 0.074 mM and the specific activity was 34 units/mg.

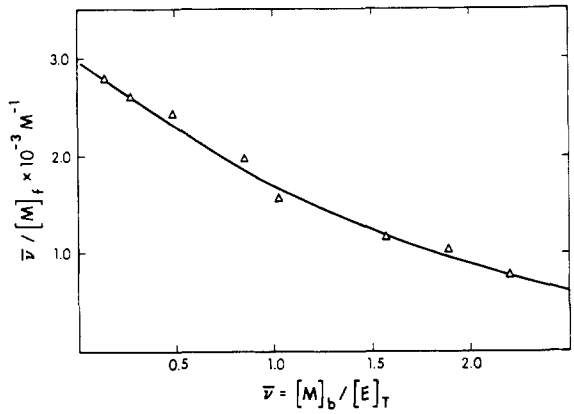

FIGURE 2: The Scatchard plot of the data obtained from an EPR M titration experiment on a lower specific activity enzyme sample. The enzyme concentration was 0.096 mM. Other experimental conditions are the same as for Figure 1.

temperature dependence of the exchange lifetime τ_m is expressed as:

$$\tau_m^{-1} = \frac{kT}{h} \exp\left[-\frac{\Delta H^{\ddagger}}{RT} + \frac{\Delta S^{\ddagger}}{R}\right] \quad (8)$$

$\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are, respectively, the activation enthalpy and entropy for the exchange.

Results

Stoichiometry of the Phospho-succinyl-CoA Synthetase-Mn(II) Complex.

EPR Titration Experiments. The number of metal ion binding sites and their dissociation constants were determined by EPR using the M-titration procedure. For high-activity enzyme samples (29–34 units/mg), a straight line Scatchard plot is observed (Figure 1). The experiments were repeated three times with samples from separate homogeneous preparations and the results can all be analyzed in terms of one group of metal ion binding sites using eq 1. The average value for the dissociation constant, K_D, is 6.9 ± 2.5 × 10^{-4} M and the number of metal ion binding sites per mole of the enzyme is 3.5 ± 0.7. Similar values (K_D = 7.1 × 10^{-4} M; n = 3.4) have previously been obtained by Buttlaire, Cohn, and Bridger (1971).[2]

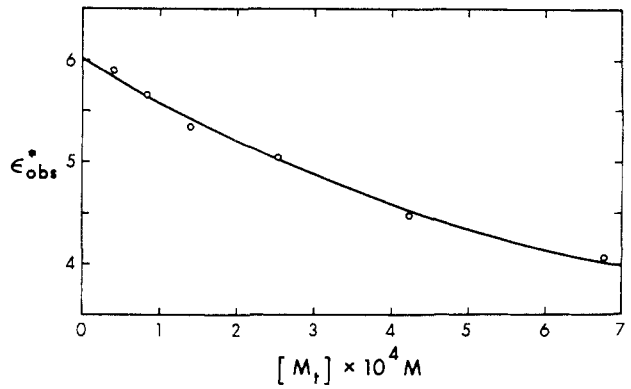

FIGURE 3: Water proton relaxation enhancements from the NMR M titration experiments. The measurements were carried out at 25.15 MHz and at 25 °C. The total enzyme concentration was 0.11 mM.

When enzyme samples in the lower activity range (18–27 units/mg) (also from homogeneous preparations) were studied, the titration data could not be fitted with a straight line (Figure 2). The nonlinearity of the Scatchard plot indicates that there are several types of metal ion binding sites with different dissociation constants. Following the procedure of Miziorko and Mildvan (1974) and Grisham and Mildvan (1974), the data were analyzed using a nonlinear least-squares program (Marquardt, 1964). The results in Figure 2 show that there are two different groups of metal ion binding sites. For the first group, the number of metal ions n_1 is equal to 1.6 ± 0.4. For the second group, n_2 is equal to 2.0 ± 0.5. The solid curve in Figure 2 represents the best fit to the data. The stoichiometric constants used to compute the curve are listed in Table I.

NMR Titration Experiments. To support the EPR results and to determine the enhancement for the enzyme-manganese(II) complex, water proton relaxation rates (PRR) were studied using NMR techniques in an M titration (proton frequency of 25.15 MHz) and in an E titration (100.0 MHz). Both experiments were carried out on enzyme samples in the lower specific activity range.

In the M titration, the water proton relaxation rates in the presence of the enzyme were measured as a function of the Mn(II) ion concentration. The Mn(II) ion concentration ranged from 6.7 × 10^{-4} to 4.2 × 10^{-5} M and the enzyme concentration was 0.11 mM. The enzyme specific activity was 25 units/mg. The results are shown in Figure 3 and the relaxation rates are expressed as enhancement ratios ϵ_{obsd}^* ($T_{1p_f}/T_{1p_{obsd}}^*$). The solid curve in the figure is the calculated best fit to the data obtained using eq 9, which is obtained from eq 2 for the present case:

$$\epsilon_{obsd}^* = \frac{[M_{b1}]}{[M_t]}\epsilon_{b1} + \frac{[M_{b2}]}{[M_t]}\epsilon_{b2} + \frac{[M_f]}{[M_t]} \quad (9)$$

The ϵ_{b1} and ϵ_{b2} terms are, respectively, the binary enhancement constants for the first and the second groups of metal ion binding sites (n_1 and n_2). $[M_{b1}]$ and $[M_{b2}]$ are the enzyme-bound Mn(II) ion concentrations for the corresponding groups. These metal ion concentrations were calculated as a function of the Mn(II) ion concentration making use of the four stoichiometric constants in Table I. Using the above equation and

[2] D. H. Buttlaire, M. Cohn, and W. A. Bridger, unpublished results. These data have also been referred to by Buttlaire and Cohn (1974a).

TABLE I: Stoichiometric Constants for the Manganese(II)–Succinyl-CoA Synthetase System.[a]

Sp Act. (units/mg)	No. of Mn(II) Binding Sites (per mol of enzyme)		Dissociation Constants (M)	
	n_1	n_2	K_{D_1}	K_{D_2}
18–27 (lower range)	1.6 ± 0.4	2.0 ± 0.5	$6.6 \pm 1.4 \times 10^{-4}$	$4.0 \pm 2 \times 10^{-3}$
29–34 (higher range)	3.5 ± 0.7		$6.9 \pm 2.5 \times 10^{-4}$	

[a] The experiments were carried out in 0.05 M Tris-HCl buffer (pH 7.2), 0.1 M KCl, and at 25 °C.

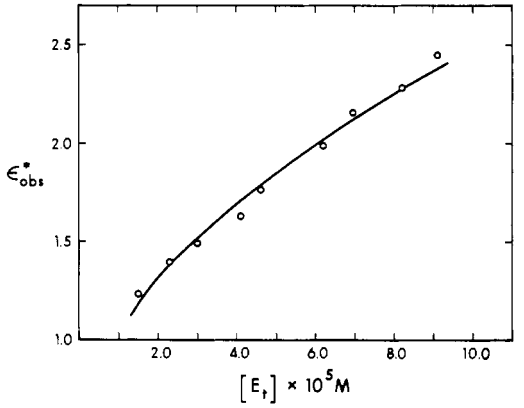

FIGURE 4: Water proton relaxation enhancements from the NMR E-titration experiments. The measurements were carried out at 100.0 MHz and at 25 °C. The total Mn(II) ion concentration was 5.03×10^{-5} M.

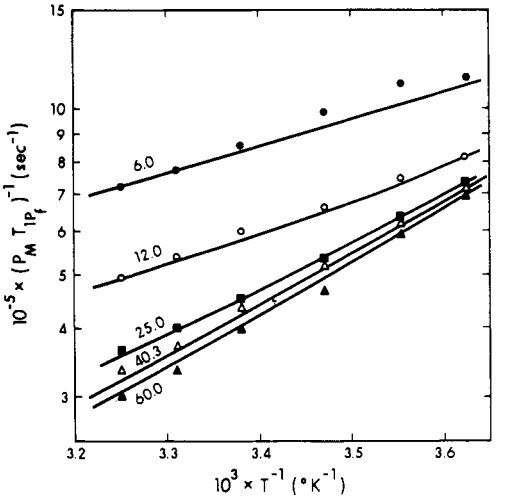

FIGURE 5: Frequency (MHz) and temperature dependence of $(P_M T_{1P_f})^{-1}$ due to the $Mn(II)\cdot(H_2O)_6^{2+}$ complex in Tris buffer. $P_M = [Mn(II)]_t/[H_2O] = [Mn(II)]_t/(55.5)$. The solution (pH 7.2) contains 0.1 M KCl, 0.05 M Tris buffer, and 0.14 mM Mn(II) ions. Solid curves represent the computer fit to the data.

the nonlinear least-squares technique, the best-fit curve to the data was obtained (Figure 3). The analysis of the data gives a value of 25 ± 2 for ϵ_{b1} and 3.7 ± 3 for ϵ_{b2} (at 25 °C).

In the E titration (100.0 MHz), the total Mn(II) ion concentration was 5.03×10^{-5} M, while the enzyme concentration varied from 0.092 to 0.015 mM. The enzyme activity was 25 units/mg. The water proton relaxation rates, again expressed as the observed enhancements, ϵ_{obsd}*, are plotted as a function of the total enzyme concentration in Figure 4. The solid curve in the figure is the computed result obtained by fitting the data using eq 9 as for the preceeding M titration. The binary enhancement constants (at 100.0 MHz) are 9.8 ± 1 for ϵ_{b1} and 1.0 ± 0.5 for ϵ_{b2} (at 25 °C).

Frequency and Temperature Dependence of the Water PRR for the Strong Metal-Binding Sites

Water PRR of the Control $MnCl_2$ Solutions. The water PRR of the hexaaquomanganese(II) ion complex was studied as a function of temperature (4–38 °C) and frequency (6.0, 12.0, 25.0, 40.3, and 60.0 MHz) (Figure 5). Using eq 3, 4, and 7 and the fact that $\tau_c = \tau_R$ and $\tau_m \ll T_{1M}$ (Bloembergen and Morgan, 1961), a least-squares analysis of the data was carried out. The computed curves are shown in Figure 5 and the best-fit values[3] are 4.6 kcal/mol for the rotational activation energy E_{R_f} and 1.7×10^{-14} s for τ_{R_f}° (calculated using a value of 2.87 Å for R).

[3] These results are in agreement with previous studies on aqueous Mn(II) ion solutions (Bloembergen and Morgan, 1961; Pfeifer, 1962; Reuben and Cohn, (1970). However, the results from the present control experiments were used in the following enzyme–metal ion study to eliminate systematic errors.

Water PRR of the Manganese(II)–Enzyme Complex. The presence of the strong Mn(II) ion binding sites (n_1) and their large enhancement constants suggested that further experiments be carried out on the enzyme in the lower activity range. Hence, variable frequency and temperature studies were carried out on enzyme samples with a specific activity of 22.9 units/mg and at a concentration of 0.078 mM. The Mn(II) ion concentration was 0.14×10^{-3} M. Under these conditions and using the values of the stoichiometric constants in Table I, the product $\epsilon_{b2}[M_{b2}]/[M_t]$ can be shown to be less than 8% of $(\epsilon_{b1}[M_{b1}]/[M_t])$. For this calculation, the enhancement constants at 25.15 MHz were used. Therefore, eq 9 simplifies into the following form:

$$T_{1P_{obsd}}^{-1} = \frac{[M_f]}{[M_t]} T_{1P_f}^{-1} + \frac{[M_{b1}]}{[M_t]} T_{1P_{b1}}^{-1} \qquad (10)$$

and is used in the analysis of the following data. The total $[M_b]$ and $[M_f]$ metal ion concentrations were measured directly using EPR techniques at each temperature. The value used for $[M_{b1}]$ was 0.81 of the total bound metal ion concentration $[M_b]$ as the remaining bound metal ions correspond to $[M_{b2}]$ (based on data in Table I).

Hence, the water PRR $T_{1P_{b1}}^{-1}$ for the manganese(II)–enzyme complex was calculated using eq 10. The results are plotted in Figure 6, and differ significantly from the results in Figure 5 (for the enzyme-free complex). This behavior characterizes the participation of the electronic relaxation in the

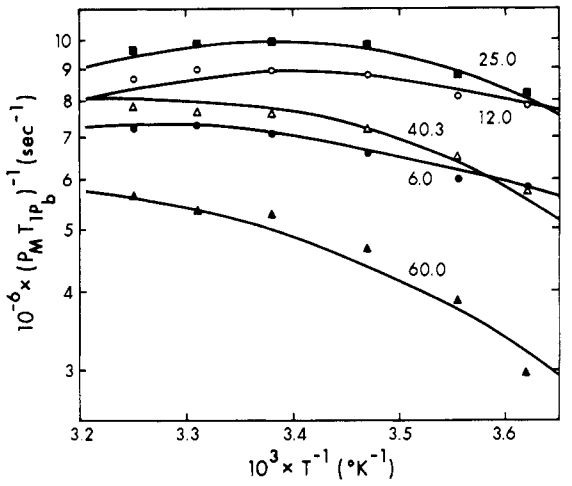

FIGURE 6: Frequency (MHz) and temperature dependence of $(P_M T_{1P_b})^{-1}$ due to the manganese(II)-succinyl-CoA synthetase complex. $P_M = [Mn(II)]_t/(55.5)$. The solution (pH 7.2) contains 0.1 M KCl, 0.05 M Tris-HCl, 0.14 mM Mn(II) ions, and 0.078 mM phospho-succinyl-CoA synthetase. Solid curves represent the computer fit to the data using the parameters in Table II.

dipolar relaxation of the water protons, as observed in many other enzyme-metal ion systems (Reuben and Cohn, 1970; Koenig, 1972). The data in Figure 6 were analyzed using the relevant equations for the enzyme-bound metal ion complexes (eq 3-8). All parameters in these equations were varied until the root mean square deviation between the experimental and calculated values reached 6%. The best-fit values for the parameters from the least-squares analysis are listed in Table II, together with the corresponding values for the enzyme-free Mn(II) complex. To check that all the data are self-consistent, the binary enhancement constant, ϵ_{b1}, was computed using the parameters in Table II at two frequencies. The values are 23 ± 3 and 7.5 ± 2 for 25.15 and 100.0 MHz, respectively. These values are in agreement, within experimental error, with the results obtained in the NMR titration experiments.

Discussion

The presence of two types of binding sites on the enzyme succinyl-CoA synthetase may be easily reconciled with its $\alpha_2\beta_2$ subunit structure (Bridger, 1971). The presence of multiple metal ion binding sites has been noted in previous studies on other enzymes (Mildvan and Cohn, 1963; Grisham and Mildvan, 1974; Jouve et al., 1975; Maggio et al., 1975). The present results on the enzyme with high specific activity (Figure 1) indicate that 3.5 metal ion binding sites exist on the enzyme with indistinguishable dissociation constants. However, when the specific activity of the enzyme decreases, two different classes of metal ion binding sites become evident (Figure 2). Thus, there are 1.6 strong metal ion binding sites ($K_D = 6.6 \times 10^{-4}$ M) and 2.0 weak metal ion binding sites ($K_D = 4.0 \times 10^{-3}$ M) in low specific activity enzyme. These results complement the studies of Bowman and Nishimura (1975), who observed that 1 mol of the enzyme with high specific activity binds 2 mol of CoA and incorporates 1.7 mol of ^{32}P following phosphorylation by [γ-^{32}P]ATP. These investigators noted that when the specific activity decreases, both the capacity for binding CoA and for phosphorylation decrease and level off at about 1 mol/mol of the enzyme. Our present results strongly suggest that the existence of 4 high-affinity metal-binding sites per enzyme molecule is related to the integrity or configuration of that portion of the molecule which interacts with substrates. The relationship is clearly indirect and complex; however, an apparently symmetrical loss of high-affinity sites ($2/\alpha_2\beta_2$ tetramer) is accompanied by the appearance of asymmetric properties in the enzyme, such as half-sites reactivity. While it is possible that the remaining high affinity sites may be associated with substrate-binding regions, they cannot be confidently located on the basis of available data. Accordingly, we have undertaken a study of the effects of substrates on the water PRR in this system.

TABLE II: Parameters for Water Molecules in the Enzyme-Bound and the Hexaaquomanganese(II) Complexes.

Parameter	Enzyme-Bound Mn(II) Ions (Strong)	Hexaaquo-manganese(II) Ions (Enzyme Free)
$\tau_R°$ (s)	4.6×10^{-12}	$(1.7, 1.6^a; 2.1^b) \times 10^{-14}$
E_R (kcal/mol)	4.1	4.6; 4.5[a]; 4.3[b]
$\Delta H^{\ddagger}$ (kcal/mole)	8.0	7.5[c]; 7.82[d]
$\Delta S^{\ddagger}$ (eu)	1.6	1.3[c]; 1.38[d]
q (water molecules)	3[e]	6
$\tau_v°$ (s)	2.4×10^{-14}	3.5×10^{-15} [a]
E_v (kcal/mol)	3.7	3.9[a]
B (rad^2/s^2)	0.7×10^{19}	1.0×10^{19} [f]

[a] Value from Bloembergen and Morgan (1961). [b] Value from Pfeifer (1962) and Pfeifer et al. (1966). [c] Value from Bloembergen and Morgan (1961) as calculated by Swift and Connick (1962). [d] Value from Zetter et al. (1972). [e] Value based on R = 2.87 Å. [f] Value from Reuben and Cohn (1970) and references therein.

The NMR titration experiments (Figures 3 and 4) support the conclusions based on the EPR experiments that there are two different types of metal ion binding sites on the enzyme. The strong metal ion binding sites have a large value of the binary enhancement constant, whereas for the weak metal ion binding sites it is very small. Due to the limited solubility of the enzyme (the most concentrated enzyme solution available was about 22 mg/ml), the range over which the titration experiments can be carried out is restricted. The data in Figure 4 were also analyzed using the double-reciprocal plot method (Mildvan and Cohn, 1963), as well as by plotting $(\epsilon_{obsd}{}^{*-1})^{-1}$ vs. E_t^{-1} (Dwek, 1974). The present analysis gives the best fit to the data.

The water PRR for the manganese(II)-enzyme complex are shown in Figure 6. The results are analyzed assuming that only the metal ions that bind strongly affect the water PRR (as discussed under Results). A comparison of the data in Figure 6 with the PRR results for the hexaaquomanganese(II) ion complex (Figure 5) indicates that, in the presence of the enzyme, the normalized water PRR behave quite differently. First of all, at a given temperature, these values do not vary monotonically with frequency. Secondly, at a given frequency, there is no sharp increase of the PRR with an increase in $1/T$, as observed in Figure 5.

The water protons in the hexaaquomanganese(II) complex relax via the dipolar mechanism, which arises mainly from the tumbling motion of the complex. However, for the manganese(II)-enzyme complex, the rotational correlation time decreases and the frequency dependence of τ_{1S} must be considered (eq 5 and 6). Using the results in Table II, it is instructive to examine the frequency dependence of τ_c; $(1 + \omega_I{}^2\tau_c{}^2)^{-1}$ and the product $\tau_c \cdot (1 + \omega_I{}^2\tau_c{}^2)^{-1}$ which appear in eq 4 for the water protons in the enzyme-manganese(II) complex. The results of these calculations over the frequency range studied at 25 °C are shown in Figure 7. From the figure it is seen that

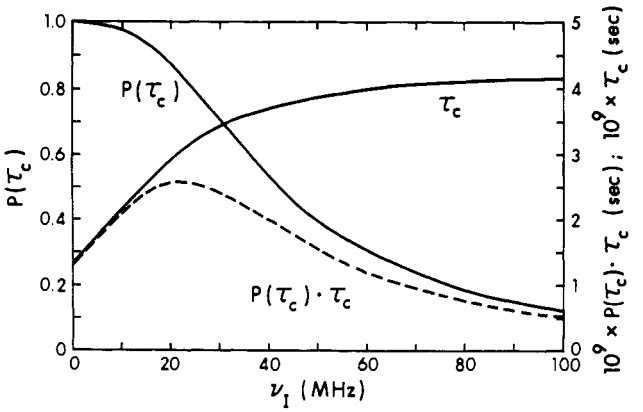

FIGURE 7: Frequency dependence of τ_c, $P(\tau_c)$ and the product $\tau_c P(\tau_c)$ for the enzyme-bound Mn(II) complex. These curves are calculated using the parameters for the enzyme-manganese(II) complex listed in Table II (25 °C). $P(\tau_c) = (1 + \omega_I^2\tau_C^2)^{-1}$.

the frequency dependence of the product accounts for the observed frequency dependence of $T_{1P_{bk}}^{-1}$ (Figure 6). These principles have been discussed in detail previously (Peacocke et al., 1969; Reuben and Cohn, 1970; Mildvan and Engle, 1972; Dwek et al., 1974; Dwek, 1973).

A comparison of the enzyme-manganese(II) complex with the hexaaquomanganese(II) ion complex may now be made (Table II). In the hexaaquomanganese(II) ion complex, there are six water molecules in the first coordination sphere of the metal ion. For the manganese(II)-enzyme complex, this number decreases to three when the distance between the water protons and the Mn(II) ion (R) is 2.87 Å. Morgan (1963) determined a value of R equal to 2.86 Å for the hexaaquo complex, and Reuben and Cohn (1970) estimated from crystallographic data (Zalkin et al., 1964; Morosin and Groeber, 1965; Montgomery et al., 1966; Chidambaram, 1962) a lower limit of R to be 2.815 Å and an upper limit of 2.923 Å. Hence, the average value 2.87 Å is used (Reuben and Cohn, 1970; Mildvan and Engle, 1972; Buttlaire and Cohn, 1974b) in computing the parameters in Table II. Hence, for each strongly bound metal ion on the enzyme, three coordination sites are occupied by water molecules. In this calculation, any effects due to anisotropic motion (Peacocke et al., 1969) and from the weak metal ion binding sites (eq 10) are neglected. Therefore, the uncertainty in the value of q may be as large as ±1.

Finally, the value of $\tau_v°$ increases significantly on binding of the metal ion to the enzyme (Reuben and Cohn, 1970; Jones et al., 1974a,b), possibly reflecting a restricted access of water molecules in the complex. The values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are very similar for both complexes. This indicates that the water molecules can readily exchange between the first coordination sphere of the enzyme-bound Mn(II) ion and the bulk solvent.

Acknowledgments

The authors thank Mr. E. Brownie for valuable technical assistance and Dr. R. B. Jordan for valuable suggestions.

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Oxidation–Reduction Potential Measurements on Chloroperoxidase and Its Complexes†

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ABSTRACT: The oxidation–reduction potential of chloroperoxidase, an enzyme which catalyzes peroxidative chlorination, bromination, and iodination reactions, has been investigated. In addition to catalyzing biological halogenation reactions, chloroperoxidase is unusual in that the carbon monoxide complex of ferrous chloroperoxidase shows the typical long wavelength Soret absorption associated with P-450 hemoproteins. The pH dependence of the chloroperoxidase oxidation–reduction potential shows a discontinuity around pH 4.7. Similarly, measurements of the affinity of ferrous chloroperoxidase for carbon monoxide monitored both by spectroscopic and potentiometric titration exhibit a discontinuity in the pH 4.7 region. Oxidation–reduction potential measurements on chloroperoxidase in a CO atmosphere also show a discontinuous pH profile. These results suggest that ferrous chloroperoxidase undergoes reversible modification at low pH and that these changes are reflected in the oxidation–reduction potential. The oxidation–reduction potential of chloroperoxidase at pH 6.9 is −140 mV, close to that measured for cytochrome P-450$_{cam}$ in the presence of substrate. The oxidation–reduction potential of chloroperoxidase at pH 2.7, the pH optimum for enzymatic chlorination, is +150 mV. The oxidation–reduction potentials of the halide complexes of chloroperoxidase (chloride, bromide, and iodide) are essentially identical with the potential measurements on the native enzyme. These observations suggest that, although halide anions bind to the enzyme, they probably do not bind as an axial ligand to the heme ferric iron.

Chloroperoxidase is a unique hemoprotein which catalyzes the chlorination reactions involved in the biosynthesis of caldariomycin (Morris and Hager, 1966). In the presence of suitable halogen donors and acceptors, chloroperoxidase catalyzes the peroxidatic formation of a variety of halometabolites (Thomas et al., 1970a). Chloroperoxidase also catalyzes the oxidation of hydrogen donors such as guaiacol and ascorbate (Thomas et al., 1970a) and exhibits high catalase activity for hydrogen peroxide decomposition with oxygen evolution (Thomas et al., 1970a). Although chloroperoxidase is similar to plant and animal peroxidases in many of its catalytic functions, recent studies have drawn attention to numerous similarities between bacterial and microsomal P-450 type hemoproteins and chloroperoxidase. The physicochemical properties of chloroperoxidase, studied by optical, EPR,[1] and Mössbauer spectroscopy, are very similar to those of P-450$_{cam}$ in both the ferric and ferrous states (Champion et al., 1973, 1975; Hollenberg and Hager, 1973; Chiang et al., 1975). These similarities suggest that chloroperoxidase and P-450$_{cam}$ share a common heme environment.

The oxidation–reduction potentials of several isozymes of horseradish peroxidase and turnip peroxidase have been measured (Harbury, 1957; Yamada et al., 1975; Ricard et al., 1972). Here we report the oxidation–reduction potentials of the ferric–ferrous couple of native and liganded states of chloroperoxidase.

Materials and Methods

Enzyme Preparations. Chloroperoxidase was isolated from *Caldariomyces fumago* and purified as reported previously (Morris and Hager, 1966). The preparations used in this study had R_z values of 1.35 to 1.4. Horseradish peroxidase was ob-

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§ Recipient of a Postdoctoral Fellowship from the National Institutes of Health.

[1] Abbreviations used: EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance; NADPH, reduced nicotinamide adenine dinucleotide phosphate; FAD, flavin adenine dinucleotide; M.V., methyl viologen.