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THE MAGNETIC SUSCEPTIBILITY OF REDUCED CYTOCHROME P-450$_{cam}$

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Summary

The magnetic susceptibility of singly reduced, camphor-complexed cytochrome P-450 from *Pseudomonas putida* has been measured at various temperatures. Computer analysis using a spin Hamiltonian gives values of D between 15K and 25K, which agrees with a value of D = 20K found by low-field variable temperature Mössbauer spectroscopy. This result is compared with those from deoxyhemoglobin and deoxy-myoglobin.

Cytochrome P-450$_{cam}$ is a heme protein ($M_r \approx 45\,000$) that has been isolated from *Pseudomonas putida* [1]. During the catalytic process (hydroxylation of camphor), the heme iron passes through a variety of charge and spin states. One of the most interesting of the stable intermediates is the singly reduced camphor-complexed P-450 molecule. The heme iron of this complex is in the high-spin (S=2) ferrous state and is ready to bind molecular oxygen as the next step in the catalytic cycle. Upon oxygen binding, P-450$_{cam}$ shows no paramagnetism and has Mössbauer parameters [2] at 4.2 K and that are very similar to those of oxyhemoglobin [3]. We have measured the

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** In order to reduce the number of free parameters, the electronic g-values were calculated via a simple 2nd order perturbation expansion [11] for $D > 0$ (values of $D < 0$ are not compatible with the data):

$$g_x = 2.0\,[1+k(D-E)/\lambda]$$

$$g_y = 2.0\,[1+k(D+E)/\lambda]$$

$$g_z = 2.0$$

The orbital reduction factor, k was set equal to 0.7 while the spin-orbit coupling, λ, was set equal to the free ion value of 100 cm^{-1}. The effect of g on the analysis is quite small.

magnetic susceptibility of the high-spin ferrous of P-450$_{cam}$ as part of an extensive investigation into the electronic structure of the high spin ferrous heme iron [4,5]. To our knowledge, deoxyhemoglobin and deoxymyoglobin are the only other high-spin ferrous heme proteins that have been investigated in this manner [6,7].

The methods used in the purification of P-450$_{cam}$ from *Pseudomonas putida* have been described previously [8]. P-450$_{cam}$ susceptibility samples of reduced P-450$_{cam}$ were prepared under argon by dialysis against sodium dithionite. The samples were then frozen in small (sample volume = 0.5 ml) Delrin* susceptibility cells and stored under liquid nitrogen. Two separate experiments were performed using different batches of enzyme. The first batch was purified and concentrated to 1.72 mM (0.86 μmol of Fe in the sample cell) while the second batch had a concentration of 1.52 mM (0.76 μmol of Fe in the sample cell). The concentrations were determined by dilution of several aliquots of sample, reoxidation and measurement of absorbance at 390 nm (ϵ_{mM} = 87). Cross-checks were made by forming the reduced carbon monoxide complex and measuring the absorbance at 446 nm (ϵ_{mM} = 106 [9]).

Variable temperature magnetic susceptibility measurements were made as described previously [10] with a superconducting coil vibrating magnetometer designed by Dr A. Redfield of IBM Watson Laboratory. The susceptibility of the samples at a given temperature was taken from the linear, low-field region of the moment vs. field curve (generally fields of 0—3 kG). The susceptibility of the buffer and the protein backbone was shown to be temperature independent by performing the experiment using a sample of carbon monoxide-complexed P-450$_{cam}$. (Mössbauer measurements in strong applied fields have shown that the heme iron in the CO-complex is in a diamagnetic low-spin ferrous state [4].

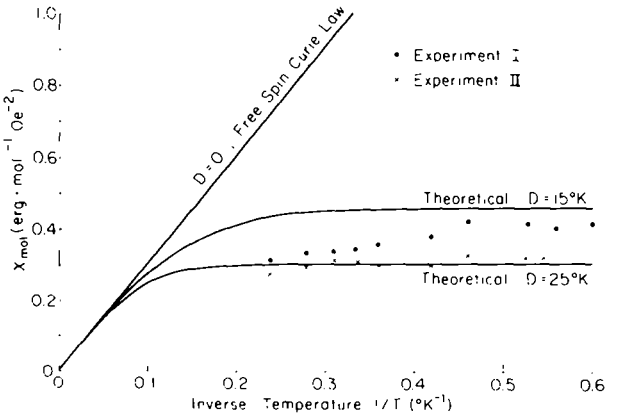

Fig.1. The temperature dependence of the magnetic susceptibility of reduced cytochrome P-450$_{cam}$. The theoretical curves are generated by a computer program that uses Eqn 1 to calculate powder averaged susceptibility curves. The curves shown have E/D = 0, although a small range of E/D values is compatible with the data. See ref 4 for details.

Fig. 1 displays the molar magnetic susceptibility of the reduced camphor-complexed cytochrome as a function of inverse temperature. There is a strong deviation from the linear Curie law at low temperatures. This implies that the degeneracy of the ground state spin quintet has been lifted by the spin-orbit coupling in conjunction with the ligand field. The resultant zero field splitting is adequately and conveniently described by a spin Hamiltonian:

$$H = D[S_z^2 - 1/3 \cdot S(S+1) + E/D(S_x^2 - S_y^2)] + \beta \vec{S} \cdot \tilde{g} \cdot \vec{H} \qquad (1)$$

where D and E are coefficients describing the zero field splitting, $\vec{H}$ is the applied magnetic field, and $\tilde{g}$ expresses the anisotropic Zeeman interaction **. The solid theoretical curves in Fig. 1 are the results of a computer analysis that utilizes the Hamiltonian of Eqn 1 and calculates powder averaged susceptibility and magnetization curves.

Both experiments indicate values of D that are greater than 15 K and less than 25 K. Fits to measurements at fixed temperature (2.2 K) as a function of field (0—15 kG) put similar bounds on D. However, the experimental accuracy is not sufficient to allow precise determinations of D or E/D. For this reason we have checked the results using a different technique, low-field, variable temperature Mössbauer spectroscopy. This method has been described elsewhere [4,5] and, when applied to the reduced P-450$_{cam}$ complex, results in values of D that are close to 20 K.

The large value of D obtained from these experiments contrasts markedly with the values of D = 7.5 K found for deoxyhemoglobin and deoxymyoglobin [6,7]. This is somewhat surprising in view of the fact that all three of these proteins form a unique diamagnetic complex with molecular oxygen. If we look more closely at the Mössbauer results, however, we see that both deoxyhemoglobin and deoxymyoglobin display strongly temperature dependent quadrupole splittings [12,13] while the quadrupole splitting of reduced P-450$_{cam}$ is essentially temperature independent [2,14]. Similarly, the quadrupole splitting of oxyhemoglobin depends more strongly on the temperature than that of oxy P-450$_{cam}$ [14]. This difference in the quadrupole interaction, while not necessarily directly related to the zero-field splitting, warns us that there are distinct differences between reduced P-450$_{cam}$ and the deoxy forms of hemoglobin and myoglobin.

This brings us to a final comment concerning the applicability of the spin Hamiltonian presented in Eqn 1. Since the spin has a value S = 2 in this formalism, we can apply Eqn 1 only when there is a well defined spin quintet associated with the ground state oribital. It is particularly risky to apply this formalism when there is the possibility of strong coupling to low-lying orbital states via the spin-orbit interaction. If this occurs, the entire ^5D manifold of the high-spin ferrous ion must be treated in a coupled representation (i.e. the spin and orbital wavefunctions are not separable) and a ground state "spin quintet" does not exist [4,5].

One relatively simple way to check for the presence of low-lying orbital states involves a measurement of the temperature dependence of the quadrupole splitting. A large and temperature-independent (over the range 4.2 K to 200 K) quadrupole splitting is a good indication that the first excited state is well separated from the ground state [15]. It therefore appears that the

high-spin iron atom in reduced P-450 has an isolated ground state oribital (with spin quintet) conducive to the spin Hamiltonian formalism, while the heme iron of deoxyhemoglobin and deoxymyoglobin might be better described using a different approach [12].

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