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Magnetic State of the a_3 Center of Cytochrome *c* Oxidase and Some of Its Derivatives†

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ABSTRACT: The temperature dependence of the magnetic susceptibility was used to investigate the nature of the coupling between cytochrome a_3 and Cu_B in resting and oxidized cyanide- and formate-bound cytochrome oxidase. Resting and formate-bound enzymes were found to have strong antiferromagnetic coupling with an $S = {}^5/_2$ cytochrome a_3, results that were independent of the dispersing detergent and the enzyme isolation method. The cyanide-bound enzyme was heterogeneous, with a minor fraction showing intermediate strength antiferromagnetic coupling. The magnitude of this coupling was independent of the enzyme isolation method and depended moderately on the identity of the dispersing detergent. The major fraction of the cyanide-bound enzyme had a lowest energy state of $M_s = 0$. The coupling constant for this fraction did not depend on the isolation technique or on the identity of the dispersing detergent. The use of glucose–glucose oxidase to deoxygenate samples influenced the susceptibility behavior of some preparations of both the resting and formate-bound enzymes, with results indicating an $S = {}^3/_2$ cytochrome a_3 in the resting enzyme samples. Retention of a 417-nm Soret band for formate-bound enzyme concomitant with peroxide-induced changes in susceptibility behavior indicates different sites of enzyme interactions for the formate ion and hydrogen peroxide.

It was suggested over 20 years ago (Van Gelder & Beinertt, 1969) that the lack of EPR signals from cytochrome a_3 and Cu_B in oxidized, resting cytochrome oxidase is due to their magnetic coupling but the nature of that coupling is still unresolved [for general reviews of cytochrome oxidase, see Wikstrom et al. (1981), Naqui et al. (1986), Scott (1989), and Chan and Li (1990)]. Both ferromagnetic coupling (Thomson et al., 1981; Kent et al., 1982) and antiferromagnetic coupling (Tweedle et al., 1978) have been reported for the resting or cyanide-bound enzyme as a result of MCD, Mössbauer, and magnetic susceptibility measurements. As with much of the work done with cytochrome oxidase, the various experiments were carried out by using enzyme isolated with different methods, different dispersing detergents, or different solvents. In addition, oxygen was removed from the magnetic susceptibility samples by addition of glucose oxidase and glucose, a technique that produces peroxide that can bind to the enzyme.

The enzyme isolation method used affects the reduction and ligand-binding properties of cytochrome oxidase (Jones et al., 1983; Naqui et al., 1984; Halaka et al., 1984; Baker et al., 1987; Hartzell et al., 1988), and the identity of the dispersing detergent can affect the enzyme activity (Rosevear et al., 1980;

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Robinson et al., 1985). The present work addresses whether the magnetic states of the cytochrome a_3–Cu_B center ("a_3 center") of resting and cyanide-bound enzyme isolated with Hartzell and Beinert (1974) and Yonetani (1966) methods differ. It also examines whether the dispersing detergent identity affects the magnetic coupling of resting or cyanide-bound enzyme and whether the interactions of hydrogen peroxide or formate ion with the resting enzyme affect its a_3 center magnetic state.

Experimental Procedures

Cytochrome oxidase was extracted from the membrane by either the Yonetani or the Hartzell–Beinert isolation method. EDTA was used in buffer solutions to ensure the removal of adventitious metal ions. Concentrations were determined by using ϵ_{605} = 21.2 cm^{-1} mM^{-1} cytochromes aa_3 for the reduced enzyme. Enzyme isolated with the Hartzell–Beinert method was dispersed in either lauryl maltoside or Tween–20, and enzyme isolated with the Yonetani method was dispersed in Tween-20. All enzyme was dispersed in HEPES or phosphate buffer at pH 7.4. Samples were made anaerobic by one of two methods: repeated cycles of evacuation and argon equilibration ("outgassing") or the use of glucose oxidase and glucose. Cyanide binding to the Hartzell–Beinert enzyme was accomplished by its overnight incubation at 4 °C with a 120-fold excess of sodium cyanide. Formation of the cyanide-bound Yonetani enzyme required a 3-day incubation at 4 °C with a 120-fold excess of sodium cyanide. In both cases, binding was judged complete when the optical spectrum of a diluted aliquot showed no shoulder at 655 nm. Formate binding of the Hartzell–Beinert enzyme was accomplished by its overnight incubation at 4 °C with a 100-fold excess of sodium formate and was judged complete when the Soret peak of the spectrum of a diluted aliquot shifted to 417 nm.

Magnetic susceptibilities were measured at 7 or 9 kG with an SHE Corp. (now Biomagnetic Technologies Corp.) SQUID variable-temperature susceptometer. A Brüker ER200D spectrometer and an Oxford ESR-9 Helium cryostat were used to record EPR spectra at 8 K. Enzyme concentrations were 0.6–1.1 mM in cytochromes aa_3. No hysteresis was visible in the data. Corrections were made for the sample holder and buffer by subtraction of their independently determined susceptibilities. The enzyme signal varied from 20 to 40% of the total background signal, depending on the enzyme concentration and temperature. Interpretations were based on the temperature dependence of the magnetic susceptibility rather than the absolute value of the enzyme susceptibility, which is more difficult to measure. Because the accuracy of using D_2O solutions (Day et al., 1987) was not necessary for the purpose of this study, buffers were dissolved in H_2O. The value of the slope of the χ vs $1/T$ plot found by using this procedure for a myoglobin fluoride standard corresponds to results that are within 3% of the value found by Kotani (1966). Nonlinear least-squares data analyses were done with KINFIT4, a modification of a program by Dye and Nicely (1971).

Results

Resting Enzyme. As shown in Figure 1, the magnetic susceptibility of resting cytochrome oxidase obeyed the Curie Law from 20 to 180 K; that is, it followed the expression:

$$\chi = \frac{Ng^2\beta^2S(S+1)}{3kT} = \frac{Ng^2\mu_{eff}^2}{3kT} \qquad (1)$$

where χ is the magnetic susceptibility, β is the Bohr magneton number, and μ_{eff} is the effective magnetic moment. The slope of the χ vs $1/T$ plot for the resting enzyme corresponds to a

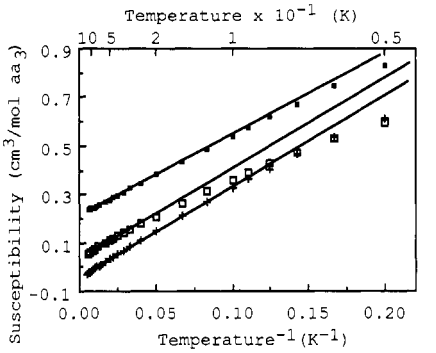

FIGURE 1: Susceptibility vs reciprocal temperature for resting and formate-bound cytochrome oxidase in lauryl maltoside. Resting (□) and formate-bound (+) enzyme deoxygenated by outgassing. Resting (■) deoxygenated with glucose–glucose oxidase (enzyme preparation 1). Susceptibility is expressed in cubic centimeters per mole of aa_3.

Table I: Squares of Effective Magnetic Moments for the Possible Magnetic States for the Cytochrome Oxidase a_3 Center with an S = $^1/_2$ Cu_B (Tweedle et al., 1978)

cyt a_3 spin	coupling	a_3 center μ_{eff}^2[a] (μ_B^2)
$^1/_2$	antiferromagnetic	0 (strong), 6 (weak)
$^1/_2$	none	6
$^1/_2$	ferromagnetic	8 (strong), 6 (weak)
$^3/_2$	antiferromagnetic	8 (strong), 18 (weak)
$^3/_2$	none	18
$^3/_2$	ferromagnetic	24 (strong), 18 (weak)
$^5/_2$	antiferromagnetic	24 (strong), 38 (weak)
$^5/_2$	none	38
$^5/_2$	ferromagnetic	48 (strong), 38 (weak)

[a] $\mu_{eff}^2 = \bar{g}^2S(S+1)$, $\bar{g}$ = 2.0.

μ_{eff}^2 value of 32.0 ± 0.5. The a_3 center contribution is calculated by subtracting 7.2, the sum of the contributions of cytochrome a and Cu_A, which were calculated from their g values as given by Aasa et al. (1976). The a_3 center contribution is thus 24.8.

Comparing 24.8 with the calculated values of the spin-only μ_{eff}^2 for the possible magnetic states of an iron(III) ion and a copper(II) ion (Table I) permits a determination of the actual magnetic state of the resting enzyme. Since cytochrome a_3 in the resting enzyme is high-spin (Babcock et al., 1976), the only possible state having a μ_{eff}^2 value close to 24.8 is the high-spin iron ion coupled antiferromagnetically to the copper ion. This S = 2 ground state is the result of exchange coupling, and the appropriate Hamiltonian is equal to $-2JS_1S_2$, with $2J$ being the energy difference between the S = 3 excited state and the S = 2 ground state. Since the χ vs $1/T$ plot remains linear through 180 K, the energy difference between the ground and next highest magnetic state must be large compared to kT, that is, $-J$ > 200 cm^{-1}. These observations confirm those of Tweedle et al. (1978).

The magnetic susceptibility of the resting enzyme deviated from the Curie Law below 20 K, with a χ vs $1/T$ slope that corresponds to an average total μ_{eff}^2 value of 11 between 2 and 5 K, in good agreement with the value of 10 obtained over that temperature range from data reported by Moss et al. (1978). The deviation from the Curie Law may be explained by considering the effect of zero-field splitting. Griffith (1971) has derived an equation that describes the temperature dependence of a coupled ion pair consisting of an S = $^5/_2$ ion and an S = $^1/_2$ ion. This equation may be used to calculate the expected total susceptibility of all metal centers in cytochrome oxidase by adding $7.2/T$ to account for the contribution of cytochrome a and Cu_A to the effective magnetic moment. The susceptibilities calculated with $-J$ = 200 cm^{-1}

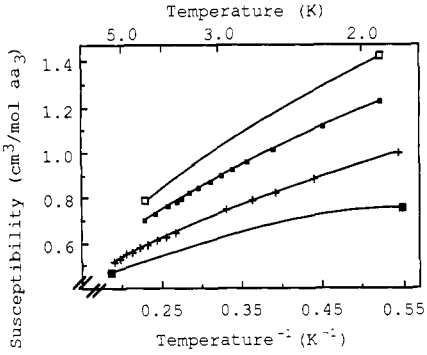

FIGURE 2: Calculated and experimental susceptibilities for resting and formate-bound cytochrome oxidase in lauryl maltoside. Calculated: (□) D = 3 cm^{-1} and (large ■) D = 10 cm^{-1}. Experimental: (small ■) formate bound and (+) resting. Deoxygenation was accomplished by outgassing. Susceptibility is expressed in cubic centimeters per mole of aa_3.

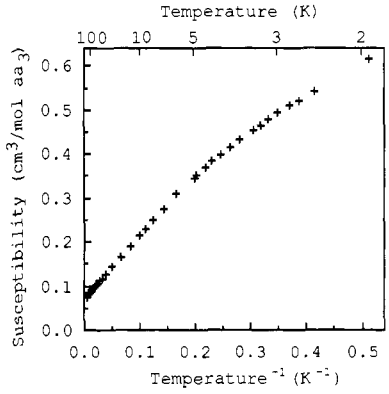

FIGURE 3: Susceptibility vs reciprocal temperature for cyanide-bound cytochrome oxidase in lauryl maltoside. Deoxygenation was accomplished by outgassing. Susceptibility is expressed in cubic centimeters per mole of aa_3.

and various values of the axial zero-field splitting factor, D, are shown in Figure 2, along with the experimental susceptibilities. The data have not been offset, but it is the relative slopes at a given temperature that are of primary interest rather than the actual susceptibility values. The slope of the experimental data curve falls between the slopes of the theoretical curves calculated by using D values of 3 and 10 cm^{-1}, a range typical of that found for other heme proteins (Tasaki et al., 1966; Uenoyama et al., 1968; Behere et al., 1979). This indicates that a reasonable value of the zero-field splitting parameter is sufficient to explain the deviation from the Curie Law at low temperatures for the resting enzyme.

Cyanide-Bound Cytochrome Oxidase. Figure 3 shows the temperature dependence of the susceptibility of the cyanide-bound enzyme from 2 to 180 K. Since cytochrome a_3 goes from high- to low-spin when cyanide binds to the enzyme (Babcock et al., 1976; Thomson et al., 1981; Palmer, 1987), couplings between two $S = {}^1/_2$ ions should be considered when interpreting these data.

If the lowest energy state was $M_s = 0$ and any $M_s = \pm 1$ states were significantly higher in energy, then the effective magnetic moment of the a_3 center would be zero. If the $M_s = \pm 1$ and both $M_s = 0$ states were close in energy, then the a_3 center would have a thermally randomized population distribution and would contribute 6.0 to the a_3 center μ^2_{eff}. If the energy states were spread moderately in energy with the $M_s = 0$ state lowest, then the distribution of states would change as the temperature increased, with the high and low temperature limiting behavior corresponding to a_3 center μ^2_{eff} values of 6.0 and 0, respectively. The observed behavior most closely matches the last case, with a_3 center μ^2_{eff} values of 8.8 and 0.2 at high and low temperature, respectively. The deviation from 6.0 would be expected due to the g anisotropy of cytochrome a_3. The $g_z = 3.55$ signal seen by Jenson et al. (1984) for the partially reduced cyanide derivative indicates that the cytochrome a_3–cyanide complex contribution to the effective magnetic moment would be larger than that of cytochrome a. If this remains true for the fully oxidized cyanide derivative, then the μ^2_{eff} value for the cytochrome a_3–Cu_B center would be larger than 7.2.

Table II: Coupling Constants and Curie Constants for Oxidized Cyanide-Bound Cytochrome Oxidase

preparation	temp range (K)	J value (cm^{-1})	C
Yonetani (Tween-80)	5–190	−29.6 ± 11.6	1.61 ± 0.04
	2–190	−1.26 ± 0.13	0.8 ± 0.4
Hartzell–Beinert (Tween-20)	5–180	−30.1 ± 6.4	1.5 ± 0.1
Hartzell–Beinert (lauryl maltoside)	20–201	−56.5 ± 5.7	1.60 ± 0.04
	2–201	−1.31 ± 0.11	0.69 ± 0.03

The equation that describes the susceptibility of two coupled $S = {}^1/_2$ ions is

$$\chi = \frac{2g^2N\beta^2}{3kT}\left[1 + \frac{1}{3}\exp\left(\frac{-2J}{kT}\right)\right]^{-1} \qquad (2)$$

where $2J$ is the energy difference between the two spin states. In order to fit this equation to the cytochrome oxidase susceptibility data, a Curie term, C/T, was added for the cytochrome a–Cu_A contribution, and a temperature-independent term was added for the diamagnetism of the protein. When the modified equation was fit to the data collected between 5 and 180 K, the resulting J value ranged from −30 to −57 cm^{-1} (Table II). A substantially lower J magnitude, −1.3 cm^{-1}, was obtained when the equation was fit to data collected from 2 to 180 K.

Different apparent J values for different temperature ranges would result if the enzyme were heterogeneous with two forms present, one with a moderate-strength coupling and the other with weak coupling. At intermediate and high temperatures, the weakly coupled form of the enzyme would show Curie Law behavior and would contribute to the Curie term found by fitting the data in this region rather than to the J value. At low temperatures, the moderately coupled form of the enzyme would be largely in the $S = 0$ state, and the signal would be due to the weakly coupled form. A fit over the entire temperature range for a single value of J would be dominated by the low-temperature data due to the smaller variances of the measured temperatures in this region. The values of the Curie constants found by fitting the equation (Table II) support this hypothesis; when the low-temperature data are excluded, the constants are larger than the value of 0.9 expected from the a center (calculated by using the Curie Law and a μ^2_{eff} value of 7.2), indicating additional Curie contributors at higher temperatures. When data from the entire temperature range are used, the Curie constants obtained are close to 0.9.

Data from the entire temperature range were used to fit an equation with two weighted terms of the type given in eq 2, a fixed Curie term of $0.9/T$ and a temperature-independent term for protein diamagnetism. The coupling constants and the diamagnetic term were allowed to vary, along with the weighting factors, which represent the fraction of each form of the enzyme. The resulting fit showed that 78 ± 1% of the enzyme had $-J = 1.3 \pm 0.1$ cm^{-1} and the remainder of the enzyme had $-J = 44 \pm 22$ cm^{-1}. These percentages may vary

somewhat since program convergence was achieved on only one data set and the low-temperature points are weighted more heavily, but it is interesting to note that Jensen et al. (1984) reported an 80:20 heterogeneity in the EPR detectability of the cyanide complex of ferric cytochrome a_3 in partially reduced, cyanide-bound cytochrome oxidase.

Formate-Bound Cytochrome Oxidase. When formate-bound samples were made anaerobic by outgassing, their susceptibility behavior was very similar to that of the resting enzyme. The susceptibility followed the Curie Law from 20 to 180 K, with an a_3 center μ_{eff}^2 value of 24.0. As with the resting enzyme, cytochrome a_3 is high-spin (Nicholls, 1976; Babcock, 1988), so this behavior matches an a_3 center composed of an $S = {}^5/_2$ iron ion antiferromagnetically coupled to an $S = {}^1/_2$ ion. The linearity of the χ vs $1/T$ plot at temperatures as high as 180 K indicates that $-J > 200$ cm^{-1}. The low-temperature susceptibility deviates from the Curie Law, but calculations show that this can be explained by an axial zero-field splitting parameter between 3 and 5 cm^{-1}. Figure 2 shows the experimental data and the expected values of the susceptibility of all four metal centers in cytochrome oxidase for different D values and for $-J = 200$ cm^{-1}. These were calculated with Griffith's (1971) equation, modified as discussed above. A comparison shows that the zero-field splitting parameter of the formate-bound enzyme is smaller than that of the resting enzyme.

Effect of Oxygen Removal with Glucose Oxidase. Use of glucose oxidase to deoxygenate resting and formate-bound enzyme samples caused dramatic changes in the temperature dependence of the susceptibilities of some samples. The magnetic susceptibility behavior of the resting enzyme then fell into two categories. Three Hartzell–Beinert and one Yonetani preparation showed Curie Law behavior corresponding to an average a_3 center μ_{eff}^2 value of 18 ± 2. The 428-nm band that is present in the optical spectrum of diluted aliquots of these samples is red-shifted from the 420-nm maximum characteristic of the resting enzyme and indicates an interaction of the enzyme with hydrogen peroxide produced by the glucose–glucose oxidase system. The Soret band position and susceptibility behavior did not change upon addition of excess hydrogen peroxide, indicating maximum hydrogen peroxide–enzyme interaction.

Following reoptimization of the Triton X-114:protein ratio in the isolation procedure upon receipt of a new detergent lot, the susceptibility of three Hartzell–Beinert preparations deoxygenated with glucose–glucose oxidase showed Curie Law behavior corresponding to an average a_3 center μ_{eff}^2 value of 26.4 ± 0.7. In all cases, the 428-nm Soret band of diluted aliquots indicated hydrogen peroxide–enzyme interaction. Both Soret position and susceptibility behavior remained unchanged upon addition of excess hydrogen peroxide, again indicating maximum hydrogen peroxide–enzyme interaction.

The susceptibility behavior of formate-bound enzyme samples changed when glucose–glucose oxidase was used for deoxygenation and when hydrogen peroxide was added to the sample and deoxygenation was accomplished by outgassing alone. The Soret band of diluted aliquots remained at 417 nm in all cases. In each case, the Curie Law was obeyed at temperatures above 20 K, but the a_3 center μ_{eff}^2 values varied from 22.4 to 54.7, depending on the concentration of added hydrogen peroxide, the presence or absence of glucose oxidase, and the Triton X-114 used (Table III).

Table III: Effective Magnetic Moment Squared of Formate-Bound Cytochrome Oxidase in the Presence of Glucose–Glucose Oxidase and Hydrogen Peroxide

Triton X-114:protein ratio (mg/mg)	equiv of added H_2O_2	a_3 μ_{eff}^2 (μ_B^2)
2.0[a]	0	36.6
2.0[a]	20	44.7
2.0[a]	50	54.7
1.6[a]	37	22.4

[a] When deoxygenation was accomplished with glucose–glucose oxidase, the a_3 μ_{eff}^2 values observed for the Hartzell–Beinert enzyme isolated with detergent:protein ratios of 2.0 and 1.6 mg/mg were 18 ± 2 and 26.4 ± 0.7 μ_B^2, respectively.

Table IV: Effective Magnetic Moments Squared for Resting Cytochrome Oxidase

preparation	temp range (K)	a_3 μ_{eff}^2 (μ_B^2)
Yonetani (Tween-80)	12–169	26 ● 3
Hartzell–Beinert (Tween-20)	20–201	23 ● 1
Hartzell–Beinert (lauryl maltoside)	30–180	24.8 ± 0.5

Discussion

The temperature dependence of the susceptibilities of the Hartzell–Beinert and the Yonetani resting enzyme clearly indicates that in both cases the a_3 center is antiferromagnetically coupled and that the magnitude of J is large. The low-temperature deviation from the Curie Law shown by the resting enzyme susceptibility is adequately explained by a reasonable value of the axial zero-field splitting parameter. These results agree with those reported by Tweedle et al. (1978) for the Hatzell–Beinert resting enzyme and by Brudvig et al. (1986).

The effective magnetic moments of the Yonetani and Hartzell–Beinert resting enzyme are the same, within experimental error (Table IV), showing that the a_3 center of these enzymes is magnetically the same. This is at variance with a report by Powers et al. (1987) that the Cu_B environment in the Hartzell–Beinert enzyme differs from that of the Yonetani enzyme, but in agreement with a report by Scott et al. (1986), which indicates similar environments regardless of isolation method. The similar magnetic behavior of the a_3 center in the Yonetani and Hartzell–Beinert enzyme also suggests that the fast vs slow binding of cyanide to the enzyme as isolated (Hartzell et al., 1988) is unlikely to be determined by large-scale differences in the structure of the binding site. In addition, the effective magnetic moment of enzyme isolated with the Hartzell–Beinert method was not affected by the identity of the dispersing detergent, so our results do not support the hypothesis that conflicting literature reports of the coupling of the a_3 center in the resting enzyme are due to differences caused by either the isolation method or the dispersing detergent.

The cyanide-bound enzyme exhibits magnetic heterogeneity, with the major fraction of the enzyme having $-J = 1.3$ cm^{-1} and a minor fraction of the enzyme having $-J = 44$ cm^{-1}. This latter coupling constant is close to the value of -38.5 cm^{-1} obtained by Tweedle et al. (1978), though they found the cyanide-bound enzyme to be homogeneous.

The $-J = 44$ cm^{-1} value we find for the minor fraction of the enzyme clearly indicates antiferromagnetic coupling, but interpretation of the coupling constant of the major fraction is more difficult. The negative sign of its coupling constant indicates a lowest energy state of $M_s = 0$, and the low-temperature a_3 center μ_{eff}^2 value of 0.2 indicates that essentially all of the enzyme is in this state. However, since the coupling is weak, this population distribution could arise either from antiferromagnetic coupling or from ferromagnetic coupling with a zero-field splitting that results in a lowest energy $M_s = 0$ state. In either case, the lowest energy states with $M_s =$

0 found for both forms of the cyanide-bound enzyme are at a variance with the model proposed by Thomson et al. (1981). On the basis of their MCD data, they suggested that the lowest energy states had $M_s = \pm 1$, which would require a low-temperature a_3 center μ^2_{eff} value of 8 rather than the value of 0.2 obtained for our samples. Yonetani enzyme was used in the MCD studies, and the results presented here indicate that this is an unlikely source for the discrepancy between the MCD and susceptibility data. We do note that ethanediol was used as a glassing agent by Thomson et al. (1981). Given the sensitivity of the exchange coupling that we observe, it is possible that solvent differences may be responsible for the different conclusions reached in that work and from the results previously presented (Tweedle et al., 1978).

The effect on cyanide-bound enzyme of different enzyme isolation methods and of different dispersing detergents can be assessed by examining the coupling constants of the two forms of the enzyme (Table II). The a_3 magnetic states of enzyme isolated with the Hartzell–Beinert and Yonetani methods are the same, as shown by their effective magnetic moments. The use of lauryl maltoside as a dispersing detergent rather than Tween-20 had no effect on the coupling constant of the weakly coupled form of the enzyme and resulted in an approximate doubling of the coupling constant value for the moderately coupled form of the enzyme. This change indicates a detergent-induced effect on bond angle or orbital overlap at the a_3 center with the use of a different detergent, but since antiferromagnetism is retained, it is insufficient to explain ambiguities in the literature concerning the coupling in the cyanide-bound oxidized enzyme a_3 center.

The existence of several forms of cytochrome oxidase has previously been shown (Antonini et al., 1977; Kent et al., 1983; Maison-Peteri & Malmstrom, 1989). Heterogeneity in the kinetics of reduction of the resting enzyme [see, for example, Halaka et al. (1984)] and in the interactions of resting enzyme with hydrogen peroxide (Bickar et al., 1982), NO (Brudvig et al., 1981), and cyanide (Naqui et al., 1984; Baker et al., 1987; Schoonover et al., 1988) has been reported. In addition to the heterogeneity of the resting enzyme, Jensen et al. (1984) reported a heterogeneity in the EPR properties of the partially reduced, cyanide-bound enzyme. The work reported here shows that this heterogeneity can also be present in the fully oxidized, cyanide-bound enzyme. This postbinding heterogeneity indicates structural differences more fundamental to the a_3 center than kinetic accessibility. The difference may be due to a variation in ligand bond angle or may be evidence for more than one cyanide-binding site, as suggested by Yoshikawa and Caughey (1990) for the partially reduced cyanide-bound enzyme.

This magnetic heterogeneity of the cyanide-bound enzyme was not reported by Tweedle et al. (1978), possibly because they had no data at temperatures less than 20 K. The similarity of the coupling constant that they found (-38.5 cm^{-1}) with that found by using our higher temperature data (-44 cm^{-1}) supports this possibility. The heterogeneity we observe is also at odds with the report by Yoshikawa and Caughey (1990) of only one CN stretching frequency in the IR spectra of the fully oxidized cyanide-bound enzyme. They do report more CN stretching frequencies for the cyanide-bound samples when extraneous copper ions were present or when the bound enzyme was partially reduced upon standing for 40 h, but it is improbable that either of these situations was the case with our samples. EDTA was used during the enzyme isolation to remove extraneous metal ions, and none of the Hartzell–Beinert enzyme samples were incubated more than 12 h before they were frozen. While the optical absorption spectrum of concentrated samples of cyanide-bound enzyme was not checked, the visible region of the spectrum of neat samples of resting enzyme showed no evidence of autoreduction during deoxygenation by outgassing.

All resting enzyme that was isolated in our laboratory and deoxygenated with glucose–glucose oxidase showed a Soret maximum at 428 nm, indicating enzyme–hydrogen peroxide interaction. Direct addition of hydrogen peroxide itself did not affect the susceptibility behavior of these samples, and the linearity of all χ vs $1/T$ plots indicates the lack of a temperature-dependent spin equilibrium. The EPR spectra of these samples showed no cytochrome a_3 or Cu_B signals. When deoxygenated with glucose–glucose oxidase, our early batches of enzyme preparations showed a Curie Law susceptibility behavior corresponding to an a_3 center μ^2_{eff} value of 18 ± 2. While this value would be expected if the a_3 center were in a ground energy state resulting from weak coupling between an $S = {}^3/_2$ cytochrome and the $S = {}^1/_2$ copper ion, the linearity of the χ vs $1/T$ plot from 30 to 180 K indicates that the distribution between energy states remains constant with increasing temperature. Thus, a better explanation is that the a_3 center exists as a thermally randomized distribution between an $S = 1$ and a higher energy $S = 2$ state, which would result from very weak antiferromagnetic coupling of an $S = {}^3/_2$ cytochrome iron with the copper ion. The predicted a_3 center μ^2_{eff} value for this case would be 16. A spin state of ${}^3/_2$ for cytochrome a_3 in the peroxide-bound resting enzyme has also been suggested by resonance Raman data (Carter et al., 1981; Babcock, 1988).

Resting enzyme prepared with the Triton X-114 lot that we obtained later in our study showed a susceptibility behavior corresponding to an average μ^2_{eff} value of 26.4 when deoxygenated with the glucose–glucose oxidase system, and hydrogen peroxide was added directly to the resting enzyme. The origin of the change in magnetic behavior as a function of the Triton X-114 is unclear, though it may be due to differences in the degree of protonation of the peroxide ion. Chan and coworkers (Blair et al., 1985), Vygodina and Konstantionov (1988), and Wikstrom (1989) have discussed protonation/deprotonation reactions associated with peroxide and peroxide-derived ligands.

The peroxide-induced susceptibility behavior observed with the latter samples in our study most closely matches what would be observed for either strong antiferromagnetic coupling of an $S = {}^5/_2$ cytochrome and an $S = {}^1/_2$ copper ion or strong ferromagnetic coupling of an $S = {}^3/_2$ cytochrome and an $S = {}^1/_2$ copper ion. The former would require a change of both iron ion spin state and type of coupling as a result of a change in the detergent lot or detergent:protein ratio during enzyme isolation, but an $S = {}^3/_2$ cytochrome would require only a change in the strength of the coupling. The lack of a high-spin cytochrome signal in the Raman spectra of peroxide-bound cytochrome oxidase (Carter et al., 1981; Babcock, 1988) makes ferromagnetic coupling between the unusual $S = {}^3/_2$ cytochrome and the copper ion the preferred explanation. In addition, the $S = 2$ a_3 center indicated for these resting enzyme–hydrogen peroxide samples is not possible for the iron(IV)–Cu(II) center that has been reported (Chan & Li, 1990) to form when hydrogen peroxide is added to pulsed enzyme. Whether this is due to a difference in resting and pulsed enzyme is unclear.

Tweedle et al. (1978) used glucose–glucose oxidase as a deoxygenation procedure and observed susceptibility behavior ($\mu^2_{eff} = 24.3$) that is consistent with our results for the resting

enzyme. This difference can be explained in three ways: they may have had an enzyme form that is similar to that which we observe after the protein-to-detergent ratio was changed, their enzyme may not have bound peroxide in the time before the samples were frozen, or their glucose oxidase may have been contaminated with catalase, as commercial preparations often are.

Regardless of spin-state interpretation, the 428-nm Soret band observed in enzyme spectra upon addition of glucose–glucose oxidase indicates that this deoxygenation procedure should be used with extreme caution. Also, the variation in magnetic moment seen for cytochrome oxidase with a 428-nm Soret band indicates the high sensitivity of this form of the enzyme to changes in isolation and handling procedures.

The susceptibility behavior of the Hartzell–Beinert formate-bound enzyme indicates strong antiferromagnetic coupling between a high-spin cytochrome a_3 and the Cu_B, and the low-temperature deviations from the Curie Law are explained by a reasonable zero-field splitting value. Further information can be obtained about the possible binding site of the formate ion by comparing the susceptibilities of the resting and formate-bound enzymes.

Since cytochrome a_3 in the resting enzyme has a histidine in the axial site proximal to the copper ion (Salmeen et al., 1978; Stevens & Chan, 1981; Ogura et al., 1983), the formate must bind either away from the cytochrome a_3 or between the cytochrome a_3 and Cu_B, as proposed for oxygen in the "front-binding" model of the a_3 center (Blumberg & Peisach, 1979). In the latter case, the resulting structural disruption would be expected to lead to a large change in the magnetic susceptibility behavior relative to that of the resting enzyme. Since there is no difference in the temperature dependence of the resting and formate-bound enzymes, it may be inferred tha the front-binding model is incorrect for formate and that it binds at a site other than the iron ion. This view is supported by the lack of substantial difference between the Raman spectra of the resting and oxidized formate-bound enzymes (Woodruff et al., 1981; Babcock, 1988).

The retention of the 417-nm Soret band concurrent with the susceptibility changes that occur upon addition of hydrogen peroxide to the formate-bound enzyme is further evidence for a non-heme-binding site of the formate ion. Simultaneous interactions of the enzyme with hydrogen peroxide and formate ion indicate two sites of interaction. Since hydrogen peroxide binds to cytochrome a_3 in the resting enzyme (Wrigglesworth, 1984), this implies either that the formate ion interacts away from the a_3 center, but close enough to affect the value of the zero-field splitting factor, or that it binds to Cu_B.

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The Conserved, Buried Aspartic Acid in Oxidized *Escherichia coli* Thioredoxin Has a pK_a of 7.5. Its Titration Produces a Related Shift in Global Stability†

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ABSTRACT: Aspartic acid 26 in *Escherichia coli* thioredoxin is located at the bottom of a hydrophobic cavity, near the redox-active disulfide of the active site. Asp 26 is embedded in the protein except for part of the surface of one carboxyl oxygen. The high degree of evolutionary conversion of Asp 26 suggests that it plays a critical role in thioredoxin function. We have determined the pK_a of Asp 26 by a novel electrophoretic method based on the relative electrophoretic mobilities of wild-type thioredoxin and of D26A thioredoxin (with Asp 26 replaced by alanine). The pK_a of Asp 26 determined by this technique is 7.5, more than 3 units above the pK_a of a solvated carboxyl side chain. The titration of Asp 26 is thermodynamically linked to the stability of thioredoxin. As expected if thioredoxin stability depends on the ionization state of Asp 26, $\Delta G°_{WT}$, the free energy of the cooperative denaturation reaction of wild-type thioredoxin by guanidine hydrochloride, varies with pH in a sigmoidal fashion in the vicinity of pH 7.5. Over the same pH range, the free energy for D26A folding, $\Delta G°_{D26A}$, is pH independent and D26A is highly stabilized compared to wild type. From the thermodynamic cycle describing the linkage of Asp 26 titration to thioredoxin stability, the difference in free energy between wild-type thioredoxin with protonated Asp 26 and wild-type thioredoxin with deprotonated Asp 26, $\Delta\Delta G°_{(COOH \rightarrow COO^-)}$, is calculated to be 4.9 kcal/mol. In good agreement with this value, we find that the difference in free energy between wild type and D26A denaturation, $\Delta\Delta G°_{(WT \rightarrow D26A)}$, is 4.6 kcal/mol at pH 8.5, where Asp 26 is mostly deprotonated. This indicates that the stabilization of D26A compared to wild type is primarily due to the electrostatic effects of removing the abnormally titrating aspartic acid and corroborates the pK_a of 7.5 obtained for Asp 26 by the electrophoretic method. In the following paper [Langsetmo, K., Fuchs, J., Woodward, C., & Sharp, K. (1991) *Biochemistry* (following paper in this issue)], the role of Asp 26 titration in thioredoxin structure is further described. In that paper the excellent agreement of the experimental pH dependence of $\Delta G°_{WT}$ with a general expression for the linkage of protein titration groups and protein stability is reported.

Escherichia coli thioredoxin is a 108 amino acid, soluble protein that participates in diverse redox and regulatory reactions (Holmgren et al., 1975; Holmgren, 1985). It has an α/β structure (Katti et al., 1990; Eklund et al., 1984), of which the most prominent feature is the central five-stranded, twisted β-sheet (Figure 1). The only two cysteine residues in the molecule, Cys 32 and Cys 35, form the redox-active disulfide/dithiol in a reverse turn between the middle β-strand and the α2-helix. Thioredoxin is purified in the oxidized form, with the active site cysteines in a disulfide bond. In the presence of reducing agents, the cysteines are in the dithiol form. Although reduced thioredoxin is much less stable than oxidized (Kelley et al., 1987; Langsetmo et al., 1989), reduction produces only small, local changes in the vicinity of the cysteines (Brown et al., 1987; Dyson et al., 1990).

E. coli thioredoxin has high sequence homology to other thioredoxins (Eklund et al., 1984; Gleason, 1986; Gleason & Holmgren, 1988). In addition to the active site sequence Trp-Cys-Gly-Pro-Cys, the completely conserved residues include two others of unusual conformation in the crystal structure. These are Pro 76, which is in a cis peptide bond, and Asp 26, which has a buried carboxyl group. We are

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