Sodium fluoride induces changes in the heme $d_i$ components of both the frozen solution EPR and room temperature optical absorption spectra of oxidized *Pseudomonas* cytochrome oxidase. The EPR resonances of the heme $d_i$ (iron-chlorin) moiety(ies) appear to undergo a rhombic to axial transition ($g = (2.53, 2.42, 1.73) \to (2.54, 2.54, 1.61)$) with increasing concentrations of fluoride ($0 \to 0.1 M$, pH 7.0) while the $g$ values remain within the range usually attributed to low spin iron-porphyrin complexes ($3.75 > g > 0.5$). The axial spectrum which results is apparently low spin and suggests a degeneracy of two of the three iron $d$ orbitals ($d_x, d_y, d_z$). The optical difference spectrum induced by sodium fluoride exhibits pronounced features at 475 and 645 nm which correspond to positions of heme $d_i$ optical absorption bands. The spectral alterations induced by fluoride are not due to a dimer $\to$ monomer transformation since gel chromatography of the oxidase in the absence and presence of sodium fluoride (0.1 M, pH 7.0) gives the same molecular weight. The heme $c$ resonances of the oxidase are not altered substantially by the addition of sodium fluoride, and the axial ligation of the heme $c$ moiety(ies) of the unperturbed oxidase is investigated using the observed $g$ values.

The interaction of oxidized *Pseudomonas* cytochrome oxidase with potassium cyanide causes distinct changes in both the heme $d_i$ and heme $c$ components of the frozen solution EPR spectrum in which the former appears to retain its rhombic shape. Plausible modes of interaction of the enzyme with both fluoride and cyanide are discussed and, through the use of $d$ orbital energy level diagrams, compared with energy levels calculated from published $g$ values of iron-porphyrin and iron-chlorin moieties complexed with known axial ligands. Consistent differences between $d$ orbital energy levels of iron-porphyrin and -chlorin (or chlorin-like) complexes are outlined.

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**Pseudomonas* cytochrome oxidase (ferrocyanochrome $C_{60}$; oxygen oxidoreductase, EC 1.9.3.2) functions as a terminal electron carrier in *Pseudomonas aeruginosa* (1, 2) and in its purified form exhibits both oxidase and nitrite reductase activities (3, 4). Gel electrophoresis shows the enzyme to be composed of two apparently equivalent subunits, each of molecular weight 60,000 (5, 6) and each containing one heme $d_i$ and one heme $c$ moiety. Spectral alterations occur at both hemes upon incubation of the enzyme with several exogenous ligands (7, 8) and one such ligand, cyanide, strongly inhibits enzymatic activity (1). The interaction at the heme $d_i$ moiety is of special interest in that it appears to be the site of preferential ligand binding (9–11) and terminal catalytic activity (11, 12).

Heme $d_i$ is structurally unique in that it is an iron-chlorin (13) in which addition across the periphery of a single pyrrole alters both the conjugation and optical absorption spectra from that normally found with iron-porphyrins (14). The $g$ values of EPR spectra obtained from bis-imidazole complexes of ferrous iron-porphyrins and iron-chlorins differ substantially (15), which suggests that the disruption of conjugation can influence the electronic states of the iron. In contrast, the EPR spectra of nitric oxide complexes of ferrous iron-porphyrins and iron-chlorins (as both models and in proteins) exhibit quite similar spectral parameters (10, 16).

Previous EPR studies of oxidized *Pseudomonas* cytochrome oxidase by Gudat et al. (5) assigned the structure at $g = 2.45$ and resonance at $g = 1.71$ to the heme $d_i$ moiety(ies) and the resonances at $g = 2.93, 2.31, and 1.4$ to those of the heme $c$. The assignment (excluding the $g = 1.4$ resonance) was confirmed by Walsh et al. (17) in which they suggested, through a combination of EPR and magnetic circular dichroism techniques, that the ferric ions of both the heme $d_i$ and heme $c$ moieties in the absence and presence of KCN are in the low spin state. The present study focuses on a unique change in the $g$ values of the heme $d_i$ moiety(ies) of the oxidase induced by NaF and re-examines changes induced by KCN at both the heme $d_i$ and heme $c$ moieties. The changes in $g$ values are interpreted in terms of alterations in the crystal field interactions which govern the electron distribution of the $t_{2g}$ levels of ferric low spin iron.

**Experimental Procedures**

**Materials**—All inorganic reagents used were of analytical reagent grade except for Na$_2$S$_2$O$_3$ which was technical grade (90%+) from Aldrich. Of the reagents employed in altering the spectral characteristics of the purified oxidase, both NaF and NaN$_3$ were purchased from Fisher and KCN was obtained from Mallinckrodt Chemical Works. Both the (NH$_4$)$_2$HCO$_3$ and KNO$_3$ needed for the cell growth medium were purchased from Matheson, Coleman and Bell and the yeast extract was a product of the BBL division of Becton, Dickinson and Company. Ethylenediaminetetraacetic acid, disodium 1The abbreviations used are: Im, imidazole (charge state unspecified or added to solution); Im$^-$, imidazolate; $\lambda$, spin orbit coupling constant (approximately 400 cm$^{-1}$); TPP, tetraphenylporphyrin; TPC, tetraphenylchlorin.
salt, was procured from Fisher, Sephadex G-100, G-150, CM-Sephadex (C-50), and blue dextran were obtained from Pharmacia. Water was the press of swollen microgranular DEAE-cellulose (DE-52) and CM-cellulose (CM-52). Horse skeletal myoglobin, type I, and bovine alkaline phosphatase, type I, were purchased from Sigma, and chicken egg white ovalbumin (twice recrystallized) and beef heart lactate dehydrogenase were obtained from Worthington.

Methods—Both the source of and maintenance procedures for cultures of P. aeruginosa are described by Gudat et al. (5). Two stages were employed for the large scale production of cells of P. aeruginosa. Both stages employed a citrate growth medium composed of the following per liter: (NH₄)₂HC₆H₅O₇, 5 g; KN0₃, 20 g; KH₂PO₄, 11 g; MgSO₄·7H₂O, 0.18 g; yeast extract, 10 g; Na₂B₆H₄O₇·2H₂O, 0.51 g; NaCl, 0.5 g; and 10 g of Hutner’s salts. The Hutner’s salts contained per liter: ethylenediaminetetraacetic acid, disodium salt, 2.50 g; ZnSO₄·7H₂O, 10.95 g; FeSO₄·7H₂O, 5.00 g; MnCl₂·H₂O, 1.54 g; CuSO₄·5H₂O, 0.39 g; Co(NO₃)₂·6H₂O, 0.25 g; Na₂SO₄·10H₂O, 0.18 g; a few drops of concentrated H₂SO₄ were added to retard precipitation. In addition, 0.4 ml of 10% Na₂S was added to each stage of the culture. The pH of this solution was adjusted with H₃PO₄ to 6.4 and the culture was incubated at 37 °C for 20–24 h, the cells were harvested by centrifugation at 13,700 × g for 45 min at 4 °C. The supernatant was recovered and taken to 95% of saturation (39.8 g of cell paste which then was stored frozen at -20 °C). Total protein was recovered and dialyzed against 0.01 M potassium phosphate buffer, pH 6.0, with repeated changes of buffer until absorption from the dialysed buffer was negligible. The oxidase was precipitated from the crude extract using (NH₄)₂SO₄ following the procedure outlined by Gudat et al. (5) with the following modifications. The extract was brought to 40% of saturation with (NH₄)₂B₄O₇·7H₂O and centrifuged at 13,700 × g for 45 min at 4 °C. The supernatant was recovered and taken to 85% of saturation (29.8 g/100 ml) followed by centrifugation at 27,600 × g for 45 min at 4 °C. The procedure for purification of the pellet and its dialysis is as described by Gudat et al. (5).

Further purification of the oxidase employed chromatography using four distinct columns which differed somewhat from those employed previously (5). The dark green dialysate was applied to the top of a DEAE-cellulose column (5.0 × 30 cm) equilibrated with 0.02 M potassium phosphate, pH 6.4. The oxidase adhered to the top while the other two proteins washed through. The oxidase was eluted from the column with 0.04 M potassium phosphate, pH 6.9, and then washed through the column with the same buffer. The effluent was collected in fractions (8.0 ml) and those with a ratio of A₅₈₀/A₅₅₀ greater than 1.0 were pooled. The oxidase was then applied to a second DEAE-cellulose column (5.0 × 30 cm) equilibrated with 0.01 M potassium phosphate, pH 6.0, and was eluted by gravity flow using the same buffer. Fractions (4.0 ml) were collected and those with a ratio of A₅₈₀/A₅₅₀ greater than 1.0 were pooled and concentrated. The stock solution of enzyme was stored frozen (-20 °C) until ready for use, and multiple freeze/thaw cycles were avoided.

Preparation for EPR spectroscopy, the enzyme was concentrated further to approximately 0.3 mM through the use of a vacuum dialysis apparatus obtained from Schleicher & Schuell. Samples were dialyzed at 4 °C for a minimum of 6 h and maximum of 24 h against 0.04 M potassium phosphate buffer, pH 7.0, which contained the appropriate concentration of ligand. Dialysis against potassium phosphate buffer in the absence of ligands and in the presence of NaF, NaN₃, or NaCl (<1.0 M) produced little or no precipitation. The variable amount of precipitate found after dialysis of Pseudomonas cytochrome oxidase against 0.1 M KCN in 0.04 M potassium phosphate, pH 7.0, was removed by centrifugation before EPR spectra were obtained.

Optical absorption and difference spectra were recorded at room temperature using a Cary model 14 spectrophotometer. Addition of buffered 0.5 M NaF to the sample cell was matched in volume by addition of buffer to the reference cell. EPR spectra were obtained with a Varian E-4 X-band spectrometer equipped with either an Air Products model LTD-3-110 helli-tran accessory or an E-257 variable temperature device to obtain temperatures near those of liquid helium or liquid nitrogen, respectively. The samples for EPR spectroscopy (approximately 0.25 ml each) were frozen in dry ice/acetone and stored for a short time in liquid nitrogen before use.

The molecular weight of the oxidized enzyme was determined with a Sephadex G-150 column (110 × 1.0 cm) equilibrated with 0.1 M NaF in 0.04 M potassium phosphate, pH 7.0. The enzyme sample was dialyzed against this same solution for 12 h before application to the top of the column. After equilibrium was achieved, the column was calibrated with the following proteins: lactate dehydrogenase, alkaline phosphatase, ovalbumin, and myoglobin. The void volume was determined with the blue dextran.

The concentration of Pseudomonas cytochrome oxidase was calculated using an extinction coefficient of 3.02 mm⁻¹ cm⁻¹ (19) per heme c at 550 nm after addition of a few grains of Na₂S₂O₄ and is expressed in terms of total enzyme (not heme c) concentration.

RESULTS

Addition of NaF to an aqueous solution of oxidized Pseudomonas cytochrome oxidase caused changes in both the room temperature optical and frozen solution EPR spectra. Fig. 1 shows the alterations in the EPR spectrum of the purified oxidase at 90 K in 0.04 M potassium phosphate buffer, pH 7.0, induced by dialysis against NaF concentrations increasing from 0 to 0.1 M. The spectrum is initially rhombic in shape and exhibits the g values (2.51, 2.42, 1.73) which, as the shoulder gradually attenuates and the spectrum becomes axial, change to (2.53, 2.53, 1.62). The inset to Fig. 1 clearly shows significant alterations in the high field resonance upon adding 0.01 M NaF with centrosymmetrical transformation (to axial) occurring at 0.1 M fluoride. Furthermore, as the fluoride concentration increases from 0 to 0.1 M, the low field structure (which consists of one or more resonances) decreases from a peak to trough width of 160 to 105 G, while the high field resonance appears to broaden. The axial shape of the EPR spectrum of the oxidase induced with NaF is unique in that, although it is commonly reported for Fe(III) heme proteins, it is expressed in terms of total enzyme (not heme c) concentration.

The frozen solution EPR spectrum of the purified oxidase at pH 7.0 in 0.04 M potassium phosphate and recorded at 12 K exhibits resonances in addition to those found at 90 K (see

2 The precise assignment of g values to either of the two heme d₃ moieties is difficult as will become evident in the discussion. At this point, the convention which will be followed is that the three distinct g values which can be obtained from the heme d₃ component of the spectra of the native oxidase in the absence and presence of cyanide will be referenced as if they originate from two electronically equivalent iron centers and labeled sequentially (g₁, g₂, g₃). Furthermore, no reference to molecular (heme) axes is implied until specifically discussed.

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subtle differences in the purified oxidase preparations from the two laboratories.

The effect of 0.01 M \( \text{NaNO}_2 \) in 0.04 M potassium phosphate, pH 7.0, on the frozen solution spectrum (12 K) of the purified oxidase is seen in Fig. 2D. There is almost complete attenuation of the pronounced structure evident in the native oxidase near \( g = 2.5 \), while resonances at \( g = (2.95, 2.30, 1.42) \) remain clearly visible. A small shoulder at \( g = 2.71 \), as well as a resonance near \( g = 6 \), also can be seen in the spectrum. The overall shape of the spectrum which results is clearly similar to that of the heme c component of the unperturbed oxidase. The attenuation of both the structure near \( g = 2.5 \) and the resonance at \( g = 1.73 \) induced by \( \text{NaNO}_2 \) can be observed at 90 K. Sodium azide also drastically reduces the intensity of these two features (spectrum not shown) and this change has been observed by others (21).

**Fig. 1.** EPR spectra of *Pseudomonas* cytochrome oxidase in the presence of varying concentrations of NaF. The enzyme was dissolved in 0.04 M potassium phosphate, pH 7.0, and had a final concentration of 0.20 mM. A, 0.0 M NaF; B, 0.001 M NaF; C, 0.01 M NaF; D, 0.1 M NaF. Enzyme samples were dialyzed against appropriate solutions for 6 h before freezing. The settings of the spectrometer were: microwave power, 100 milliwatts; field modulation, 5 G; time constant, 0.3 s; microwave frequency, approximately 9.16 GHz; scanning speed, 8 min; temperature, 90 K. The EPR signal in the inset was amplified by a factor of 5 relative to that of the wide field trace.

**Fig. 2A.** Resonances at \( g = (2.51, 2.43, 1.73) \) are seen at both temperatures with little variation in position (\( |\Delta g| < 0.01 \)), but only at the lower temperature are resonances found at \( g = (2.97, 2.26, 1.40) \). Spectra A and B of Fig. 2 show that the introduction of 0.1 M NaF alters the position and shape of the resonances at \( g = (2.51, 2.43, 1.73) \) in a manner almost identical with that found at the higher temperature while those at \( g = 2.97 \) and 2.26 appear almost unaltered. It is possible that the structure near \( g = 1.5 \) induced with NaF is due to partial overlap of a resonance near \( g = 1.4 \) and one near \( g = 1.6 \), though an independent resonance cannot be ruled out. The separation between the two resonances at \( g = 1.63 \) and 1.48 is over 400 G, which is much greater than the doubling separation of 44 G reported for high spin ferric myoglobin fluoride near liquid helium temperatures (19). Low temperature EPR studies of the interaction of fluoride with the oxidase were performed by Walsh et al. (21); however, the unique change induced by this reagent was not reported. The only apparent difference between their work and ours is their use of a saturating amount of sucrose.

The spectral changes induced in the purified oxidase observed at 12 K in 0.04 M potassium phosphate, pH 7.0, with 0.1 M KCN are evident in Fig. 2C. The spectrum is similar to that published by Walsh et al. (17), although we observe resonances at \( g = 3.31, 2.47, 2.35, \) and 1.61 while they report a resonance at \( g = 2.58 \), even though the positions of their lowest and highest field resonances only differ from ours by a maximum of \( |\Delta g| = 0.01 \). Furthermore, the ratio of the distance from peak to inflection to the distance from peak to trough of the \( g = 2.5 \) structure is 0.18 in our spectrum, but 0.07 in their spectrum. A similar difference in detail of the structure near \( g = 2.5 \) in the purified unperturbed oxidase occurs between our spectrum in Fig. 2A and that published by Walsh et al. (17), even though in this experiment the enzyme was frozen in identical buffering systems and spectra were recorded at almost identical spectrometer settings. Such results suggest
the former. The enzyme was incubated for 1.5 h in 0.04 M potassium phosphate, pH 7.0, before recording the difference spectrum; both spectra were recorded at 23°C.

Dialysis of purified Pseudomonas cytochrome oxidase against up to 1 M NaCl (in 0.04 M potassium phosphate, pH 7.0) did not induce a rhombic to axial transition in the frozen solution (90 K) EPR spectrum. However, small changes in the definition of the resonances which compose the structure near $g = 2.5$ (possibly changes in line width) were observed within this concentration range. Above 1 M NaCl the enzyme precipitates, which precludes an accurate study at higher salt concentrations. No resonances distinct from those normally seen at 90 K were introduced by interaction with the salt. It is possible that a small fraction of the resonating centers undergo a transition to a spectrum with an axial shape. However, their high field resonance must be contained within that seen in the spectrum of the purified enzyme or be of an intensity that is difficult to detect.

The optical difference spectrum of oxidized Pseudomonas cytochrome oxidase with 0.1 M NaF minus the unperturbed oxidized enzyme (both in 0.04 M potassium phosphate, pH 7.0) did not induce an axial or rhombic transition in the frozen solution (90 K) EPR spectrum. However, small changes in the definition of the resonances which compose the structure near $g = 2.5$ (possibly changes in line width) were observed within this concentration range. Above 1 M NaCl the enzyme precipitates, which precludes an accurate study at higher salt concentrations. No resonances distinct from those normally seen at 90 K were introduced by interaction with the salt. It is possible that a small fraction of the resonating centers undergo a transition to a spectrum with an axial shape. However, their high field resonance must be contained within that seen in the spectrum of the purified enzyme or be of an intensity that is difficult to detect.

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The EPR spectrum of oxidized Pseudomonas cytochrome oxidase can be divided into resonances associated with the haem $c$ ($g = (2.98, 2.27, 1.38)$) and haem $d_1$ ($g = (2.52, 2.42, 1.73)$) moieties (5). Though both sets of resonances are visible at 12 K, those associated with the haem $c$, as with beef and tuna cytochrome $c$ (24, 25), become obscure at 90 K, while those of the haem $d_1$ remain clearly defined.

Examination of the spectrum in Fig. 2A suggests the possibility of overlap of the $g$ resonances of the haem $c$ and haem $d_1$ moieties causing the determination of the former resonance to be in error. This can be clarified through the use of a relationship given by Bohan (26) from which a third $g$ value can be calculated from the knowledge of two others. When this equation is applied to the EPR spectrum of the haem $c$, the magnitudes of the $g$ values at 2.98 and 1.38 predict a third, 2.28, which is in close agreement with that measured for the oxidase.

The identity of the axial ligands of the haem $c$ moieties of the oxidase can be considered by using the crystal field diagrams of Blumberg and Peisach (27, 28) with crystal field parameters calculated for ferric low spin iron by the method of Taylor (29). Fig. 4 shows that the parameters of the haem $c$ component of the oxidase fall closest to those of horse, bovine, tuna, and yeast cytochrome $c$ which all contain the Met-Fe-His coordination (42). Assuming reasonable errors in the assignment of $g$ values, the calculated error in tetragonal crystal field distortion for the haem $c$ component is $\pm 0.085$. This interval is about 1.5 times the separation along the tetragonal axes for values representing tuna cytochrome $c$ from two separate reports (25, 30), but does not allow for overlap between values given for the oxidase and those of horse and bovine cytochrome $c$. It can be argued that this error interval is insufficient and should be increased or that

3 Unless the $g$ values mentioned in the text are associated directly with a figure, the average $g$ values obtained from several spectra of the same system will be given. These average values may differ slightly from those found in the figures.

4 To calculate the error in tetragonal component, the $g$ values from four distinct preparations of Pseudomonas cytochrome oxidase (0.04 M potassium phosphate buffer, pH 7.0) were averaged and the standard deviations of the mean were determined: $g = (2.979 \pm 0.006, 2.265 \pm 0.008, 1.384 \pm 0.011)$. The standard deviations for $g_1$ and $g_2$ were rounded to 0.01 and for $g_3$ increased liberally to 0.02 (because of the broadness of the high field resonance). These values are substituted into the error equations given by Taylor (29) to calculate the variance in the tetragonal crystal field distortion.

**DISCUSSION**

Pseudomonas cytochrome oxidase contains two different prosthetic groups: haem $c$, which is covalently bound to the protein via thioether bonds, as well as being ligated through the axial positions, and haem $d_1$, which appears to be linked only axially. Recent work in which NO was bound (10, 16) strongly suggests that, at least in the reduced enzyme, both the haem $c$ and haem $d_1$ moieties are axially coordinated to nitrogenous bases donated by the protein. However, the modes of axial ligation of the haem moieties in the oxidized enzyme have not yet been clearly defined and even the number of ligands bound to each haem $d_1$ moiety is still in question (17). It is within this context that the spectrum of the unperturbed oxidase and the spectral changes induced by NaF and KCN are examined.

The EPR spectrum of oxidized Pseudomonas cytochrome oxidase can be divided into resonances associated with the haem $c$ ($g = (2.98, 2.27, 1.38)$) and haem $d_1$ ($g = (2.52, 2.42, 1.73)$) moieties (5). Though both sets of resonances are visible at 12 K, those associated with the haem $c$, as with beef and tuna cytochrome $c$ (24, 25), become obscure at 90 K, while those of the haem $d_1$ remain clearly defined.

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the heme c moieties of the oxidase have axial ligation which differs from the aforementioned cytochromes c. However, the variation in crystal field parameters could result from mechanisms independent of ligand exchange. For example, it has been observed that solvent conditions can cause the low field resonance of horse heart cytochrome c to vary from $g_\parallel = 2.47$ to $g_\parallel = 3.00$ (43). Changes of this type probably result from small deformations of distance or angle in protein-iron bonds induced by strain through small conformational changes of the protein. Alternatively, similar conformational differences might be induced by a variation in the amino acid sequence of the heme c-containing proteins. Intrinsically broadening in both EPR (25) and Mössbauer (44) spectra of low spin ferric heme proteins appears to originate in a distribution of crystal field parameters. Furthermore, Fig. 4 shows a Met:Fe:His (heme c) complex which lacks intact protein conformational constraints with its tetragonal crystal field parameter approximately midway between those of the aforementioned group of cytochromes and those with His:Fe:His coordination (complex 6) (32).

If the nitrogenous base bound to the heme c in the oxidase is assumed to be histidine, the protonization/deprotonization of the N-1 nitrogen could also alter the crystal field parameters. In Fig. 4 it can be seen that the direction of change from the aforementioned group of cytochromes to the heme c of the oxidase is the same as that found for pig liver cytochrome $b_5$ (33) and heme-Im$^+$ (15, 45) under hydroxyl ion perturbation. However, less easily explained changes in the parameters have been characterized (31) between the different classes of cytochromes c (see Fig. 4).

In many prokaryotic and eukaryotic types of cytochromes c the presence of an absorption band near 695 nm is usually indicative of a Met:Fe linkage (42, 46). Unfortunately, hemec$^+$ in aqueous solution can absorb in that region (47, 48), thus precluding an unequivocal assignment. Furthermore, the nitrite reductase of *Micrococcus denitrificans* can exhibit a significant band at 705 nm (49) which probably originates from protein-bound heme $d_1$. If the axial ligation of the heme c moieties of the oxidase is not Met:Fe:His the next most likely choice is His:Fe:His as found for cytochrome $b_5$ near pH 7 (50). Another possibility supported by the ligation of an endogenous nitrogenous base, as found in the complex of NO with the heme c of the reduced enzyme, would be Met:Fe:LyS. Unfortunately the $g$ values of this complex have not yet been reported.

Interaction of the Oxidase with Exogenous Ligands—In contrast to the changes seen in the resonances attributable to heme $d_1$ in the oxidase when NaF is added, the low field heme c resonance ($g_\parallel$) shifts only from $g_\parallel = 2.98$ to 2.95. The $g_\parallel$ resonance is similar to that seen in the native enzyme but that of $g_z$, is not clearly defined. The latter appears to be a broad resonance onto which a relatively sharp heme $d_1$ signal is superimposed. Furthermore, the heme c resonances might be shifted by NaF through a solvent effect (43). No clearly distinguishable set of resonances is introduced by exposure to NaF which would suggest alteration of the unperturbed heme c axial ligation. The optical difference spectrum at room temperature induced by fluoride shows little amplitude associated with heme c visible bands, but large changes with those of heme $d_1$. This is in agreement with the low temperature EPR results. The small signal of variable amplitude at $g_\parallel = 6$ is probably caused by an iron-fluoride coordination in some of the enzyme which becomes denatured.

Addition of KCN to the oxidase causes extensive changes in the EPR resonances of both heme $d_1$ and heme c. The heme c peak at $g_\parallel = 2.98$ shifts to 3.31, a value close to that found in the cyanide complexes of horse heart cytochrome c (31) and sperm whale myoglobin (36). However, assignment of the entire structure with a peak at $g_\parallel = 2.47$ and a trough at 1.61 to the interaction of the heme $d_1$ moiety(ies) with cyanide requires some justification. Indeed, the inflection between $g_\parallel = 2.47$ and 2.35 might originate from the cyanide complex of the heme c moiety(ies) of the oxidase while the heme $d_1$ moiety(ies) exhibit an axial EPR spectrum as with NaF. A careful comparison of the $g$ values, amplitudes, and line widths of the EPR resonances of the cyanide complex of sperm whale myoglobin (36) with that of the oxidase suggests that the inflection found cannot be accounted for by the $g_\parallel$ resonance of the heme c. Furthermore, the $g_\parallel$ resonance of the myoglobin complex falls at a substantially higher magnetic field than that of the oxidase. Additional support for the interaction of cyanide with heme $d_1$ comes from the optical difference spectrum at room temperature which shows significant changes of the bands attributable to heme $d_1$ as well as heme c (8). Such arguments strongly suggest that the resonances ($2.47, 2.55$,


1.61) belong to a rhombic EPR spectrum originating from the heme $d_i$ moiety(ies) of the oxidase complexed with KCN. Other experiments show the oxidase without the heme $c$, but in the presence of KCN, exhibits both the inflection mentioned above and $g$ values almost identical with those assigned to the heme $d_i$ moiety(ies) of the intact oxidase with KCN.

Examined of the Heme $d_i$ Resonances—The assignment of $g$ values to the heme $d_i$ resonances of the unperturbed oxidase is ambiguous since the enzyme contains two heme $d_i$ moieties which demonstrate aspects of inequivalence and since NaF can induce an axial EPR spectrum. The unperturbed spectrum can be interpreted as: 1) two equivalent heme $d_i$ moieties, each exhibiting an identical set of rhombic $g$ values, or 2) the superposition of two slightly different axial EPR spectra which originate from two inequivalent heme $d_i$ moieties. The induction of an axial EPR spectrum with the oxidase provides the basis for the second interpretation to be operative. Among the several factors which suggest inequivalence are Hill coefficients significantly greater than one (8, 51) and the interpretation of reductive titration of the oxidase as being cooperative (52). In addition, recent electrophoretic evidence of a slight difference in subunit molecular weight could explain inequivalent spectra of heme $d_i$ without a cooperative interaction.

The transition from a rhombic to an axial EPR spectrum induced by NaF might be caused by a change from a dimer to a monomeric structure in which the inequivalence between the subunits (and the heme $d_i$ moieties) in the dimer is relaxed. However, the inability to find any evidence for a NaF-induced dissociation suggests that this does not occur. Indeed, it is difficult to imagine how an inequivalence could cause such a large difference between low field resonances with no detectable difference at high field.

With the interpretation that the EPR spectrum of the unperturbed oxidase originates from two equivalent heme $d_i$ sites (see "1" above), both axial and rhombic spectra are intrinsic to the metal centers. To our knowledge no axial EPR spectrum of a heme protein has been reported within the range $3.75 > g > 0.5$. Recently spectra of iron-chlorin model complexes with sulfur-containing ligands have been reported (53) which are axial. However, the range of $g$ values is much smaller than with the oxidase. Those with a $g$ value spread closer to the oxidase have nitrogens and/or oxygen as ligands; however, these spectra are rhombic and not axial. Data assembled from several sources (36, 53–55) suggest that a bound thiol(ate) causes a minimum in the range of $g$ values of iron-porphyrins and chlorins compared with other ligands and that in cases of similar axial ligation, iron-chlorins may give axial spectra and have a narrower range of $g$ values than corresponding iron-porphyrins.

Each set of $3g$ values for the various ferric low-spin iron complexes can be used to calculate (26, 29) the separations between the $d$ orbitals ($d_{xz}, d_{yz}, d_{z^2}$) before mixing by spin orbit interaction. As discussed by Blumberg and Peisach (27, 28, 54) these separations are related to both the symmetry and strengths of the iron-ligand bonds. Such separations can be calculated for heme $d_1$ of Pseudomonas cytochrome oxidase and then compared with those of other iron-porphyrins with and without modified rings. However, the $3g$ values from a particular heme protein complex can give several different sets of orbital separations (26, 54, 56), depending upon the choice of their numerical signs and upon their assignment to a particular direction with respect to the molecular (heme) axes. Therefore, a basis for a consistent choice

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**Fig. 5.** Relative one-electron orbital energy levels for the heme $d_1$ component of Pseudomonas cytochrome oxidase in the (A) absence and (B) presence of KCN, and for both (C) sulfhemoglobin and (D) myoglobin in the presence of KCN. The energy level separations are calculated from the $g$ values by the method of Taylor (29) and expressed in units of $\lambda$. The $g$ values for each heme system and the article from which each set was obtained are listed as follows: Pseudomonas cytochrome oxidase in the absence (2.52, 2.42, 1.73) and presence (2.47, 2.35, 1.61) of KCN, this work; sulfhemoglobin + KCN (2.65, 2.43, 1.65) (61); myoglobin + KCN (3.45, 1.89, 0.93) (36).

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5 B. Muhoberac, unpublished results.
6 P. Horowitz, unpublished results.
7 Mims and Peisach (57) point out that myoglobin hydroxide may be an exception in which the bonding is not represented by minimal covalency.

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Pseudomonas Cytochrome Oxidase

The energy level separations are calculated as shown in Reference 29 with choice of sign and ordering as described under "Discussion." The separations \(|d_\gamma-d_\gamma|\) and \(|d_\alpha-d_\alpha|\) are in units of \(\lambda\), and per cent changes between iron-porphyrins and iron-chlorins are listed in the last column. The \(g\) values for the sulfmyoglobin and sulfhemoglobin complexes were taken from Reference 26. The only exception was that of the cyanide complex of myoglobin obtained from Reference 36. The \(g\) values for the model complexes were obtained from Reference 15.

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| hydroxide | hemoglobin       | 2.59     | 7.58     | 2.44     | 7.80     | +2.8
|          |                  | 2.19     | 4.32     | 2.21     | 3.45     | -20.1
| cyanide   | myoglobin        | 2.60     | 5.51     | 2.44     | 8.03     | -5.6
|          |                  | 2.15     | 5.13     | 2.20     | 3.70     | -30.6
| cyanide   | myoglobin*       | 3.45     | 3.77     | 2.65     | 3.72     | -1.3
|          |                  | 1.89     | 2.85     | 2.43     | 0.96     | -66.3
| sulfide   | hemoglobin       | 2.52     | 7.60     | 2.36     | 7.79     | +42.5
|          |                  | 2.22     | 3.79     | 2.25     | 2.14     | -43.5
| sulfide   | myoglobin        | 2.58     | 6.32     | 2.38     | 7.21     | +10.6
|          |                  | 2.25     | 3.14     | 2.26     | 1.99     | -36.0
| azide     | myoglobin        | 2.82     | 6.22     | 2.61     | 6.44     | +3.5
|          |                  | 2.19     | 3.91     | 2.23     | 3.31     | -15.3
| benzimidazole | TPP(Fe\(^{3+}\)) | 2.92     | 4.25     | 2.49     | 4.22     | -0.7
|          |                  | 2.30     | 2.27     | 2.39     | 0.61     | -73.1

* The rationale behind using the ferric myoglobin cyanide \(g\) values in this calculation is as follows: 1) the two published sets of values for the cyanide complexes of ferric horse heart cytochrome c (31) and sperm whale myoglobin (36) are identical and differ significantly in high field \(g\) value from the set communicated to the authors of Reference 29 for the hemoglobin cyanide complex; 2) from the published spectrum of ferric myoglobin cyanide (36), it is clear that the amplitude of the high field \(g\) value makes its precise determination difficult.
myoglobin, sulfmyoglobin, and Pseudomonas cytochrome oxidase in the presence of KCN.

To investigate these changes further, the oxidase was incubated with several ligands (including the substrate nitrite) with the object of systematically varying the heme δ axial ligation. However, in studies which cover the power and temperature range available with our instrument, the addition of NaN₃ causes almost complete attenuation of the heme δ resonances without clearly distinguishable replacement of lost signal intensity. Fig. 2D shows that the spectrum which remains closely resembles that of the heme c of the unperturbed oxidase. We previously mentioned (10) significant changes in the optical absorption spectrum of the oxidase upon addition of NaN₃ and observed, as have others (21), that azide can also cause an attenuation of EPR signal intensity. Several factors such as relaxation effects, electron donation, dipolar broadening (68, 69), disorder at the heme δ sites (70, 71), or even antiferromagnetic coupling between two iron centers (72, 73) could preclude detection of the EPR signal.

A bonding model which explains the effect of NaF on Pseudomonas cytochrome oxidase and encompasses a true rhombic to axial transition is difficult to construct. Complications arise because the exact state of heme δ ligation prior to addition of NaF is unknown. Fluoride bound to iron in hemoglobin or myoglobin causes a high spin EPR spectrum (19). However, lack of the latter in the case of the oxidase does not necessarily imply that the fluoride interacts elsewhere, since both the chlorins differ substantially from those of the porphyrin and since Momenteau et al. (35) have observed a low spin (rhombic) spectrum with fluoride and Im as axial ligands with iron-porphyrin. They also suggest that fluoride ions can interact with a bis-Im iron-porphyrin compound at the uncomplexed nitrogens of the bound Im's and produce significant spectral changes.

It is clear from parts C and D of Fig. 6 that, under the δ value assignments previously outlined, the dₓ and dᵧ orbitals of the heme δ become degenerate upon addition of a sufficient quantity of NaF. This change is not easily interpreted as a symmetric attachment of a fluoride ion as a sixth ligand of the iron since the introduction of degeneracy in energy of dₓ and dᵧ could only be fortuitous. If the δ value assignments were altered such that an inversion of orbital energy levels occurred between iron-porphyrins and iron-chlorins, the effect of fluoride could be interpreted as generate dₓ and dᵧ orbitals. This may seem more appealing in terms of symmetric interaction of a fluoride ion with the dₓ and dᵧ orbitals. However, the effects of differential electron population as a function of direction in the chlorin and the contribution from the fifth position (axial) ligand must then be assumed to cancel. Indeed, what is of importance is that the chlorin ring tends to move the δ values closer together apparently caused by a large δ(dₓ-dᵧ) separation. It is within this framework that the modest perturbation provided by fluoride induces an axial spectrum.

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Pseudomonas Cytochrome Oxidase

Electron paramagnetic resonance study of the interaction of some anionic ligands with oxidized Pseudomonas cytochrome oxidase.
B B Muhoberac and D C Wharton


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