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Conformational Substates of Bovine Heart Cytochrome *c* Oxidase: The Modified Volpe–Caughey Variant[†]

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ABSTRACT: Kinetic and equilibrium binding studies using optically correlated, quantitative electron paramagnetic resonance (EPR) spectroscopy are reported for the reaction of a modified Volpe–Caughey preparation of bovine heart cytochrome *c* oxidase with anionic (F^-, CN^-) and gaseous (NO) ligands. A fast phase of cyanide and fluoride ligation can be attributed to an EPR-silent conformer(s), while the slow and medium phases of cyanide binding are correlated with the $g = 12$ conformer(s). Using dioxygen or ferricyanide, it is possible to modulate reciprocally the relative amounts of these two species, that together account for at least 95% of the active-site conformers of the resting form of the enzyme.

Bovine heart cytochrome *c* oxidase (ferrocytochrome *c*: oxygen oxidoreductase, EC 1.9.4.1) is an integral multisubunit metalloenzyme (2 heme *a*/2 Cu/1 Zn) of the inner mitochondrial membrane catalyzing the reduction of dioxygen to water. There are a wide variety of preparative methods currently used to extract the enzyme, most of which employ some combination of bile salts, nonionic detergents, and ammonium sulfate. Four studies have specifically investigated whether active-site heterogeneity exists in the enzyme as isolated.

In the first investigation, optical spectroscopy showed that anaerobiosis, aging, and detergents could perturb the electronic spectrum of the isolated oxidase (Muisjers et al., 1971). The authors concluded that the enzyme had an O_2-stabilized "oxidized" form with a Soret maximum at 424 nm and a so-called "420 nm" form which was stable under anaerobiosis. They further suggested that the "normal oxidized" form of Lemberg (1966) and King (1966) with a Soret maximum at 418 nm was a third species.

Brudvig et al. (1981) employed a combination of optical and electron paramagnetic resonance (EPR)[1] spectroscopies to depict four active-site conformers, e.g., a "$g = 5$" species (Shaw et al., 1978) that was formed by rapid oxidation of the fully reduced enzyme with dioxygen, and which then relaxed into an "oxygenated" form.[2] Some fraction of the latter species gave rise to a "$g = 12$" conformer, while a "resting"[3] conformer, defined by its reactivity with NO, was found only in the enzyme as isolated. The authors investigated two preparative variants of cytochrome *c* oxidase (Hartzell & Beinert, 1974; Yu et al., 1975) and quantitated the conformers in each preparation.

Naqui et al. (1984) used extended X-ray absorption fine structure (EXAFS) data and cyanide binding kinetics to show active-site differences among five preparations of cytochrome *c* oxidase (Yonetani, 1961; Frey et al., 1978; Babcock et al., 1981; Hartzell & Beinert, 1974; Yoshikawa et al., 1977).

Most recently, Baker et al. (1987) examined several preparative variants using optical and EPR methodologies like those to be described shortly and comparable to the ones first developed by Brudvig et al. (1981). The authors equated a "rapid" enzyme (referring to a component in the reaction of the oxidase with cyanide) with a "pulsed" form and a "slow" enzyme (again referring to a cyanide binding component) with a "$g = 12$" species.

The modified Volpe–Caughey enzyme (Yoshikawa et al., 1977) examined here has two major active-site components, but there may be up to three minor species if there is no "conformer overlap". Significant structural perturbations are caused by exposure of the resting enzyme to oxidants, but it is as yet not possible to definitively correlate a particular structural type with a particular chemical reactivity profile. Evidence will be presented to support the hypothesis that subsets of both the EPR-silent and the $g = 12$ conformers exist.

MATERIALS AND METHODS

Cytochrome *c* oxidase was isolated from bovine heart by using a methodology outlined previously (Yoshikawa et al., 1977) and then stored at 77 K in 0.01 M sodium phosphate, pH 7.4 (standard buffer), at a concentration of 0.5–1.0 mM in heme *a*. Enzyme concentrations are given in terms of heme *a* unless stated otherwise and are calculated by using the extinction coefficients of Yoshikawa. Enzyme activity was assayed by the method of Smith (1955) with values of 10–12 $s^{-1}\ mg^{-1}\ (3\ mL)^{-1}$ being obtained. Chemicals were of reagent or analytical grade and were not purified further unless so stated. $KF{\cdot}2H_2O$ was used in solid form while NaN_3, $NaCO_2H$, and KCN were added as neutralized 1 M solutions (the latter being freshly prepared before each experiment).

The kinetics of cyanide binding to the oxidase at 20 °C were followed at either 428 or 650 nm, starting 15 s after manually mixing an aliquot of the cyanide solution with the enzyme. The reaction of the enzyme with fluoride was monitored by measuring the change of intensity at 640 nm. The extent of

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[1] Abbreviations: Cu *a*, copper associated with cytochrome *a*; Cu a_3, copper associated with cytochrome a_3; EPR, electron paramagnetic resonance; EXAFS, extended X-ray absorption fine structure; MCD, magnetic circular dichroism.

[2] Oxygenated oxidase was originally defined as the end product observed a few minutes after a vigorous aeration of a solution of dithionite-reduced oxidase (Okunuki & Sekuzu, 1954) while pulsed oxidase (Antonini et al., 1977) was produced milliseconds after the oxygenation of a solution of oxidase reduced with excess ascorbate in the presence of cytochrome *c*. The distinction between these two forms is not advocated in this paper.

[3] Resting oxidase is defined as the end product of the procedure used to prepare cytochrome *c* oxidase.

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reaction was calculated by using the relationship $[\Delta A(640\text{–}700)_{F^-} - \Delta A(640\text{–}700)_{\text{control}}]/\Delta A(640\text{–}700)_{\text{control}}$ and expressed as either "ΔA 640–700" or "fluoride reactivity". In a typical experiment, potassium fluoride was added to 0.5 mL of a 100 μM solution of oxidase to give a final concentration of 0.5 M. After manual mixing, the sample was placed in a spectrometer thermostated at 20 °C and the optical spectrum recorded 5 min following fluoride addition.

The enzyme was always passed through a 0.22-μm Millipore filter for oxidative experiments requiring long incubations at room temperature (23–26 °C). Saturation with O_2 was achieved either by a continuous gentle flow of wet O_2 gas (Union Carbide, 99.6%) or by vigorous flushing of a 100-mL round-bottom flask for 30 min with O_2, after which the vessel was sealed.

In experiments with NO, anaerobiosis was achieved with conventional flush/evacuation techniques employing a mechanical pump and a glass gas train purged with BASF catalyst-treated, high-purity argon (Union Carbide, 99.999%). An all-glass titrator or modified Thunberg-type cuvette was attached to the train with a Cajun fitting and a 2-in. length of butyl rubber tubing. Then with the enzyme sample on ice, there followed 10 successive 4-min cycles of flush/evacuation to exchange the stale gas. An optical spectrum was recorded with the enzyme under vacuum after which the titrator was connected to an argon–NO gas train. This apparatus consisted of a tank of O_2-free argon (Union Carbide, 99.996%, 0.5 ppm of O_2) in series with a tank of NO (Union Carbide, 98.5%). The total time for sample preparation after attachment of the evacuated titrator until mixing of NO with the oxidase was about 1 h. By use of a mercury manometer, the pressure of NO admitted to the titrator was found to be 0.6 atm.

EPR spectra were obtained by using a Varian E6 spectrometer with temperature control provided by an Air Products Helitran refrigeration system. The spectrometer is on-line to an Interdata minicomputer allowing collection, storage, and retrieval of individual spectra. Spectra were recorded at X-band frequency with a modulation amplitude of 10 G, a time constant of 0.3 s, and a temperature of 12 K. The amount of adventitious copper in this preparation has been found to be 10–15% of the visible copper (Greenaway et al., 1977). Quantitation of high-spin signals was done by using metmyoglobin fluoride, while for low spins the methods of Aasa and Vanngard (1975) or DeVries and Albracht (1979) were used. MCD spectra were recorded with a Jasco-500C spectropolarimeter in a magnetic field of 1.3 T. Optical spectra were taken on a Model 17 Cary Spectrophotometer.

Pulsed oxidase (Antonini et al., 1977) was prepared by mixing 40 mM ascorbate plus 2 mM cytochrome *c* with 400 μM oxidase. This mixture was then removed from a modified Thunberg cuvette using a Hamilton gas-tight syringe and applied to a column of G-50 Sephadex overlaid with Whatman CM-52 cellulose in air-equilibrated standard buffer.

As described in detail recently (Young & Palmer, 1986), "redox-cycled" oxidase was produced by mixing an excess of purified, reduced cytochrome *c* with oxidase, incubating for 5 min, and then exposing the mixture to air. This form of the enzyme has a Soret band at 416 nm (419 nm in resting oxidase) and a diminished α-band intensity. In the experiments described here, 2 mM reduced cytochrome *c* was mixed with 200 μM oxidase, and after incubation, the two components were separated by chromatography on CM-cellulose.

RESULTS

Experiments with NO plus Anions. (*A*) *NO Reaction.* Two minutes after addition of NO to a sample of resting oxidase, the optical spectrum (Figure 1A) showed a red shift of the Soret to 423 nm, an increase of α-band and Soret band intensities, loss of the intensity of the 655-nm band, and enhancement of the β band at 550 nm.[3] At much longer times (16 h), the spectrum was qualitatively similar to that reported by Brudvig et al. (1981) with a Soret maximum at 428 nm and a 595-nm α band of increased intensity. The EPR spectrum (Figure 1B) of the 2-min sample showed that NO produced a loss of 25% in the $g = 3$ and $g = 12$ intensities,[4,5] a 5% loss of the Cu *a* intensity (not shown), and a new rhombic high-spin species accounting for no more than 1% of a heme. Examination of five different preparations of modified Volpe–Caughey enzyme failed to demonstrate a rhombic, high-spin signal with an intensity like that found for a Hartzell–Beinert preparation (Stevens et al., 1979). Pulsed and redox-cycled (Young & Palmer, 1986) modified Volpe-Caughey enzyme gave no appreciable amount of any high-spin form in the presence of NO, as has been noted by others (Brudvig et al., 1981; Stevens et al., 1979). In no experiment with NO was a signal at $g = 2.09$ seen.

(*B*) *Fluoride/NO Reaction.* When fluoride was added to a solution of resting oxidase, and the optical spectrum recorded after a 5-min incubation (Figure 2A), distinct and reproducible changes were seen which will be elaborated on later. The

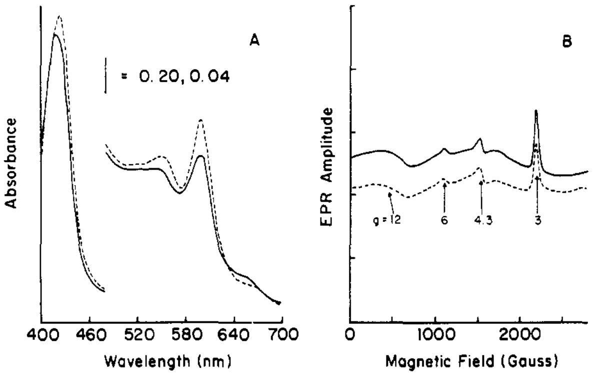

FIGURE 1: (A) Optical spectrum of 100 μM cytochrome *c* oxidase in standard buffer, before (solid lines) and 2 min after (dashed lines) addition of NO to 0.6 atm. This and all other optical spectra were taken at 20 °C. (B) EPR spectra of the same samples of enzyme, taken on an aliquot frozen in liquid N_2 immediately following optical spectroscopy. The power was 3 mW with other running conditions as outlined under Materials and Methods.

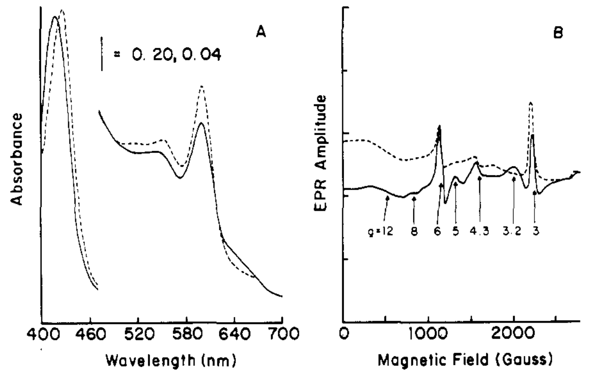

FIGURE 2: (A) Optical spectrum of 120 μM oxidase in standard buffer + 500 mM KF, before (solid lines) and 2 min after (dashed lines) addition of NO. (B) EPR spectra of the same samples of enzyme, taken as in Figure 1B.

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[4] The reversibility of the effect of NO on the optical spectrum was not determined.

[5] The *g* value used to denote the position of this transition is frequency dependent.

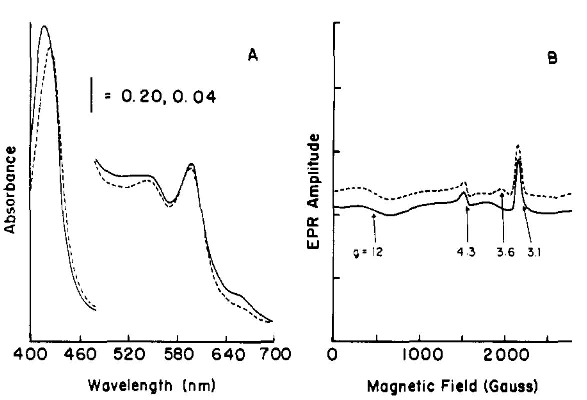

FIGURE 3: (A) Optical spectrum of 110 μM oxidase in standard buffer, before (solid lines) and after (dashed lines) addition of 90 mM KCN and NO. A 10% dilution due to the addition of KCN has not been corrected for. The spectrum was recorded 2 min after addition of NO and 25 min after addition of KCN. (B) EPR spectra of 100 μM oxidase plus 90 mM KCN (optical spectrum not shown) before (solid lines) and after (dashed lines) addition of NO, with samples taken as in Figure 1B.

choice of this time interval for the rapid binding of fluoride to the enzyme is based on the experiments of Muisjers et al. (1974). The EPR spectrum (Figure 2B) showed new features at g = 8, 6, 5, 4.3, and 3.2, like those reported previously (Brudvig et al., 1981). The g = 12, 3, and 2 intensities were found to be smaller than those in the resting enzyme by 25–35%, 25–35%, and 5–10%, respectively. NO abolished the g = 8, 5, 4.3, and 3.2 signals, diminished the height of the g = 6 species, and left the remaining signals essentially unchanged. The optical spectrum recorded 3 min after addition of NO to the fluoride-treated enzyme (Figure 2A) showed an increase in the intensity of both the α and Soret bands plus a shift of the latter to 423 nm. The 655-nm band was restored in form but not intensity, and two β bands were seen at 555 and 520 nm.

Only very small axial, g = 6 signals (2–5% of a heme) were seen with both the redox-cycled and pulsed forms of the modified Volpe–Caughey preparation in the presence of 500 mM F^- plus NO. In contrast, a significant intensity (25–58% of a heme) has been reported for an axial, g = 6 signal in oxygenated Yu oxidase in the presence of 100 mM F^- and NO (Brudvig et al., 1981).

(*C*) *Cyanide/NO Reaction.* The optical spectrum of resting oxidase (Figure 3A) was noticeably perturbed following the addition of cyanide, with the α and Soret bands (the latter located at 424 nm) demonstrating an increase of intensity, while a loss was observed for the 655-nm band. No further change occurred on addition of NO. EPR spectroscopy of cyanide-treated oxidase (Figure 3B) demonstrated a loss of intensity for the g = 12, 3, and 2 signals, as was found after addition of fluoride. Interestingly, there was an apparent shift in the g value of cytochrome a to lower field, from g = 3 to g = 3.1. NO produced no further changes in the three resonances but did give a new feature at g = 3.57.

Experiments with the g = 12 and g = 5 Species. The g = 12 transition in the EPR spectrum of bovine heart cytochrome c oxidase is well-known, and recently Hagen (1983) has reported that ferrous hexahydrate produces a similar low-field feature. This result was confirmed qualitatively, but the quantitative aspects developed by Hagen cannot be commented on since imperfections in the glass formed by 10 mM $FeSO_4$ in 10% glycerol–water gave rise to significant variability in the intensity. A preliminary study of the saturation characteristics of the g = 12 transition suggested that a feature at g = 3.8 (see Figure 5B) showed a parallel dependence on temperature and power, confirming the findings of Greenaway et al. (1977).

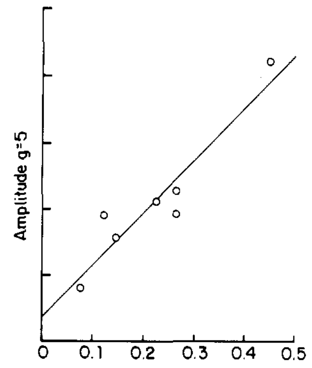

FIGURE 4: Correlation between the normalized height of the fluoride EPR signal at g = 5 (12 K) and the fluoride reactivity [ΔA(640–700) (273 K)]. Oxidase concentrations were from 70 to 155 μM in standard buffer with fluoride at 500 mM. Smaples were from five different preparations treated with air, 100% O_2, ferricyanide, or a "pulsing" protocol (see Table I).

Small and variable amounts of a species with g = 5, 1.78, and 1.69 (Armstrong et al., 1983) have been found in the resting enzyme, which is abolished by adding azide, cyanide, or NO.

Experiments with Fluoride and Cyanide: Effect of Oxidants. (*A*) *Fluoride Reaction.* The most striking effect of 0.5 M fluoride on the optical spectrum of resting oxidase was to reproducibly shift the 655-nm band to 640 nm and increase its intensity (Figure 2A). In contrast to these effects on the 655-nm band, little or no change was observed in the intensity of the α band while the Soret band underwent a 5% loss of absorbance and a blue shift of 3 nm (419 to 416 nm). There does appear to a reasonable correlation between the optical changes (the "ΔA 640–700" parameter) and the intensity of the F^--induced g = 5 EPR signal (but not the g = 6 feature), as shown in the linear regression plot of Figure 4 (r^2 = 0.87). Experiments with Cl^-, Br^-, and I^-, each at 0.5 M, indicated that the effect of F^- on the 655-nm band was specific but that minor intensity changes of the Soret and α bands could also occur with Cl^-. Passage of the fluoride-treated oxidase over an anionic exchange resin reversed the optical changes. Addition of 90 mM formate, azide, and cyanide gave half-times for loss of the fluoride feature that were on the order of minutes, with the rate of azide ~ nitric oxide > formate ~ cyanide. However, even after a 20-h incubation, 0.5 M Cl^- did not change the optical spectrum of the fluoride adduct.

The significant diminution of the intensity of the g = 12, 3, and 2 resonances which occurred when fluoride was added to the resting oxidase was not reversed by ferricyanide at 100 μM or 10 mM, whether it was added immediately before or after introduction of fluoride. Under such conditions, there was also no effect of ferricyanide on the 830-nm band.

It was possible to modulate the extent to which resting oxidase would rapidly react with fluoride (Table I). Incubation of air-equilibrated oxidase with 10 mM ferricyanide for 20 min produced a 20% loss of fluoride reactivity, increasing to 40% after 1 h and to 75% after 2 h. If the reaction with 10 mM ferricyanide was done anaerobically, there was almost a complete loss of F^- reactivity in 5 min. Incubation of resting or redox-cycled oxidase under an atmosphere of 100% O_2 at room temperature also diminished the extent of reaction. The pulsed oxidase (with no g = 12 signal) showed the highest reactivity toward F^-. In a parallel experiment, cyanide eliminated the 655-nm band of the pulsed enzyme

Table I: Fluoride Reactivity [ΔA(640–700)] for Various Species of Cytochrome *c* Oxidase

sample[a]	time (h)/temp	oxidation technique	ΔA(640–700)
prepn 1			0.235
prepn 2			0.233
prepn 3[c]			0.240
prepn 4			0.235
prepn 5[b]			0.219
pulsed 1		air	0.450
pulsed 2		air	0.490
resting 1	0.3/room temp	$Fe(CN)_6^{3-}$/air	0.192
resting 2	1.0/room temp	$Fe(CN)_6^{3-}$/air	0.146
resting 3[b]	1.8/room temp	$Fe(CN)_6^{3-}$/air	0.064
resting 4	0.08/room temp	$Fe(CN)_6^{3-}$/anaerobic	0.025
resting 5[b]	20.6/room temp	100% O_2	0.067
resting 6	21.5/room temp	100% O_2/$Fe(CN)_6^{3-}$	0.029
resting 7	22.3/room temp	100% O_2	0.076
resting 8	20.9/room temp	100% O_2	0.056
resting 9[b,c]	60.0/ice	air	0.105
resting 10[b]	20.5/room temp	air	0.147
cycled 1	22.9/room temp	100% O_2	0.059

[a] Numbers after the word prepn denote different preparations, but with other entries, numbers refer to different samples. [b] Activities were done on these samples. [c] These samples were used in the cyanide binding experiments.

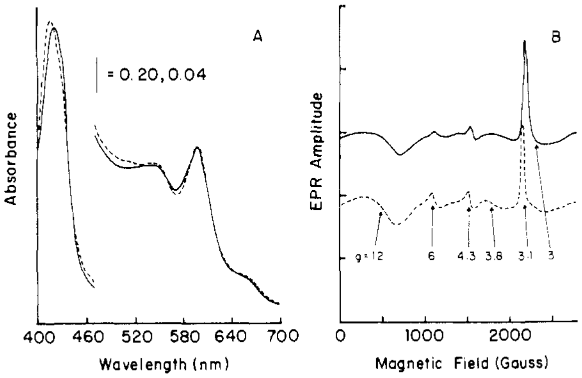

FIGURE 5: (A) Optical spectrum of 115 μM resting oxidase in standard buffer, before (solid lines) and after (dashed lines) 20 h at room temperature. (B) EPR spectra of the same samples of enzyme, taken as in Figure 1B.

(present at 90% of its resting intensity) within 5 min. This was the first indication that there was a correlation between rapid binding of cyanide and fluoride.

(*B*) *Dioxygen Reaction of Resting Oxidase.* The optical and EPR spectra of resting oxidase changed upon incubation of the enzyme under oxygen to a degree which depended on the temperature, the length of incubation, and the ratio of dioxygen to enzyme. For example, when the enzyme was incubated under 100% O_2 at room temperature for 20 h, there was a blue shift of the Soret from 419 to 416 nm, an increase of its intensity by 5%, and a decrease in the α/β ratio[6] from 1.09 to 1.06. This can be contrasted with the results of a 20-h room temperature (Figure 5A) or a 60-h ice temperature incubation under air, both of which gave an identical blue shift of the Soret band but with only a 2% increase in its intensity and no change in the α/β ratio. In all of these samples, the intensity of the 655-nm band either did not change or exhibited a small (1–2%) increase.

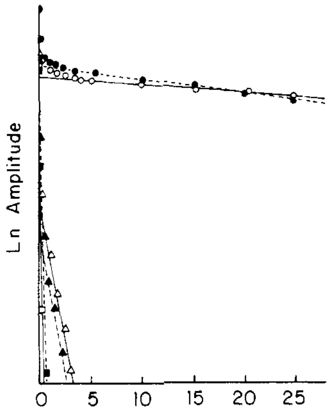

FIGURE 6: Normalized semilog plots of the kinetics of cyanide binding to resting enzyme at 428 nm (solid symbols, dashed line) and 650 nm (open symbols, solid lines). The squares, triangles, and circles denote the fast, medium, and slow phases which show rate constants (min^{-1}) at 428 and 650 nm of 3.8 and 7.0, 0.7 and 0.6, and 0.016 and 0.009, respectively. In each run, the enzyme concentration was 92 μM in standard buffer with KCN at 85 mM. Data were taken at 20 °C.

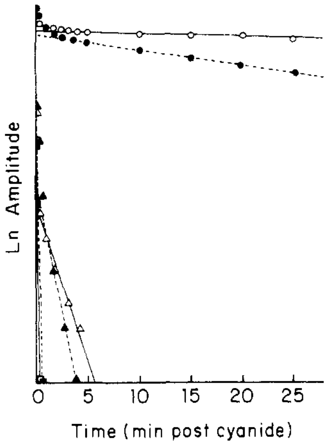

FIGURE 7: Normalized semilog plots of the kinetics of cyanide binding to "aerobically-treated" oxidase (60-h incubation on ice of the same sample of enzyme used in Figure 6) at 428 and 650 nm. The lines and symbols have the same meaning as in Figure 6. The rate constants (min^{-1}) at 428 and 650 nm for the fast, medium, and slow phases are 3.1 and 5.5, 0.6 and 0.4, and 0.018 and 0.003, respectively. Enzyme was dissolved in standard buffer plus 90 mM KCN with an oxidase concentration of 89 μM for the 428-nm run and 85 μM for the 650-nm run. Data were taken at 20 °C.

The EPR spectrum of the air-treated oxidase (Figure 5B) indicated that this treatment had increased the g = 12 amplitude by 20%, decreased that of the g = 3 resonance by 15%, and shifted the g value of the latter to lower field. Quantitation of the "g = 3" resonances showed no change in the amount of ferric cytochrome *a*. The optical and EPR changes seen with the 100% O_2 sample showed a similar but quantitatively larger trend. Enzyme activity was decreased by 10% after either hyperbaric oxygen or 10 mM ferricyanide but was unchanged by other treatments (Table I).

(*C*) *Cyanide Reaction.* The finding that oxidative perturbations of the resting enzyme could significantly reduce its ability to react rapidly with fluoride suggested that other anions might likewise be affected, and so the kinetics of cyanide binding to samples of resting (Table I, entry 3) and "aerobic" (Table I, entry 16) enzyme were examined (Figures 6 and 7). The reaction was found to be multiphasic with at least three phases present for each form. The phase distributions agree well, e.g., 44% and 48%, 8% and 10%, and 48% and 42% for the fast, medium, and slow phases at 428 and 650 nm, respectively, in the resting run (Figure 6) and, similarly, 18% and 19%, 12% and 9%, and 70% and 72% in the aerobic run (Figure 7). The agreement of the rate constants (see figure legends) is less impressive due to the steep slope of the fast

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[6] The α/β ratio is defined as $\Delta A_{598-700nm}/\Delta A_{540-700nm}$.

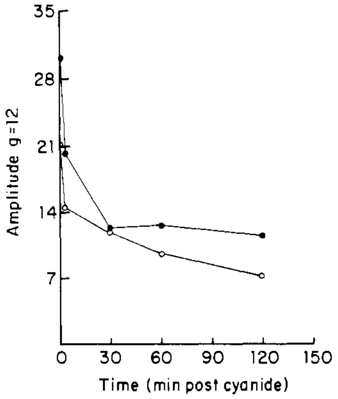

FIGURE 8: Measured height of the $g = 12$ signal as a function of time after addition of KCN (final concentration 90 mM) to a solution of resting (open circles) or "aerobically-treated" (closed circles) enzyme both with oxidase dissolved in standard buffer at 92 μM. Running conditions were as for Figure 1B.

phase and the lower signal to noise ratio in the 650-nm runs.

EPR spectra were obtained on the samples of enzyme used in the kinetic experiments, and in Figure 8, the intensity of the $g = 12$ signal has been plotted as a function of time after the addition of cyanide. The aerobic perturbation increased the initial intensity of the resting enzyme's $g = 12$ resonance by almost 50% from 21 au to 30.5 au (an au is an arbitrary unit obtained by measuring the peak to trough height of the $g = 12$ signal) and is to be compared to the 55% loss of fluoride reactivity and the 60% decrease in the intensity of the fast phase of cyanide binding. These data suggested that an inverse relation existed between the height of the $g = 12$ signal and either the fast phase of cyanide binding or the fluoride reactivity.

If the amount of slow-phase cyanide conformer (expressed in terms of heme) is equated to the $g = 12$ intensity (0.45–21 for resting enzyme and 0.71–30.5 for aerobic enzyme), values of 47 and 43, respectively, are obtained for the maximum intensity of the $g = 12$ signal. If instead the slow and medium phases are postulated to produce the $g = 12$ transition, values of 39 and 37 are found. Almost the same numbers result when "1 – the fluoride reactivity" (expressed in terms of heme with a $\Delta A_{640-700}$ of 0.5, corresponding to complete reaction with one heme) is equated to the $g = 12$ intensity, e.g., 40 for the resting sample and 38 for the aerobic sample.

These data suggest that "$g = 12$" conformers are responsible for both the slow and medium phases of cyanide binding, fluoride reactivity is correlated to a fast phase of cyanide binding, and the $g = 12$ signal cannot represent all of the cytochrome a_3 present in this preparative variant of cytochrome *c* oxidase.

DISCUSSION

This work attempts to describe a method whereby the distribution of active-site conformers present in any preparation of the protein can be determined. If the protocol is to be generally applicable, there should be nothing artifactual about either the modified Volpe–Caughey enzyme itself or the techniques used to probe the active site.

The resting enzyme can routinely be obtained in high yield (0.6 g/kg of heart muscle mince), with a high level of phospholipid (20% w/w) and excellent activity in buffer containing no added detergents (Volpe & Caughey, 1974; Yoshikawa et al., 1977; Young, 1981). Even after several weeks at room temperature under argon, sterile samples show no evidence of turbidity, and there is no loss of activity in sterile samples after 6 days on ice or 2 days at 30 °C. Velocity sedimentation[7] of the enzyme in standard buffer showed that it is polydisperse with apparent molecular weights of 321K (70%), 480K (5%), and 594K (20%). The precise phospholipid composition and degree of polydispersity (percent of monomer, dimer, etc.) of this enzyme are probably different from those of other preparative variants. As yet, no correlation (let alone a direct cause and effect relation) between either of these variables and a particular set of active-site conformers has been demonstrated; i.e., high phospholipid enzyme whether polydisperse (Yoshikawa et al., 1977) or monodisperse (Li et al., 1987) and low phospholipid enzyme whether dissolved in Tween 20 (Hartzell & Beinert, 1974) or β-dodecyl maltoside (Baker et al., 1987) have been found to contain a mixture of active-site conformers.[8]

Previous investigators have used 90 mM KCN [see Van Gelder and Beinert (1969), Thomson et al. (1977), Frey et al. (1978), Kojima and Palmer (1983), Scott et al. (1985), and Baker et al. (1987) for data obtained using 60–100 mM KCN] and 500 mM KF (Eglinton et al., 1980). As is the case here, the effects of cyanide and fluoride were found to be both specific and qualitatively identical with those found at lower concentrations (Ericinska & Wilson, 1980; Brudvig et al., 1981; Baker et al., 1987). The concentrations of fluoride and cyanide used in this study were chosen to ensure the absence of artifacts due to incomplete binding.

It seems reasonable then to state that there is nothing unusual about either the preparation or the techniques used to probe the active site. That is not to say that the only active-site conformers which can be present in cytochrome *c* oxidase are those which have been identified here, but rather that if such are present there is a way in which they can be quantified.

Reaction with NO plus Anions. The failure to observe a significant rhombic $g = 6$ signal immediately after addition of NO to this oxidase implies that only a small amount of the resting conformer defined by Brudvig et al. (1981) is present. It was originally proposed by Antonini et al. (1970) that the term "resting" be used to designate the product of whatever methodology was used to prepare the oxidase. It thus is suggested that Brudvig's resting conformer be named the "NO-induced, rhombic, high-spin" conformer.

The optical and EPR findings (in particular, the EPR silence) for the reaction of NO with resting enzyme are open to several interpretations, none of which can be favored by the results presented here, e.g., simultaneous ligation of NO to oxidized cytochrome a_3 and Cu a_3, antiferromagnetic coupling between reduced cytochrome a_3 nitrosyl and oxidized Cu a_3, or weak antiferromagnetic coupling between oxidized Cu a_3 and a ferromagnetically coupled ferricytochrome a_3 nitrosyl [see Baker et al. (1987) for a discussion of the latter].

Taken together, the optical and EPR data strongly suggest that NO and fluoride can bind to the same conformer(s) and that NO can displace fluoride. The most plausible site for fluoride binding is cytochrome a_3, given that the changes in the optical spectrum following addition of fluoride are similar to those seen for hemoglobin and myoglobin (Antonini & Brunori, 1971), while the rates for the effect of azide, formate, and cyanide on the 640-nm band are in keeping with what would be expected from the rate constant data for ligation of

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[7] M. Himmel, L. J. Young, P. G. Squire, and W. S. Caughey, unpublished results.

[8] It has yet to be demonstrated that phospholipids can interact directly with any component of the O_2 binding site. Furthermore, while "propagated" conformational effects (secondary to intramolecular protein–lipid interactions or intermolecular lipoprotein–lipoprotein interactions) may be present, they do not provide a unique explanation of all of the results presented here.

these anions to cytochrome a_3 (Ericinska & Wilson, 1980).

Only 2–3% of the oxygenated conformer of Brudvig et al. (1981) was detected in resting modified Volpe–Caughey oxidase in spite of the fact that fluoride reacts with about 50% of the cytochrome a_3 sites (Table I). The pulsed variant (not conformer) of the enzyme showed the highest fluoride reactivity and totally bound cyanide within the required 5 min. This rapid binding of cyanide to pulsed (or oxygenated) oxidase has been reported by Brittain and Greenwood, (1976). An aliquot of the same sample of pulsed enzyme mentioned above gave a $g = 6$ signal quantitating to only 5% of a heme when reacted with NO and fluoride. Thus, the oxygenated conformer of Brudvig cannot be the only one which reacts with fluoride, and it is proposed that it be named the "NO-induced, F^- high-spin" conformer.

Cyanide, like fluoride and NO, diminished the intensities of the $g = 12$, 3, and 2 resonances of the resting enzyme. When NO was introduced to the cyanide-complexed oxidase, a low-spin signal was produced which quantitated to 0.45 spin, but the kinetic data predicted a value of 0.70 spin at this time. If this difference is real, the possibility either of a competition between NO and cyanide for the same conformer or of a reductive side reaction of NO with the cyanide-bound oxidase must be considered.

Effect of Oxidants on Cyanide and Fluoride Binding. The observation that fluoride reactivity can be markedly diminished by procedures which are oxidative in nature is difficult to explain as being due to partial reduction of the resting enzyme since room temperature optical and MCD spectra show evidence of no more than 5% reduction (in total) of cytochromes a and a_3 and Cu a with fluoride and only 2–3% without it.[9] EPR spectroscopy of resting oxidase does, however, indicate a significant decrease in the intensities of the $g = 12$ and $g = 3$ signals after addition of fluoride, even though it has already been shown (Yoshikawa & Caughey, 1982) that 4 equiv is required to reduce resting modified Volpe–Caughey oxidase. Thus, these cryptic reducing equivalents must be "invisible" at 12 and 273 K in the absence of ligands and "visible" only at 12 K in the presence of ligands.

A possible hiding place could be Cu a_3, but what the chemical composition of the active site would be under such circumstances is not clear. Possibilities include an "inter-site" ferryl-cuprous/ferric-cupric equilibrium or an "intra-site" ferric-cuprous–dioxygen complex. On the other hand, the missing $g = 3$ and $g = 12$ intensities could be explained by the combination of a subset of fluoride-sensitive cytochrome a conformers plus multiple $g = 12$ conformers. The initial part of the hypothesis finds support in the observation that treatment of resting oxidase with cyanide (Figure 3; Brudvig, 1980), exposure to dioxygen (Figure 5), and reaction with fluoride (Figure 2B) all produce an apparent shift of the cytochrome a signal to lower field. The second part requires that there be a fast equilibrium between conformers which rapidly bind fluoride and some of the $g = 12$ species.

The greatest fluoride reactivity was found for the pulsed enzyme (Table I), but it does not follow that this variant of the fully oxidized (Williams et al., 1968; Brunori et al., 1979) oxidase accounts for the resting enzyme's ability to rapidly bind fluoride, if only because the Soret band of the resting oxidase is at 419 nm while that of the pulsed form is at 428 nm. This species was here used only to provide an optical end point for the reaction of the enzyme with fluoride. EPR spectroscopy and cyanide binding kinetics of samples of known fluoride reactivity indicated that a rapid phase of cyanide binding and the fluoride reactivity were measuring the same "EPR-silent" conformer(s) and that the slow and medium phases of cyanide binding were most reasonably attributed to the $g = 12$ conformer(s). Use of the term "EPR-silent" follows from the finding that in both resting and aerobic forms of the enzyme, the $g = 12$ species could not account for all of the cytochrome a_3, and thus a species which had no EPR signal had to be present. There is no necessary identity between EPR-silent conformer(s) of resting oxidase and either the pulsed variant of resting oxidase or the NO-induced, F^- high-spin conformer of resting oxidase.

Table II: Conformer Composition of Some Forms of Oxidized Cytochrome *c* Oxidase

	conformer			
species	NO/hs[a] (%)	NO/F^-/hs[b] (%)	$g = 12$[c,d] (%)	EPR-silent[e] (%)
resting	<1	2–3	46, 9	45
aerobic	nd[f]	nd	71, 10	18
pulsed	<1	2–5		100

[a] NO-induced, rhombic, high-spin conformer equivalent to the resting conformer of Brudvig et al. (1981) and the NO-induced, $g = 6$ conformer of Baker et al. (1987). [b] NO-induced, F^-, high-spin conformer equivalent to the oxygenated conformer of Brudvig et al. (1981) and may be equivalent to some fraction of the rapid conformer of Baker et al. (1987). [c] The first entry is the percentage of slow phase and the second the percentage of medium phase. [d] Equivalent to the $g = 12$ conformer of Brudvig et al. (1981) and Baker et al. (1987). [e] Some fraction may be equivalent to the rapid conformer of Baker et al. (1987). [f] Not determined.

Conformers of Modified Volpe–Caughey Cytochrome c Oxidase. The data for the resting, aerobic, and pulsed forms of the oxidized enzyme are summarized in Table II (percentages may be in error by as much as 15% of themselves). The $g = 12$ and EPR-silent species account for about 95% of the active-site conformers in the resting enzyme. These two entities are interconvertible and reversibly so with respect to the effects of redox cycling (Young & Palmer, 1986) on the resting enzyme. The reversible interconversion of resting and aerobic forms has yet to be demonstrated.

The results obtained in this investigation are in general qualitative agreement with those found by Brudvig et al. (1981) and Baker et al. (1987); however, neither of these groups stated the yield of enzyme which they obtained from any preparation of cytochrome *c* oxidase used in their studies. This would be important to know when in the future the question of the etiology and physiological significance of the various active-site conformers is investigated. The methodology used by Brudvig et al. (1981) to quantitate the $g = 12$ conformer may be subject to some amount of error due to the shift in the g value of cytochrome a in the presence of cyanide, since the quantitation formula uses an area, not an integrated intensity, for cytochrome a. It also requires that measurements be made at 16 K and 9.24 GHz because of the sensitivity of the $g = 12$ signal to frequency and temperature.

It is not immediately clear why Baker et al. (1987) were unable to reproducibly isolate the modified Volpe–Caughey enzyme (see their Table I). The major thrust of the work has to do with a new preparative variant of cytochrome *c* oxidase called HB-LC (Hartzell–Beinert, low cholate) which shows a small "NO-induced, $g = 6$" conformer (see Table II of this paper) and apparently homogeneous cyanide binding kinetics. The resting forms of the modified Volpe–Caughey and Yu et al. enzymes likewise show little immediate reaction with NO. The results presented here also indicate that the EPR-silent resting conformer(s) and the pulsed variant of modified

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[9] L. J. Young and G. Palmer, unpublished results.

Volpe–Caughey oxidase react rapidly with cyanide and fluoride but form only 0.02–0.05 spin/heme of axial, high-spin signal on addition of NO to the fluoride-treated enzyme. However, the resting Yu et al. preparation, which also reacts rapidly with fluoride, gives 0.25–0.58 spin/heme of axial, high-spin signal when NO is subsequently added. It must be then that there can be at least two EPR-silent, resting conformers and/or pulsed forms for preparations of bovine heart cytochrome *c* oxidase, and hence the assertions of Baker et al. (1987) are open to question; e.g., the homogeneous cyanide binding kinetics which they saw may be more apparent than real, either due to a failure of their methods (the "adder–mixer" technique) to discriminate between a pair of fast phases or because two structurally different conformers show similar cyanide binding kinetics.

CONCLUSIONS

The resting, modified Volpe–Caughey enzyme has been found to contain approximately equal amounts of the $g = 12$ and EPR-silent conformers, which together account for about 95% of the active sites in the enzyme. The $g = 12$ species give rise to slow and medium phases in the cyanide binding reaction of the resting enzyme. Oxidative perturbations increase the amount of such species at the expense of EPR-silent species, which are responsible for the fast phase in the reaction of the resting oxidase with either cyanide or fluoride. A methodology for quantitating the various active-site conformers of the enzyme is proposed which involves monitoring the reaction of the resting enzyme with both cyanide and fluoride, in the presence and absence of NO, using quantitative optical and EPR spectroscopy of equilibrium forms as well as optical and EPR kinetics of ligand binding. It is suggested that this protocol can be used to quantitate active-site conformers of other preparative variants of cytochrome *c* oxidase.

Registry No. F^-, 16984-48-8; CN^-, 57-12-5; NO, 10102-43-9; cytochrome *c* oxidase, 9001-16-5.

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