Diazene—A Not so Innocent Ligand for the Binuclear Center of Cytochrome *c* Oxidase†

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ABSTRACT: Diazene reacts rapidly with cytochrome *c* oxidase to reduce cytochrome *a* and Cu_A and to form a charge-transfer complex with ferric cytochrome a_3; the diazene may serve to bridge the heme iron of this cytochrome and Cu_B. The complex is characterized by an intense, optically active absorbance located at 847 nm. A similar band had been observed previously upon reduction of cytochrome oxidase with hydrazine [Markossian, K. A., Paitian, N. A., and Nalbandyan, R. M. (1983) *FEBS Lett. 156*, 235−238], but it appears that this band is actually due to the diazene produced as a result of the oxidation of the hydrazine that occurs in this process. A similar diazene to iron charge-transfer band is found following the reaction of diazene with ferric horseradish peroxidase and with hemin chloride but not with metmyoglobin.

Cytochrome *c* oxidase (CcO)[1] is the terminal enzyme in the respiratory chain of all eukaryotes and many prokaryotes. It is responsible for catalyzing the four-electron reduction of dioxygen to water; this reaction is accompanied by a transmembrane movement of protons and results in the conservation of some of the free energy dissipated in water production.

This enzyme contains two heme centers, denoted cytochrome *a* and cytochrome a_3, and two copper centers. One copper center, conventionally referred to as Cu_A, is quite anomalous in its spectroscopic properties, a consequence of its structure as a binuclear copper center (*1*, *2*) with the formal representation of $[Cu(1.5)]_2$. Oxidized Cu_A possesses a strong and characteristic EPR signal at $g = 2$ and a weak, broad optical absorbance at 830 nm. The second copper center, commonly referred to as Cu_B, is ligated by three histidine residues disposed in a somewhat unusual T-like arrangement (*1*, *2*). However, in at least one regard Cu_B is quite unique for it is located adjacent to cytochrome a_3 and indeed might well be coordinated to the iron atom of that heme by a yet to be identified, bridging ligand. This proximity of Cu_B and cytochrome a_3 results in a strong antiferromagnetic interaction between these two centers (ref *3* and references cited therein) with the consequence that both metal ions are EPR silent. While cytochrome a_3 can still be visualized by its intense optical properties, this coupling has made the experimental visualization of Cu_B problematic. Thus, despite the many spectroscopic studies on this enzyme, little can be reliably concluded about the physical properties of this second copper center.

In 1983, Markossian et al. (*4*) reported that the addition of hydrazine to cytochrome oxidase, both in the presence and absence of oxygen, led to the reduction of the enzyme, and more interestingly, the reaction was accompanied by the appearance of a previously unreported, relatively intense absorption band at ca. 845 nm. It was suggested that this new band reflected changes induced in Cu_B by hydrazine. However neither the mechanism of the interaction of hydrazine with this enzyme nor the possible mode of interaction of hydrazine with Cu_B was established.

In view of the potential value of a simple spectroscopic probe of Cu_B, we have pursued these observations. Our data show that the important reagent in this system is not hydrazine per se but diazene (also called diimide), the product that arises from the two-electron oxidation of hydrazine. We will show that both hydrazine and diazene can function as electron donors to cytochrome oxidase but that only diazene can form high affinity complexes with the enzyme and that these complexes involve the cytochrome a_3 component of the binuclear center.

MATERIALS AND METHODS

Cytochrome *c* oxidase preparation was isolated from beef heart by the modification of the method of Hartzell and Beinert (*5*) developed in this laboratory (*6*); this procedure yields enzyme that reacts in a single rapid phase with cyanide (cf. ref *6*) and has an absorbance maximum at 424 nm. Enzyme concentration is stated in terms of cytochrome aa_3 using an absorbance coefficient of 158 mM^{-1} cm^{-1} at 424 nm. Fully reduced enzyme was prepared by anaerobic addition of a slight excess of dithionite (in 100 mM Hepes, pH 8.0) to enzyme dissolved in 50 mM Hepes and 0.1% DM, pH 8.0, and incubating for 30 min at 4 °C.

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[1] Abbreviations: CcO, cytochrome *c* oxidase; UV−vis, ultraviolet−visible; EPR, electron paramagnetic resonance; mcd, magnetic circular dichroism; cd, circular dichroism; HOMO, highest occupied molecular orbital; LUMO, lowest unoccupied molecular orbital; HALS, highly anisotropic low-spin spectrum; DM, dodecylmaltoside; HRP, horseradish peroxidase; Ches, 2-(cyclohexylamino)ethanesulfonic acid; TMPD, tetramethyl *p*-phenylenediamine.

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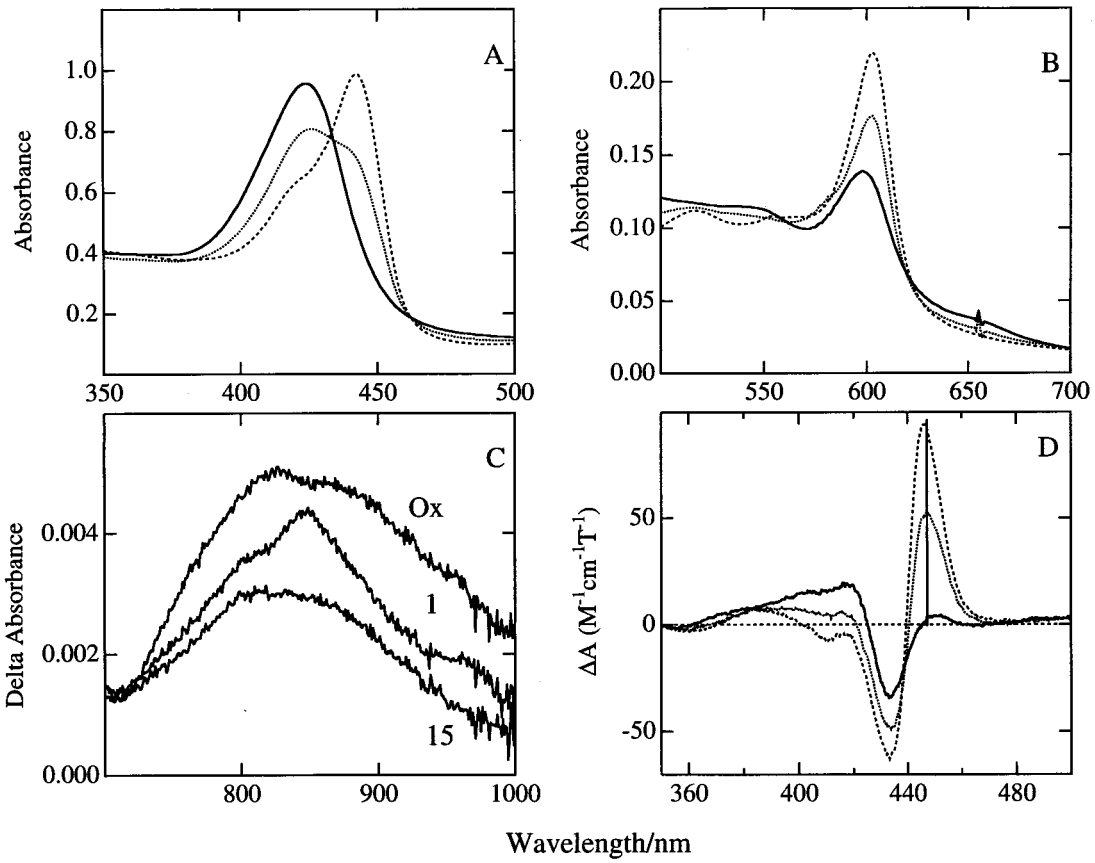

FIGURE 1: Reaction of an equimolar quantity of diazene with cytochrome oxidase under anaerobic conditions. The enzyme concentration was 6 μM for the spectra shown in panels A, B, and D and 7.5 μM for the near-IR measurements shown in panel C. Panels A, B, and D: (—) oxidized enzyme; (•••) 1 min after addition of diazene (as azodiformate); (- - -) 180 min after addition of diazene. The vertical line in panel D indicates 447.5 nm. Panel C: The spectra are indicated by Ox, oxidized enzyme; 1, 1 min after addition of diazene; 15, 15 min after addition of diazene. The spectra in panel C are differences calculated with respect to the spectrum recorded after 60 min. The UV−vis spectra were recorded at the time indicated while the mcd spectra were initiated at the time stated and required 10 min to be recorded. The buffer was 50 mM Hepes, pH 8.0, containing 0.1% DM, and the temperature was 23 °C.

Water was purified by passage through a Milli-Q system. Hydrazine was bought from Aldrich Chemical Co. and was used without further purification. Horseradish peroxidase, myoglobin, and hemin chloride were purchased from Sigma. All other materials were of reagent or AR grade and were used as received.

Potassium azodiformate was prepared from azodicarbonamide (Aldrich) and aqueous KOH as described by Thiele (*7*). The yellow solid was isolated by precipitation from ethanol, washed with methanol and ether, air-dried, and stored in a desiccator. The material appears to be stable indefinitely provided it is kept dry. Stock solutions were prepared in 0.2 M KOH and standardized spectrophotometrically using a molar absorbance at 403 nm of 33 M^{-1} cm^{-1} (*8*).

Diazene was generated in situ from the acid-catalyzed hydrolysis of potassium azodiformate; this was accomplished by adding small portions of a stock solution of potassium azodiformate in 0.2 M KOH to the appropriate enzyme sample dissolved in 50 mM Hepes and 0.1% DM, pH 8.0. It was verified that these small additions of KOH did not affect the pH of the reaction mixture.

Enzyme solutions were made anaerobic in a Thunberg-style optical or EPR cuvettes by exposure to 4−5 cycles of vacuum and highly purified argon finishing under argon. Solutions of diazene and hydrazine were made oxygen-free by extensive purging with prepurified argon. Reagents were loaded into nitrogen-purged Hamilton syringes that were mounted onto the cuvettes using a positive pressure of argon to minimize the entrance of air.

Absorbance data were recorded in a variety of conventional and diode array spectrophotometers. CD and mcd spectra were recorded with a Jasco-500C spectrometer equipped with a 1.3 T electromagnet. EPR spectra were obtained using a Varian E-6 spectrometer operating at 9.27 GHz and a temperature of 12 K. EPR samples were frozen by plunging the EPR tube into an acetone−solid CO_2 slush; freezing was complete within 1 s or so.

Calculations were carried out using MacSpartan Plus version 1.17 (Wavefunction Inc., Irvine CA). The vendor has enhanced the PM3 semiempirical method to handle transition metal ions.

RESULTS

Reaction of Oxidized Cytochrome Oxidase with Diazene. In our initial studies on the reaction of hydrazine with cytochrome oxidase, it became readily apparent that hydrazine is indeed an effective reductant for cytochrome oxidase, as had been observed previously (*4*). However from comparisons of the reaction of oxidized and reduced enzyme with hydrazine, we came to suspect that the formation of the 847 nm species observed by Markossian et al. (*4*) required the presence of an oxidation product of hydrazine.

The most likely candidate for this product is diazene, which can be obtained from the two-electron oxidation of hydrazine according to

$$H_2N{-}NH_2 \Leftrightarrow HN{=}NH + 2H^+ + 2e^- \quad (1)$$

Because diazene is very unstable in neutral aqueous solution, the only practical way to introduce this reagent is via the addition of azodiformate. This latter compound is quite stable in alkaline solution but at neutral pH decomposes to diazene via an acid-catalyzed reaction with a rate of ca. 40 s^{-1} (*8*):

$$[CO_2N{=}NCO_2]^{2-} + 2H^+ \Leftrightarrow HN{=}NH + 2CO_2 \quad (2)$$

Consequently, all references to the addition of diazene to the enzyme should be understood to mean that it is the corresponding quantity of azodiformate that is actually added.

Diazene itself is a potent two-electron reductant:

$$HN{=}NH \Leftrightarrow N_2 + 2H^+ + 2e^- \quad (3)$$

and can also decay by both dismutation

$$2N_2H_2 \rightleftharpoons N_2H_4 + N_2 \quad (4)$$

with a rate constant of about 2×10^4 M^{-1} s^{-1} (*8*) and by decomposition

$$2N_2H_2 + 2H^+ \rightleftharpoons 2H_2 + N_2 \quad (5)$$

Because of reactions 3−5, the lifetime of diazene at neutral pH is quite short. From the data of Stanbury (*8*), we estimate that the half-life of 1 mM diazene at pH 8 is no more than about 5 s.

The reaction of diazene with cytochrome oxidase was probed initially by incubating separate samples of oxidized enzyme with fixed molar ratios of diazene in the absence of oxygen and recording the appropriate spectra.

A UV−vis spectrum recorded 1 min after the addition of 1 equiv of diazene (i.e., 2 electron equiv) leads to partial reduction of the enzyme (Figure 1). In the Soret region, there is a large decrease in absorbance at 424 nm (the wavelength maximum of the oxidized enzyme) and a corresponding increase at 443 nm, a large increase in the α-band at 605 nm, some decrease of the broad feature at 830 nm due to Cu_A, and loss of the 655 nm feature of oxidized a_3. However only a slight trace of the 847 nm band is apparent with this stoichiometric ratio. Subsequently, the 426 and 830 nm features decay further with concomitant increases at 443 nm and at the α-band and the disappearance of both the 830 and 847 nm features.

Immediately after the addition of diazene, the Soret mcd had a maximum at 447.5 nm (Figure 1D). This wavelength is characteristic of the mcd spectrum of fully reduced enzyme in which both hemes contribute equally to the observed spectrum; the individual spectra of reduced cytochrome *a* and (high-spin) reduced cytochrome a_3 exhibit individual Soret maxima at 452 and 445 nm, respectively (*9*). It would appear that with 1 equiv of diazene both hemes are reduced to about the same extent; from a comparison of the observed mcd intensity at 447 nm with that of fully reduced enzyme (*9*), this is estimated to be about 35%. Note that it took about 8 min to record the mcd spectrum; there were only small additional absorbance changes during this time.

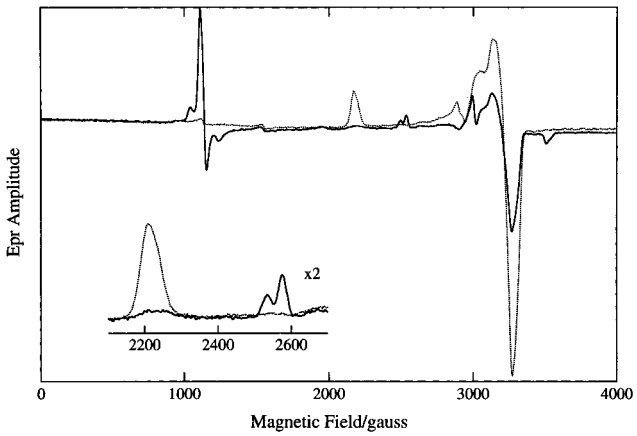

FIGURE 2: Effect of an equimolar amount of diazene on the EPR spectrum of cytochrome oxidase under anaerobic conditions. 50 μM diazene (as azodiformate) was mixed with 50 μM oxidized cytochrome oxidase under argon in an anaerobic EPR tube and the reaction mixture frozen after 120 s; (···) oxidized enzyme; (—) enzyme + diazene. The inset shows the low-spin region enlarged 2×. The EPR spectra were recorded at 12 K using a microwave power of 1 mW and a modulation amplitude of 20 G. The spectrometer frequency was 9.273 GHz.

However, an EPR spectrum (recorded at 12 K) of a sample rapidly frozen about 120 s following the anaerobic addition of 1 equiv of diazene revealed almost complete loss of the $g = 3$ low-spin signature of cytochrome a^{+3} and a 60% decrease in the amplitude of the EPR signal due to Cu_A (Figure 2). The difference in the degree of reduction of cytochrome *a* and Cu_A as assessed optically at room temperature with that observed at 12 K implies that significant electron rearrangement has occurred upon lowering the temperature.

In addition to the changes in EPR of cytochrome *a* and Cu_A, a large $g = 6$ high-spin and two new low-spin signals appeared (Figure 2). The latter had g_z values of 2.61 and 2.65; their combined area is about 16% of that of the low-spin feature of the cytochrome *a* present in the oxidized sample. A crude estimate of the relative amounts of the $g = 2.6$ and the $g = 6$ signals suggests that they are present in approximately equal concentrations.

Two minutes after the addition of 2 equiv of diazene to cytochrome oxidase, the intensity of the 847 nm band is substantial with an absorbance of about 4300 M^{-1} cm^{-1}, close to the value of 4900 M^{-1} cm^{-1} obtained when this band is maximally developed (Figure 6B); at this time the absorbance changes in the Soret and α-band reveal only a small additional reduction of heme relative to the 1:1 case. With diazene to enzyme ratios of 2:1 (or larger) the initial mcd spectrum peaks at 451−452 nm (Figure 3D), implying that it is mainly cytochrome *a* that is reduced. With this 2:1 ratio, the mcd intensity at 452 nm is 51 M^{-1} cm^{-1} T^{-1}. While this value is similar to that found in the 1:1 case, when combined with the wavelength maximum it implies that cytochrome *a* is ca. 73% reduced and that there is relatively little reduction of a_3. The subsequent changes in mcd that occur over the next 60 min yield a difference spectrum that has the shape and intensity consistent with the reduction of 70% of cytochrome a_3. During this time the 847 nm band also decays. Immediately following the addition of diazene

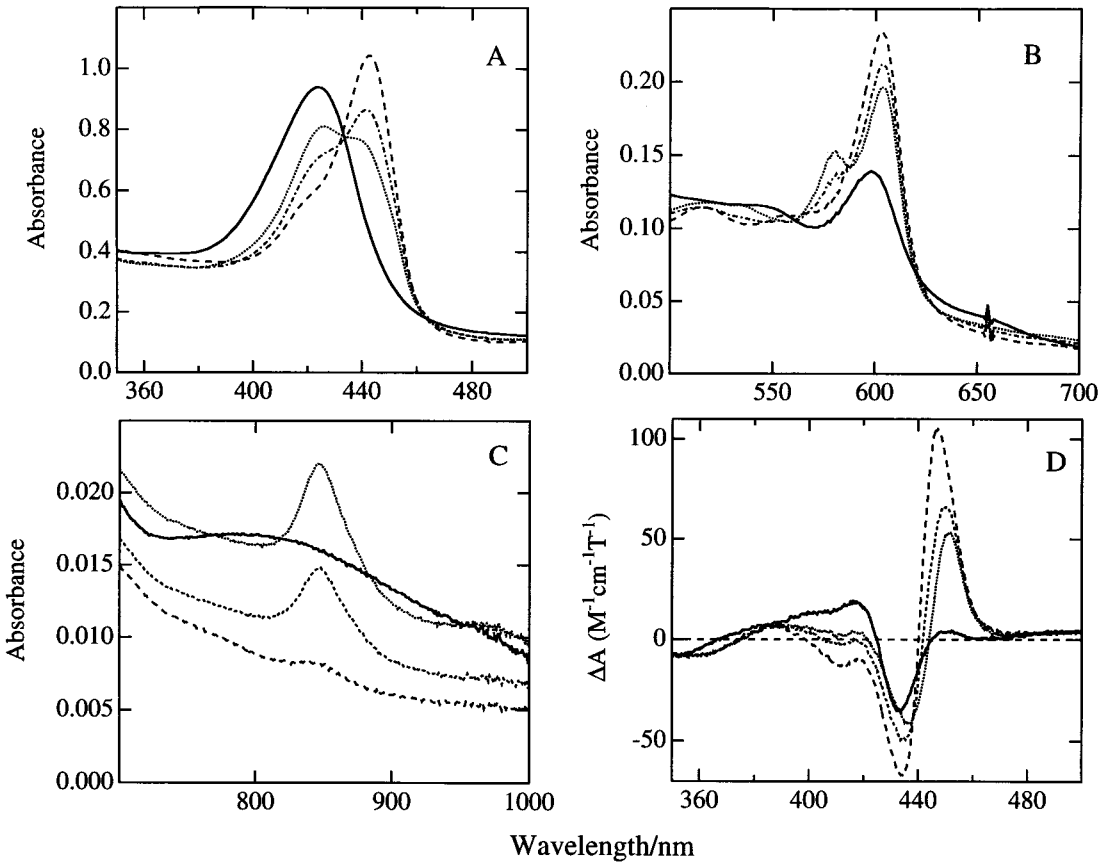

FIGURE 3: Reaction of cytochrome oxidase with 2 equiv of diazene under anaerobic conditions. The enzyme concentration was 6 μM for the spectra shown in panels A, B, and D and 7.5 μM for the near-IR measurements shown in panel C. Panels A, B, and D: (—) oxidized enzyme; (···) 1 min after addition of diazene (as azodiformate); (- - -) 11 min after addition of diazene; (— —) 180 min after addition of diazene. Panel C: (—) oxidized enzyme; (···) 1 min after addition of diazene; (- - -) 15 min after addition of diazene; (— —) 60 min after addition of diazene. The spectra in panel C are absolute spectra and not difference spectra as were shown in Figure 1C. Other conditions as in Figure 1.

the α-band shows a small shoulder at 578 nm, which is characteristic of a complex between ferrous cytochrome a_3 and diazene, as described below. This peak decays rapidly with time.

An EPR spectrum of a sample of enzyme reacted with 2:1 diazene for about 80 s showed that both the $g = 3$ signal and the Cu_A signal had been eliminated while the intensity of both the $g = 6$ signal and the low-spin signals at $g = 2.6$ were about one-half that observed with the equimolar sample (data not shown).

Increasing the ratio of diazene to cytochrome oxidase to 4:1 or 8:1 yields optical data similar to that obtained with the 2:1 ratio, with only slight additional changes at 426, 443, and 605 nm. At an initial ratio of 8:1 the mcd spectrum recorded immediately peaked at 452 nm and had a Soret intensity of 70 M^{-1} cm^{-1} T^{-1} (Figure 4); these values imply that cytochrome *a* is fully reduced.

This was verified by comparison of the mcd spectrum of this rapidly reduced component with that of the mixed-valence formate derivative (*9*). This latter species has the redox configuration $a^{+2}a_3{}^{+3}$, resulting from the stabilization of cytochrome a_3 in the ferric state following the binding of formate. The Soret mcd spectra of the mixed-valence formate derivative produced by addition of TMPD and ascorbate to enzyme pretreated with 0.1 M sodium formate together with enzyme reduced with 8 equivalents of diazene have essentially identical amplitudes and line shapes (Figure 4). As the mcd spectrum of the mixed-valence formate derivative is diagnostic of cytochrome a^{+2} (*9*), it can be

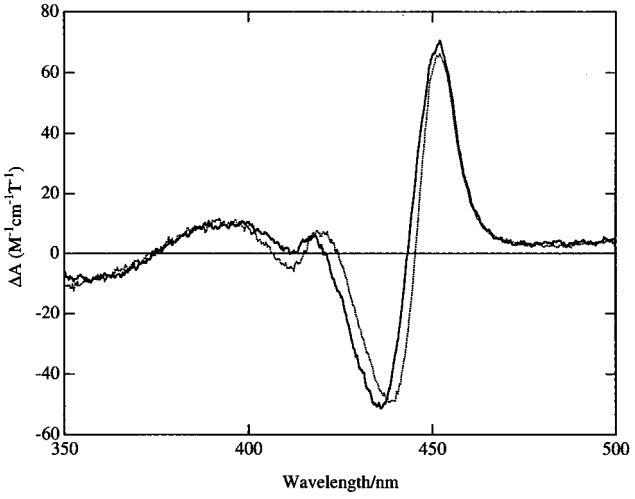

FIGURE 4: Comparison of the mcd spectrum of the cytochrome oxidase reacted with an excess of diazene with the mixed-valence formate derivative of cytochrome oxidase. (—): mcd spectrum of 7.5 μM cytochrome oxidase 1 min after addition of 60 μM diazene (as azodiformate) at pH 8.0 under anaerobic conditions; (···) mcd spectrum recorded immediately after addition of 100 μM TMPD and 1.0 mM ascorbate to 7.3 μM cytochrome oxidase under aerobic conditions. This enzyme had been incubated overnight with 0.1 M sodium formate at 4 °C. The buffer was 50 mM Hepes at pH 8.0 containing 0.1% DM.

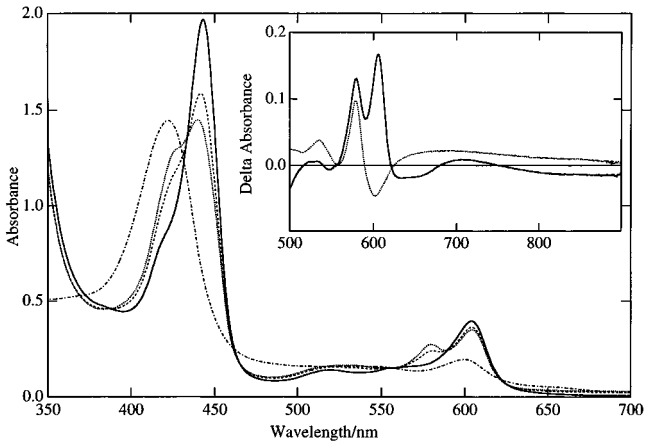

FIGURE 5: Reaction of excess diazene with reduced cytochrome oxidase. 10 μM cytochrome oxidase was made anaerobic and reduced with a slight excess of a stock solution of dithionite, and the optical spectra were monitored until the enzyme was fully reduced. Then diazene (as azodiformate) was added anaerobically to a final concentration of 80 μM. (− · −) oxidized enzyme; (···) 1 min after diazene; (- - -) 60 min after diazene; (—) reduced enzyme. Inset: Difference spectra after 1 min of reaction calculated with respect to oxidized enzyme (—) and reduced enzyme (···). The buffer wa 50 mM Hepes at pH 8.0 containing 0.1% DM.

confidently concluded that cytochrome *a* is essentially completely reduced in the early stages of reaction with this small excess of diazene.

Reaction of Diazene with Reduced Cytochrome Oxidase. Addition of 1 mM diazene to cytochrome oxidase previously reduced with a small excess of dithionite produces a marked decrease in the amplitude of the Soret maximum with the development of a pronounced shoulder at 426 nm, a slight decrease in the amplitude of the α-band, and the development of a well-resolved new peak at 578 nm that appears to be associated with a weak feature at 534 nm (Figure 5). These 534 and 578 nm features are particularly apparent in the difference spectrum calculated with respect to reduced enzyme (Figure 5, inset); in this difference spectrum the strongest feature is at 579 nm. In addition, a weak band centered at 690 nm develops immediately following the addition of diazene to the reduced enzyme. Optical features similar to the new 578 nm band and the broad 690 nm absorption have been previously observed upon addition of diazene to deoxymyoglobin (*10*).

By analogy to the qualitatively similar changes that are observed when cyanide or carbon monoxide are added to reduced enzyme, the 578 and 534 nm chromophores are presumed to be the α and β bands, respectively, of a low-spin complex of cytochrome a_3^{2+} coordinated by diazene (the energy difference of these two wavelengths is 1480 cm^{-1}). These new spectral features are not stable; after about 1 h the enzyme had reverted about 30% of the way back to the fully reduced unliganded state.

Optical Activity of the 847 nm Species. The formation of the 847 nm species is accompanied by an intense negative Cotton effect (Figure 6A); this cd has a similar width as the parent absorption (Figure 6B) but its maximum is slightly blue-shifted, from 847 to 840 nm. This shift may be an instrument artifact because the data were recorded at fixed slit-width of 200 μm, which results in a fairly large, wavelength dependent, spectral dispersion. The cd intensity is -15 M^{-1} cm^{-1}, which leads to a Kuhn anisotropy factor

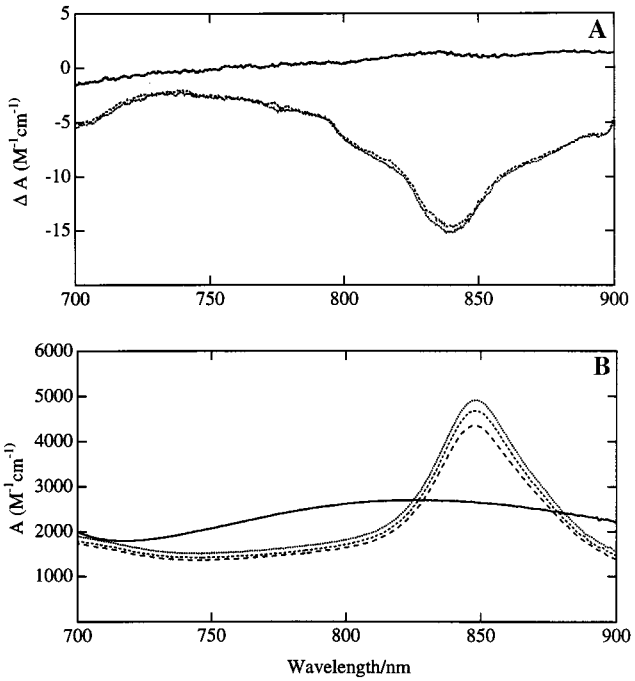

FIGURE 6: Comparison of near-IR CD and absorption of 100 μM enzyme reacted with 1.5 mM diazene (as azodiformate) anaerobically. Panel A, CD: (—) oxidized enzyme; (···) 5 min after mixing; (- - -) 10 min later. Panel B, UV−vis: (—) oxidized enzyme; (···) before first CD spectrum; (- - -) after first CD spectrum; (− −) after second CD spectrum.

($\Delta A/A$) associated with this Cotton effect of -0.003; thus, the parent optical transition has both significant electric dipole (absorption) and magnetic dipole (cd) contributions. No mcd could be detected within this absorption band at room temperature.

Effect of Cyanide on the Reaction of Cytochrome Oxidase with Diazene. To confirm that the species represented by the 847 nm absorption band is associated with the binuclear center, we have tested the effect of cyanide on the ability of diazene to induce this spectrophotometric change.

Enzyme that had been treated with 1 mM cyanide overnight on ice exhibited the normal spectrum of the cyanide derivative (Figure 7) most clearly seen as a shift of the Soret maximum from 424 to 428 nm. Subsequent addition of 1 mM diazene had very little effect on the spectrum (Figure 7) with only 3% decrease in absorbance at 428 nm immediately while the increase in absorbance at 443 nm after 65 min of reaction (Figure 7, inset) was only 15% of that observed within 1 min when cyanide was omitted (Figure 3). A similar result was obtained when hydrazine was used as reductant.

In a complementary experiment, cytochrome oxidase was reacted with 10 mM hydrazine for 1 min and a UV−vis spectrum recorded; a large 847 nm absorbing species was observed. Then 1 mM cyanide was added and a second spectrum recorded immediately. The absorbance at 847 nm was completely eliminated within 1 min, the time taken for the spectrophotometer used to scan from 900 to 847 nm (data not shown).

Other common inhibitors of cytochrome oxidase have a much less dramatic effect. Thus following pretreatment of cytochrome oxidase with 10 mM azide the observed spectral changes produced by the addition of diazene are quite similar

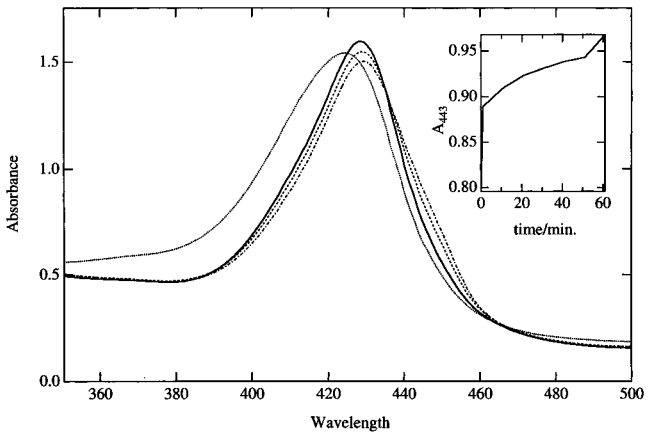

FIGURE 7: Effect of cyanide on the reaction of cytochrome oxidase with diazene. 10 μM cytochrome oxidase was incubated overnight on ice with 1 mM potassium cyanide. The solution was made anaerobic, the optical spectrum recorded, 80 μM anaerobic diazene (as azodiformate) added, and spectra recorded with time. (···) oxidized enzyme; (—) cyanide treated enzyme; (- - -) after 1 min with diazene; (— · —) 60 min with diazene. The inset shows the time dependence of the increase in absorbance at 443 nm; the slight jump is taken to be the reaction of enzyme unreacted with cyanide. The buffer was 50 mM Hepes at pH 8.0 containing 0.1% DM.

to those observed with uninhibited enzyme. There are however two important differences: First the presence of azide almost completely suppresses the formation of the 578 nm species, and second, the rate of decay of the 847 nm species is accelerated by about a factor of 2. Addition of 1 mM azide to enzyme treated with diazene for 15 min also leads to the immediate loss of the weak 578 nm band together with a decreased stability of the 847 nm species.

Reaction of Oxidized Cytochrome Oxidase with Diazene in the Presence of Oxygen. During the first 30 s following addition of 1 mM diazene to oxidized cytochrome oxidase under aerobic conditions, there are only small absorbance changes in the Soret region and no detectable decrease of the 830 nm band. Subsequently, there is fairly rapid partial reduction of the enzyme ($k \sim 0.3$ min^{-1}) as judged by the increase in absorbance at 443 nm and at 605 nm and by the loss of absorbance at 830 nm, the latter being replaced by the 847 nm band with a molar absorbance of 4600 M^{-1} cm^{-1}. The feature at 580 nm apparent in the data obtained under anaerobic conditions (Figure 5) is not seen in these spectra.

The enzyme reduced in this way is relatively stable. No change in absorbance at 443 nm is detected after 70 min while the 847 nm band had only lost about 20% of its maximum intensity in this time. These data are consistent with the enzyme entering an aerobic steady state that lasts for 30 s before conversion to the diazene-stabilized intermediate.

Reaction of Horseradish Peroxidase, Myoglobin, and Heme with Diazene. In an effort to establish the identity of the metal ion coordinated with diazene, we have examined the reaction of diazene with several heme systems. Upon treatment of oxidized horseradish peroxidase with 0.5 mM diazene under anaerobic conditions, the Soret shifts from 403 to 423 nm; the weak charge-transfer band at 647 nm is replaced by a pair of poorly resolved features at 540 and 560 nm; and the broad weak feature centered at 900 nm is replaced by a narrow, relatively intense band at 850 nm with an absorbance of 5500 M^{-1} cm^{-1} (Figure 8). The rate of

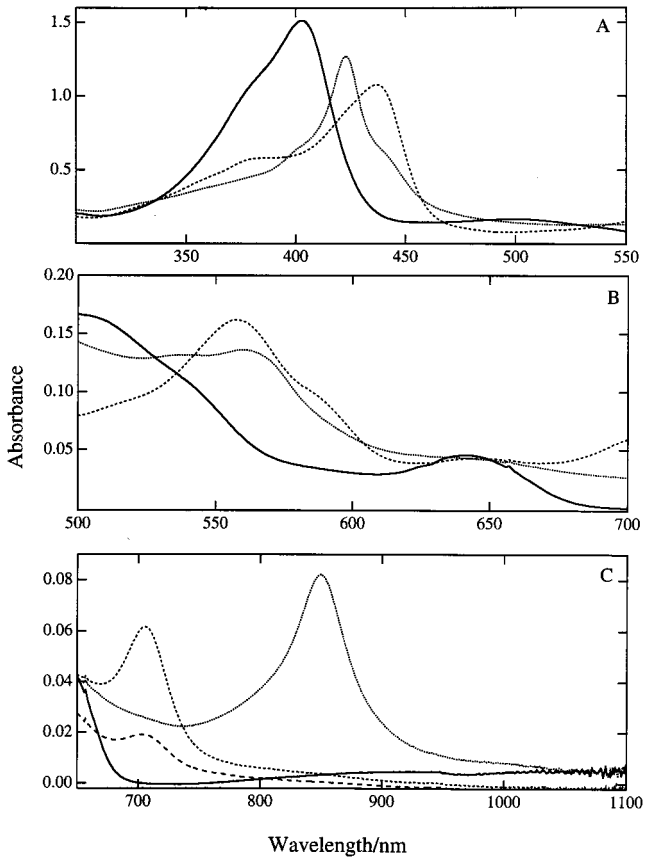

FIGURE 8: Optical changes following reaction of 500 μM diazene (as azodiformate) with 15 μM horseradish peroxidase in the Soret (A), visible (B), and near-IR (C) regions. (—) oxidized HRP; (···) after 1 min; (- - -) after 40 min; (— —) after 60 min (panel C only). The buffer was 0.1 M potassium phosphate, pH 7.4.

formation of the 850 nm band is quite pH dependent with both the rate of formation and decay being slowed upon raising the pH; at pH 7.4 the 850 nm absorbing species is formed immediately but has essentially disappeared by 20 min being replaced by a second narrow and intense band at 705 nm, which in turn decays with a half-time of about 1 h (Figure 8). During this time the Soret band slowly shifts from 423 to 437 nm, and the pair of bands at 550 and 560 are converted to a strong band at 558 nm with a shoulder at 585 nm; these wavelengths are characteristic of reduced horseradish peroxidase (*11*). Because of the instability of the 850 nm species, it was not possible to record a high-quality CD spectrum; however, we were able to demonstrate that this band is optically active and to estimate its Kuhn anisotropy factor to be at least −0.0004.

An EPR sample on a pH 9.4 sample frozen 1 min after the addition of diazene showed that the large anisotropic $g = 6$ high-spin signal characteristic of ferric horseradish peroxidase was substantially eliminated and replaced by two low-spin signals (Figure 9). One low-spin signal had g-values of 2.97, 2.13, and 1.67; the other was of the HALS type (*12*) and had a low-field g-value of 3.58. The non-HALS species had a rhombicity and a tetragonal field similar to the azide complex of cytochrome a_3. From the areas of the two low-spin species and after correction for differences in transition probability (*13*), an estimate of the relative amounts of the two species suggests that the $g = 2.9$ species is about 25% more intense that the $g = 3.58$ species. In addition to the two heme contributions, a radical-like signal

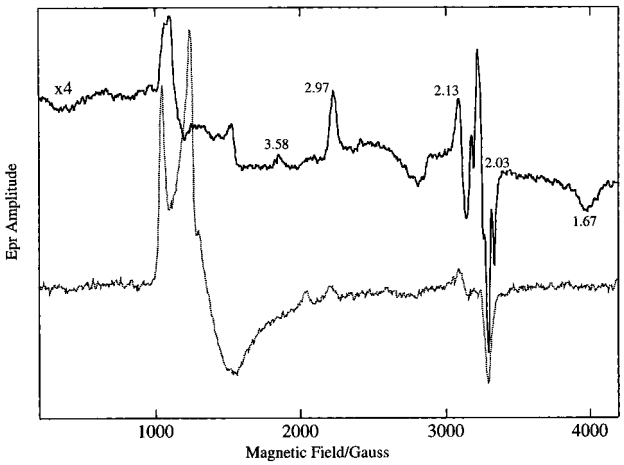

FIGURE 9: EPR spectra of 74 μM ferric HRP (···) and 100 μM HRP + 1 mM diazene (as azodiformate) recorded at 4× sensitivity (—). Enzyme and diazene were mixed together anaerobically and frozen after 1 min of reaction. EPR spectra were recorded at 12 K using a microwave power of 1 mW and a modulation amplitude of either 5 (HRP) or 20 G (HRP + diazene). The microwave frequency was 9.273, and the buffer was 0.1 M Ches-KOH, pH 9.4. Selected *g*-values are displayed on the figure

of unknown origin is observed at $g = 2.027$; this signal is relatively symmetric and shows well-resolved structure.

When the same experiment is conducted with metmyoglobin, strikingly different absorbance changes are observed. There is a major loss of Soret absorption with a shift in the maximum from 409 to 435 nm and the development of broad and relatively unresolved bands from 500 to 1000 nm (data not shown). Hanstein et al. (*10*) had previously shown that diazene binds to reduced myoglobin and other ferrous heme systems in a straightforward manner.

A neutral solution of hemin chloride exhibits a split Soret band due to the formation of μ-oxo-dimers; the maxima are at 365 and 385 nm. Addition of 1 mM diazene to such a solution converts the Soret to a single peak at 395 nm, produces a well resolved pair of bands in the visible at 528 and 559 nm, and produces an intense band at 850 nm with an absorbance of 6400 M^{-1} cm^{-1} (data not shown). However, we were unable to detect any CD within this band; the Kuhn *g*-factor must be smaller than 0.00004. The characterization of the reaction of diazene with heme proteins other than cytochrome oxidase is being pursued.

Reaction of Cytochrome Oxidase with Hydrazine. Essentially identical spectral changes to those described above have been obtained with hydrazine, in confirmation of the earlier work of Markossian et al. (*4*) and Kubota and Yoshikawa (*14*). The only additional piece of information is that hydrazine is a much weaker ligand for ferrous cytochrome a_3 than is diazene. Thus, we find that the 578 nm band of low-spin ferrous cytochrome a_3 is fully developed using 1 mM diazene. By contrast, we find that the corresponding band elicited by hydrazine is undetectable at 1 mM hydrazine but is clearly present using 100 mM hydrazine. From the data of Kubota and Yoshikawa (*14*), we calculate that the dissociation constant for the binding of hydrazine to reduced oxidase is ca. 150 mM.

DISCUSSION

While the incentive for undertaking this study was the expectation that a possible optical probe of the Cu_B component of the binuclear center would be forthcoming, the data described above make it most probable that cytochrome a_3 is the principal site of attachment of diazene. The inhibition by cyanide of the reaction between cytochrome oxidase and diazene clearly implicates the binuclear center as the relevant locus while the marked similarity of the optical properties of the near-IR band elicited by diazene in cytochrome oxidase, horseradish peroxidase, and hemin chloride identify heme iron rather than Cu_B as the principal site of ligation. These similarities include the exceptionally close match in wavelengths, the similar intensities, and the observation that in both proteins the band has strong optical activity with the same sense of chirality.

Although diazene is highly reactive as a free molecule having a lifetime measured in seconds at neutral pH (*8*), it appears to be substantially stabilized when coordinated to a metal, for the half-time for decomposition of the 847−850 nm absorbing species is 15−20 min with cytochrome oxidase and several minutes with horseradish peroxidase and hemin chloride. Indeed Lehnert and colleagues (*15*) have prepared two μ-1,2-iron(II)−diazene complexes that were sufficiently stable to be characterized by X-ray crystallography. This stabilization is undoubtedly due to the nature of the metal−ligand bond.

The inherent instability of diazene precludes an accurate measurement of the dissociation constant; nevertheless, the observation that almost complete formation of the 847 nm species is accomplished at a 2:1 ratio of ligand to enzyme allows us to estimate that the dissociation constant approaches 1 μM. This puts diazene as among the most avid of ligands for the binuclear center. For comparison, for fully oxidized enzyme the dissociation constant for cyanide is 1 μM (*16*), that for azide is 64 μM (*17*), and that for formate is about 500 μM (*18*; W. Li and G. Palmer, unpublished); the dissociation constant of cyanide for partially reduced enzyme is less than 0.1 μM (*19*).

The origin of this strong binding can be understood from the nature of the frontier molecular orbitals of diazene (Figure 10). The HOMO is a good σ-donor and can make a strong bond with the vacant d_{z2} of the low-spin iron of a_3; the LUMO is a π-acceptor and is able to accept electrons from a filled d_π orbital of this metal ion. This combination of good σ-donation together with π-back-bonding is similar to the behavior of carbon monoxide in ferrous carbonyls though in the present case the iron is in the ferric oxidation state; this is evident by the absence of the mcd of high-spin ferrous a_3 and the 578 nm absorbance of low-spin ferrous a_3.

The strong stabilization provided by the HOMO rationalizes why iron rather than copper is the preferred target. In Cu(II), d_{z2} is fully occupied and thus not able to function as a σ-acceptor. However as copper can still function as a π-donor, it is entirely possible that diazene is able to bridge the two metal ions with a strong bond to a_3 and a weaker bond to Cu_B. Such a bridging arrangement would promote an exchange interaction between the two metal ions and explain why there is not a substantial EPR signal due to either cytochrome a_3 or Cu_B in the 847 nm species.

The stable form of diazene has the trans stereochemistry in solution, and this stereochemistry is maintained in the two symmetric μ-1,2-iron(II)−diazene complexes that have been structurally characterized (*15*). To determine whether this is likely to be the case when diazene bridges a heterometallic

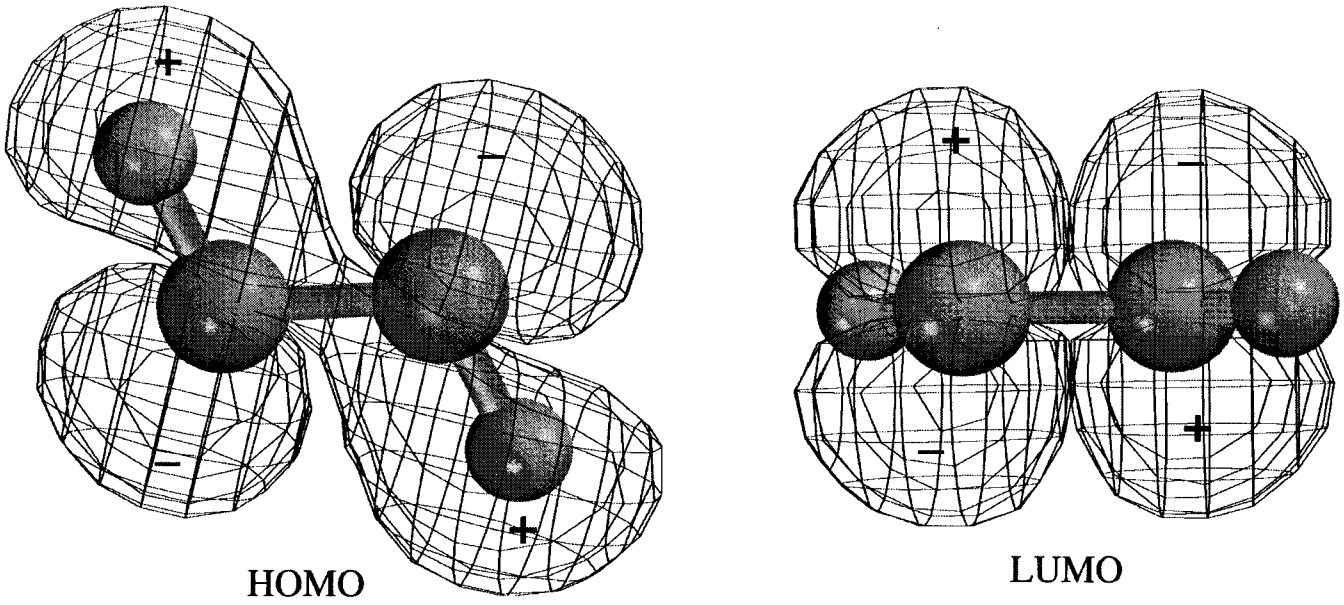

FIGURE 10: Frontier molecular orbitals of diazene. Left: Highest occupied molecular orbital, HOMO; right, lowest unoccupied molecular orbital, LUMO. The orbitals were generated using the PM3 semiempirical method of MacSpartan Plus. Color versions of this figure and Figure 11 can be found on the cytochrome oxidase web site (http://www-bioc.rice.edu/~graham/CcO).

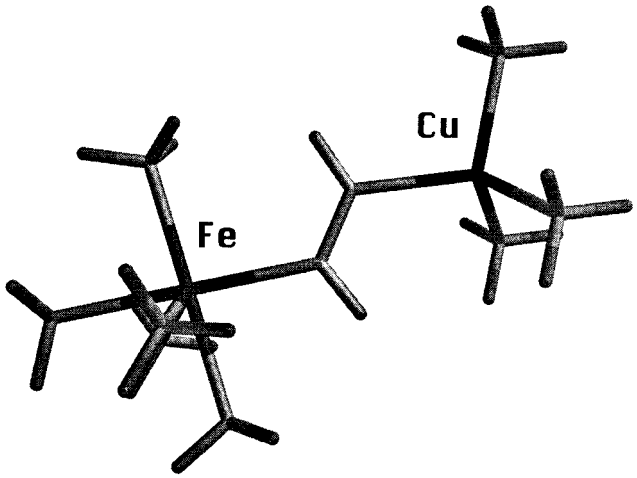

FIGURE 11: Optimized structure of $Fe(III)(NH_3)_5(N_2H_2)Cu(II)(NH_3)$ used as a model for the iron−copper binuclear structure of cytochrome oxidase bridged by diazene. The structure was minimized using the PM3 semiempirical method of MacSpartan Plus.

center, we have performed semiempirical calculations on $Fe(III)(NH_3)_5(N_2H_2)Cu(II)(NH_3)_3$ (Figure 11) as a simplified model for diazene bound in a bridging mode between the Fe and Cu centers of cytochrome oxidase. The structure was first constructed using the graphical builder of MacSPARTAN Plus, and the structure was initially refined by molecular mechanics using the SYBYL force field. Refinement was then continued using the PM3 semiempirical method, which has been modified for use with transition metal ions. Upon optimization, the model refines to a structure in which the bridging diazene has trans stereochemistry (Figure 9) as was observed in the two μ-1,2-iron(II)−diazene complexes (*15*). In this simplified model, the iron to copper distance optimizes to 4.9 Å, a distance comparable to the separation of the two metal ions in the X-ray structures of the enzyme (*1, 2*). This value clearly supports the notion that diazene bridges the two metal ions even though coordination to iron is likely to be stronger than it is to copper.

Given that the ligand is a strong reductant and the iron is in the ferric oxidation state, it is reasonable to believe that the 847 nm band is a diazene to iron (ligand to metal) charge-transfer band. To rationalize both the large absorbance and the high Kuhn factor of the 847 nm transition, we use the following logic. The LUMO [called π^*_v by Lehnert et al. (*15*)] will overlap strongly with one of the two dπ orbitals of the low-spin ferric iron; following Lehnert et al. (*15*), we assign this orbital as d_{yz}. This overlap results in the formation of two orbitals with composition $\pi^*_v-d_{yz}$ and $d_{yz}-\pi^*_v$. We postulate that the former is more stable and is constituted primarily by the ligand orbital while the latter, at higher energy, has a much larger contribution from the metal d orbital. Thus a transition from $\pi^*_v-d_{yz}$ to $d_{yz}-\pi^*_v$ will have significant amounts of electric dipole character from that part which involves the π orbitals as well as magnetic dipole character from the change in participation of the d orbitals. The former contribution accounts for the intense absorbance while the combined contributions of the electric dipole and magnetic dipole components results in the observed optical activity. In this way, the spectroscopic signature resulting from diazene coordination to the two heme proteins can be plausibly rationalized. The presence of optical activity in the protein−diazene complexes and not in the complex of diazene with free heme simply reflects the fact that a dissymmetric environment is still required for optical activity to exist, in this instance, for the transition induced by right circularly polarized light to be preferred over that with left circularly polarized light.

The absence of mcd can be rationalized within this proposal because the ground state ($\pi^*_v-d_{yz}$) resides energetically below the highest occupied orbital of the complex (which is where the unpaired electron is located) while the excited state is nondegenerate. The former circumstance ensures that mcd C-terms will be absent while the latter means that mcd A-terms will also be absent. As these are the only source of significant mcd intensity in heme

complexes, the absence of any detectable mcd is understandable.

One puzzle is the inability to demonstrate a 847 nm absorbance when diazene is reacted with myoglobin. The observed spectral changes of a severe loss in Soret absorbance and acquisition of a broad and diffuse absorbance persisting to 1000 nm suggest that the porphyrin ring has been modified. It may be relevant that cytochrome oxidase and horseradish peroxidase both contain a positive charge in the distal pocket-Cu_B and an arginine residue, respectively. This positive charge is missing in horse heart myoglobin. We plan to examine the reaction of a myoglobin engineered to introduce an arginine residue into this location.

The potency of diazene as reductant is apparent from the experiments with graded amounts of this reagent, which also point to complications in the reaction. Thus mcd spectra recorded after the addition of 1 equiv had peaked at 447.5 nm, the wavelength maximum characteristic of the fully reduced enzyme implying that both hemes contribute in comparable amounts to the observed spectrum. Comparing the intensity of this peak with that of fully reduced enzyme suggests that the hemes are each about 35% reduced. This is to be expected for equilibrium potentiometry at room temperature, which shows that both hemes are reduced equally over the whole range of applied potential (*20*).

However EPR data recorded 2 min after the addition of 1 equiv of diazene show that all of cytochrome *a* and more than half of Cu_A is reduced; furthermore, the appearance of heme signals reasonably attributed to uncoupled cytochrome a_3 suggest that about 30% of Cu_B is also reduced, thus accounting for essentially all of the diazene added. From this crude electron counting, it would seem that neither $a_3^{2+}-Cu_B^{2+}$ nor $a_3^{2+}-Cu_B^{1+}$ is present to any appreciable extent. The absence of the former is confirmed by the lack of any signals reasonable attributed to Cu_B in the EPR spectrum. It thus appears that lowering the temperature shifts the electron distribution resulting in further reduction of cytochrome *a* at the expense of oxidizing cytochrome a_3. Such a temperature-dependent rearrangement is not inherently surprising, and indeed Jensen et al. (*21*) observed similar shifts in the redox state of cytochrome *a* and Cu_A upon the relatively slow freezing of oxidase, which was in an aerobic steady state.

A related puzzle is the observation that the reduction of the enzyme by the first equivalent of diazene is rapid while the second equivalent produces a stable product in which the binuclear center is oxidized. At first sight, mechanisms in which the initial reaction is the reduction of the binuclear center followed by the redistribution of electrons among all the redox centers would appear to be ruled out because such a mechanism implies that the reduction of the binuclear center is thermodynamically favorable and kinetically allowed. The data suggest that the adduct of the oxidized binuclear center and diazene is relatively stable but nevertheless can function as an reductant for cytochrome *a*. Thus when cytochrome *a* is oxidized, bound diazene can be oxidized, but once cytochrome *a* is reduced, diazene bound to cytochrome a_3 remains (relatively) intact. As the reduction potential for diazene is extremely negative, this stability implies that the reduction of the binuclear center by diazene is kinetically forbidden. The origin of the kinetic restriction is by no means clear.

However, this cannot be the whole story because enzyme treated with 2 equiv of diazene does show the transient formation of the 578 nm absorbance of $a_3^{2+}N_2H_2$. The available data do not allow us to distinguish between the alternative possibilities: $a^{+3}Cu_A^{+2}a_3^{+2}-N_2H_2-Cu_B^{1+}$ and $a^{+2}Cu_A^{+1}a_3^{+2}-N_2H_2-Cu_B^{1+}$. The former configuration implies that $a^{+2}Cu_A^{+1}$ and $a_3^{+3}Cu_B^{2+}$ are in equilibrium and that the equilibrium can be displaced toward a reduced binuclear center upon ligation of diazene. But this equilibrium is not observed at higher molar ratios of diazene to enzyme. Clarifying the mechanism of this complicated behavior will undoubtedly require extensive kinetic analysis.

The ability to block the reduction of the enzyme by preincubating it with cyanide is an unusual result as most reductants are believed to donate electrons their electrons first to Cu_A and then to cytochrome *a*, i.e., by the physiological route. However, the absence of any substantial spectrophotometric changes at 443, 605, or 830 nm following the addition of diazene to the cyanide-treated enzyme implies that electron transfer from diazene to cytochrome oxidase is almost completely inhibited in this derivative. This relatively unique behavior can be attributed in part to the small size of this reagent, which is comparable to that of hydrogen peroxide, a molecule known to react readily with the binuclear center (*22*−*25*); commonly used reductants are significantly larger and thus unlikely to access the pocket containing the binuclear center. However this does not explain the inability of diazene to reduce Cu_A when access to the binuclear center is blocked. While the two-electron reaction at the binuclear center leading to the production of N_2 is undoubtedly energetically favorable, the one-electron reaction with Cu_A would yield $N_2H_2^+$ (or possibly N_2H) and not dinitrogen as the product; the energetics of this one-electron reaction are not known. A further consideration is the stability of free diazene. The reaction of hydrazine with anaerobic oxidized enzyme is complete in less than 1 min; however, reaction of hydrazine with cyanide-treated enzyme requires 40−60 min for completion. Thus hydrazine, which can also react readily with the binuclear center, also reduces Cu_A, though at about 2% of the former rate. As hydrazine is fairly stable in aqueous solution, it can survive this length of incubation; as already mentioned, free diazene has a lifetime of only a few seconds (*8*) and would thus decompose before any significant reaction had occurred.

This also raises the issue of the origin of the optical changes, which occur over many minutes. These appear to be a combination of two processes; a slow electron rearrangement and further reduction. Slow electron rearrangements are well-known. For example, while the reduction of cytochrome *a* by cytochrome c^{+2} occurs on the millisecond time scale, the subsequent transfer of the electron to cytochrome a_3 is much slower (*26*); likewise, it is well-known that the full reduction of the enzyme by reagents such as dithionite can take as long as 20 min, again because of a slow reduction of cytochrome a_3. Further reduction can have two origins: (i) reaction of the diazene stabilized by virtue of its attachment to cytochrome a_3 and (ii) reaction of any hydrazine produced by the dismutation of the diazene when used in significant excess.

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