Cytochrome cd_1 from *Paracoccus pantotrophus* Exhibits Kinetically Gated, Conformationally Dependent, Highly Cooperative Two-Electron Redox Behavior†

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ABSTRACT: Each monomer of the dimeric cytochrome cd_1 nitrite reductase from *Paracoccus pantotrophus* contains two hemes: one *c*-type center and one noncovalently bound d_1 center. Potentiometric analysis at 20 °C shows substantial cooperativity between the two redox centers in terms of their joint co-reduction (or co-oxidation) at a single apparent potential with an *n* value of 1.4 ± 0.1. Reproducible hysteresis is demonstrated in the redox titrations. In a reductive titration both centers titrate with an apparent midpoint potential of +60 ± 5 mV while in the oxidative titration the apparent potential is +210 ± 5 mV. However, at 40 °C the reductive and oxidative titrations are shifted such that they almost superimpose; each has $n = 2$. A kinetically gated process that can be correlated with oxidation/reduction-dependent ligand changes at the two heme centers, previously seen by crystallography, is implicated. In contrast, a semi-apoenzyme, lacking the d_1 heme, exhibits a reversible redox titration with a midpoint potential of +242 ± 5 mV ($n = 1$). The data with the holoenzyme show how redox changes can themselves generate a gating of the type that is minimally required to account for redox-linked proton pumping by membrane-bound cytochromes.

Cytochrome cd_1 is a respiratory nitrite reductase, the reaction product being nitric oxide, found in the periplasms of many denitrifying bacteria. Electrons are delivered to the enzyme via the cytochrome bc_1 complex and water-soluble *c*-type cytochromes and/or cupredoxins; the latter are also located in the periplasm (*1*). In this enzyme the *c*-type cytochrome acts as the electron-accepting center while X-ray crystallography has shown that the d_1 heme provides the active site (*2*). High-resolution crystal structures have been obtained by X-ray diffraction for the cytochromes cd_1 from *Paracoccus pantotrophus* [formerly *Thiosphaera pantotropha* (*3*)] and *Pseudomonas aeruginosa* (*4*, *5*). Contrary to the expectation that a single class of enzymes should always have the same central structural features, the enzymes from these two sources differ significantly. The oxidized *P. pantotrophus* enzyme has His/His axial ligation at the *c* heme iron and Tyr/His axial ligation at the d_1 heme iron. Upon reduction, the ligation of the *c* heme iron switches to His/Met concomitant with dissociation of the tyrosine ligand to leave the d_1 heme iron 5-coordinate (*2*). In contrast, the enzyme from *P. aeruginosa* has His/Met coordination at the *c*-type cytochrome center in both oxidation states while the d_1 heme coordination is hydroxide/His in the oxidized state and thought to be vacant/His in the reduced state (*5*, *6*). Spectroscopic studies in solution have either directly confirmed, or been consistent with, these different ligation patterns in the enzymes from the two species, in either oxidation state (*7*−*10*).

Several determinations of the reduction potentials of the redox centers in cytochrome cd_1 from *P. aeruginosa* have been reported. There are some discrepancies between these reports, but in general the potential of the *c*-type cytochrome center is given as approximately +290 mV (*11*), consistent with it receiving electrons from a pathway involving the cytochrome bc_1 complex plus soluble periplasmic redox proteins. Reduction potentials of +234 mV for the *c* heme and +199 mV for the d_1 heme have been reported for the homologous enzyme from *Pseudomonas nautica* (*12*). In contrast, there are no literature reports of the reduction potentials of the heme centers in the cytochrome cd_1 from either *P. pantotrophus* or *Paracoccus denitrificans*, even though the latter has been studied extensively for many years and has identical heme ligands to the former (*13*). His/His ligation of a *c*-type cytochrome heme center usually correlates with a reduction potential much lower than +200 mV because histidine is less effective than methionine in stabilizing the ferrous state (*14*). For example, the potentials of the His/His-coordinated *c*-type cytochrome centers in *Desulfovibrio vulgaris* cytochrome c_3 have midpoint potentials that range from −80 to −380 mV vs SHE[1] (*15*). A potential of considerably below +200 mV would not readily be compatible with a role for the *c*-type cytochrome center as a point

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[1] Abbreviations: PES, phenazine ethosulfate or *N*-ethyldibenzopyrazine ethyl sulfate salt; PMS, phenazine methosulfate or *N*-methylphenazonium methosulfate; DAD, 2,3,5,6-tetramethyl-1,4-phenylenediamine; SHE, standard hydrogen electrode; EPR, electron paramagnetic resonance.

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of entry of electrons into *P. pantotrophus* cytochrome cd_1 from the respiratory chain. Of the putative in vivo physiological electron donors to this nitrite reductase, pseudoazurin has a reduction potential of +230 mV (*16*) and cytochrome c_{550} +265 mV (*17*). This consideration, together with the need to understand the energetics of the ligand switching from His/His to His/Met upon reduction, required that the redox properties of the cytochrome cd_1 from *P. pantotrophus* be investigated. This is the subject of the present paper.

EXPERIMENTAL PROCEDURES

Cytochrome cd_1 from *P. pantotrophus* was purified as described elsewhere (*16*, *18*). The semi-apo form of the enzyme was prepared as described by Kobayashi et al. (*18*). Potentiometric titrations of the holoenzyme were carried out at 20 °C in a Belle Technology glovebox under a nitrogen atmosphere, with O_2 maintained at less than 2 ppm. A 3 μM cytochrome cd_1 solution was prepared in 0.1 M sodium phosphate buffer (8 mL, pH 7.0). To this were added the following soluble mediators (*19*) to concentrations of 15 μM: anthraquinone-2-sulfonic acid sodium salt ($E^{\circ\prime} = -225$ mV), 2-hydroxy-1,4-naphthoquinone ($E^{\circ\prime} = -145$ mV), phenazine ethosulfate ($E^{\circ\prime} = +55$ mV), and phenazine methosulfate ($E^{\circ\prime} = +80$ mV). DAD [2,3,5,6-tetramethyl-1,4-phenylenediamine ($E^{\circ\prime} = +260$ mV)] was also added to a final concentration of 15 μM from a stock solution in 33% ethanol.

The solution was titrated electrochemically according to the method of Dutton (*19*) using NADH or sodium dithionite as reductant and potassium ferricyanide as oxidant. Robinson et al. (*20*) reported efficient reduction of cytochrome cd_1 from *P. denitrificans* by addition of NADH in the presence of PMS. After each reductive/oxidative addition, a 10−15 min equilibration time was allowed. Spectra, between 800 and 300 nm, were recorded on a Shimadzu 1201 UV−visible spectrophotometer contained within the anaerobic environment. The electrochemical potential of the sample solutions was monitored using a CD740 meter (WPA) coupled to a Pt/calomel electrode. The electrode was calibrated using the Fe^{III}/Fe^{II} EDTA couple as a standard [+108 mV vs SHE (*21*)], and all values are reported versus the standard hydrogen electrode.

The enzyme was initially titrated in the reductive direction. It was fully reduced at ca. −50 mV vs SHE and was then reoxidized up to potentials of ca. +350 mV vs SHE. The holoenzyme was cycled between its fully oxidized and reduced states, typically three times (with negligible signs of enzyme denaturation), to confirm that observed differences in the reductive and oxidative directions were reproducible. Complete acquisition of data required 16−20 h.

Data were analyzed using Origin (Microcal). The degree of reduction, measured by the absorbance at a given wavelength, Abs_x, was fitted to eq 1, derived by extension

$$Abs_x = A_x + B_x\left[\frac{10^{[(E_o-E)RT]/nF}}{1+10^{[(E_o-E)RT]/nF}}\right] \qquad (1)$$

of the Nernst equation in combination with the Beer−Lambert law. A_x is the absorbance of the fully oxidized enzyme, B_x is the total increase in absorbance on reduction,

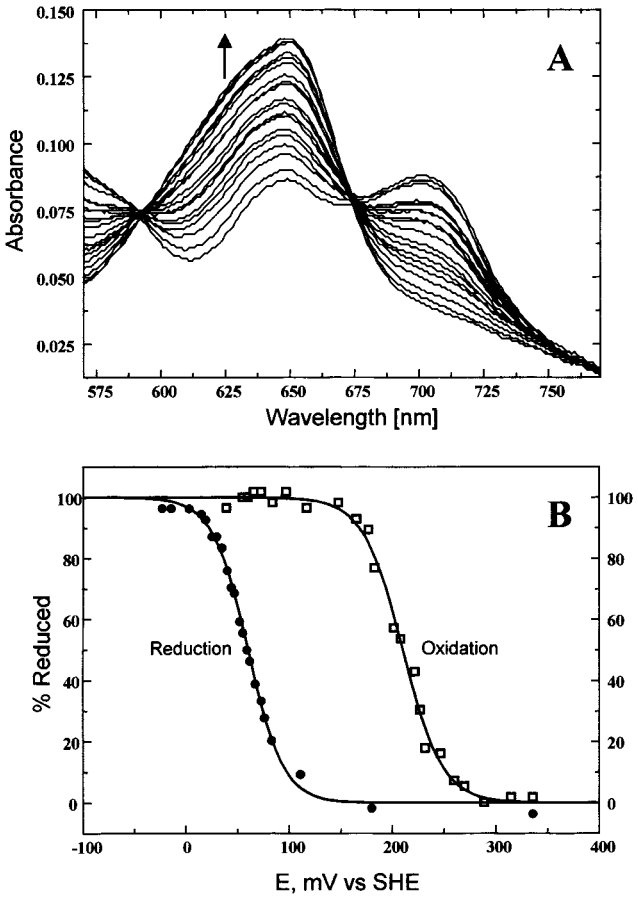


FIGURE 1: (A) Reductive potentiometric titration of cytochrome cd_1 from *P. pantotrophus* conducted in 0.1 M sodium phosphate buffer, pH 7.0 at 20 ± 2 °C. The arrow indicates the direction of the absorbance change at 624 nm during the course of the titration with NADH. (B) Plot of absorption at 624 nm versus electrode potential fitted to eq 1 as described in Experimental Procedures. Data recorded at 20 ± 2 °C. From the reductive data (●), $E'_{red} = 60 \pm 5$ mV and $n = 1.5 \pm 0.1$, and from the oxidative data (□), $E'_{ox} = 210 \pm 5$ mV and $n = 1.3 \pm 0.2$.

E_o is the formal oxidation or reduction potential (E_{ox} and E_{red}, respectively, mV vs SHE), and E is the measured potential (mV vs SHE). The value of RT/nF represents the slope of the Nernst plot, where n is the number of electrons transferred in the process and RT/F is equal to 58.1 mV at 20 °C.

Potentiometric titrations of the semi-apoenzyme were carried out according to Dutton (*19*) using a cuvette over which a stream of humidified argon was continually passed; absorbance changes were measured with an Aminco DW2 spectrophotomer. PES, PMS, and DAD were chosen as mediators (*19*). Sodium dithionite was used as reductant and potassium ferricyanide as oxidizing agent. A silver/silver chloride electrode connected to a Hanna Instruments HI9321 meter was used to monitor electrochemical potential. The electrode was calibrated using a $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ standard (*22*).

RESULTS

When cytochrome cd_1 from *P. pantotrophus*, in the oxidized form as purified, was exposed to a reductive titration at 20 °C, the series of visible absorption spectra shown in Figure 1A was acquired. The wavelength range covers that

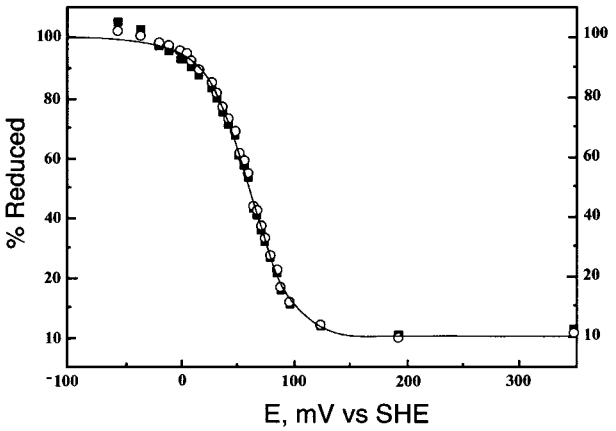


FIGURE 2: Reductive titration of cytochrome cd_1 at 20 °C followed at wavelengths diagnostic of the *c* (420 nm, ○) and d_1 (463 nm, ■) type hemes. Data were fitted to eq 1.

over which the spectra can be unambiguously assigned to the d_1 heme center (*18*). The reductive titration monitored at 624 nm (and at other wavelengths shown in Figure 1A) could be fitted to the Nernst equation with a midpoint potential of $+60 \pm 5$ mV and an *n* value of 1.5 ± 0.1 (Figure 1B). During an oxidative titration of the fully reduced enzyme, reoxidation was not observed until significantly more positive potentials, giving a titration curve quite distinct from the reductive titration. In this case a fit to the Nernst equation of $+210 \pm 5$ mV was obtained with an *n* value of 1.3 ± 0.2. Further cycles of oxidation and reduction (up to three) generated identical titration curves. The lack of direct reversibility was further demonstrated by the finding that it was impossible to reverse a reductive or oxidative titration when it was stopped part way. For example, if an oxidative titration was stopped when the enzyme was approximately 50% in the oxidized state, and the potential decreased by ca. 100 mV, no changes in the spectrum were observed. Similar reductive, and subsequent oxidative, titrations were observed with both NADH and dithionite as reductant. This indicates that any perturbing effect of sulfite [an oxidation product of the dithionite shown in crystals to bind to the d_1 heme (*2*)] is small and that the reductive titration is independent of the chemical nature of the reductant.

As d_1 heme is a one-electron center, $n = 1.4$ implies cooperativity between the redox behavior of the *c* and d_1 hemes and/or the two d_1 hemes in the dimeric enzyme. By cooperativity, we mean that there is a very strong thermodynamic driving force for a second electron transfer to follow the first (*23*); i.e., in the present case, reduction of a single, one-electron, heme center leads spontaneously to the reduction of the second. Successive electron transfers may proceed cooperatively where there is structural compensation for the accumulation of charge (*24*). Evidence for the cooperativity being between the *c* and d_1 hemes was obtained when titrations were compared at wavelengths that are indicative of redox events at either the *c*-type or d_1-type centers (*18*). Figure 2 shows that reductive titrations at two such wavelengths, 463 nm characteristic of the d_1 heme and 420 nm characteristic of heme *c*, were superimposable. The same result was obtained at other wavelengths, and inspection of the whole visible spectrum gave no evidence for a detectable difference in the titration of the two types of heme center. Furthermore, the superimposability of absorbance changes

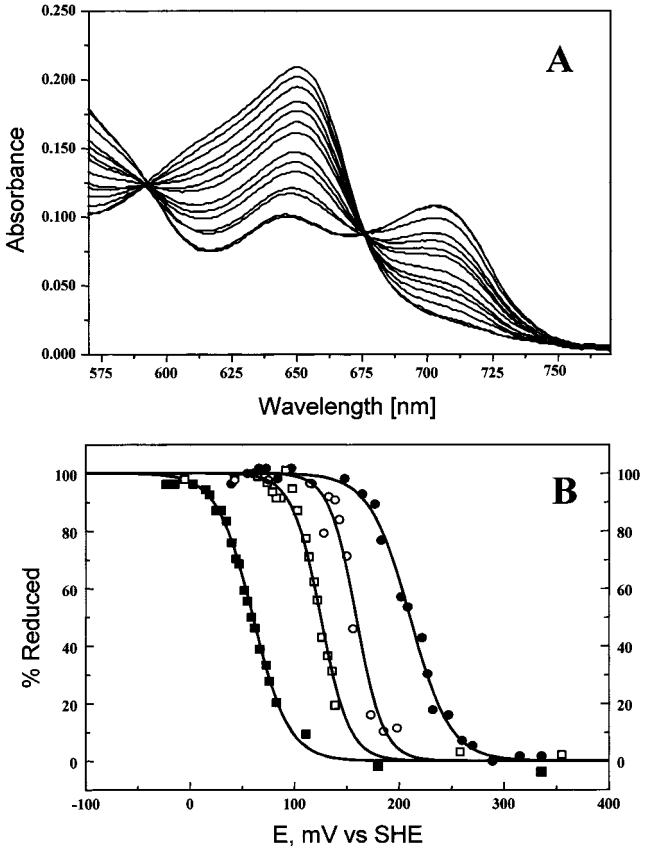


FIGURE 3: (A) Reductive potentiometric titration of cytochrome cd_1 from *P. pantotrophus* conducted in 0.1 M sodium phosphate buffer, pH 7.0 at 40 ± 2 °C. (B) Comparisons of the redox titrations of *P. pantotrophus* cytochrome cd_1 at 20 and 40 °C. For the 40 °C data, the plot of absorption at 624 nm versus electrode potential was fitted to eq 1 as described in Experimental Procedures. From the reductive data (□), $E'_{red} = 125 \pm 5$ mV and $n = 2$, and from the oxidative data (○), $E'_{ox} = 159 \pm 5$ mV and $n = 2$. The 20 °C data are as in Figure 1B [reductive titration (■), oxidative titration (●)].

at different wavelengths was also clear when the protein was titrated in the oxidative direction.

The potentiometric investigation of cd_1 from *P. pantotrophus* at 20 °C clearly showed that reduction and oxidation of the enzyme occurs at different potentials. In addition, the two redox centers act as a highly cooperative system with $n = 1.4 \pm 0.1$, and a titration cannot be reversed part way. These data suggest that a kinetic phenomenon results in the separation of the oxidative and reductive titrations; i.e., although these are potentiometric titrations, they are not equilibrium titrations since kinetic effects mean that equilibrium could not be reached at each of the points. The corollary of such a suggestion is that a change in temperature would alter the redox titrations. Fortunately, we established that the cytochrome cd_1 was stable at 40 °C for up to 16 h in the oxidized form, and 98% of the protein was recovered after heating at 40 °C for 5 h in the dithionite reduced form, as judged from visible absorption spectra. We estimated that 40 °C was the highest viable temperature for the 16−20 h period required to complete a potentiometric titration.

Redox titrations at 40 °C are shown in Figure 3A. Both oxidative and reductive titrations were significantly shifted compared with those at 20 °C, the respective apparent midpoint potentials being $+160 \pm 5$ mV and $+125 \pm 5$ mV

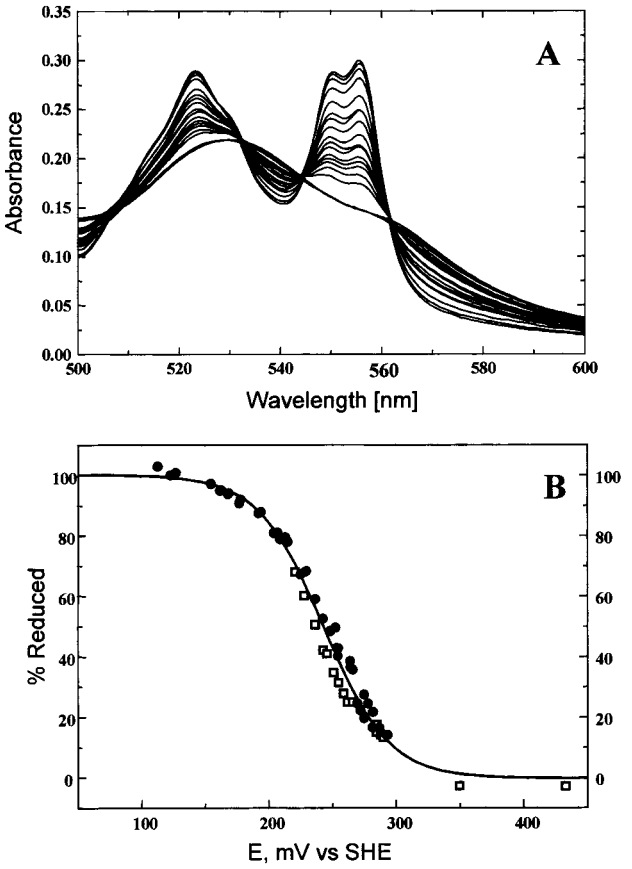


FIGURE 4: (A) Redox titration of the semi-apocytochrome cd_1 from *P. pantotrophus* conducted in 50 mM HEPES−KOH buffer at pH 7.0 and 25 ± 2 °C. Data shown include reduction with dithionite and oxidation with ferricyanide. (B) Plot of absorption at 555 nm versus electrode potential for the semi-apo form of cytochrome cd_1, which contains only the *c*-heme center. Data were fitted to eq 1. Reductive data (●) and oxidative data (□) are shown; $E'_m = 242 \pm 5$ mV and $n = 1$.

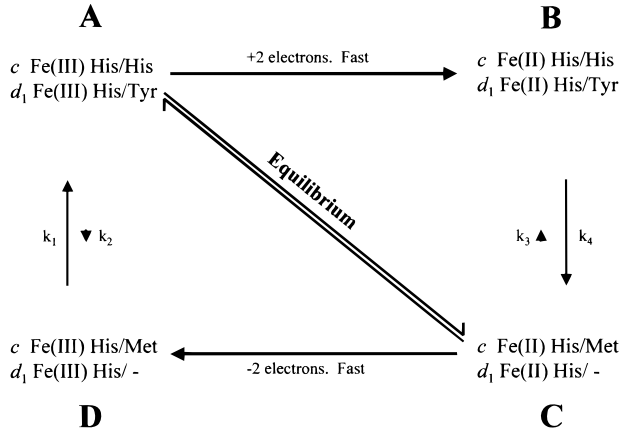
(Figure 3B). In each case the best fit was to $n = 2$, demonstrating a very high degree of cooperativity in electron transfer. Importantly, at this temperature a titration could now be reversed midway through, with both the spectrum and potential changing as anticipated. For comparison with the 20 °C data, both sets of titration curves are shown in Figure 3B.

A semi-apo form of cytochrome cd_1, lacking the d_1 heme, has been shown by a charge-transfer band at 695 nm in the near-infrared absorption region and by nuclear magnetic resonance spectroscopy (*25*, *26*) to have His/Met axial ligation of the heme iron in the oxidized form of the protein. When this form of the enzyme was examined by redox potentiometry, the reductive and oxidative titrations coincided (Figure 4A). The data fitted a Nernst plot with a midpoint potential of +242 ± 5 mV and, as expected, $n = 1$ (Figure 4B). Thus none of the complexity associated with the holoenzyme was observed, and at least in this semi-apo form, there was no indication for cooperativity between the two *c*-type cytochrome centers in the dimeric molecule.

DISCUSSION

A number of complexities in the oxidation/reduction behavior of cytochrome cd_1 nitrite reductase from *P. pantotrophus* are readily observed. It might have been expected

Scheme 1: Proposed Oxidation States and Heme Ligations That Occur during Redox Titrations of *P. pantotrophus* Cytochrome cd_1



that the *c* and d_1 cytochrome centers would have titrated with different reduction potentials that could be resolved, if not by eye, then by fitting. However, the data presented in the present paper, taken together with knowledge of the structural changes that occur around the *c* and d_1 heme centers upon reduction of the enzyme, establish that in the absence of substrate these two centers cannot undergo independent redox transitions. Thus in analyzing the redox behavior of cytochrome cd_1, it is assumed that only the four states of the enzyme shown in Scheme 1, each with both of the hemes in one monomer either oxidized or reduced, need to be considered. Two of these states, A and C, respectively, correspond to the oxidized and reduced equilibrium states of the enzyme that are seen by crystallography. State B corresponds to the enzyme having the structure observed for the oxidized state, but with all of the heme groups reduced, while state D corresponds to the "reduced structure", but with all of the heme groups oxidized (Scheme 1). The redox behavior of a protein that can be described by such a square scheme will depend on several kinetic factors including the rate constants for the various horizontal and vertical transitions shown in Scheme 1 and the time scale of the measurement procedure. A recent study of a mutant form of yeast iso-1-cytochrome *c* which has His/His coordination in the oxidized state and His/Met in the reduced state illustrates the point. Rapid scanning cyclic voltammetry permitted the isolation of two reduction potentials corresponding to two horizontal transitions analogous to those shown in Scheme 1. $E^{\circ\prime}$ values of +43 and +247 mV were obtained for oxidation/reduction of the His/His and His/Met forms, respectively (*27*). These data were obtained because conformational changes required for the His/His to His/Met conformational transitions, and vice versa, were slow relative to the measuring time. When a much longer "measuring time" was employed via digital simulation, an $E^{\circ\prime}$ value of +176 mV was obtained (*27*), corresponding to an equilibrium situation, analogous to the diagonal transition (A ⇌ C) shown in Scheme 1.

In the present work, a relatively slow method for following oxidation/reduction behavior of cytochrome cd_1 has been used; all attempts to employ cyclic voltammetry have been unsuccessful (J. W. A. Allen et al., unpublished observations). It might, therefore, have been expected that an equilibrium titration, corresponding to the diagonal in

Scheme 1, would have been observed. Only if all the vertical transitions in Scheme 1 were extremely slow relative to the measurement time would one expect to obtain independent equilibrium titrations for the two conformational states of the enzyme. However, it is clear that this condition is not satisfied in the present work. If it were, then at 20 °C it would be possible to reverse either the oxidative or reductive titrations, contrary to observation. The consequence is that the present data must, therefore, be explained on the basis that the rate of interconversion (k_4) from ferrous His/His (B) to ferrous His/Met (C) occurs on a time scale (ca. 10 min) that is coincidentally similar to the time allowed for the establishment of a steady signal in the redox titration procedure. The same argument must apply to the upward transition (k_1) at the left-hand side of Scheme 1. The back-reactions of these rearrangements (k_2 and k_3) must occur significantly more slowly, perhaps over hours. Thus, true equilibrium is not reached on the time scale of the experiment at 20 °C, and the reduction potentials derived from the data shown in Figures 1 and 2 are apparent rather than real values. It is possible that the values obtained are biased toward the real values corresponding to the horizontal transitions in Scheme 1. As with the yeast iso-1-cytochrome *c* mutant, a less positive value is expected for the reduction of the His/His coordinated form of the *c*-type center relative to the His/Met form (*27*).

If the observations shown in Figures 1 and 2 do not represent true equilibrium data, it might be predicted that the visible absorption spectrum would reveal a time-dependent variation. However, we were not able to identify any such effect. It is possible that any changes in the visible absorption spectrum associated with upward or downward transitions in Scheme 1 were small and therefore escaped detection. Precedent shows that only very small changes in the α-band region are associated with a change from His/His to His/Met coordination (*28*). Another point to consider is that on each addition of an aliquot of oxidant or reductant only a relatively small fraction of the total sample undergoes a redox change; this makes it difficult to detect any small spectroscopic change that follows the larger change associated with the horizontal transitions in Scheme 1. Nevertheless, the set of spectra in Figure 1A does not give exact isosbestic points, as would be expected for a two-state equilibrium system.

If the hysteresis evident in Figure 1B is a kinetic effect, then it was predicted that if the redox titration was conducted either much more slowly at 20 °C or over the same time scale at a higher temperature, then a shift toward a single reversible titration should be observed. Prolonging the time scale of the experiment was impractical, but the data obtained at 40 °C support the prediction. The extent of the hysteresis is much less. Furthermore, two well-defined isosbestic points are observed, suggesting that the displacement from equilibrium conditions is much less than at 20 °C. An approximate extrapolation from the data at 40 °C suggests that the equilibrium potential, i.e., the diagonal in Scheme 1, would be $+140 \pm 15$ mV. $E^{\circ\prime}$ values are usually temperature dependent, and assuming a variation with temperature comparable with that for other cytochromes (*29*), we may expect an equilibrium value of $+170 \pm 15$ mV at 20 °C.

At 40 °C, $n = 2$ for both of the oxidative and reductive titrations shown in Figure 3. Even when two one-electron redox centers undergo a cooperative change, n can be <2. An n value of 2 at 40 °C raises the possibility of a higher n value for the true equilibrium titration. Cytochrome cd_1 is a dimer, and thus, in principle, oxidation/reduction of one monomer may promote the same process in the other monomer, contributing to an n value >2. Our titrations of semi-aponitrite reductase imply that there is no cooperativity between the *c* heme domains within the dimer. This contrasts with the observations of Silvestrini et al. (*30*), who found that a recombinant form of semi-aponitrite reductase from *P. aeruginosa* showed functional inequivalence between monomers in electron-transfer experiments. Blatt and Pecht (*31*) reported no cooperativity within a subunit in the cytochrome cd_1 from *P. aeruginosa* but did observe some cooperativity between subunits, quantified as +30 mV between the d_1 hemes and −30 mV between the *c*-type centers. It is noteworthy that in their enzyme the N-terminus of one subunit crosses over to the other, interacting with it significantly and coordinating through residue Tyr-10 to a hydroxyl ligand bound to the oxidized heme d_1 (*5*). This provides a structural basis for a mechanism of intersubunit cooperativity, which would be absent in the enzyme from *P. pantotrophus*, for which there is no apparent structural incompatibility between the conformations of oxidized and reduced subunits (V. Fülöp, personal communication). The latter suggests that there is no reason to expect intersubunit cooperativity in the *P. pantotrophus* enzyme, although this possibility cannot be completely excluded. Therefore, we take $n = 2$ to imply very strong cooperativity between two hemes on one polypeptide.

The apparent mechanism of electron-transfer cooperativity within a monomer in the *P. pantotrophus* enzyme is that one axial ligand to each heme in the oxidized state is displaced upon reduction (*c* heme His-17 and d_1 heme Tyr-25). These residues are joined by a very short amino acid loop. It is reasonable to suggest that their side chains bind cooperatively to the two redox centers in the absence of substrate and such "all or nothing" ligation would concomitantly require complete two-electron reduction or oxidation.

A form of *P. pantotrophus* cytochrome cd_1 with one electron on the monomer has been observed in pulse radiolysis experiments (*18*). Initial selective reduction of the *c*-type cytochrome center was followed by an essentially complete intermolecular electron transfer, on the millisecond time scale, to the d_1 heme. This observation was interpreted as meaning that the reduction potential of the d_1 center must be approximately 100 mV more positive than that of the *c*-type center. At first sight that result is at odds with the observations in the present paper. However, in the pulse radiolysis work the reduction of the *c* cytochrome was so fast that a low-potential, His/His-coordinated, ferrous state would very probably have been obtained, from which the electron would have passed to the d_1 heme. The cooperativity seen in the present paper implies that, under pulse radiolysis conditions, the reduction of the d_1 heme would have caused dissociation of the tyrosine ligand and cooperative ligand rearrangement at the *c*-type cytochrome center; this hypothesis is consistent with the data presented by Kobayashi et al. (*18*). In the case of *P. aeruginosa* cytochrome cd_1, Nurizzo et al. (*32*) have demonstrated crystallographically that it is reduction of the d_1 heme, which occurs after reduction of heme *c*, that triggers the conformational changes observed on reduction of the enzyme.

The redox titration data presented here under steady-state conditions explain why we have been unable to prepare a half-reduced enzyme with electrons residing only on either the *c* or d_1 heme centers. In contrast, it was possible (*31*, *33*; A. Koppenhöfer, unpublished observations under conditions similar to those used in the present work) to reduce the *c*-type cytochrome center in a solution of *P. aeruginosa* cytochrome cd_1 while the d_1 heme remained oxidized. Similarly, it has been possible to obtain the structure of *P. aeruginosa* cytochrome cd_1 with only the *c* heme reduced (*32*), in agreement with those literature reports which indicate that the *c* heme center has the more positive reduction potential. Thus the considerable structural differences between the enzymes from *P. aeruginosa* and *P. pantotrophus* are also reflected in their redox behavior.

What are the physiological implications of the redox titration reported for cytochrome cd_1 in this paper? Cytochrome cd_1 receives electrons after the cytochrome bc_1 complex of the electron transport chain; cytochrome c_{550} and pseudoazurin are probably the direct electron donor proteins. The latter have $E^{\circ\prime}$ values in the range of +250 mV and so appear unlikely electron donors to a form of cytochrome cd_1 with $E^{\circ\prime}$ of the order of +60 mV. Thus, in vivo it is not clear how the enzyme in the oxidized state with His/His coordination at the *c*-type heme could be reduced. Perhaps there is an activation factor in the bacterial periplasm; alternatively, the enzyme may only be functional when the ratio of reduced to oxidized electron donor proteins is very high. It is also conceivable that if the enzyme is synthesized in reduced form, then in the steady-state presence of electrons and substrate it need never reach conformation A (Scheme 1), which would, therefore, be a resting state. Crystallographic data suggest that the His/His-coordinated state of the *c*-heme does occur during steady-state catalysis (*2*), but its catalytic significance cannot be considered as settled. If it does not normally occur during catalysis, it would be the His/Met-coordinated form of cytochrome cd_1 that is reduced by cytochrome c_{550} or pseudoazurin in the steady state. Clearly, the redox cycling we report here is too slow to be catalytically relevant ($k_{cat} = 4\ s^{-1}$). Furthermore, the reduction potential of the semi-apoenzyme, where the *c* heme is His/Met ligated, is +242 mV, which is much more compatible with receiving electrons from the putative electron donors pseudoazurin and cytochrome c_{550}. The enzyme may be kinetically trapped on the bottom of Scheme 1 during turnover, and thus *c* His/Met ligation retained. A further consideration is that, during the titrations described in the present paper, heme d_1 either is ligated by tyrosine-25 or is pentacoordinate, according to crystallography and EPR spectroscopy (*2*, *4*, *7*, *8*). However, during turnover it will bind nitrite and nitric oxide, which must change the reduction potentials significantly. Presumably when heme d_1 receives electrons from heme *c*, it must have a similar or higher reduction potential than the latter.

The hysteretic redox titration reported here is the first example, to our knowledge, of this type of behavior. It shows how a protein can be designed to have appropriate energetics and rate constants for the interconversion of the different liganded forms of heme groups such that oxidation follows a path different from that of reduction. This is what is required for a membrane-bound heme protein that drives proton (or other ion) translocation across a membrane. Such differences between the oxidative and reductive pathways can ensure that one state can be limited to proton release at one side of the membrane and another state to proton uptake at the opposite side.

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