^{1}H NMR Investigation of Cytochrome cd_1: Complexes with Electron-Donor Proteins†

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ABSTRACT: Mixtures of the dissimilatory nitrite reductase cytochrome cd_1 from *Pseudomonas aeruginosa* and potential electron-donating proteins were prepared in both fully oxidized and fully reduced states and examined by ^{1}H NMR spectroscopy. The relatively narrower lines of the donor proteins enabled them to be clearly observed in spectra in the presence of significant amounts of the high molecular weight cd_1. Mixtures of the physiological donor (*Pseudomonas* ferrocytochrome *c*-551) and ferrocytochrome cd_1 showed specific line-broadening effects on the resonances of *c*-551 that depended on the mole ratio of *c*-551 to cd_1. The experimental broadening fit a model in which *c*-551 is in intermediate or fast exchange between free solution and a complex with cd_1, with an association constant for the complex in excess of 10^4 M^{-1}. The model yields a minimum estimate for the forward bimolecular rate constant of 5×10^7 M^{-1} s^{-1} and suggests that the actual value may be much larger. The complexation was independent of pH in the range of 6–8, was independent of ionic strength over a salt concentration range of 20–1000 mM, and possessed a low thermal activation barrier. Mixtures of ferricytochrome *c*-551 and ferricytochrome cd_1 showed no observable NMR perturbations, indicating that any hypothetical complex involving the oxidized forms must follow different dynamical and/or equilibrium conditions. No observable NMR perturbations existed in spectra of mixtures of cd_1 and mammalian cytochrome *c* or *Pseudomonas* azurin in either oxidation state.

Cytochrome cd_1 functions as the dissimilatory nitrite reductase in many chemoautotrophic bacteria. It is a soluble protein composed of two identical subunits each of about 60 000 daltons. Each subunit has two prosthetic groups, a covalently attached *c*-type heme and a noncovalently associated heme d_1 (Kuronen et al., 1975). The latter has an unusual macrocycle structure (Timkovich et al., 1984a,b). The enzyme accepts electrons from a reduced donor at a heme *c* site, undergoes internal electron-transfer events from heme *c* to heme d_1, and donates reducing equivalents to nitrite, reducing it predominantly to nitric oxide (Wharton & Weintraub, 1980). In a nonphysiological reaction, it may also reduce oxygen to water. The physiological electron donor is a small, soluble *c*-type cytochrome. In the commonly studied strain *Pseudomonas aeruginosa*, this is an 8700-dalton cytochrome *c*-551, whose crystal structure is known (Almassy & Dickerson, 1978; Matsuura et al., 1982). The *Pseudomonas* cytochrome cd_1 is also capable of being reduced by the blue copper protein azurin as well as artificial electron donors.

The kinetics of the reactions catalyzed by cytochrome cd_1 have been extensively studied, but many mechanistic questions remain unanswered. Turnover reactions with different donors have been studied (Yamanaka & Okunuki, 1963, 1964; Barber et al., 1976; Saraste & Kuronen, 1978; Silvestrini et al., 1979; Robinson et al., 1979; Timkovich et al., 1982; Dhesi & Timkovich, 1984). Rapid kinetics have been measured for electron transfer with some donors and acceptors (Wharton & Gibson, 1976; Barber et al., 1977, 1978; Greenwood et al., 1978; Schichman & Gray, 1981). With the exception of azurin (Wharton et al., 1973), the donors studied have been low molecular weight models. Azurin itself may not be a major donor to cd_1 in vivo (Martinkus et al., 1980). Data on the rapid, bimolecular reaction(s) of the physiological donor ferrocytochrome *c*-551 with cd_1 have not been obtained. Rapid reactions of cytochromes have been often and precisely measured by following the rich visible spectra of these heme proteins. But in the present case the *c*-type heme of cd_1 overlaps spectrally with *c*-551 and has precluded optical studies.

^{1}H NMR has provided an avenue to obtain information on the dynamics of this interaction. It is well-known that, in certain kinetic windows, NMR is capable of affording rate data on complexation reactions because of the effects of chemical exchange on resonance positions or line shapes. Numerous examples exist for the studies of enzyme–substrate interactions. There is no difference in principle when the substrate becomes a macromolecule itself [for examples, see Gupta & Yonetani (1973) and Chan et al. (1983)]. It has proven possible to study mixtures of cd_1 and potential protein electron donors. Although both have distinctive ^{1}H NMR spectra, the very broad cd_1 resonances allow a clear observation of the narrow resonances of the donor, with the observation of specific complexation events that lead to data on the association dynamics.

Materials and Methods

Cytochrome cd_1, cytochrome *c*-551, and azurin from *P. aeruginosa* strain ATCC 19429 were purified and kinetic assays performed as described previously (Dhesi & Timkovich, 1984). Horse heart cytochrome *c*, type III, was purchased from Sigma Chemical Co.

Protein samples for NMR studies were dialyzed vs. 50 mM ammonium bicarbonate buffer, pH 7.8, lyophilized, and redissolved as concentrated stock solutions in 99.8% deuterium oxide buffered with 20 mM potassium phosphate to an appropriate pH. Values of pH reported are the direct reading of a glass microelectrode previously calibrated vs. protic standards and will be designated pH*. Concentrations of stock protein solutions were determined by UV–visible spectrophotometry. The reported concentrations of cytochrome cd_1 will refer to the subunit concentration. Aliquots were mixed and diluted with buffer if necessary to give the final mixtures.

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For mixtures of reduced proteins, the final solution was deoxygenated by repeated cycles of vacuum degassing and argon flushing, reduced under argon with a fresh aliquot of dithionite from a concentrated solution, and then sealed in the 5-mm NMR tube under argon. Because of possible problems with dithionite as reductant (Barber et al., 1976), ascorbate was substituted in some experiments, but the NMR observations were identical. There is a potentially serious problem for reduced mixtures involving *Pseudomonas* c-551. It has been shown that the relatively narrow lines of ferrocytochrome c-551 are broadened by the presence of even 1% ferricytochrome c-551, due to chemical exchange broadening by a facile self-exchange process (Keller et al., 1976). Therefore, the observation of the relatively narrow lines of ferrocytochrome c-551 in a sample by itself was a convenient and consistent indicator that the technique led to complete reduction. For mixtures involving cd_1, use of the same technique ensured that the observed line broadening was not due to the self-exchange reaction. This was further confirmed by opening the sealed tube at the end of the experiment and examining the visible spectrum under an argon atmosphere immediately after opening and then after the addition of a further aliquot of reductant. No further optical changes were seen. It should also be pointed out that the self-exchange broadening would not be a function of the mole ratio of cd_1 to c-551 as was observed in the experiments and the self-exchange reaction causes chemical shift differences that were not observed in the present samples. For other reduced donors this issue is not as critical, since self-exchange does not cause line broadening, but they were also examined by optical spectroscopy after the NMR experiments.

Spectra were obtained on Nicolet spectrometers operating at 200 or 300 MHz for protons. Dioxane and acetate were used as internal standards, but chemical shifts will be reported on a nominal scale with respect to sodium 4,4-dimethyl-4-silapentanesulfonate as zero parts per million (0 ppm). Prior to experiments, the magnetic field was shimmed to give a constant minimal width to the low molecular weight internal standards. The internal standards indicated that bulk viscosity was not a line-broadening mechanism until the concentration of cd_1 exceeded 0.5 mM.

The resonances of the high molecular weight cd_1 (Timkovich & Cork, 1982; Timkovich et al., 1985) are so much broader and overlap to such a greater extent than those of c-551, horse cytochrome c, or azurin that the latter are readily resolvable in mixtures. Indeed, up to 0.5 mM in cd_1, the resonances of cd_1 are not apparent beyond a gradual rise in the base line. Data consist of observations of effects on the resonances of the donors in the presence of cd_1. Although the donor was present in excess (in the range of 0.2–10 mM), the concentration of cd_1 (0.05–0.5 mM) was significantly greater than a catalytic amount.

Line widths were computed from observed spectra by a Lorentzian curve-fitting routine that is a standard feature of Nicolet software. Statistical relative standard deviations on the computed width were typically less than 10%. However, even the better resolved resonances of donors do have neighboring peaks, and the computed widths became sensitive to the choice of base line region especially when the addition of cd_1 began to cause serious line broadening. This leads to additional experimental scatter of data points.

The excess line widths to be reported are the differences between the line width of a resonance of the donor when it was present in a sample with cd_1 and the line width when the donor spectrum was recorded in a sample of the pure donor at the same concentration. The pure donors did not show a concentration line width dependence over the concentration ranges employed in this study. Each data point in this study represents an individual sample tube with virgin proteins. Protein samples were not reused or recycled. Control samples of pure donors were freshly prepared from the same batch when a titration series with variable cd_1 or variable donor was to be examined.

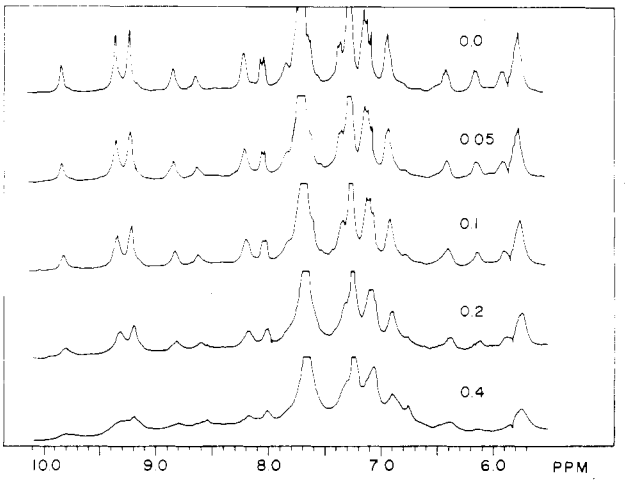

FIGURE 1: Aromatic region of the ^1H NMR spectrum of ferrocytochrome c-551 in the presence of increasing amounts of *Pseudomonas* ferrocytochrome cd_1, given as the millimolar concentration next to individual spectra, at 25 °C and pH* 6.0. The vertical scale was adjusted to emphasize the fine structure in the control spectrum, and the tops of two strong peaks at 7.2 and 7.6 ppm have been cut off.

RESULTS

The resonances of ferrocytochrome c-551 were systematically broadened by increasing concentrations of ferrocytochrome cd_1. Figure 1 illustrates a typical effect for a portion of the c-551 spectrum. The effect was most apparent when the mole ratio (c-551 to cd_1) was in the range of 10–100. At higher ratios the excess width caused by cd_1 was less than the reproducibility of line width measurements for pure c-551 samples. At lower ratios, the widths were so large that the spectrum became a featureless envelope, and it was not possible to make quantitative width measurements. No chemical shift differences were seen, although this assessment became problematic as the lines drastically broadened. The critical parameter was the mole ratio. When the ratio was held constant, but the absolute concentrations were diluted 2–3-fold, the amount of broadening remained the same within experimental error. When the concentration of cd_1 was held constant, and the concentration of c-551 was varied, the excess line width appeared to be a linear function of reciprocal c-551 concentration. This is illustrated for the *S*-methyl resonance of the sixth ligand Met-61 in Figure 2. When the concentration of c-551 was held constant and the concentration of cd_1 was varied, the excess line width appeared to be a linear function of cd_1 concentration. This is illustrated for select resonances in Figure 2. Above cd_1 concentrations of 0.5 mM, the bulk viscosity increased sufficiently to cause nonspecific line width effects, and so higher concentrations of cd_1 were not studied. Also illustrated in Figure 2 is the general trend that the excess line width increase was of the same order of magnitude for different resolved resonances but was different for each. The general correlation was that the broader resonances broadened more rapidly with increasing cd_1 concentration. It is possible in principle for complexation to perturb select resonances at the actual interface sufficiently so that some information

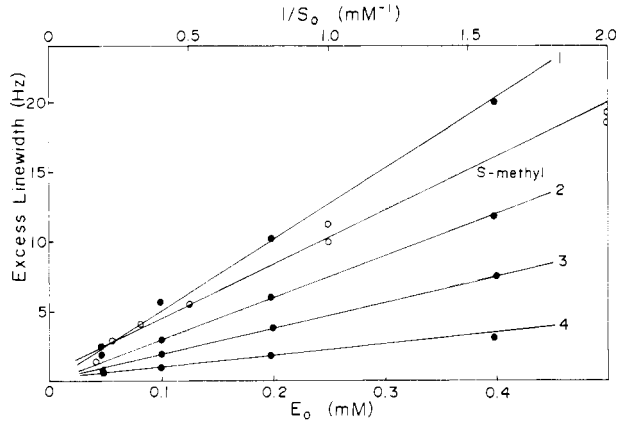

FIGURE 2: Concentration dependence of the excess line widths for select resonances of *Pseudomonas* ferrocytochrome *c*-551. Representative data have been taken from two series of titrations at 25 °C and pH* 6.0. The open circles, the curve marked *S*-methyl, and the *upper* abscissa ($1/S_0$) are excess line widths for the Met-61 *S*-methyl resonance at −2.9 ppm as a function of the reciprocal of the total *c*-551 concentration where the cd_1 concentration was constant. The closed circles, curves 1–4, and the *lower* abscissa (E_0) are excess line widths for select resolved resonances in *c*-551 as a function of the total cd_1 concentration where the *c*-551 concentrations was constant: curve 1, the heme δ meso proton at 9.8 ppm; curve 2, a nonexchangeable (at 25 °C) amide NH proton at 8.9 ppm; curve 3, a thioether bridge methine proton at 6 ppm; curve 4, a heme ring methyl proton at 3.3 ppm. As shown in the supplementary material and summarized under Discussion, in the case of very tight binding, the excess line width is represented by $1/T_{2E}$, which is given by $1/T_{2E} = [E_0/(T_{2m} + \tau)](1/S_0)$, where the actual binding constant has dropped out of the explicit equation.

identifying the binding site may be obtained. In the present case it was not possible to discern this type of effect. This is not to say it does not occur, but many resonances are obscured even in a small protein like *c*-551 by overlap or hindered rotation broadening.

The effects of pH* were examined over the pH range of 6–8. Over this span, the turnover activity of cd_1 with *c*-551 as donor falls from a maximum to less than 10% of this. The excess line widths, however, were not sensitive to pH*. The effects of temperature were determined over the range of 3–35 °C. The resonances of *c*-551 narrowed at the higher temperatures. For example the *S*-methyl resonance narrowed by 3 Hz over this span. However, the excess line width induced by cd_1 remained the same.

The effects of ionic strength were examined by varying the salt concentration with either NaCl or phosphate, at pH* 6, from 20 mM to 1 M. The excess line width caused by cd_1 was not dependent on ionic strength. This insensitivity was very surprising, especially in light of the fact that this laboratory had previously published turnover kinetic data with the related cytochrome cd_1 from *Paracoccus denitrificans* in which steady-state transfer rates were markedly diminished as the ionic strength increased (Timkovich et al., 1982). The kinetic data had been interpreted as disruption of key interface salt bridges between cd_1 and its donor. Ionic strength effects on the turnover activity of *Paracoccus* cd_1 with horse cytochrome *c* as donor and that of *Pseudomonas* cd_1 with *Pseudomonas* *c*-551 as donor, and either oxygen or nitrite as acceptor, were measured. As shown in Figure 3, the *Paracoccus* enzyme reactivity was markedly sensitive to salt whereas the *Pseudomonas* reactivity was virtually insensitive. There thus appears to be a real difference in this aspect of the kinetics of the two otherwise rather closely related enzymes. Unfortunately, insufficient quantities of purified *Paracoccus* cd_1 were available to extend the comparison to the NMR experiments.

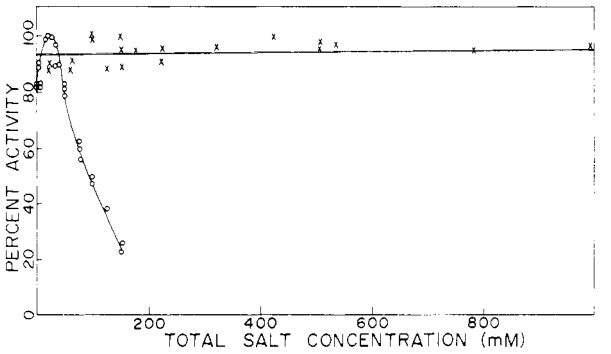

FIGURE 3: Ionic strength dependence of the relative turnover rates from initial rate assays for the oxidation of *Pseudomonas* ferrocytochrome *c*-551 by *Pseudomonas* cd_1 (crosses) and the oxidation of horse ferrocytochrome *c* by *Paracoccus* cd_1 (open circles). The highest observed rate in either series was taken as 100%, and the remaining assays were expressed relative to this maximal rate.

Mixtures of reduced *Pseudomonas* cd_1 and other potential donors such as reduced horse heart cytochrome *c* and reduced *Pseudomonas* azurin were prepared. Mixtures of oxidized cd_1 and either oxidized *c*-551, horse *c*, or azurin were also prepared and examined. In all these cases, there were no observable effects on the NMR spectrum of the donor (with the trivial exception of nonspecific viscosity-related broadening at very high cd_1 concentrations as mentioned under Materials and Methods). Mixed oxidation states (one component reduced, the other oxidized) could not be studied by the technique because of kinetically facile electron-transfer reactions.

Discussion

The supplementary material reviews a conventional theoretical background for the observed NMR effects (see paragraph at end of paper regarding supplementary material). The observed excess line widths caused by reduced cd_1 on the resonances of reduced *c*-551 can be related to an excess spin–spin relaxation rate by

$$1/T_{2E} = \frac{E_0}{(T_{2m} + \tau)} \frac{1}{S_0}$$

where $1/T_{2E}$ is the excess relaxation rate, E_0 is the total concentration of cd_1, S_0 is the total concentration of *c*-551, T_{2m} is the spin–spin relaxation time of the observed *c*-551 resonance in the complex, and τ is the average lifetime of the complex. The data appear to fit the case of fast or intermediate exchange between free solution *c*-551 and *c*-551 bound in a complex with cd_1, where the association is covered by an equilibrium constant $K = k_{on}/k_{off}$ that is large so that the product KS_0 is greater than 1. Although the association constant K has explicitly dropped out of the above equation for excess line width, it and favorable values for the individual microscopic rate constants k_{on} and k_{off} are the prime reasons for observing the NMR phenomenon. It is reasonable to take the reciprocal of values of Michaelis–Menten K_m derived from turnover kinetics as a lower estimate of the association constant K. While a spread of values has been reported (Barber et al., 1976; Dhesi & Timkovich, 1984), probably because of differences in assay techniques, a K_m of 20 μM is representative, leading to an estimated K of 5×10^4 M^{-1}, which in turn does show that KS_0 is greater than 1 for the concentrations employed in the study.

The operative dynamic parameter responsible for excess line widths is the sum $T_{2m} + \tau$. If τ dominated in this sum, all resonances should broaden by the same amount. Since different resonances show different line broadening, this argues that T_{2m} is significant compared to τ and may even be greater. T_{2m} is likely to be dominated by the increased rotational

tumbling time when free *c*-551 (M_r ca. 8700) becomes involved in a complex (M_r in excess of 120 000). Paramagnetic line broadening is not likely to be a major factor for several reasons. The ferrocytochrome hemes *c* are both diamagnetic. Although the heme d_1 of ferrocytochrome cd_1 is paramagnetic (high spin, $S = 2$) [Timkovich & Cork (1983) and references cited therein], the initial electron-transfer reaction from a donor is to heme *c*, and this bespeaks greater proximity to the diamagnetic heme site. Paramagnetic broadening falls off very rapidly with distance, and the resolved *c*-551 resonances from interior protons (the heme meso protons and the ligand *S*-methyl) are highly unlikely to be close to the paramagnetic heme d_1 iron.

A very reasonable relaxation model for the *S*-methyl resonance of Met-61 in *c*-551 when *c*-551 is in a complex with cd_1 is the *S*-methyl resonance of the diamagnetic heme *c* of cd_1 itself. This has been observed previously at −2.8 ppm in the spectrum of pure ferrocytochrome cd_1 (Timkovich et al., 1985). The *S*-methyl resonance of cd_1 has a line width of 90 Hz, corresponding to an estimated T_{2m} of 3.5×10^{-3} s. A plot of the excess line width vs. cd_1 concentration for the *S*-methyl of *c*-551 when in the mixture has a slope of 22 Hz mM^{-1}, from which the sum $T_{2m} + \tau$ for *c*-551 is calculated to be 3.6×10^{-3} s. The correspondence indicates that T_{2m} is the dominant factor in the sum $T_{2m} + \tau$ as applied to *c*-551 resonances when complexation has occurred.

In a study of complexes of cytochrome *c* with cytochrome peroxidase, Gupta and Yonetani (1973) showed that the operative correlation time was proportional to the molecular weight and that the observed line width in the complex was proportional to a paramagnetic contribution plus the dipole–dipole contribution in the unassociated cytochrome times a factor of the molecular weight of the complex divided by the free molecular weight. This treatment may be applied to the *S*-methyl resonance of *c*-551, which has an observed line width of 7.4 Hz in the uncomplexed state. Once again, no paramagnetic contribution is assumed, and the molecular weight increase is 141 400 [124 000 for the cd_1 dimer (Kuronen et al., 1975; Silvestrini et al., 1979) plus 2×8700 for *c*-551] to 8700 (the unit containing a free *c*-551 *S*-methyl). The predicted line width for an *S*-methyl in the complex becomes 99 Hz. As described in the supplementary material (eq 4), for any mixture of *c*-551 and cd_1, the observed excess line width will be proportional to the mole fraction of *c*-551 bound. In the case of tight binding (large association *K*), the fraction, for example, is 0.125 at 4 mM *c*-551 and 0.5 mM cd_1 (subunits) and the predicted excess line width is 12.4 Hz. All experimental observations for these relative concentrations fell within the range of 11–15 Hz.

These considerations make it seem likely that τ is less than T_{2m}, at least for the *S*-methyl resonance. Certainly it is reasonable to take the calculated $T_{2m} + \tau$ of 3.6×10^{-3} s as an *upper* estimate of τ. The dynamic k_{off} is given as the reciprocal of τ, or a *lower* estimate of k_{off} is 2.8×10^2 s^{-1}. Previously, a lower estimate of the association constant *K* was given as 5×10^4 M^{-1}. Placing all these together, a lower bound estimate of the forward rate constant, k_{on}, of 1.4×10^7 M^{-1} s^{-1} is made. In other words, k_{on} must be at least that fast, and probably is faster. This minimum estimate is within 3 orders of magnitude of diffusion control and highlights the extremely favorable association dynamics that must exist for this segment of the electron transport chain. Bimolecular rate constants on the order of 10^6 M^{-1} s^{-1} or faster [calculated from data in Figure 9 of Wharton et al. (1973)] have been observed for the reaction of reduced azurin with oxidized cd_1. These spectrophotometrically observed rates may have been composed of a faster initial binding followed by the optically observable event of electron transfer [see Discussion in Wharton et al. (1973)].

The insensitivity of the NMR broadening to pH* is perhaps not surprising. The association is expected to be the result of interactions among surface residues and topology, and this is not likely to drastically change over a narrow range near neutrality. A previous ^1H NMR investigation (Timkovich & Cork, 1982) of the hyperfine-shifted proton spectrum of ferricytochrome cd_1 revealed a structural transition near the heme d_1 site that roughly paralleled the enzymatic activity decrease from pH* 6–8, and thus there exist hypotheses to account for the kinetic rate diminution that do not involve association with donor. The temperature insensitivity may or may not be surprising, depending upon one's preconceptions as to what constitutes an activation barrier for protein–protein recognition. The lack of temperature dependence could be due to the fact that in the term $T_{2m} + \tau$ only τ strongly depends on thermal activation in general and, as has been discussed, it appears that T_{2m} dominates in the sum. The salt insensitivity was a surprise in view of the previous kinetic studies with the *Paracoccus* system. There is an extensive literature [for example, see Staudenmayer et al. (1977)] indicating that cytochrome oxidases are highly dependent upon lysine residues on *c*-type cytochromes for recognition and binding. The *Pseudomonas* system may have significant differences when compared to protein–protein interactions involving mammalian cytochrome aa_3 or even *Paracoccus* cd_1. It is known that the specificity of the *Pseudomonas* cd_1 is markedly different from other cytochrome oxidases (Yamanaka & Okunuki, 1964; Yamanaka, 1967; Timkovich et al., 1982). The crystal structure of *Pseudomonas c*-551 does show an increase in the hydrophobicity of residues near the heme crevice and a decrease in the number of positively charged lysine residues (Almassay & Dickerson, 1978; Matsuura et al., 1982).

It is possible to rationalize the lack of any observable NMR effects for mixtures of cd_1 and either horse cytochrome *c* or azurin by evoking arguments that these donors may not be physiological in nature and hence have less favorable kinetics that fall outside the rather narrow NMR time window. A more disturbing paradox is why mixtures of oxidized cd_1 and oxidized *c*-551 do not show evidence of complexation. It is known that the oxidized form of *c*-551 is a potent inhibitor of transfer turnover with an apparent inhibition constant K_i substantially less than K_m (Barber et al., 1976; Robinson et al., 1979; Dhesi & Timkovich, 1984). Crystal structure data indicate virtually no conformational change between ferri- and ferrocytochrome *c*-551 (Matsuura et al., 1982). Less is known about the conformational differences of ferri- and ferrocytochrome cd_1, and these could account for differing interaction statics as well as dynamics. Previous kinetic results complement the present NMR results. The time course of oxidation of ferricytochromes *c* by cd_1 indicates that some redox state (or states) of cd_1 discriminates between ferri- and ferrocytochrome *c*-551 (Robinson et al., 1979; Timkovich et al., 1982). The apparent competitive inhibition constant for ferricytochrome *c*-551 inhibiting further oxidation of ferrocytochrome *c*-551 is significantly less that the apparent K_m for ferrocytochrome (Barber et al., 1976; Dhesi & Timkovich, 1984). The two independent approaches of steady-state kinetics and NMR point toward redox state related differences in the protein interactions of cytochrome cd_1.

ACKNOWLEDGMENTS

Many of the protein isolations required for this study were

capably performed by Priscilla Taylor. Robert Kubas contributed data for the assays summarized in Figure 3.

Supplementary Material Available

Description of the underlying theory and assumptions for the equations presented under Discussion (4 pages). Ordering information is given on any current masthead page.

Registry No. Cytochrome cd_1, 9027-00-3; cytochrome *c*-551, 9048-77-5; nitrite reductase, 9080-03-9.

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