Implications of the Integrated Rate Law for the Reactions of *Paracoccus denitrificans* Nitrite Reductase†

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ABSTRACT: The integrated rate law for the reaction of the nitrite reductase of *Paracoccus denitrificans*, a cytochrome *cd*, has been established for turnover assays using donor ferrocytochromes *c* and either nitrite or molecular oxygen as the ultimate acceptor. The time course for the concentration of ferrocytochrome follows the law

$$S = S_0 \exp\left[-u_1 t - \frac{u_2(S - S_0)}{1 + u_3(S - S_0)} \right]$$

where S is the concentration of donor ferrocytochrome *c*, S_0 is the initial concentration, t is time, and u_1, u_2, and u_3 are empirical parameters that are constant for a given experiment but depend upon the initial substrate concentration. In particular, all the u_i increase with decreasing initial ferrocytochrome concentration. Saturation of reaction rates at high donor ferrocytochrome concentrations was not observed. The parameter u_1 was proportional to the enzyme concentration while u_2 and u_3 were not. The form of the integrated rate law and the behavior of the u_i impose severe restrictions on possible kinetic schemes for the activity of the enzyme. Contemporary mechanisms that have been proposed for mitochondrial oxidase aa_3 are examined and found to be inadequate to explain the reactivity of cytochrome *cd*. The simplest interpretations of the cytochrome *cd* data suggest that the enzyme does not bind the ferri and ferro forms of donor cytochromes *c* with equal affinity and that the enzyme is subject to inhibition by a product of reaction. Eucaryotic horse cytochrome *c* reacts with the *Paracoccus* cytochrome *cd* with 77% of the activity when *Paracoccus* cytochrome c_{550} is used as the electron donor.

Dissimilatory nitrite reductase activity in chemoautotrophic bacteria has been attributed to a soluble, multiheme enzyme ferrocytochrome *c*:nitrite (or oxygen) oxidoreductase (EC 1.9.6.4). The enzyme has been studied in most detail as isolated from *Pseudomonas aeruginosa* (Horio et al., 1961; Kuronen & Ellfolk, 1972; Gudat et al., 1973; Parr et al., 1976) and to a lesser extent from *Paracoccus* (formerly *Micrococcus*) *denitrificans* (Newton, 1969; Lam & Nicholas, 1969). Its molecular weight is circa 100 000, consisting of two circa 50 000 subunits, each of which contains one *c*-type and one *d*-type heme (Kuronen et al., 1975). The enzyme, termed cytochrome *cd*, utilizes small, soluble bacterial cytochromes *c* as electron donors or, at least in the case of Pseudomonads, a small copper-containing protein, azurin (Wharton et al., 1973). It catalyzes the reduction of nitrite to nitric oxide or molecular oxygen to presumably water. The latter reaction is nonphysiological and plays no role in the aerobic respiration of the denitrifying bacteria (Yamanaka et al., 1963; Yamanaka, 1964).

Because of its multiheme nature, the similarity of heme *d* to heme *a*, its specificity toward cytochromes *c* as electron donors, and its ability to reduce O_2, cytochrome *cd* can be and has been taken as analogous to the mitochondrial cytochrome oxidase aa_3. Early reports on cytochrome *cd* emphasize this analogy, and contemporary studies still note the similarities of the bacterial and mitochondrial oxidases. One goal of this paper is to investigate whether the kinetic behavior of cytochrome *cd* supports an analogy to mitochondrial cytochrome oxidase aa_3.

An appropriate approach is to make a systematic examination of the turnover or steady-state kinetics of cytochrome *cd*. Wharton and co-workers have studied the bimolecular steps between *Pseudomonas* cytochrome *cd* and oxygen and also the blue copper protein azurin (Wharton & Gibson, 1976; Wharton et al., 1973). Model inorganic compounds reacting with cytochrome *cd* have also been studied (Barber et al., 1977). Early work by Yamanaka and co-workers explored the specificity of *Pseudomonas* cytochrome *cd* toward various donor cytochromes *c*. However, only initial rates were employed to monitor reactivity, and an implicit assumption was made that the kinetic mechanism of cytochrome *cd* was analogous to that of mitochondrial cytochrome aa_3 oxidase (Yamanaka & Okunuki, 1964; Yamanaka, 1967). Other kinetic studies have been confined to artificial donors or employed only initial rates. In particular, the time course of reaction with nitrite as the terminal acceptor has not received much attention, in part, because of recognition of complexities in the reaction (Newton, 1969). One purpose of this present report is to extend kinetic studies on cytochromes *cd* by using natural electron donating cytochromes *c*, by examining the entire time course of reaction, and by determining any reaction differences with nitrite or oxygen as the terminal acceptors. The cytochrome *cd* from *Paracoccus denitrificans* was chosen for study to compliment the more extensive studies on *Pseudomonas* cytochrome *cd*.

The first step in a systematic study of the turnover kinetics of *Paracoccus* cytochrome *cd* was the determination of the integrated rate law for the oxidation of ferrocytochromes by cytochrome *cd*. The history of kinetic investigations on mitochondrial oxidases has shown that the integrated rate law is a critical starting point for the elucidation of oxidase mechanisms. It places severe constraints on mechanistic proposals and establishes appropriate parameters by which reactivity may be measured and expressed (Minnaert, 1961). A major goal of this work was the demonstration of the correct integrated law for *Paracoccus* cytochrome *cd*. This may then be used to understand the influence of assay conditions on reactivity and to measure reactivity in an accurate and meaningful manner.

During the course of this work, some new methodology was employed for the rapid and facile determination of total

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cytochrome *cd* nitrite reductase activity in either crude or purified preparations and for convenient photoreduction of cytochromes *c* to be used in strictly anaerobic reactions. These techniques may be of general value to other workers.

Materials and Methods

Paracoccus denitrificans (ATCC 13543) was grown on the nitrate medium of Vernon (1956) for 72 h before harvesting. Cytochrome *cd* was purified either as described by Newton (1969) or by a new procedure which will be described elsewhere. The purity of samples used in the assays ranged from crude, cell-free extracts to homogeneous preparations with a ratio of 410- to 280-nm absorbance of greater than 1. The enzyme content of pure samples was determined by using an approximate extinction coefficient of 150 mM^{-1} cm^{-1} at 410 nm in the oxidized state and for crude samples was determined by activity comparisons to standard preparations.

The small bacterial cytochrome c_{550} from *Paracoccus* was purified as described by Scholes et al. (1971). Eucaryotic cytochromes were purchased from Sigma, rechromatographed on Sephadex G-75, and dialyzed against 20 mM Tris base plus 10 μM EDTA. NADH was purchased from Calbiochem. The photoreductive deazaflavin, 3,10-dimethyl-5-deazaisoalloxazine, was the generous gift of Professor V. Massey, University of Michigan.

All anaerobic assays were performed in Thumberg-type cuvettes obtained from Kontes or Rho Scientific. Cuvettes for titrations were constructed from standard designs (Burleigh et al., 1969). Solutions were deoxygenated by alternating vacuum degassing with flushing by argon that had been scrubbed of residual oxygen by passing through a catalytic copper catalyst (Shriver, 1969). Optical measurements were performed on Gilford or Cary spectrophotometers equipped with chart recorders.

Nitrite reduction was directly monitored by colorimetric determinations of residual nitrite after incubation of a standard solution with enzyme (Lam & Nicholas, 1969; Nicholas & Nason, 1957). An alternative assay quantitated the amount of enzyme present by measuring the total time required for oxidation of a stock amount of NADH. Phenazine methosulfate (PMS) was present to act as a mediator between NADH and cytochrome *cd* and to serve as an indicator. The assay relies on NADH maintaining a steady state in which the PMS is in the colorless, reduced form until the enzyme utilizes all reducing equivalents, leading to the appearance of yellow, oxidized PMS. The standard reaction mixture contained in the main well 5.00 μmol of sodium nitrite, 0.30 μmol of PMS, and an enzyme aliquot in a total volume of 2.4 mL of 50 mM phosphate buffer, pH 6.0, and in the side arm 2.0 μmol of NADH in 0.2 mL of buffer. After deoxygenation, the contents were mixed and the yellow color of oxidized PMS immediately disappeared. The mixture was monitored at 430 nm, and the time required for the reappearance of oxidized PMS was noted. The end point is sufficiently sharp that it can be estimated visually. For precise work, the absorbance corresponding to half-oxidized PMS was determined, as shown in Figure 1.

The time course for the oxidation of ferrocytochromes *c* by *Paracoccus* cytochrome *cd* with oxygen as the ultimate acceptor was determined by the standard assay in open cuvettes developed for mammalian cytochrome oxidases (Smith et al., 1974). With nitrite as the terminal acceptor, two systems were used. The main system employed a synthetic deazaflavin as an in situ photoreductant for cytochrome *c*. Massey et al. have shown that deazaflavins can act in catalytic amounts as photoreductants under mild illumination with a primary amine

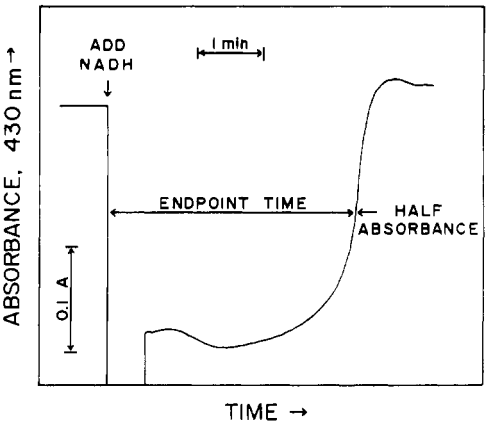

FIGURE 1: Absorbance changes for the NADH/PMS assay of nitrite reductase activity in a crude, cell-free extract. The standard assay mixture described under Material and Methods was used at 25 °C. Reaction was initiated at the arrow by tipping in NADH from the side arm. The dead time represents mixing and operations with the spectrophotometer. The half-time for reaction was taken as the time for reappearance of half of the absorbance due to oxidized phenazine methosulfate. The particular assay shown contained cytochrome *cd* at a final concentration of 16 nM.

as the ultimate source of electrons (Massey & Hemmerich, 1978). The standard assay in this study consisted of 500 μM sodium nitrite in a volume of 0.01 mL of buffer in the side arm while the main well contained 1–100 μM cytochrome *c* that was initially oxidized, 0.8 μM deazaflavin, and 10 mM sodium EDTA in total volume of 2.79 mL of 50 mM phosphate buffer, pH 6.0. The cuvette was sealed, deoxygenated, and illuminated 3 min in a Rayonet carosel photochemical reactor equipped with 350-nm lamps with a 40-nm band width at half-height. The half-time for reduction of 10 μM cytochrome *c* is approximately 20 s, and 3 min of irradiation ensures quantitative reduction. Cytochrome *cd* is itself reduced by deazaflavin in the absence of cytochrome *c* but with half-times estimated to be on the order of 5 times as long. Cuvettes were constructed of Pyrex which further filters the shorter ultraviolet radiation. This system eliminated problems with traces of oxygen which distort the kinetics. Residual oxygen is reduced by the enzyme–cytochrome *c* mixture during illumination to that level where it is no longer a kinetically effective substrate, and then illumination regenerates the substrate ferrocytochrome *c*. It is also convenient to produce mixtures of ferro- and ferricytochrome by controlling the time of illumination.

In control experiments, cytochrome *c* was alternatively reduced with dithionite and the excess removed by gel filtration on a short column of Sephadex G-25. The enzyme was added to the side well before thorough deoxygenation. Assay results agreed with those employing photoreduction.

Concentrations of ferrocytochrome *c* were calculated from the absorbance difference at 550 nm between the sample and the sample fully oxidized, either by enzymatic action or after addition of ferricyanide, by using a difference extinction coefficient of 18.5 mM^{-1} cm^{-1}. Data analyses and curve fitting were performed by a general, nonlinear least-squares program adapted for use on a dedicated PDP 11/03 minicomputer. The computer program has been documented in detail by its authors (Dye & Nicely, 1971) and was obtained from the Michigan State University Computer library. It can fit arbitrary functions of up to 20 parameters to data sets containing up to 99 points. The generality of the program and its computational flexibility did not limit attempts to fit an integrated rate law. On the PDP 11/03 execution time for the final functional form was approximately 5 s/data set. The

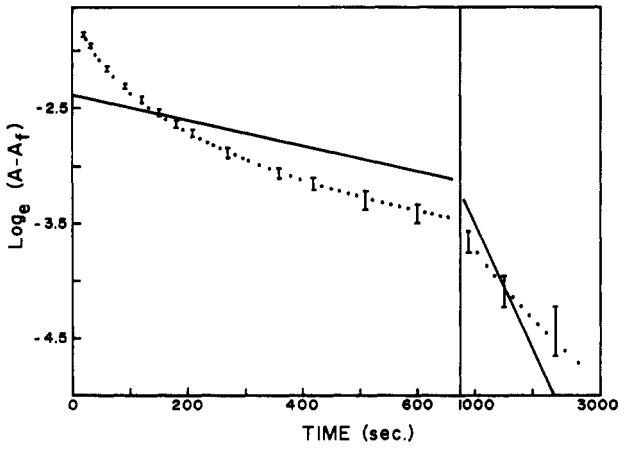

FIGURE 2: The time course of oxidation of 10.2 μM ferrocytochrome *c* by *Paracoccus* cytochrome *cd* in a typical anaerobic nitrite assay, at 25 °C, in 50 mM phosphate buffer at pH 6.0. The natural log of the absorbance A minus A_f, the final absorbance of completely oxidized cytochrome *c*, is plotted vs. time. The time scale has been changed at 700 s for brevity of display. Observed data points are plotted as vertical line segments where the heights of the error bars correspond to error limits, assuming a constant error of ±0.003 in any measurement of absorbance A. The limits increase with increasing time because of the relative contribution of error in A as $A - A_f$ becomes zero. The solid line represents the least-squares fit to data points between 18 and 3000 s for a simple exponential law. The dotted line was calculated from the integrated rate law with the following parameter values: $u_1 = 3.67 \times 10^{-4}$ s^{-1}, $u_2 = 9.07 \times 10^{-2}$ μM^{-1}, $u_3 = -5.86 \times 10^{-2}$ μM^{-1}, and a difference extinction coefficient for cytochrome *c*, ferro to ferri, of 0.0185 μM^{-1}.

parameters u_i are output by the program along with statistical measures of the goodness of fit.

Results

The NADH/PMS clock reaction provides a facile assay for total nitrite reductase activity. Segel has discussed the conditions under which an enzyme may be quantitated by measuring the total time for reaction of a standard quantity of substrate (Segel, 1975). The integrated rate equation discussed later satisfies these conditions. The enzyme concentration is predicted to be proportional to the reciprocal of the end point time, and this was confirmed between 0 and 50 nM enzyme. In control experiments, the concentration of residual nitrite at the end of the clock reaction was measured by the colorimetric dye procedure and found to agree with the expected concentration based on the amount of NADH consumed.

In spectrophotometric assays of purified or partially purified *Paracoccus* cytochrome *cd*, the time course of the decrease in ferrocytochrome absorbance with either nitrite or oxygen as the terminal acceptor superficially resembled an exponential curve. However, careful examination of logarithmic plots, Figure 2, indicated the failure of a simple first-order law to describe the data. In assays on crude cell-free extracts, the fit may be much closer. However, such extracts also demonstrate much greater activity with oxygen as the terminal acceptor than with nitrite and tend to be opulescent. They may be contaminated with minute membrane fragments arising from cell disruption and containing the membrane-bound oxygen respiratory enzyme (Scholes & Smith, 1968).

Several lines of evidence support the conclusion that the complexities of the reaction truly reflect enzyme kinetics and not heterogeneity in the preparations. (1) Progression curves have the same form during the isolation process for cytochrome *cd*. Extensive experiments on alternative purification steps have been performed, and the general form of the progression curve has remained constant. (2) Attempts were made to fit the progression curves as the sum of two exponentials by using four adjustable parameters (two preexponential factors plus two decay constants). Agreement was poor with clear systematic error and low confidence levels. Further, data sets obtained under conditions where only the concentration of catalytic amounts of enzyme was varied refined to parameters which did not vary in a systematic, monotonic fashion with cytochrome *cd* concentration. (3) Published data of an independent investigator from an earlier study of *Paracoccus* cytochrome *cd* [Figure 1B of Newton (1969)] also fail to fit a simple exponential yet can be effectively explained by the equation to be described.

At the highest ferrocytochrome concentrations employed, 100 μM, the progression curves did not show saturation, that is, a zero-order behavior or constant reaction rate. The most accurate initial slopes required extrapolation to zero time. An alternative approach was the deduction of the integrated rate equation.

Empirical evaluation of the data plus consideration of the factors mentioned under Discussion led to an integrated rate equation

$$S = S_0 \exp\left[-u_1 t - \frac{u_2(S - S_0)}{1 + u_3(S - S_0)} \right] \qquad (1)$$

where S is the concentration of ferrocytochrome *c* at time t, S_0 is the initial concentration of ferrocytochrome, and u_1, u_2, and u_3 are adjustable parameters. As will be discussed, these are constants for identical experimental runs, but they do change in a systematic manner with initial substrate concentration.

As shown in Figure 2, the equation accounted for the progression curve over at least 95% of its extent. Ignoring u_3 or setting it to zero still predicted a good approximation to the observed time course but led to a small systematic underestimation at the beginning of the reaction. Accurate estimation of the parameters required accurate values for time, ferrocytochrome concentration, and the final absorbance at "infinite" time. The latter was especially important since S and hence $S - S_0$ were calculated from observed absorbance differences.

Statistical percent standard deviations for the fit of the parameters in a single experiment were typically 4% for u_1, 1% for u_2, and 3% for u_3. The significance level of the fit was better than 0.001. Percent standard deviations for multiple experiments under presumably identical conditions were greater, typically 10%. In part, this may have been due to the correlation between the u_i; i.e., an inaccurate value for one u_i may be compensated by incorrect adjustment of the others to obtain nearly the minimal sum of residuals. This is an inherent and unavoidable consequence of any multiparameter data-fitting algorithm.

Both horse cytochrome *c* and *Paracoccus* cytochrome c_{550} were effective donors to the enzyme, and both obeyed the same integrated rate law. Expressed in terms of the parameter u_1, horse cytochrome *c* reacted with 77% of the activity of *Paracoccus* cytochrome c_{550}.

Specific values for the parameters varied depending on assay concentrations. Examples of this behavior are shown in Table I. The parameter u_1 varied proportionally with enzyme concentration and hence was of the form

$$u_1 = \text{constant} \cdot e$$

where e is total enzyme concentration. u_1 also decreased with increasing total concentration of cytochrome *c* (ferro plus

Table I: Concentration Effects on the Rate Parameters of the Integrated Rate Law for *Paracoccus* Nitrite Reductase

substrate mixture[a] (μM)	acceptor[b]	enzyme (nM)	$u_1 \times 10^3$ (s^{-1})	$u_2 \times 10^2$ (μM^{-1})	$u_3 \times 10^2$ (μM^{-1})
10.0 *c*(II)	NO_2^-	8.5	0.71	7.9	−3.0
10.0 *c*(II)	NO_2^-	21.2	1.35	8.0	−5.6
10.0 *c*(II)	NO_2^-	29.7	2.23	7.9	−5.3
10.0 *c*(II)	NO_2^-	42.4	3.24	8.5	−5.3
2.5 *c*(II)	NO_2^-	25.3	5.00	47.3	−95.4
10.0 *c*(II)	NO_2^-	25.3	1.24	7.4	−4.1
40.0 *c*(II)	NO_2^-	25.3	1.05	1.7	0.02
5.0 *c*(II)	NO_2^-	13.0	1.21	12.6	−7.3
5.0 *c*(II) + 5.0 *c*(III)	NO_2^-	13.0	1.03	14.3	−8.3
5.0 *c*(II) + 15.0 *c*(III)	NO_2^-	13.0	0.56	12.9	−7.1
5.0 *c*(II) + 35.0 *c*(III)	NO_2^-	13.0	0.01	16.5	−7.6
5.0 *c*(II)	O_2	134	1.39	17.4	−13.3
5.0 *c*(II) + 5.0 *c*(III)	O_2	134	0.56	18.0	−12.8
5.0 *c*(II) + 15.0 *c*(III)	O_2	134	0.30	17.1	−12.5
5.0 *c*(II) + 35.0 *c*(III)	O_2	134	0.02	17.9	−13.6
10.0 *c*(II)	NO_2^-	135	5.09	7.2	−5.2
10.0 *c*(II)	O_2	135	0.76	9.4	−5.7
10.0 *c*(II)	NO_2^-	35	1.7	8.6	−5.1
10.0 c_{550}(II)	NO_2^-	35	2.2	8.3	−6.3

[a] Symbols used: *c* stands for horse cytochrome *c* and c_{550} stands for *Paracoccus* cytochrome c_{550}; (II) denotes ferrocytochrome and (III) denotes ferricytochrome. [b] The concentration of O_2 was always 270 μM and that of NO_2^- was 500 μM.

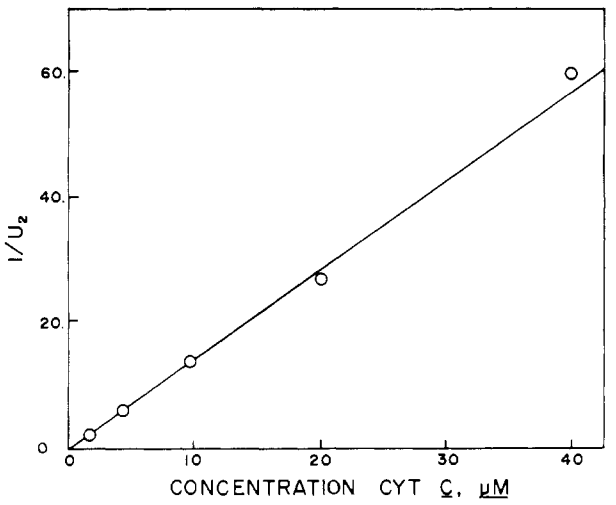

FIGURE 3: The inverse of parameter u_2 is plotted vs. the initial concentration of the substrate ferrocytochrome for reactions in which nitrite is the terminal acceptor. The value for the intercept at zero ferrocytochrome concentration is numerically too small to be obtained graphically from the plot. It was calculated from eq 8 for each value of S_0 and averaged to obtain 2.7×10^5 M^{-1}.

ferri). The parameter u_2 varied inversely with ferrocytochrome concentration as shown in Figure 3. It was constant with variable cytochrome *cd* concentration. The parameter u_3 was also independent of cytochrome *cd* concentration but showed a marked dependence on cytochrome *c* concentration. All three parameters were independent of nitrite concentration over the range of 50 μM–50 mM.

The product of nitrite reduction, NO dissolved in solution, was an inhibitor of the reaction. When aliquots of NO-saturated buffer were added to the reaction vessel through a septum prior to mixture, the rate of reaction diminished. Because of the nonequilibrium distribution of NO in the system with this technique, the precise concentration of NO in solution was unknown, and a thorough numerical analysis of product inhibition has yet to be completed. Reaction in buffer saturated with NO was greater than 95% inhibited when compared with the case of no initial NO.

Difference spectra were obtained for oxidized cytochrome *cd* plus dissolved NO vs. oxidized cytochrome *cd* as well as reduced cytochrome *cd* plus NO vs. reduced cytochrome *cd* with both the sample and reference in anaerobic cuvettes in 50 mM phosphate, pH 6.0. The dominant feature in the oxidized case was a shift of the Soret band maximum from 409 to 412 nm. Oxidized cytochrome *cd* was titrated anaerobically with aliquots of NO-saturated buffer injected through a septum and monitored at either 405 or 420 nm, the difference spectrum extrema. Double-reciprocal plots of observed absorbance changes vs. estimated NO concentration gave an approximate association binding constant of 2×10^5 M^{-1}. Data scatter was severe, and it could not be ascertained whether binding was of a simple, single-site nature or more complex. The reduced case has yet to be studied in detail.

Discussion

The enzyme activity assay using NADH plus PMS as indicator/mediator is an extremely facile technique for monitoring nitrite reductase activity in cytochrome *cd* containing preparations. It is convenient for monitoring multiple fractions obtained during purification steps. Although it provides only a relative determination, it is far less time consuming than nitrite colorimetric assays. In our hands, it has proven to be less susceptible to interference by oxygen and by turbidity in the sample. Side reactions between electron donors and the nitrite colorimetric reagents need not be of concern. The assay has accelerated concurrent research on the purification and isolation of cytochromes *cd* from *Paracoccus denitrificans* and *Micrococcus halodenitrificans*. Of course, colorimetric assays for nitrite continue to be of importance because they directly monitor nitrite concentrations. A rational strategy for monitoring cytochrome *cd* activity is to use the colorimetric assay as a control or spot check and the NADH/PMS reaction for rapid, routine determinations.

The integrated rate law for nitrite-linked electron transfer affords a firm basis for a detailed kinetic analysis of cytochrome *cd*. The parameters u_i are appropriate observables against which kinetic mechanisms may be tested. There is clearly a functional dependence between the u_i and the reaction conditions, and a correct correspondence between observed and predicted behavior will be a stringent requirement for a successful mechanistic scheme.

Examination of the rate parameters for the reactions of horse cytochrome *c* and *Paracoccus* cytochrome c_{550} with *Paracoccus* cytochrome *cd* (Table I) shows that cytochrome *c* is 77% as effective as cytochrome c_{550} as an electron donor, expressed relative to the parameter u_1. The X-ray crystal structure of cytochrome c_{550} (Timkovich & Dickerson, 1976) has determined that cytochrome c_{550} is structurally homologous to eucaryotic cytochrome in internal polypeptide folding and in the surface region around the heme crevice. Other surface areas on cytochrome c_{550} contain insertions or deletions of the polypeptide chain or localizations of acidic residues not found in cytochrome *c*. It is possible that the active interface between cytochrome *cd* and donors cytochrome *c* is the common heme crevice region, although the present kinetic data are not conclusive evidence for this hypothesis.

A fundamental conclusion of this report is that the electron transfer reactions between cytochromes *cd* and *c* are distinct from the kinetics that characterize mitochondrial oxidases. Mitochondrial cytochrome oxidase aa_3 reacts with a simple exponential progression curve in a spectrophotometric assay

(Smith et al., 1974). This has been interpreted as indicating equal binding constants for ferrocytochrome as well as ferricytochrome and subsequent product inhibition by ferricytochrome (Yonetani & Ray, 1965; Errede et al., 1976; Errede & Kamen, 1978). The complexities of the cytochrome *cd* reaction indicate that this is not the case for cytochrome *cd* or that binding and ferricytochrome inhibition are not the dominant rate-determining characteristics. Either alternative makes the enzyme action distinct from that of mitochondrial cytochrome oxidase aa_3.

If one considers that cytochrome *cd* has three possible substrates (ferrocytochrome, NO_2^-, and O_2), three possible products (ferricytochrome, NO, and the reduction product of O_2), and two subunits with the logical possibility of cooperativity between subunits, then the complete kinetic description would require consideration of a minimum of 54 rate constants and equations. This is an extremely ambitious task. A rational beginning is to consider the simplest mechanisms which correctly predict general trends in the assay data. Later refinements in the proposals may then be introduced to account for unexplained complexities, additional kinetic data over expanded conditions, or deviations between predicted and observed parameters. At the present time our main concern is whether commonly proposed kinetic models for mitochondrial oxidases can be applied to cytochrome *cd*. The remaining discussion is intended to demonstrate the inadequacies of mitochondrial oxidase kinetic proposals when applied to cytochrome *cd*. Although a final mechanistic proposal for cytochrome *cd* is not intended, we can eliminate certain schemes from consideration and illuminate others, even though incomplete, as more attractive for future study.

Under the conditions of steady-state assays (nitrite concentrations greater than 50 μM or oxygen concentrations greater than 200 μM), the concentrations of nitrite and oxygen do not affect the rate of the reaction [data from this study and also from Gudat et al. (1973) and Lam & Nicholas (1969)]. Hence, they may be neglected in considering rate-limiting steps.

The simplest Michaelis–Menten scheme

$$\mathrm{E} + \mathrm{S} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES} \xrightarrow{k_3} \mathrm{E} + \mathrm{P} \qquad (2)$$

generally predicts non-first-order integrated rate laws. Here E is the free enzyme cytochrome *cd*, S is any of the possible substrates, P is any of the possible products, ES is either a true enzyme–substrate complex or a reaction intermediate, EP is the enzyme–product complex or a second intermediate, e is the total cytochrome *cd* concentration, S_0 and P_0 are the initial concentrations, and S and P are now ferro- and ferricytochrome.

$$S = S_0 \exp\left[-\frac{k_1 k_3 e t}{k_2 + k_3} - \frac{k_1 (S - S_0)}{k_2 + k_3} \right] \qquad (3)$$

However, the coefficients of t and $S - S_0$ are true constants, independent of S or P, and this is not the case for the parameters u_1 and u_2 for cytochrome *cd*.

All kinetic proposals for mitochondrial oxidases introduce the concept of product inhibition. Minnaert (1961) and Errede & Kamen (1978) have discussed various alternatives in detail. A general consequence is the appearance of terms proportional to $1/S_0$ and $1/P_0$. For example, Minnaert's mechanism IV

$$\mathrm{S} + \mathrm{E} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES} \xrightarrow{k_3} \mathrm{EP} \underset{k_6}{\overset{k_5}{\rightleftharpoons}} \mathrm{E} + \mathrm{P} \qquad (4)$$

integrates to

$$S = S_0 \exp\left[\frac{-k_1 k_3 k_5 e t}{(k_2 + k_3)(k_5 + k_6)(P_0 + S_0)} - \frac{[k_1(k_3 + k_5) - k_6(k_2 + k_3)](S - S_0)}{(k_2 + k_3)[k_5 + k_6(P_0 + S_0)]} \right] \qquad (5)$$

For the special case of equal affinity for ferrocytochrome (S) and ferricytochrome (P), $k_1 = k_6$ and $k_2 = k_5$, and the second term disappears. The integrated law becomes a pure exponential in time, but the first-order constant decreases with either increasing S_0 or increasing P_0. This is the proposed behavior for mitochondrial oxidase. Schemes other than eq 4, involving product inhibition of some form, are possible (Minnaert, 1961), but the general form

$$S = S_0 \exp\left[\frac{-\text{constant} \cdot e t}{\text{constant} + (P_0 + S_0)} - \frac{\text{constant}(S - S_0)}{\text{constant} + (P_0 + S_0)} \right] \qquad (6)$$

is obtained with the second term disappearing under conditions of equal ferri–ferro binding and a decrease in both terms with increasing P_0 or S_0.

For the case of cytochrome *cd*, the appearance of the parameter u_2 in the integrated rate law would support the hypothesis of unequal binding constants for ferro- and ferricytochrome. As predicted, both u_1 and u_2 decrease with increasing S_0 (ferrocytochrome). However, it appears that only u_1 varies with P_0 (ferricytochrome) while u_2 remains approximately constant.

X-ray structure determinations have revealed highly similar average conformations for the two oxidation states of cytochrome *c* (Mandel et al., 1977). It is therefore reasonable to expect that an oxidase would bind ferri- and ferrocytochrome with equal strength. However, chemical and spectroscopic evidence supports the hypothesis that there are structural differences between the two states (Margoliash & Schejter, 1966). Such differences may be dynamic in nature, due to differences in flexibility or vibrational modes in the cytochrome chain which are not determined by the static X-ray structures. Bacterial cytochrome *cd* may be able to discern this difference in cytochrome *c* as an electron-donating substrate.

If one considers the reaction with nitrite as the terminal acceptor, the ultimate product NO must be considered as a potential inhibitor. Equilibrium binding studies in the absence of substrates indicated NO bound to oxidized cytochrome *cd* with an association constant of 2×10^5 M^{-1}. If the cytochrome *cd*–NO complex is nonreactive, increasing concentrations of NO will decrease the reaction rate. A pseudo-first-order law with respect to ferrocytochrome coupled to rapid equilibrium between cytochrome *cd* and NO

$$\text{cytochrome } cd + \mathrm{NO} \underset{}{\overset{K_1}{\rightleftharpoons}} \text{cytochrome } cd\text{–NO} \qquad (7)$$

in which there is no initial NO predicts an integrated law

$$S = S_0 \exp\left[-\frac{k_a e t}{1 + K_I S_0} - \frac{K_1}{1 + K_I S_0}(S - S_0) \right] \qquad (8)$$

where k_a can be a collection of constants describing reactions between cytochromes *c* and *cd*. Although this is product inhibition at the level of NO, it is expressed in terms of S_0 and S, ferrocytochrome concentrations, by the stoichiometry of the reaction and the fact of no initial NO. For reasons discussed under Results, our experiments are constrained to this latter condition. The integrated rate law predicts the observed inverse relations between u_1, u_2, and S_0. Furthermore, it

predicts that a plot of $1/u_2$ vs. S_0 should be a straight line of unit slope such that the reciprocal of the intercept is K_I. The solid line fitting the inverse plot of u_2 in Figure 3 corresponds to a slope of 1.33 and a predicted K_I of 2.7×10^5 M^{-1}.

Neither the unequal cytochrome *c* binding scheme, eq 6, nor the NO inhibition scheme, eq 8, by itself satisfies all the observed behavior. The former predicts u_1 and u_2 should decrease with increasing P_0, ferricytochrome, and this is obeyed for u_1 but not u_2. The latter accounts for the behavior of parameters in nitrite assays but does not explain the existence of nonexponential progression curves in O_2 assays where inhibitory NO is not present. The reduction product of O_2 by cytochrome *cd* has never been definitively shown to be H_2O.[1] Indeed, hydrogen peroxide, H_2O_2, has not been ruled out as the product, and it is logically possible to postulate that peroxide could act as a product inhibitor in O_2 assays as does NO in nitrite assays. The interactions of peroxide and cytochrome *cd* are complex, but it has been shown that peroxide initially added to an O_2 assay mixture is not inhibitory (M. Robinson and R. Timkovich, unpublished experiments).

It is possible that the O_2 reactions represent the kinetics influenced solely by ferricytochrome *c*–ferrocytochrome *c* while the nitrite assays have just added the aspect of NO inhibition. The parameter u_2 is smaller for nitrite reactions than for oxygen reactions. This roughly corresponds to greater inhibitory effects which may be due to summing two modes of inhibition.

The origin of the parameter u_3 remains obscure. It affects predominantly the initial stages of the time course. If omitted or set to zero, the resultant fit is still good over most of the time course, but with systematic error at short times. Its existence could arise from a differential rate law involving powers of substrate concentration. Such a form is common for multiple subunit enzymes demonstrating allosteric controls (Segel, 1975). The binding of a first molecule of ferrocytochrome to one subunit could possibly affect interactions at the second subunit.

The relative reactivities of nitrite and oxygen bear on this question. Other conditions equal, reactions with nitrite are 6 times faster than with oxygen, expressed relative to u_1 (see Table I). Yet, under the conditions employed, the rates are independent of the oxygen or nitrite concentration. It is possible that wholly different rate-limiting steps are involved, but then it is surprising to find similar integrated rate laws and similar parameter behavior. Other alternatives are that nitrite is an activator of the system or oxygen is a deactivator.

The existence of some form of allostery in cytochrome *cd* would explain why it appears as a multisubunit enzyme with four metal centers when the reduction of nitrite to NO is a unit electron transfer. It is reasonable to expect that the production of toxic NO within the cell would be subject to strict control mechanisms.

[1] Recently, evidence has been presented that water is indeed the reduction product of O_2 for *Paracoccus* cytochrome *cd* [Timkovich, R., & Robinson, M. K. (1979) *Biochem. Biophys. Res. Commun. 88*, 649].

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