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Reduction of Cytochrome Oxidase by 5,10-Dihydro-5-methylphenazine: Kinetic Parameters from Rapid-Scanning Stopped-Flow Experiments†

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ABSTRACT: The kinetics of the reduction of resting cytochrome oxidase and of its cyanide complex by 5,10-dihydro-5-methylphenazine (MPH) have been characterized by rapid-scan and fixed-wavelength stopped-flow spectrophotometry in the Soret, visible, and near-IR spectral regions. In this study, we focused on a form of the resting enzyme that is characterized by a Soret absorption maximum at 424 nm. These experiments complement earlier work on the reduction of a 418 nm absorbing form of the resting enzyme [Halaka, F. G., Babcock, G. T., & Dye, J. L. (1981) *J. Biol. Chem. 256*, 1084–1087]. The reduction of cytochrome *a* is accomplished in a second-order reaction with a rate constant of 3×10^5 M^{-1} s^{-1}. The reduction of the 830-nm absorber, Cu_a, is closely coupled to but lags the reduction of cytochrome *a*; we have resolved a rate constant of about 20 s^{-1} for the copper reduction. The reduction of cytochrome *a* proceeds with a rate constant that is nearly independent of the spectral properties of the resting enzyme and of the ligation state of cytochrome a_3. The reduction of cytochrome a_3 occurs by slow, intramolecular electron transfer. We have resolved two phases for this process that have rate constants of $\sim$0.2 s^{-1} and $\sim$0.02 s^{-1} for both the 418- and 424-nm forms of the resting enzyme. It appears, therefore, that spectroscopic heterogeneity at the cytochrome a_3 site in the resting enzyme exerts very little influence on the kinetics of the anaerobic reduction of the oxidase metal centers. From this we conclude that the rate of electron transfer to the a_3 site is probably controlled by the protein conformation and not primarily by local factors within the a_3 environment.

Redox reactions are of central importance to the understanding of the catalytic activity of cytochrome *c* oxidase in electron transport. In biological systems, the enzyme catalyzes the four-electron reduction of molecular oxygen to water [for reviews see Malmström (1979) and Wikström et al. (1981)]. The reduction of the protein, either under anaerobic conditions or during turnover in the presence of oxygen, has been extensively studied, particularly by using its natural substrate, cytochrome *c* [for example, Gibson et al. (1965), Andreasson et al. (1972), Andreasson (1975), Wilms et al. (1981) and Antalis & Palmer (1982)]. The reduction of the oxidase has also been carried out by using the positively charged metal ion complexes hexaaquochromium(II) (Greenwood et al., 1977) and hexaammineruthenium(II) (Scott & Gray, 1980; Reichardt & Gibson, 1982) and by using the negatively charged species $SO_2^{-}\cdot$ (Halaka et al., 1981). Those studies suggest that the charge type of the reductant plays an important role in the kinetics of reduction and in determining the site of interaction with the oxidase.

A second conclusion that has been drawn from this earlier work is that the rate of intramolecular electron transfer from

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cytochrome *a* and its functionally associated copper, Cu_a, to cytochrome a_3 and the second copper, Cu_{a_3}, is a sensitive function of the pretreatment of the enzyme. In resting cytochrome oxidase, these intramolecular rates are sluggish and much too slow to sustain the high turnover rates observed for the fully active enzyme. Prior oxidation/reduction cycles remove this kinetic restriction to an extent and accelerate the intramolecular rates. A phenomenological model describing this activation process has appeared recently (Wilson et al., 1981). Paralleling the diverse kinetic behavior that the oxidized enzyme can exhibit is a variability in its spectroscopic properties (Lemberg, 1969; Orii & King, 1976; Brudvig et al., 1981). Although the Soret absorption maximum of the turnover-activated, or oxygen-pulsed (Wilson et al., 1981), enzyme is red-shifted relative to the resting form, it is unclear at present whether this spectroscopic property is a reliable indication of a more reactive form of the enzyme.

The reductant 5,10-dihydro-5-methylphenazine (MPH)[1] was used in the present work to study the reduction of different forms of the oxidized enzyme by rapid-scanning stopped-flow spectrophotometry. MPH was chosen because of its spectral properties (Halaka et al., 1982), which can be used to monitor oxygen, if present either deliberately as a reagent or accidentally as a contaminant, as well as the number of electrons used for the reduction of the oxidase. MPH is a neutral molecule whose reduction potential (~80 mV at neutral pH; Jagendorf & Margulies, 1960) is sufficient to drive the four-electron reduction of the oxidase. Thus we are able to monitor both the rate of electron transfer between MPH and cytochrome oxidase and rates of intramolecular electron redistribution within the protein.

In a previous paper (Halaka et al., 1981), we characterized the spectral changes that accompany the reduction of resting (418-nm species, see below) cytochrome oxidase by MPH in the wavelength region 330–520 nm. We interpreted our results to indicate that cytochrome *a* of the oxidase is reduced faster by MPH than is cytochrome a_3. Spectral changes arising from the oxidase, as well as from the MPH–MP^+ couple (commonly known as PMS), were followed in the above-mentioned wavelength region. From these spectral changes, we concluded that the stoichiometry of the reduction was two MPH (four electrons) per one oxidase molecule. In the present work, we report rapid-scan results over the 400–650-nm spectral range for the reduction by MPH. We present details of the kinetics of this reduction monitored at several fixed wavelengths, including some in the 830-nm region. We have also investigated the relationship between the spectral properties of the resting enzyme and the rate of reduction by MPH. These results, in conjunction with the conclusions we reached from the principal component analysis of this reaction presented elsewhere (Halaka, 1981), allow us to construct an overall model for the reduction of cytochrome oxidase by MPH.

Materials and Methods

Cytochrome oxidase was prepared by the method of Hartzell & Beinert (1974). In the course of preparing the enzyme over a 4-year period, we have noticed a preparation to preparation variation of about 6 nm in the wavelength maximum of the Soret band of the freshly isolated enzyme. Similar variations have been noted and commented upon by others (Wharton & Gibson, 1968; Lemberg, 1969; Kuboyama et al., 1972). In the work presented here and previously (Halaka et al., 1981), we have focused on two of these enzyme preparations, one with λ_{max} = 418 nm and designated in the text as the "418-nm species" and the other with λ_{max} = 424 nm and designated in the text as the "424-nm species", in order to assess the relationship between spectral and kinetic properties. In agreement with previous observations by Wharton & Gibson (1968), we note little difference in catalytic activity of the various resting forms of the enzyme. Other details of the handling of the enzyme have been described previously (Babcock et al., 1976). Cytochrome oxidase concentration is expressed on a per aa_3 basis. The oxidized CN-bound complex was prepared by the addition of a concentrated KCN solution (pH 8.0) to the oxidase solution in order to give a final CN^- concentration of about 500 μM. The resulting solution was incubated at 4 °C in an air-tight bottle for about 10 h. The spectral properties of the CN-bound oxidase were checked by using a Cary 17 spectrophotometer. MPH was prepared as previously described (Halaka et al., 1982). Anaerobicity was achieved by a series of evacuations and fillings with purified argon. Solutions were kept under about 3 psig argon pressure. Unless otherwise mentioned, all reagents were prepared in 50 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer containing 0.5% Tween 20, pH 7.4. Argon gas was purified by passage through a 1-m BASF catalyst column heated to 100 °C. Water was doubly distilled in glass. All other reagents were of analytical grade and were used without further purification unless stated otherwise.

A double-beam, vacuum-tight, rapid-scanning stopped-flow apparatus was used throughout this study (Papadakis et al., 1975; Coolen et al., 1975; Suelter et al., 1975). The apparatus was made anaerobic by at least four cycles of evacuations (<0.001 mmHg), each followed by flushing with argon, and was kept under ~1 psig of argon pressure throughout the course of the stopped-flow experiments. For anaerobicity tests and other details on the performance of the stopped-flow system, see Halaka (1981). Although scanning experiments produce data with high signal-to-noise ratio and can be used to obtain rate parameters, kinetic analyses were usually carried out with data collected in a fixed-wavelength mode. This was chosen to ensure collecting more data points, which could not be done in the scanning mode due to computer memory limitations. The reproducibility in the fixed-wavelength mode was such that the same rate constants to within ±7% could be obtained in different experiments. Nonlinear least-squares fitting was carried out by using the program KINFT4 (a modified version of the program KINFIT; Dye & Nicely, 1971).

Results

Reduction of the Cyanide-Bound Cytochrome Oxidase by MPH. When ferric cytochrome a_3 binds to cyanide, the Soret absorption peak of the oxidized protein, regardless of its position in the resting enzyme, occurs at 427 nm. This is attributed to a shift in the cyt a_3^{3+} peak to 427 nm upon formation of its low-spin cyano complex. Under these conditions, electron transfer to the a_3 site is inhibited, and the reduction of cytochrome a^{3+} and its associated copper, Cu_a can be isolated.

Reduction of the CN complex is fast and monophasic. The spectra collected during the reaction show, by comparison of absorbance changes at 388 and 444 nm, with $\Delta\epsilon_{388}$ = 21 mM^{-1} cm^{-1} and $\Delta\epsilon_{444}$ = 25 mM^{-1} cm^{-1} (Van Buuren et al., 1972), that the stoichiometry of the reduction in this case is

$$MPH + [\text{cyt } a^{3+}\cdots Cu_a^{2+}] \rightarrow MP^+ + [\text{cyt } a^{2+}\cdots Cu_a^{+}] \quad (1)$$

The reduction of Cu_a is deduced since two electrons are provided to the oxidase–CN complex by MPH. Only one electron

[1] Abbreviations: HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; MPH, 5,10-dihydro-5-methylphenazine (reduced PMS); MP^+, 5-methylphenazinium ion; PMS, phenazine methosulfate.

Table I: Rate Constants for the Reduction of Various Cytochrome Oxidase Species by MPH[a]

enzyme prepn	k_1 (M^{-1} s^{-1})	k_2 (s^{-1})	k_3 (s^{-1})
oxidized + KCN[b]	(2.7 ± 0.3) × 10^5		
418-nm species[c]	(1.8 ± 0.3) × 10^5	0.19 ± 0.03	0.020 ± 0.001
424-nm species[d]	(3.2 ± 0.5) × 10^5	0.19 ± 0.02	0.021 ± 0.003

[a] For details, see Halaka (1983). [b] Average obtained from experiments at two different oxidase and two different MPH concentrations. [c] From Halaka et al. (1981). [d] From the experiments described in this paper; average obtained from experiments at five different oxidase concentrations and three different MPH concentrations; data analysis at 605 or 444 nm gave the same rate constants within error limits.

is detected in the reduced heme band at 444 nm [see also Blair et al. (1982)].

The rate constant of the reaction was determined from analysis of fixed-wavelength experiments at two different reactant concentrations. Analysis of the data at 430, 444, and 605 nm showed that the reaction is a simple second-order process (first-order in both reactants and monophasic). The rate constant is given in Table I. Deviations from the calculated curves at three wavelengths were small and random.

The behavior with MPH as a reductant is different from that found by Scott & Gray (1980) for reduction by $Ru(NH_3)_6^{2+}$, as they observed biphasic kinetics and attributed the fast phase to the reduction of cytochrome *a* and, by implication, the slow phase to the reduction of cytochrome a_3.

Inspection of their Figure 3 shows that the amplitude of the absorbance change during the slow phase is only about half that in the fast phase, which suggests either incomplete reduction of the CN-bound cytochrome a_3 or substantial changes in the extinction coefficients. Since only relative absorbance changes were given, it is not possible to determine whether cytochrome *a* was fully reduced during the fast phase. If not, biphasic reduction of cytochrome *a*, with no reduction of a_3, would also explain the observed kinetics. Since $Ru(NH_3)_6^{2+}$ is a one-electron donor while MPH donates two electrons, a difference in the mechanism of reduction of cytochrome *a* would not be surprising. In any case, our results show that MPH reduces only cytochrome *a* in the CN-bound oxidase.

Reduction of Oxidized Cytochrome Oxidase by MPH. Selected spectra from the time–wavelength–absorbance surface for the anaerobic reduction of cytochrome oxidase (424-nm species) by MPH are shown in Figure 1. In this wavelength region (410–640 nm), the MPH–MP^+ couple does not contribute appreciably to the absorbance. In the α-band region (580–630 nm), ~80% of the absorbance change is completed during the fast-reduction phase (see below). Note also the shift in the peak position in the α-region to shorter wavelengths in the final spectrum, a phenomenon that, although small, is reproducible. This shows that the component that is reduced in the last stage of the reaction (cytochrome a_3) has its peak at around 595 nm compared to 604 nm for cytochrome *a*. This observation is further confirmed by the study of the reduction of the oxidase by sodium dithionite (Halaka et al., 1981).

Nonlinear least-squares fitting of absorbance changes at 444 nm, as well as at several other wavelengths, showed that the anaerobic reduction of the oxidase by MPH is triphasic. The initial fast phase is best described by a second-order reaction between MPH and cytochrome *a*. This is followed by two slower first-order processes, as discussed below.

Analysis of the Fast Phase. Since the rates of the three processes were sufficiently different to allow their separation, the first phase was analyzed by simply using the first 200 ms of the reaction. Analysis of the full time course (which extends

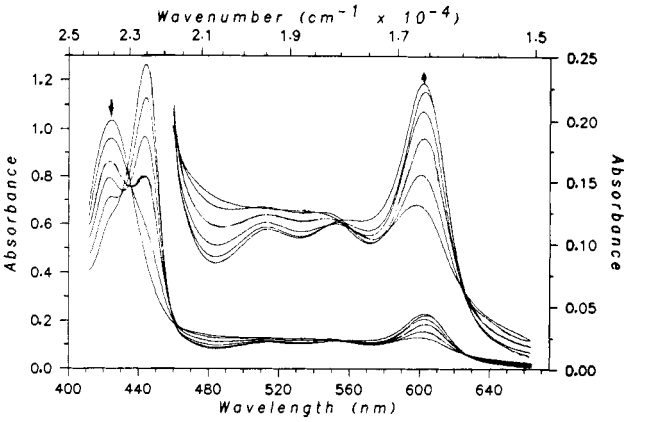

FIGURE 1: Selected spectra in the reaction of MPH with cytochrome oxidase. Concentrations after mixing: cytochrome oxidase, 3.2 μM; MPH, 26 μM. Note the shift in the α-peak in the last spectrum to a shorter wavelength. Times of spectra in seconds from bottom to top at 600 nm are 0.014, 0.110, 0.354, 0.914, 25.87, and 292.

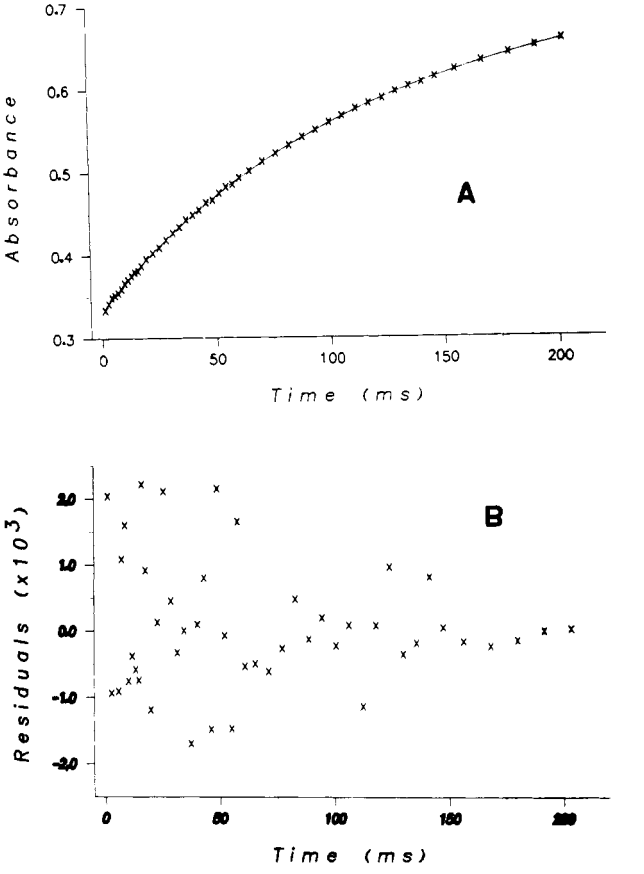

FIGURE 2: (A) Fit of the fast phase for the reduction of cytochrome oxidase by MPH. ×'s are the experimental data points, and the solid line is the calculated curve. Data were collected in a fixed-wavelength mode. Concentrations after mixing were (oxidase) 3.1 μM and (MPH) 19 μM. (B) Residuals of the fit shown in (A). Residuals are defined as *A*(calculated)−*A*(observed).

to more than 5 min) was also done by using a second-order phase followed by two first-order processes (Halaka, 1981). Full time course analysis becomes easier when the MPH concentration is high compared to that of the oxidase, so that a three-exponential equation representing the three phases can be used. Figure 2 displays a tyical fit of a second-order equation to the initial phase of the reduction (in a fixed-wavelength experiment at 444 nm). The residuals are random. The same second-order rate constant, to within experimental errors, was obtained for data collected at 605 nm (see Table I). The change in absorbance at 444 nm corresponding to the

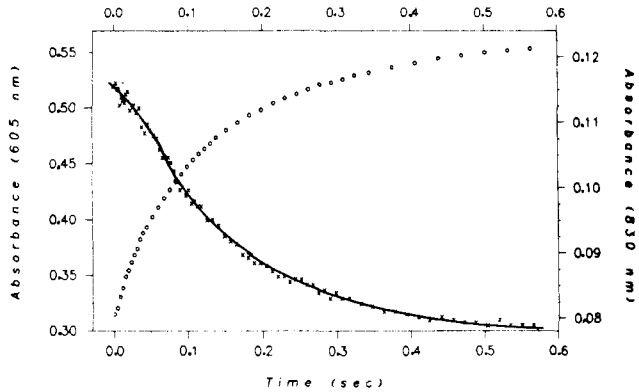

FIGURE 3: Decay at 830 (×) and growth at 605 nm (○) during the reduction of 10.8 μM cytochrome aa_3 by 27 μM MPH. The fit of eq 4 to the data at 830 nm is drawn as the solid line.

first phase was about 50% of the total change at that wavelength. By contrast, at 605 nm the fast phase accounted for about 80% of the total absorbance change, in agreement with the results of Scott & Gray (1980). This suggests that cytochrome *a* contributes more to the absorbance at 605 nm (of the reduced oxidase) than does cytochrome a_3, in agreement with earlier suggestions (Vanneste, 1966; Nicholls & Chance, 1974; Wikström et al., 1976) and with the results of principal component analysis (F. G. Halaka et al., unpublished results). The results obtained in a similar way for the 418-nm species of the oxidized enzyme (Halaka et al., 1981) are also given in Table I. For all three species, the cyanide-bound enzyme and the two different resting forms, the second-order rate constants for the fast-reduction phase are similar.

Kinetics of the Fast Phase at 830 nm. The kinetics of the reduction of cytochrome oxidase by MPH was studied by following the absorbance changes at 830 nm as well at 605 nm. The kinetics of the growth at 605 nm showed the expected second-order behavior in the fast phase with essentially the same second-order rate constant as that determined at 444 nm (Table I). The rate of the 830-nm band decay, however, showed a detectable lag under identical conditions (Figure 3). A simple scheme that accounts for this observed lag at 830 nm and agrees with the kinetic measurements at 605 nm can be represented as

$$\underset{}{\mathrm{MPH}} + \underset{\mathrm{A}}{[\mathrm{cyt}\ a^{3+}\cdots \mathrm{Cu}_a^{2+}]} \xrightarrow{k_1^f} \underset{\mathrm{B}}{\mathrm{MPH}^{+}\cdot[\mathrm{cyt}\ a^{2+}\cdots \mathrm{Cu}_a^{2+}]} \xrightarrow{k_2^f} \mathrm{MP}^{+} + \underset{\mathrm{C}}{[\mathrm{cyt}\ a^{2+}\cdots \mathrm{Cu}_a^{+}]} \quad (2)$$

This scheme assumes that most, if not all, of the absorbance change at 830 nm is due to the reduction of Cu_a^{2+} (Beinert et al., 1980). Assuming that $MPH^{+}\cdot$ can transfer an electron very rapidly to any oxidized heme *a* formed when an electron is transferred to Cu_a^{2+}, the heme iron will, in effect, remain reduced while transferrring electrons to its "associated" copper. This would make the observed rate at 605 nm proportional to the rate of production of species B + C, defined in eq 2. Since this rate is second order, as discussed above, the concentration of species B + C at time *t* (measured at 605 nm) would be given by

$$([\mathrm{B}] + [\mathrm{C}])^t = \frac{[\mathrm{L}^0](1 - e^{-k't})}{[\mathrm{L}^0]/[\mathrm{M}^0] - e^{-k't}} \quad (3)$$

where $[L^0]$ and $[M^0]$ are the initial concentrations of MPH and cytochrome oxidase, respectively, and $k' = k_1^f([L^0] - [M^0])$. At 830 nm, rate of change in absorbance is proportional to the production of species C and is given by

$$d[\mathrm{C}]/dt = k_2^f([\mathrm{B}] + [\mathrm{C}] - [\mathrm{C}]) \quad (4)$$

The term [B] + [C] was replaced by its value from eq 3. The resulting differential equation was solved numerically, and k_2^f was adjusted. The fit of the data from two separate time courses (Figure 3) yielded $k_2^f = 17.8 \pm 0.5\ s^{-1}$.

Kinetics of the Slow Phase. In previous work on the reduction of cytochrome oxidase (418-nm species) by MPH, we showed that the slow "phase" of the anaerobic reduction was best fitted by two first-order processes, with rate constants that are independent of both MPH and cytochrome oxidase concentrations (Halaka et al., 1981). This indicates that the slow phases represent intramolecular redistribution of electrons from the reduced cytochrome a/Cu_a pair to the cytochrome a_3/Cu_{a_3} pair. During this process, both Cu_a and cytochrome *a* are maintained in their reduced states by MPH so that the spectral changes observed reflect only the reduction of the metal components at the O_2 reducing site. We have now carried out a similar analysis for the reduction of cytochrome a_3 by MPH in the 424-nm form of the resting enzyme.

The data were fit by three rate laws: a single first-order process, two parallel first-order reactions, and a single second-order process. Visual inspection of the goodness of fit provided little information since the maximum deviations were only ±0.004 absorbance unit out of a total change of ~0.3 unit. As shown in Figure 4, however, the residuals provided a clear choice. Only for the fit by two parallel first-order processes are the residuals random, with maximum deviations of ±0.0008 absorbance unit. This indicates that intramolecular electron-transfer reactions (the slow phases) occur by two first-order processes. This conclusion is strongly supported by the fact that the rate constants are independent of the oxidase and MPH concentrations (Table I).

The rate constants, obtained by least-squares analysis of the fast and slow phases of reduction of the 424-nm species by MPH, are collected in Table I, along with the corresponding data reported earlier for the 418-nm species (Halaka et al., 1981). The two first order rate constants for the slow phases are essentially the same for the two different spectral forms of the enzyme. We also find that the relative amplitude contributions of the two slow phases to the total slow phase magnitude are about the same for the two forms of the resting enzyme. The faster of the two processes (rate constant k_2 in Table I) contributes between 10 and 20% of the absorbance change, and the second (rate constant k_3 in Table I) is responsible for the remainder of the change.

Discussion

It is known that positively charged reductants such as $Cr(H_2O)_6^{2+}$ (Greenwood et al., 1977) and $Ru(NH_3)_6^{2+}$ (Scott & Gray, 1980; Reichardt & Gibson, 1982) preferentially reduce the cytochrome *a* site of the oxidase. We here add that MPH, a neutral molecule, also reduces only the *a* site directly. These reductants can thus be used as artificial electron donors that imitate the biological substrate, cytochrome *c*.

The anaerobic reduction of resting cytochrome oxidase by MPH is a triphasic process. Figure 5 presents a schematic interpretation of these phases in relation to the various metal centers of the protein. The fast initial phase, which arises from a second-order reaction between the cytochrome a^{3+} site on the enzyme and the reductant, accounts for about 50% of the total absorbance change at 444 nm and for 75–80% of the total absorbance change at 605 nm. Although we have not yet quantified these relative contributions in detail, we have noticed that they are a function of the initial state of the oxidized protein. The 424-nm species, for example, has a slightly higher

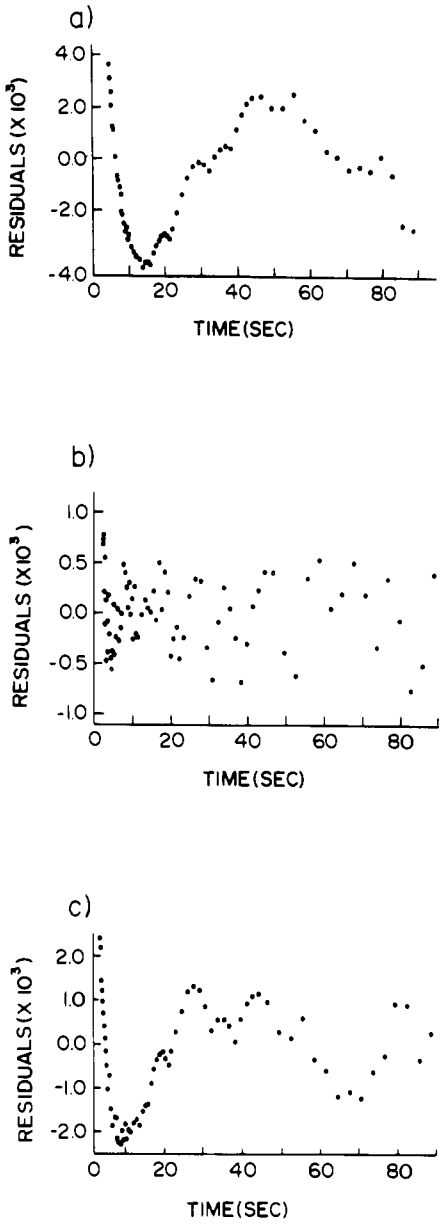

FIGURE 4: Residual plots for the fit of the "slow phase" to one exponential (a), to two exponentials (b), and to a second-order process (c). Residuals are defined as A(calculated) − A(observed).

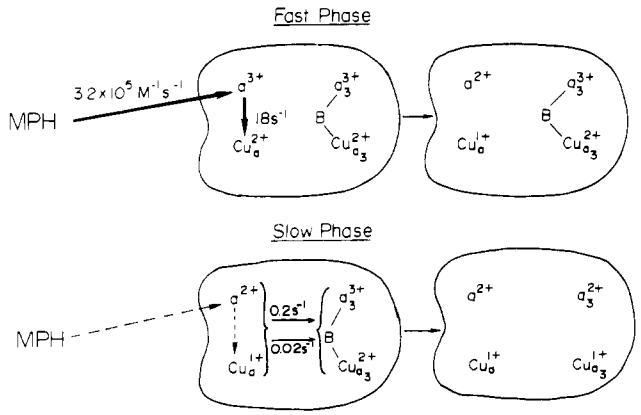

FIGURE 5: Model for the reduction of resting cytochrome oxidase by MPH. See text for details.

absorbance at 600 nm than does the 418-nm species (G. T. Babcock, unpublished results). Since the spectrum of the fully reduced protein is essentially the same for both of these forms, the percent contribution of the fast phase in the α-band region is somewhat greater for the 424-nm species than for the 418-nm species.

The rate data show that the second-order reduction of cytochrome a^{3+} (k_1 in Table I) is relatively insensitive to either the spectral form of the resting enzyme (418-nm or 424-nm species) or the spin state of the cytochrome a_3 iron. This observation is consistent with data presented previously for the reduction of cytochrome oxidase by chromous ion in which second-order rate constants for the reduction of cytochrome a in resting oxidase, in oxidized cyanooxidase, and in mixed-valence, carbonmonoxy cytochrome oxidase were within a factor of 2 of each other (Greenwood et al., 1977). Similarly, rate constants for the reduction of cytochrome a in resting and oxygen-pulsed oxidase by cytochrome c also show agreement to within a factor of 2 (Wilson et al., 1981). These results indicate that the entry of electrons into cytochrome oxidase by way of cytochrome a is nearly independent of both the conformation of the protein and perturbations to the local environment of cytochrome a_3.

Accompanying the fast, second-order reduction of cytochrome a is the reduction of the Cu_a site. The present data on the kinetics in the near-IR region (830 nm) show that Cu_a lags in the anaerobic reduction with respect to the cytochrome a component (measured only for the 424-nm form of the enzyme). Thus, when MPH, a two-electron reductant, reacts with oxidase, the first electron is transferred to cytochrome a. The very fast equilibration between cytochrome a and Cu_a observed with cytochrome c as reductant [addressed in detail by Antalis & Palmer (1982)] does not occur with MPH. Rather, cytochrome a appears to remain reduced during transfer of a second electron to Cu_a, similar to the behavior observed for the cyanide-bound enzyme discussed above. Our data, however, agree with the observations by Wilson et al. (1975), who show that during the reduction of the oxidase by cytochrome c in the presence of oxygen, the decay at 830 nm either lagged or coincided with the growth at 605 nm depending on the reductant concentration. From the lag we observe, we have extracted a rate constant for the reduction of Cu_a of 18 s^{-1}. This number, although somewhat lower, can be compared to results reported by Greenwood and co-workers (1976, 1977), who used two methods to estimate rate constants pertinent to the reduction of Cu_a. In the first paper, they used temperature-jump studies of partially reduced carbonmonoxy oxidase in the presence of cytochrome c to estimate a value of 40–100 s^{-1} for the sum of the forward and reverse rate constants for electron exchange between cytochrome a and Cu_a. In the later experiments, they used stopped-flow studies of the reduction of cytochrome oxidase by hexaaquochromium(II) ion to obtain a value of 40–70 s^{-1} for the forward rate constant for reduction of Cu_a. Thus, the Cu_a rate constants obtained in the three different experiments show a fair degree of consistency.

The latter two phases in the reduction of cytochrome oxidase by MPH are extremely slow relative to enzyme turnover rates, and reflect intramolecular transfer from the a/Cu_a couple to the a_3 and (presumably) Cu_{a_3} sites (Halaka et al., 1981; see above). Our observations are not unique to the MPH reduction process; similar internal rates have been observed for hexaaquochromium(II) (Greenwood et al., 1977), hexaammineruthenium(II) (Reichardt & Gibson, 1982), dithionite (Halaka et al., 1981), and cytochrome c (Gibson et al., 1965). As noted above, however, these rates of internal transfers appear to be susceptible to modulation by structural factors within the protein (Wilson et al., 1981). As an example, Reichardt & Gibson (1982) showed that hexaammine-

ruthenium(II) reduced the resting enzyme slowly, the intramolecular transfers proceeding with a rate constant of ~0.019 s^{-1} ($t_{1/2}$ = 37 s). This slow rate is comparable to that which we observe for MPH. Upon turnover activation of the enzyme, however, this intramolecular rate constant increased by a factor of more than 10^3 (to 70 s^{-1}). Although Wilson et al. (1981) have proposed a kinetic model that provides a plausible first-order mechanistic explanation for this activation phenomenon, its molecular basis remains obscure.

We have attempted to provide some insight into this question by characterizing k_2 and k_3 for two different spectral forms of the resting enzyme. The rationale for this approach is based upon the fact that in turnover-activated, or oxygen-pulsed, cytochrome oxidase, a considerable red shift in the Soret maximum (to 428 nm) occurs. There also appears to be a substantial shift in the EPR (electron paramagnetic resonance) characteristics of this form of the enzyme (Shaw et al., 1978; Brudvig et al., 1981). This suggests, in turn, that the activation process may involve a local perturbation to the cytochrome a_3 site, which produces both the changes in optical and EPR properties and changes in the activated state. In this model, the 424-nm species of the resting enzyme may contain a greater proportion of activated oxidase than the 418-nm species and consequently may be expected to show faster intramolecular electron transfer. That this is not the case, at least under anaerobic conditions, is shown clearly by the data in Table I. The intramolecular rates, in terms of both rate constants and relative contribution to the slow phase, are essentially the same for the two different spectral forms of the resting enzyme.

It appears, therefore, that the kinetic properties of the enzyme as isolated are determined by structural effects that may be distributed throughout the protein rather than concentrated at the cytochrome a_3 site. Additional evidence that this is the case has been provided by Bonaventura et al. (1978), who showed that cross-linking of the resting protein was sufficient to block the resting to activated transition. Wilson et al. (1981), arguing from this observation and others, also concluded that it is the enzyme conformation that is key to the kinetic state of the enzyme.

The basis for the altered spectral properties of the various forms of the oxidized enzyme remains obscure. On the basis of EPR (Brudvig et al., 1981) and, to some extent, of Raman (Babcock et al., 1981) data, it appears that the heterogeneity is restricted to the a_3 site. While a shift in the Soret maximum does accompany aging of the protein, it is clear that the various spectral forms do not, in general, result from degradation or denaturation of the protein [see comment on enzyme activity above and the more detailed study by Wharton & Gibson (1968)]. Muijsers et al. (1971) have cited the presence or absence of O_2 as influencing the position of the Soret maximum, but we have noted variations of up to 6 nm in the position of this band for different, but identically isolated, preparations of the enzyme. From our data, however, it is clear that the different spectral forms of the resting enzyme behave kinetically in an indistinguishable fashion and that, with MPH as the reductant, the fast phase corresponds to cytochrome *a* and Cu_a reduction, while the slow phases represent intramolecular transfer to the O_2 reducing site.

The origin of *two* slow intramolecular electron-transfer rates rather than one remains obscure. The two rate constants differ by a factor of 10, with the faster process accounting for only 15–20% of the total absorbance change of cytochrome a_3. This may be due to the enzyme heterogeneity described above. If so, the essentially identical kinetic results for the 418-nm and the 424-nm forms show that the heterogeneity responsible for the different electron-transfer rates is not the same as that which shifts the a_3 band from 418 to 424 nm. It should be noted that the same types of enzyme preparation also show heterogeneity in the rate of reduction by dithionite (F. G. Halaka et al., unpublished results). The absorbance due to cytochrome a_3 is rapidly decreased approximately 20% by dithionite, followed by slower reduction of both sites. By contrast, Jones et al. (1983), using enzyme prepared by the Yonetani (1960) method, found no corresponding fast phase and concluded that reduction by dithionite occurs preferentially at the cytochrome *a* site. Clearly, the effects of the method of preparation of the enzyme on the spectral and kinetic properties need further study.

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Kinetics of Tyramine Transport and Permeation across Chromaffin-Vesicle Membranes†

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ABSTRACT: Tyramine permeates chromaffin-granule membranes via a reserpine-insensitive mechanism. The rate is unsaturable and increases with pH, indicating permeation of the unprotonated form of the amine. Reserpine-insensitive dopamine uptake is at least 10 times slower, consistent with dopamine's lesser lipophilicity. Dopamine is transported into chromaffin-granule membrane vesicles via a saturable, reserpine-sensitive, proton-linked mechanism. Tyramine inhibits dopamine transport with a K_i of 5–10 μM. Tyramine is not accumulated nearly as well as dopamine because inward transport is opposed by outward permeation. Nevertheless, the velocity of reserpine-sensitive tyramine transport can be deduced from the steady-state level of tyramine accumulation and the rate of permeation. V_{max} for tyramine transport is about one-third of the value for dopamine transport. Therefore, two aromatic hydroxyls are not needed for monoamine transport but are required for efficient accumulation and storage.

In studying the substrate specificity of membrane transport systems, one is generally limited to impermeant substrates although the behavior of more lipophilic molecules might be of interest. For example, catecholamine storage vesicles transport dopamine [2-(3,4-dihydroxyphenyl)ethylamine] but only poorly accumulate its more lipophilic analogue tyramine [2-(4-hydroxyphenyl)ethylamine]. It is thought that tyramine is transported, but because of its greater lipophilicity, it leaks back across the membrane in its unprotonated form (Scherman & Henry, 1980; Johnson et al., 1982). Because of this permeation, the rate of tyramine transport cannot be measured as the rate of net uptake. Nevertheless, it is important to measure the rate of tyramine transport for two reasons. First, it is necessary to clarify the substrate specificity of the amine translocator. Second, it may explain the well-known ability of tyramine to deplete vesicular catecholamine stores.

Transport into catecholamine storage vesicles has been most completely characterized in the chromaffin granules of the adrenal medulla (Carmichael, 1983). Chromaffin granules take up catecholamines via an H^+-linked transport system (Njus et al., 1981). The granule membrane has an inwardly directed H^+-translocating adenosinetriphosphatase (ATPase). Catecholamines are taken up by exchange diffusion for H^+ with an H^+/catecholamine stoichiometry of 2 (Knoth et al., 1980, 1981a; Phillips & Apps, 1980; Johnson et al., 1981). This exchange is thought to be mediated by a reserpine-sensitive amine translocator. The translocator has a rather broad specificity since chromaffin granules exhibit reserpine-sensitive uptake of epinephrine, norepinephrine, dopamine, and serotonin (DaPrada et al., 1975).

The amine translocator presumably catalyzes reserpine-sensitive tyramine transport as well [Figure 1(2)]. Tyramine can also cross the membrane by deprotonating [Figure 1(5)] and permeating in its unprotonated form [Figure 1(3)]. Tyramine fluxes via these two pathways establish a steady state in which inward transport is opposed by outward permeation. We have employed the following strategy to characterize the kinetics of tyramine transport and permeation across chromaffin-granule membranes. We can block transport using

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