Structure and Reactivity of Multiple Forms of Cytochrome Oxidase As Evaluated by X-ray Absorption Spectroscopy and Kinetics of Cyanide Binding†

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ABSTRACT: The extended X-ray absorption fine structure (EXAFS) data show differences between the active site structures of different cytochrome oxidase preparations. In the resting (as isolated) state of the Yonetani preparation, the bridging atom between $Fe^{3+}_{a_3}$ and $Cu^{2+}_{a_3}$ is present [Powers, L., Chance, B., Ching, Y., & Angiolillo, P. (1981) *Biophys. J. 34*, 465], whereas in another preparation (e.g., Hartzell–Beinert), this atom seems to be bound only to $Fe^{3+}_{a_3}$ in a significant fraction of the molecules. Both preparations bind cyanide in a multiphasic fashion, suggesting that the resting cytochrome oxidase is not homogeneous but rather is a mixture of several forms. The proportion of these forms as detected by cyanide binding kinetics differs for different preparations. However, upon reduction and reoxidation (conversion to the "oxygenated" form) the cyanide binding kinetics become monophasic and all preparations of the oxygenated form bind cyanide at the same rate. Thus, a combination of structural and kinetic approaches seems necessary for evaluation of the nature of the active site of cytochrome oxidase in its various forms.

In the resting state solubilized cytochrome oxidase (ferrocytochrome *c*:O_2 oxidoreductase EC 1.9.3.1) isolated by different methods, and by the same method at different times, is known to be heterogeneous. Such heterogeneity has been observed in the reaction of the enzyme with hydrogen peroxide (Bickar et al., 1982; Chance et al., 1983; Kumar et al., 1983) and with other ligands (Brudvig et al., 1981). X-ray absorption studies of the enzyme purified by different methods also have presented evidence for structural heterogeneity (Scott et al., 1981; Powers et al., 1981). Spectroscopically, the Soret peak of the isolated enzyme may be found anywhere between 418 and 424 nm in the enzyme as isolated (Lemberg & Barrett, 1973). Such variability can be simply interpreted in terms of purified cytochrome oxidase being a variable mixture of more than one distinct molecular form of the enzyme.

Other than the structural variability of the "resting" enzyme, a distinct form known as the "oxygenated" form is formed when cytochrome oxidase is reduced with excess dithionite and reoxidized with oxygen (Orii & Okuniki, 1963). This form has a Soret peak at 428 nm and enhanced absorption in the α region.

X-ray absorption studies suggest that the sulfur atom bridging Fe_{a_3} and Cu_{a_3} found in the resting form of the Yonetani preparation is missing in the oxygenated form which has an Fe_{a_3} structure similar to that of the peroxidases (Chance et al., 1983). Recently, we have established that the oxygenated form is a peroxide derivative of the enzyme (Kumar et al., 1984). Antonini et al. (1977) have shown that this form possesses greater enzymatic activity than the resting form. Such recently reoxidized oxidases have been given different names by different authors: "oxygenated" (Orii & Okuniki, 1963), "pulsed" (Antonini et al., 1977), "oxygen pulsed" (Armstrong et al., 1983), "oxy–ferri" (Nicholls & Chanady, 1981), and "peroxidatic" or "non-sulfur bridged" (Chance et al., 1983). However, we shall retain the term oxygenated for the reduced and reoxidized form throughout this paper.

A sensitive and convenient method of analyzing structural heterogeneity would be to study the kinetics of a ligand that slowly reacts with the enzyme. Different molecular species may react with the ligand at different rates. With this consideration, we have analyzed in detail the reaction of cyanide with different cytochrome oxidase preparations. Earlier studies suggest that cyanide binds at the cytochrome a_3 active site of cytochrome oxidase [for a review, see Erecinska & Wilson, (1981)]. These results are compared to structural differences observed by X-ray absorption spectroscopy of the same enzyme preparations.

Preliminary reports describing some of this work have appeared previously (Kumar et al., 1983; Powers et al., 1983).

Experimental Procedures

Biochemical Methods. Cytochrome oxidase was prepared from beef heart by different methods: (a) modified Hartzell–Beinert method, prepared by Dr. G. T. Babcock (preparation 1), and Hartzell–Beinert method (Hartzell & Beinert, 1974), donated by Dr. C. R. Hartzell (preparation 2); (b) Yonetani method (Yonetani, 1961), prepared in our laboratory (preparations 3 and 4); (c) modified Volpe and Caughey method (Yoshikawa et al., 1977), prepared in our laboratory (preparations 5 and 6); (d) Frey et al. method (Frey et al., 1978), prepared by Dr. T. G. Frey (preparation 7).

In the first six preparations cytochrome oxidase is solubilized in different detergents. In the seventh, however, the cytochrome oxidase is membrane bound and hence called membranous oxidase.

Fresh cyanide solutions, prepared in 1 mM KOH, were used in all experiments. The membranous oxidase was used as a dilute solution in 0.1 M phosphate buffer, pH 7.4. All the other preparations were diluted in 0.1 M phosphate buffer, pH 7.4, containing 0.5% Tween 20. Cytochrome oxidase concentrations were measured from reduced (by sodium di-

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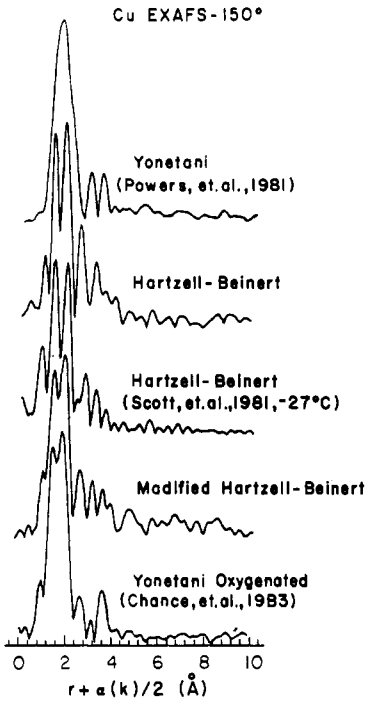

FIGURE 1: Fourier transforms of the Cu EXAFS obtained by background subtraction and k^{+3} (wave vector) multiplication for oxidized cytochrome oxidase. From top to bottom: Yonetani (preparation 3) (Powers et al., 1981); Hartzell–Beinert (preparation 2); Hartzell–Beinert (Scott et al., 1981); modified Hartzell–Beinert (preparation 1); oxygenated form of Yonetani preparation (Chance et al., 1983).

thionite) minus oxidized spectra by using $\Delta\epsilon = 20.5$ mM^{-1} cm^{-1} at 605–630 nm (Yonetani, 1960). A Johnson Foundation dual wavelength spectrometer (Chance et al., 1972) and stoppered cells of 10-mm path length were used for all measurements.

Cyanide was added to the enzyme solution and mixed thoroughly, and difference spectra were run at desired time intervals, with the spectrum of the enzyme before cyanide addition serving as the base line. The reference wavelength used was 522 nm.

The oxygenated enzyme was prepared by reduction under argon of a concentrated enzyme solution by dithionite for 20 min followed by gentle oxygenation for 1 min. Oxygenated oxidase, thus prepared, was immediately diluted and used for cyanide binding studies.

X-ray Absorption Methods. Extended X-ray absorption fine structure (EXAFS)[1] measurements were made at the Stanford Synchrotron Radiation Laboratory during dedicated operation of the SPEAR storage ring. Conditions and methods of data collection and analysis were identical with those previously described (Powers et al., 1981; Chance et al., 1983). Optical spectroscopy and EPR spectroscopy were used to ensure sample integrity (Chance et al., 1980). [For a detailed description of methods and terminology, see Powers (1982).]

Results and Discussion

Copper EXAFS Studies. Figure 1 summarizes the results of X-ray absorption studies on the copper atoms of the different preparations described under Experimental Procedures. Examination of the data presented in Figure 1 shows clearly that the various preparations are different in their local structure at the copper sites. The sulfur-bridged resting form of the Yonetani preparation gives a single first coordination shell (Powers et al., 1981); the non-sulfur-bridged oxygenated form

[1] Abbreviations: EXAFS, extended X-ray absorption fine structure; EPR, electron paramagnetic resonance.

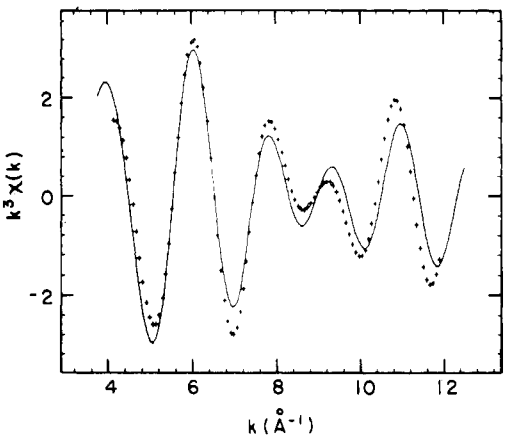

FIGURE 2: Comparison of the copper first shell data normalized to one copper atom of the EXAFS data: Hartzell–Beinert (preparation 2) (—) and that reported by Scott et al. (1981) (+).

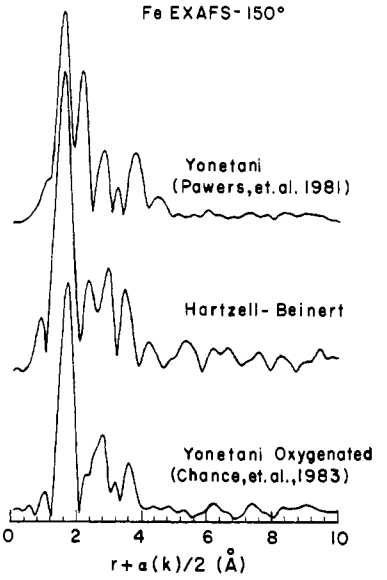

FIGURE 3: Fourier transforms of the Fe EXAFS obtained by background subtraction and k^{+3} (wave vector) multiplication for oxidized cytochrome oxidase. From top to bottom: Yonetani (preparation 3) (Powers et al., 1981); Hartzell–Beinert (preparation 2); oxygenated form of Yonetani preparation (Chance et al., 1983).

(Chance et al., 1983) gives a split first shell. The Hartzell–Beinert preparation in the resting state also gives a split first shell; however, detailed comparison of the filtered first shell data show that it is different from, and cannot be fitted to by a combination of, the resting and oxygenated forms of the Yonetani preparation. The modified Hartzell–Beinert preparation is more similar to the oxygenated form of the Yonetani preparation. The poor agreement between the data on different preparations clearly suggests the presence of molecular heterogeneity and significant structural differences among these preparations. These differences not only are found in the first coordination shell but also extend throughout the higher shells. It is worth noting that on the basis of our studies of Yonetani preparations we have identified an outer shell contribution in Cu EXAFS to be from Fe_{a_3}. Scott et al. (1982), studying a Hartzell–Beinert preparation, find no evidence for the presence of iron in their Cu EXAFS. [Scott et al. (1984) now assign a peak in their Cu EXAFS to Fe atom.] Our present data on a Hartzell–Beinert preparation and the reported EXAFS data by Scott et al. also differ from each other (Figure 2).

We observe higher shells in both copper (Figure 1) and iron (Figure 3) EXAFS data of the Hartzell–Beinert preparation

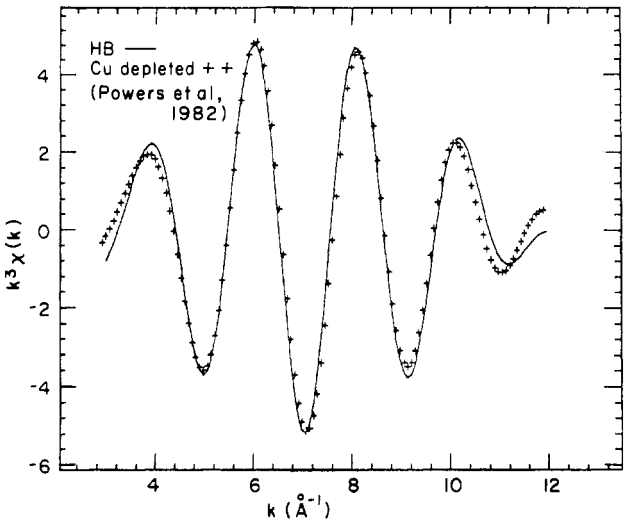

FIGURE 4: Comparison of the iron first shell filtered data normalized to one iron atom for Hartzell–Beinert (preparation 2) (—) and copper-depleted preparation (Powers et al., 1982) (+).

used in this study. However, these higher shell peaks do not meet the criteria that were used to identify the iron of the active site, Fe_{a_3}, in copper EXAFS of the resting and oxygenated forms of the Yonetani preparation. It should also be noted that the two sets of copper EXAFS data presented on samples of the Hartzell–Beinert enzyme by Scott et al. (1981) (at −27 and −50 °C) differ from each other significantly. While part of these differences between the different sets of data may be due to signal-to-noise limitations, we believe that structural heterogeneity in different samples of the enzyme prepared by the same method is also a significant contributing factor.

Iron EXAFS. Results on iron X-ray absorption studies of different preparations are summarized in Figure 3, and they further extend our observations on enzyme heterogeneity. The Yonetani preparation shows a split first coordination shell in the resting form, the second of which results from the bridging sulfur atom (Powers et al., 1981) in the active site. This bridging sulfur is missing from the first shell of the oxygenated form of the Yonetani preparation where this sulfur is coordinated only to the active site copper, resulting in a single shell and a structure of the active site iron that is similar to that of peroxidase (Chance et al., 1983). The Hartzell–Beinert preparation also shows a single first shell peak in its resting form. However, this peak occurs at a different distance from that of the oxygenated form of the Yonetani enzyme. The higher coordination shells of the Hartzell–Beinert preparation are also different from both the resting and oxygenated forms of the Yonetani enzyme. Our attempts to assign a structure to the heme a_3 active site in the Hartzell–Beinert preparation show that the first shell of this preparation is similar to that observed for the copper-depleted oxidase (Weintraub & Wharton, 1981) (Figure 4) and suggest that a large fraction of this preparation may have a similar iron environment (Powers et al., 1982). The copper-depleted oxidase (Weintraub & Wharton, 1981) has been shown (Powers et al., 1982) to have a cytochrome a_3 similar to cytochrome c, with five nitrogen and one sulfur ligands. This suggests that the sulfur atom is ligated only to Fe_{a_3} in the Hartzell–Beinert preparation whereas in the resting state of the Yonetani preparation it bridges the Fe_{a_3} and Cu_{a_3}. In the oxygenated state of the Yonetani preparation, the sulfur atom is bound to Cu_{a_3} only.

The structural similarity suggested by EXAFS between cytochrome c and cytochrome a_3 in the Hartzell–Beinert preparation is a somewhat surprising result since other studies (Babcock et al., 1981; Kent et al., 1983; Tweedle et al., 1978) show that cytochrome a_3 is a high-spin heme in the resting state. However, a Raman study does suggest that cytochrome a_3 is six-coordinated (Babcock et al., 1981).

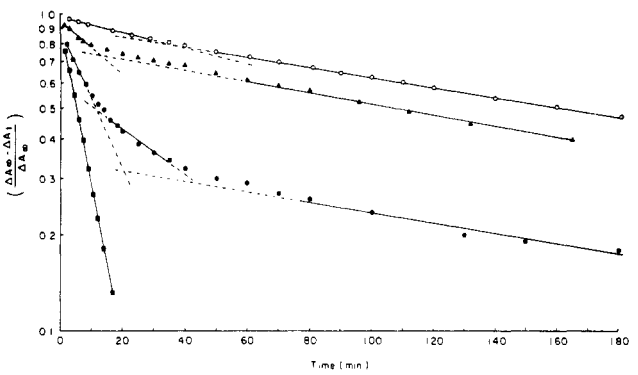

FIGURE 5: Kinetics for the reaction of cyanide with different preparations of cytochrome oxidase: (●) preparation 1; (○) preparation 3; (▲) preparation 4; (■) preparation 7 (for specification of preparations, see Experimental Procedures). Concentration of cytochrome oxidase was in the range 6–8 μM. The reaction was initiated by the addition of 1.33 mM cyanide. Change of absorbance was monitored in the Soret region (432–411 nm), 0.1 M phosphate buffer containing 0.5% Tween 20, pH 7.4, with a temperature of 21 °C.

Cyanide Binding to Resting Cytochrome Oxidase. The reaction of resting oxidase with cyanide is very slow. The spectral changes observed in the wavelength region studied are similar to those observed by van Buuren et al. (1972a). The difference in ΔA between the maxima and minima in the Soret region difference spectra (432 minus 411 nm) was used for all calculations of ligand binding kinetics. ΔA_∞ (absorbance change in infinite time) measurements were made about 20 h after initiation of the cyanide reaction.

Figure 5 shows the progress of reaction with cyanide of cytochrome oxidase prepared by different methods. The kinetic curve for the reaction of cyanide with the modified Hartzell–Beinert preparations, for example, is nonlinear in a semilog plot. Analysis of the data shows that the kinetic curve may be decomposed into three separate exponentials of different amplitude. Each of the exponents, plotted separately in Figure 6, shows a simple first-order reaction of one part of the oxidase with cyanide which can simply be represented as

$$A = \sum_i A_i e^{-k_i^{\text{eff}} t} \qquad (1)$$

where A is the change in absorbance, k_i^{eff} values are the effective pseudo-first-order constants, and t is the time. Presence of three exponentials in the progress curve, in a simple sense, suggests the presence of three different forms of cytochrome oxidase, each with a different rate of cyanide binding.

The fraction of the total absorbance change that corresponds to each of the three exponents varies significantly from preparation to preparation as shown in Table I. This suggests that the three exponents are not due to successive reaction of a sequence of intermediates since a sequence of reactions such as $E_1 + CN \rightarrow E_2 \rightarrow E_3$ would require that amplitude of the absorbance changes in the different steps be in a fixed ratio. Further, as mentioned in the introduction, heterogeneity has been observed in cytochrome oxidase by other methods, and we are only attempting to quantify the heterogeneity here. In addition, it is unlikely that the multiexponential nature of the reaction is due to the binding of more than one cyanide ligand to each of the enzyme molecules. A cyanide to oxidase stoichiometry of 1:1 has been suggested by earlier studies (Yoshikawa & Orii, 1972; van Buuren et al., 1972b). Hence,

Table I: Rate Constants and Proportion of Different Species in Cytochrome Oxidase As Isolated[a]

method of preparation[b]	$k_1^{eff} \times 10^3$ (s^{-1})	E_1 (%)	$k_2^{eff} \times 10^3$ (s^{-1})	E_2 (%)	$k_3^{eff} \times 10^3$ (s^{-1})	E_3 (%)
(1) modified Hartzell–Beinert	3.72	18	1.14	44	0.061	38
(2) Hartzell–Beinert	5.90	6	1.17	19	0.055	75
(3) Yonetani			0.79	9	0.057	91
(4) Yonetani	2.66	15			0.066	85
(5) modified Volpe–Caughey	2.53	6			0.059	94
(6) modified Volpe–Caughey	2.80	16	1.04	33	0.059	51
(7) membranous	2.26	100				
average value[c]	3.31 ± 1.36[d]					

[a] The values of the effective pseudo-first-order rate constants and the proportion of different species are calculated from data in Figure 5. $k_1^{eff(I)}$, $k_2^{eff(I)}$, and $k_3^{eff(I)}$ are effective first-order rate constants for cyanide binding in different preparations. E_1, E_2, and E_3 are proportions of these species in the resting cytochrome oxidase determined under our experimental conditions (see legend to Figure 5): $E_1 = A_1/\sum_i A_i$, $E_2 = A_2/\sum_i A_i$, and $E_3 = A_3/\sum_i A_i$ where A_1, A_2, and A_3 are preexponential coefficients in eq 1. KCN at 1.33 mM was used in all experiments. [b] For details, see Experimental Procedures. [c] The mean and the standard deviation are shown. [d] The mean second-order rate constant is then 2.5 M^{-1} s^{-1}.

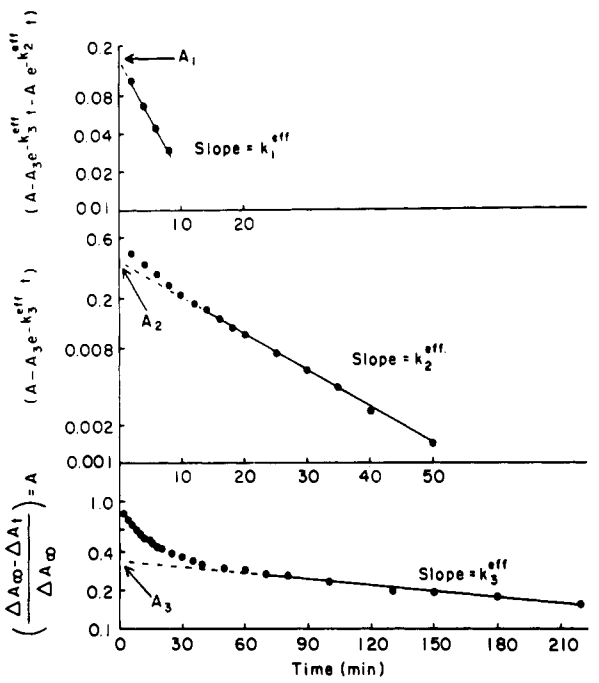

FIGURE 6: Method used to separate the individual exponents and determine the rate constants and preexponential coefficients is shown. The data are for preparation 1 (in Figure 5) and was taken as an example. All other conditions are as in Figure 5.

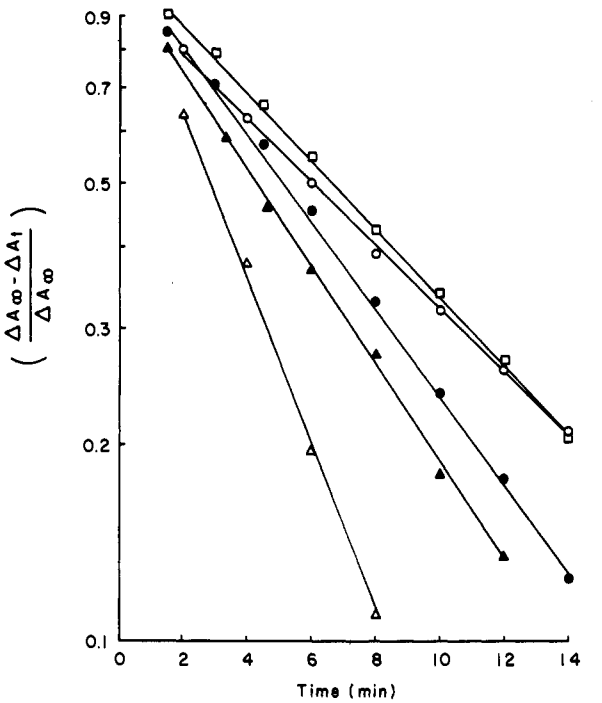

FIGURE 7: Kinetics of the reaction of cyanide with different preparations of cytochrome oxidase, which was reduced and reoxidized (oxygenated form): (●) preparation 1; (△) preparation 2; (○) preparation 3; (□) preparation 5; (▲) preparation 6 (for specifications of preparations, see Experimental Procedures). Concentration of cytochrome oxidase varied from 6 to 8 μM, and the reaction was initiated by the addition of different concentrations of cyanide: 1.33 mM to preparations 1 (●) and 6 (▲), 1.0 mM to preparations 3 (○) and 5 (□), and 2.0 mM to preparation 2 (△). All other conditions are as in Figure 5.

we interpret our data to mean that the different preparations in the resting state consist of a mixture of different amounts of three distinct forms of the enzyme.

Cyanide Binding to Oxygenated Oxidase. Figure 7 shows the kinetics of cyanide binding to different preparations of cytochrome oxidase that have been all converted to the oxygenated form. It is clear from Figure 7 that all preparations are converted to one homogeneous form, showing simple pseudo-first-order kinetics of reaction with cyanide. In Table II, the relevant rate constants are listed. It may be noted from Table II that not only are the rates of cyanide reaction very similar for the various oxygenated preparations but they are also fairly similar to the rate constant of the reaction of the fastest reacting fraction of resting oxidase with cyanide. This suggests that a portion of the resting oxidase is similar to oxygenated oxidase as far as the cyanide reaction is concerned, but its proportion varies from preparation to preparation.

Similar forms of cytochrome oxidase with rates of cyanide reaction faster than resting have been obtained by the reaction of mixed valence oxidase with O_2 (Brittain & Greenwood, 1976) or of the fully reduced oxidase with O_2 (Shaw et al., 1978). These two forms have second-order rate constants of the order of 20 and 2500 M^{-1} s^{-1}, respectively. The second value has been estimated from the data reported by Shaw et al. (1978).

Table II: Rate Constants for Reaction of Oxygenated Form of Cytochrome Oxidase with Cyanide[a]

methods of preparation[b]	k^{II} (M^{-1} s^{-1})	E (%)
(1) modified Hartzell–Beinert	2.0	100
(2) Hartzell–Beinert	2.0	100
(3) Yonetani	1.8	100
(4) Yonetani	2.4	100
(5) modified Volpe–Caughey	1.8	100
(6) modified Volpe–Caughey	2.1	100
(7) membranous	4.0	100
average[c]	2.3 ± 0.7	

[a] The values of second-order rate constants (k^{II}) are calculated from data from Figure 7. E is the proportion of enzyme that accounts for the reaction with cyanide with rate constants shown; i.e., E is the preexponential coefficient in eq 1 if $i = 1$. [b] For details, see Experimental Procedures. [c] The mean and the standard deviations are shown.

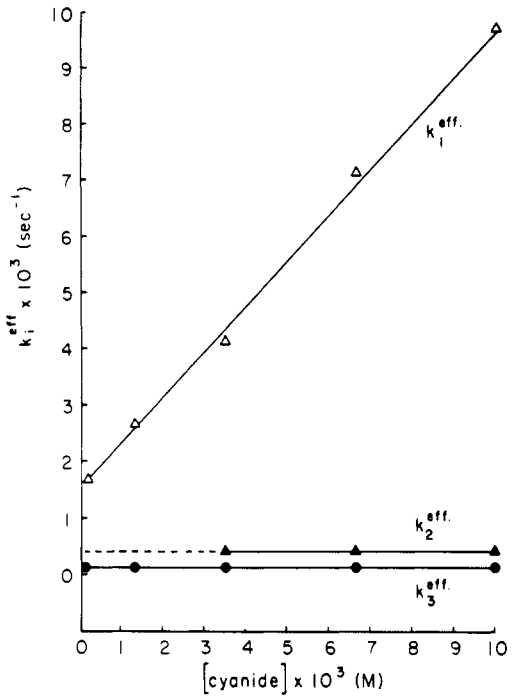

FIGURE 8: Cyanide concentration dependence of the pseudo-first-order effective rate constants: k_1^{eff} (△); k_2^{eff} (▲); k_3^{eff} (●). All data are given for preparation 4, and rate constants are calculated as in Figure 2. All other conditions are as in Figure 5.

Model for the Cyanide Reaction. Figure 8 shows the cyanide concentration dependence of the pseudo-first-order rate constants of these three forms in a sample of preparation 4 (Yonetani method). It is clear from Figure 8 that the first-order rate constant of the fast reacting (with cyanide) form (k_1^{eff}) is directly dependent on cyanide concentration, but the two slower reacting forms show first-order rates independent of cyanide concentration. This may be interpreted to mean that only the fast reacting component reacts with cyanide directly, and the two slowly reacting populations must convert to the fast reacting form before reaction with cyanide can occur. A detailed kinetic model, presented in the Appendix, suggests that the data presented in Figures 5 and 8 can be interpreted in terms of an equilibrium between the enzyme forms

$$E_1 \rightleftharpoons E_2 \rightleftharpoons E_3$$

with only the enzyme in the E_1 form reacting directly with cyanide.

Conclusions

It is clear from the results, structural and kinetic, presented in this paper that there exists significant molecular heterogeneity and differences between preparations of cytochrome oxidase by different methods and by the same method at different times. Forms of cytochrome a_3 possessing a sulfur atom as a sixth ligand (essentially all resting oxidase preparations) seem to bind cyanide more slowly than those forms in which the sulfur is not ligated to the cytochrome a_3 (essentially all oxygenated forms).

It would be ambitious to attempt to correlate *directly* the heterogeneity we find in cyanide binding kinetics to the structural modulations revealed by EXAFS. However, we believe that the two methods here reveal complementary aspects of the same phenomena. While our studies here have delineated and underlined the differences between and heterogeneity of different preparations, their similarities are underlined by the observation that all these enzyme preparations are slower in their cyanide binding rates, by a factor of about 10^3, than the enzyme in mitochondria or under turnover conditions.

Acknowledgments

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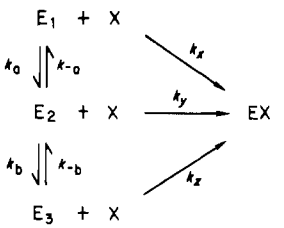
Appendix

Kinetic Model for the Reaction of Cyanide with Cytochrome Oxidase in the Resting State (Enzyme As Isolated). As we stated earlier, there are at least three different forms of the enzyme in cytochrome oxidase as isolated, so we can propose the following simple model for cyanide binding to cytochrome oxidase:



(2)

where E_1, E_2, and E_3 are different conformations of the enzyme as isolated and X is the ligand (cyanide); we assume that all products (E_1X, E_2X, and E_3X) are spectrally identical and hence shown as EX.

The following equation can easily be derived for scheme 2:

$$A = A_1 e^{-(k_a + k_x[X])t} + A_2 e^{-(k_{-a} + k_b + k_y[X])t} + A_3 e^{-(k_{-b} + k_z[X])t} \quad (3)$$

where A is the activity of the cytochrome oxidase toward cyanide (in our case, it is change in absorbance). Now comparing eq 1 and 3, one can see that k_i^{eff} values are not elementary constants; they are effective constants and are related to the constants of scheme 2 in the following manner:

$$k_1^{eff} = k_a + k_x[X] \quad (4)$$

$$k_2^{eff} = (k_{-a} + k_b) + k_y[X] \quad (5)$$

$$k_3^{eff} = k_{-b} + k_z[X] \quad (6)$$

The dependence of these effective constants (k_1^{eff}, k_2^{eff}, and k_3^{eff}) on cyanide concentration would be useful in determining the elementary constants and interpreting scheme 2. As it can be seen from expression 5, k_{-a} and k_b cannot be determined separately. Thus, the equilibrium constants cannot be determined either. However, their limiting values can be estimated.

As stated earlier and shown in Figure 8, the k_1^{eff} increases with the increase of cyanide concentration. However, k_2^{eff} and k_3^{eff} are independent of cyanide concentration, suggesting that

$$k_2^{eff} \approx (k_{-a} + k_b) \qquad k_y = 0 \quad (7)$$

$$k_3^{eff} \approx k_{-b} \qquad k_z = 0 \quad (8)$$

Thus, these two slower phases in the cyanide binding kinetics are not due to binding of cyanide to species E_2 and E_3; k_3^{eff} represents the conversion of E_3 to E_2, and k_2^{eff} is the sum of the two separate steps: formation of E_1 from E_2 and conversion of E_2 to E_3. However, k_1^{eff} is dependent on cyanide concentration, and the values of k_a and k_x, which can be calculated from eq 4 and data of Figure 8, are found to be 1.5×10^{-3} s^{-1} and 0.8 M^{-1} s^{-1}, respectively.

Now let us estimate the limiting values of the equilibrium constants. It can be seen from Figure 8 and eq 7 that

$$k_{-a} + k_b = 4 \times 10^{-4}\ s^{-1}$$

so both k_{-a} and k_b are less than $4 \times 10^{-4}\ s^{-1}$.

Then if the equilibrium constants for the two steps are expressed as K_1 and K_2, then

$$K_1 = \frac{k_{-a}}{k_a} = \frac{<4 \times 10^{-4}\ s^{-1}}{1.5 \times 10^{-3}\ s^{-1}} < 2.7 \times 10^{-1}$$

and

$$K_2 = \frac{k_{-b}}{k_b} = \frac{8 \times 10^{-5}\ s^{-1}}{<4 \times 10^{-4}\ s^{-1}} > 2 \times 10^{-1}$$

These values show that, at the resting state, the equilibrium is shifted toward the "nonreactive with cyanide forms" (E_2 and E_3).

Registry No. Fe, 7439-89-6; Cu, 7440-50-8; cyanide, 57-12-5; cytochrome oxidase, 9001-16-5.

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