Kinetics and Mechanism for the Binding of HCN to Cytochrome *c* Oxidase[†]

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ABSTRACT: The kinetics of cyanide binding to cytochrome *c* oxidase were systematically studied as a function of [HCN], [oxidase], pH, ionic strength, temperature, type and concentration of solubilizing detergent, and monomer−dimer content of oxidase. On the basis of these results a minimum reaction mechanism is proposed in which the spectrally visible rapid and slow cyanide binding reactions are two consecutive first-order reactions, not parallel reactions with different conformers of cytochrome *c* oxidase. The fast reaction (k'_{obs}) follows saturation type kinetics to form an HCN complex that subsequently undergoes a slow reaction (k''_{obs}). The fast k'_{obs} reaction is independent of ionic strength but is strongly dependent upon pH. Two pK values were evaluated from the bell-shaped rate versus pH profile; one is due to an ionizable group on the protein (pK_a = 7.45), while the other is that of HCN (pK_{HCN} = 9.15). Therefore, oxidase is reactive toward HCN only when the group on the protein is unprotonated. The slow k''_{obs} reaction is not a reaction of oxidase with either CN^- or HCN; in fact, the product formed by the fast k'_{obs} reaction, the oxidase−HCN complex, still undergoes the slow k'' process even if all of the excess KCN is removed. The apparent rate constant of the slower phase (k''_{obs}) is independent of all the variations done in this study, and it probably corresponds to either a slow conformational change in the protein or a change in ligand coordination at one of the metal centers after HCN binds to the bimetallic center of oxidase. Based upon the bell-shaped pH dependence of the fast phase and the pH independence of the slow phase, the mechanism also predicts that a single conformer of cytochrome *c* oxidase can exhibit either monophasic or biphasic cyanide binding kinetics depending upon the pH. At either very low or very high pH, the two rates become comparable in magnitude, which makes the reaction appear to be monophasic even though both reactions still occur. The amount of monomeric or dimeric oxidase only slightly affects the magnitude of k'_{obs} and k''_{obs} values, and both processes are clearly present in both types of oxidase.

Cytochrome *c* oxidase (EC 1.9.3.1) is a multisubunit enzyme of the mitochondrial respiratory chain that transfers electrons from cytochrome *c* to dioxygen. Its function is to couple these electron transfers to the translocation of protons across the inner membrane (Wikström et al., 1981; Naqui & Chance, 1986; Papa et al., 1987; Vänngård, 1988; Kadenbach et al., 1987; Beinert, 1988; Copeland & Chan, 1989; Capaldi, 1990; Azzi & Muller, 1990; Malmström, 1990a; Saraste, 1990). Mammalian cytochrome *c* oxidase comprises 13 dissimilar polypeptide subunits (Zhang & Capaldi, 1988) and four redox components: two heme A groups and two coppers (Wikström et al., 1981; Naqui & Chance, 1986; Papa et al., 1987; Vänngård, 1988; Kadenbach et al., 1987; Copeland & Chan, 1989; Malmström, 1990b). The two heme A groups are associated with subunit I and are known as cytochromes *a* and a_3 (Holm et al., 1987). One of the coppers, Cu_B, and cytochrome a_3 form a bimetallic center that is the oxygen binding site (Wikström et al., 1981; Naqui & Chance, 1986; Papa et al., 1987; Vänngård, 1988; Kadenbach et al., 1987; Beinert, 1988; Copeland & Chan, 1989; Capaldi, 1990; Azzi & Muller, 1990; Malmström, 1990a; Saraste, 1990; Zhang & Capaldi, 1988). The other copper, Cu_A, is associated with subunit II (Malmström, 1990b; Holm et al., 1987) and participates in electron transfers from bound cytochrome *c* to cytochrome *a*.

The oxygen binding site of cytochrome *c* oxidase has been probed with two common inhibitors of respiration, carbon monoxide and cyanide. Both inhibitors bind at the binuclear cytochrome a_3−Cu_B site but react quite differently. Bound CO can be oxidized to CO_2 and subsequently released from the binuclear center (Young & Caughey, 1986), whereas CN^- (or HCN) is irreversibly bound to the binuclear center (Malmström, 1990). The mechanism of CO ligand binding to cytochrome *c* oxidase is complex but follows well-defined kinetics (Woodruff et al., 1991; Hallén et al., 1994; Georgiadis et al., 1994; Basu et al., 1994; Einarsdóttir et al., 1995; Ädelroth et al., 1995). However, a unified mechanism that explains the widely divergent cyanide binding data is not available. Nevertheless, the chemistry of cyanide binding to cytochrome *c* oxidase is quite well understood from a variety of spectroscopic techniques, i.e., Cu X-ray (Scott et al., 1985), EXAFS (Naqui et al., 1984), EPR (Baker et al., 1987; Schoonover & Palmer, 1991; Day et al., 1993; Lodder & Van Gelder, 1994), resonance Raman (Schoonover et al., 1988; Larsen et al., 1989), infrared (Yoshikawa & Caughey, 1990; Caughey et al., 1993; Tsubaki & Yoshikawa, 1993; Li & Palmer, 1993), UV−visible (Fabian & Malmström, 1989; Berka et al., 1993; Gullo et al., 1993; Lodder & Van Gelder, 1994) and magnetic circular dichroism (Babcock et al., 1976; Papadopoulous et al., 1991; Schoonover & Palmer, 1991).

The major complication in understanding the cyanide binding is the fact that the kinetics are sometimes monophasic (Hill & Robinson, 1986; Baker et al., 1987; Schoonover &

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Palmer, 1991; Gullo et al., 1993; Baker & Gullo, 1994; Soulimane & Buse, 1995; Geier et al., 1995) but are usually either biphasic or multiphasic (Naqui et al., 1984; Moody et al., 1991; Cooper et al., 1993), suggesting either multiple cyanide binding sites or enzyme heterogeneity. The most accepted explanation is that cytochrome *c* oxidase is a mixture of two conformers that differ in their cyanide reactivity (Naqui et al., 1984; Malmström 1990a,b; Yoshikawa & Caughey, 1990; Moody et al., 1991; Cooper et al., 1993; Day et al.; 1993; Li & Palmer, 1993; Tsubaki & Yoshikawa, 1993; Baker & Gullo, 1994; Fabian & Palmer, 1995). In fact, the relative amplitudes of the two phases are often used to estimate the percentage of the "slow" ($k''_{obs} = (1-3) \times 10^{-4}$ s^{-1}) and "fast" ($k'_{obs} = (0.5-2) \times 10^{-2}$ s^{-1}) conformer in a particular preparation (Naqui et al., 1984; Gullo et al., 1993; Baker & Gullo, 1994; Geier et al., 1995). According to this hypothesis, preparations that contain predominantly the fast conformation show fast monophasic kinetics, whereas preparations that contain a significant amount of the slow conformer usually exhibit biphasic or multiphasic behavior (Schoonover & Palmer, 1991; Moody et al., 1991; Cooper et al., 1993; Baker & Gullo, 1994). The origin of conformer heterogeneity is not known, but differences in the amounts of the two conformers have been attributed to a shift in the equilibrium between the two forms. For this to be true, the interconversion between the two conformers must be nearly irreversible since the relative amounts of the slow and fast forms do not change with time (Naqui et al., 1984; Babcock et al., 1976; Baker et al., 1987; Papadopoulous et al., 1991; Schoonover & Palmer, 1991; Moody & Cooper, 1991; Day et al., 1993; Lodder & Van Gelder, 1994). However, enzyme that exhibits biphasic kinetics can be slowly converted into enzyme with monophasic kinetics by incubation at either slightly acidic pH or with formate at pH > 8 (Moody et al., 1991; Schoonover & Palmer, 1991; Papadopoulous et al., 1991; Cooper et al., 1993; Gullo et al., 1993; Baker & Gullo, 1994). No adequate, generally accepted kinetic scheme is consistent with all of the widely divergent cyanide binding data (Malmström, 1990a,b). Apparent K_m, k_{cat}, and V_{max} values have been evaluated, but these are based upon the [KCN] dependence of the reaction at only a single pH (Van Buuren et al., 1972a,b; Musatov & Konstantinov, 1988; Andreev & Konstantinov, 1983). These values should not be interpreted as intrinsic constants until a complete study of all factors that influence the kinetics is performed (Malmström, 1990a,b). Lodder and Van Gelder (1994) pointed out that such a detailed kinetic study of the slow conformer is needed for the biphasic binding kinetics to be diagnositic of conformer heterogeneity in different oxidase preparations. For these reasons, we examined in detail the cyanide binding kinetics of a cytochrome *c* oxidase preparation that exhibits two distinct phases in its KCN reactivity. The results of this study have permitted us to develop a minimum reaction mechanism that predicts that the monophasic or biphasic kinetics can be due to the pH dependence of the reaction and are not necessarily diagnostic of multiple enzyme conformers.

MATERIALS AND METHODS

Cytochrome *c* oxidase was prepared from Keilin-Hartree beef heart muscle particles (Yonetani, 1960) by the method of Fowler et al. (1962) with the modifications described by Mahapatro and Robinson (1990). Usually it was necessary to precipitate the cholate-solubilized enzyme at least 2 times with 40−42% saturated ammonium sulfate to obtain a purified preparation. The final oxidase pellet was then solubilized in 1% sodium cholate containing 0.1 M NaH_2PO_4 and 1.0 mM EDTA at pH = 7.4 to make stock solutions of approximately 100 μM and stored in liquid nitrogen.

Cytochrome *c* oxidase concentrations were based upon a value of $\epsilon_{422} = 1.54 \times 10^5$ M^{-1} cm^{-1} for the oxidized enzyme (Van Gelder, 1978). The phospholipid content (30 nmol of PL/nmol of oxidase), heme content (9.0−9.3 nmol/mg of protein), enzyme activity (330−360 s^{-1}), and percentage of monomeric and dimeric oxidase were each determined as previously described (Robinson & Talbert, 1986; Dale & Robinson, 1988; Mahapatro & Robinson, 1990). The three different preparations used in this study were each very similar except for the content of monomeric and dimeric oxidase: two were mostly dimeric (<10% monomers), while the other was predominantly monomeric (>70% monomers). Controlling the amount of dimer in a preparation is difficult, but in general repeated solubilization with cholate and precipitation with ammonium sulfate favors monomerization, as does exposure to high concentrations of detergent, e.g., a detergent to protein ratio > 5 (Robinson & Talbert, 1986; J. Ortega-Lopez and N. C. Robinson, unpublished results).

Detergents were obtained from the following sources: dodecyl β-D-maltoside (DM) was from Calbiochem, Triton X-100 (TX-100) was from Boehringer Mannheim, *n*-dodecyl octa(ethylene glycol) monoether ($C_{12}E_8$) was from Calbiochem, and polyoxyethylenesorbitan monooleate (Tween 80) and polyoxyethylene-10-oleyl ether (Brij 96) were each obtained from Sigma. These detergents and all other chemicals (Fisher ACS grade) were used without further purification. The sodium salts of 7-deoxycholic acid (NaDOC) and cholate (free acid) were obtained from Sigma, and both were purified by recrystallization from ethanol.

For kinetic studies in the various detergents, cholate that is present in the stock enzyme solution was exchanged for the desired detergent, e.g., DM, by adding 1 mg of the new detergent per milligram of protein and dialyzing overnight at 5 °C against a 100-vol excess of the appropriate buffer containing the new detergent with one change of dialysate after 4−6 h.

UV−visible spectra were recorded on a Shimadzu UV−visible spectrophotometer (Model UV-260) with 0.1-nm resolution. The spectra of the cyanide-bound cytochrome *c* oxidase complex at "infinite" time were recorded after 5 h, at which time changes were no longer observed in the spectra.

Kinetics. Kinetics were monitored at 418 nm (near the Soret maximum of resting oxidase) and at 428 nm (the maximum for cyanide-reacted oxidase) for the disappearance of oxidase and the formation of the cyanide product(s), respectively. The two wavelengths were monitored simultaneously using a Hewlett Packard 8452A diode array UV−visible spectrophotometer equipped with a temperature-controlled cuvette holder and HPIB interface to collect the data. In a typical experiment the reaction was initiated by quickly mixing a few microliters of 1.0 M KCN with 1 mL of 5 μM enzyme. The absorbance data as a function of time were fitted to first-order equations of the form

$$A_t = A_\infty + \sum_{i=1}^{i=n} A_i e^{-k_i(t)} \quad (1)$$

where A_t = absorbance at time t, k_i values are the pseudo-first-order rate constants; and A_∞ and A_i values are the variable parameters. An iterative nonlinear least squares fit to the data was done with MINSQ, a commercially available program (Micro Math Scientific Software, Salt Lake City, UT) on a 386DX microcomputer equipped with a math coprocessor. For a biphasic reaction, this fitting procedure gives best fit values for both rate constants (k_1 and k_2) and the three parameters (A_∞, A_1, and A_2); i.e., no assumptions need to be made concerning the end point of the reaction. We want to emphasize that the values of A_1 and A_2 are only fitting parameters and are not directly related to the fractional amounts of enzyme participating in the the slow and fast reactions. The value of n was either 1 or 2 depending on whether the data were fitted to a single or a biphasic reaction, respectively. Acceptable fits had a value for $r > 0.998$. Most of the experiments were done in duplicate, and the results from each pair agreed within 2−5%.

Complete Oxidation of Cytochrome c Oxidase by Ferricyanide. The ferricyanide treatment of cytochrome *c* oxidase was done at three different pH values: pH = 6.60, 7.32 and 7.80, similar to the treatment reported by Mitchell et al. (1992). In each case 500 μL of 50 μM enzyme in phosphate buffer containing 2.0 mM dodecyl maltoside ($[\text{phosphate}]_{tot}$ = 0.1 M; I = 0.3 M with NaCl) was reacted with 10 mM $K_3Fe(CN)_6$ for 2.5 h to oxidize reduced species that might be present. $K_3Fe(CN)_6$ and its reduced products were removed by exhaustive dialysis at 4 °C vs phosphate buffer (pH 6.50, 7.32, or 7.80) containing 2 mM dodecyl maltoside.

Preparation of HCN-Bound Oxidase Complex. Seven micromolar oxidase was reacted with 16 mM $[HCN]_{tot}$ in 20 mM Tris buffer, pH 7.80, containing 1 mM EDTA, 0.1 M NaCl, and 2 mM dodecyl maltoside. After reaction at room temperature for 6 min (long enough to ensure completion of the fast phase of the reaction since $t_{1/2} \cong 35$ s under these conditions) excess KCN was removed by gel filtration on a PD-10 Sephadex G-25M column (Pharmacia LKB, Uppsala, Sweden) previously equilibrated with the reaction buffer. The cyanide−oxidase complex (~5 μM) in cyanide-free buffer was used immediately.

RESULTS

Cytochrome *c* oxidase reacts with KCN to causes a time-dependent red shift in the Soret maximum from 418 to 428 nm (Figure 1). The kinetic spectra have a single isosbestic point at 423 nm which is indicative of a two-state process. The kinetics of the reaction were monitored at 418 nm for the disappearance of unreacted enzyme and at 428 nm for the formation of the cyanide-bound product. At pH 7.8, the change in absorbance with respect to time at either 418 or 428 nm was clearly biphasic with the faster reaction lasting about 5−10 min and the slower phase continuing beyond 6 h (Figure 2). At either wavelength, the amplitude of the faster phase is about 2-fold greater than the amplitude of the slower phase.

Evaluation of the kinetic rate constants of such a biphasic reaction from the inital and final slopes of a semilogarithmic plot of the data is difficult, if not impossible. Such an analysis requires that one know the final absorbance of both

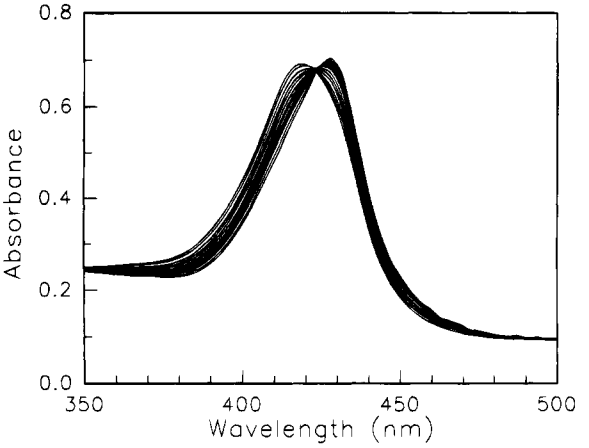


FIGURE 1: Effect of reaction with KCN upon the spectral properties of cytochrome *c* oxidase. Spectral scans are at 0 (λ_{max} = 420 nm), 0.25, 0.75, 1.33, 2, 3, 4.5, 6, 10, 20, 40, 80, and 120 min (λ_{max} = 428 nm). Reaction conditions: [oxidase] = 5.5 μM; [KCN] = 5.0 mM; [Tris] = 20 mM; I = 0.1 M with NaCl; [EDTA] = 1.0 mM; pH = 7.8; [DM] = 2.0 mM; T = 25 °C.

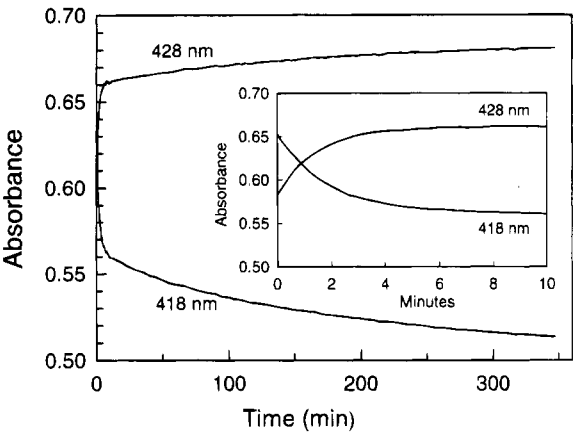


FIGURE 2: Kinetics of the reaction of cytochrome *c* oxidase with KCN monitored at 418 and 428 nm. Biphasic kinetics become clearly apparent after 10 min. Inset: The initial 600 s (10 min) of the reaction in which the monophasic k' kinetics predominate. Reaction conditions: [oxidase] = 5.5 μM; [KCN] = 5.0 mM; [Tris] = 20 mM; I = 0.1 M with NaCl; [EDTA] = 1.0 mM; pH = 7.8; [DM] = 2.0 mM; T = 25 °C.

the fast and the slow reaction phase, something that is not possible to ascertain from the absorbance data. Futhermore, the rapid phase is always "contaminated" by the slower phase, making determination of the apparent first-order rate constant for the faster reaction subject to error (Espenson, 1981; Jencks, 1987). Problems that arise from this type of analysis are best illustrated by Figure 3 in which the same data were analyzed using different end points, i.e., different values for A_∞. The reaction appears to be monophasic if it is assumed to be complete after 3 or 10 min, multiphasic if it is assumed to be complete by 60 or 90 min, and biphasic if it is assumed to be complete only after 6 h. For these reasons, we chose to directly fit the absorbance versus time data to a multiple-exponential equation as suggested by Bunnett (1986) (refer to eq 1 in Materials and Methods). This approach has the advantage that no assumptions need to be made regarding the end points for either phase, and the iterative nonlinear least squares fitting procedure yields pseudo-first-order rate constants that are not contaminated by each other.

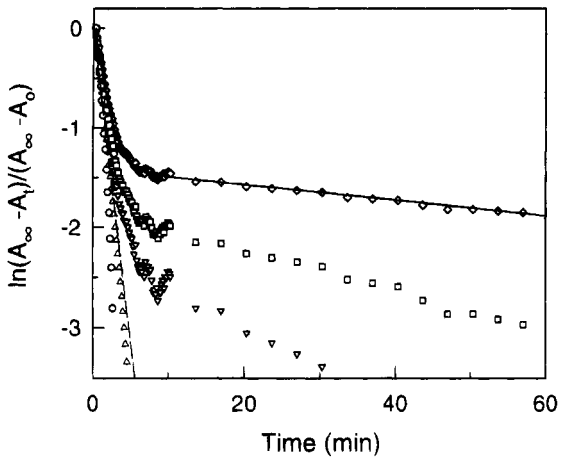


FIGURE 3: Effect of choosing different end points upon the semilogarithmic first-order rate plot for the cyanide−cytochrome *c* oxidase reaction. Data analyzed are those shown in Figure 2. A_∞ values were chosen as the absorbance value at 3 (○), 10 (△), 60 (▽), 90 (□), and 360 min (◇). The solid line corresponds to the fitted biphasic line generated by fitting the data to eq 1, i.e., $k_1 = 9.3 \times 10^{-3}$ s^{-1} and $k_2 = 1.0 \times 10^{-4}$ s^{-1}. The dashed line corresponds the theoretical monophasic fast phase which would occur in the absence of the slower second phase, i.e., $k_1 = 9.3 \times 10^{-3}$ s^{-1}, initial absorbance $= A_1 + A_2 + A_\infty$, and final absorbance $= A_\infty + A_2$. Note that the initial slope of the biphasic curve (solid line) is not equal to the slope of the theoretical monophasic line (dashed line).

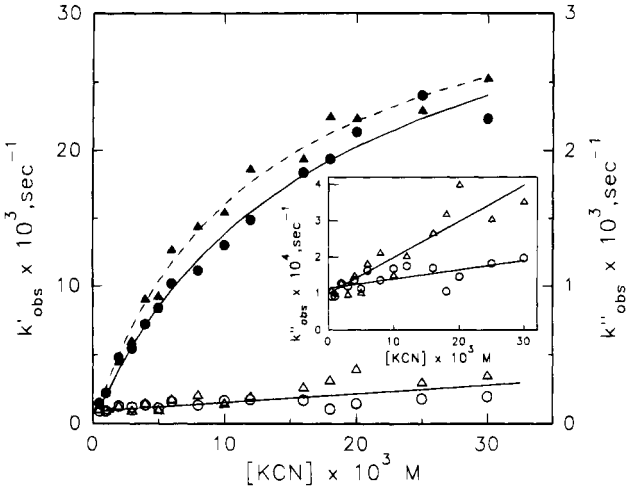


FIGURE 4: [KCN] dependence of the fast k'_{obs} reaction (●, 418 nm; ▲, 428 nm) and the slow k''_{obs} reaction (○, 418 nm; △, 428 nm). [Oxidase] = 5.5 μM; [EDTA] = 1.0 mM; [Tris] = 20 mM; pH = 7.8; I = 0.1 M with NaCl; [DM] = 2.0 mM; $T = 25 \pm 0.5$ °C. The solid and dashed curves through the k'_{obs} data are the fitted nonlinear least square regression lines according to eq 3 for the k'_{obs} vs [KCN] data obtained at 418 and 428 nm, respectively. The nearly flat line drawn through the k''_{obs} data illustrates the near absence of dependence of k''_{obs} upon [KCN].

The rate constants for the fast phase, as evaluated by the nonlinear least squares fitting procedure, were nearly identical whether the kinetics were monitored at 418 or 428 nm; therefore, no intermediate accumulated during this phase. The fast rate (k'_{obs}) is strongly dependent upon the concentration of KCN when measured in pH 7.8 buffer containing 2 mM dodecyl maltoside (Figure 4) and appears to be hyperbolic, corresponding to saturation type kinetics. The cyanide dependence of k'_{obs} for three different preparations of oxidase that contained different percentages of monomeric and dimeric oxidase were nearly identical to that shown in Figure 4. In contrast to the strong [KCN] dependence of the faster rate (k'_{obs}), the slower rate (k''_{obs}) was nearly independent of [KCN] between 0.5 and 30 mM. The slower rate does not follow pseudo-first-order kinetics since it increases only 2-fold (418-nm data) to 4-fold (428-nm data) as the [KCN] is increased 60-fold (inset to Figure 4). Furthermore, neither set of data extrapolates to $k''_{obs} = 0$ at [KCN] = 0. Once again, similar results were obtained with the three preparations of oxidase that contained different amounts of monomer and dimer.

The k'_{obs} values, as a function of $[HCN]_{tot}$, were analyzed according to a saturation type kinetic mechanism (eq 2).

$$\text{oxidase} + \text{HCN} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [\text{complex}] \xrightarrow{k_2} \text{products} \qquad (2)$$

The rate law for such a mechanism, which is analogous to classical Michaelis−Menten, enzyme kinetics, is given by eq 3.

$$\text{rate} = k'_{obs}[\text{oxidase}]_o = \frac{k_2[\text{oxidase}]_o[\text{HCN}]_{tot}}{(K_m + [\text{HCN}]_{tot})} \qquad (3)$$

where $[\text{oxidase}]_o$ is the total concentration of uncomplexed and complexed oxidase, $[\text{HCN}]_{tot}$ is the concentration of all cyanide species, and $K_m = (k_{-1} + k_2)/k_1$. The data in Figure 4 were fitted to eq 3 and resulted in $K_m = 17.5 \pm 2$ mM and $k_2 = 3.81 \pm 0.25 \times 10^{-2}$ s^{-1} using the 418-nm data, and $K_m = 12.4 \pm 1.2$ mM and $k_2 = 3.60 \pm 0.16 \times 10^{-2}$ s^{-1} using the 428-nm data.

The observed rates for both k'_{obs} and k''_{obs} (measured at either 418 or 428 nm) were independent of the oxidase concentration or the ionic strength and were only slightly dependent upon the concentration or the type of detergent that was used to solubilize the enzyme, i.e., 2−20 mM dodecyl maltoside, Triton X-100, NaDOC, $C_{12}E_8$, Tween 80, or Brij 96 (Table 1). The independence of both the slow and the fast rate upon enzyme concentration (1−23 μM) indicates that the rate equations for both phases involve only first-order concentration terms (Jencks, 1987). The lack of dependence of the rates upon ionic strength (0−1.0 M NaCl) reveals that the transition state for the formation of the enzyme−substrate complex does not involve the interaction of two charged species (Espenson, 1981); i.e., the species binding to the positively charged metal center(s) of oxidase is HCN, not CN^-. It is known that cyanide binding to cytochrome *c* oxidase is accompanied by uptake of one proton (Konstantinov et al., 1986; Rousseau et al., 1993). Therefore, HCN as the reacting species is consistent with the suggestion of Mitchell and Rich (1994); i.e., charge compensation at the bimetallic center must be provided for by a protonation reaction.

Although changing the solubilizing detergent can alter electron-transfer rates from cytochrome *a* to a_3 by as much as 2 orders of magnitude (Robinson et al., 1985; Mahapatro & Robinson, 1990), k'_{obs} for the HCN reaction was changed by less than 2-fold for the detergents that were tested (Table 1). In each detergent the reaction with KCN was biphasic; however, in Brij 96 the enzyme began to aggregate after ~1200 s, which prevented evaluation of k''_{obs}.

It has been reported that preparations of oxidase often contain a small amount of a one-electron-reduced species (Moody, 1991; Mitchell et al., 1992). This species (about

Table 1: Variation of Detergent Identity[a]

detergent	mg/mL	418 nm $k'_{obs} \times 10^3$ (s^{-1})	418 nm $k''_{obs} \times 10^4$ (s^{-1})	428 nm $k'_{obs} \times 10^3$ (s^{-1})	428 nm $k''_{obs} \times 10^4$ (s^{-1})
DM	2.0	10.89 ± 0.33	1.44 ± 0.16	10.54 ± 0.12	1.93 ± 0.21
	5.1	10.74 ± 0.46	1.70 ± 0.25	10.90 ± 0.32	1.92 ± 0.31
TX-100	2.0	8.52 ± 0.19	1.68 ± 0.04	11.65 ± 0.39	not seen
	10.0	8.56 ± 0.28	1.42 ± 0.10	9.56 ± 0.46	not seen
NaDOC	2.0	5.85 ± 0.12	1.83 ± 0.15	8.35 ± 0.24	1.23 ± 0.11
	10.0	10.35 ± 0.51	1.04 ± 0.07	7.69 ± 0.21	not seen
$C_{12}E_8$	2.0	13.08 ± 0.38	2.21 ± 0.11	19.58 ± 0.64	2.92 ± 0.27
	10.0	18.85 ± 0.85	2.90 ± 0.11	19.34 ± 0.68	not seen
Tween 80	2.0	8.87 ± 0.18	2.38 ± 0.16	10.49 ± 0.27	0.91 ± 0.07
	10.0	10.29 ± 0.48	1.20 ± 0.16	11.22 ± 0.30	2.20 ± 0.08
Brij 96[b]	2.0	12.20 ± 0.37		10.57 ± 0.23	
	10.0	18.17 ± 0.37		13.01 ± 0.36	

[a] [oxidase] = 5.5 μM; [KCN] = 5.0 mM; [EDTA] = 1.0 mM; [Tris] = 20 mM; pH = 7.80; I = 0.1 M with NaCl; T = 25 ± 0.5 °C. [b] Note: In Brij 96 the slower reactions are obscured by light scattering after ~1200 s.

Table 2: Reaction of Ferricyanide Treated and Nontreated Cytochrome *c* Oxidase with KCN[a]

	418 nm $k'_{obs} \times 10^3$ (s^{-1})	418 nm $k''_{obs} \times 10^4$ (s^{-1})	428 nm $k'_{obs} \times 10^3$ (s^{-1})	428 nm $k''_{obs} \times 10^4$ (s^{-1})
pH = 6.50				
A	5.16 ± 0.13	1.01 ± 0.02	6.82 ± 0.11	1.16 ± 0.09
B	4.70 ± 0.23	1.12 ± 0.04	5.09 ± 0.15	1.31 ± 0.09
pH = 7.32				
A	9.66 ± 0.20	1.41 ± 0.03	15.62 ± 0.33	1.87 ± 0.14
B	7.11 ± 0.09	1.12 ± 0.03	7.24 ± 0.09	1.45 ± 0.06
pH = 7.80				
A	15.90 ± 0.33	1.42 ± 0.01	28.58 ± 0.61	2.53 ± 0.08
B	14.84 ± 0.23	1.40 ± 0.06	20.02 ± 0.46	1.35 ± 0.07

[a] A = ferricyanide treated; B = untreated cytochrome *c* oxidase. [oxidase] = 5.5 μM; [KCN] = 5.0 mM; [EDTA] =1.0 mM; $[Phosphate]_{tot}$ = 10 mM; I = 0.3 M with NaCl; [DM] = 2.0 mM. T = 25 ± 0.5 °C.

3% of cytochrome *a* is Fe^{2+} and referred to as the E-state by Mitchell) is not oxidizable by O_2 but can be eliminated by oxidizing the enzyme with $K_3Fe(CN)_6$ (Mitchell et al., 1992). To test whether a small amount of one-electron-reduced species is responsible for the slow k''_{obs} reaction, the KCN kinetics of the fully oxidized enzyme were investigated (Table 2). The rate constants for the ferricyanide-treated oxidase were slightly greater than for the untreated oxidase, but the completely oxidized enzyme still exhibited the characteristic biphasic reaction with HCN.

pH Dependence of the HCN Reaction. The kinetics of the HCN reaction, monitored at both 418 and 428 nm, were studied in the pH range 6.2−9.5. At pHs below 8.90, the kinetics are clearly biphasic. However, at pHs greater than 8.90, the slower phase of the reaction was observed only at 418 and not at 428 nm (Table S5). The k'_{obs} reaction was slower at both low and high pH with an optimum pH of approximately 7.8 (Figure 5). Such a bell-shaped dependence of k'_{obs} upon pH indicates that at least two ionizable groups participate in the reaction (Laidler, 1965; Fromm, 1975), one with pK_a = 7.0−7.5 and another with pK_a = 9.0−9.5. HCN has a pK_a of 9.21 at 25 °C (Dean, 1985) and is almost certainly responsible for the higher pK_a. Therefore, the species that binds and reacts with oxidase seems certain to be HCN, which is consistent with the independence of the rate upon ionic strength. The lower pK_a must be due to

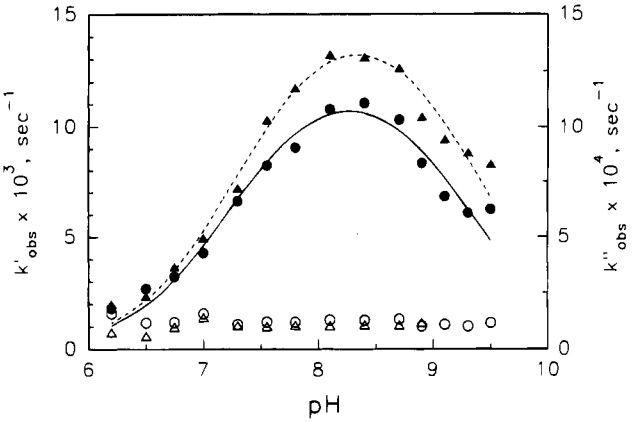
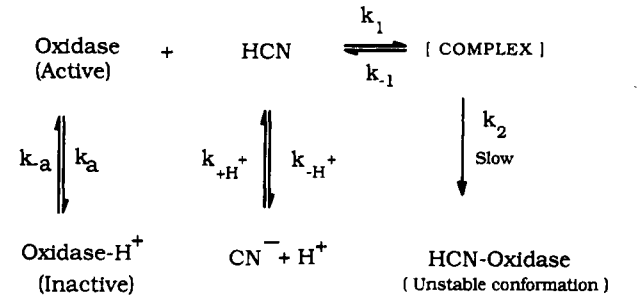


FIGURE 5: Reaction of cytochrome *c* oxidase with HCN: pH dependence of the k'_{obs} rate (●, 418 nm; ▲, 428 nm) and pH independence of k''_{obs} rate (○, 418 nm; △, 428 nm). Reaction conditions: [oxidase] = 5.5 μM; [KCN] = 5.0 mM; [EDTA] = 1.0 mM; [Tris], [phosphate] or [borate] = 0.1 M; I = 0.1 M with NaCl; [DM] = 2.0 mM; T = 25 ± 0.5 °C. Solid and dashed curves are the fitted, nonlinear least squares regression lines calculated according to eq 6 (refer to Materials and Methods).

Scheme 1: Mechanism of the Fast (k') Process



one or more ionizable groups on the enzyme with only the unprotonated form reactive towards HCN.

Once HCN binds to the unprotonated form of oxidase, the steady-state complex proceeds to give the product in a slow rate-limiting step, k_2 (Scheme 1). Using this type of equilibrium approach and assuming that $k_{-1} \gg k_2$, the rate law for the reaction of oxidase with HCN is

$$\text{rate} = \frac{k_2 K_1 [\text{oxidase}]_{tot} [\text{HCN}]_{tot}}{(K_{HCN}/K_a) + (K_{HCN}/[H^+]) + ([H^+]/K_a) + K_1[\text{HCN}]_{tot} + 1} \quad (4)$$

where rate = $k'_{obs}[\text{oxidase}]_{tot}$ $[\text{oxidase}]_{tot}$ = sum of the concentrations of all species of uncomplexed and complexed oxidase, $[\text{HCN}]_{tot}$ = total concentration of free [HCN] and $[CN^-]$, $K_1 = k_1/k_{-1}$, $K_a = k_a/k_{-a}$, and $K_{HCN} = k_{+H^+}/k_{-H^+}$.

The k'_{obs} data, as a function of pH, were iteratively fitted to eq 4, and the parameters k_2, K_{HCN}, K_1, and K_a were evaluated. Using this approach, k_2 was computed to be (3.12 ± 0.22) × 10^{-2} and (3.00 ± 0.14) × 10^{-2} s^{-1} as evaluated from the 418 - and 428-nm data, respectively. These values are somewhat smaller than those obtained from the [KCN] dependence of k'_{obs}, i.e., (3.81 ± 0.25) × 10^{-2} s^{-1} at 418 nm and (3.60 ± 0.16) × 10^{-2} s^{-1} at 428 nm (Figure 4). Therefore, the larger k_2 values were used as constants, and refined values for K_{HCN}, K_1, and K_a were computed. The solid and dashed lines in Figure 5 are the least squares lines generated using these parameters. The theoretical semilog

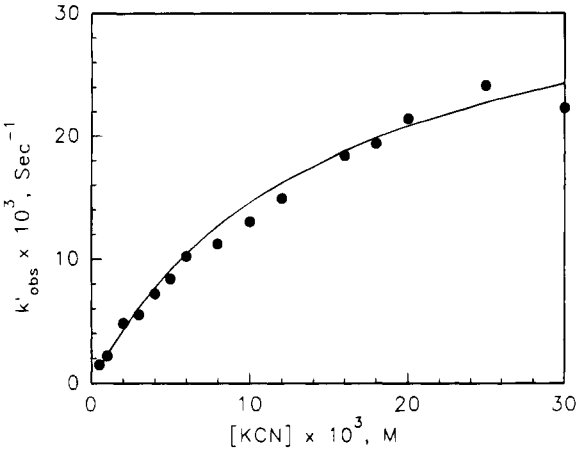


FIGURE 6: Reaction of cytochrome *c* oxidase with HCN: Comparison of experimental data for k'_{obs} monitored at 418 nm (●) with theoretical curve generated according to eq 4. Data are for the dependence of k'_{obs} upon $[KCN]_{tot}$. Parameters used in the simulation: $k_2 = 8.0 \times 10^3$ s^{-1}, pK_{HCN} = 9.15, pK_a = 7.4, and pH = 7.8. Experimental conditions: [oxidase] = 5.5 μM; [EDTA] = 1.0 mM; [Tris] = 20 mM; pH = 7.8; I = 0.1 M with NaCl; [DM] = 2.0 mM; T = 25 ± 0.5 °C.

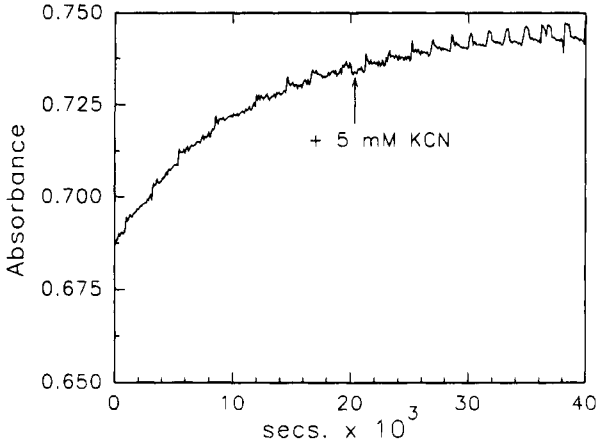


FIGURE 7: Absence of dependence of the slow k'' reaction upon free HCN. Cyanide-free oxidase (5.5 μM) was prepared as described in Materials and Methods. First 2000 s (~5.5 h): absorbance changes that occur in the absence of HCN; at 2000 s, 5.0 mM KCN was added. Absorbance changes occurring after the addition of KCN were identical to those that occurred if no KCN was added. Reaction conditions: [Tris] = 20 mM; [EDTA] = 1.0 mM; pH = 7.8; I = 0.1 M with NaCl; [DM] = 2.0 mM; T = 25 ± 0.5 °C

first-order rate plots corresponding to both the fast and the slow phase and the fast phase alone were also evaluated and are compared with the data (Figure 3).

The equilibrium constants evaluated from fitting the 418-nm data to eq 4 were $K_a = (4.0 \pm 0.6) \times 10^{-8}$ M (pK_a = 7.39), $K_{HCN} = (6.9 \pm 1.5) \times 10^{-10}$ M (pK_{HCN} = 9.16), and $K_1 = 98 \pm 4$ M. The constants evaluated from the 428-nm data were $K_a = (2.9 \pm 0.3) \times 10^{-8}$ M (pK_a = 7.54), $K_{HCN} = (7.4 \pm 1.7) \times 10^{-10}$ M (pK_{HCN} = 9.13), and $K_1 = (157 \pm 6)$ M. The two values for pK_{HCN} are in excellent agreement and are close to the known value for the pK_a of HCN of 9.2 (Dean, 1985). To test the validity of eq 4, theoretical rate values were generated using this equation at various KCN concentrations and were found to be in reasonable agreement with the experimental values for k'_{obs} at pH = 7.8 (Figure 6). Such agreement suggests that Scheme 1 for the fast process satisfactorily explains the experimental results.

The Slow Second Phase Reaction of HCN-Bound Oxidase Complex. In contrast to the fast k'_{obs} process, the slower k''_{obs} values were completely independent of pH with an average value of $(1.2 \pm 0.2) \times 10^{-4}$ s^{-1} for all the data collected between pH 6.2 and 9.5. Therefore, it appears that the slow reaction (k'') is not influenced by the equilibrium between CN^- and HCN and is unlikely to be due to the reaction of HCN with a small amount of a slow-binding form of cytochrome *c* oxidase. The lack of dependence of k'' upon HCN was confirmed by reacting oxidase with HCN for 6 min followed by removal of unreacted HCN and KCN by rapid gel filtration. Isolation of the cyanide-inhibited complex by gel filtration is possible since dissociation of cyanide from the oxidized enzyme is exceedingly slow with an off rate of 1.4×10^{-6} s^{-1} (Van Buuren et al., 1972), corresponding to a $t_{1/2}$ of 138 h. The isolated product still underwent the slow, second phase, k''_{obs} process with a pseudo-first-order rate constant equal to $(9.8 \pm 0.2) \times 10^{-5}$ s^{-1} even though the [KCN] or [HCN] had been decreased more than 20-fold (Figure 7). Furthermore, addition of KCN to this product did not increase the k''_{obs} rate (Figure 7). The observed first-order rate measured in the absence of HCN is similar to the average k''_{obs} value of $(1.21 \pm 0.19) \times 10^{-4}$ s^{-1}

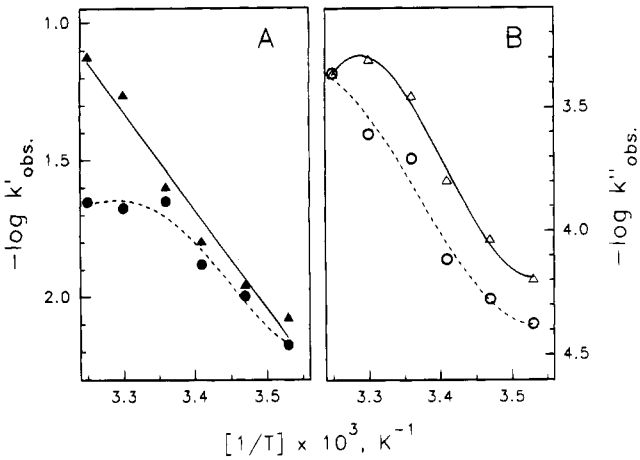


FIGURE 8: Temperature dependence of k'_{obs} (●, 418 nm; ▲, 428 nm) and k''_{obs} (○, 418 nm; △, 428 nm) for reaction of cytochrome *c* oxidase with HCN. Reaction conditions: [oxidase] = 5.5 μM; [KCN] = 3.0 mM; [EDTA] = 1.0 mM; [Tris] = 20 mM; I = 0.1 M with NaCl; [DM] = 2.0 mM; pH = 7.8.

that was obtained from fitting the biphasic data in Figure 2. Clearly, the slow k'' reaction is not due to a parallel reaction of HCN with a subpopulation of a slow-binding form of oxidase.

Effect of Temperature upon Reaction with KCN. The kinetic constants for the reaction of oxidase with saturating amounts of KCN, i.e., 30 mM, were measured in the temperature range 10−35 °C . Arrhenius plots of log k'_{obs} and log k''_{obs} vs T^{-1} were nonidentical (Figure 8). The faster process (k'_{obs}) measured at 428 nm was well behaved and gave the expected linear increase in log k'_{obs} with decreasing $1/T$. However, at 418 nm log k'_{obs} increased linearly only until 25 °C; above 25 °C, log k'_{obs}(418 nm) was nearly independent of temperature. Computed thermodynamic parameters for the overall rate process, monitored at 428 nm, are $\Delta E_a^{\ddagger} = 68.2$ kJ mol^{-1}, $\Delta H_a^{\ddagger} = 65.7$ kJ mol^{-1}, and $\Delta S^{\ddagger} = -12.8$ eu at 298 K. The $\Delta E_a^{\ddagger}$ value is in good agreement with the value of ~59 kJ mol^{-1} (14 kcal mol^{-1}) reported by Van Buuren et al. (1972b).

At higher temperatures, i.e., 30−35 °C, the absorbance vs time plots did not give acceptable fits to an equation with

two exponentials; therefore, a third reaction, that may or may not involve HCN, must occur. Even in the absence of HCN, small temperature-induced spectral changes occur in oxidase at 35 °C at both 418 and 428 nm with a first-order rate constant of 1.0×10^{-5} s^{-1}. Whether such temperature induced spectral changes in oxidase explain the nonlinearity in the Arrhenius plot at the higher temperatures, or whether they are identical to the postulated third phase of the KCN reaction at 35 °C, is difficult to ascertain due to the extremely small amplitudes associated with this process. However, these temperature-induced changes in oxidase at 35 °C are irreversible and permanently alter its subsequent reactivity with HCN and Soret maximum (λ_{max} shifts from 418 to 422 nm). Enzyme that was incubated for 24 h at 35 °C in the absence of KCN and repurified by DEAE ion-exchange chromatography exhibited purely monophasic HCN kinetics in pH 7.8 buffer containing dodecyl maltoside with a k'_{obs} value that was nearly identical to that obtained for the untreated enzyme.

DISCUSSION

The reaction of cyanide with cytochrome c oxidase has been studied for many years, but this is the first detailed investigation of the pH dependence of each phase of the biphasic kinetics. We have evaluated the pH dependence of both the fast (k'_{obs}) and slow (k''_{obs}) rate constants and have evaluated the intrinsic K_m and k_{cat} (k_2 in our nomenclature) values for the fast reaction. From these results we find that only the fast k' reaction is dependent on [HCN], [oxidase], pH, ionic strength, temperature, monomer−dimer content of oxidase, and concentration and type of solubilizing detergent. We conclude that the two spectrally distinct fast and slow phases of the cyanide reaction are two consecutive first-order reactions; i.e., the slow, cyanide-independent reaction occurs only after the fast, cyanide-dependent reaction. Furthermore, the fast reaction exhibits a strong pH dependence with a bell-shaped profile that is characteristic of two independent ionizable groups, while the second slow reaction is unaffected by pH. Each of the three preparations of oxidase exhibited biphasic kinetics at pH = 7.8 but nearly monophasic cyanide reactivity at either low (pH < 6) or high pH (pH > 9). We therefore believe that the conversion of the fast enzyme into the slow enzyme by lowering the pH may be no more that the expected response of the fast k' process to changes in pH and may not represent the conversion of a heterogeneous mixture of cytochrome *c* conformers to a single fast-reacting species.

Kinetic Analysis of the Cyanide Reaction. The rate constants for the reaction of cyanide with cytochrome *c* oxidase are often evaluated from the slopes of semilogarithmic, first-order rate plots. However, this approach is subject to inherent methodological errors. First of all, the final absorbance of the reaction when all phases are complete must be known, something that is almost impossible to visually determine from the data. Improper choice of A_∞ can easily make the biphasic reaction appear to be monophasic (refer to Figure 3). Second, if the semilogarithmic plot is biphasic, the initial slope is not equal to the true value of the faster rate constant, k'_{obs}. The intial slope is contaminated by the slower reaction and makes k'_{obs} appear to be slower than it really is; e.g., compare the slope of the dashed line with the initial slope of the solid line in Figure 3. Evaluation of the rate constants from these types of semilogarithmic plots should be avoided. A far more accurate method for obtaining the cyanide rate constants is to directly fit the absorbance versus time data that has been collected for at least 2 h by iterative nonlinear least squares. Lastly, data must be collected over a long enough time period to detect the slower second phase before conclusions are made regarding its presence or absence, i.e., at least 2 h for the reaction of cyanide with cytochrome *c* oxidase. Following the reaction for only a very short time period can easily make the biphasic reaction appear to be purely monophasic (Figure 3). It is certainly possible that the slower reaction phase was missed in many of the reports of homogeneous fast cyanide reacting cytochrome *c* oxidase since the reaction was often followed for less than 10 min.

The Fast k' Reaction. In all cases, the fast phase of the reaction showed well-behaved saturation type kinetics, corresponding to the formation of an enzyme−HCN complex that is in fast equilibrium with HCN and is defined by K_m. The values obtained for K_m (17.5 and 12.4 mM evaluated from the 418- and 428-nm data, respectively) are in good agreement with previously reported values, e.g., 5−10 mM at pH 7.4 with Tween 80 as the solubilizing detergent (Van Buuren et al., 1972; Andreev & Konstantinov, 1983). Our values for the apparent second-order rate constant of the fast k' reaction also agree with the average value of 2.5 M^{-1} s^{-1} that Naqui et al. (1984) obtained for seven different preparations of oxidase (evaluated at pH = 7.4 in 0.1 M phosphate buffer at 21 °C). We evaluated an apparent second-order rate constant of 2.2−2.3 $M^{-1}s^{-1}$ either from k'_{obs}/[KCN] at 1.0 mM KCN or from the limiting rate as the concentration of HCN approaches zero, i.e., k_2/K_m. The kinetically determined K_m is a good estimate of the K_d for the dissociation of HCN from the steady-state intermediate since the relatively slow k_2 is certainly much smaller than k_{-1} (refer to eq 2). However, we need to emphasize that K_m does not correspond to the equilibrium constant for the dissociation of HCN or cyanide from the product of the essentially irreversible k_2 reaction. The product of the fast reaction is very stable with an off rate of 1.4×10^{-6} s^{-1} (Van Buuren et al., 1972), corresponding to a $t_{1/2}$ of 138 h.

Effect of pH on the Fast k' Reaction. pH greatly affects the observed rate constant for the fast phase of the cyanide reaction (k'_{obs}), as is evident from the bell-shaped pH rate profile (refer to Figure 5). Such bell-shaped plots are common in enzyme kinetics when two or more pK_as of titratable groups are involved (Laidler, 1965; Fromm, 1975). From the nonlinear least squares fitting of experimental data to the rate equation, two apparent pK_as were evaluated. The pK_a of 9.15 is almost certainly that of HCN, while the other pK_a of 7.5 must be an ionizable group on cytochrome *c* oxidase, most likely an imidazole, a thiol, or an α-amino group. These results are summarized in the cyanide reaction mechanism proposed in Scheme 1 in which only an unprotonated form of oxidase is reactive toward HCN to form either $Cu_B^{2+}-CN^-$ or $Fe^{3+}-(CN^-)-Cu_B^{2+}$ at the bimetallic center of oxidase (Scott et al., 1985; Naqui et al., 1984; Papadopoulous et al., 1991). HCN as the reactive species is in agreement with earlier studies (Wilson et al., 1972; Van Buuren et al., 1972a,b; Jones et al., 1984; Musatov & Konstantinov, 1988; Papadopoulous et al., 1991; Gullo et al., 1993) and is consistent with the lack of dependence of k'_{obs} upon ionic strength. Interestingly, the kinetically determined pK_a of 7.45 is quite close to a pK_a value of 7.8

reported for a tyrosine residue that is responsible for pH-induced changes in the Soret maximum of cytochrome a_3 (Papadopoulous et al., 1991). More recently, Hallén et al. (1994) have also reported a kinetic pK_a of 7.7 that was obtained from following the pH dependence of CO dissociation from mixed-valence cytochrome *c* oxidase. The excellent agreement of pK_a values from these three entirely different types of studies seems to confirm the presence of a titratable group near the bimetallic center that is in equilibrium with the bulk solvent (Hallén et al., 1994; Konstantinov et al., 1986; Rousseau et al., 1993; Mitchell & Rich, 1994).

The Slow k″ Reaction. Our studies clearly indicate that the second, slow-phase reaction (k''_{obs}) is nearly independent of $[HCN]_{tot}$ and all of the other experimental variables that were tested. Increasing the concentration of cyanide 60-fold causes only a 2−4-fold increase in k''_{obs} (refer to inset in Figure 4). In fact, the slow k'' phase occurs even in the absence of any free cyanide once the initial k' process is complete (Figure 7). The lack of dependence of k''_{obs} upon cyanide concentration is consistent with the findings of both Naqui et al. (1984) and Baker et al. (1987). In all cases the slow k'' reaction has a pseudo-first-order rate of $(1-3) \times 10^{-4}$ s^{-1}. The alternative mechanism proposed by both Naqui et al. (1984) and Konstantinov et al. (1988), in which a form of the enzyme that does not bind cyanide is in a slow equilibrium with the active deprotonated form, is not supported by our data. First, the HCN−oxidase complex that is free of exogenous HCN still exhibits the slow, k''_{obs}, unimolecular reaction (refer to Figure 7). Second, slightly acidic pH does not induce the predicted spectral changes as a function of time in the absence of HCN. It seems unlikely that a slow preequilibrium reaction would occur only in the presence of HCN, yet be independent of the cyanide concentration. We conclude that the k'' process is not a parallel direct reaction with cyanide, but is a consecutive reaction that occurs only after the rapid reaction is complete. The slow second phase is most probably either (1) a slow conformation change in the enzyme after the fast k' reaction with cyanide, (2) a change in ligand coordination at one of the metal centers after HCN binds to the bimetallic center, (3) a unimolecular decomposition of the cyanide-bound oxidase; (4) a change in the spin state of heme a_3^{3+} at the bimetallic center after formation of the initial product, or (5) some other spectral change that occurs only after the initial rapid reaction with HCN. The hypothesis that the slow phase results from a conformation change in oxidase after its reaction with HCN seems most logical since it is consistent with most of the previous data and the fact that only 1 mol of cyanide is bound per mole of cytochrome *c* oxidase, even after long reaction times (Van Buuren et al., 1972b).

Effect of Detergent Environment upon Reaction with Cyanide. Changes in the type of detergent used to solubilize oxidase cause less than a 2-fold change in the fast k' reaction. Detergent effects are known to alter the steady-state electron transfer kinetics by as much as 2 orders of magnitude (Robinson et al., 1985; Mahapatro & Robinson, 1990). Therefore, the detergent-induced conformational changes in oxidase that greatly alter electron transfer from cytochrome *a* to a_3 do not perturb the bimetallic center enough to significantly alter its reaction with HCN. In all of the detergents except Brij 96, both phases are clearly present. The second slow phase probably also occurs when oxidase is solubilized with Brij 96; however, light scattering arising from the slow aggregation of the enzyme in Brij 96 prevented spectral detection and analysis of the data after 20 min. Increasing the concentration of the solubilizing detergent is known to induce monomerization of oxidase (Robinson & Talbert, 1986; J. Ortega-Lopez and N. C. Robinson, unpublished results); however, such increases in detergent concentration only slightly affect the reaction rates of oxidase with HCN. Clearly, the aggregation state of oxidase has little influence upon the reactivity of oxidase toward HCN.

Effects of Temperature upon Reaction with HCN. Both the fast k' phase (monitored at 428 nm) and the slower k'' phase (monitored at either 418 or 428 nm) exhibit classical Arrhenius type behavior from 10 to 30 °C. Interpretation of data for the fast k' reaction is complex since the results obtained at 418 nm do not agree with those obtained at 428 nm. Above 25 °C, k'_{obs}(428 nm) continues to increase as expected, but k'_{obs}(418 nm) becomes independent of temperature (refer to Figure 8A). This is inconsistent with any expected behavior and implies that the product forms more rapidly (428 nm data) than the unreacted oxidase disappears (418 nm data). Temperature-induced changes in the visible spectrum above 30 °C, presumably due to the first stages of thermal denaturation, greatly complicate monitoring of the reaction, and the data no longer could be adequately fitted to a simple biphasic reaction.

The induction of monophasic fast HCN reaction kinetics by incubation of the enzyme at 35 °C followed by DEAE purification at first seems inconsistent with the sequential mechanism, i.e., Scheme 1. However, disappearance of the slow second phase is almost certainly due to dissociation of subunit III from the complex at the higher temperature (Rigell & Freire, 1987). Removal of subunit III from the resting enzyme is known to induce monophasic kinetics (Hill & Robinson, 1986), probably due to altered ligand configuration at the a_3-Cu_B site. Exactly how removal of subunit III would prevent the slow, consecutive, second-phase reaction is unknown, but it is tempting to speculate that subunit III may directly participate in the unimolecular change that occurs in the HCN−oxidase complex.

Fast and Slow Cyanide-Reactive Conformers of Oxidase. A mixture of fast- and slow-reacting conformers of oxidase has been proposed to explain both the nonidentical cyanide reactivities of different oxidase preparations and the accepted biphasic cyanide kinetics at neutral pH (Naqui et al., 1984; Baker et al., 1987; Malmström, 1990a,b; Schoonover et al., 1988; Larsen et al., 1989; Schoonover & Palmer, 1991; Day et al., 1993; Lodder & Van Gelder, 1994). Even though more than one conformer of oxidase may exist in different oxidase preparations, our kinetic model suggests that a single conformer of oxidase could exhibit either monophasic or biphasic cyanide kinetics depending upon the pH of the reaction.

According to the two-conformer hypothesis, the biphasic cyanide reactivity at pH 7.4−8.0 is due to a mixture of the fast and slow forms, while the monophasic reactivity at lower pHs is due to a predominance of the slow form (Baker et al., 1987; Schoonover et al., 1988; Larsen et al., 1989; Schoonover & Palmer, 1991). In some cases, this conversion from the fast to the slow form is slow and requires as long as 24 h (Schoonover & Palmer, 1991). We have proposed an alternative kinetic explanation for the transition from

biphasic kinetics at pH 7.5−8.0 to monophasic kinetics at low or high pH (refer to Figure 5 and Scheme 1); however, this scheme does not explain the very slow conversion of biphasic to monophasic kinetics. Nevertheless, we would expect the reaction of cytochrome *c* oxidase to become nearly monophasic at pH < 6.5 or pH > 9 on the basis of the pH dependence of k'_{obs}. At either of these extremes in pH the value for the rate constant of the fast k'_{obs} reaction becomes approximately the same as the value for the rate constant of the slow, cyanide-independent k''_{obs} reaction, provided that the concentration of KCN is sufficiently small. The result would be two kinetically indistinguishable rates. Interestingly, both our kinetic model and the two-conformer model are dependent upon a group with a pK_a near 7.5 (Papadopoulous et al., 1991; Hallén et al., 1994). This may be no more than a coincidence since the kinetic model does not require a pH-dependent conformational change, only that the protonation of a group affects the rate-limiting step.

Although our model can explain the apparent conversion of enzyme exhibiting biphasic kinetics into enzyme with monophasic kinetics, it does not explain the nonidentical cyanide reactivities of different enzyme preparations at a single pH (Naqui et al., 1984; Papadopoulous et al., 1991; Gullo et al., 1993; Lodder & Van Gelder, 1994). The pH dependence of the kinetics is fully reversible, while the different behavior of separate enzyme preparations implies a nonequilibrium type of stability. The only apparent way to reconcile these different results is to conclude that these are independent processes; i.e., multiple conformers of oxidase exist that have nonidentical cyanide reactivities. However, conversion of a fast conformer into a slow conformer is not responsible for the change from biphasic to monophasic kinetics at low pH. A possible explanation is that the intrinsic, irreversible, k_2 rate, which has a value of $(3.6-3.8) \times 10^{-2}$ s^{-1} for our preparation of oxidase, may be either faster or slower with other preparations. A decrease in k_2 would decrease both the apparent first-order rate constant and the preexponential term for the fast reaction. If the second, slow reaction had the same rate in all oxidase conformers, a decrease in k_2 would appear to change the "percentage of enzyme" that reacts rapidly with cyanide.

At present, our results cannot explain the altered cyanide reactivity of different preparations of cytochrome c oxidase, but it is clear that a single type of oxidase can exhibit either monophasic or biphasic kinetics depending upon the pH. We therefore conclude that the presence of monophasic kinetics at either low or high pH, or biphasic cyanide kinetics at neutral pH, is not a good indicative test of conformational homogeneity or heterogeneity for bovine cytochrome *c* oxidase.

ACKNOWLEDGMENT

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SUPPORTING INFORMATION AVAILABLE

Tables of k'_{obs} and k''_{obs} values as a function of [KCN], [oxidase], ionic strength, [dodecyl β-D-maltoside], pH, and temperature (6 pages). Ordering information is given on any current masthead page.

APPENDIX: Derivation of the Rate Expression

The rate expression in eq 3 was derived on the basis of Scheme 1 as follows. The equilibrium constants are given by the following equations:

$$K_{HCN} = k_{+H^+}/k_{-H^+} = [CN^-][H^+]/[HCN] \quad (a)$$

$$K_a = k_a/k_{-a} = [\text{oxidase}][H^+]/[\text{oxidase}H^+] \quad (b)$$

$$K_1 = k_1/k_{-1} = [\text{complex}]/[\text{oxidase}][HCN] \quad (c)$$

$$[HCN]_{tot} = [HCN]\,(1 + (K_{HCN}/[H^+])) \text{ or}$$
$$[HCN] = [HCN]_{tot}[H^+]/(K_{HCN} + [H^+]) \quad (d)$$

Assuming a steady-state condition for the complex, its rate of formation is the same as the rate of disappearance, i.e., k_1[oxidase] [HCN] = k_{-1} [complex] + k_2 [complex], i.e.,

$$[\text{complex}] = k_1[\text{oxidase}][HCN]/(k_{-1} + k_2) \quad (e)$$

The total oxidase concentration from mass balance is

$$[\text{oxidase}]_{tot} = [\text{oxidase}] + [\text{oxidase } H^+] + [\text{complex}] \quad (f)$$

Substituting for [oxidase] and [oxidase H^+] from (b) and (c) into (f) and then rearranging gives

$$[\text{oxidase}]_{tot} = [\text{complex}]\,\{(1/[HCN]K_1)(([H^+]/K_a) + 1) + 1\}$$

Therefore,

$$[\text{complex}] = [\text{oxidase}]_{tot}/\{(1/[HCN]K_1)(([H^+]/K_a) + 1) + 1\} \quad (g)$$

Substituting for [HCN] in terms of $[HCN]_{tot}$ from (d) in (g) and by algebraic manipulation,

$$[\text{complex}] = \frac{K_1[\text{oxidase}]_{tot}[HCN]_{tot}}{\{(K_{HCN}/[H^+]) + 1\}\{([H^+]/K_a) + 1\} + K_1[HCN]_{tot}} \quad (h)$$

The rate of formation of product is

$$\text{rate} = k_2[\text{complex}] \quad (i)$$

Therefore, substituting the expression of [complex] from (h) into (i) and on further simplification we obtain

$$\text{rate} = \frac{k_2K_1[\text{oxidase}]_{tot}[HCN]_{tot}}{(K_{HCN}/K_a) + (K_{HCN}/[H^+]) + ([H^+]/K_a) + K_1[HCN]_{tot} + 1} \quad (j)$$

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