Ohtsuka, E., & Ikehara, M. (1988) *Protein Eng. 2*, 55-61.
Heinemann, U., & Saenger, W. (1982) *Nature (London) 299*, 27-31.
Hoffmann, E., & Rüterjans, H. (1988) *Eur. J. Biochem. 177*, 539-560.
Hurlbert, R. B. (1957) *Methods Enzymol. 3*, 785-805.
Inagaki, F., Kawano, Y., Shimada, I., Takahashi, K., & Miyazawa, T. (1981) *J. Biochem. (Tokyo) 89*, 1185-1195.
Inagaki, F., Shimada, I., & Miyazawa, T. (1985) *Biochemistry 24*, 1013-1020.
Jeener, J., Meier, B. H., Bachmann, P., & Ernst, R. R. (1979) *J. Chem. Phys. 71*, 4546-4553.
Kanaya, S., & Uchida, T. (1981) *J. Biochem. (Tokyo) 89*, 591-597.
Kyogoku, Y., Watanabe, M., Kainosho, M., & Oshima, T. (1982) *J. Biochem. (Tokyo) 91*, 675-679.
Nagai, H., Kawata, Y., Hayashi, F., Sakiyama, F., & Kyogoku, Y. (1985) *FEBS Lett. 189*, 167-170.
Osterman, H. L., & Walz, F. G., Jr. (1979) *Biochemistry 18*, 1984-1988.
Piantini, U., Sorensen, O. W., & Ernst, R. R. (1982) *J. Am. Chem. Soc. 104*, 6800-6801.
Richarz, R., & Wüthrich, K. (1978) *J. Magn. Reson. 30*, 147-150.
Rüterjans, R., & Pongs, O. (1971) *Eur. J. Biochem. 18*, 313-318.
Sato, K., & Egami, F. (1957) *J. Biochem. (Tokyo) 44*, 753-767.
States, D. J., Haberkorn, R. A., & Reuben, D. J. (1982) *J. Magn. Reson. 48*, 286-292.
Sugio, S., Amisaki, T., Ohishi, H., Tomita, K., Heinemann, U., & Saenger, W. (1985a) *FEBS Lett. 181*, 129-132.
Sugio, S., Oka, K., Ohishi, H., Tomita, K., & Saenger, K. (1985b) *FEBS Lett. 183*, 115-118.
Sugio, S., Amisaki, T., Ohishi, H., & Tomita, K. (1988) *J. Biochem. (Tokyo) 103*, 354-366.
Takahashi, K. (1961a) *J. Biol. Chem. 240*, 4117-4119.
Takahashi, K. (1961b) *J. Biochem. (Tokyo) 60*, 239-245.
Takahashi, K. (1972) *J. Biochem. (Tokyo) 72*, 1469-1481.
Takahashi, K., & Moore, S. (1982) *Enzymes* (Boyer, P. D., Ed.) pp 453-468, Academic Press, New York and London.
Wüthrich, K. (1976) *NMR in Biological Research: Peptides and Proteins*, North-Holland, Amsterdam, Oxford/American Elsevier, New York.

Effect of Changing the Detergent Bound to Bovine Cytochrome *c* Oxidase upon Its Individual Electron-Transfer Steps†

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Received June 27, 1989; Revised Manuscript Received September 6, 1989

ABSTRACT: The influence of the detergent environment upon individual electron-transfer rates of cytochrome *c* oxidase was investigated by stopped-flow spectrophotometry. The effects of three detergents were studied: lauryl maltoside, which supports a high turnover number (TN = 350 s^{-1}), *n*-dodecyl octaethylene glycol monoether ($C_{12}E_8$), which supports an intermediate TN (150 s^{-1}), and Triton X-100 in which oxidase is nearly inactive (TN = 2-3 s^{-1}). Under limited turnover conditions (cytochrome *c*:cytochrome *c* oxidase ratio = 1:1 to 8:1), the rate of oxidation of cytochrome *c* was measured and compared with the fast reduction of cytochrome *a* and its relatively slow reoxidation. Two reducing equivalents of cytochrome *c* were rapidly oxidized in a burst phase; the remaining two to six equivalents were oxidized more slowly, concurrent with the reoxidation of cytochrome *a*; i.e., the percent reduced cytochrome *a* reflects the percent reduced cytochrome *c*. With the resting enzyme, the bimolecular reaction between reduced cytochrome *c* and cytochrome *a* was rapid, was insensitive to the detergent environment, and was not the rate-limiting step in the presence of any detergent. The rate of internal electron transfer from cytochrome *a* to cytochrome a_3 in the resting enzyme was slow and only slightly affected by the detergent environment: 1.0-1.1 s^{-1} in Triton X-100, 5-7 s^{-1} in $C_{12}E_8$, and 5-12 s^{-1} in lauryl maltoside. With the pulsed enzyme, the intramolecular electron transfer between cytochrome *a* and cytochrome a_3 increased 4-5-fold in the lauryl maltoside enzyme but did not increase in the Triton X-100 enzyme (intermediate values were obtained with the $C_{12}E_8$ enzyme). We conclude that cytochrome *c* oxidase acquires the pulsed conformation only in those detergents that support high TN's, e.g., lauryl maltoside and $C_{12}E_8$, but it is locked in the resting conformation in those detergents which result in low TN's, e.g., Triton X-100.

Cytochrome *c* oxidase is an integral membrane protein complex that spans the inner mitochondrial membrane. The complex catalyzes the four-electron reduction of oxygen to water by four transmembrane electron transfers from reduced cytochrome *c*. In steady-state experiments, the turnover number (TN)[1] of the enzyme is sensitive to the apolar envi-

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[1] Abbreviations: $C_{12}E_8$, *n*-dodecyl octaethylene glycol monoether; LM, lauryl maltoside (dodecyl β-D-maltopyranoside); TN, enzymatic turnover number; TX, Triton X-100; Tris-acetate buffer, tris(hydroxymethyl)-aminomethane base titrated to the appropriate pH with acetic acid; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; $C_{12}E_8$-enzyme, cytochrome *c* oxidase with bound $C_{12}E_8$; LM-enzyme, cytochrome *c* oxidase with bound LM; TX-enzyme, cytochrome *c* oxidase with bound TX.

0006-2960/90/0429-764$02.50/0

ronment surrounding the membrane-spanning domains of the complex. A proper apolar environment can be supplied either by the fluid fatty acid tails of a phospholipid bilayer or by appropriate choice of detergent used to solubilize the complex. Alteration of either the fatty acid composition of the phospholipids or the hydrocarbon tail of the bound detergent greatly affects the maximum rate of electron transport. TN's can change by more than a factor of 100 depending on the nature of the bound detergent, or phospholipid. For example, the enzyme is maximally active in lauryl maltoside (LM),[1] Tween 20 or 80, or phospholipids with at least 50% unsaturation (TN = 300–600 s^{-1}) (Hartzell et al., 1978; Rosevear et al., 1980; Vik & Capaldi, 1980; Thompson & Ferguson-Miller, 1983; Robinson et al., 1985), is partially active in *n*-dodecyl octaethylene glycol monoether ($C_{12}E_8$), decyl glucoside, or phospholipids with less than 50% unsaturation (TN = 80–150 s^{-1}) (Robinson et al., 1985; De Cuyper & Joniau, 1980), and is nearly inactive in Triton X-100 (TX), sodium cholate, and other detergents that have bulky hydrocarbons as part of their apolar structure (TN = 2–4 s^{-1}) (Robinson & Capaldi, 1977; Robinson et al., 1985). These reduced activities in $C_{12}E_8$, TX, or cholate are reversible, and maximal electron-transfer rates can be regenerated by exchange of these detergents with LM. Interestingly, TX and/or cholate are routinely used to extract the membrane-bound complex in the standard preparative protocols (Fowler et al., 1962; Hartzell et al., 1978).

We have previously reported the temperature dependence of the steady-state activity of cytochrome *c* oxidase in a variety of detergents that have substantial variation in their structures (Robinson et al., 1985). The activation enthalpy in many detergent systems (e.g., alkyl maltosides, $C_{12}E_8$, decyl glucoside, Zwittergent 3-12, or TX) was nearly the same (ΔH_{act} = 11.2 kcal mol^{-1}). Therefore, the previously proposed rate-limiting product dissociation (Gibson et al., 1965; Sinjorgo et al., 1984) seemed to be an unlikely explanation for the widely different turnover numbers.

We had suggested that cytochrome *c* oxidase might be locked in the unreactive "resting" conformation in those detergents in which it is essentially inactive, e.g., TX, whereas the more reactive "pulsed" form might be favored in those detergents that support higher activity, e.g., LM, other alkyl maltosides, poly(oxyethylene) ethers, or Tween 20 and 80 (Robinson et al., 1985). We now describe stopped-flow kinetic studies that confirm this hypothesis and identify electron-transfer steps that are expressly influenced by the detergent environment.

Materials and Methods

Keilin Hartree muscle particles were prepared from beef heart by the method of Yonetani (1960). Cytochrome *c* oxidase was isolated by the method of Fowler et al. (1962), as modified by Capaldi and Hayashi (1972). After the final ammonium sulfate precipitation, the oxidase pellet was dissolved in 1% sodium cholate, 0.1 M NaH_2PO_4, and 1 mM EDTA, pH 7.4, buffer and stored in liquid nitrogen at approximately 20 mg/mL. The isolated complex contained 9.2–10.0 nmol of heme *a*/mg of protein on the basis of $\Delta\epsilon_{606-630}$(red) = 16.5 mM^{-1} cm^{-1} (Griffiths & Wharton, 1961), had the Soret maximum at 420–422 nm, and had a molecular activity of 350–370 μmol of cytochrome *c* oxidized (μmol of cytochrome *c* oxidase)$^{-1}$ s^{-1} when assayed in 0.025 M phosphate buffer pH 7.0, 25 °C, containing 2 mM LM. Initial concentrations of oxidase were determined from measured absorbances using ϵ_{422}(ox) = 154 mM^{-1} cm^{-1} (van Gelder, 1978) and $\Delta\Delta\epsilon_{605-630}$(red–ox) = 13.5 mM^{-1} cm^{-1} (Nicholls, 1979). Sodium cholate in the stock oxidase was exchanged for either LM or TX by addition of a 10 mg/mL aliquot of the second detergent and extensive dialysis vs phosphate buffer (0.1 M, pH 7.4, 0.2 mg/mL LM or TX-100) to completely remove the sodium cholate.

Cytochrome *c* (type III from horse heart) was obtained from Sigma Chemical Co. Reduced cytochrome *c* was freshly prepared by dithionite reduction, and excess dithionite was removed on a G-25 Sephadex column. Initial concentrations of ferrocytochrome *c* were determined by using ϵ_{550} = 29.5 mM^{-1} cm^{-1}. Ultrapure Triton X-100 was purchased from Boehringer-Mannheim. Lauryl maltoside and $C_{12}E_8$ were from Calbiochem.

The rapid kinetic measurements were performed on a stopped-flow unit with a 20-mm cell path length (Kinetic Systems, Ann Arbor, MI) that was attached to a DU2 Beckman spectrophotometer. Absorbance changes at selected wavelengths (444, 550, and 606 nm) were measured over a 50-ms to 10-s time period using a Metrabyte DASH-16 Analog I/O interface board, an AT&T 6300 microcomputer, and ASYSTANT+(tm) data collection and fitting software. This allowed routine collection of 500 data points within a preselected time base. The reservoir syringes, the optical cell, and the entire flow system were submerged in a thermostatically controlled water bath maintained at 25 °C. The dead time of the instrument was found to be 3–4 ms using the standard reaction of 2,6-dichlorophenolindophenol and ferricyanide reduction by L-ascorbate (Tonomura et al., 1978).

The reduction of cytochrome *a* and its reoxidation were followed at either 444 or 606 nm. The number of reducing equivalents delivered by ferrocytochrome *c* was determined from the change in absorbance at 550 nm, where the contribution of oxidase is less than 5% (Antalis & Palmer, 1982). The degree of reduction of cytochrome *a* was calculated from the absorbance changes at 444 and 605 nm, after correcting for the contribution of cytochrome *a*, cytochrome a_3, and ferro- and ferricytochrome *c* to the absorbance at each wavelength (Antalis & Palmer, 1982; Greenwood & Gibson, 1967). Steady-state levels of reduced cytochrome *c* and cytochrome *a* in experiments with ascorbate and TMPD as the electron donors, e.g., Figure 1, were calculated by using $\Delta\epsilon_{444}$(red–ox) = 56.8 mM^{-1} cm^{-1} for cytochrome *a*, $\Delta\epsilon_{550}$(red–ox) = 21.2 mM^{-1} cm^{-1}, and $\Delta\epsilon_{444}$(red–ox) = −9.5 mM^{-1} cm^{-1} for cytochrome *c* (Antalis & Palmer, 1982).

Under aerobic conditions, cytochrome a_3 remains essentially oxidized (Antonini et al., 1970). Cytochrome *a* and cytochrome a_3 are often considered to contribute equally to the Soret maximum of the oxidized (418–420 nm) and reduced oxidase (444 nm), based upon extinction coefficients measured with ligands bound to cytochrome a_3 (Yonetani, 1960). However, Gibson and Greenwood have reported that cytochrome *a* contributes about 33% of the kinetic difference spectrum at 444 nm in the absence of ligands (Greenwood & Gibson, 1967). This has also been confirmed by Antalis and Palmer (1982). Our absorbance changes were also in agreement with a 33% contribution by cytochrome *a*. Calculations based upon absorbance changes at 606 nm are less ambiguous since cytochrome *a* is generally accepted to contribute 75% of the absorbance change at 605 nm upon complete reduction of oxidase (Yonetani, 1960).

Preparation of Oxygenated and Pulsed Oxidase. (*A*) *Oxygenated oxidase* was prepared by dithionite reduction of the resting oxidase (2.5 mL, 40 μM enzyme in 0.1 M phosphate buffer, pH 7.4, containing 5 mg/mL LM). The sample was allowed to stand for 1 h on ice to ensure complete reduction, which was monitored at 606 nm. The sample was then rapidly

passed through a PD-10 Sephadex G-25 column in the same buffer to remove dithionite, and the oxidase was reoxidized by dioxygen. The Soret maximum at 428 nm was characteristic of the oxygenated enzyme (Naqui et al., 1984; Chance et al., 1983; Okunuki & Sekuzu, 1954).

(*B*) *420-nm pulsed oxidase* (Kumar et al., 1984a,b) was prepared by reducing resting oxidase (2.5 mL, 40 μM enzyme) by ascorbate (5 mM) and cytochrome *c* (2 μM), under an atmosphere of argon, for 2 h in phosphate buffer, (0.1 M, pH 7.4) which contained either 2 mM LM or 3 mM TX. Complete reduction was observed spectrophotometrically at 606 nm. The sample was rapidly passed through CM-52 to remove cytochrome *c* and then through a PD-10 (G-25) column to remove excess ascorbate (both steps were performed in the same buffer as the initial reduction step). The sample was then gently reoxygenated. The resulting pulsed oxidase showed a Soret maximum at 420–422 nm, and it was immediately used for the stopped-flow studies.

Anaerobic Experiments. Anaerobic solutions of cytochrome *c* oxidase and buffers were obtained by thorough deoxygenation of the solutions on a vacuum-line manifold. Reactant solutions were kept under an atmosphere of argon or purified nitrogen. The reservoir syringes were sealed with rubber septa and purged with nitrogen for more than 1 h to remove oxygen. The waste outlet of the stopped-flow unit was connected to a tower containing a solution of dithionite to minimize oxygen contamination. The reservoir syringes and the entire flow system were thoroughly flushed with deoxygenated and nitrogen-purged buffer which contained catalytic amounts of glucose oxidase and catalase, and an excess of β-D-glucose (Englander et al., 1987).

Results

Cyanide Binding Studies. Resting oxidase is known to be a mixture of slow and fast cyanide-reactive forms which are evident from the biphasic cyanide binding kinetics (Hill & Robinson, 1986; Baker et al., 1987). The fully reduced and the pulsed-oxidized enzyme, however, each react as a single, fast-reacting population (van Buuren et al., 1972; Naqui et al., 1984). In either LM or TX, our preparation of resting oxidase reacted rapidly with cyanide; 75% of the total absorbance change occurred in less than 5 min in either detergent. The fast reaction was followed by a slow second phase extending over 12 h. The fast cyanide binding phase gave a second-order rate of 2.5–4.0 M^{-1} s^{-1} at 25 °C. The rate of cyanide binding was independent of the nature of the bound detergent (LM or TX).

Anaerobic Reduction of Cytochrome c Oxidase. The reduction of cytochrome aa_3 by cytochrome *c* and by other reducing agents was studied in order to understand the influence bound detergent has upon the electron-transfer rates from reduced cytochrome *c* to cytochrome *a*–Cu_A, and from cytochrome *a*–Cu_A to the oxygen binding cytochrome a_3–Cu_B site. The reduction of cytochrome *c* oxidase by cytochrome *c*/ascorbate–TMPD in low ionic strength buffer (Tris–acetate, 25 mM, pH 7.8) was studied by stopped-flow spectrophotometry at 444 nm. Resting cytochrome *c* oxidase and cytochrome *c* were mixed with ascorbate and TMPD in the stopped-flow apparatus. Strictly anaerobic conditions were maintained by thorough deoxygenation of all buffers and reactant solutions on a vacuum-line manifold as described under Materials and Methods. Stopped-flow traces for the anaerobic reduction of oxidase in LM and in TX are given in Figure 1. The reduction of cytochrome *a* was extremely rapid and was essentially complete in the dead time of the instrument (3–4 ms). The burst phase accounted for approximately 34% of the total absorbance change at 444 nm, in agreement with Antalis and Palmer (1982) and Greenwood and Gibson (1967). This rapid reduction of cytochrome *a* was then followed by a slow second phase in which the absorbance increased for several seconds. First-order rate constants for the second phase, i.e., the reduction of cytochrome a_3, were 1–5 s^{-1} for resting oxidase; this rate was not very sensitive to the detergent environment of the enzyme.

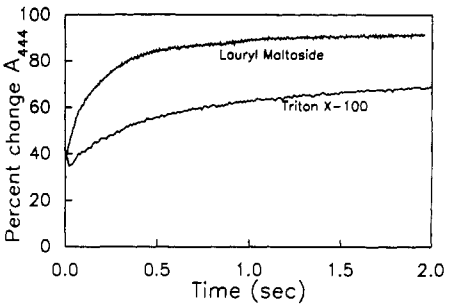

FIGURE 1: Anaerobic reduction of cytochrome *c* oxidase by cytochrome *c*, ascorbate, and TMPD. Reaction conditions: [oxidase] = 2.5 μM; [cyt *c*] = 2.5 μM; 5 mM ascorbate, 0.25 mM TMPD, and 25 mM Tris–acetate buffer, pH 7.80, 25 °C, containing 2 mM LM or 3 mM TX. Upper trace, pulsed enzyme in LM; lower trace, resting enzyme in TX.

Table I: Effect of Bound Detergent upon Aerobic Reduction of the Cytochrome *a* in Resting Oxidase by Reduced Cytochrome *c*[a]

detergent	[cyt *c*]/[oxidase]	equiv of reduced cyt *a*[b] (444 or 606 nm)	equiv of oxidized cyt *c*[c] (550 nm)
Triton X-100	4	0.6–0.7	1.8–2.0
	1	0.7–0.8	>0.9
lauryl maltoside	4	0.25–0.30	2.8–3.0
	1	0.20–0.25	>0.95

[a] Measured at 25 °C in 0.1 M phosphate buffer, pH 7.40. [b] Equivalents of cytochrome *a* that became reduced in the fast phase of the aerobic reaction, e.g., Figures 2, 3B, and 4B. [c] Equivalents of cytochrome *c* that were oxidized in the fast initial phase of the aerobic reaction, e.g., Figures 3A and 4A.

Aerobic Studies. Cytochrome *c* oxidase catalyzes the four-electron reduction of oxygen:

$$4\text{cyt } c^{2+} + O_2 + 4H^+ \rightarrow 4\text{cyt } c^{3+} + 2H_2O \qquad (1)$$

Therefore, stopped-flow kinetics studied under aerobic conditions were done using ratios of cytochrome *c* to oxidase that were at or near the stoichiometry represented by eq 1 (cytochrome *c*:cytochrome *c* oxidase ratio = 1:1, 2:1, 4:1, and 8:1). All reactions were done at 25 °C in 0.1 M phosphate buffer (pH 7.4, 0.22 M ionic strength) which contained either 2 mM LM or 3 mM TX.

Under very limited turnover conditions (cytochrome c: oxidase ratio = 1:1) and in the presence of LM and oxygen, all of the ferrocytochrome *c* was rapidly oxidized in an initial burst phase (50–100 ms) but only 20–25% of the cytochrome *a* became reduced (Table I). Presumably, the remaining 75% of the reducing equivalents were trapped in Cu_A. With an increased ratio of reduced cytochrome *c* to cytochrome *c* oxidase (4:1), 2 equiv of ferrocytochrome *c* was rapidly oxidized in the burst phase, but still only 25–30% of the cytochrome *a* was reduced. Similar experiments in TX gave somewhat different results with steady-state reduction of cytochrome *a* reaching about 70% (Table I). This fast reduction of cytochrome *a* to a steady-state level of 25–30% reduced cytochrome *a* in LM, or 70–80% reduced cytochrome *a* in TX is illustrated in Figure 2. Notice that the rate of reduction of cytochrome

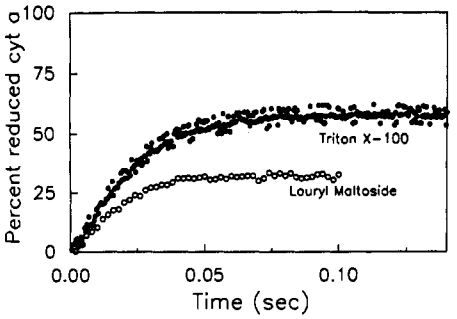

FIGURE 2: Rapid reduction of cytochrome *a* by reduced cytochrome *c* under aerobic conditions. Reaction conditions: $[oxidase]_{resting}$ = 3 μM; $[cyt\ c]_{red}$ = 12 μM; 0.10 M phosphate buffer, pH 7.40, 25 °C, containing either 2 mM LM or 3 mM TX. The percent reduction of cytochrome *a* was calculated from the change in absorbance at 606 nm.

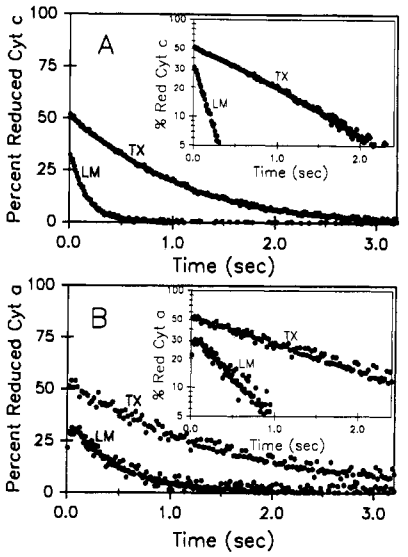

FIGURE 3: Resting cytochrome *c* oxidase: reoxidation of reduced cytochrome *a* and oxidation of reduced cytochrome *c* during the slower second phase of the aerobic reaction. (The initial fast phase of this reaction is shown in Figure 2.) Reaction conditions were the same as described in Figure 2. Panel A: Oxidation of cytochrome *c* calculated from the change in absorbance at 550 nm. The inset to panel A is the first-order rate plot of the data. Panel B: Reduction of cytochrome *a* calculated from the change in absorbance at 606 nm. The inset to panel B is the first-order rate plot of the data.

a is similar in each detergent, about 25–40 s^{-1} with these concentrations of cytochrome *c*. After the initial burst phase, the remaining 2 equiv of ferrocytochrome *c* was oxidized in a slow second phase extending over several seconds (Figure 3A), and this was concurrent with the reoxidation of cytochrome *a* (Figure 3B) by electron transfer to cytochrome a_3 and then rapidly to oxygen.

Pseudo-first-order rates for the formation of reduced cytochrome *a* and its slow reoxidation were calculated from the semilog plots of the change in absorbance at 444 and 605 nm versus time (insets to Figure 3A,B). First-order rates for the oxidation of ferrocytochrome *c* were observed in the presence of either LM or TX. Notice that the first-order decay of cytochrome *c* oxidation is approximately twice the first-order decay of cytochrome *a* oxidation, which is consistent with a stoichiometry of two cytochrome *c*'s oxidized per cytochrome *a* reduced. The absorbance–time curves were fitted to a first-order exponential by using the ASYSTANT+ data-fitting routine which allowed us to view the distribution of the residuals. Rate data are given in Table II for the resting enzyme in the two detergent systems. It is obvious from the first-order

Table II: Effect of Bound Detergent upon Aerobic Intramolecular Electron-Transfer Rates within Resting Cytochrome *c* Oxidase[a]

detergent	[cyt *c*]/[oxidase]	k_{obs} (s^{-1}) at 550 nm[b]	444 nm[c]	606 nm[d]
Triton X-100	1	no 2nd phase	1.0	1.1
	4	1.2	0.49	0.54
	8	0.84		0.60
$C_{12}E_8$	4	5–7	1.8	
lauryl maltoside	4	5[e]	1.3–1.4	1.6

[a] All rates are interpreted to give intramolecular electron transfer from cytochrome *a* to cytochrome a_3 under aerobic conditions. Initial concentrations: $[cyt\ c\ oxidase]_{oxidized}$ = 3 μM; $[cyt\ c]_{red}$ = 12 μM. Rates were measured at 25 °C in 0.1 M phosphate buffer, pH 7.40. [b] The rate was determined by linear regression analysis of the oxidation of cytochrome *c* measured at 550 nm, e.g., inset to Figure 3A. [c] The rate was determined by linear regression analysis of the oxidation of cytochrome *a* measured at 444 nm, e.g., inset to Figure 3B. [d] The rate was determined by linear regression analysis of the oxidation of cytochrome *a* measured at 606 nm. [e] Three preparations of enzyme gave rates of 5.2, 9.5, and 12.3 s^{-1}; the three preparations each had a rate of 1.2 s^{-1} in TX. The rates of oxidation of reduced cytochrome *a* (444- and 606-nm rates) were also higher with preparations having the higher rates for cytochrome *c* oxidation.

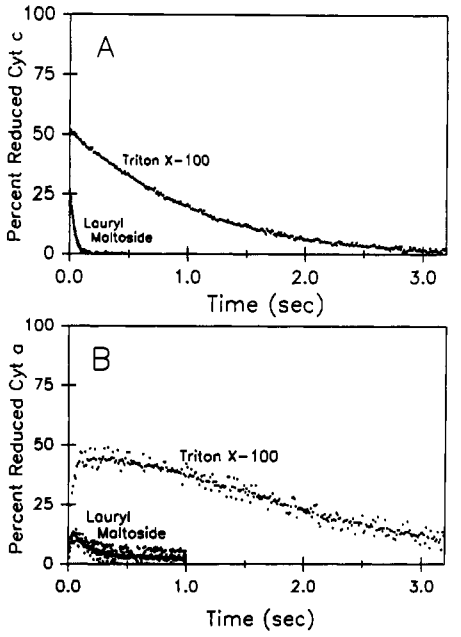

FIGURE 4: Pulsed cytochrome *c* oxidase: reoxidation of reduced cytochrome *a* and oxidation of reduced cytochrome *c* during the slower second phase of the aerobic reaction. Reaction conditions and calculations were the same as described in Figures 2 and 3.

plots that the initial reduction of cytochrome *a* is not the same in the two detergents (50–60% in TX, but only 30–40% in LM). This finding is consistent with the data in Table I and most likely indicates that the rate of electron transport from cytochrome *a* to a_3 has become significant compared with the rate of reduction of cytochrome *a* by cytochrome *c* when the enzyme is in LM. A steady-state level of 30–35% reduced cytochrome *a* is what would be predicted by kinetic modeling using the rate of reduction of cytochrome *a* (25–40 s^{-1}; Figure 2) and the rate for electron transfer from cytochrome *a* to a_3 (5–10 s^{-1}; Figure 3B) that we obtained for the LM–enzyme.

Cytochrome oxidase was converted into two activated forms (428 nm oxygenated and 420 nm pulsed), and the rate of reoxidation of cytochrome *a* and the rate for the slow second phase of cytochrome *c* oxidation were measured in the presence of the two detergents (Figure 4A,B). The effect of pulsing the enzyme did not lead to any significant rate enhancement in the internal electron transfer between cytochrome *a* and

Table III: Effect of Bound Detergent upon Aerobic Intramolecular Electron-Transfer Rates of Resting, Oxygenated, and Pulsed Cytochrome *c* Oxidase[a]

detergent	type of oxidase	k_{obs} (s^{-1}) at 550 nm[b]	444 nm[c]	606 nm[d]
Triton X-100	resting	1.2	0.49	0.54
	oxygenated	1.3	0.80	0.80
	pulsed	1.0	0.50	0.55
$C_{12}E_8$	resting	5–7	1.8	
	pulsed	12–15		
lauryl maltoside	resting	5[e]	1.3	1.6
	oxygenated	25		
	pulsed	30		

[a] Measured at 25 °C in 0.1 M phosphate buffer, pH 7.40. [b] The rate was determined by linear regression analysis of the oxidation of cytochrome *c* measured at 550 nm, e.g., inset to Figure 3A. [c] The rate was determined by linear regression analysis of the oxidation of cytochrome *a* measured at 44 nm, e.g., inset to Figure 3B. [d] The rate was determined by linear regression analysis of the oxidation of cytochrome *a* measured at 606 nm. [e] In some preparations of enzyme, this rate was as high as 12 s^{-1} as discussed in the legend to Table II.

cytochrome a_3 with TX bound to oxidase (compare Figures 3B and 4B). The observed rate constants are given in Table III. Results obtained with oxygenated and pulsed LM–oxidase were more interesting. The level of reduced cytochrome *c* after the initial burst was less than 20% (Figure 4A). The observed rate of oxidation of the remaining cytochrome *c* was in the range of 25–30 s^{-1}, i.e., 20–30 times faster than the pulsed enzyme in TX. The steady-state level of reduced cytochrome *a* was less than 10% which also indicates a more rapid rate of internal electron transfer (Figure 4B). Kinetic modeling, using the rate of reduction of cytochrome *c* (25–40 s^{-1}) and the rate of internal electron transfer from cytochrome *a* to cytochrome a_3 (25–30 s^{-1}), once again predicts this low steady-state level for reduced cytochrome *a*. The reoxidation of reduced cytochrome *a* was so fast that accurate measurement of the rate of formation of reduced cytochrome *a* by the stopped-flow system was not possible. Results obtained with the oxygenated form of the enzyme were nearly identical with those obtained with the 420-nm pulsed form.

Steady-state levels of reduced cytochrome *c* and cytochrome *a* at the relatively flat plateau in Figure 2 were calculated by using the appropriate extinction coefficients as described under Materials and Methods. From these steady-state levels, the stoichiometries for cytochrome *a* reduction and cytochrome *c* oxidation were calculated and are summarized in Table IV.

Discussion

Antonini et al. were first to report that reoxidation of reduced cytochrome *a* is slow under aerobic conditions. The reoxidation rate is limited by the slow intramolecular electron transfer from cytochrome *a*–Cu_A to the oxygen binding site, cytochrome a_3–Cu_B (Chance, 1981; Antonini et al., 1970). In this work, we have chosen to take advantage of the slow reoxidation of cytochrome *a* to probe the effect of changing the detergent environment at the apolar surface of cytochrome *c* oxidase upon the individual electron transfers between reduced cytochrome *c* and oxygen.

The steady-state levels of reduced cytochrome *a*, the extent of the oxidation of cytochrome *c* in the burst phase (2 cytochromes *c* are oxidized for 1 cytochrome *a* reduced), and the approximately 2-fold difference between the rate of oxidation of cytochrome *c* and cytochrome *a* all suggest a stoichiometry for the initial burst phase of

$$a^{3+}\text{–}Cu_A^{2+} + 2\text{cyt } c^{2+} \rightarrow a^{2+}\text{–}Cu_A^{1+} + 2\text{cyt } c^{3+}$$

This stoichiometry implies that reduced cytochrome *a* must

Table IV: Effect of Bound Detergent upon Aerobic Reduction of Cytochrome *a* in Resting, Oxygenated, and Pulsed Oxidase[a]

detergent	type of oxidase	equiv of reduced cyt *a*[b] (444 or 606 nm)	equiv of reduced cyt *c*[c] (550 nm)
Triton X-100	resting	0.60–0.70	1.8–2.0
	oxygenated	0.60–0.65	1.8–2.0
	pulsed	0.65–0.70	1.8–2.0
lauryl maltoside	resting	0.25–0.30	2.8–3.0
	oxygenated	<0.10	3.0–3.4
	pulsed	<0.10	3.0–3.4

[a] Measured at 25 °C in 0.1 M phosphate buffer, pH 7.40. All experiments were done with [cyt *c*]:[cyt *c* oxidase] = ratio 4:1. [b] Equivalents of cytochrome *a* that became reduced in the fast phase of the aerobic reaction, e.g, Figures 2, 3B, and 4B. [c] Equivalents of cytochrome *c* that were oxidized in the fast initial phase of the aerobic reaction, e.g., Figures 3A and 4A.

be rapidly equilibrated with reduction of Cu_A (Beinert & Palmer, 1964). Morgan et al. (1989) recently reported that such an electron transfer from cytochrome *a* to Cu_A is very rapid with an apparent rate of 17 000 s^{-1} and that the equilibrium constant between cytochrome *a* and Cu_A is near unity for the CO-inhibited enzyme. However, the data that we obtained for the enzyme in LM (Table I) suggest that the equilibrium may lie toward Cu_A for the noninhibited enzyme. Under the conditions of a single turnover (cyt *c*:cyt *c* oxidase ratio = 4:1), the four reducing equivalents are partitioned into two stoichiometric, two-electron processes. The two processes are sufficiently separated in time to permit an evaluation of the second rate constant, i.e., the rate of electron transfer from the cytochrome *a*–Cu_A center to the cytochrome a_3–Cu_B center. Either the reoxidation of cytochrome *a* or the second phase of the oxidation of cytochrome *c* can be monitored, but reduction of cytochrome a_3 is not directly observable due to its extremely rapid rate of reoxidation by oxygen (Greenwood & Gibson, 1967).

Reduction and reoxidation of cytochrome *a* were clearly defined with the TX–enzyme but were more difficult to measure with the LM–enzyme due to the small steady-state reduction levels of cytochrome *a* in LM (refer to Figures 2 and 3B). With the pulsed or oxygenated LM–enzyme, internal electron transfer was so fast that steady-state levels of reduced cytochrome *a* were less than 10% (refer to Figure 4B). The very low steady-state level of reduced cytochrome *a* observed with the pulsed LM–enzyme is consistent with a much higher rate of internal electron transfer and a high steady-state turnover number for oxidase in this detergent.

The rate of cytochrome *c* oxidation after the initial burst phase also gives the intramolecular electron-transfer rate between cytochrome *a* and cytochrome a_3 unless reduced cytochrome *c* could be oxidized by a direct electron transfer to cytochrome a_3. The rates for the second phase of the oxidation of cytochrome *c* by TX– or LM–oxidase correlated well with the steady-state levels of reduced cytochrome *a* (refer to Figures 3 and 4 and Tables II and III) and indicate that the bound detergent alters the internal electron transport within oxidase.

The mechanism of the reoxidation of cytochrome *a* merits further discussion. Reoxidation of cytochrome *a* occurred concurrently with the oxidation of the remaining 2–6 equiv of reduced cytochrome *c* (the number of equivalents depends upon the initial starting conditions). This means the reduction level of cytochrome *a* reflects the reduction level of cytochrome *c* as would be predicted by the Nernst equation and their fairly close E^0 values. This observation together with the rapid burst phase reduction of the *a*–Cu_A center suggests rapid exchange

of reduced and oxidized cytochrome *c* at the binding site on oxidase and indicates neither the initial electron transfer from cytochrome *c* to cytochrome *a* nor the off-rate for oxidized cytochrome *c* is rate limiting. A secondary consequence of concurrent oxidation of cytochromes *a* and *c* is that it is not possible to maintain a constant steady-state level of reduced cytochrome *a* during aerobic experiments when cytochrome *c* is the sole reductant.

Reoxidation of the cytochrome *a*–Cu_A center involves a net two-electron transfer to O_2 at the oxygen binding site (a_3–Cu_B). It is the resulting peroxide complex that seeks the remaining 2 reducing equiv. Whether the reducing equivalents are funnelled through the primary acceptor, cytochrome *a*, or directly from cytochrome *c* to the peroxide complex is presently not known. Direct donation of electrons from cytochrome *c* to the bound peroxide at low temperature (−80 °C) has been reported (Chance et al., 1979). Hill and Greenwood (1984) also inferred such a mechanism in the reoxidation of the reduced, photolabile CO–oxidase complex in the presence of an equivalent amount of cytochrome *c*. Our experiments favor the pathway through cytochrome *a* for two reasons. First, the simultaneous first-order oxidation of both cytochromes *c* and *a* indicates an electron-transfer pathway through cytochrome *a*. Second, electron-transfer rates were slow enough within the TX–enzyme that we were able to compare the rate of oxidation of cytochrome *c* by resting, pulsed, and oxygenated (428-nm Soret) forms of oxidase (refer to Table III). In each case, the rate was nearly identical, suggesting that reduction of the H_2O_2 adduct (oxygenated form) occurs via the same pathway as the initial reduction of O_2 to H_2O_2 in the resting or pulsed forms.

Several other important conclusions can be reached from the present study and can be summarized as follows: First, neither shifts in the Soret maximum nor increased cyanide reactivity correlated well with converting the enzyme to a higher turnover form. These parameters were very similar whether we studied the TX- or LM–enzyme, but only the LM–enzyme seemed to acquire a truly "pulsed" conformation as defined by an increased rate of electron transfer. Second, the intramolecular electron-transfer rate of the resting form of the enzyme is affected to only a limited extent by changing the detergent environment; the rate of intramolecular electron transfer increased by only a factor of 5–10 as TX was replaced by either $C_{12}E_8$ or LM. This is far less than the 75–200-fold change in the steady-state turnover number as the detergent is changed. Third, the intramolecular electron-transfer rate from cytochrome *a* to cytochrome a_3 increases by an additional factor of 3–6 if the $C_{12}E_8$- or LM–enzyme is oxygenated (428 nm) or pulsed (420 nm). This increase was not seen with the TX–enzyme. Therefore, the stopped-flow measurement of changes in the internal electron transfer accounts for at least a 25–30-fold increase in the steady-state turnover number when TX is replaced by LM. The factor of 25–30 is a lower limit, and the electron-transfer rate for the LM–enzyme could be higher since we only observed the very end of a very fast reaction. The enhanced activity of cytochrome *c* oxidase in $C_{12}E_8$ or LM could be due to subtle conformational changes that can occur only if the enzyme is pulsed or oxygenated when it is surrounded by the proper hydrophobic environment. These results reinforce our earlier suggestion that oxidase is locked in an inactive conformation when TX is bound at its surface but is conformationally more flexible in $C_{12}E_8$, LM, and other detergents that support high steady-state activity (Robinson et al., 1985).

Lastly, the effect of bound detergent upon the conformational flexibility of cytochrome *c* oxidase could also explain both its reversible inactivation by TX and its nearly identical enthalpy of activation in a wide variety of detergents; i.e., an entropic, not an enthalpic, factor influences the rate of intramolecular electron transfer (Robinson et al., 1985). The simplest scheme to explain the electron-transfer properties of cytochrome *c* oxidase must then include both inactive and potentially active forms for the resting enzyme. The equilibrium between these two forms would then be affected by the detergent bound to the enzyme. Only the potentially active form, which occurs in detergents supporting high turnover numbers, could then be effectively pulsed or oxygenated to the highly active, high turnover conformation.

ACKNOWLEDGMENTS

We thank Linda Talbert for expert technical assistance.

Registry No. $C_{12}E_8$, 3055-98-9; LM, 108400-13-1; TX, 9002-93-1; cytochrome *c* oxidase, 9001-16-5; cytochrome *c*, 9007-43-6.

REFERENCES

Antalis, T. M., & Palmer, G. (1982) *J. Biol. Chem. 257*, 6194–6206.
Antonini, E., Brunori, M., Greenwood, C., & Malmstrom, B. G. (1970) *Nature 228*, 936–937.
Baker, B. M., Noguchi, M., & Palmer, G. (1987) *J. Biol. Chem. 262*, 595–604.
Beinert, H., & Palmer, G. (1964) *J. Biol. Chem. 239*, 1221–1227.
Capaldi, R. A., & Hayashi, H. (1972) *FEBS Lett. 26*, 261–263.
Chance, B. (1981) *Curr. Top. Cell. Regul. 18*, 343–360.
Chance, B., Waring, A., & Powers, L. (1979) in *Cytochrome Oxidase* (King, T. E., et al., Eds.) pp 353–360, Elsevier, New York.
Chance, B., Kumar, C., Powers, L., & Ching, Y. C. (1983) *Biophys. J. 44*, 353–363.
De Cuyper, M., & Joniau, M. (1980) *Eur. J. Biochem. 104*, 397–405.
Englander, S. W., Calhoum, D. B., & Englander, J. J. (1987) *Anal. Biochem. 161*, 300–306.
Fowler, L. R., Richardson, S. H., & Hatefi, Y. (1962) *Biochim. Biophys. Acta 64*, 170–173.
Gibson, Q. H., Greenwood, C., Wharton, D. C., & Palmer, G. (1965) *J. Biol. Chem. 240*, 888–894.
Greenwood, C., & Gibson, Q. H. (1967) *J. Biol. Chem. 242*, 1782–1787.
Griffiths, D. E., & Wharton, D. C. (1961) *J. Biol. Chem. 236*, 1850–1856.
Hartzell, C. R., Beinert, H., van Gelder, B. F., & King, T. E. (1978) *Methods Enzymol. 53*, 54–66.
Hill, B. C., & Greenwood, C. (1984) *FEBS Lett. 166*, 362–366.
Hill, B. C., & Robinson, N. C. (1986) *J. Biol. Chem. 261*, 15356–15359.
Kumar, C., Naqui, A., & Chance, B. (1984a) *J. Biol. Chem. 259*, 11668–11671.
Kumar, C., Naqui, A., & Chance, B. (1984b) *J. Biol. Chem. 259*, 2073–2074.
Morgan, J. E., Li, P. M., Jang, D.-J., El-Sayed, M. A., & Chan, S. I. (1989) *Biochemistry 28*, 6975–6983.
Naqui, A., Kumar, C., Ching, Y.-C., Powers, L., & Chance, B. (1984) *Biochemistry 23*, 6222–6227.
Nicholls, P. (1979) *Biochem. J. 183*, 519–529.

Okunuki, K., & Sekuzu, I. (1954) *Seitai no Kagaku 5*, 265-272.
Robinson, N. C., & Capaldi, R. A. (1977) *Biochemistry 16*, 375-380.
Robinson, N. C., Neumann, J., & Wiginton, D. (1985) *Biochemistry 24*, 6298-6304.
Rosevear, P., VanAken, T., Baxter, J., & Ferguson-Miller, S. (1980) *Biochemistry 19*, 4108-4115.
Sinjorgo, K. M. C., Meijling, J. H., & Muijsers, A. O. (1984) *Biochim. Biophys. Acta 767*, 48-56.
Thompson, D. A., & Ferguson-Miller, S. (1983) *Biochemistry 22*, 3178-3187.
Tonomura, B., Nakatani, H., Ohnishi, M., Yamaguchi-Ito, J., & Hiromi, K. (1978) *Anal. Biochem. 84*, 370-383.
van Buuren, K. J. H., Zuurendonk, P. F., van Gelder, B. F., & Muijsers, A. O. (1972) *Biochim. Biophys. Acta 256*, 243-257.
van Gelder, B. F. (1978) *Methods Enzymol. 53*, 125-128.
Vik, S., & Capaldi, R. A (1980) *Biochem. Biophys. Res. Commun. 94*, 348-353.
Yonetani, T. (1960) *J. Biol. Chem. 235*, 845-852.

Observation of a Kinetic Slow Transition in Monomeric Glucokinase†

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Received May 30, 1989; Revised Manuscript Received August 31, 1989

ABSTRACT: Rat liver glucokinase (EC 2.7.1.2) is a monomeric enzyme with positive cooperativity for glucose phosphorylation for which several kinetic mechanisms have been proposed. We have observed a slow kinetic transition when the enzyme is assayed in the presence of 30% glycerol. When the enzyme had been preincubated or stored in 50 mM glucose, the initially rapid activity decayed, via a first-order process, to a new steady-state velocity. The glucose-induced process is reversible since if the enzyme is preincubated without glucose, an initially low activity accelerates over minutes to the same steady-state velocity. This final velocity is independent of the preincubation conditions and is determined solely by the glucose and ATP concentrations in the assay. Possible artifacts which might cause nonlinear progress curves have been ruled out. The transition has a half-time of 2-10 min depending on glucose and ATP concentrations and temperature. In the steady-state kinetics, positive cooperativity occurs with glucose with a Hill coefficient (n_H) = 1.3 at high ATP concentrations, approaching unity as the ATP concentration decreases. This pattern is similar to that seen in the linear velocities in the absence of glycerol. Similarly, negative cooperativity with MgATP is seen in the steady-state velocities at low glucose concentrations with the Hill coefficient approaching 1 as the glucose concentrations approach saturation. The initial velocity for enzyme preincubated in high glucose concentration was either Michaelis-Menten as a function of glucose at high MgATP concentration or heterogeneous ($n_H < 1$, negatively cooperative) at low MgATP concentration. Preincubation with the competitive inhibitor *N*-acetylglucosamine eliminated the cooperativity in the absence or in the presence of glycerol and also eliminated the slow transition, presumably by preferentially binding to one of the active conformations of glucokinase. Other monosaccharide substrates had individualized transition times and cooperativity patterns. We propose that this hysteretic behavior observable in glycerol is due to a conformational change and is the molecular basis for the kinetic cooperativity with the monomeric enzyme, glucokinase, under normal assay conditions. Our data require two catalytic cycles and thus support the slow transition model [Neet, K. E., & Ainslie, G. R., Jr. (1980) *Methods Enzymol. 64*, 192-226; Cardenas, M. L., Rabajille, E., & Niemeyer, H. (1984) *Eur. J. Biochem. 145*, 163-171] that is consistent with the known kinetic and physical properties of glucokinase.

Glucokinase (EC 2.7.1.2) is a hepatic enzyme which participates in the short-term regulation of blood glucose levels. Evidence from several laboratories (Storer & Cornish-Bowden, 1977; Cardenas et al., 1979) has shown that glucokinase kinetics are positively cooperative with glucose. Functionally, this cooperativity (Hill coefficient = 1.5) probably allows glucokinase activity to have an increased sensitivity to fluctuating blood glucose levels in the physiological range (Bontemps et al., 1978). Mechanistically, however, it is difficult to explain this cooperativity in classical terms, since several studies have shown glucokinase to be a 50000-dalton monomer (Holroyde et al., 1976; Cardenas et al., 1978) with only a single glucose binding site and no evidence of oligomerization.

Ligand-induced slow transitions (Ainslie et al., 1972) and mnemonic mechanisms (Ricard et al., 1974) have been utilized to explain possible kinetic cooperativity in monomeric enzymes. Hysteretic enzymes (Frieden, 1970) that display a slow transient during assay have been related to this kinetic cooperativity through the mechanism of Ainslie et al. (1972). The basic assumptions of either a mnemonic or a ligand-induced slow transition mechanism in a monomeric enzyme are the existence of at least two kinetically distinct conformational

† This investigation was supported in part by National Institutes of Health Grant AM12881 and by the Diabetes Association of Greater Cleveland. A portion of this work is taken from a thesis submitted by P.S.T. to Case Western Reserve University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
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0006-2960/90/0429-770$02.50/0