Effect of Subunit III Removal on Control of Cytochrome *c* Oxidase Activity by pH†

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ABSTRACT: Studies were undertaken to assess the postulated involvement of subunit III in the proton-linked functions of cytochrome *c* oxidase. The effect of pH on the steady-state II-containing parameters of subunit III containing and subunit III depleted cytochrome oxidase was determined by using beef heart and rat liver enzymes reconstituted into phospholipid vesicles. The TN_{max} and K_m values for the III-containing enzyme increase with decreasing pH in a manner quantitatively similar to that reported by Thornstrom et al. [(1984) *Chem. Scr. 24*, 230–235], giving three apparent pK_a values of <5.0, 6.2, and 7.8. The maximal activities of the subunit III depleted enzymes (beef heart and rat liver) show a similar dependence on pH, but the K_m values are consistently higher than those of the III-containing enzyme, an effect that is accentuated at low pH. The pH dependence of TN_{max}/K_m for both forms of the enzyme (±subunit III) indicates that protonation of a group with an apparent pK_a of 5.7 lowers the affinity for substrate (cytochrome *c*) independently of a continued increase in maximal velocity. *N,N'*-Dicyclohexylcarbodiimide (DCCD) decreases the pH responsiveness of the electron-transfer activity to the same extent in both III-containing and III-depleted enzymes, indicating that this effect is mediated by a peptide other than subunit III. Control of intramolecular electron transfer by a transmembrane pH gradient (or alkaline intravesicular pH) is shown to occur in cytochrome oxidase vesicles with cytochrome *c* as the electron donor, in agreement with results of Moroney et al. [(1984) *Biochemistry 23*, 4991–4997] using hexaammineruthenium(II) as the reductant. Subunit III removal does not alter this effect, and DCCD treatment inhibits the response of subunit III containing and III-depleted enzymes to a transmembrane pH gradient in a similar manner. These studies demonstrate that subunit III is not involved in the pH control of electron-transfer activity of cytochrome oxidase.

Hydrogen ions participate directly in the catalytic functions of cytochrome *c* oxidase (EC 1.9.3.1). Protons are consumed in the reduction of dioxygen to water, while in a coupled reaction the enzyme translocates protons across the membrane in which it is incorporated (Wikstrom, 1977). In order to clarify the mechanism of these reactions, it is important to define the nature and extent of proton modulation of the enzyme's measurable parameters. Steady-state electron-transfer activity is highly pH dependent, exhibiting increasing turnover rates as pH decreases (Wilms et al., 1980). The availability of hydrogen ions for the reduction of oxygen intermediates in highly buffered solutions does not appear to be rate limiting (Lindskog & Coleman, 1973). However, analysis of steady-state kinetic data suggests that proton binding steps may be required for intramolecular electron transfer and, in turn, may be involved in the proton translocation mechanism (Thornstrom et al., 1984). Transmembrane pH gradients have been shown to regulate the level of steady-state reduction of cytochrome *a* in the enzyme, also suggesting that pH controls the rate of electron transfer from this center to cytochrome a_3 (Moroney et al., 1984). The location of the proton binding sites that mediate this pH-dependent behavior has not been determined.

Subunit III, a membrane-spanning component of cytochrome oxidase, has been implicated in proton translocation [see review by Prochaska and Fink (1987)] and in regulation of intramolecular electron transfer by pH (Moroney et al., 1984), activities that may be mechanistically related. The role of this peptide in the former process appears to be facilitative rather than essential, based on retention of up to 50% of proton translocating activity following its removal (Thompson et al., 1985; Puettner et al., 1985; Finel & Wikstrom, 1986; Gregory et al., 1987). It is possible that subunit III provides a link necessary for the efficient coupling of electron transfer to proton translocation, such as a proton conducting channel. Indeed, under conditions where *N,N'*-dicyclohexylcarbodiimide (DCCD)[1] causes substantial inhibition of proton translocating activity, subunit III shows the highest level of modification (Casey et al., 1980; Prochaska et al., 1981). At similar levels, DCCD also causes increased steady-state reduction of cytochrome *a* and decreased regulation by pH, suggesting that covalent modification of subunit III by DCCD interferes with a proton binding site involved in control of intramolecular electron transfer (Moroney et al., 1984).

We removed subunit III from cytochrome oxidase and investigated the effects of its depletion on pH-modulated characteristics of cytochrome oxidase: kinetic parameters, absorbance spectra, reduction of cytochrome *a*, and DCCD inhibition. Subunit III depleted oxidase retains a similar sensitivity to pH with respect to its maximal velocity and the steady-state reduction of cytochrome *a*, demonstrating that

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[1] Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; H^+/e^-, ratio of protons translocated per electron transferred by cytochrome oxidase; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; RCR, respiratory control ratio; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; Δ pH, transmembrane pH gradient; $\Delta\psi$, transmembrane electrical gradient.

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subunit III is not the site of regulation by pH.

Materials and Methods

Materials. Asolectin (Associated Concentrates, Long Island, NY) was repurified as described by Sone et al. (1977) using two precipitation cycles and dithiothreitol rather than butyrated hydroxytoluene as a reducing agent. The following chemicals were obtained from the sources indicated: DCCD (Pierce Chemical Co., Rockford, IL); acrylamide (Serva, Westbury, NY); Acrylaide and GelBond PAG film (FMC Bioproducts, Rockland, ME). Sources of all other materials were listed by Thompson and Ferguson-Miller (1983).

Cytochrome Oxidase Preparations. Subunit III containing cytochrome oxidase was prepared from beef heart according to Suarez et al. (1984) omitting the ethanol wash step. The resultant purified protein can be reconstituted to give good respiratory control ratios[2] [RCR = 5.9 ± 0.7 (21 determinations)] and good ratios for proton translocation [H^+/e^- = 0.8 ± 0.1 (12 determinations)]. This preparation is considered the control enzyme in the following studies. Subunit III depleted cytochrome oxidase (less than 2% subunit III; Thompson et al., 1985) was prepared from rat liver by using cytochrome *c* affinity chromatography in lauryl maltoside as described by Thompson and Ferguson-Miller (1983). The reconstituted enzyme exhibits excellent respiratory control [RCR = 13.0 ± 3.1 (21)], and its proton translocating activity [H^+/e^- = 0.4 ± 0.1 (8)] is 50% of that demonstrated by the subunit III containing control.

SDS–PAGE. Subunit composition of the oxidase preparations was evaluated by SDS–PAGE using a modification of the procedure described by Merle and Kadenbach (1980). The gels were cross-linked with 0.38% Acrylaide instead of 0.56% methylenebis(acrylamide) and attached to GelBond PAG film. This modification enhanced separation between subunits II and III. Sample treatment was as described by Thompson and Ferguson-Miller (1983) except that β-mercaptoethanol was omitted from the dissociation buffer.

Reconstitution. Cytochrome oxidase was reconstituted into phospholipid vesicles by using a modification of the cholate dialysis procedure described by Casey et al. (1979). Asolectin (40 mg/mL) was sonicated for 1 min/mL in 2% cholate and 75 mM K-Hepes, pH 7.4. Oxidase was added at a ratio of 0.1 nmol of enzyme/mg of phospholipid, and the suspension was dialyzed for 40 h into a final buffer of 25 mM K-Hepes, pH 7.4, 40 mM KCl, and 20 mM sucrose (kinetic studies) or into 10 mM K-Hepes, pH 7.4, 41 mM KCl, and 38 mM sucrose (proton translocation and cytochrome *a* reduction studies). After reconstitution, the fraction of oxidase accessible to cytochrome *c* was determined (usually between 80 and 90%) by comparing the absorbance at 605 nm in the absence and presence of permeable reductant (2.3 μM TMPD), and enzyme activities were corrected accordingly.

pH Dependence of Cytochrome Oxidase Activity. The uncoupled electron-transfer activities of the reconstituted enzymes (containing 1.1 μM valinomycin and 5.7 μM CCCP) were measured by using a Gilson oxygraph (Model 5\6H; Middleton, WI) at 25 °C. The high ionic strength (I = 0.55 M) conditions used by Thornstrom et al. (1984) were modified for polarographic analysis by adding ascorbate (2.9 mM) and TMPD (0.6 mM) to maintain complete reduction. All buffers were prepared so that ionic strength was constant over the entire pH range. Before use, repurified cytochrome *c* was reduced with sodium dithionite and chromatographed on a Sephadex G-50-40 (Pharmacia, Uppsala, Sweden) column in 50 mM K_2SO_4/10 mM MES, pH 6.0, for the pH 5.0–6.5 assays or in 50 mM K_2SO_4/10 mM Hepes, pH 7.6, for the pH 7.0–8.5 assays.

Spectrophotometric assays of electron-transfer activity were performed according to the conditions of Thornstrom and co-workers using a 0.5-cm path-length cell with a Perkin-Elmer Lambda 4B spectrophotometer. After addition of cytochrome oxidase vesicles, enzyme concentration was 5.5 nM in the pH 7.5 assays and 9.3 nM in the pH 8.5 assays. Initial rates were calculated by using the integral method described by Yonetani and Ray (1965) and then divided by enzyme concentration to give turnover rates.

Kinetic parameters and their standard deviations were estimated by linear regression analysis with the computer program WILMAN4 (Brooks & Suelter, 1986) using the intermediate weighting option.

Steady-State Reduction Studies. Reduction of cytochrome *a* under steady-state conditions was recorded with an Aminco DW2a spectrophotometer using cells of 1-cm path length. All assays were performed at 18 °C in 10 mM K-Hepes, pH 7.4, 41 mM KCl, and 38 mM sucrose. Examination of steady-state spectra of cytochrome oxidase vesicles revealed that when a transmembrane electrical gradient was present, oxidized TMPD (Wurster's blue) electrophoretically migrated to the vesicle interiors and contributed to the absorbance (Gregory and Ferguson-Miller, unpublished experiments). Spectra of coupled and pH-equilibrated states were corrected in proportion to the anomalous absorbance at 630 nm. Complete reduction of cytochrome oxidase and cytochrome *c* was determined approximately 5 min after anaerobiosis. Cytochrome *a* was assumed to contribute 80% of the absorbance at 605 nm (from a base line drawn from 650 to 565 nm) upon complete reduction of the enzyme [reviewed in Wikstrom et al. (1981)] and 100% of the absorbance under steady-state conditions where cytochrome a_3 is totally oxidized (Gibson et al., 1965). The absorbance at 550 nm from a base line drawn from 565 to 540 nm was used to measure the reduction levels of cytochrome *c*.

Miscellaneous Procedures. Respiratory control ratios (Thompson & Ferguson-Miller, 1983) were determined in buffers identical with the final dialysis solution. Proton translocating activity was measured as described by Thompson et al. (1985) with the following exceptions: (1) the assay buffer was composed of 50 μM K-Hepes, pH 7.4, 45 mM KCl, 50 mM sucrose, and 2.5 $MgCl_2$; (2) assay temperature was 22 °C; and (3) 0.24 nM CCCP was present in the acidification phase.

Results

pH Dependence of Kinetic Parameters of Reconstituted Cytochrome Oxidase with and without Subunit III. The effect of pH on the kinetics of steady-state electron-transfer activity in reconstituted cytochrome oxidase with and without subunit III was studied at high ionic strength (I = 0.5 M) in the presence of uncouplers, conditions similar to those described by Thornstrom et al. (1984). Reconstitution into lipid vesicles stabilizes the enzyme at lower pH values (Gregory & Ferguson-Miller, 1988) and eliminates any complicating effects of differing lipid contents of the isolated enzymes. However, vesicle aggregation at low pH appeared to be a problem which necessitated the use of high ionic strength in order to obtain reproducible data.

To determine the assay method most advantageous for this study, kinetic parameters of subunit III containing cytochrome

[2] For RCR and H^+/e^- ratios, the mean values are quoted ± the standard deviation. The number in parentheses indicates the number of experimental determinations.

Table I: Comparison of Steady-State Kinetic Parameters Obtained by Spectrophotometric and Polarographic Assays[a]

assay	pH 7.5		pH 8.5	
	TN_{max} (s^{-1})	K_m (μM)	TN_{max} (s^{-1})	K_m (μM)
spectrophotometric[b]	280 ± 19[c]	60 ± 7	48 ± 5	25 ± 5
polarographic	295 ± 7	82 ± 4	57 ± 2	22 ± 2

[a] Results for reconstituted subunit III containing cytochrome oxidase. [b] Assay methods are described under Materials and Methods. [c] Kinetic parameters and their standard deviations were calculated by using the computer program WILMAN4 (Brooks & Suelter, 1986).

oxidase were obtained at several pHs using spectrophotometric and polarographic assays and then compared (Table I). The maximal activity (TN_{max}) and apparent K_m values at pH 7.5 and 8.5 calculated from polarographic data were found to be very similar to those computed from spectral data at corresponding pHs. At low pH, however, very high concentrations of cytochrome *c* (at least 400 μM) are needed to obtain good estimates of kinetic parameters due to the high K_m values for cytochrome *c* under acidic conditions. The resulting high absorbances interfere with spectrophotometric activity determinations, making the data relatively inaccurate, whereas the polarographic assay is not limited by high substrate concentrations and thus offers a significant advantage for accurately assessing the kinetic constants over a wide pH range. Addressing the reservations expressed concerning the use of the polarographic assay in this type of study (Smith et al., 1979; Wilms et al., 1980; Thornstrom et al., 1984), we found that cytochrome *c* remained greater than 97% reduced under the high salt conditions used. Therefore, on the basis of its superior accuracy at low pH and good agreement with the spectral method, the polarographic assay was deemed more desirable for investigation of pH dependence. (See Discussion for further consideration of apparent discrepancies between these assay systems.)

To determine the pH range over which the enzyme was stable, the reconstituted enzymes were incubated in buffers ranging from pH 4.5 to 8.5 for 10 min and then assayed for activity at pH 7.4 (Gregory & Ferguson-Miller, 1988). Each enzyme tested retained greater than 85% of its activity after incubation at pHs 5.0 through 8.5, establishing the pH range used in subsequent studies. At pH 4.5, significant inactivation occurred.

When the kinetic data at various pHs are visualized by double-reciprocal plots (Figure 1), all forms of the oxidase (beef heart, rat liver, ±subunit III) display qualitatively similar patterns. Under the high ionic strength conditions of these assays, the plots at all pHs were monophasic, probably representing the interaction of cytochrome *c* at only the high-affinity site of the enzyme (Sinjorgo et al., 1986). Data obtained at pH 5.0 through pH 6.0 form a series of plots of decreasing slopes while data from pH 6.5 to 8.5 describe a series of nearly parallel plots. This phenomenon results in nonlinear log (TN_{max}/K_m) versus pH plots (see Discussion; Figure 7B).

For all reconstituted oxidase preparations tested, TN_{max} (Figure 2A) and K_m values (Figure 2B) for uncoupled electron-transfer activity increase continuously with decreasing pH, as observed by Thornstrom et al. (1984) using the reconstituted oxidase and previously by Wilms et al. (1980) using detergent-solubilized enzyme. The degree of stimulation of activity at the lowest pH (5.0) was independent of the presence of subunit III, but the activity of the subunit III depleted enzyme decreased more dramatically as the pH increased from 5.0 to 5.5 and was consistently lower than the activity of the III-containing enzyme from pH 5.5 to 8.5. In contrast, K_m

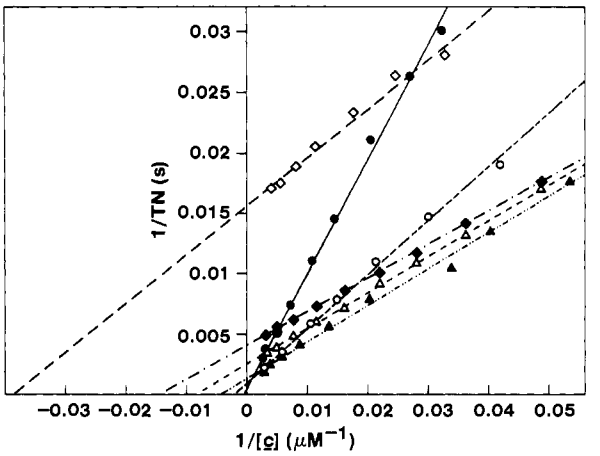

FIGURE 1: Double-reciprocal plots showing steady-state uncoupled activity of reconstituted subunit III containing cytochrome oxidase (beef heart) as a function of cytochrome *c* concentrations at various pH values. Rates of oxygen consumption were measured as described under Materials and Methods using cytochrome oxidase vesicles (0.015–0.03 nmol of aa_3) in 1.75 mL of 167 mM K_2SO_4 and 50 mM MES for pH 5.0–6.5 or 50 mM Hepes for pH 7.0–8.5. The reaction mixture contained 2.9 mM ascorbate, 0.6 mM TMPD, 1.1 μM valinomycin, and 5.7 μM CCCP. Cytochrome *c* concentration ranged from 18 to 410 μM. pH 5.0 (●); pH 5.5 (○); pH 6.0 (▲); pH 7.0 (△); pH 7.5 (♦); and pH 8.5 (◇).

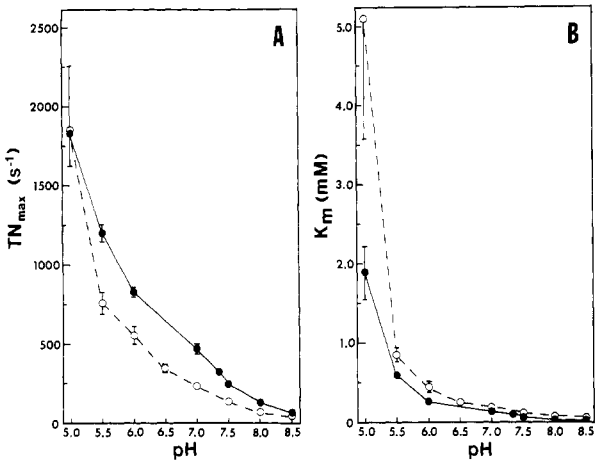

FIGURE 2: pH dependence of (A) maximal activity (TN_{max}) and (B) K_m for reconstituted cytochrome oxidase [subunit III containing beef heart enzyme (●); subunit III depleted rat liver enzyme (○)]. Kinetic parameters and standard deviations were estimated by linear regression analysis.

values are higher for the subunit III depleted oxidase when compared to the complete enzymes, an effect that is accentuated at pH 5.0. The plot of log (TN_{max}/K_m) (Figure 7B) is similar for the two forms of the oxidase (±subunit III) but shifted down by 0.5 log unit for the III-depleted enzyme as a result of its lower TN_{max} and higher K_m values.

Kinetic studies at lower ionic strength with the different forms of the enzyme in reconstituted or soluble states show biphasic kinetics typical of cytochrome oxidase (Ferguson-Miller et al., 1976). Interestingly, while the K_m values are dramatically different (30–40-fold greater at I = 0.5 M compared to I = 0.07 M), the maximal activities are very similar (within 15%) for a particular pH. This observation is in agreement with the findings of Wilms et al. (1980) showing that at a single pH, TN_{max} is unaffected by increases in ionic strength.

Effect of pH on the Spectral Properties of Cytochrome Oxidase with and without Subunit III. The possibility was considered that the increase in TN_{max} at low pH might be due

Table II: Effect of Varying Subunit III Content on the Kinetic Parameters of Cytochrome Oxidase

tissue source	subunit III content (%)	TN_{max} (s^{-1}) pH 5.0	TN_{max} (s^{-1}) pH 7.4	K_m (μM) pH 5.0	K_m (μM) pH 7.4
beef heart	100	1900[a] (±150)	320 (±4)	2100 (±200)	100 (±3)
	39	1900 (±400)	200 (±4)	3700 (±800)	140 (±5)
	23	1900 (±300)	120 (±2)	6000 (±900)	120 (±4)
rat liver	<2	1800 (±400)	130 (±4)	5100 (±1500)	120 (±10)

[a] Kinetic parameters and their standard deviations were calculated by using the computer program WILMAN4 (Brooks & Suelter, 1986).

to an alteration in the redox potential of one of the metal centers. A candidate was suggested by the observations of Callahan et al.[3] which indicate that low pH disrupts a hydrogen bond between the protein and the formyl group of the heme a^{2+} porphyrin ring, resulting in a blue shift of the Soret and α-peaks and a predicted lower redox potential of heme *a*. When we compared the absorbance maximum for the α-peak of reduced, reconstituted oxidase at pH 7.4 to that of the same enzyme exposed to pH 5.0 for 1 min, we also found a small blue shift (from 605 to 604 nm) that increased to 3 nm after 5 min. These shifts were minimal compared to those seen with the soluble enzyme (immediate shifts of 4 nm at pH 5.5 and 10 nm at pH 4.5), and the presence or absence of subunit III had no effect. In addition, the results were independent of the presence or absence of uncouplers which equilibrate pH across the vesicle membrane. It is evident that the lipid environment of the vesicle affords the enzyme a large degree of protection against the pH effect observed by Callahan and co-workers. This suggests that the increase in maximal activity at low pH, measured during the first minute, is not the result of loss of this protein to heme *a* hydrogen bond. It is interesting to note that the α-peak of the reconstituted subunit III depleted oxidase (603 nm) is already blue shifted 1–2 nm when compared to subunit III containing oxidase, in agreement with findings of Penttila (1983) and Puettner et al. (1985) for the Triton X-100 solubilized oxidase depleted of subunit III. However, the III-depleted enzyme in lauryl maltoside does not show a blue shift, and resonance Raman analysis reveals that the formyl hydrogen bond is still intact (Babcock et al., 1981). Thus, it appears that the lipid environment not only protects against extremes of pH but also stabilizes a different conformation of the enzyme than that seen in lauryl maltoside. Further studies are under way to establish the significance of this effect.

Comparison of Beef Heart and Rat Liver Oxidases Depleted of Subunit III. In the studies discussed above, subunit III depleted oxidase originated from rat liver while subunit III containing oxidase was prepared from beef heart. Buge and Kadenbach (1986) have proposed that tissue isozymes of cytochrome oxidase demonstrate kinetic differences [however, see Sinjorgo et al. (1987)]. To examine the possibility that the observed differences were related to species or tissue variations rather than subunit III content, the beef heart enzyme was depleted of subunit III using lauryl maltoside and affinity chromatography as described by Hill and Robinson (1986). Different extents of removal were achieved by varying detergent and salt concentrations during treatment. As subunit III content decreased, respiratory control ratios increased accompanied by some reduction in proton translocating ability (Figure 3), characteristics similar to those exhibited by subunit III depleted oxidase from rat liver. Under all conditions tested, the kinetic parameters of the highly III-depleted beef heart enzyme closely resembled the III-depleted rat liver oxidase.

[3] P. M. Callahan, J. Centeno, R. T. Ingle, and G. T. Babcock, unpublished experiments.

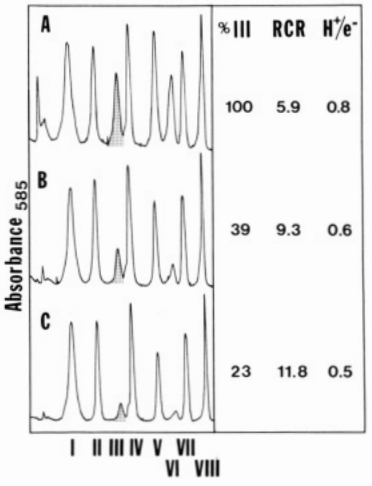

FIGURE 3: Densitometric scans of Coomassie blue stained SDS–polyacrylamide gels for beef heart cytochrome oxidase before and after treatment to remove subunit III. (A) Untreated subunit III containing oxidase. (B) Oxidase after a 24-h incubation in 1% lauryl maltoside (9 mg/mg of protein) and 0.5 M NaCl followed by cytochrome *c* affinity chromatography. (C) Oxidase after a 24-h incubation in 1.9% lauryl maltoside (15 mg/mg of protein) and 1 M NaCl followed by affinity chromatography. Ranges of values obtained for the functional properties of these enzymes are as follows: (A) RCR = 4.2–7.0, H^+/e^- = 0.7–0.9 (using two to four turnovers); (B) RCR = 9.2–9.4, H^+/e^- = 0.5–0.6 (using five turnovers); (C) RCR = 9.4–14.0, H^+/e^- = 0.5 (using four turnovers).

Kinetic studies of reconstituted beef heart preparations of varying subunit III content were carried out at pH 5.0 and 7.4 (Figure 4). At pH 5.0, decreasing levels of subunit III resulted in increases in K_m values without changes in maximal activity, while at pH 7.4, the K_m was only slightly affected by removal of subunit III whereas maximal activity decreased significantly. The effect of subunit III removal on V_{max} at pH 7.4 is consistent with the observed difference in activity between the beef heart control oxidase and the subunit III depleted rat liver enzyme. Our results indicate that the lower activity of the rat liver oxidase at higher pH, as well as its higher K_m at low pH, is related to subunit III removal rather than species- or tissue-specific differences. The values of the kinetic constants for the different forms of beef heart oxidase are summarized and compared to the III-depleted rat liver enzyme in Table II.

Effect of DCCD on the Kinetic Parameters of Cytochrome Oxidase with and without Subunit III. The effects of DCCD treatment on the kinetics of cytochrome oxidase were examined to determine whether glutamate-90 of subunit III, which is highly modified by DCCD and postulated to have a role in proton translocation (Casey et al., 1980; Prochaska et al., 1981), could be implicated in the observed pH-dependent kinetic differences between the III-containing and III-depleted oxidases. The reconstituted enzymes were treated with a 300 molar excess of DCCD using the method of Prochaska et al. (1981). Upon DCCD modification, the two enzymes (±subunit III) showed similar changes in kinetics at pH 5.0 and 7.4 (Table III). Each exhibited significant losses in activity at

Table III: Effect of DCCD on Kinetic Parameters of Reconstituted Cytochrome Oxidase with and without Subunit III

enzyme	treatment	RCR	TN_{max} (s^{-1}) pH 5.0	TN_{max} (s^{-1}) pH 7.4	K_m (μM) pH 5.0	K_m (μM) pH 7.4
aa_3(+III)	control[a]	6.3	2200[c] (±400)	330 (±10)	2200 (±500)	100 (±6)
	DCCD[b]	6.8	1200 (±150)	250 (±4)	1500 (±200)	100 (±4)
aa_3(−III)	control	13.4	1300 (±400)	150 (±10)	5000 (±1500)	200 (±30)
	DCCD	7.3	600 (±100)	110 (±5)	3200 (±700)	170 (±10)

[a] Control for DCCD treatment using the solvent (ethanol) only. [b] Treated with 300 mol of DCCD/mol of oxidase according to Prochaska et al. (1981). [c] Kinetic parameters and their standard deviations were calculated by using the computer program WILMAN4 (Brooks & Suelter, 1986).

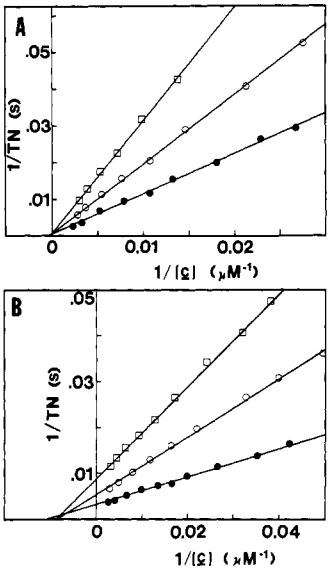

FIGURE 4: Double-reciprocal plots for reconstituted beef heart cytochrome oxidase of varying subunit III content at (A) pH 5.0 and (B) pH 7.4. Activities were assayed as described in Figure 1. Untreated enzyme (100% subunit III) (●); oxidase with 39% subunit III (○); oxidase with 23% subunit III (□). Kinetic parameters and standard deviations are listed in Table II.

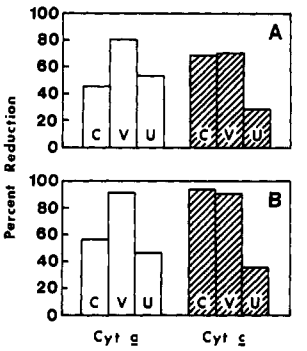

FIGURE 5: Levels of reduction of cytochrome *a* (open bars) and cytochrome *c* (hatched bars) were determined from difference spectra (steady-state minus oxidized) as described under Materials and Methods. (A) Subunit III containing oxidase from beef heart. (B) Subunit III depleted oxidase from rat liver (<2% III). Ascorbate (12 mM) and TMPD (0.3 mM) were added to maintain steady-state activity of 0.6 μM vesicular cytochrome oxidase in the presence of 2 μM cytochrome *c*. Reduction levels are presented for: (C) coupled activity; (V) activity after electrical equilibration by the addition of valinomycin (4 μM); and (U) uncoupled activity after subsequent addition of CCCP (20 μM).

pH 5.0 (approximately 50%) with lesser inhibition at pH 7.4 (25%), suggesting a loss of sensitivity to pH stimulation. The results show that the kinetic alterations and attenuation of pH effects resulting from DCCD treatment cannot be attributed to modification of subunit III.

Effect of Subunit III Content on the Control of Steady-State Reduction Levels of Cytochrome a. In studies of the effects of ΔpH and $\Delta\psi$ on the electron-transfer activity of reconstituted cytochrome oxidase, Moroney et al. (1984) concluded that the pH on the matrix side of the membrane regulates the steady-state level of reduced cytochrome *a*, in agreement with Artzatbanov et al. (1978). Using hexaammineruthenium(II) as an electron donor, Moroney and co-workers found that cytochrome *a* was about 80% reduced in the coupled steady state, and this level remained unchanged when valinomycin was added to abolish $\Delta\psi$. However, when nigericin or permeable acid was added to diminish the Δ pH, the level of reduction dropped significantly with little change in the rate of respiration. Treatment with DCCD lessened the sensitivity of the redox state of cytochrome *a* to additions of proton equilibrating agents. From these observations, the authors proposed a proton-limited step between cytochromes *a* and a_3 and an involvement of subunit III in mediating the regulation.

To test this possibility, we examined the behavior of reconstituted cytochrome oxidase with and without subunit III under coupled and uncoupled steady-state conditions. Using low levels of cytochrome *c* in conjunction with ascorbate and TMPD as reductants (Wrigglesworth & Nicholls, 1978), we measured the relative changes in levels of reduction of cytochrome *a* and cytochrome *c* upon sequential additions of valinomycin and the uncoupling agent CCCP. The two forms of the enzyme (±subunit III) gave similar responses to perturbations of the electrical and pH components of the gradient. Figure 5 shows the results of a typical experiment. We find that for a particular vesicle preparation, results agree within 10% upon duplication but that the sizes of the changes observed in different vesicle preparations are dependent on the respiratory control ratio (Gregory & Ferguson-Miller, 1989). In general, the results were in agreement with the studies of Moroney and co-workers, with the exception that our steady-state spectra showed that cytochrome *a* was less reduced in the coupled state (40–60% versus 80%) and that levels of reduction increased to 70–90% upon addition of valinomycin, concomitant with a 30–50% increase in oxygen consumption rates. Cytochrome *c* remained 70–90% reduced under these conditions and changed no more than 10% upon the addition of valinomycin. Our results indicate that elimination of the electrical gradient releases the control of electron transfer to cytochrome *a* and suggest that cytochrome *a* reduction by cytochrome *c* (unlike the artificial substrate hexaammineruthenium) is limited by $\Delta\psi$ (Hinkle & Mitchell, 1970; Gregory & Ferguson-Miller, 1989). With subsequent additions of a protonophore, CCCP or nigericin, abolishing the pH component of the membrane potential, oxygen consumption was stimulated 3–8-fold (accompanied by oxidation of cytochrome *c*), and cytochrome *a* became more oxidized again, indicating a release of control on electron transfer within the enzyme. The behavior of cytochrome *a* is in agreement with the observations of Moroney and co-workers, suggesting that electron transfer from cytochrome *a* to a_3 is regulated by Δ pH, but our results do not indicate a role for subunit III in this regulation.

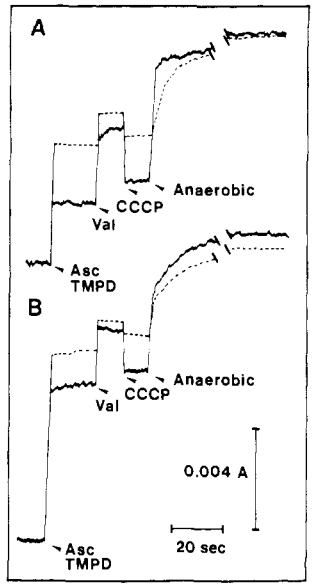

FIGURE 6: Effect of DCCD treatment on the steady-state level of reduced cytochrome *a* (monitored at 605–630 nm) in the presence and absence of ionophores. Cytochrome oxidase vesicles (0.5–0.6 nmol of aa_3) were added to 0.85 mL of buffer containing 3.5–5.5 μM cytochrome *c*. Ascorbate (12.5 mM) and TMPD (0.2 mM) were added to maintain steady-state activity (coupled). Valinomycin (Val; 4 μM) was added to abolish $\Delta\psi$ followed by CCCP (20 μM) to equilibrate the ΔpH. Complete reduction was measured 5 min after the system became anaerobic. (A) Reconstituted subunit III depleted oxidase (rat liver). (B) Reconstituted subunit III containing oxidase (beef heart). Solid traces from untreated vesicles; dashed traces from DCCD-treated vesicles (1400 mol of DCCD/mol of oxidase).

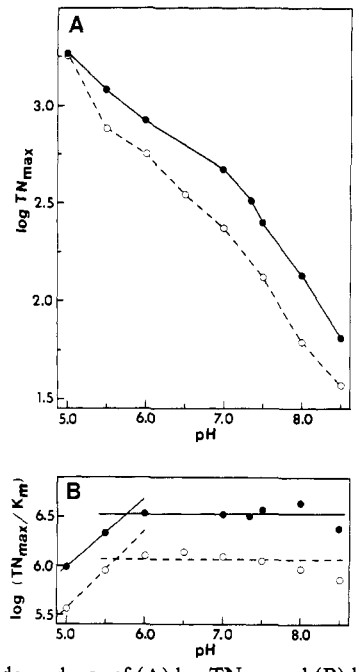

FIGURE 7: pH dependence of (A) log TN_{max} and (B) log (TN_{max}/K_m) using data from Figure 2. Subunit III containing oxidase from beef heart (control) (●); subunit III depleted oxidase from rat liver (○).

To further investigate the possibility of subunit III involvement in control of oxidase activity, the reconstituted enzyme with and without subunit III was treated with DCCD over a range of 0–1900 mol of DCCD per mole of oxidase. Both preparations showed loss of activity with increasing DCCD, but the subunit III depleted oxidase was somewhat more affected. Respiratory control ratios (RCR) of subunit III containing oxidase were relatively unaffected by the treatment, while those of the subunit III depleted enzyme dropped from 15 to 10 after treatment with a 1770-fold excess of DCCD (data not shown). Examination of the steady-state levels of reduction of cytochrome *a*, measured by the absorbance at 605 minus 630 nm (Figure 6), revealed that DCCD treatment caused increased reduction of cytochrome *a* in all phases (coupled, electrically equilibrated, and pH equilibrated). As observed for the loss in activity [also noted by Prochaska et al. (1980)], the increase in reduction was dependent on the concentration of DCCD used for the modification. This may indicate that both phenomena are due to increasing modification of a residue essential for intramolecular electron transfer. It is also apparent that the redox state of cytochrome *a* becomes less responsive to changes in the $\Delta\psi$ and Δ pH following DCCD treatment. These effects are observed in the presence or absence of subunit III and indicate that these DCCD-induced changes are not caused by modification of subunit III.

DISCUSSION

pH Dependence of Steady-State Kinetic Parameters. Many studies have demonstrated that the electron-transfer activity of cytochrome oxidase is pH dependent. Although pH optima have been reported by a number of investigators (Yonetani, 1961; Davies et al., 1964; Ferguson-Miller et al., 1976; Maurel et al., 1978), more recent studies indicate that maximal activity increases continuously with decreasing pH, exhibiting no pH optimum (Wilms et al., 1980; Thornstrom et al., 1984; Sinjorgo et al., 1986). The observation of an optimal pH can be attributed to one or more of the following: (1) the use of only one substrate concentration over the pH range without compensation for increases in K_m with decreasing pH; (2) instability of the enzyme preparation at pH extremes; (3) inability to achieve the high substrate concentrations needed to obtain the most accurate kinetic parameters; or (4) insufficient ionic strength to prevent enzyme or particle aggregation. Studies of the pH dependence of cytochrome oxidase activity in Keilin–Hartree particles at low ionic strength (I = 0.01–0.05 M) demonstrated that activity was optimized in the alkaline region (Ferguson-Miller et al., 1976). Although Thornstrom et al. (1984) attributed this to the use of the polarographic assay, Maurel et al. (1980) observed the same relationship using the spectrophotometric assay with submitochondrial particles at low ionic strength (I = 0.01 M). In both cases, it is likely that the apparent pH optimum is strongly influenced by aggregation of the membrane particles at low pH and low ionic strength. In the polarographic assay, the high-pH optimum is further accentuated by a major contribution to the activity by the rapid turnover of bound cytochrome *c* (through efficient re-reduction by TMPD) at high pH and low cytochrome *c* concentrations (Brautigan et al., 1978). At the high ionic strengths and high substrate concentrations used in the present study, neither of these phenomena influences the measured activity. Indeed, in agreement with the recent spectrophotometric analysis of the pH dependence of cytochrome oxidase activity, we find that TN_{max} increases continuously with decreasing pH when rates of oxygen consumption are measured in the presence of added reductants (ascorbate and TMPD) under conditions otherwise similar to those described by Thornstrom et al. (1984). At lower ionic strength (I = 0.075 M; data not shown), we observed some discrepancies in TN_{max} between the two assay methods, but these differences disappear at high ionic strength, and the kinetic parameters obtained by the two methods become nearly identical.

A plot of log TN_{max} versus pH (Figure 7A) reveals a complex relationship from which pK_a values are not readily de-

termined. However, when the model of Dixon and Webb (1964), as adapted by Wilms et al. (1980), is applied to these data, the relationship of activity to pH is found to be described best by at least three pK_a values: <5.0, 6.2, and 7.8 for the subunit III containing enzyme and <5.0, 6.0, and 7.9 for the subunit III depleted enzyme. Our values agree with those obtained by Wilms et al. (1980) for the detergent-solubilized enzyme (4.8, 6.5, and 8.0) and by Thornstrom et al. (1984) for the reconstituted enzyme (4.5, 6.8, and 7.8). The significance of the pK_a values has been discussed by these investigators, but there is no consensus or experimental basis for assigning them to specific groups or relating them, as yet, to specific functions. However, our studies at least exclude subunit III as the location of the protonation sites since there is no significant change in the pK_a values or the magnitude of the response in its absence.

Analysis of the kinetic data reveals a marked pH dependence of the K_m as well as TN_{max}. These studies, which were carried out at ionic strengths of 0.5 M, show monophasic kinetics in agreement with the observation that the low-affinity phase of the kinetics disappears with increasing ionic strength ($I > 0.1$ M; Veerman et al., 1980; Brooks & Nicholls, 1982; Sinjorgo et al., 1986). Therefore, only one K_m, that of the high affinity or primary site, is obtained at each pH. Parallel to the change in TN_{max}, the K_m is also found to increase continuously with decreasing pH. In this case, however, a comparison of III-containing and III-deficient enzymes reveals that the latter has significantly higher K_m values at low pH, regardless of tissue source. Although the major site of interaction of cytochrome oxidase with cytochrome *c* appears to be subunit II (Bisson et al., 1982; Millett et al., 1983), subunit III has also been implicated in cytochrome *c* binding (Birchmeier et al., 1976; Fuller et al., 1981). Previous studies have shown that, at pH 7.4, there is no change in K_m for cytochrome *c* upon removal of subunit III (Prochaska & Reynolds, 1986). However, our studies suggest that in the highly protonated form of the enzyme, subunit III (or subunit VI-a, -b, or -c which are also removed when III is depleted) helps to maintain a higher affinity binding domain for cytochrome *c*. Indeed, in contrast to Thornstrom et al. (1984), who report that log k_{cat}/K_m is independent of pH, we find that this parameter becomes pH dependent in the more acidic region with a slope close to +1 (Figure 7B), indicating that groups with pK values of approximately 5.7–5.8 affect the rate of association of the substrate and enzyme. Although removal of subunit III increases the effect of low pH, it does not alter the apparent pK_a, indicating that the group or groups involved in determining this change in association are not on this subunit.

Analysis of pH dependence data using a minimal kinetic scheme, as carried out by Malmstrom and Andreasson (1985), indicates that the rate of dissociation of cytochrome *c* is faster than the k_{cat} and, therefore, not limiting in the lower pH range when using high ionic strength. When treated similarly, our data also suggest that the dissociation constant, while very similar to maximal turnover rates at high pH, becomes increasingly faster than the TN_{max} as pH is decreased. In contrast to Sinjorgo et al. (1984), who state that substrate dissociation is rate limiting under steady-state conditions and suggest that decreasing pH allows increases in TN_{max} as a result of increases in this dissociation (Sinjorgo et al., 1986), our results as well as those of Malmstrom and co-workers (Thornstrom et al., 1984; Malmstrom & Andreasson, 1985) suggest that increasing activity with increasing [H^+] reflects control of one or more intramolecular electron-transfer events. Since the subunit III depleted enzyme, which is only 50% efficient at proton translocation, shows no major changes in its response to pH, our data do not provide direct support for the further hypothesis that regulation by pH occurs at an electron-transfer step that is involved in coupling that process to proton translocation. However, since removal of subunit III may not, in fact, alter the basic mechanism that couples proton translocation to electron transfer, this lack of change in pH dependence may not be a definitive test of the relationship.

pH Dependence of Steady-State Reduction of Cytochrome a. In agreement with the results of Moroney et al. (1984), we observe a decrease in the level of reduction of cytochrome *a* upon elimination of the transmembrane pH gradient, indicating an increased rate of electron transfer from cytochrome *a* to cytochrome a_3. Our studies show that this effect is also observed in the subunit III depleted oxidase and, further, that its sensitivity to DCCD is not diminished by removal of subunit III. These results refute the hypothesis (Moroney et al., 1984) that subunit III contains the proton binding site(s) involved in control of electron transfer. In addition, the retention of DCCD inhibition indicates that other subunits must be involved in mediating the DCCD effect on pH sensitivity. This conclusion is strongly supported by our kinetic analysis which shows that DCCD causes greater loss of activity at pH 5.0 than at pH 7.4 regardless of subunit III content. Thus, the effect of DCCD is to attenuate H^+ stimulation of activity in a subunit III independent manner, indicating a DCCD-modifiable residue elsewhere on the enzyme involved in mediating this effect. Indeed, in addition to its major modification site in subunit III, DCCD has also been shown to covalently label cytochrome oxidase in subunits II and IV (Prochaska et al., 1980; Gregory & Ferguson-Miller, 1988) and may form protein cross-links in self-eliminating reactions [see Nalecz et al. (1986)].

In summary, the pH dependence of the kinetic parameters of cytochrome oxidase and the level of reduction of cytochrome *a* support the conclusion that there is a proton-regulated internal electron-transfer step. However, it is clear from our studies that subunit III does not contain the protonatable group(s) responsible for this regulation. Moreover, the inhibitory effect of DCCD on the pH sensitivity suggests that a site of modification on a peptide other than subunit III may mediate this control. Thus, subunit III can be ruled out as the primary structure responsible for pH regulation of electron transfer. It remains to be determined how this large hydrophobic peptide contributes to oxidase function in general and the efficiency of proton translocation in particular.

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Registry No. DCCD, 538-75-0; cytochrome *c* oxidase, 9001-16-5; cytochrome *c*, 9007-43-6; cytochrome *a*, 9035-34-1.

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