Protonmotive Activity of Cytochrome *c* Oxidase: Control of Oxidoreduction of the Heme Centers by the Protonmotive Force in the Reconstituted Beef Heart Enzyme†

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Received April 24, 1989; Revised Manuscript Received September 12, 1989

ABSTRACT: This paper contributes to the characterization of partial steps of electron and proton transfer in mitochondrial cytochrome *c* oxidase with respect to their membrane arrangement and involvement in energy-linked protonmotive activity. It is shown that $\Delta\psi$ controls electron flow from cytochrome *c* to heme *a* and from the latter and heme a_3 to oxygen. The back-pressure exerted by $\Delta\psi$ on electron flow from cytochrome *c* to heme *a* is consistent with the view that the latter center is buried in the membrane in a central position. The pressure exerted by $\Delta\psi$ on oxidation of heme a_3 by O_2 indicates also that this center is buried in the membrane at some distance from the inner side and is consistent with observations showing that protons consumed in the reduction of O_2 to H_2O derive from the inner space. Electron flow from heme *a* to heme a_3 is shown to be specifically controlled by ΔpH and in particular by the pH of the inner phase. Analysis of the effect of DCCD treatment of oxidase vesicles reveals that concentrations of this reagent which result in selective modification of subunit III (Prochaska et al., 1981) produce inhibition of redox-linked proton release. Higher concentrations of DCCD which result also in modification of subunits II and IV (Prochaska et al., 1981) cause inhibition of the pH-dependent electron-transfer step from heme *a* to heme a_3.

Reduction of O_2 to H_2O catalyzed by cytochrome *c* oxidase (EC 1.9.3.1) is organized anisotropically (Mitchell, 1966) in the coupling membranes of mitochondria and bacteria, electrons being donated by cytochrome *c* located at the outer side of the membrane and protons utilized from the inner aqueous phase (Mitchell & Moyle, 1970; Papa et al., 1974; Konstantinov et al., 1986; Wikström, 1988). This results directly in the generation of transmembrane protonmotive force (PMF).[1]

In the oxidase of eukaryotes and various prokaryotes, electron flow from cytochrome *c* to oxygen is also associated with proton ejection (Wikström, 1976). Thorough studies of this process in rat liver mitochondria and reconstituted beef heart cytochrome *c* oxidase are generally considered to provide evidence that it represents electrogenic uphill proton translocation (H^+ pumping) from the inner to the outer aqueous space [Wikström et al., 1981; Wikström & Saraste, 1984; Mitchell et al., 1985; for an extensive discussion of this issue, see Papa (1988)]. The actual mechanism of protonmotive activity of cytochrome *c* oxidase remains, however, still to be established. Various direct (Mitchell et al., 1985; Mitchell, 1987) and indirect models (Wikström et al., 1981; Malmström, 1985; Gelles et al., 1986) have been developed, and attempts are being made to verify the possible participation in the process of the heme *a* (Wikström et al., 1981) Cu_A (Gelles et al., 1986) and the heme a_3 Cu_B oxygen reduction centers (Mitchell, 1987).

This paper is intended to characterize partial steps of electron and proton transfer in bovine heart cytochrome *c* oxidase reconstituted in phospholipid vesicles (COV) with respect to their membrane arrangement and involvement in energy-linked protonmotive activity. Evidence is available indicating that electron distribution between cytochrome *c* and heme *a* is controlled by the $\Delta\psi$ component of PMF (Hinkle & Mitchell, 1970; Gregory & Ferguson-Miller, 1989) and that internal electron transfer in the oxidase represents a rate-limiting step controlled by pH and transmembrane ΔpH (McGovern Moroney et al., 1984; Thörnström et al., 1984; Gregory & Ferguson-Miller, 1988, 1989).

The present results show that $\Delta\psi$ controls electron flow from cytochrome *c* to heme *a* and from the latter and heme a_3 to oxygen. The $\Delta\psi$-controlled step in electron flow from cytochrome *c* to heme *a* is found to be pH independent. Electron flow from heme *a* to heme a_3 is controlled by ΔpH and, in particular, by the pH of the inner (matrix) space. Low concentrations of DCCD, which result in predominant modification of subunit III, cause inhibition of redox-linked proton ejection and apparent enhanced reduction of heme a_3. At high concentrations of DCCD, resulting also in modification of subunits II and IV, there occurs inhibition of the pH-dependent electron-transfer step from heme *a* to heme a_3.

Experimental Procedures

Chemicals. TMPD was from BDH Chemicals Ltd.; valinomycin, nigericin, CCCP, horse heart cytochrome *c* (type VI), and soybean phospholipids (type II) were from Sigma Chemical Co.; DCCD was from Kock-Light Laboratories. All other

† This work was supported in part by Grants 88.00376.11 and 88.00794.44 from the Consiglio Nazionale delle Ricerche, Italy.

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[1] Abbreviations: COV, cytochrome oxidase vesicle(s); $\Delta\psi$, transmembrane electrical gradient; ΔpH, transmembrane pH gradient; PMF, protonmotive force; DCCD, *N,N'*-dicyclohexylcarbodiimide; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; HEPES, 4-(2-hydroxyethyl)-1-piperazineethasulfonic acid; EDTA, ethylenediaminetetraacetic acid; H^+/e^-, ratio of protons translocated per electron transferred by cytochrome oxidase; q^+/e^-, ratio of electrical charges translocated per electron transferred by cytochrome oxidase; RCR, respiratory control ratio; MES, 2-(*N*-morpholino)ethanesulfonic acid.

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reagents were of the highest purity grade available.

Preparation of Cytochrome c Oxidase and Oxidase Vesicles. Cytochrome *c* oxidase, 10–12 nmol of hemes $a + a_3$/mg of protein, was prepared from beef heart mitochondria as in Errede et al. (1978). Cytochrome oxidase vesicles (COV) were prepared by cholate dialysis (Casey et al., 1979). The percentage of total cytochrome oxidase incorporated "right-side out" was calculated (Casey et al., 1980, 1982) to be always higher than 80%.

Cytochrome Assay. Hemes $a + a_3$, in the soluble oxidase or in reconstituted vesicles, were measured spectrophotometrically at room temperature using a $\Delta\epsilon$ at 605–630 nm of 12 $mM^{-1}\cdot cm^{-1}$ (Nicholls & Kimelberg, 1972). Ferrocytochrome *c*, prepared as in Casey et al. (1979), was measured by using a $\Delta\epsilon_{550}$(red–ox) = 21 $mM^{-1}\cdot cm^{-1}$ (Massey, 1959).

Measurement of Proton Translocation and Oxygen Uptake. Proton translocation was measured with a pH combination electrode connected to a rapidly responding electrometer amplifier (Papa et al., 1979). Oxygen consumption was measured with a Clark-type electrode. Measurements were carried out in all-glass cells, thermostatically controlled at 25 °C.

For measurement of proton translocation, 0.25-mL vesicles (final concentration 0.5 μM oxidase) were suspended in 100 mM choline chloride, 0.2 mM choline–HEPES, 0.1 mM choline–EDTA, 5.0 mM KCl, and 2.0 μg of valinomycin/mL, pH 7.0 [see Papa et al. (1987)].

Respiratory activity was measured in a medium containing 40 mM KCl, 0.1 mM EDTA, and 20 mM MES (for pH 5.0–6.0) or 20 mM HEPES (for pH 6.5–8.0).

Measurement of Steady-State Redox Levels of Cytochromes. COV (1.0 μM cytochrome oxidase) were suspended in 180 mM sucrose, 20 mM KCl, and 20 mM MES (for pH 6.0–6.5) or 20 mM HEPES (for pH 7.0–8.5) in the presence of 1.0 μM cytochrome *c*. Redox changes of cytochromes were monitored with a Johnson Foundation dual-wavelength spectrophotometer at 550–540 nm for cytochrome *c* and at 605–630 and 443–470 nm for the α and γ peaks of cytochromes $a + a_3$. At the concentration of cytochrome *c* used, the mutual optical overlap in the Soret region of cytochromes was verified to be negligible. Full reduction of cytochromes was obtained with sodium dithionite. Specific redox levels of hemes *a* and a_3 were calculated from the spectrophotometric traces assuming the relative contribution of heme *a* at 605–630 and 443–470 nm to be 72.5% and 35%, respectively (Blair et al., 1986), and using the equations [see Papa et al. (1981)]:

$$\% \text{ reduction heme } a = 73.4(2.36f_\alpha - f_\gamma)$$

$$\% \text{ reduction heme } a_3 = 93.5(2.07f_\gamma - f_\alpha)$$

where

$$f_\alpha = \Delta A_{605-630}/\Delta A_{t,605-630};\ f_\gamma = \Delta A_{443-470}/\Delta A_{t,443-470}$$

ΔA represents the measured absorbance changes; ΔA_t represents the total absorbance changes (dithionite-reduced minus fully oxidized oxidase).

Chemical Modification of Cytochrome c Oxidase by DCCD. COV (3 μM cytochrome oxidase) were incubated with DCCD (from 0.5 μM ethanolic solution), at the concentrations indicated in the figures, at 12–14 °C for 90 min. The reaction was stopped in ice, and samples were diluted in the suspending media. The control was treated in the same way with an amount of ethanol equivalent to that of the highest concentration of DCCD used. Controls showed that DCCD treatment did not affect the extent of right-side-out incorporation of the oxidase in the vesicles.

Measurement of the Bohr Effect Linked to Heme a. KCN-treated soluble cytochrome *c* oxidase was subjected to a cycle of reduction–oxidation by successive additions of ferrocytochrome *c* and ferricyanide. Redox changes of cytochromes *a* and *c* were measured at 605–630 and 550–540 nm, using extinction coefficients of 20.4 and 19.1 $mM^{-1}\cdot cm^{-1}$, respectively. pH changes associated with the reduction–oxidation cycle of cytochrome *a* were monitored electrometrically with a measuring setup providing accuracy better than 0.001 pH unit (Papa et al., 1979, 1986). When necessary, correction for small effects from adding the same amount of cytochrome *c* and ferricyanide to the reaction mixture without cytochrome oxidase was made.

The Bohr effect linked to heme *a* was expressed as moles of H^+ released per mole of heme *a* oxidized. At alkaline pHs due to the Bohr effect of cytochrome *c*, the measured H^+/e^- ratio was corrected for proton release associated with oxidation of the residual ferrocytochrome *c* by ferricyanide (Papa et al., 1977). The pH dependence of the observed H^+/e^- ratio was best fitted with a curve obtained by resolving the equation:

$$H^+/e^- = \sum_i \left| \frac{1}{10^{pH-pK_{i,ox}} + 1} - \frac{1}{10^{pH-pK_{i,red}} + 1} \right|$$

which refers to the theoretical pH dependence of the H^+/e^- ratio for a redox-linked Bohr effect attributable to one or more protolytic group(s) with different values of pK_a in the two redox states of the metal center.

Results

Crossover Effects of Δψ and ΔpH. Respiratory activity and cytochrome redox levels were examined in turning over COV, supplemented with cytochrome *c*, TMPD, and excess ascorbate. In the presence of nigericin, all of the PMF is present as Δψ, and the respiratory control this component exerts on electron flow is evaluated from the enhancement of respiration caused by further addition of valinomycin. To measure the respiratory control exerted by ΔpH, the same procedure was applied, adding first valinomycin followed by nigericin. Since electron flow in the oxidase is pH dependent (Wilms et al., 1980; Thörnstrom et al., 1984; Gregory & Ferguson-Miller, 1988; Einardsdottir et al., 1988), ionophores were tested in a pH range from 5.5 to 8.0.

The respiratory activity of fully uncoupled oxidase vesicles (both ionophores present) was maximal at acidic pHs and declined as the pH of the suspension was raised from 7.0 to 8.0 (Figure 1). A similar pH dependence was also observed with the soluble oxidase (data not shown). The overall respiratory control exerted by PMF increased with pH, giving at pH 7.5–8.0 a respiratory control index as high as 12. The control exerted by ΔpH exhibited the same pH dependence as that given by total PMF. The respiratory control exerted by Δψ was practically pH independent. These results indicate that Δψ and ΔpH exert predominant control effects on different redox steps.

The crossover sites for the action of Δψ and ΔpH on electron flow in cytochrome oxidase were identified by monitoring the effects exerted by the two ionophores on the steady-state redox levels of cytochromes in COV. Aerobic cytochrome oxidase, supplemented with cytochrome *c*, TMPD, and ascorbate, goes, in a few seconds, through many turnovers and is conceivably all in the pulsed state (Brunori et al., 1979). The steady-state redox levels of cytochrome *c* at 550–540 nm and that of cytochromes $a + a_3$ at 605–630 and 443–470 nm were continuously monitored with a dual-wavelength spectrophotometer (Figure 2A). Control measurements at the wavelength couple 575–630 nm, which is isosbestic for cytochromes and give a

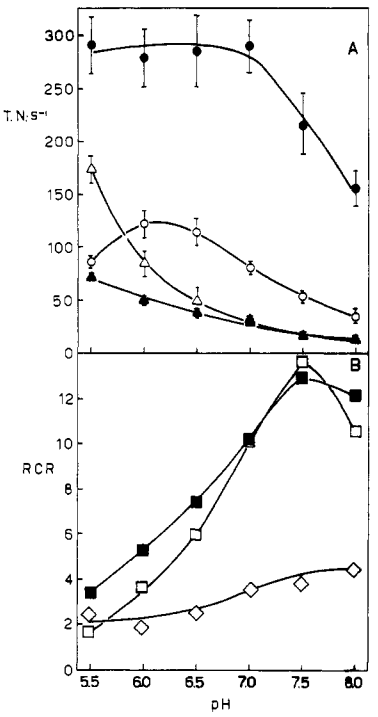

FIGURE 1: pH dependence of the respiratory activity of COV. Respiratory activity was measured in the media described under Experimental Procedures supplemented with 50 μM cytochrome *c* and 10 mM ascorbate *plus* 0.2. mM TMPD; final volume 3.3 mL. The reaction was started by addition of COV (final oxidase concentration 36.4 nM). The rates of oxygen consumption were corrected for the autoxidation of ascorbate + TMPD measured in the absence of COV. Panel A presents respiratory activities expressed as turnover number (T.N.) (moles of cytochrome *c* per second per mole of cytochrome aa_3) in the control (▲), in the presence of 2 μg/mL nigericin (○) or 2 μg/mL valinomycin (△), and in the presence of both ionophores (●); the values are means ± SEM of three to five experiments. Panel B gives the respiratory control ratios exerted by $\Delta\psi$ (◇), ΔpH (□), and the total PMF (■) calculated as described in the text from the values of panel A.

$\Delta\epsilon_{mM}$ of 5.1 for $TMPD^+$ (oxidation product of TMPD) (Papa et al., 1983), showed that under the experimental conditions used there was no detectable accumulation of $TMPD^+$ which could interfere with the measurement of oxidoreduction of cytochromes. From the relative contributions of hemes *a* and a_3 to the overall absorbance changes at the two wavelength couples (Blair et al., 1986), the specific redox changes of the two hemes were estimated (Papa et al., 1981) (Figure 2B). It should be recalled that in previous studies on the effect of ionophores on the steady-state redox levels of cytochromes in COV (Wrigglesworth et al., 1978; McGovern Moroney et al., 1984; Gregory & Ferguson-Miller, 1989), the overall absorbance changes of cytochromes $a + a_3$ were monitored without resolution of the specific redox changes of hemes *a* and a_3. The addition of reductants to the oxidized enzyme resulted in 75% reduction of cytochrome *c*. Heme *a* was rapidly reduced up to 60% while heme a_3 was reduced to around 10% (Figure 2B). $\Delta\psi$ collapse by valinomycin caused a rapid and significant oxidation of cytochrome *c*, with a rise time around 1 s, accompanied by a small oxidation of heme a_3 with no net change in this interval of heme *a*. These changes were then followed during an interval of about 15 s in which $\Delta\psi$ was expected to be replaced by generation of extra ΔpH, by extensive reduction of heme *a*, well above the initial level and partial re-reduction of cytochrome *c*. Further collapse of ΔpH by nigericin caused immediate definite oxidation of cytochrome *c* and even larger oxidation of heme *a* accompanied by small reduction of heme a_3.

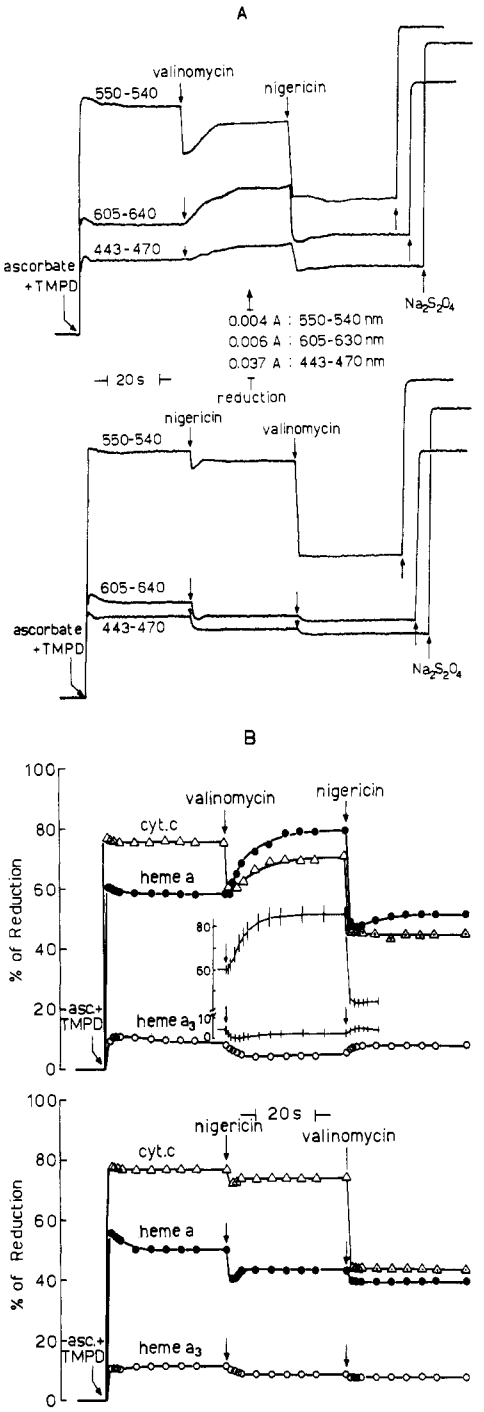

FIGURE 2: Redox transitions of cytochromes induced by ionophores in steady-state respiring COV. Steady-state redox levels of cytochromes in COV were monitored spectrophotometrically in the conditions described under Experimental Procedures. Final pH of the suspension was 7.5. (A) Absorbance changes of cytochrome *c* and hemes $a + a_3$. Where indicated, 10 mM ascorbate *plus* 0.1 mM TMPD, 2 μg/mL valinomycin, and 2 μg/mL nigericin were added. (B) Specific redox levels of cytochrome *c* and hemes *a* and a_3 calculated from the spectrophotometric traces as described under Experimental Procedures. The inset gives the mean values ± SEM of 16 experiments.

When the order of addition of the two ionophores was reversed, nigericin, added in the absence of valinomycin, caused definite oxidation of heme *a* with only small oxidation of cytochrome *c* and heme a_3. Collapse of $\Delta\psi$ by further addition of valinomycin resulted in large oxidation of cytochrome *c* and small oxidation of heme *a*.

In Figure 3, the dependence on the pH of the suspending medium of the crossover effects exerted by ionophores on the

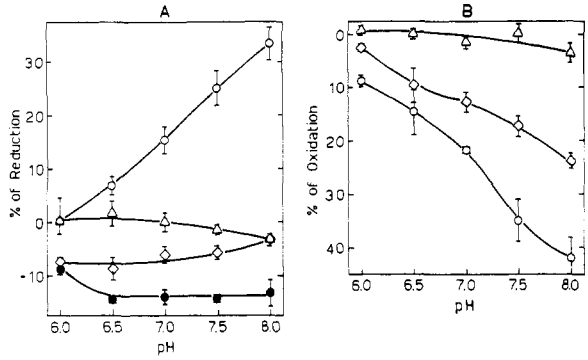

FIGURE 3: pH dependence of changes in steady-state redox levels of cytochrome *c* and hemes *a* and a_3 induced by ionophores in respiring COV. For experimental conditions, see Experimental Procedures and legend to Figure 2. The values plotted represent the changes in the steady-state redox levels caused by addition of valinomycin to substrate-supplemented oxidase vesicles (A) or by addition of nigericin to vesicles supplemented with substrates and valinomycin (B). The values are means of six different experiments ± SEM. (A) (○) Extra reduction of heme *a*; (△) oxidation of heme a_3; (●) transient oxidation of cytochrome *c*; (◇) steady-state oxidation of cytochrome *c* attained after partial re-reduction. (B) (○) Oxidation of heme *a*; (◇) oxidation of cytochrome *c*; (△) oxidation of heme a_3.

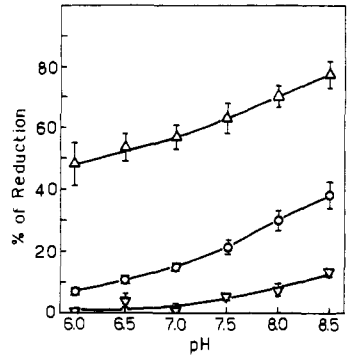

FIGURE 4: pH dependence of changes in steady-state redox levels of cytochrome *c* and hemes *a* and a_3 in respiring soluble cytochrome *c* oxidase. 1 μM soluble cytochrome *c* oxidase was suspended in the same media used for COV experiments (see Experimental Procedures and legend to Figure 2) supplemented with 0.5% Tween 80. The values shown are the specific redox levels of cytochrome *c* (○), heme *a* (△), and heme a_3 (▽) measured at the steady state after addition of 10 mM ascorbate *plus* 0.1 mM TMPD and represent the means of six experiments ± SEM. For further details, see Experimental Procedures.

steady-state redox levels of cytochromes is shown. The rapid oxidation of cytochrome *c* caused by valinomycin was pH independent in the range from 6.5 to 8.0. The accompanying extra reduction of heme *a* increased, markedly, as the pH of the medium was raised from 6.0 to 8.0 (Figure 3A).

There was some increase with pH of the steady-state reduction level of cytochrome *c* which was attained with the delayed re-reduction following the initial rapid oxidation caused by valinomycin. An increase with pH was also observed for the extent of oxidation of heme *a* and the accompanying oxidation of cytochrome *c* caused by addition of nigericin to valinomycin-supplemented COV (Figure 3B).

In respiring soluble cytochrome oxidase (Figure 4), the reduction level of heme *a* was significantly higher than that of cytochrome *c* and much higher than that of heme a_3. The reduction levels of hemes *a* and a_3 and of cytochrome *c* increased with pH.

Redox Bohr Effect. The E_m of hemes *a* and a_3 is pH dependent, thus showing that the redox transitions of these centers are linked to protolytic processes in the enzyme (redox Bohr effects) (Papa et al., 1986). Redox Bohr effects linked to heme *a* were directly determined in the soluble oxidase with

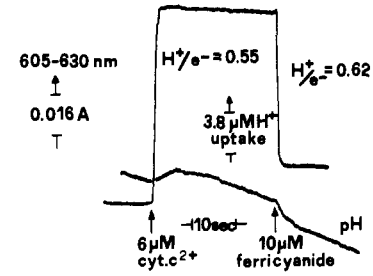

FIGURE 5: Bohr effect of heme *a* in KCN-inhibited soluble cytochrome *c* oxidase. 8 μM cytochrome *c* oxidase was incubated overnight at 4 °C, with 2 mM KCN in 150 mM KCl; a 1.5-mL sample was adjusted to pH 7.6 and transferred into a stirred cuvette. After the cycle of reduction–oxidation of heme *a* induced by successive pulses of ferrocytochrome *c* and ferricyanide, 30 μM ferricyanide was added to oxidize completely the cytochromes, and the absorbance levels thus reached were taken as base line for calculations. Heme *a* oxidoreduction was monitored spectrophotometrically, pH changes electrometrically. For further details, see Experimental Procedures.

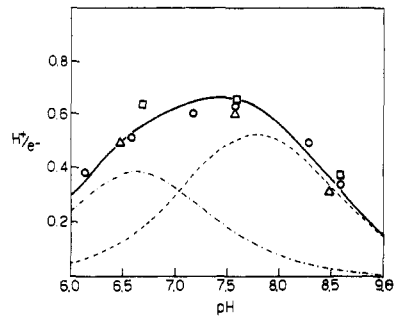

FIGURE 6: pH dependence of the redox Bohr effect of heme *a* (H^+/e^- or s/n coupling number). For experimental details, see the legend to Figure 5 and Experimental Procedures. The experimental value resulting from three different experiments was best-fitted by a curve (solid line) obtained by combining those derived from equations expressing the theoretical pH dependence of the H^+/e^- ratio for a redox-linked protolytic group, one with pK_{ox} = 6.3 and pK_{red} = 7.0 (–·–) and the other with pK_{ox} = 7.3 and pK_{red} = 8.3 (– – –).

heme a_3 blocked by CN^-. Figure 5 shows that reduction of heme *a* by ferrocytochrome *c* resulted in the uptake of protons, which were released in the same amount upon reoxidation of heme *a* by ferricyanide with an H^+/e^- ratio of 0.6 at pH 7.6. The pH dependence of this ratio, presented in Figure 6, is suggestive of contribution of more than one protolytic group [cf. Wilms et al. (1980) and Thörnstrom et al. (1984)]. The best fit of the experimental points was, in fact, obtained by a curve resulting from a combination of those constructed for two hypothetical redox-linked protolytic groups, one with pK_{ox} of 6.3 and pK_{red} of 7.0 and the other with pK_{ox} of 7.3 and pK_{red} of 8.3.

Effect of Modification by DCCD. Treatment of bovine heart cytochrome oxidase with DCCD modifies preferentially glutamic-90 in subunit III, followed at higher reagent concentrations by modification of subunits II and IV (Prochaska et al., 1981). Modification of the oxidase by DCCD depresses proton translocation elicited by the ferrocytochrome *c* pulse of aerobic COV with an apparently smaller effect on electron flow (Casey et al., 1979; Prochaska et al., 1981).

The mechanism by which DCCD modification causes this effect is as yet unclear (Wikström, 1988).

Figure 7 shows the effect of DCCD modification on respiratory activity and proton translocation by COV. Modification by DCCD concentrations from 50 to 750 mol/mol of aa_3 practically effected all the depression of proton ejection elicited by ferrocytochrome *c* pulses of valinomycin-supplemented aerobic COV and caused some decrease of the respiratory control exerted by the ΔpH component of PMF.

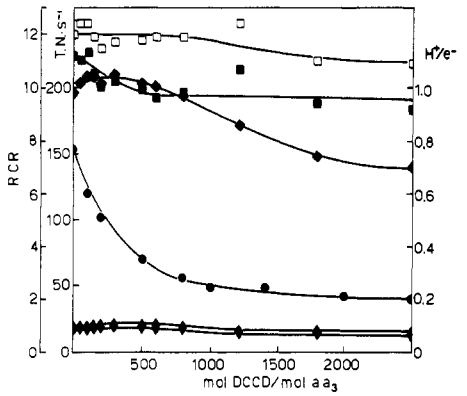

FIGURE 7: Effect of DCCD on the respiratory activity and redox-linked proton ejection in COV. Respiratory activity measurement: the conditions are those described in the legend to Figure 1. T.N. values expressed as e^- equivalents per mole of cytochrome *a*, a_3 per second: (▼) control; (▲) *plus* 2 μg/mL valinomycin; (♦) *plus* valinomycin and 2 μg/mL nigericin; (□) respiratory control ratio exerted by total PMF; (■) respiratory control ratio exerted by ΔpH. Mean values of three experiments. (●) Proton ejection: COV (0.5 μM cytochrome *a*, a_3) were suspended in the medium described under Experimental Procedures. The aerobic COV suspension was pulsed with 4 μM ferrocytochrome *c*, and proton release was measured as described under Experimental Procedures. For DCCD treatment of COV, see Experimental Procedures. Mean values of two experiments.

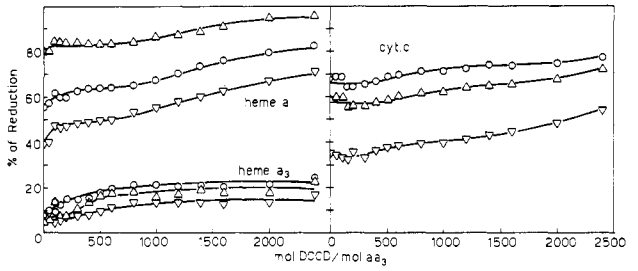

FIGURE 8: Effect of DCCD treatment on steady-state redox levels of cytochrome *c*, heme *a*, and heme a_3 in steady-state respiring COV. For conditions and measurements, see Experimental Procedures and legend to Figure 2. (○) Control; (△) *plus* 2 μg/mL valinomycin; (▽) *plus* valinomycin and 2 μg/mL nigericin. The values are the mean of three experiments.

Higher concentrations of DCCD caused inhibition of respiration in all the conditions tested with only little further depression of the H^+/e^- ratio.

Figure 8 illustrates the effect of oxidase modification by a range of DCCD concentrations on the redox level of cytochromes in turning-over COV. Low concentrations of DCCD, sufficient to cause significant depression of the H^+/e^- ratio, enhanced the steady-state reduction levels of heme a_3, in particular, and also of heme *a*, attained after addition of substrates to aerobic COV, without significant net change in the reduction level of cytochrome *c*. Increasing the DCCD concentration above that practically sufficient to produce maximal depression of the H^+/e^- ratio (see Figure 7) resulted in enhancement of the steady-state reduction level of heme *a* and cytochrome *c* without any further effect on the redox level of heme a_3 (Figure 8).

Figure 9 shows that DCCD at a concentration (500 mol/mol of aa_3) causing extensive decoupling of redox-linked proton translocation had no effect on the pH dependence of the respiratory rate measured in the uncoupled state.

Discussion

This study provides direct insight into the involvement of redox centers of cytochrome *c* oxidase in the protonmotive activity of the enzyme. The results show that in turning-over COV Δψ controls electron flow from cytochrome *c* to heme

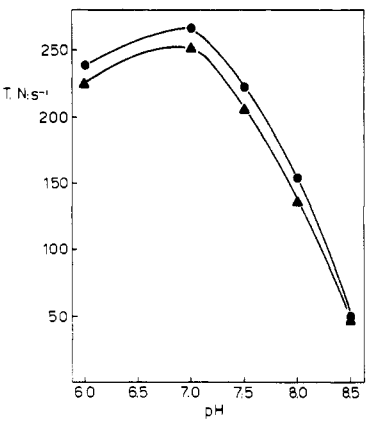

FIGURE 9: pH dependence of the respiratory activity of control and DCCD-treated COV. For conditions, see Experimental Procedures and legend to Figure 1. The T.N. reported refer to uncoupled COV supplemented with 2 μg/mL valinomycin *plus* 2 μg/mL nigericin. (●) Control; (▲) COV treated with 500 mol of DCCD/mol of cytochrome aa_3. The values are the mean of three experiments.

a and from the latter and heme a_3 to oxygen. Electron flow from heme *a* to heme a_3 is, in addition, shown to be controlled by ΔpH and in particular by the pH of the M phase [cf. McGovern Moroney et al. (1984)]. This step, which is maximally activated at acidic pHs, represents the pH-dependent internal rate-limiting step described for soluble and reconstituted cytochrome *c* oxidase by Thörnstrom et al. (1984).

Our observations [see also Papa (1988)] substantiate and extend those presented in a very recent paper of Gregory and Ferguson-Miller (1989), who concluded, on the basis of measurements of the overall absorbance changes of cytochromes $a + a_3$ at a single wavelength couple (605–630 nm), that Δψ controls electron transfer from cytochrome *c* to heme *a* and ΔpH controls electron transfer from heme *a* (Cu_A) to heme a_3 (Cu_B) [see also McGovern Moroney et al. (1984)]. Gregory and Ferguson-Miller (1989) did not resolve the specific redox changes of hemes *a* and a_3.

The back-pressure exerted by Δψ on electron flow from cytochrome *c* to heme *a* and from this to oxygen is consistent with the reported effect of Δψ on the E_m of heme *a* in the CO-inhibited enzyme and the view that this center is buried in the membrane in a central position across the dielectric barrier (Hinkle & Mitchell, 1970; Rich et al., 1988). The back-pressure exerted by Δψ on oxidation of heme a_3 by O_2 is consistent with observations showing that also this heme is buried in the membrane at some distance from the M side (Ohnishi et al., 1984) and that protons consumed in the reduction of O_2 to H_2O derive from the M aqueous phase (Mitchell & Moyle, 1970; Papa et al., 1974; Papa, 1976).

The Δψ-controlled step in electron flow from cytochrome *c* to heme *a* is found to be pH-independent (see Figures 1 and 3). Evidently, the protonation step associated with reduction of heme *a* (redox Bohr effect) is much faster than the electron-transfer step controlled by Δψ. The lack of effect of pH and ΔpH on electron flow from cytochrome *c* to heme *a*, together with the observation of half the effect of the ATP-induced Δψ on the E_m of heme *a* and the negligible response of this shift to pH (Rich et al., 1988), militates against the proposal (Gelles et al., 1986) that Cu_A, apparently located between cytochrome *c* and heme *a* (Rich et al., 1988; Holm et al., 1987), acts as a proton-pumping redox center.

Replacement of Δψ by extra ΔpH, induced by valinomycin addition to respiring oxidase vesicles, inhibits electron flow from heme *a* to heme a_3, and collapse of ΔpH by nigericin

releases this inhibition. The respiratory control exerted by pH between hemes *a* and a_3 increases with the pH of COV suspension (Figure 3). In respiring soluble oxidase, the reduction level of heme *a*, which is much higher than that of heme a_3 and even higher than that of cytochrome *c*, increases with pH (Figure 4), thus showing that the actual pH in the aqueous phase controls electron flow from heme *a* to heme a_3. This control is released in COV by nigericin addition which in the presence of valinomycin is expected to cause large acidification of the small internal space of the vesicles, but has negligible effect on the pH of the external space. Thus, it is the pH of the inner (M) space that affects primarily electron flow from heme *a* to heme a_3.

It might be recalled that redox Bohr effect linked to heme *a* involves protons from the inner aqueous phase (Artzatbanov et al., 1978; Konstantinov et al., 1986). Yet this effect cannot account for the particular pH dependence of electron flow from heme *a* to heme a_3 described here. In this case, pH effects just opposite to those observed should be expected.

The analysis of the concentration dependence of the effect of DCCD modification on the redox and protonmotive activity of cytochrome *c* oxidase reveals a dual mechanism of action. Low concentrations of DCCD, which results in selective modification of subunit III (Prochaska et al., 1981), produced depression of proton translocation with no inhibition of respiratory rate and even some decrease of the respiratory control exerted by the ΔpH component of PMF. Interestingly enough, these effects of DCCD were accompanied by enhancement of the steady-state reduction level of heme a_3. These observations taken together provide evidence showing that modification of subunit III is responsible for inhibition by DCCD of redox-linked H^+ release by COV. We would like to propose that modification of Glu-90 in subunit III results, either in a direct manner or through changes in the oligomeric state of the oxidase (Finel & Wikström, 1986), in a perturbation of the asymmetric access of protons from the matrix space to the binuclear a_3 Cu_B center where the peroxy intermediate is protonated with H_2O formation (Wikström, 1988). Depression of this protonic access, which might be responsible for the enhancement of the steady-state reduction level of heme a_3, can be accompanied by enhanced proton leakage from the cytoplasmic side into the binuclear center [see also Wikström (1988)].

Modification of subunits II and IV, which takes place at higher concentrations of DCCD (Prochaska et al., 1981), seems, on the other hand, to be responsible for inhibition of the pH-dependent electron-transfer step from heme *a* to heme a_3 [Figure 8; cf. Gregory and Ferguson-Miller (1988)].

ACKNOWLEDGMENTS

The skillful technical assistance of M. Minuto is gratefully acknowledged.

Registry No. O_2, 7782-44-7; cytochrome *c* oxidase, 9001-16-5; heme, 14875-96-8.

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Effect of Chemical Modification of Lysine Amino Groups on Redox and Protonmotive Activity of Bovine Heart Cytochrome *c* Oxidase Reconstituted in Phospholipid Membranes†

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Received April 24, 1989; Revised Manuscript Received September 12, 1989

ABSTRACT: A study is presented of the effect of chemical modification of lysine amino groups on the redox and protonmotive activity of bovine heart cytochrome *c* oxidase. Treatment of soluble oxidase with succinic acid anhydride resulted in succinylation of lysines in all the subunits of the enzyme. The consequent change of surface charges from positive to negative resulted in inversion of the orientation of the reconstituted enzyme from right-side-out to inside-out. Reconstitution of the oxidase in phospholipid vesicles prevented succinylation of subunits III and Vb and depressed that of other subunits with the exception of subunits II and IV which were predominantly labeled in a concentration-dependent manner by succinic acid anhydride. This modification of lysines produced a decoupling effect on redox-linked proton ejection, which was associated with a decrease of the respiratory control exerted by the ΔpH component of PMF. The decoupling effect was directly shown to be exerted at the level of the pH-dependent rate-limiting step in intramolecular electron flow located on the oxygen side of heme *a*.

Mammalian cytochrome *c* oxidase (EC 1.9.3.1) is composed of 13 polypeptides (Kadenbach et al., 1985; Takamiya et al., 1987). Subunits I, II, and III are mitochondrial-encoded and respectively analogous to the three subunits of *Paracoccus denitrificans* (Buse et al., 1987; Finel et al., 1987). The other subunits are encoded by the nuclear genoma [see Papa et al. (1987a,b)].

Reduction of dioxygen to H_2O by ferrocytochrome *c* is arranged in cytochrome oxidase in the membrane so as to result directly in the generation of protonmotive force (PMF)[1] (Mitchell, 1966; Papa, 1976; Wikström, 1988). In mitochondria and certain bacteria, including *Paracoccus denitrificans*, aerobic oxidation of ferrocytochrome *c* by the oxidase is also associated with proton release (Wikström, 1976; Wikström et al., 1985; Papa, 1988). This process is generally considered to represent uphill proton translocation (H^+ pumping) from the inner to the outer aqueous phase, its mechanism remaining, however, to be established (Wikström et al., 1985; Mitchell et al., 1985; Mitchell, 1987; Malmström, 1985; Gelles et al., 1986; Papa, 1988).

Subunit I may perform basic processes of redox catalysis (Buse et al., 1987; Müller et al., 1988) and protonmotive activity (Hon-nami & Hoshima, 1984). Subunit II mediates reaction of cytochrome *c* with the oxidase (Capaldi et al., 1982). Subunit III is apparently involved in the protonmotive activity of the mitochondrial oxidase (Wikström et al., 1985). The function of the other subunits of mitochondrial cytochrome *c* oxidase is as yet unclear. Supernumerary subunits can be involved in membrane assembly of the enzyme (Patterson et al., 1987) and/or, in what may be tissue specific, regulatory properties of cytochrome oxidase (Kadenbach, 1986; Bisson et al., 1987).

It is likely that the quaternary structure and activity of mitochondrial cytochrome *c* oxidase, which can also exist as dimer in the membrane (Finel & Wikström, 1986), are governed by electrostatic interactions between the constituent subunits and between these, membrane phospholipids, and polar solutes. Effects of anions (Hüther & Kadenbach, 1987;

† Supported by an EMBO short-term fellowship to D.S., by Grants 88.00376.11 and 88.00794.44 from the Consiglio Nazionale delle Ricerche, Italy, S.P., and by grants from the Deutsche Forschungsgemeinschaft (SFB 103,42) and Fonds der Chemischen Industrie to B.K.

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[1] Abbreviations: COV, cytochrome oxidase vesicle(s); CCCP, carbonyl cyanide, *m*-chlorophenylhydrazone; DMSO, dimethyl sulfoxide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; TRIS, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; PMF, protonmotive force; $\Delta\psi$, transmembrane electrical potential; ΔpH, transmembrane pH gradient; RCR, respiratory control ratio; H^+/e^-, ratio of protons translocated per electron transferred by cytochrome oxidase.

0006-2960/90/0429-2945$02.50/0