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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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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;

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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.

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Bisson et al., 1987; Hüther et al., 1988) on the redox activity and spectra of soluble and reconstituted oxidase suggest that positively charged residues located at the surface of the enzyme modulate the catalytic activity.

In this paper, 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. The results show that general modification of lysines in the soluble oxidase prevents its proper reconstitution in phospholipid vesicles. In reconstituted oxidase vesicles (COV), succinylation of lysine residues exposed at the cytosolic (C) side in a restricted number of subunits results in specific inhibition of electron flow from cytochrome *c* to heme *a* and decoupling of the protonmotive activity without effect on the incorporation of the oxidase in the vesicles.

Experimental Procedures

Chemicals. Soybean phospholipids (type II), cytochrome *c* (type VI from horse heart), valinomycin, and CCCP were obtained from Sigma; dodecyl β-D-maltoside was from Calbiochem. Succinic acid anhydride was purchased from Serva. [1,4-^{14}C]Succinic acid anhydride (111 μCi/mol) was from Amersham.

Isolation and Reconstitution of Cytochrome c Oxidase. Cytochrome *c* oxidase, isolated from bovine heart mitochondria as in Errede et al. (1978), was reconstituted into phospholipid vesicles by cholate dialysis (Casey et al., 1979; Capitanio et al., 1990). The orientation of the oxidase in the vesicles and the remaining fraction of soluble enzyme were measured as described by Casey et al. (1979).

Succinic Acid Anhydride Modification. Soluble cytochrome oxidase or COV were treated with succinic acid anhydride in DMSO at the final concentrations specified in the legends. Succinic acid and DMSO were added to the samples in order to get the same final concentrations of DMSO and succinic acid released by hydrolysis of the anhydride. After 1-h incubation at 4 °C, samples were dialyzed for 4 h against 10 mM K-HEPES, pH 7.2, 27 mM KCl, and 73 mM sucrose and overnight against 1 mM K-HEPES, pH 7.2, 30 mM KCl, and 79 mM sucrose.

Measurements of Oxygen Uptake and Proton Translocation. Oxygen consumption was measured with a Clark-type electrode in 10 mM K-HEPES, pH 7.4, 40 mM KCl, 0.1 mM EDTA, 25 mM potassium ascorbate, 0.02 μM oxidase, and various cytochrome *c* concentrations in the presence and absence of 1 μg/mL valinomycin, 1 μg/mL nigericin, and 3 μM CCCP at 25 °C. Proton translocation was measured electrometrically (Papa et al., 1979; Capitanio et al., 1990).

Labeling of COV and Soluble Enzyme with [^{14}C]Succinic Acid Anhydride. COV (7.5 μM oxidase) were 2-fold concentrated by dialysis against 100 mM K-HEPES, pH 7.6, and 10% poly(ethylene glycol) 20000 for 5 h. After 1-h incubation at 4 °C with [^{14}C]succinic acid anhydride, the enzyme was isolated from COV by sucrose gradient centrifugation (Zhang et al., 1984). The protein pellet was dissolved in 62.5 mM Tris-HCl, pH 6.8, 8% SDS, 15% glycerol, and 2% mercaptoethanol, and SDS–polyacrylamide gel electrophoresis was performed as previously described (Kadenbach et al., 1983). The soluble enzyme (30 μM) was modified with [^{14}C]succinic acid anhydride (2 μCi/mol) in 100 mM K-HEPES, pH 8.5, and 0.02% dodecyl β-D-maltoside. The samples, treated as described above, were subjected to electrophoresis, protein bands were dissolved in 30% H_2O_2 and 3% NH_4Cl, and radioactivity was counted by liquid scintillation.

Measurement of Steady-State Redox Levels of Cytochromes. Oxidoreductions were monitored with a dual-wavelength spectrophotometer at 550–540 nm for cytochrome *c* and at 605–630 and 443–470 nm for cytochrome oxidase, and the specific changes of hemes *a* and a_3 were calculated as in Capitanio et al. (1990).

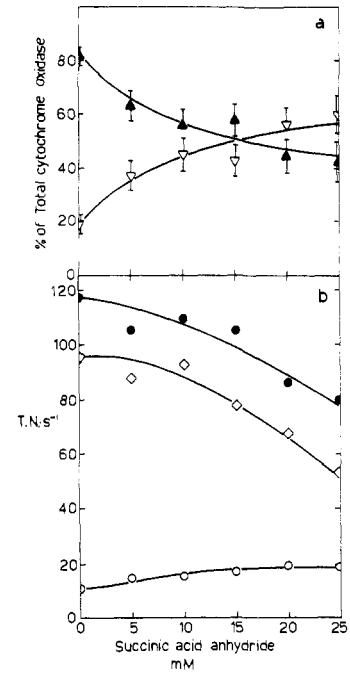

FIGURE 1: Effect of succinic acid anhydride modification of soluble cytochrome oxidase on the orientation and respiratory activity of COV. (a) The percentage of right-side-out (▲) and inside-out (▽) oriented cytochrome oxidase in vesicles was calculated after reduction by impermeant and permeant reducing agents as described under Experimental Procedures; the values are means of four different experiments, ±SEM. (b) Respiratory activity of oxidase vesicles measured as decreased under Experimental Procedures in the absence (○) and in the presence (●) of valinomycin *plus* nigericin; (◇) respiratory activity of soluble oxidase measured in the presence of 0.025% dodecyl β-D-maltoside. Mean values of two experiments.

Results

In the experiment presented in Figure 1, soluble cytochrome *c* oxidase was treated with succinic acid anhydride, which produces specific succinylation of lysine amino groups (Klapper & Klotz, 1972). This treatment caused inversion of the orientation in which the oxidase was incorporated in phospholipid vesicles. Reaction with 25 mM succinic acid anhydride decreased the percentage of the oxidase molecules incorporated right-side-out from 82% in control COV to 40% and increased the percentage of oxidase molecules incorporated in the vesicles in an inside-out orientation from 18% in the control to 60% (Figure 1a). Succinic acid anhydride caused depression of the respiratory rate both in the soluble and in the reconstituted state in the presence of uncouplers. In COV, stimulation of the respiratory rate in the absence of uncouplers (Figure 1b) was produced by succinic acid anhydride with consequent decrease of the respiratory control.

Succinylation of individual subunits was determined by labeling with [^{14}C]succinic acid anhydride. In the soluble enzyme, all the subunits of the oxidase were modified by succinic acid anhydride (Figure 2a). The largest radioactive labeling was observed in subunit IV for which 3 mol of lysine out of a total of 18 *per* mole of protein (Buse et al., 1987) was estimated to be modified after reaction with 20 mM succinic acid anhydride.

Effect of Succinylation of Lysine Residues in Reconstituted Oxidase. Figure 3 shows that treatment with succinic acid anhydride of cytochrome oxidase already reconstituted in

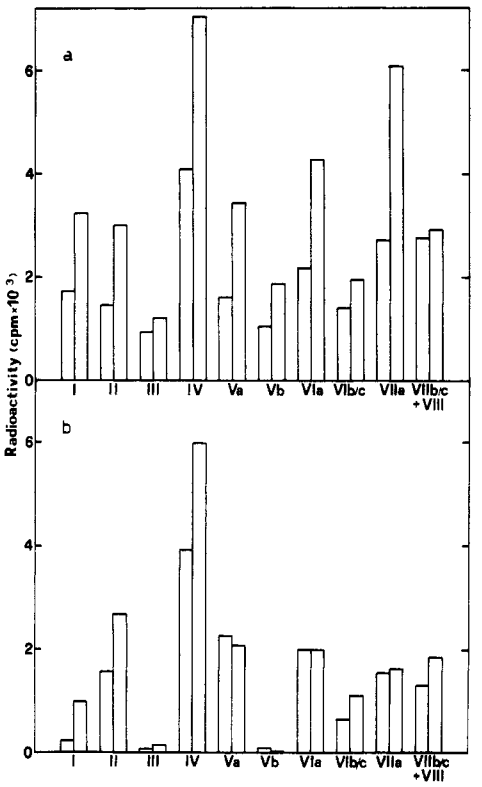

FIGURE 2: [^{14}C]Succinic acid anhydride labeling of soluble and reconstituted cytochrome *c* oxidase. Soluble enzyme (a) and cytochrome *c* oxidase vesicles were modified with 10 mM (left bar) and 20 mM (right bar) [^{14}C]succinic acid anhydride. Samples were run on SDS–polyacrylamide gels, the Coomassie-blue stained protein bands were cut out, and radioactivity was counted as described under Experimental Procedures. The radioactivity, given as cpm per band slices of 2 mm, was corrected for the background calculated from 2-mm slices of the gel without protein bands.

phospholipid vesicles had no significant effect on the incorporation of the oxidase in the vesicles. The respiratory activity of COV in the absence of ionophores was practically unaffected by the treatment which depressed, however, uncoupled respiration.

It has been reported that anions stimulate at low concentrations and cause almost complete inhibition at high concentrations of the redox activity in both soluble and reconstituted cytochrome *c* oxidase (Hüther et al., 1988). In Figure 4, the influence of COV treatment with succinic acid anhydride on the anion sensitivity of uncoupled respiration in reconstituted bovine heart oxidase is presented. The chemical modification did not suppress the inhibition of respiratory activity observed at high chloride concentrations, but less chloride was required to obtain the same degree of inhibition. However, modification abolished the stimulatory effect exerted on respiration by low chloride concentrations.

Proton translocation elicited by a ferrocytochrome *c* pulse of aerobic COV, supplemented with a saturating concentration of valinomycin plus K^+ (Casey et al., 1979), was inhibited after modification of the vesicles with succinic acid anhydride in a concentration-dependent manner (Figure 5). Treatment with 20 mM succinic acid anhydride decreased proton ejection from a H^+/e^- ratio of 0.78 ± 0.05 in the control [this is the value generally observed in bovine heart COV under the standard conditions used here, I = 0.1 M, pH 7.0; see Wikström and Saari (1977), Casey et al. (1979), and Papa et al. (1987 a,b)] to a H^+/e^- ratio of 0.30 ± 0.06 (Figure 5). The apparent $t_{1/2}$ for the decay of the acidification, either measured directly from the potentiometric traces or computed from first-order plots of the process, amounted at 20 °C to

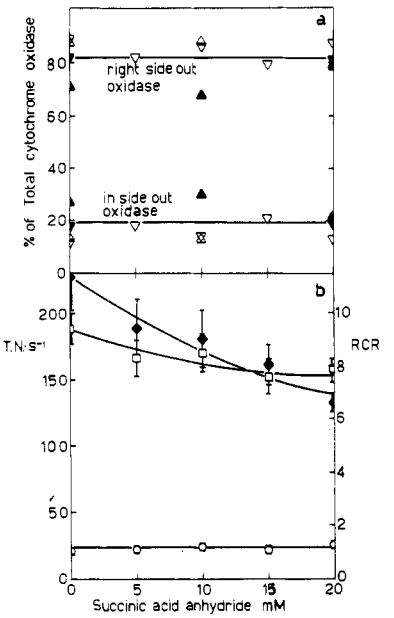

FIGURE 3: Effect of succinic acid anhydride modification of COV on the respiratory activity of cytochrome *c* oxidase. (a) The percentage of right-side-out and inside-out oriented cytochrome oxidase in vesicles was calculated as described under Experimental Procedures; each symbol refers to a different experiment. (b) Respiratory activity was measured as described under Experimental Procedures with 10 mM ascorbate *plus* 0.1 mM TMPD and 50 μM cytochrome *c* in the absence (○) and in the presence (□) of 1 μg/mL valinomycin + 3 μM CCCP; (♦) respiratory control ratio. The values are the means ± SEM of 9–13 different experiments.

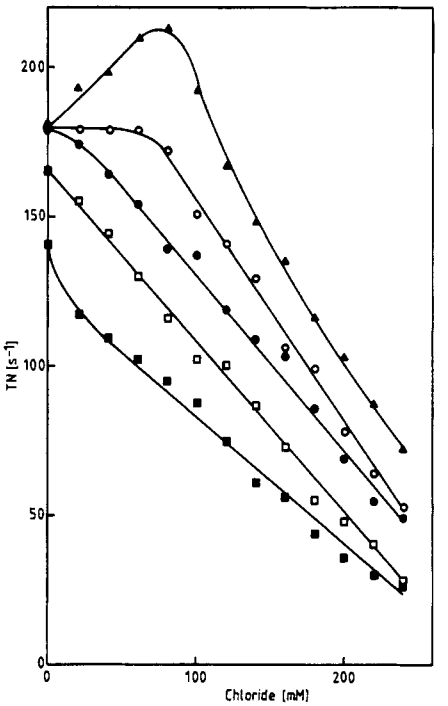

FIGURE 4: Effect of chloride on the activity of COV modified by succinic acid anhydride. The activity was measured polarographically in 10 mM K-HEPES, pH 7.5, 0.1 mM EDTA, 25 mM potassium ascorbate, 40 μM cytochrome *c*, and 0.005 μM reconstituted cytochrome oxidase with the given concentration of chloride, added as KCl, in the presence of 1 μg/mL valinomycin *plus* 3 μM CCCP. (▲) Control; (○) COV modified by 5 mM; (●) COV modified by 10 mM; (□) COV modified by 15 mM; (■) COV modified by 20 mM succinic acid anhydride.

10–15 s both in control and in treated COV. Furthermore, direct measurements showed that the rate of passive proton uptake induced by valinomycin-mediated K^+ efflux from COV,

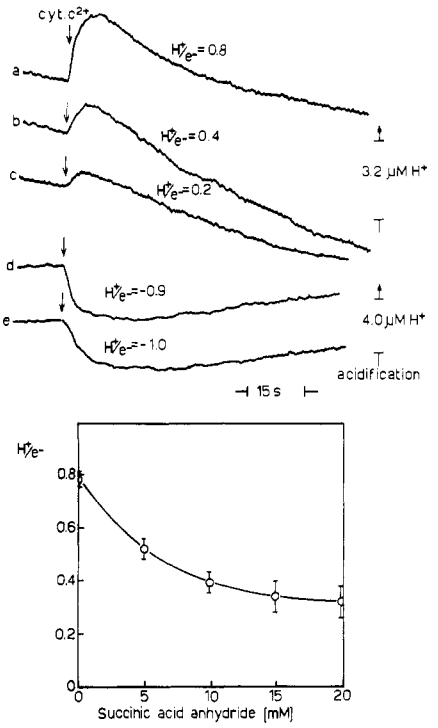

FIGURE 5: Effect of succinic acid anhydride modification of COV on proton translocation elicited by ferrocytochrome *c* pulses of aerobic vesicles. Vesicles (0.5 μM cytochrome oxidase) were suspended in 100 mM choline chloride, 0.1 mM EDTA, 5 mM KCl, pH 7.0, and 1 μg/mL valinomycin, temperature 25 °C. (a–c) Electrometric pH traces of COV pulsed with 4.25 μM ferrocytochrome *c*. (d, e) Traces of COV pulsed with 5.35 μM ferrocytochrome *c* in the presence of 3 μM CCCP. (a and d) Control COV; (b) COV treated with 10 mM succinic acid anhydride; (c and e) COV treated with 20 mM succinic acid anhydride. In the panel, the mean H^+/e^- ratios of 8–11 different experiments ± SEM for redox-linked proton ejection in coupled control and modified COV are presented.

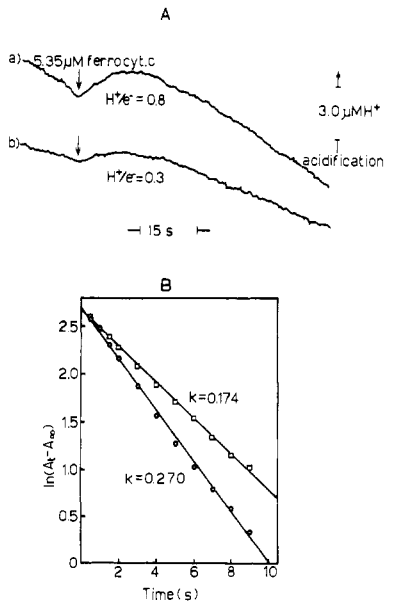

FIGURE 6: Effect of succinic acid anhydride modification on redox-linked proton ejection and kinetics of ferrocytochrome *c* oxidation in COV at 4 °C. The experimental conditions are those of Figure 5 with the only difference that the measurements were carried out at 4 °C. (A) Proton translocation elicited by pulses of 5.35 μM ferrocytochrome *c*: (a) control; (b) COV treated with 20 mM succinic acid anhydride. (B) Semilog plot of the kinetics of ferrocytochrome *c* oxidation monitored by a dual-wavelength spectrophotometer at 550–540 nm. (○) Control; (□) COV treated with 20 mM succinic acid anhydride.

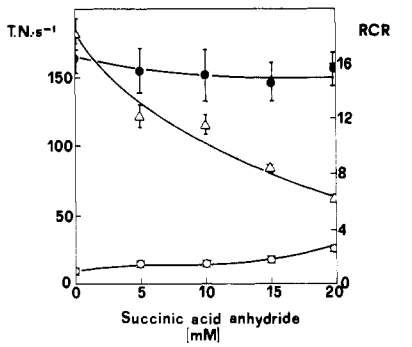

FIGURE 7: Effect of succinic acid anhydride modification on the control exerted by ΔpH on the respiratory activity in COV. The experimental conditions are those described in the legend of Figure 3. (○) Respiratory activity in the presence of 2 μg/mL valinomycin; (●) respiratory activity in the presence of valinomycin *plus* 2 μg/mL nigericin; (△) respiratory control ratio. The values are mean of 7–11 different experiments ± SEM.

in the absence of electron flow, was unaffected by succinic acid treatment. The expected direct proton consumption observed with ferrocytochrome *c* pulses of aerobic vesicles supplemented with CCCP was practically unaffected by treatment with succinic acid anhydride (Figure 5).

In the experiment of Figure 6, the incubation temperature was lowered to 4 °C, so that oxidation by aerobic COV of pulsed ferrocytochrome *c* could be followed spectrophotometrically. Treatment of COV with succinic acid anhydride practically resulted, at 4 °C, in the same extent of inhibition of proton translocation as that observed at 20 °C (Figure 6A). The apparent first-order constant of ferrocytochrome *c* oxidation was also lowered but to a much lower extent than proton translocation (Figure 6B).

The PMF generated by cytochrome *c* oxidation is expected to exert back-pressure on electron-transfer steps involved in the protonmotive activity of the oxidase. It has been found that $\Delta\psi$ exerts a controlling back-pressure on electron flow from cytochrome *c* to heme *a* and from the latter to heme a_3. ΔpH, and specifically the pH of the inner space, controls electron flow from heme *a* to heme a_3 [Capitanio et al., 1990; see also McGovern Moroney et al. (1984)].

In Figure 7, the effect of COV treatment with succinic acid anhydride on the specific control exerted by ΔpH on electron flow in the oxidase is shown. The steady-state respiratory activity supported by an excess of ascorbate *plus* TMPD and cytochrome *c* in the presence of valinomycin, i.e., under conditions in which proton translocation is measured and electron flow is controlled by ΔpH, was enhanced by chemical modification with succinic acid anhydride (about 80% stimulation at 20 mM succinic acid anhydride). The respiratory control exerted by ΔpH—measured by the enhancement of respiration produced by further addition of nigericin—was markedly depressed in the treated COV in a concentration-dependent manner similar to what was observed for the depression of redox-linked proton ejection.

Figure 8 shows the effects exerted by COV modification with succinic acid anhydride on the redox levels of cytochromes in turning over vesicles. Succinic acid anhydride treatment inhibited the rapid oxidation of cytochrome *c* induced by valinomycin addition to steady-state respiring COV and depressed the extra reduction of heme *a* which ensued in the interval in which $\Delta\psi$ was expected to be replaced by extra ΔpH. As a consequence of these effects, modification resulted in a specific depression of the steady-state reduction level of heme *a* in COV respiring in the presence of valinomycin (Figure 9). Also when ΔpH was abolished by further addition

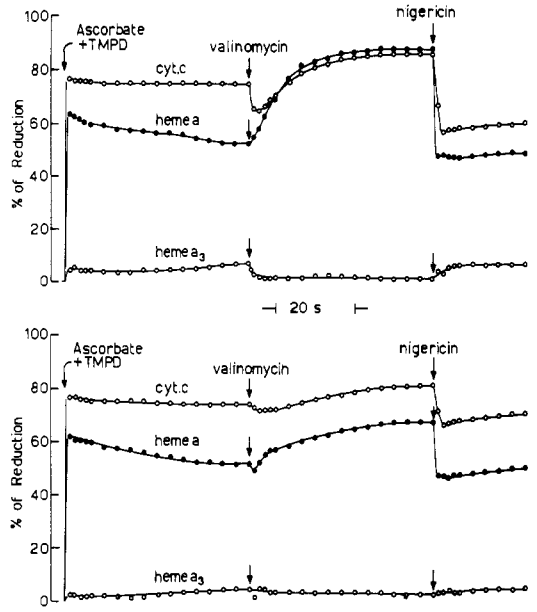

FIGURE 8: Effect of succinic acid anhydride modification on redox transitions of cytochromes caused by ionophores in steady-state respiring COV. COV (1 μM cytochrome oxidase) were suspended in 180 mM sucrose, 20 mM KCl, and 20 mM K-HEPES, pH 7.5, in the presence of 1 μM cytochrome *c*. Where indicated, 10 mM ascorbate *plus* 0.2 mM TMPD, 2 μg/mL valinomycin, and 2 μg/mL nigericin were added. Specific redox levels of hemes *a* and a_3 were calculated from the spectrophotometric traces as described under Experimental Procedures.

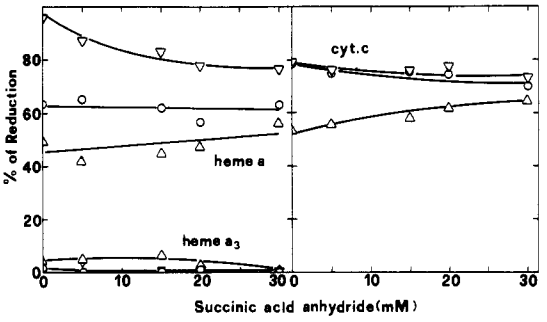

FIGURE 9: Effect of succinic acid anhydride on the steady-state redox levels of cytochrome *c* and hemes *a* and a_3 in respiring oxidase vesicles. The experimental details are those of Figure 8; the values are the average of three to four different experiments and refer to the steady-state redox levels reached in the presence of ascorbate *plus* TMPD (○), after the addition of valinomycin (▽), and in the presence of valinomycin *plus* nigericin (△).

of nigericin, chemical modification of COV with succinic acid anhydride did not affect the steady-state reduction level of heme *a* but that of cytochrome *c* was slightly enhanced (Figure 9).

Analysis of the labeling of the individual subunits of the oxidase with [^{14}C]succinic acid anhydride showed that the reagent produced succinylation of the enzyme also when added after its incorporation in the vesicles (Figure 2b). Reconstitution of the oxidase prevented succinylation of subunits III and Vb and depressed modification of the other subunits with the exception of subunits II and IV which were equally labeled by succinic acid anhydride in the soluble and reconstituted oxidase.

Discussion

Positively charged amino groups located at the surface of bovine heart cytochrome *c* oxidase are apparently critical for proper anisotropic incorporation of the enzyme in phospholipid vesicles. Treatment of the soluble oxidase with succinic acid anhydride resulted in modification of all the subunits of the enzyme. Succinylation of the free amino groups replaces the positively charged groups at the surface of the protein with negatively charged carboxylic groups. This change of surface charge from positive to negative caused inversion of the orientation of the reconstituted enzyme from right-side-out to inside-out. Thus, the anisotropic right-side-out insertion of the oxidase in the phospholipid membrane is critically governed by electrostatic interactions between positively charged free amino groups, essentially ε-amino groups of lysine residues, located at the surface of the oxidase and charged polar heads of phospholipids. This may explain why with the cholate dialysis method cytochrome *c* oxidase (Casey et al., 1979) and also cytochrome *c* reductase molecules (Papa et al., 1983) are essentially all right-side-out incorporated in phospholipids vesicles.

Subunit IV, which is the more abundant in lysine residues (Buse et al., 1987), is the one to be predominantly succinylated. However, all the subunits of the oxidase are modified in the soluble enzyme; thus, the present results do not allow us to specify which subunit is critically involved in the effects produced by succinic acid anhydride.

Reconstitution of the oxidase in phospholipid vesicles (80–90% right-side-out orientation) prevented succinylation of subunits III and Vb, and depressed that of the other subunits with the exception of subunits II and IV whose labeling was the same in the soluble and reconstituted oxidase [cf. McMillen et al. (1986)].

From the hydropathic profile and membrane folding, it has been predicted that no lysine of subunit III protrudes in the cytosol but that three lysines are exposed at the matrix side in this subunit (Wikström et al., 1985). This orientation is in fact directly supported by the absence of labeling of subunit III in the reconstituted enzyme. The present results furthermore suggest that intraliposomal amino groups are shielded from reactions with succinic acid anhydride. Thus, of the 6 lysine residues of subunit II and 18 lysines of subunit IV, only those located at the C side are accessible and reactive to succinic acid anhydride.

Modification by succinic acid anhydride of reconstituted oxidase produced a decoupling effect on proton translocation. The depression of proton ejection was in fact associated with enhancement of the steady-state respiratory rate in the presence of valinomycin, under which conditions net redox-linked proton ejection is maximally promoted and all of the PMF is present as transmembrane ΔpH. Analysis of the effects of the modification on the steady-state redox levels of cytochromes shows that the decoupling effect is exerted at the level of the pH-dependent rate-limiting step in intramolecular electron flow located on the oxygen side of heme *a* (McGovern Moroney et al., 1984; Thörnstrom et al., 1988; Capitanio et al., 1990). Modification by succinic acid anhydride also caused inhibition of the rapid Δψ-controlled electron flow from cytochrome *c* to heme *a* (see Figures 8 and 9).

Succinylation of COV abolished the activation effect exerted by anions on the redox activity of the oxidase. Evidently, positively charged groups in the oxidase, involved in anion binding (Hüther et al., 1987; Bisson et al., 1987), are modified by succinic acid anhydride. In particular, evidence has been presented indicating that ATP, under conditions in which it inhibits electron flow, binds to subunit IV and one of the smallest subunits of the oxidase (Montecucco et al., 1986). The fact that, in COV, labeling by succinic acid anhydride of subunits II and IV is predominant and increases with concentration, similarly to the decoupling of redox-linked

proton translocation, would indicate that this effect results from modification of one or both of these two subunits. The decoupling effect would result from disorganization of the anisotropic vectorial arrangement of proton translocation in the oxidase from the water phase to the protonmotive redox catalytic center. The present results do not allow us, however, to differentiate between a direct involvement of the modified residues in proton translocation and an indirect effect on enzyme conformation.

Registry No. Lys, 56-87-1; H^+, 12408-02-5; O_2, 7782-44-7; heme, 14875-96-8; cytochrome *c* oxidase, 9001-16-5.

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