*Hypothesis*

# Characteristics of redox-linked proton ejection in cytochrome *c* oxidase reconstituted in phospholipid vesicles

## New observations support mechanisms different from proton pumping

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Experimental observations reveal a number of characteristics of the redox-linked proton ejection from cytochrome *c* oxidase vesicles, which apparently cannot be explained by a proton pumping activity of the oxidase. These observations seem, on the other hand, to provide useful elements for alternative explanation(s) of the proton ejection. It is proposed here that the process is *scalar* and not *vectorial* and can derive from redox-linked rupture of protonated salt-bridges in the oxidase–lipid complex.

*Cytochrome oxidase vesicle* *Redox-linked proton translocation* *Redox proton-pump* *Redox-linked deprotonation* *Protolytic rupture, of salt-bridge*

## 1. INTRODUCTION

Aerobic oxidation of ferrocytochrome *c* by cytochrome *c* oxidase (EC 1.9.3.1) isolated from mitochondria [1–7] or certain bacteria ([8,9], cf. [10]) and reconstituted in phospholipid vesicles results in a rapid proton ejection. This reaction is widely considered as representing definite evidence for a proton pumping function of cytochrome oxidase ([3,4], cf. [11,12]).

Wikström et al. [3,13] and others (review [3]) obtained evidence for proton pumping by cytochrome oxidase also 'in situ' in the native membrane.

However, Mitchell et al. [12,14] and Papa et al. [11,15–17] have produced a number of observations which concur to show that cytochrome oxidase 'in situ' in coupling membranes does not function as a proton pump. These authors maintain [11,14] that in mitochondria, generation of transmembrane $\Delta\mu_{H^+}$ associated with aerobic oxidation of cytochrome *c* by the oxidase derives from the anisotropic arrangement of the reduction of dioxygen to $H_2O$, whereby electrons are donated by cytochrome *c*, located at the outer surface of the membrane, and protons derive from the matrix aqueous phase [18,19]. This being the case, the redox-linked proton ejection observed in cytochrome oxidase vesicles would not be related to the process of transmembrane $\Delta\mu_{H^+}$ generation by the enzyme 'in situ' in the native membrane. Such a possibility will undoubtedly be strengthened if alternative explanation(s) for the proton ejection in cytochrome *c* oxidase can be found.

This paper analyses experimental observations, from this and other laboratories, which reveal a number of characteristics of the redox-linked proton ejection in oxidase vesicles which apparently

*Abbreviations:* DCCD, *N,N'*-dicyclohexylcarbodiimide; FCCP, carbonyl-cyanide-*p*-trifluoromethoxyphenylhydrazone; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EDTA, ethylenediaminetetracetic acid

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cannot be explained by a proton pumping activity. These characteristics provide, on the other hand, useful elements for different explanations of the process. It is proposed that proton ejection is *scalar* and not *vectorial* and derives from redox-linked rupture of protonated salt-bridges in the oxidase–lipid complex involving carboxylic group(s), modified by DCCD (cf. [7]).

## 2. EXPERIMENTAL OBSERVATIONS

### 2.1. *Reductant pulses*

#### 2.1.1. General characteristics

The general characteristics of proton ejection induced in cytochrome oxidase vesicles by ferrocytochrome *c* pulses, as reported [1–9] and reproduced by us (fig.1), can be summarized as follows:

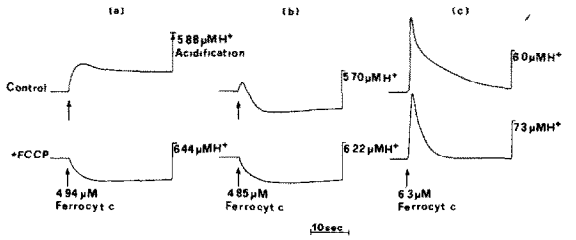


Fig.1. Proton translocation induced by ferrocytochrome *c* pulses in aerobic suspension of cytochrome oxidase vesicles. Cytochrome oxidase, containing 10–12 nmol hemes $a + a_3$/mg protein, was prepared from beef-heart mitochondria [20]. Cytochrome oxidase vesicles were prepared as in [5]. Valinomycin (1 μg/ml) plus FCCP (3 μM) stimulated respiration around 10 times, measured polarographically in 40 mM KCl, 10 mM HEPES (pH 7.4), 0.1 mM EDTA, 25 mM ascorbate, 15 μM cytochrome *c* and 15 μl vesicles (0.05 nmol heme $a + a_3$) in 3.25 ml. Ferrocytochrome *c* was prepared from ferricytochrome *c* (Sigma, type VI) as in [5]. Proton translocation was measured electrometrically [21]. In expt. (a) and (c) 0.17 ml vesicles (0.5 μM oxidase) and in expt. (b) 0.085 ml vesicles were suspended in: 100 mM choline–Cl, 0.2 mM choline–HEPES, 0.1 mM choline–EDTA, 5 mM KCl, 1 μg valinomycin/ml (pH 7 ± 0.01); final vol. 1 ml. In expt. (c) choline–Cl was replaced by 300 mM sucrose. Where added, FCCP was 3 μM. Statistical analysis for 49 expt (a) gave a mean value for proton ejection/cytochrome *c* oxidized of 0.81 (± 0.02) and $H^+/e^-$ ratio for proton uptake, in the presence of FCCP, of 0.83 (± 0.02, 39 expt).

(i) The addition of an amount of ferrocytochrome *c* sufficient for 2–3 turnovers, results, in a well-coupled system (respiratory control around 10), in the presence of saturating concentrations of valinomycin and $K^+$ and ionic strength of the medium of $10^{-1}$, in the rapid ejection of protons at an $H^+/e^-$ stoicheiometry that can vary, at pH 7.0, from 0.5–1.0 (see fig.1, [2]).

(ii) The $H^+/e^-$ ratio varies with the concentration of cytochrome oxidase vesicles. Values of around 1 are found with 0.5 μM oxidase (49 expt as in fig.1a, gave in our hands a mean value of 0.81 ± 0.02) (cf. [2,5]). At this concentration of oxidase the acidification process is quite persistent (cf. [2,5]). At lower oxidase concentrations the acidification is smaller and is followed by a net alkalinisation, as expected from proton consumption in the reduction of dioxygen to $H_2O$. However, this alkalinisation is smaller than the amount of ferrocytochrome *c* added and oxidized (fig.1b) (cf. [2,5]).

(iii) In the presence of saturating concentrations of FCCP the addition of ferrocytochrome *c* results, in the experiments at high ionic strength, in direct proton consumption [1–9]. Statistical analysis of the values for the $H^+/e^-$ ratio measured by us in 39 expt (fig.1a) gave a mean value of 0.83 ± 0.02.

#### 2.1.2. Effect of ionic strength

Proton ejection induced by ferrocytochrome *c* pulses is affected by the ionic strength of the medium [2,22]. At low ionic strength ($6.2 \times 10^{-3}$) the addition of ferrocytochrome *c* results in a much larger acidification than that observed at ionic strength $1.06 \times 10^{-1}$ (fig.1c, fig.2). This acidification, which apparently amounts to 2 $H^+/e^-$, decreased rapidly to the baseline. Thus 33% of the reaction is irreversible and compensates for net proton consumption in the aerobic oxidation of ferrocytochrome *c*. The transient acidification observed under these conditions is practically insensitive to FCCP. As the ionic strength of the medium is enhanced (fig.2), the $H^+/e^-$ ratio for proton release in the absence of FCCP progressively decreases, the reaction becomes more depressed by FCCP and, concomitantly, the irreversible fraction of the acidification diminishes so that the $H^+/e^-$ ratio for

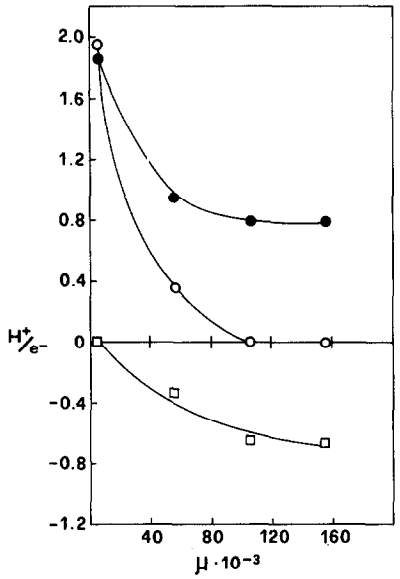


Fig.2. Effect of ionic strength on proton translocation induced by ferrocytochrome *c* pulses in aerobic cytochrome oxidase vesicles. The vesicles (0.5 $\mu$M cytochrome oxidase) were suspended in: 0.2 mM choline–HEPES, 0.1 mM choline–EDTA, 5 mM KCl, 1 $\mu$g valinomycin/ml and different concentrations of sucrose and choline–Cl providing an osmolarity of 0.3 and the ionic strength shown on the abscissa. The vesicles suspension was pulsed with 6.3 $\mu$M ferrocytochrome *c*; (●—●) $H^+/e^-$ ratio for proton release in the absence of FCCP; (○—○) $H^+/e^-$ ratio for transient proton release in the presence of FCCP (see fig.1c); (□—□) $H^+/e^-$ ratio for net proton uptake in the presence of FCCP. The $H^+/e^-$ ratios for $H^+$ release were obtained from the peak of the transient acidification process. For other details see fig.1.

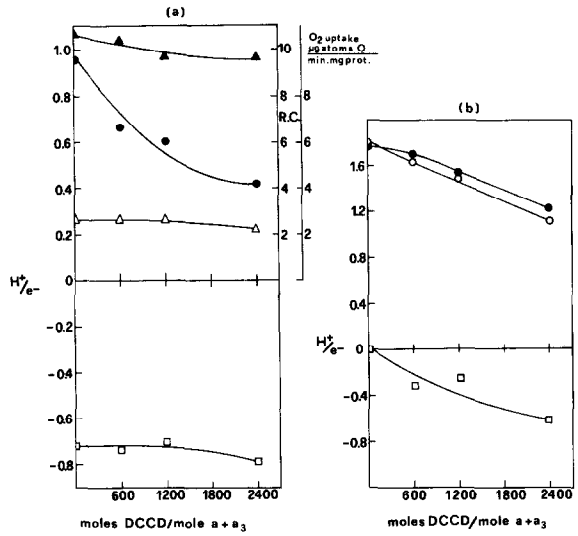


Fig.3. Effect of DCCD on respiratory activity and proton translocation in cytochrome oxidase vesicles. Vesicles (0.5 $\mu$M cytochrome oxidase) were treated with DCCD, at the concentrations shown, for 2 h at 15°C. In experiments of fig.3(a) the medium was that of expt. (a) and (b) of fig.1 (ionic strength $1.06 \times 10^{-1}$). In experiments of fig.3b the medium was that described for fig.1(c) (ionic strength $6 \times 10^{-3}$). The concentration of ferrocytochrome *c* was 6 $\mu$M; (●—●) $H^+/e^-$ ratio for proton release in the absence of FCCP; (○—○) $H^+/e^-$ ratio for transient proton release in the presence of FCCP; (□—□) $H^+/e^-$ ratio for net proton uptake in the presence of FCCP. For other details see fig.1 and 2; (△—△) respiratory rate in the absence of FCCP and valinomycin; (▲—▲) respiratory control.

proton uptake in the presence of FCCP tends to increase.

### 2.1.3. Effect of DCCD

Pre-treatment of cytochrome oxidase vesicles with DCCD results in a depression of the redox-linked proton ejection [7,23]. Fig.3a shows the effect of DCCD on proton release induced by ferrocytochrome *c* pulses of the vesicles in a medium of ionic strength $1.06 \times 10^{-1}$. Increasing concentrations of DCCD cause progressive inhibition of proton ejection. This is accompanied by a small enhancement of the $H^+/e^-$ ratio for proton consumption in the presence of FCCP. It is noteworthy that, under the conditions used, DCCD has no significant effect on the rate of electron flow measured at the steady-state either in the coupled or in the uncoupled state (cf. [7]).

Fig.3b shows that DCCD inhibits also the acidification reaction elicited by ferrocytochrome *c* pulses of vesicles in a medium of low ionic strength ($6 \times 10^{-3}$). This inhibition is accompanied by a depression of the irreversible part of the acidification reaction, so that in the presence of FCCP, as the concentration of DCCD is increased, net proton uptake progressively becomes more apparent. Thus, DCCD inhibits the irreversible scalar acidification, contributing to proton release elicited by ferrocytochrome *c* pulse.

2.1.4. pH dependence and valinomycin requirement (fig.4)

Proton translocation elicited by ferrocytochrome *c* pulses varies with pH. In coupled vesicles, supplemented with valinomycin plus $K^+$, there is low proton ejection at pH 6. The acidification becomes larger as the pH of the medium is raised and reaches a maximum of 1.1 $H^+/e^-$ at pH 7.7. Controls show that at acidic pH-values there is no change in the rate of electron flow in the coupled state neither in the passive proton conduction caused by valinomycin-induced $K^+$ discharge from the vesicles, as compared to neutral pH-values.

In the absence of valinomycin the addition of ferrocytochrome *c* results at pH 6.0 in a direct proton consumption, stoicheiometric with the reductant added. As the pH of the medium is raised net proton consumption starts to be obscured by an acidification process, until, at pH 7.7, there is a net transient acidification amounting to an $H^+/e^-$ ratio of 0.4. Both in the presence and absence of valinomycin, the acidification process shows an apparent p*K* around 6.3. Proton consumption measured in the presence of FCCP is, at pH 6.0, practically stoicheiometric with added ferrocytochrome *c*. However, as proton ejection in the coupled state augments with pH, there appears a significant decrease in the $H^+/e^-$ ratio for proton consumption.

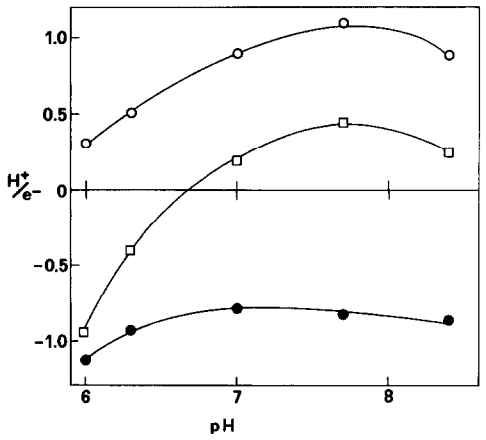


Fig.4. pH dependence of proton translocation elicited by ferrocytochrome *c* pulses of aerobic cytochrome oxidase vesicles. The experimental conditions are those of fig.1a; (○—○) $H^+/e^-$ ratio for proton release in the presence of valinomycin; (□—□) $H^+/e^-$ ratio for proton uptake (pH 6.0 and pH 6.3) and transient proton release (higher pH-values) in the absence of valinomycin; (●—●) $H^+/e^-$ ratio for proton uptake in the presence of valinomycin plus FCCP.

## 2.2. *Oxygen pulses*

It is interesting to examine the behaviour of oxidase vesicles when electron flow is activated by oxygenation of the reduced anaerobic enzyme. In this case, the enzyme is converted in one turnover to the pulsed active state [24]. This is functionally [24] and structurally [25] different from the oxidized or resting enzyme, which is the condition exploited in the reductant pulse experiments so far reported.

In the oxygen pulse experiments, oxidase vesicles are made anaerobic by a flux of argon. Oxidation of added ferrocytochrome *c* by a pulse of oxygen causes a transient acidification with an $H^+/e^-$ ratio of 0.29 (fig.5a-2). This small acidification is followed by net proton consumption with an

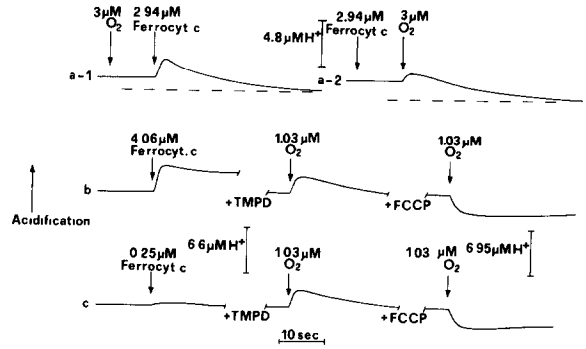


Fig.5. Proton translocation elicited by ferrocytochrome *c* or oxygen pulses in cytochrome oxidase vesicles. The vesicles were suspended in the medium of fig.1a,b at 0.3 μM (a) and 0.5 μM oxidase (b,c). Fig.5a-1. The aerobic system was pulsed with ferrocytochrome *c*. Fig.5a-2. Vesicles, made anaerobic with an argon flux, were first pulsed with deoxygenated ferrocytochrome *c* and then with $O_2$ as air-saturated medium. In expt 5b and 5c the aerobic vesicles suspension was first pulsed with ferrocytochrome *c*, then the system was made anaerobic with a flux of argon, supplemented with 200 μM deoxygenated TMPD and pulsed with oxygen in the absence and then in the presence of 3 μM FCCP. The direct alkalinisation observed in the presence of FCCP exhibited an $H^+/e^-$ ratio of 0.7, which, when corrected for proton release in the conversion of $TMPDH^+$ to $TMPD^+$, approached unity.

$H^+/e^-$ ratio of 0.65, which, with the addition of the initial acidification, amounts to 0.94. This has to be compared with a reductant pulse which results, with the same vesicles and amount of ferrocytochrome *c*, in proton ejection with an $H^+/e^-$ ratio of 0.60. The acidification is followed, in this experiment, by net alkalinisation, amounting to an $H^+/e^-$ ratio of 0.45, without including the initial acidification, and 1.05 when this is included.

Fig.5b shows an experiment in which aerobic vesicles, after being pulsed with 4 $\mu$M ferrocytochrome *c* (proton release with an $H^+/e^-$ of 0.9) are made anaerobic and then supplemented with TMPD. The addition of an amount of oxygen corresponding to 4 nequiv. $e^-$ causes a transient acidification. In this system the ultimate reductant of oxygen is TMPD, which prevents net oxidation of ferrocytochrome *c*. Correction for protons released directly in the conversion of TMPD to its oxidized form [15] gives an $H^+/e^-$ ratio of 0.23. The same ratio is found when added ferrocytochrome *c* is 0.25 $\mu$M.

## 3. EXPLANATION OF THE EXPERIMENTAL DATA

### 3.1. *Can proton pumping explain the experimental data?*

If reduction of dioxygen by ferrocytochrome *c* results in a vectorial proton translocation, the ultimate net result of the redox reaction, once electron flow is terminated and translocated protons are re-equilibrated across the membrane, has to be 1-to-1 proton consumption with respect to ferrocytochrome *c* added and oxidized according to:

$$4\ \text{cyt.}c^{2+} + O_2 + 4\ H^+ \longrightarrow 2\ H_2O + 4\ \text{cyt.}c^{3+} \quad (1)$$

It is, however, generally found that in the coupled state and relatively high concentration of oxidase vesicles (~0.5 $\mu$M oxidase) the acidification process caused by ferrocytochrome *c* pulses is quite persistent (see fig.1a) ([2,5]; cf. [12]). At low oxidase concentration the acidification is reversible to a variable extent, but generally not completely, so that it obscures part of the scalar proton consumption expected from reaction (1)

It has been reported [1–6] that, in the presence of saturating concentrations of FCCP, proton consumption is practically equivalent to ferrocytochrome *c* added and oxidized. We have, however, obtained from a large number of experiments a mean value of 0.83 ± 0.02 for the $H^+/e^-$ ratio in the presence of FCCP and high ionic strength which is significantly different from one. Thus the acidification process is in part irreversible, although to a smaller extent, also in the uncoupled state. The question arises whether all of the proton ejection may represent a scalar reaction, rather than a vectorial, energy conserving process, which is more or less reversible depending upon the prevailing experimental conditions.

Such a possibility seems to be supported by the following:

(i) The extent of the redox linked acidification diminishes whilst its reversibility and degree of sensitivity to FCCP increase continuously as the ionic strength of the medium is enhanced until, at the ionic strength routinely used to demonstrate redox-linked acidification, the usual values are found (see fig.2) (cf. [2,22]). Thus it seems difficult to accept the view [2] that, whilst at low ionic strength the interaction of cytochrome *c* with the vesicles leads to a net production of protons, this deprotonation could be excluded at relatively high ionic strength, where the proton ejection would only represent transmembrane proton pumping. Rather, it seems likely that the proton release observed at high ionic strength is what remains of a scalar deprotonation maximal at low ionic strength.

(ii) Under the conditions of fig.3a, DCCD depressed proton ejection induced by ferrocytochrome *c* pulses, leaving the steady-state electron flow in the coupled and uncoupled state unaffected; i.e., the respiratory control was unaffected. Others have reported inhibition by DCCD of the rate of electron flow in the uncoupled state under conditions where the acidification reaction was inhibited [7,23]; we also obtained similar results using different preparations of cytochrome oxidase and vesicles. However, it is evident that if the redox-linked proton ejection represents a vectorial process primarily associated with energy conservation, the proton ejection should not be inhibited unless electron transport is either decoupled (enhancement of the rate of electron flow in the coupled state), or inhibited (depression of the uncoupled rate of electron

flow). It should be noted that DCCD also inhibits the redox-linked scalar acidification process observed at low ionic strength (fig.3b).

(iii) The proton ejection elicited by ferrocytochrome *c* pulses of the oxidase vesicles requires $\Delta\psi$ collapsing agents [1,2,5]. The supporters of a proton pumping activity of the oxidase consider this as a strong piece of evidence in favour of electrogenic proton pumping [2–9]. However, it should be recalled [12] that generation of transmembrane $\Delta\mu_{H+}$ by electron conduction from the outer side of the membrane to protons from the inner aqueous phase [18], corresponds to the effective translocation from inside to outside of one positive electrical charge/$e^-$. In the absence of a charge-compensating flow of other positive ions up to 1 $H^+/e^-$ could be pulled from the outer to inner space and this could obscure redox-linked proton dissociation at the outer surface.

The experiments on the pH dependence of redox-linked proton ejection support this explanation. At pH 6.0, where in the presence of valinomycin the ferrocytochrome *c* pulse resulted only in a small proton release, a direct proton consumption of around 1 $H^+/e^-$ was observed in the absence of valinomycin. As, by increasing pH net acidification became larger in the presence of valinomycin, in the absence of valinomycin, a short-fall in proton consumption appeared, followed by a small transient acidification at pH > 7. Thus there occurs, in the absence of valinomycin, a proton release which is clearly scalar and corresponds to the acidification measurable in the presence of valinomycin.

### 3.2. *Can alternative explanations be offered?*

In [12], Mitchell and Moyle concluded that the redox-linked proton ejection in cytochrome oxidase vesicles may not be attributable to proton pumping. They have proposed, as an explanation, that oxidation of ferro- to ferricytochrome *c* results in a scalar deprotonation of a complex, at the surface of the vesicles, of cytochrome *c* with proton-binding phospholipids.

Our data provide evidence that the proton ejection is, indeed, *scalar* and not *vectorial* and may derive from redox-linked rupture of protonated complexes. The present results, and other related observations, indicate, however, that the conversion of ferro- to ferricytochrome *c* is not the primary cause of the acidification reaction. In fact:

(i) Oxidation of ferro- to ferricytochrome *c* by ferricyanide in oxidase vesicles treated with azide does not cause significant acidification comparable to that observed upon aerobic oxidation of ferrocytochrome *c* by the vesicles (observation reported in [5] and confirmed by us).

(ii) Net oxidation of ferrocytochrome *c* (at concentrations equivalent to those added in the reductant pulse) by oxygenation of anaerobic vesicles results in a proton release amounting only to one third of that observed in the reductant pulse (see fig.5a).

(iii) A pulse of oxygen in the amount needed to produce electron flow equivalent to that induced in the reductant pulse, causes, in vesicles supplemented with an excess of TMPD, a proton ejection which amounts to around one third of that observed in the reductant pulses independently from the concentration of cytochrome *c* added. It should be recalled that the excess of TMPD prevents any net oxidation of ferrocytochrome *c*.

### 3.3. *Redox-linked rupture of salt-bridges as a possible source of proton ejection*

We propose that the acidification process results primarily from rupture of protonated salt-bridges in the cytochrome oxidase–lipid complex, as a consequence of changes in the tertiary and quaternary structure of the oxidase as the enzyme changes from the inactive to the active state. The oxidase is made up of a large number of polypeptides [26], many of which protrude outside of the phospholipid surface [27]. It is feasible that a number of salt-bridges can be formed between ionizable amino acid residues of the oxidase polypeptides (cf. [28]) or between these residues and ionizable groups of phospholipids. The oxidase apparently undergoes protolytic [28] changes in its conformation during functional transitions [25]. In this context, it is noteworthy that the acid reaction appears to be smaller when the enzyme starts from the reduced state than from the oxidized state.

It is feasible that one group involved in the salt-bridge is the carboxylic group modified by DCCD

(glutamic residues [7,23]). The experience of oxygen Bohr-effects in hemoglobin [29] has shown that electrostatic engagement of a protolytic with a carboxylic group results in enhancement of its p$K$ and proton binding. Inversely, when the salt-bridge is broken the p$K$ is lowered and proton release takes place.

Deprotonation of the salt-bridges leaves extra negative charges on the membrane which can attract lipophilic cations. This could explain the extra-uptake of $K^+$ in the presence of valinomycin or of lipophilic cations reported to occur in excess of 1 charge/$e^-$ flowing from cytochrome $c$ to oxygen [6].

The p$K$ of 6.3 observed here for the acidification process in oxidase vesicles may refer to the proton-releasing group in the salt-bridge. We are now attempting to verify a possible role of other amino acid residue and/or phospholipids (cf. [12]) in the redox-linked proton ejection in oxidase vesicles. It is likely that when the salt-bridge opens up, the protolytic group(s) becomes superficially exposed in the vesicles. Inversely, when, in the resting state, the salt-bridge closes up, the ionizable group could become embedded in the membrane. The degree of superficial exposure of the ionizable group and the presence of protonophoric substances (cf. [12]) would determine the extent of reversibility of the proton ejection.

This examination seems to exclude the possibility that cooperative proton transfer reactions result in proton pumping by cytochrome oxidase. However, cooperative proton transfer in redox enzymes may play a role in their redox and proton-motive function [19,30–32]. It could be involved in proton transfer from the inner aqueous phase to the catalytic center of cytochrome oxidase for protonation of reduced oxygen to $H_2O$ [32] or in asymmetric protonation–deprotonation of protein-bound quinone systems supposedly involved in transmembrane proton translocation in the $b$–$c_1$ complex [31,32].

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