MEASUREMENTS OF THE COMPONENTS OF THE PROTONMOTIVE FORCE GENERATED BY CYTOCHROME OXIDASE IN SUBMITOCHONDRIAL PARTICLES

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1. Introduction

There are two conflicting views as to the mechanism of the mitochondrial cytochrome oxidase reaction within the general context of the chemiosmotic hypothesis. The original model of Mitchell visualises all the components of the cytochrome *c* to oxygen segment of the mitochondrial respiratory chain as mediators of only vectorial electron transport [1–3], while in a more recent proposal cytochrome oxidase is suggested to pump protons across the mitochondrial membrane with a stoichiometry of one proton translocated per electron passing through the oxidase molecule [4,5]. According to the latter view, but not to the former, in submitochondrial particles the donation of reducing equivalents to cytochrome *c* by means of ascorbate plus the redox couple *N,N,N',N'*-tetramethyl-*p*-phenylene-diamine / Wurster's Blue (TMPD / WB^+) in the presence of antimycin A, should generate both a membrane potential ($\Delta\psi$, positively charged inside) and a pH gradient (ΔpH, acid inside) as a result of the cytochrome oxidase proton pump activity [4–7], but contrast [8].

We have reported [7] the generation in submitochondrial particles, resuspended in a low osmolarity P_i/Tris medium, of a membrane potential of approx. 100–120 mV with ascorbate plus TMPD. However we were unable to detect a pH gradient even with the permeant NO_3^- ion present to maximise the ΔpH component of the protonmotive force.

We report here with ascorbate plus TMPD as substrate:

1. The detection of both a pH gradient of approx. 1.5 units and a membrane potential of approx. 100 mV in submitochondrial particles incubated in a high osmolarity medium containing 50 mM KCl.

2. That a small pH gradient has now been detected in submitochondrial particles suspended in the P_i/Tris medium.

These data reinforce the view that cytochrome oxidase is proton translocating [4–7].

2. Materials and methods

$^{14}CH_3NH_2$ and $S^{14}CN^-$ distributions across the membrane of submitochondrial particles for the ΔpH and $\Delta\psi$ determinations were followed by means of the flow-dialysis technique, as detailed in [7]. Further additional reagents and the protein concentration in each experiment are given in the legends.

ATP-Mg bovine heart submitochondrial particles, prepared following the method in [9], were suspended in either of the two reaction mixtures used in the flow-dialysis experiments, a low osmolarity one composed of 10 mM P_i/Tris, 5 mM magnesium acetate, pH 7.3, and a high osmolarity one composed of 200 mM sucrose, 10 mM Na^+Hepes, 2 mM $MgCl_2$,

Abbreviations: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylene-diamine; WB^+, Wurster's Blue; $\Delta\psi$, membrane potential; ΔpH, pH gradient; Δp, protonmotive force; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-ethanesulphonate; NEM, *N*-ethyl-maleimide; FCCP, carbonylcyanide *p*-trifluoromethoxy-phenylhydrazone

5 mM potassium phosphate and 50 mM KCl, pH 7.5. The antimycin A concentration used in each experiment was the minimum amount necessary to give maximal inhibition of succinate oxidation, as routinely checked at the oxygen electrode. Protein concentration was determined with the biuret method [10] and respiratory rates were determined with a Clark-type O_2-electrode.

All the experiments were performed at room temperature.

The values of the internal volume of the particles used for calculating the magnitude of $\Delta\psi$ and ΔpH were 1.3 μl/mg protein in the P_i/Tris buffer and 1 μl/mg protein in the sucrose/Hepes/KCl medium, as determined [7].

TMPD.2 HCl (BDH Ltd, Poole, Dorset) was recrystallised from ethanol. Solutions of TMPD were made immediately before use (unless indicated otherwise) and titrated to pH 7.0 with NaOH. (Solutions of TMPD that were stored at -20°C for more than approx. 1 month were observed to darken in colour although their activity did not appear to alter.) Poly(L-lysine) hydrobromide (Type 1-B, mol. wt 70 000) was obtained from Sigma. All other reagents were of the highest grade commercially available.

3. Results

Submitochondrial particles, suspended in the sucrose/Hepes/KCl medium and respiring with ascorbate plus TMPD as substrate, generated a substantial ΔpH as shown by the efflux of $^{14}CH_3NH_2$ from the particles upon addition of an uncoupler (fig.1). Table 1 reports the values of both $\Delta\psi$ and ΔpH in the same medium. The detection of both the membrane potential and pH gradient in this buffer, also used in [7,11], is probably a result of movement of Cl^- into the particle lumen, thus increasing ΔpH at the expense of

Table 1
Magnitudes of $\Delta\psi$ and ΔpH linked to oxidation of ascorbate plus TMPD in sucrose/Hepes/KCl medium

Additions	60 ΔpH (mV)	$\Delta\psi$ (mV)
None	85	100
Valinomycin	50	45
NEM	85	105

Estimates of $\Delta\psi$ and ΔpH were obtained from flow-dialysis measurements as described in [7]. The reaction mixture contained, in final vol. 1 ml, 200 mM sucrose, 10 mM Na^+Hepes, 2 mM $MgCl_2$, 5 mM potassium phosphate, 50 mM KCl pH 7.5, and submitochondrial particles (9.75 mg protein and 5.85 mg protein for determination of ΔpH and $\Delta\psi$, respectively). The suspension was pre-incubated for 5 min at room temperature where NEM (0.37 mM) or valinomycin (0.48 μg/mg protein) were present. Antimycin A was always present at a concentration of 0.15 μg/mg protein. Sodium D-iso-ascorbate (20 mM) and TMPD (0.1 mM) were added immediately prior to the addition of 20 μM $^{14}CH_3NH_2$.HCl or 20 μM $KS^{14}CN$. A water-saturated stream of oxygen was blown over the surface of the reaction mixture throughout the experiments. FCCP was added approx. 7.5 min after the addition of $KS^{14}CN$ or 10 min after the addition of $^{14}CH_3NH_2$ to final conc. 5 μM. ΔpH and $\Delta\psi$ values were calculated, respectively from

$$\log \frac{[^{14}CH_3NH_2]_{in}}{[^{14}CH_3NH_2]_{out}} \text{ and } \log \frac{[S^{14}CN^-]_{in}}{[S^{14}CN^-]_{out}}$$

as detailed in [7]

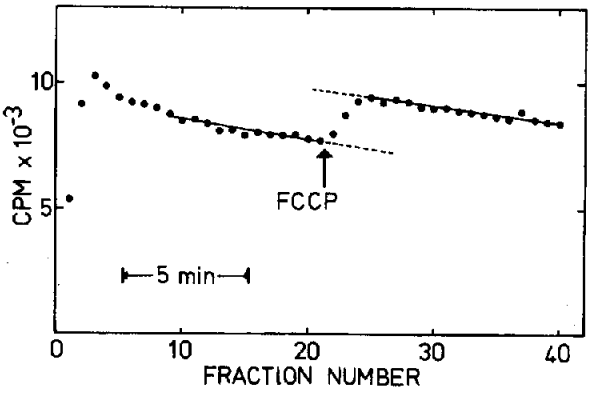

Fig.1. Ascorbate plus TMPD-dependent uptake of $^{14}CH_3NH_2$ by submitochondrial particles as determined by flow-dialysis. The upper chamber of the flow-dialysis cell [7] contained, in final vol. 1 ml, 20 μM $^{14}CH_3NH_2$.HCl (55.5 μCi/μmol), 20 mM sodium D-iso-ascorbate, 0.1 mM TMPD, 200 mM sucrose, 10 mM Na^+Hepes, 2 mM $MgCl_2$, 5 mM potassium phosphate, 50 mM KCl, pH 7.5, 1.5 μg antimycin A and submitochondrial particles (9.75 mg protein). 5 μM FCCP was added 10 min after the experiment had been started by adding $^{14}CH_3NH_2$ and initiating the collection of fractions from the flow-dialysis cell. The pH of the suspending medium changed by less than 0.1 unit during ascorbate oxidation.

$\Delta\psi$. The addition of the ionophore valinomycin, which should induce a greater expression of the chemical component of the protonmotive force [12], failed to raise the pH gradient (table 1); in fact the total protonmotive force value decreased by 50% with respect to the one obtained in the absence of the ionophore, although ΔpH was less affected than $\Delta\psi$. Comparative experiments performed in the presence and absence of *N*-ethylmaleimide (NEM) showed no variation in the resulting protonmotive force (table 1). NEM was added as an inhibitor of the P_i-carrier in an attempt to prevent any perturbation of ΔpH by P_i movements [5]. The lack of effect of NEM seems to indicate that either no P_i movement occurs, or NEM cannot react with the inhibitor-sensitive site of the asymmetrically orientated P_i-carrier because of restricted permeability of NEM, as suggested in [13] (but see [14]). NEM treatment had no effect on the rate of ascorbate plus TMPD oxidation.

We mentioned [7] how the magnitude of the membrane potential was subject to variability when submitochondrial particles, suspended in a P_i/Tris buffer, were respiring on ascorbate plus TMPD. Further experiments (table 2), performed under similar conditions except for the presence of NEM, confirmed that indication, the value of $\Delta\psi$ varying from 85–130 mV. The estimation of the pH gradient in P_i/Tris is more difficult, its value being frequently below the sensitivity of the flow-dialysis technique for an accurate and reliable estimation (for detailed discussion see [7]). However in some instances a pH gradient close to 30 mV could be detected (table 2). More suitable conditions were sought under which the pH gradient could be fully expressed at the expense of the membrane potential. As already reported [7] with NADH as substrate, the inclusion of 2 mM NO_3^- in the P_i/Tris reaction mixture resulted in the appearance of a substantial ΔpH at the expense of $\Delta\psi$. However, as shown in table 2, the addition of either 2 mM or 5 mM NO_3^- did not increase the magnitude of ΔpH when ascorbate plus TMPD was substrate.

The basis of the experiments reported in the present paper was that TMPD acted solely as an electron carrier from ascorbate outside the particles to cytochrome *c* which is located inside submitochondrial particles. Therefore it was important to exclude the possibility that ascorbate itself could permeate the membrane and reduce cytochrome *c* directly, as such ascorbate oxidation would release protons inside. The submitochondrial particles were found to respire with ascorbate alone at approx. 5% of the rate when 100 μM TMPD was added as well. This TMPD-independent respiration was not inhibited by poly(L-lysine) at a

Table 2
Magnitudes of $\Delta\psi$ and ΔpH linked to TMPD oxidation in P_i/Tris medium

Exp.	[TMPD] (μM)	Additions	$\Delta\psi$ (mV)	ΔpH (mV)
1a	100	none	100	35
1b	100	NO_3^- 2 mM	90	40
2a	300	none	130	< 30
2b	300	NO_3^- 5 mM	90	< 30
3a	100	none	85	⩽ 30
3b	5000	none	60	40

All the experiments were performed in a flow-dialysis apparatus at room temperature. The reaction mixture contained, in final vol. 1 ml, 10 mM P_i/Tris and 5 mM magnesium acetate, pH 7.3, plus additional components as indicated. In expt. 1, 4.9 mg protein of submitochondrial particles were present for $\Delta\psi$ determination and 9.8 mg for ΔpH determination. The concentration of sodium D-iso-ascorbate was 20 mM and antimycin A was present at 0.19 μg/mg protein. In expt. 2 the concentration of particles was 6.5 mg/ml and 13 mg/ml for $\Delta\psi$ and ΔpH determinations, respectively. The concentration of sodium D-iso-ascorbate was 50 mM and antimycin A was present at 0.15 μg/mg protein. In expt. 3a the protein concentration was 5.6 mg/ml for $\Delta\psi$ determination and 11.2 mg/ml for ΔpH determination; sodium D-iso-ascorbate was 20 mM. In expt. 3b 4.2 mg particles were present for $\Delta\psi$ determination and 5.6 mg for ΔpH determination. Antimycin A was present at 0.18 μg/mg in expt. 3a/3b.

In all experiments except 3b submitochondrial particles were preincubated for 5 min with 0.37 mM NEM. Ascorbate and TMPD were added immediately before 20 μM $^{14}CH_3NH_2$ or 20 μM $KS^{14}CN$. A water-saturated stream of oxygen was blown over the surface of the reaction mixture throughout the experiments. FCCP (5 μM) was added approx. 7.5 min after the addition of $KS^{14}CN$ or 10 min after the addition of $^{14}CH_3NH_2$. For expt. 3b the procedure differed in that TMPD and 5 mM sodium dithionite were added to the reaction mixture which already contained 0.37 mM NEM, and allowed to equilibrate with the oxygen stream. The particles were then added, followed immediately by the appropriate radioactive label. Values of $\Delta\psi$ and ΔpH were calculated as in [7]. For experiments in which ΔpH was not observed the lower limit of detection is signified by < 30. (In expt. 2 the solution of TMPD had, exceptionally, been stored at −20°C for approx. 1 month before use.)

ratio of 60 μg/mg protein, and so cannot be attributed to donation of reducing equivalents to displaced cytochrome *c* on the external surface of submitochondrial particles [15,16]. Ascorbate oxidation did not generate either a membrane potential or a pH gradient when ascorbate was added to submitochondrial particles immediately before starting a flow-dialysis experiment. However, if submitochondrial particles were preincubated for 5–7 min with 20–50 mM ascorbate in either reaction mixture, a membrane potential of $\leqslant$ 40 mV was sometimes detected, but a pH gradient was never observed under these conditions. Thus in the experiments reported in tables 1 and 2, in which there was no pre-incubation with ascorbate, the generation of both $\Delta\psi$ and ΔpH can be attributed to TMPD-mediated oxidation of ascorbate.

The possibility of an interfering direct reduction of cytochrome *c* by ascorbate led us to try and detect $\Delta\psi$ and ΔpH linked to oxidation of TMPD in the absence of ascorbate. Table 2 (exp. 3b) shows that when a high concentration of TMPD was reduced by sodium dithionite, subsequent respiration generated both $\Delta\psi$ and ΔpH. The reaction medium did not become depleted of dissolved oxygen during these experiments as the blue colour of some Wurster's Blue could be detected throughout.

4. Discussion

The finding that a pH gradient as well as a membrane potential can arise from the oxidation of ascorbate plus TMPD by submitochondrial particles in the presence of suitable amounts of antimycin A, greatly favours the model of the cytochrome oxidase proton pump, as proposed in [4–6]. Alternative explanations of our data are:

(i) $\Delta\psi$ and/or ΔpH arise as a result of TMPD-independent ascorbate oxidation inside the particles.
(ii) $\Delta\psi$ is formed as a result of accumulation of the positively charged WB^+ inside the particles.
(iii) Ascorbate plus TMPD donate to a putative H^+-translocating redox carrier in the cytochrome b–c_1 region of the respiratory chain.
(iv) TMPD acts as a proton carrier either as a result of contamination by a demethylated derivative [6,7], or by crossing the membrane as $TMPDH^+$.

We believe that the experiments reported in the present paper exclude (i) and, although (ii) could conceivably explain the generation of $\Delta\psi$, the detection of ΔpH strongly suggests that $\Delta\psi$ arises as a result of H^+ translocation rather than WB^+ accumulation. We have discussed [7] why we regard (iii) as unlikely, and in the present work sufficient antimycin A was present to block electron flow through the cytochrome b–c_1 complex. The generation of ΔpH and $\Delta\psi$, when ascorbate oxidation is mediated by a freshly prepared solution of recrystallised TMPD, makes it improbable that a contaminating derivative (iv) is responsible for the observed effects. The pK_b of TMPD is 7.7 [17] and so at pH 7.3 or pH 7.5 an appreciable fraction (10%) of the TMPD is present as $TMPDH^+$. If $TMPDH^+$ acted as an electron mediator, then a proton would be released into the lumen of the particles. We believe this (iv) is improbable on at least two counts:

(1) Biological membranes are generally regarded as relatively impermeable to charged amines.
(2) Upon raising the pH of P_i/Tris from 7.3–7.8 no change in the magnitude of $\Delta\psi$ was observed, despite the decrease in the concentration of $TMPDH^+$.

Previous evidence that cytochrome oxidase pumps H^+ into the interior of submitochondrial particles was obtained from experiments in which neutral red was used as an indicator of internal pH [5] over a 1 min period. The present measurements differ from those with neutral red in two respects:

(1) $^{14}CH_3NH_2$ uptake provides a quantitative measure of ΔpH, whereas the neutral red response can be influenced by the extent of its binding to energy-transducing membranes [18,19].
(2) In our experiments a pH gradient (sustainable for up to 10 min) of substantial magnitude is detected.

It is improbable that such a ΔpH could be sustained without continuous movement of H^+ into the lumen of the particles, and thus explanations (cf. [20]) of Wikström's experiments [4–6] based on a burst of H^+ release from a reservoir of hydrogen ions possibly associated with phospholipids or cytochrome *c* are unlikely to be applicable to our observations.

Our observation that addition of valinomycin did not increase the magnitude of ΔpH (table 1) is not in agreement with experiments using neutral red [5], but a decrease in the overall protonmotive force in the presence of valinomycin (table 1) has been observed

[12], and may reflect a disruptive effect of this ionophore on the membrane [21].

The total protonmotive force with ascorbate plus TMPD as substrate is larger in the sucrose/Hepes/KCl medium than in the P_i/Tris buffer, just as was found earlier with NADH as substrate [7]. As Tris is a primary amine, it probably permeates into the lumen of submitochondrial particles, and thus restricts the extent of any internal acidification. The zwitterionic Hepes species will be much less permeant than Tris, and thus acidification of the particles' lumen can be observed in Hepes, especially at relatively high concentrations of Cl^-, when inward movement of Cl^- may increase ΔpH with a corresponding decrease in $\Delta\psi$. It will be of value to study further the effects of varying buffer systems on the magnitudes of Δp, ΔpH and $\Delta\psi$, as this is a point of controversy [22,23], and may also explain the variation in the values of Δp found in different laboratories [7,24]. Nevertheless our observation of ΔpH linked to electron flow through cytochrome oxidase under appropriate conditions provides strong support for the view that this electron carrier can translocate H^+, in agreement with other findings [4–7,25]. Some bacterial cytochrome oxidases may have similar H^+ translocating capacity [26].

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