Critical evaluation of the proton-translocating property of cytochrome oxidase in rat liver mitochondria

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1. INTRODUCTION

The mechanism and stoicheiometry of H^+ translocation by the respiratory chain is presently a subject of considerable controversy. For the cytochrome chain, catalysing oxidation of ubiquinol by O_2, the H^+/e^- stoicheiometry is suggested to be 2, 3 or 4 by different research groups. Since it is agreed that the stoicheiometry is 2 for oxidation of ubiquinol by cytochrome *c*, the disagreement concerns whether or not cytochrome *c* oxidase translocates protons, and whether the H^+/e^- ratio is 1 or 2 if such proton translocation takes place (see [1,2]).

The experimental evidence for H^+ translocation by cytochrome oxidase has been reviewed [3]. Moyle and Mitchell [4] and Papa et al. [5,6] have disputed this evidence for intact mitochondria (but cf. [1,7]) and suggested that the oxidised form of redox mediators (e.g., TMPD and ferrocyanide) used to donate electrons to cytochrome *c* is rapidly re-reduced by endogenous hydrogen donors with H^+ release into the medium. They suggested that this artefact accounts for the phenomena that Wikström [8] originally attributed to H^+ translocation by cytochrome oxidase. Moyle and Mitchell [9] further claimed that no H^+ translocation is associated with oxidation of diaminodurene or exogenous ferrocytochrome *c* by cytochrome oxidase of rat liver mitochondria.

This study was undertaken to test this criticism rigorously in order to find out whether or not cytochrome oxidase can be regarded as a proton pump, as proposed in [1–3,7,8]. The work revealed a new aspect of cytochrome oxidase functioning and control that may be of general physiological importance.

2. MATERIALS AND METHODS

Rat liver mitochondria were isolated as in [10] in 0.25 M sucrose–0.1 mM EGTA, and suspended in this medium at 10 μM cytochrome aa_3. The latter was determined at 605–630 nm by reduction with dithionite ($\varepsilon_{mM} = 27\ cm^{-1}$).

The respiratory pulse method was used, essentially as in [11], using a thermostatted reaction vessel (25 ± 0.01°C) described in [12], a pH combination electrode (Philips CA 14/02), a digital pH meter (Instrulab), and a strip chart recorder (Goerz). The 90% response time of the entire system was determined to be 0.8 s. Medium and extra additions were added to the vessel, flushed with Ar (< 3 ppm of O_2), and closed with the electrode. Further additions were made through a narrow port with calibrated microsyringes (Hamilton). Anaerobiosis occurred within a few seconds after addition of the mitochondria. Then anaerobic aliquots of valinomycin, antimycin and myxothiazol (when present) were added to 1.2 ml final vol. Anaerobic equilibration followed for 20 min. O_2 was added as a small accurately calibrated volume of pure water, care-

Abbreviations: DAD, 2,3,5,6-tetramethyl-*p*-phenylenediamine; diaminodurene, see DAD; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N'*-tetra-acetate; FeCy, potassium ferricyanide; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonate; NEM, *N*-ethylmaleimide; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; $TMPD^+$ oxidised free radical form of TMPD (Wurster's blue); $TMPDH^+$, protonated form of TMPD

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fully pre-equilibrated with moist air for >3 h at 25°C under mild agitation. At 1 atm air pressure the concentration of dissolved O_2 is then 258 μM [13]. Alternatively, an aliquot of anaerobic standard $K_3(Fe(CN)_6)$ (5 mM in water) was added as oxidant. Calibration of changes in pH in terms of ΔH^+ was carried out by adding known volumes of anaerobic standard 10 mM HCl (Titrisol, Merck). The extent of the immediate pH deflection, extrapolated to zero time, was the basis for the calibration (cf. [14,15]).

The FCCP was a gift by Dr P.G. Heytler and diaminodurene by Professor A. Trebst. Myxothiazol was obtained through the courtesy of Dr H. Reichenbach. Cytochrome *c* was from Sigma (type VI). Other reagents were commercial products of the highest purity available.

3. RESULTS

3.1. *H^+ translocation with TMPD as electron donor*

TMPD is well known to reduce endogenous cytochrome *c*, but its oxidised form $TMPD^+$ (Wurster's blue) can accept electrons from the cytochrome bc_1 segment in a reaction that is insensitive to antimycin. The TMPD/$TMPD^+$ couple is indeed known to give rise to an 'electron shunt' bypassing the antimycin site [16]. Therefore an excess of ascorbate is usually used in studies of the cytochrome *c* oxidase reaction to keep TMPD fully reduced [17,18]. Here, we deliberately allow re-reduction of $TMPD^+$ by hydrogenated reductants to determine the contribution of this reaction to H^+ translocation.

TMPD is an almost pure electron donor at pH >7, the only significant protonation between pH 6–8 being that of TMPD itself to $TMPDH^+$, with pK = 6.35–6.40, as determined in titrations with ferricyanide (not shown, but Arents and Van Dam found pK = 6.3 in unpublished experiments quoted in [19]).

When O_2 is added to anaerobic mitochondria in the presence of antimycin, there is antimycin-insensitive respiration and relatively slow H^+ ejection (fig.1A; [15]). Both activities are virtually blocked by myxothiazol in concentrations equivalent with those of the bc_1 complex (not shown). Myxothiazol is a newly discovered stoicheiometric inhibitor of the bc_1 complex that reacts with a site distinct from that of antimycin [20]. For further use

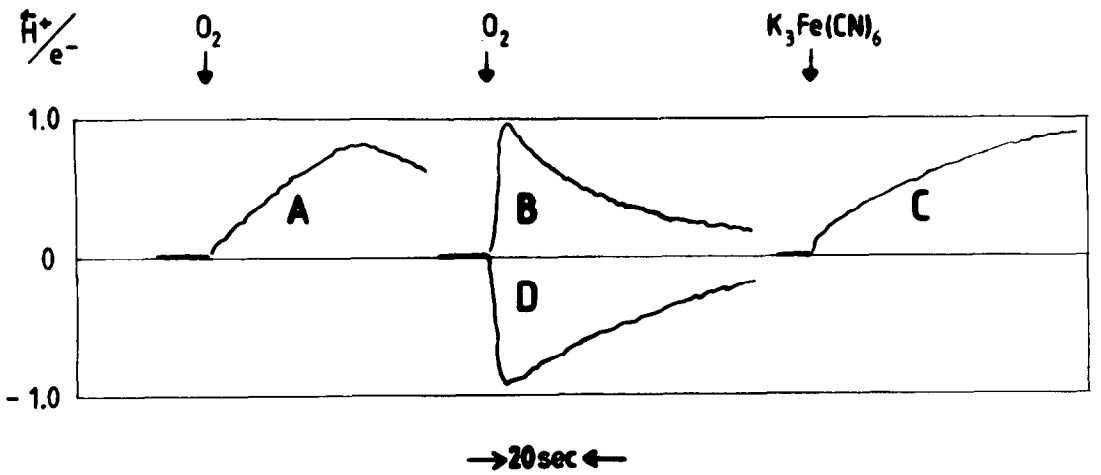

Fig.1. H^+ translocation during operation of the TMPD shunt. The medium contained 0.24 M sucrose, 10 mM $MgSO_4$, 1 mM HEPES and 1 mM EGTA (final $[K^+]$ 4 mM from pH adjustment with KOH); 2.5 μM rotenone, 4 μg/ml oligomycin, 0.25 mM NEM, 25 μg/ml carbonic anhydrase, 0.63 μg/ml valinomycin, 0.17 μg/ml antimycin, and 2 mM K^+-succinate (an additional 4 mM of K^+) were also present. The concentration of rat liver mitochondria corresponded to 0.77 μM cytochrome aa_3 (~5.5 mg protein/ml). Final pH 7.35. In: (A) 2.60 μM of O_2 was added; (B) as (A) but in the presence of 0.1 mM TMPD; (C) as (B) but with 10.4 μM anaerobic $K_3(Fe(CN)_6)$ in place of O_2; (D) as (B) but in the presence of 1 μM FCCP.

of this useful inhibitor, see below.

When TMPD is also present (fig.1B), H^+ ejection is fast with a H^+/e^- ratio of ~0.9, as shown originally by Mitchell and Moyle [21], who attributed this deprotonation of the oxidised mediator. The acid pulse decays to the baseline, showing that the O_2 must indeed be ultimately reduced by a hydrogen donor. However, since TMPD is not the hydrogen donor (at pH 7.35 the contribution of $TMPDH^+$ accounts only for 0.1 H^+/e^-; cf. above), the overall reaction must be the oxidation of succinate by O_2 mediated by the 'TMPD shunt'. Identical results were obtained without added succinate, which is understandable since rat liver mitochondria contain plenty of endogenous substrates.

The fast H^+ ejection in trace B (fig.1) may thus either be due to re-reduction of $TMPD^+$ by succinate or to proton translocation by cytochrome oxidase, linked to the oxidation phase of TMPD. Fig.1C shows that when TMPD is oxidised directly by anaerobic $K_3(Fe(CN)_6)$, in place of O_2, there is fast release of 0.1 H^+/e^- (and generation of blue colour due to $TMPD^+$), as expected from the contribution of $TMPDH^+$ at pH 7.35. This is followed by slow release of ~0.9 H^+/e^- (and synchronous fading of the colour). Since the latter reaction is completely unaffected by FCCP (not shown), the antimycin-insensitive reduction of $TMPD^+$ is clearly not coupled to proton translocation. The result suggests that the reduction of $TMPD^+$ by endogenous hydrogen donors is much slower than the H^+ ejection observed after O_2 under these conditions. In some conditions that favour accumulation of reduced ubiquinol the second phase of trace C was faster, but could be decelerated by two independent methods. This occurred either after several consecutive pulses of O_2 and/or $K_3(Fe(CN)_6)$, or in the presence of myxothiazol. Most significantly, neither treatment had any effect on the fast H^+ extrusion that followed an addition of O_2. These results therefore favour the possibility that H^+ ejection after O_2 is coupled to the oxidation of TMPD and not to its re-reduction. But with O_2 the $TMPD^+$ may be generated locally that might accelerate its re-reduction. For this reason a second control was performed.

In fig.1D, the O_2 was given in the presence of FCCP to render the inner membrane permeable to H^+. In this case there was a fast consumption of 0.9 H^+/e^- followed by much slower proton release back to the baseline. This confirms that the fast phase after O_2 is due purely to oxidation of TMPD to $TMPD^+$ by O_2 without significant re-reduction of the latter, which occurs later with kinetics similar to those after ferricyanide (cf. trace C). H^+ ejection after O_2 (or consumption with FCCP present) is fully inhibited by cyanide (not shown), suggesting that it is truly due to the cytochrome oxidase reaction. If the fast H^+ extrusion after O_2 is nevertheless due to reduction of $TMPD^+$ by a hydrogen donor, this would have to occur rapidly when the $TMPD^+$ generated with O_2, but slowly when generated with ferricyanide, or with O_2 in the presence of FCCP. This seems unlikely, and is contradicted by spectrophotometric controls indicating equal extents of $TMPD^+$ generation whether ferricyanide or an equivalent amount of 1/4 O_2 is the oxidant, and independent of the presence of FCCP (not shown). We therefore conclude that the oxidation of TMPD by O_2 catalysed by cytochrome *c* and cytochrome oxidase is linked to proton translocation.

3.2. *H^+ translocation with diaminodurene as electron donor*

Fig.2 shows an experiment similar to that of fig.1B, but with DAD in place of TMPD. Again rapid H^+ extrusion takes place after O_2, but to the

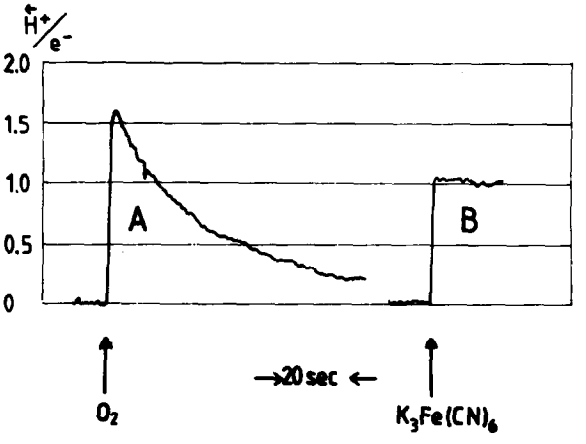

Fig.2. H^+ translocation with diaminodurene as electron donor. Conditions as in fig.1 but without succinate and with 0.56 mM diaminodurene. In (A) 2.60 μM of O_2 was added; (B) 10.4 μM anaerobic $K_3(Fe(CN)_6)$ was added, as shown; pH 7.10.

extent of 1.71 H^+/e^- (± 0.13 SD; $N = 11$). The trace relaxes to the baseline, as before, showing that a hydrogen donor is the ultimate reductant. However, in this case $K_3(Fe(CN)_6)$ causes very fast release of 1 H^+/e^- (0.98 ± 0.08 SD; $N = 11$), as also shown by Mitchell and Moyle [22]. In contrast, these authors reported extrusion of only 1 H^+/e^- also after O_2 [9]. The only essential difference between their conditions and ours is their very low concentration of K^+ and much higher concentration of O_2 (cf. [1,23] and below).

With FCCP present, O_2 caused neither H^+ release nor consumption (cf. [19]) and ferricyanide yielded a result identical to fig.2B. Both effects are expected if (as suggested in [9,22]) DAD is a pure hydrogen donor. The alternative, viz. very fast re-reduction of DAD by a mitochondrial hydrogen donor, can be excluded as shown in [22]. Previous reports purporting to incomplete deprotonation of oxidised DAD at pH 7 [22,23] can probably be ascribed to aerobic decomposition of this compound, which may be observed also in O_2 pulse experiments after several additions of high concentrations of O_2.

H^+ extrusion was also in this case completely unaffected by myxothiazol. We conclude that oxidation of diaminodurene via the cytochrome *c* oxidase system is linked to H^+ translocation, as shown above for TMPD. The very low (K^+) (see [1]) and/or high (O_2) used in [9] may account for their failure to observe H^+ translocation. 2.4 nmol O/mg protein (see [9]) would according to the proton pump model [3,8] result in translocation of 9.6 nmol H^+/mg from the matrix phase. Transport of ≥ 7 nmol H^+/mg was shown to markedly depress the observed H^+/e^- ratio in [15,21].

3.3. *H^+ translocation with exogenous cytochrome* c *as electron donor*

Wikström and Krab [3,7,18] ascribed the failure in [9] to observe H^+ translocation linked to oxidation of added cytochrome *c* to insufficient K^+ countertransport, because when the sucrose medium in [9] was replaced by 120 mM KCl H^+ translocation became observable. However, this proposal is incorrect because H^+ translocation is also not observed in media containing 4–10 mM K^+, i.e., conditions where it is readily observable with TMPD or DAD as electron donors (see above). A closer study was therefore undertaken to elucidate this unexpected complication. Myxothiazol was used (with or without antimycin) because it blocks antimycin-insensitive respiration, H^+ extrusion and re-reduction of formed ferricytochrome *c*, which are otherwise complicating side reactions.

Fig.3 shows fast H^+ extrusion (0.6–0.7 H^+/e^-) after O_2 in the presence of ferrocytochrome *c*. In its absence no such effect was observed. The trace relaxes to – 1 H^+/e^-, suggesting that there is neither significant oxidation of a hydrogen donor nor a net acidification artefact. The control with anaerobic $K_3(Fe(CN)_6)$ shows that mere oxidation of cytochrome *c* is not linked to a large acidification artefact in these conditions (contrast [22]). With FCCP present O_2 caused fast consumption of exactly 1 H^+/e^- without any acidification phase ([18]; cf. fig.4).

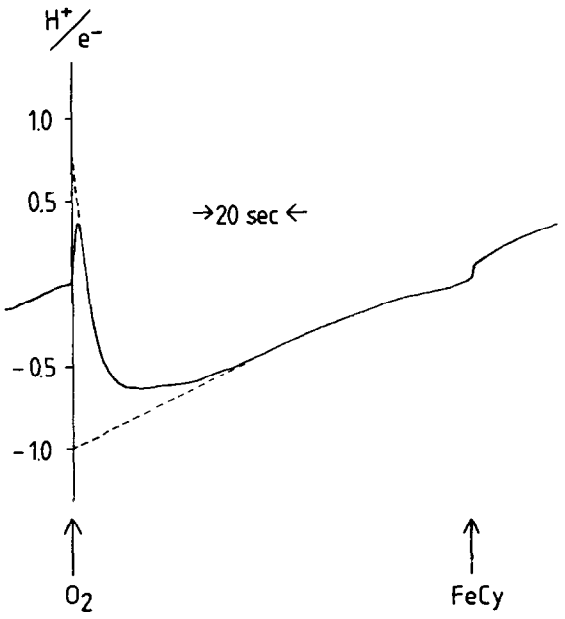

Fig.3. H^+ translocation with exogenous ferrocytochrome *c* as donor. 120 mM KCl, 1 mM HEPES, 1 mM EGTA was supplemented with 3 μM rotenone, 4 μg/ml oligomycin, 0.14 mM NEM, 1.2 μM myxothiazol, 40 μM ferrocytochrome *c* (Sigma, type VI) and rat liver mitochondria (0.77 μM cytochrome aa_3). The concentration of valinomycin was 0.21 μg/ml. One pulse of 8.3 μM $K_3(Fe(CN)_6)$ (FeCy) was added prior to the O_2 pulse (2.15 μM O_2) shown, and one is added later, as shown; pH 7.05.

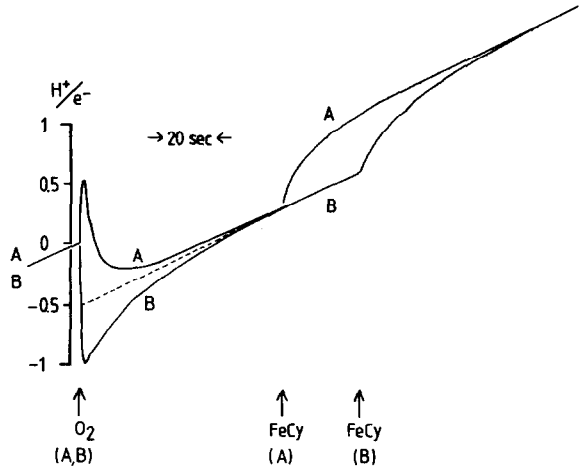

Fig.4. H^+ translocation with exogenous cytochrome *c* plus ascorbate. Conditions as in fig.3, but with an additional 0.83 mM K^+-ascorbate present. This is a continuation of the experiment in fig.3, from which 16.7 μM $K_3(Fe(CN)_6)$ is also present. (A,B) two superimposed experiments in which 2.15 μM of O_2 was added in the absence (A) and presence (B) of 1 μM FCCP. Additions of 8.3 μM potassium ferricyanide (FeCy) are also shown for both (A,B); (. . .) extrapolation backwards of the final state (in both A,B) after the addition of O_2.

With ferrocytochrome *c* as the sole reductant only very few oxidant pulses may be given in a single experiment due to the adverse effect of a high ratio between ferric and ferrous cytochrome on the rate of the reaction. Some observations also suggest that H^+ translocation may be counteracted by a rising c^{3+}/c^{2+} ratio (section 4). In practise it is difficult to prevent partial oxidation of cytochrome *c* in this type of experiment. However, this difficulty may be overcome by addition of ascorbate without disturbing the motive of the experiment, because ascorbate reduces ferric *c* much more slowly than ferrous *c* is oxidised in these conditions ([24]; cf. below). Thus full reduction of the cytochrome may be ascertained prior to the pulse of O_2 and yet the initial reaction after O_2 is due purely to oxidation of ferrous *c* without significant oxidation of ascorbate. This is clearly shown in fig.4 (trace B) from the primary consumption of 1 H^+/e^- in the presence of FCCP with much slower ($t_{1/2}$ = 15 s) release of 0.5 H^+/e^- due to reduction of *c* by ascorbate. Trace A shows, superimposed, the analogous experiment without FCCP, where there is fast extrusion of 0.6–0.7 H^+/e^- (cf. fig.3). Since there is no reason to suppose that reduction of ferric *c* by ascorbate would be faster without FCCP than with it, we conclude also from fig.4 that cytochrome *c* oxidation is linked to proton translocation.

H^+ translocation with cytochrome *c* (± ascorbate) was not observed in media containing 240 mM sucrose + 4–10 mM K^+ (contrast fig.1,2), but became detectable at higher K^+ concentrations, being half-maximal at roughly 60 mM KCl (+ 125 mM sucrose), in conditions otherwise identical to those of fig.3,4. 10 mM $MgCl_2$ was, moreover, found largely to counteract H^+ translocation in a 120 mM KCl medium. It is clear that H^+ translocation with exogenous cytochrome *c* differs considerably from that with TMPD or DAD in that the latter is virtually independent of ionic strength or high Mg^{2+} concentrations.

4. DISCUSSION AND CONCLUSIONS

We have confirmed that the cytochrome oxidase reaction is linked to H^+ translocation in intact mitochondria and rigorously excluded the alternative interpretations in [4–6]. Our contention is supported by the finding that the H^+/e^- ratio is 3 for oxidation of succinate (or ubiquinol) by O_2 [25–27], as recently corroborated in detailed O_2 pulse experiments ([28]; M.W., in preparation).

The present results led to the unexpected conclusion that oxidation of exogenous cytochrome *c* (in contrast to redox mediators) is coupled to H^+ translocation only at high ionic strength, and may be counteracted by high Mg^{2+} concentrations. Oxidation of added *c* differs from that of the mediators in that ferric *c* must dissociate from the binding site on the oxidase in the former case to be replaced by a molecule of ferrous *c*. At low ionic strength ferric *c* is bound too tightly to the so-called high-affinity site to dissociate rapidly enough for this site to engage in electron transfer. Thus electron transfer takes place with so-called low affinity site characteristics in such conditions (see e.g., [29]). In contrast, redox mediators such as TMPD or DAD are able to reduce ferric *c* directly while bound to the high-affinity site [29,30]. The activity is then independent of dissociation of *c* from the oxidase,

which is much enhanced at high ionic strength [29–31].

The results therefore suggest that H^+ translocation may be restricted to so-called high-affinity site electron transfer. Electron transfer with low affinity site characteristics is not coupled to H^+ translocation, but is yet associated with translocation of one electrical charge equivalent per transferred electron [9]. This reduction of the energy conservation efficiency by 50% is similar to that observed with the reconstituted enzyme depleted of subunit III ([32]; T.P., submitted). Interestingly, the latter has unaltered low affinity electron transfer activity [32,33], but may be drastically altered in its high-affinity site activity (in preparation).

The observed inhibitory effect of Mg^{2+} may be related to the binding of this ion to ferrous *c* [34], or to some other adverse effect on the binding of the positively charged ferrous *c* to the high-affinity site. A high c^{3+}/c^{2+} ratio may have the effect of favouring oxidation with low affinity site characteristics.

The high-affinity site is very likely to be involved in normal respiratory chain activity [35], in agreement with the H^+/e^- ratio of 3 for the oxidation of ubiquinol (see above). The function of the low-affinity site is still obscure, but may be utilised for delivery of electrons to the oxidase from auxiliary donor systems [36,37]. However, it may also function during normal respiratory chain activity to a controlled extent, then perhaps providing regulation of the efficiency of energy conservation. Such regulation may be of physiological importance in securing respiration at high phosphorylation potentials, and in adjusting ATP synthesis to an optimal efficiency [38].

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