Mechanism of proton translocation associated to oxidation of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine in rat liver mitochondria

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A kinetic analysis is presented of proton translocation, $TMPD^+$ formation and oxidation of endogenous respiratory carriers during oxygen pulses of TMPD supplemented rat-liver mitochondria. The results show that antimycin-insensitive proton ejection observed under coupled conditions derives from oxidation of endogenous respiratory carriers and re-reduction of $TMPD^+$ by hydrogenated donors and not from proton pumping by cytochrome oxidase as claimed by other investigators. The observations presented provide an example of certain interpretative difficulties in the use of redox mediators and of the methodological approaches that can be used to avoid these.

Cytochrome oxidase *Redox-linked proton translocation* *TMPD oxidation*
Cytochrome oxidation *Hydrogenated donor, oxidation of*

1. INTRODUCTION

In mitochondria and bacteria the reduction of dioxygen to H_2O by cytochrome *c*, catalysed by cytochrome oxidase (EC 1.9.3.1), results in the generation of transmembrane thermodynamic potential difference of protons ($\Delta\mu_{H^+}$) [1–3]. This was originally conceived by Mitchell [4] as a consequence of vectorial translocation by the oxidase of electrons donated by cytochrome *c*, located at the outer surface of the membrane [5], to protons deriving from the opposite inner aqueous phase (see also [2]). Experimental support for this mechanism was obtained in [2,6–8].

Abbreviations: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; $TMPD^+$, oxidised free radical form of TMPD (Wuster's blue); FCCP, *p*-trifluoromethoxy-carbonylcyanide; UHDBT, 5-(*n*-undecyl)-6-hydroxyl-4,7-dioxobenzothiazole; NEM, *N*-ethylmaleimide; EGTA, ethyleneglycol bis (*β*-aminoethyl ether)-*N,N'*-tetra-acetate; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonate

However, observations by Wikström et al. [9,10] and others (reviewed in [10–12]) have led to the proposal that cytochrome oxidase can, in addition or alternatively [10,11] to this mode of action, function as a redox-driven proton pump with effective transport of 1 or 2 H^+/e^- from the inner to the outer space [10,12].

The evidence brought forward in favour of a proton pump in cytochrome oxidase is from observations in the native membrane and purified oxidase reconstituted in liposomes [13]. In mitochondria (review [12]) and bacteria [14] the oxidation of artificial reductants of cytochrome *c* has, in fact, been found to result in antimycin-insensitive H^+ release. However, in rat-liver mitochondria [15–19] the H^+ release associated to aerobic oxidation of these artificial reductants was derived from re-reduction of their oxidised form (review [20,21]). In [22] proton translocation associated with aerobic oxidation of TMPD was considered to overcome this criticism.

Here, a detailed analysis is presented of proton

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translocation associated to aerobic oxidation of TMPD in rat-liver mitochondria, based on simultaneous direct measurement of the kinetics of formation and re-reduction of the oxidation product of TMPD ($TPMD^+$), proton translocation and redox transitions of endogenous respiratory carriers. It is shown that the antimycin-insensitive H^+ release observed results from re-reduction of $TMPD^+$ by hydrogen donors as well as from rapid oxidation of endogenous respiratory carriers, and not from proton pumping by cytochrome oxidase.

2. MATERIALS AND METHODS

UHDBT was prepared as in [23]. All the other reagents were of the highest purity grade commercially available. Rat-liver mitochondria were prepared as in [24].

Oxido-reduction of TMPD and cytochromes was monitored with a Johnson Foundation dual wavelength spectrophotometer equipped with a conventional observation chamber or a regenerative stopped flow apparatus [19]. Proton translocation was measured either with a static system or with a Roughton-type, continuous-flow pH-meter [19,25]. The spectrophotometer cuvette was thermostatically controlled at 25 ± 0.01 °C and sealed with a glass plug with a channel closed by a pH combination electrode (Beckman no.39505) and thin channel filled with the incubation mixture for insertion of microsyringe needles. Anaerobiosis occurred within a few seconds after addition of mitochondria to the reaction mixture containing succinate as respiratory substrate. Then antimycin was added (fig.1 legend). After 20 min of anaerobic preincubation, O_2 was added as a small calibrated volume of bidistilled water pre-equilibrated with moist air at 25°C; in these conditions, dissolved O_2 is 258 μM [22,26].

3. RESULTS AND DISCUSSION

Fig.1a shows proton translocation elicited by small oxygen pulses of rat liver mitochondria preincubated in anaerobiosis with TMPD, succinate and antimycin at pH 7.4. The experimental conditions were those in [22]. Oxygenation resulted in proton release with an H^+/e^- ratio of 0.8 (see table 1). The acidification then decreased to the baseline, thus showing that added O_2 was

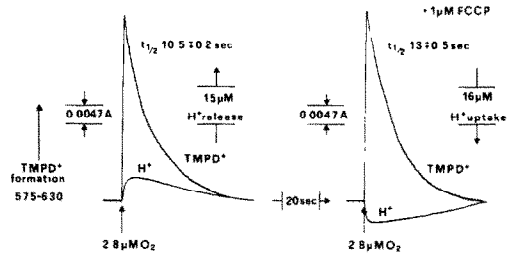

Fig.1. Simultaneous measurement of proton translocation and $TMPD^+$ formation caused by oxygen pulses of anaerobic TMPD-supplemented rat-liver mitochondria. Rat liver mitochondria (3.5 mg protein/ml) were incubated in: 0.24 M sucrose, 10 mM $MgSO_4$, 1 mM K-Hepes, 1 mM K-EGTA, 0.25 mM NEM; 2 mM K-succinate, 4 μg oligomycin/ml, 0.63 μg valinomycin/ml, 2.5 μM rotenone, 0.1 mM TMPD and 0.5 μg antimycin A/ml (pH 7.4); temp., 25°C. For experimental procedure see section 2. TMPD oxidation was monitored by $\Delta A_{575-630}$ which are isosbestic points for cytochromes. The formation of $TMPD^+$ was calculated from the absorbance changes using a $\Delta\epsilon_{mM}$ of 5.09 obtained from the oxidation of TMPD by small amounts of a standard solution of ferricyanide. The $t_{1/2}$ for re-reduction of TMPD shown on the traces are mean values of 8 expt. ± SEM.

ultimately reduced by a hydrogen donor.

In the presence of FCCP the oxygen pulse resulted directly in proton consumption (fig.1b). Also this alkalinisation reverted to the baseline.

The pattern described reproduced essentially that in [22]. These authors ascribed the proton ejection observed in the absence of FCCP (corrected for release of 0.1 H^+/e^-, arising directly from the conversion of $TMPDH^+$ to $TMPD^+$) to proton pumping by cytochrome oxidase driven by aerobic oxidation of TMPD.

Proton release could also derive from re-reduction of $TMPD^+$ by succinate and/or endogenous hydrogen donors. In [22] it was concluded that this reaction was negligible on the basis of:

(i) The consumption they observed of 0.9 H^+/e^- in the reduction of oxygen to H_2O in the presence of FCCP. The ratio found in the present study is, however, 0.78 ± 0.01 (see table 1). The shortfall in H^+ consumption derives essentially from proton release directly associated to conversion of $TMPDH^+$ to $TMPD^+$. Measurement of pH changes associated to chemical oxidation of TMPD by

Table 1

Statistical analysis of proton translocation and oxidation of TMPD caused by oxygen pulses of anaerobic, antimycin-inhibited rat-liver mitochondria

Additions (1 μM)	(i) O_2 added (nequiv. e^-)	(ii) Extent H^+ release (ng-ions)	(iii) H^+/e^-	(iv) Maximal extent $TMPD^+$ prod. (nmol)	(v) H^+/e^- (corr.)	(vi) Deficit $TMPD^+$ prod. (nmol)
–	3.23	2.58 ± 0.04 Extent H^+ uptake	0.80 ± 0.01 H^+/e^-	2.57 ± 0.03	0.65 ± 0.01 H^+/e^-	0.66 ± 0.03
FCCP	3.23	2.52 ± 0.04	0.78 ± 0.01	2.67 ± 0.03	0.94 ± 0.01	0.56 ± 0.03

Experimental conditions as in fig.1 legend. The amount of O_2 added, H^+ release or uptake and $TMPD^+$ formation refer to mg protein. The maximal $TMPD^+$ formation (line iv) was calculated at the interval of about 1 s after oxygen addition, when net formation of $TMPD^+$ was completed and its re-reduction became apparent (see fig.1). The H^+/e^- ratios in line (v) were obtained by correcting the measured H^+ translocation for proton release directly associated to conversion of $TMPDH^+$ to $TMPD^+$ (see fig.2). The deficit of $TMPD^+$ formation was calculated by subtracting maximal $TMPD^+$ formation from added oxygen. Data are the means of 8 expt. ± SEM

ferricyanide (fig.2) showed that at pH-values around neutrality TMPD is in part protonated as $TMPDH^+$, with a pK_a of 6.8. At pH 7.4, proton release deriving from conversion of $TMPDH^+$ to $TMPD^+$ amounts to 0.2 H^+/e^-.

(ii) The low rate of decay of the alkalinisation observed in the presence of FCCP towards the baseline.

(iii) The low rate of acidification observed upon oxidation of TMPD by ferricyanide in the presence of KCN.

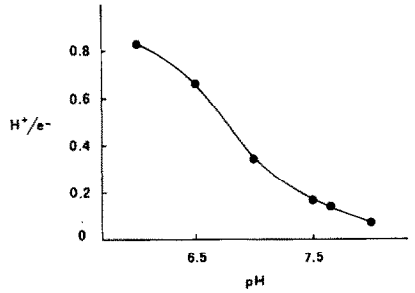

Fig.2. pH-dependence of proton release associated to chemical oxidation of TMPD by ferricyanide. The reaction mixture contained: 10 mM KCl, 1 mM Hepes and 1 mM TMPD. The pH was adjusted to the values reported in the figure by addition of few μl of 1 M KOH or 1 M HCl. H^+ release was measured potentiometrically as in section 2. The reaction was started by addition of 50 μM ferricyanide. The H^+/e^- ratio was computed from the amount of H^+ released divided by the amount of added ferricyanide.

The experiments of (ii) and (iii) indicate that the rate of proton release caused by re-reduction of $TMPD^+$ by hydrogen donors was, under the respective experimental conditions, slower than H^+ release observed upon oxygenation of coupled mitochondria. As will be shown below, the situation arising in these experimental conditions may, however, not be directly relevant to that taking place during oxygen pulses of coupled mitochondria. However, it is possible to verify, directly in the same sample, whether proton release deriving from re-reduction of $TMPD^+$ by hydrogenated donors contributes or not to the proton release observed in the coupled state, by following spectrophotometrically the kinetics of net formation of coloured $TMPD^+$ and its subsequent re-reduction to colourless TMPD.

These measurements (fig.1, table 1) revealed a number of critical points. The net formation of $TMPD^+$ after oxygen pulses of coupled mitochondria accounted for only 80% of added oxygen. Evidently, the remaining 20% oxygen was utilized for oxidation of reductants different from TMPD. Net formation of $TMPD^+$ was completed in about 1 s, after which a relatively rapid re-reduction took place.

The H^+/e^- ratio of 0.8 computed from the total extent of H^+ release is close to the value of about 0.9 reported in [22]. Correction for proton release directly associated to conversion of $TMPDH^+$ to $TMPD^+$ gives an H^+/e^- ratio of 0.65. However, it

can be noted that proton release lasted longer than net $TMPD^+$ formation; in fact a significant part of the overall acidification process was apparently associated with $TMPD^+$ re-reduction.

Also in the presence of FCCP there was a shortfall in the net formation of $TMPD^+$, amounting to 17% of the oxygen added. The actual H^+/e^- ratio for proton consumption in the presence of FCCP was 0.78 (0.94 when corrected for proton release in the conversion of $TMPDH^+$ to $TMPD^+$). Subtraction from the overall proton consumption of the amount corresponding to the aerobic oxidation of TMPD, leaves an H^+ uptake of 0.38 ng-ions/mg protein, this arising from the oxidation of 0.56 nequiv. of reductants different from TMPD. These reductants are represented by endogenous electron carriers as well as hydrogenated donors.

It should be recalled that proton release associated with subsequent re-reduction of electron carriers by hydrogenated reductants, mediated by the couple $TMPD^+$/TMPD, appears in the presence of FCCP as a distinct acidification phase which follows the initial net proton consumption. In the coupled state this acidification process, mixes up with other acidification events.

Resolution of the events contributing to proton release induced by oxygenation of coupled mitochondria was afforded by analysis with a continuous flow pH meter, with a resolution time of 10 ms [25].

The time course of proton release thus resolved was, in fact, quite peculiar and different from that of $TMPD^+$ formation (fig.3). There occurred an initial rapid phase of proton release which was almost completed in 100 ms. At this interval the ratio between proton release and $TMPD^+$ formation was 2.7. The rapid acidification was followed by a much slower phase of proton release, lasting until 300 ms. After this phase, H^+ release was again activated and continued when net oxidation of TMPD was completed, and $TMPD^+$ re-reduction was evident (cf. fig.3 and 1). The ratio between protons released from 100–300 ms and $TMPD^+$ formed in the same interval was 0.26, which is very close to that expected if proton release derived only from the conversion of $TMPDH^+$ to $TMPD^+$.

Fig.3. shows that $TMPD^+$ formation exhibited, on the contrary, a monophasic time course, either when measured with a static spectrophotometric

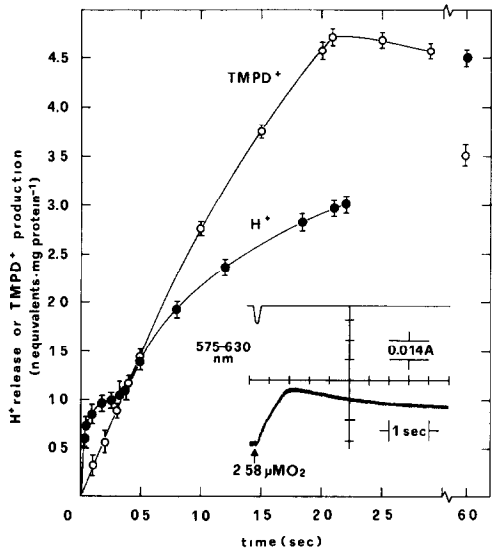

Fig.3. Kinetic analysis of proton release and $TMPD^+$ formation caused by oxygen pulses of anaerobic TMPD supplemented mitochondria. H^+ release (●—●) was measured with a continuous flow pH meter (mixing ratio, 1:58 [25]). The final concentration of oxygen added as air-saturated distilled H_2O was 5.56 nequiv. e^-/mg protein. $TMPD^+$ formation (○—○) was measured spectrophotometrically after addition of the same amount of oxygen to the anaerobic mitochondrial suspension kept in a conventional cell under vigorous magnetic stirring. The output of the spectrophotometer was fed into a storage oscilloscope providing an overall response time of about 100 ms [27]. The maximal formation of $TMPD^+$ was 4.72 ± 0.09 nmol/mg protein amounting to 85% of the oxygen added. The insert shows analysis of $TMPD^+$ formation by a stopped-flow spectrophotometer, which provided a final 1:100 dilution of the oxygenated H_2O (final oxygen conc. 3.22 nequiv. e^-/mg protein). The maximal formation of $TMPD^+$ resolved with the flow measurement was 2.63 nmol/mg protein (mean value 5 expt. was 2.5 ± 0.05, amounting to 80% of the oxygen added). For experimental conditions see section 2 and fig.1 legend. The points on the curves represent the mean of 5 expt. ± SEM.

apparatus (response time 100 ms [27]) or with a stopped-flow spectrophotometer with a response time of 5 ms [28] (see insert of fig.3). Both measurements showed that the maximal net formation of $TMPD^+$ amounted to $\leqslant$ 85% of the oxygen added (fig.3 legend).

The source of the initial rapid phase of proton release revealed by the flow analysis is clarified by

the experiments shown in fig.4. Aerobic oxidation of endogenous respiratory carriers in antimycin-treated mitochondria, without added TMPD, resulted in a rapid proton release which, at 30 ms after oxygenation, was exactly the same as that observed in the presence of 0.1 mM TMPD. At intervals from 30–170 ms, proton release in the presence of TMPD was not much higher than that observed in the absence of TMPD plus the amount of protons expected to derive directly from conversion of $TMPDH^+$ to $TMPD^+$. It should be noted that in the presence of TMPD net oxidation of endogenous carriers, like cytochrome *c* and cytochrome oxidase, was the same as that seen in the absence of TMPD. Fig.4 shows also that the rapid proton release was, both in the presence and absence of TMPD, 50% inhibited by UHDBT. It has been shown that UHDBT inhibits electron flow [29] and associated proton release in the b–c_1 complex elicited by oxygen pulses of anaerobic mitochondria [30].

Therefore, it is evident that the rapid proton release observed during the first 300 ms after oxygenation of mitochondria supplemented with TMPD has nothing to do with electron flow through cytochrome oxidase supported by aerobic oxidation of TMPD. It derives from antimycin-insensitive oxidation of endogenous respiratory carriers and in particular from electron flow in the segments of the respiratory chain on the substrate side of UHDBT [30].

The flow analysis presented in fig.3 shows that proton release at the interval of 2.1 s after the addition of 5.56 nequiv. e^- oxygen/mg protein, when net formation of $TMPD^+$ reached the maximum value, amounted to 2.95 ± 0.07 ng-ions/mg protein. Subtraction from this amount of the proton release associated to oxidation of endogenous reductants (0.84 ± 0.09 ng-ions/mg protein, see fig.4) and that directly associated to the net formation of 4.72 ± 0.09 nmol $TMPD^+$/mg protein leaves with an extent of proton release of 1.17 ± 0.08 ng-ions/mg protein. This proton release practically coincides with the shortfall of $TMPD^+$ formation with respect to the oxygen added. Thus it derives from scalar acidification associated to re-reduction of $TMPD^+$ by hydrogenated donors.

These data show unequivocally that the proton release elicited by oxygenation of TMPD supplemented mitochondria in the presence of anti-

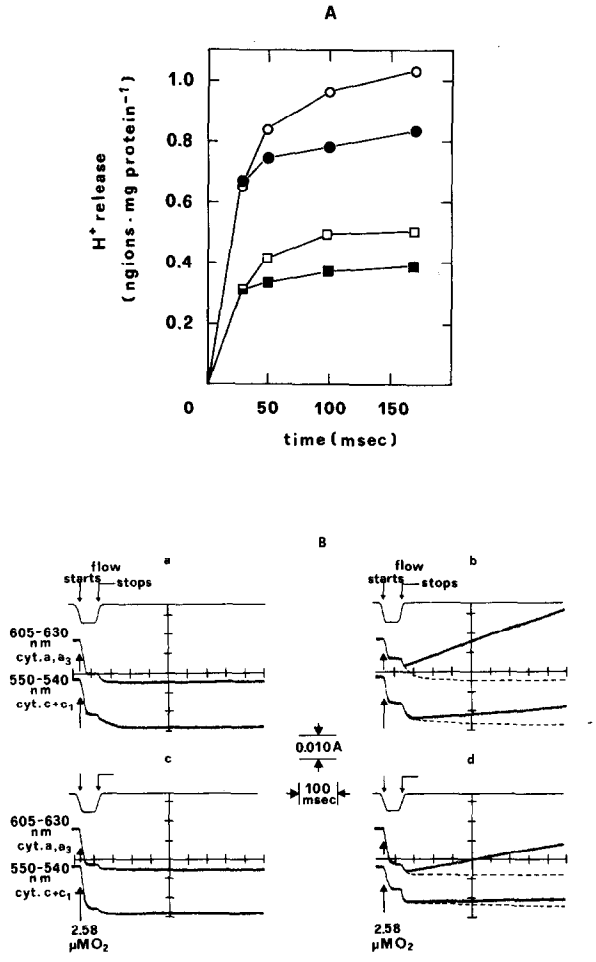

Fig.4. Kinetics of proton release (A) and oxidation of endogenous respiratory carriers (B) during oxygen pulses of anaerobic rat-liver mitochondria. Rat liver (3.5 mg/ml) was incubated in the reaction mixture described in the legend to fig.1. Where present, TMPD was 0.1 mM. Proton release (A) was measured with the flow method as in fig.3 legend: (●—●) control; (■—■) + 2 μM UHDBT; (○—○) + 0.1 mM TMPD; (□—□) + 0.1 mM TMPD + 2 μM UHDBT. The kinetics of cytochrome oxidation (B) was monitored with stopped-flow spectrophotometer [19]. The redox transitions of cytochromes in the presence of TMPD (···) were obtained by correcting the overall absorbance changes for that contributed, at the relative wavelength couples, by $TMPD^+$ formation. This was obtained from the curves of $TMPD^+$ formation (see fig.3), and the $\Delta\epsilon$ of $TMPD^+$ at the measuring wavelengths. Additions: (a) none; (b) 0.1 mM TMPD; (c) 2 μM UHDBT; (d) 0.1 mM TMPD and 2 μM UHDBT.

mycin and succinate, derives from oxidation of endogenous respiratory carriers and reduction of aerobically formed $TMPD^+$ and not from proton pumping associated to electron flow through cytochrome *c* oxidase, as claimed in [22].

It has already been pointed out [20,21] that use of artificial redox mediators to activate electron flow in a given segment of the respiratory chain may be seriously hampered by unrelated electron flow and proton translocation that can originate from re-reduction of their oxidised forms by endogenous reductants. This is demonstrated here to be the case when TMPD is used to feed electrons to the third coupling site of the respiratory chain. These unrelated redox events should be noted by monitoring directly the net formation of the oxidised form of the reductant throughout the experiment. In the absence of these controls it may be difficult to interpret correctly results obtained using artificial redox mediators.

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