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Activity of proteoliposomes containing cytochrome oxidase in the submitochondrial orientation

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Cytochrome-*c* oxidase proteoliposomes containing internally trapped cytochrome *c* can turn over on internal or external cytochrome *c*. At low TMPD levels the internal activity is significantly lower than the external activity as the functional internal cytochrome *c* is not fully reduced in the steady state. Increasing TMPD concentration increases the internal rate to equal that of the external enzyme. Internal activity results in the accumulation of $TMPD^+$. Valinomycin increases this accumulation and subsequently FCCP decreases it. In the presence of excess external cytochrome *c*, the effects of these ionophores are reversed. The internally-facing enzyme is thus capable of generating a $\Delta\mu H^+$ in proteoliposomes as well as in submitochondrial particles.

Cytochrome oxidase; Proteoliposome; *N,N,N',N'*-Tetramethyl-*p*-phenylenediamine; Cytochrome *c*; Fatty acid

1. INTRODUCTION

Cytochrome oxidase-containing proteoliposomes, when respiring on external cytochrome *c*, exhibit all the vectorial properties of the enzyme seen in mitochondria [1]. However, in the 'inverted' orientation, the turnover number and the respiratory control ratio are both lower for the proteoliposomal enzyme than for sonicated submitochondrial particles [2]. This has led to the suggestion that inverted enzyme may be inserted in the membrane in such a manner that it is automatically uncoupled [3].

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Abbreviations: COV, cytochrome-*c* oxidase reconstituted proteoliposomes; *c*-loaded COV, COV containing entrapped cytochrome *c*; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; DAD, 2,3,5,6-tetramethyl-*p*-phenylenediamine; PMS, phenazine methosulphate; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone

TMPD, which has been used to assay the internally-facing enzyme, as a donor to cytochrome *c* [4], is presumed to act as an electron carrier, unlike DAD or PMS, which move both protons and electrons across the membrane [5]. However, under some conditions the use of TMPD in enzyme-free liposomal systems can lead to the formation of a pH gradient [6].

The experiments described here show that the low turnover of internally-facing enzyme is a result of difficulties in trapping cytochrome *c* and not a function of the enzyme orientation. The steady-state behaviour of internally trapped Würster's blue ($TMPD^+$) shows that the inverted enzyme is bioenergetically fully competent, and that there are small amounts of an endogenous $TMPD^+/H^+$ antiporter in such vesicles. Differences in this activity may provide a reason for the conflicting results obtained by earlier authors [5,6].

2. MATERIALS AND METHODS

Cytochrome-*c* oxidase was prepared from beef heart essentially according to Kuboyama et al. [7],

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with Tween-80 substituting for Emasol. Cytochrome *c*-loaded cytochrome oxidase proteoliposomes were made by the sonication technique using Sigma type IV lecithin (asolectin) as described [8] in the presence of 125 μM cytochrome *c* (Sigma type VI, horse heart). Excess external cytochrome *c* was subsequently removed on a CM-25 Sephadex cation-exchange column. The internal medium was 50 mM potassium phosphate, pH 7.4, except in the experiments shown in fig.1, where 5 mM potassium phosphate, pH 7.4, was used.

The orientation of the enzyme was determined by the extent of reduction in the presence of cyanide and a small amount of external cytochrome *c* upon addition of ascorbate alone or ascorbate plus TMPD [2]. Cytochrome *c* and $TMPD^+$ (Würster's Blue) steady states were monitored using an Aminco DW-2 dual-beam spectrophotometer at 550–540 nm and 630–650 nm, respectively. Turnover numbers were measured using a Clark-type oxygen electrode (Yellow Springs Instrument Co.) attached to a suitable polarizing box and recorder.

3. RESULTS

In COV respiring on external cytochrome *c* with TMPD as reductant, maximal turnover can readily be achieved as the cytochrome *c* is almost fully reduced in the steady state at low TMPD levels [2]. Fig.1 shows that this is not the case for the internally trapped cytochrome *c*. At least three populations of cytochrome *c* molecules must be postulated: (i) one (see fig.1B) that is fully reduced at very low (nanomolar) TMPD concentrations not associated with turnover (presumably entrapped in vesicles lacking any enzyme), and two populations associated with oxidase turnover; (ii) one that is reduced at low TMPD levels (0.01–0.3 mM); and (iii) one that only approaches full reduction at very high millimolar TMPD levels (fig.1A).

In the case of submitochondrial particles Saga-Eisenberg and Gutman [9] concluded that the high K_m activity was due to direct oxidation of the oxidase by TMPD. The majority of this flux must however still proceed via internal cytochrome *c* because: (i) the turnover of the vesicular enzyme on TMPD alone is no more than 5% of the activity in the presence of saturating levels of cytochrome *c* (table 1, cf. [10]); and (ii) inducing vesicle lysis by adding the detergent lauryl maltoside sharply reduces the high TMPD rate as the effective concentration of cytochrome *c* drops dramatically (table 1).

The activity of internal compared to external enzyme can be assayed by measuring respiration in the presence and absence of external cytochrome *c* [2]. Upon increasing the TMPD concentration (allowing the high K_m internal activity to become saturated), the internal rate approaches that of the externally-facing enzyme (table 1). However, respiratory control for the internal species is still lower than that for the external one, even under conditions where uncoupled fluxes are comparable. When externally-facing enzyme is assayed alone a higher respiratory control is also seen (table 1).

If the internally-facing molecules show poor respiratory control, is it because they cannot generate an effective protonmotive force? Alternatively, Madden et al. [3] have suggested that vesicles containing 'inverted' enzyme are unusually permeable to protons. Fig.2 shows that neither of these proposals is correct. Like submitochondrial particles [9,11], *c*-loaded-COV accumulate $TMPD^+$ when respiring on ascorbate and TMPD. The addition of the K^+ ionophore valinomycin increases the $TMPD^+$ steady-state concentration. Upon anaerobiosis, which prevents further TMPD oxidation by internal cytochrome *c*, all the $TMPD^+$ is reduced by external ascorbate. In the presence of externally added cytochrome *c*, the extent of $TMPD^+$ trapping is much larger. Valinomycin now has the reverse effect, decreasing the $TMPD^+$ steady-state concentration until it reaches essentially the same level as in the absence of external cytochrome *c*.

The simplest explanation for these findings is that the $TMPD^+$ is responding to changes in the membrane potential. Internal activity generates a membrane potential, positive inside, that drives $TMPD^+$ efflux. Collapsing that potential with valinomycin allows an increase in the $TMPD^+$ steady-state level. Conversely, in the presence of external cytochrome *c*, the membrane potential becomes negative inside (due to the higher external activity at the low [TMPD] levels used) and $TMPD^+$ efflux is slowed. Once the potential is col-

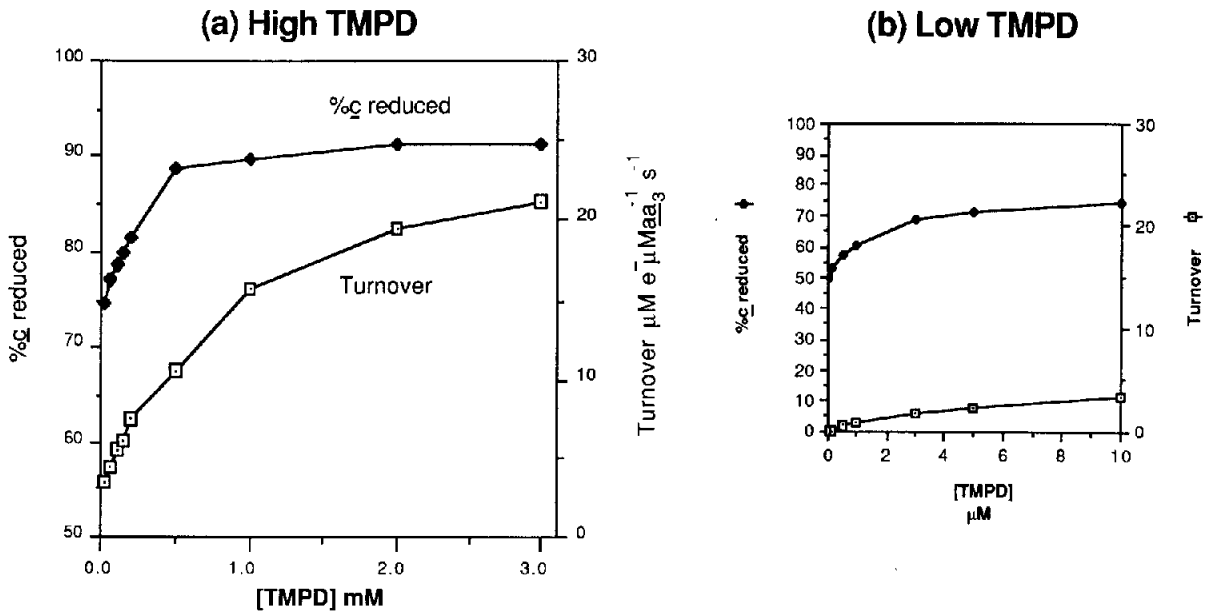

Fig.1. Effect of TMPD on the steady-state reduction of internally trapped cytochrome *c* and enzyme turnover in cytochrome oxidase proteoliposomes. Conditions: cytochrome *c*-loaded-COV (0.22 μM aa_3) in 50 mM potassium phosphate, pH 7.4, plus 5 mM ascorbate, 1.7 μM FCCP and 167 ng/ml valinomycin.

Table 1

Comparison of internally- and externally-facing enzyme activities in cytochrome *c*-loaded vesicles

Vesicle preparation	Activity monitored	Reductants	Kinetic parameters		RCR[b]	
			V_{max}[a]	K_m (app) (TMPD)	0.2 mM TMPD	2.0 mM TMPD
1. *c*-loaded COV	(a) total	asc/TMPD/*c*	179	0.1 mM	3.5	1.8
	(b) internal	asc/TMPD	77	0.5 mM	1.5	1.25
	(c) external	(a) minus (b)	102	0.02 mM	5.9	2.7
	(d) external	asc/*c*	80	–	(3.3)	–
2. *c*-loaded COV (dispersed)[c]	(total)	asc/TMPD	10	ND	ND	ND
3. COV	(total)	asc/TMPD[d]	9	ND	ND	ND

[a] Equivalent electrons $\cdot s^{-1} \cdot$ cytochrome aa_3^{-1}

[b] RCR (respiratory control ratio) = rate plus 1 μM FCCP and 0.1 μg/ml valinomycin divided by control rate

[c] By addition of lauryl maltoside (0.13%)

[d] In the absence of any cytochrome *c*

Oxygen uptake was measured polarographically with 5 mM ascorbate and 27 μM cytochrome *c* (when added), in 50 mM KP_i, pH 7.4, buffer at 30°C with varying levels of TMPD. V_{max} values are extrapolated to saturating TMPD concentrations in the presence of 1 μM FCCP and 0.1 μg/ml valinomycin. Rates are calculated per cytochrome aa_3 total in the vesicles; the orientation of vesicles was 50% cytochrome aa_3 facing-internally

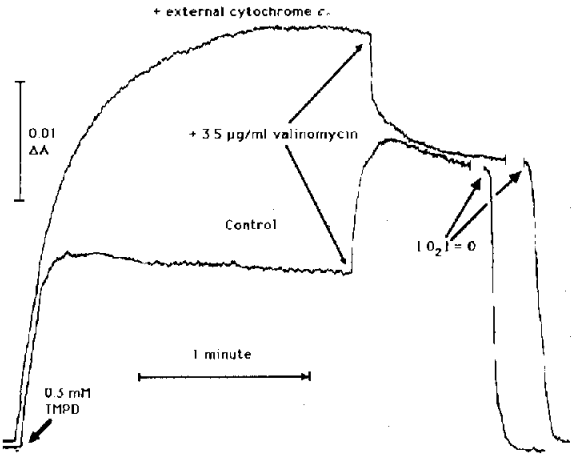

Fig.2. Accumulation of $TMPD^+$ by cytochrome *c*-loaded cytochrome oxidase vesicles. Conditions: as in fig.1, with ionophore additions as indicated [$TMPD^+$] monitored at 630–650 nm. Internal medium: 50 mM potassium phosphate, pH 7.4.

lapsed (with valinomycin) the $TMPD^+$ level is essentially independent of the presence of external cytochrome *c*. The low $TMPD^+$ steady state in the absence of valinomycin is evidence for an 'inverted' membrane potential generated by the 'inverted' enzyme. Independent measurements carried out using oxonol-V support this conclusion (not shown). The action of external cytochrome *c* also requires that a significant number of vesicles must exist containing enzyme in both orientations and that, in contrast to the suggestion of Madden et al. [3], both types can turn over simultaneously and vectorially.

External cytochrome *c* is expected to reverse the direction of the pH gradient as well as that of the membrane potential. Fig.3A shows the results of an experiment on the effect of protonophores

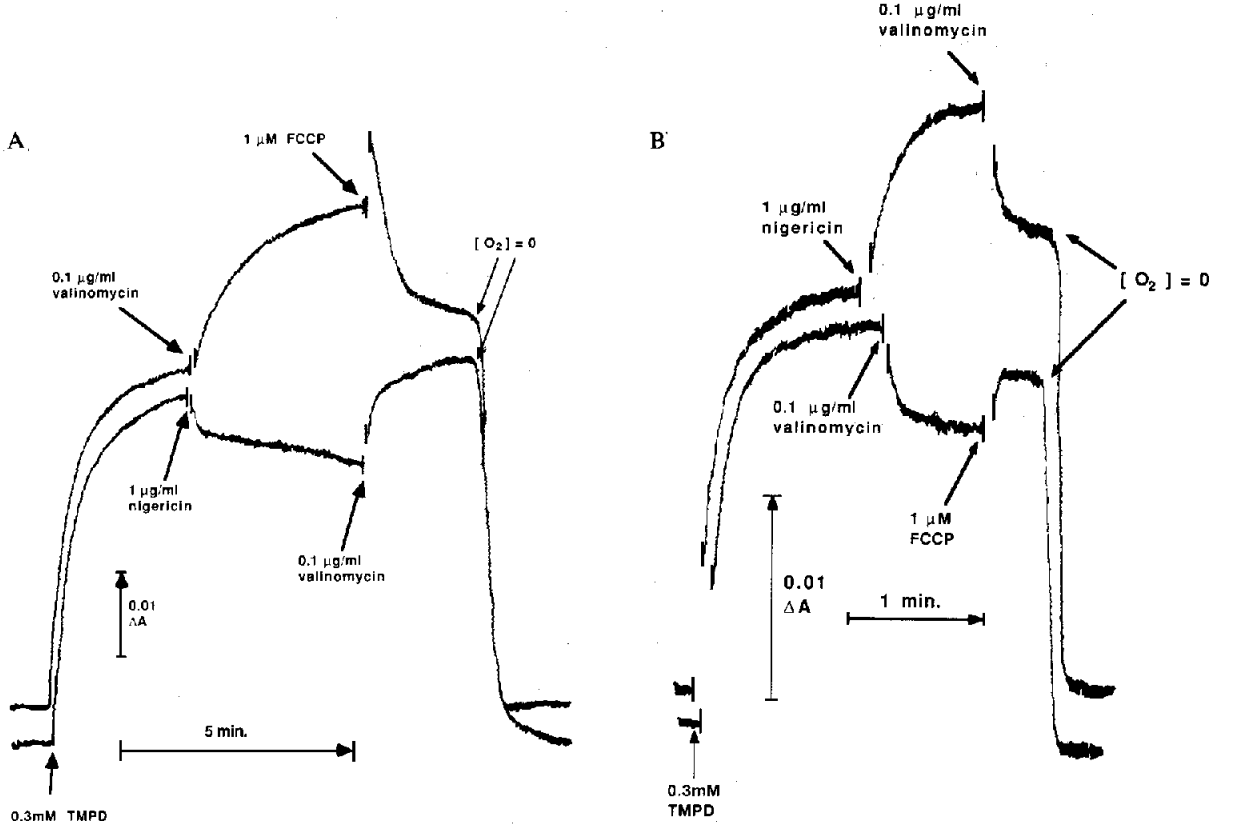

Fig.3. Effect of ionophores on $TMPD^+$ trapping by proteoliposomes. (A) In the absence of external cytochrome *c*. Conditions: cytochrome *c*-loaded-COV (0.36 μM aa_3, 68% facing-externally), otherwise as in fig.2. (B) In the presence of external cytochrome *c*. Conditions: cytochrome *c*-loaded-COV (0.24 μM aa_3, 55% facing-externally), otherwise as in fig.2.

upon the $TMPD^+$ steady state. Addition of FCCP, after valinomycin treatment, causes an initial increase in the $TMPD^+$ level followed by a drop to a new, lower, steady state. The initial increase is associated with the increase in flux through internally-facing enzyme (see table 1); similar increases are also seen when the internal flux is increased by raising the TMPD concentration (not shown). The cause of the subsequent decrease is less clear. Addition of nigericin in the absence of valinomycin also decreases the [$TMPD^+$] level. This response is biphasic, suggesting that nigericin acts to collapse the pH gradient and increase the membrane potential, as with the externally-facing enzyme [12]. Subsequent addition of valinomycin increases the [$TMPD^+$] concentration to that seen in the presence of valinomycin and FCCP.

With excess external cytochrome *c* the effects of protonophores are reversed (fig.3B). Valinomycin now decreases the [$TMPD^+$] level in the steady state and FCCP increases it. However, there is now no initial burst of $TMPD^+$ formation upon the FCCP addition; the pH gradient in the 'mixed' vesicles is of the wrong orientation (alkaline inside) to inhibit the internal flux and an increase in external flux cannot increase the $TMPD^+$ level because external ascorbate rapidly reduces external $TMPD^+$. The combination nigericin plus valinomycin also behaves in the 'opposite' way in the presence of external cytochrome *c*.

4. DISCUSSION

The high K_m for TMPD shown by cytochrome oxidase respiring on internally trapped cytochrome *c* provides an explanation for the low turnovers previously observed [2]. If a large proportion of internally trapped cytochrome *c* were in vesicles containing more oxidase than cytochrome *c* molecules, then even though the effective cytochrome *c* concentration would be high (due to the small internal volumes of such vesicles) it would not be fully reduced in the steady state unless very high TMPD concentrations were employed. Thus the 'low K_m, low V_{max}' activity seen in *c*-loaded COV (fig.1) and in submitochondrial particles [9] is associated with vesicles containing more cytochrome *c* than cytochrome oxidase while the 'high K_m, high V_{max}' activity is associated with vesicles containing more oxidase than cytochrome *c*. Evidence to support this model comes from calculations of the rate of reduction of internal cytochrome *c* by TMPD from data such as that in fig.1. The calculated rate constant at high TMPD levels is $2 \times 10^5\ M^{-1} \cdot s^{-1}$, in agreement with the value of $1.3 \times 10^5\ M^{-1} \cdot s^{-1}$ found for cytochrome *c* bound to cytochrome oxidase by Hill and Nicholls [13].

The additional effects of FCCP on $TMPD^+$ accumulation, shown in fig.3, indicate a controlling role for the pH gradient as well as the membrane potential. A small $TMPD^+$ steady-state concentration decrease is also seen at high levels of external cytochrome *c* in the presence of valinomycin, also due to an effect of the pH gradient. It must be assumed that such a pH gradient, acid inside, inhibits $TMPD^+$ efflux, independently of any effect on the enzyme activity. The simplest explanation involves the postulation of a $TMPD^+/H^+$ antiporter, which allows the $TMPD^+$ to exit electroneutrally as well as electrophoretically.

The proposal of such an antiporter may not be as surprising as it first appears. Although the vesicles are supposed to contain only cytochrome *c*, cytochrome oxidase and phospholipids, some phospholipid breakdown is likely to occur during the sonication process. Oleic acid has a nigericin-like activity when added to COV [12,14], suggesting that the uncharged free acid and cation soap are membrane permeable, but not the charged anion. A '$TMPD^+$ soap' would presumably also be permeable. This antiporter process explains satisfactorily the internal acidification seen when TMPD is oxidized by ferricyanide-loaded liposomes [6]. The proton does not enter the vesicle with the TMPD as suggested by Miller et al., but instead enters as $TMPD^+$ leaves. Fatty acid levels vary in different vesicle preparations, allowing other workers to find no internal acidification in essentially similar systems [5].

Although the internal enzyme is bioenergetically competent, it shows a low respiratory control ratio. However, proteoliposomal cytochrome oxidase activity is markedly stimulated by a decrease in pH [15]. The thermodynamically inhibitory pH gradient (acid inside) created by the 'inverted' enzyme is thus expected to be kinetically stimulatory. We conclude that cytochrome oxidase is fully competent, kinetically and bioenergetically, whichever way it is facing in the liposomal membrane.

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