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KINETICS OF CYTOCHROME *c* AND TMPD OXIDATION BY CYTOCHROME *c* OXIDASE FROM THE THERMOPHILIC BACTERIUM, PS3

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Cytochrome caa_3 (cytochrome oxidase) from the thermophilic bacterium PS3 can exhibit full catalytic activity in the presence of ascorbate and TMPD or other electron donors and in the absence of added soluble *c*-type cytochromes. It appears to possess only a low-affinity and not a high-affinity site for the soluble cytochromes. Proteoliposomal cytochrome caa_3 develops an effective membrane potential in the presence of ascorbate and TMPD or PMS, in the absence of added soluble cytochrome *c*. Reduction of the a_3 centre is blocked in the presence of cyanide. During reductive titrations of the cyanide-inhibited enzyme, electrons initially equilibrate among three centres, the *c* haem, the *a* haem and one of the associated Cu atoms. During steady-state turnover, electrons probably enter the complex via the bound *c* haem; the *a* haem and perhaps an associated Cu_A atom are reduced next. It is concluded that, despite its size and hydrophobic association with the aa_3 complex, the haem *c*-containing subunit can behave in an analogous way to that of mammalian cytochrome *c*, bound at the high-affinity site of the eucaryotic enzyme.

Introduction

Mitochondrial cytochrome *c* oxidase is known to have two binding sites (high affinity and low affinity) for its substrate cytochrome *c* [1–4]. The dissociation constant for binding at the high-affinity site is of the order of $1 \cdot 10^{-8}$ M [2,3,5] and this site was shown to be located on subunit II (the second largest subunit of mitochondrial cytochrome oxidase) by using a bifunctional imidate [6] or photosensitive azido-derivative of cytochrome *c* [7]. Recently a one-to-one complex between cytochrome *c* and bovine heart cytochrome *c* oxidase has been prepared and crystallized by Ozawa et al. [8,9]. The presence of bound cytochrome *c* at the high-affinity site is necessary for the oxidation of artificial electron donors such as TMPD; cytochrome *c* therefore seems to act as an essential molecular part of cytochrome oxidase, at least in a low ionic strength medium [2,10].

Cytochrome aa_3-type terminal oxidases containing haem *a* and copper as prosthetic groups have been purified from various bacteria (for reviews, see Refs. 11 and 12). In addition, but only in the case of thermophilic bacteria, a haem *c*-containing peptide was also copurified with the enzymes. Thus, PS3 [13,14] and *Thermus thermophilus* [15,16] preparations contained haem *c*, whereas no such haem *c* was found in the enzymes from mesophilic bacteria, including *Bacillus subtilis* [17]. Fee and co-workers [15] called *T. thermo-*

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Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Mops, 4-morpholinepropanesulphonic acid; PMS, phenazine methosulphate; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

philus enzyme cytochrome c_1aa_3, since this haem *c* was found to be attached covalently to a hydrophobic polypeptide of molecular weight 33000 and shows an EPR signal with a *g* value of 3.3.

In the thermophilic bacterium PS3, haem *c* was found attached to subunit II, the second largest polypeptide [14,18], $M_r \approx 38000$, among the three subunits. It possesses an absorption maximum in the reduced state closer to 549.5 nm than 550 nm [14]. Moreover, this bacterial subunit II was shown to crossreact immunologically with subunit II from yeast cytochrome *c* [11]; this *c*-type cytochrome therefore seems more likely to resemble cytochrome *c* than cytochrome c_1.

Here we present evidence suggesting that this covalently bound cytochrome *c* may play a role analogous to that of cytochrome *c* bound to the high-affinity site of mitochondrial cytochrome oxidase.

Experimental

Bacterial cytochrome oxidase was purified from the thermophilic bacterium PS3, as described previously [14], and bacterial cytochrome *c*-552 from *T. thermophilus* HB8 [19] was kindly donated by Dr. Hon-nami of the Mitsubishi-kasei Institute of Life Science, Tokyo. Cytochrome *c* from *Candida krusei* was a product of Sankyo Co., Tokyo. Horse heart cytochrome *c* type VI and cytochrome *c* type VIII from *Saccaromyces cerevisiae* were products of Sigma Chemical Co., St. Louis, MO, U.S.A. Hexamine ruthenium (II) chloride was a product of Alfa, Danvers, MA, U.S.A.

The cytochrome *c* oxidase activity was measured at 32°C by following changes in pH with ascorbate as a final electron donor according to Eqn. 1:

$$\text{ascorbate } H^- + H^+ + \tfrac{1}{2}O_2 \rightarrow \text{dehydroascorbate} + H_2O \qquad (1)$$

The net alkali formation was back-titrated with aliquots of 20 mM HCl. The reaction medium (2.5 ml) comprised 8 mM sodium ascorbate/10 mM KCl/2 mM potassium-Mops buffer and was stirred magnetically. The final starting pH was between 6.95 and 7.00. The enzyme turnover is expressed in terms of molecular activity, as twice the number of nmol alkali formed (equivalent to 1 electron transfer) per s and per nmol of the enzyme (aa_3). A Beckman pH meter (Φ 71) with a combination electrode (Metrohm, semi-micro type) was used and the pH changes were followed by means of a sensitive strip chart recorder (Cole-Parmer, Model 8371-10).

Membrane potential formation by PS3 cytochrome oxidase incorporated into liposomes was measured using a hand-made butyltriphenylphosphonium$^+$ electrode with a poly(vinyl chloride) membrane containing tetraphenyl boron [20]. Liposomes containing cytochrome oxidase (1 ml) were prepared by the freeze-thaw method from 40 mg acetone-washed α-tocopherol-treated asolectin [21] and PS3 cytochrome oxidase (2–4 nmol in aa_3) as described previously [14].

The steady-state redox-level of PS3 cytochrome oxidase and the associated responses of the enzyme to titrations with TMPD (in the presence of KCN) were measured using a Beckman DU-7 spectrophotometer interfaced with an Apple II plus microcomputer for data collection and analysis. The enzyme was dissolved in 50 mM sodium phosphate buffer (pH 7.4) containing 0.25% Tween-80, unless otherwise stated. Spectra (650–500 nm) were normally taken at a scanning speed of 10 nm/s using oxidized enzyme as the reference material. The following wavelength pairs were used for determination of the oxidation-reduction level: 604.5–630 nm ($\Delta E_{mM} = 23.1$) [14] or 604.5–618 nm ($\Delta E_{mM} = 20.1$) for cytochrome *a* (no correction being made for the contribution of cytochrome a_3); 550–540 nm ($\Delta E_{mM} = 4.3$) for TMPD$^+$ [22].

PS3 cytochrome oxidase (aa_3) concentration was determined using a ΔE_{mM} (reduced form, 604–630 nm) of 33.2 [14]. For the determination of cytochrome *c* concentration, the following ΔE_{mM} values (reduced minus oxidized) were used: 21.2 for *S. cerevisiae c* at 550 nm [23]; 24.3 at 549 nm for *C. krusei c* (Sankyo's manual) and 14.0 at 552 nm for HB8 cytochrome *c*-552 [16].

Results and Discussion

Oxidation of cytochrome c and TMPD

In the case of mitochondrial cytochrome *c* oxidase, electron transfer mediated by an artificial dye such as TMPD is known not to occur at an

appreciable rate unless a small amount of cytochrome *c* is added [24,10]. TMPD cannot reduce the haem of cytochrome *a* rapidly, but can reduce cytochrome *c* bound at the 'high-affinity' site [2,25].

Fig. 1 shows the results of analogous experiments carried out with PS3 cytochrome oxidase which possesses an endogenous haem *c* bound covalently upon its subunit II [14,18].

As indicated in the lower trace (closed circles), TMPD alone was able effectively to mediate ascorbate oxidation, and the addition of cytochrome *c* accelerated the oxidation only additively (upper trace, closed squares). The concentration dependence of the oxidation rate on [TMPD] in the absence of added cytochrome *c* approximated the Michaelis-Menten equation giving an apparent K_m of close to 0.27 mM and $\overline{V}_{max}$ close to 300 s^{-1} (electrons/s per *c*-aa_3).

In Fig. 2, we see the effect of cytochrome *c* concentration on the turnover of the enzyme. The results are plotted in the double-reciprocal format. Under these conditions, *Candida* cytochrome *c* is oxidized with a K_m of 3.7 μM and a maximal rate of 120 s^{-1}. The addition of TMPD to this ascorbate-cytochrome *c*-cytochrome oxidase (caa_3) system resulted in a decreased K_m for cytochrome *c* (2.4 μM) without changing the $\overline{V}_{max}$. This effect of TMPD is presumably due to its action as electron mediator between ascorbate and cytochrome *c*, as reported with the beef heart enzyme [2,24].

Fig. 3 shows the results of three similar sets of

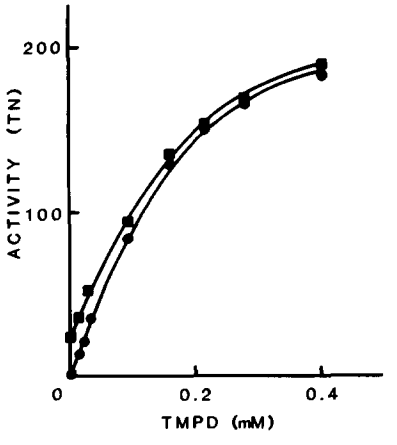

Fig. 1. Effect of cytochrome *c* addition on TMPD oxidation. The oxidation rate was followed with a glass electrode in a reaction medium (2.5 ml total volume) containing 8 mM sodium ascorbate, 10 mM KCl, 0.2% Tween-80, 80 μg/ml phospholipids and 2 mM K-Mops (pH 6.95–7.0) at 32°C. An enzyme sample containing 0.02 nmol aa_3 was used. ●, Control without cytochrome *c*; ■, plus 2.6 μM *C. krusei* cytochrome *c*. TN, turnover number.

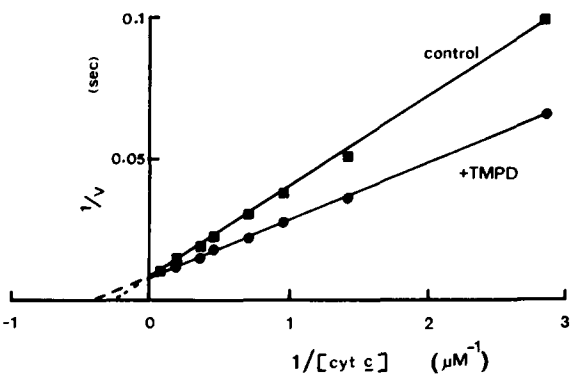

Fig. 2. Effect of TMPD on the kinetic behaviour of cytochrome *c* oxidase. Double-reciprocal plot of turnover against cytochrome *c* concentration. The assay conditions were the same as shown in Fig. 1 with the indicated concentrations of *C. krusei* cytochrome *c*. ■, control, ●, plus 54 μM TMPD.

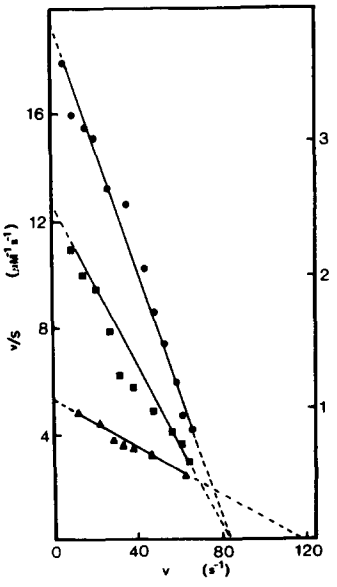

Fig. 3. Eadie-Hofstee plots showing apparent absence of a high-affinity cytochrome *c*-binding site. The assay conditions were the same as in Fig. 1, except that 0.16 nmol enzyme was used in the reaction medium at pH 7.4 in the case of hexamine ruthenium oxidation. ■, HB8 cytochrome *c*-552; ●, *S. cerevisiae* cytochrome *c*; ▲, hexamine ruthenium chloride.

experiments presented in the form of Eadie-Hofstee plots. These were carried out to determine whether there are two binding sites for exogenous cytochrome *c* on the PS3 enzyme. Two kinds of cytochrome *c*, *S. cerevisiae* cytochrome *c* and cytochrome *c*-552 from *T. thermophilus* HB8, and one unique electron mediator, hexamine ruthenium, which is also known to bind two sites in the eukaryotic enzyme [26], were employed. All three electron carriers gave straight lines. Table I summarizes the kinetic parameters obtained in Figs. 1–3. Since the K_m values listed are much larger than those known for the eucaryotic high-affinity site [2–5] but comparable to those for the eucaryotic low-affinity site, these results may indicate that the high-affinity site of the PS3 enzyme has been occupied by the intrinsic cytochrome *c* found on subunit II and only the low-affinity site can be titrated with exogenous cytochrome *c* or with hexamine ruthenium. This conclusion is also supported by the correlated fact that the addition of a small amount of cytochrome *c* does not affect TMPD oxidation (Fig. 1).

Electron transfer between cytochrome *c* and enzyme is markedly affected by the ionic strength of the reaction medium [10,25].

As shown in Fig. 4, when the oxidation of ascorbate is mediated by TMPD, the rate is not affected by high concentrations of KCl, suggesting that the binding between intrinsic cytochrome *c* and PS3 cytochrome oxidase is quite different from the salt-sensitive mammalian cytochrome-cytochrome oxidase interaction. The oxidation via cytochrome *c*-552 from HB8 was also not affected by salt. Only the oxidation rate with *S. cerevisiae* cytochrome *c* is diminished by salt addition in an analogous fashion to its reaction with mitochondrial cytochrome *c* oxidase.

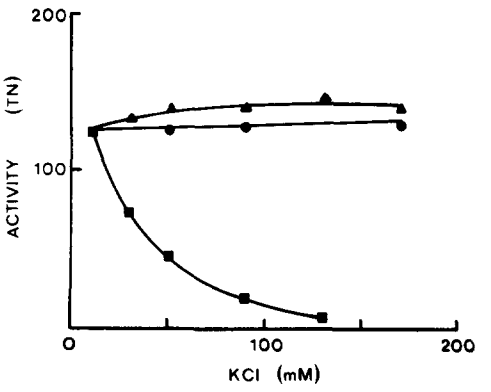

Fig. 4. Effect of ionic strength on the PS3 oxidase activity using three different substrates. The assay conditions were as shown in Fig. 1, pH 7.0 and 32°C. ▲———▲, TMPD (170 μM); ●———●, HB8 cytochrome *c*-552 (17 μM); ■———■, *S. cerevisiae* cytochrome *c* (32 μM) plus 18 μM TMPD. KCl concentrations varied from 10 to 170 mM. TN, turnover number.

TABLE I

KINETIC PARAMETERS FOR THE OXIDATION OF SEVERAL CYTOCHROMES *c* AND OTHER ELECTRON DONORS BY PS3 OXIDASE

Reaction medium contained 8 mM sodium ascorbate, 10 mM KCl, 0.2% Tween-80, 80 $\mu g/ml^{-1}$ phospholipids (asolectin), 2 mM K-Mops pH 7.0 at 32°C. Enzyme (c_1aa_3) concentration was 8 or 64 nM in $Ru(NH_3)_6$ experiments, TN, turnover number.

Electron donor	TN_{max} (s^{-1})	K_m (μM)
TMPD	≈ 300	270
$Ru(NH_3)_6$	115	110
PMS	>140	⩽ 2.5
HB8 *c*-552	85	4.3
C. krusei c	120	3.7
S. cerevisiae c	85	6.7

Kinetic behaviour of PS3 cytochrome c-aa₃ in proteoliposomes

PS3 cytochrome oxidase was incorporated into proteoliposomes as described in Experimental.

As illustrated in Fig. 5, membrane potential formation by these vesicles was recorded as a change in the external concentration of the permeant cation butyltriphenylphosphonium$^+$. Ascorbate oxidation mediated by PMS without addition of cytochrome *c* resulted in a large uptake of butyltriphenylphosphonium$^+$. If there was no internal absorption of butyltriphenylphosphonium$^+$ on liposomes, this change would correspond to a maximum membrane potential of −182 mV, assuming an internal volume of 3.5 μl/mg phospholipid. Table II summarizes the oxidation rates and the apparent membrane potentials formed. The comparatively low $\Delta\psi$ formation in the cases of both TMPD and PMS relative to their fast

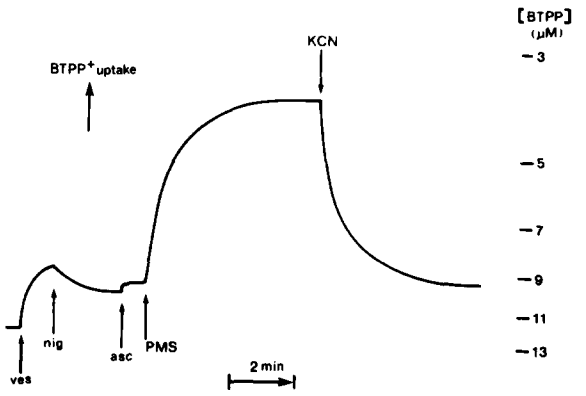

Fig. 5. Measurement of membrane potential formation coupled to ascorbate oxidation via PMS. The concentration change of butyltriphenylphosphonium$^+$ ($BTPP^+$) was measured with a butyltriphenylphosphonium$^+$ electrode at 32°C. The reaction mixture (2.5 ml) contained 25 mM K_2SO_4, 2.5 mM $MgSO_4$, 5 μM butyltriphenylphosphonium$^+$ and 5 mM Tris-Mes/2 mM K-Mops buffer at pH 7.0. The additions are: ves, a 25 μl aliquot of liposomes containing 0.10 nmol (aa_3) PS3 cytochrome oxidase and 1 mg phospholipids; nig, 0.5 μg nigericin; asc, 20 μmol sodium ascorbate; PMS, 5 nmol phenazine methosulphate; and KCN, 1 μmol KCN.

oxidation rates may be due to the membrane-permeable nature of these dyes which permits their interaction with the internally-facing enzyme molecules [2]. Nevertheless, these results show that respiration involving these mediators, in spite of the absence of cytochrome *c*, is quite competent in energy conservation.

TABLE II

RESPIRATION RATES AND MEMBRANE POTENTIAL FORMATION WITH VARIOUS SUBSTRATES BY LIPOSOMES CONTAINING PS3 CYTOCHROME OXIDASE

The membrane potential was monitored as shown in Fig. 5, and the maximal apparent values attained are shown. The oxidation rates were measured in the same medium, except that valinomycin (0.2 μg) and FCCP (0.5 μg) were substituted for butyltriphenylphosphonium$^+$ and nigericin. All measurements were made with a pH meter using ascorbate as the final electron donor. 32°C, pH 7.0. TN, turnover number.

Substrate	Concn. (μM)	Oxidation (TN, s^{-1})	Apparent $\Delta\psi$ (mV)
HB8 *c*-552	2.7	46.7	−213
C. krusei c	2.8	24.2	−171
S. cerevisiae c	2.8	25.7	−179
S. cerevisiae c	80	43.8	−207
TMPD	150	59.2	−158
PMS	2.5	138.3	−182

Titration of PS3 cytochrome caa$_3$ by TMPD in the presence and absence of cyanide

The reduction of the several redox centres in eucaryotic oxidase can be followed sequentially by adding TMPD to cyanide-inhibited enzyme [27]. It was of interest to repeat this with the PS3 enzyme which contains a *c*-type cytochrome as an extra redox centre. Firstly, the cyanide complex was formed by cyanide preincubation of the oxidized (resting)enzyme, as described in the legend to Fig.

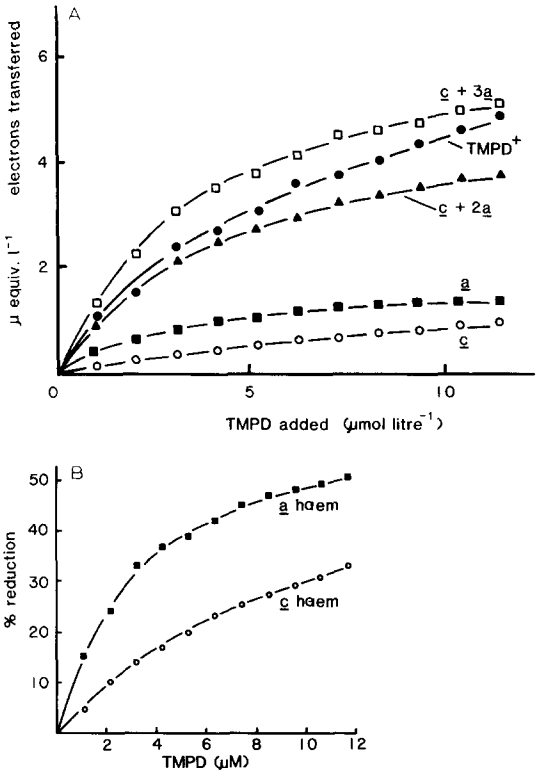

Fig. 6. (A) Titration of cyanocytochrome oxidase with TMPD. The enzyme (3.15 μM in aa_3) was incubated with 2.5 mM KCN in 50 mM sodium phosphate buffer at pH 7.4 for 10 h at 25°C and for a successive 38-h period at 4°C. Titration was carried out in a semi-micro cuvette (0.85 ml) at 31°C with sequential additions of 1 μl aliquots of 0.9 mM TMPD. The subsequent distribution of reducing equivalents was monitored with a Beckman DU-7 spectrophotometer after 20 s, recording the spectrum from 650 to 500 nm. ●, Changes at 630–650 nm ($TMPD^+$); ○, changes at 550–540 nm (c^{2+}); ■, changes at 604.5–619 nm (a^{2+}); ▲, sum of $[c^{2+}]+2[a^{2+}]$; □, sum of $[c^{2+}]+3[a^{2+}]$. (B) shows percent reduction of cytochromes *c* (○) and *a* (■) during the titration.

6. The figure then summarizes the results obtained when such a cyanocytochrome c_1aa_3 complex is titrated with small aliquots of TMPD. First, cytochrome *a* is reduced (closed squares) and then the cytochrome *c*-like haem group (open circles), as shown in Fig. 6B. During the initial phase of the titration, the amount of TMPD oxidized to $TMPD^+$, Würster's blue (closed circles) is equal to the sum of the *c* haems reduced plus twice the amount of *a* haems reduced (closed triangles). This indicates that another centre is reduced almost synchronously with cytochrome *a*, as in the case of the mammalian enzyme [27]. This centre is presumably one of the two copper atoms in the enzyme. Subsequently, the amount of $TMPD^+$ appearing increases, possibly indicating the reduction of a fourth centre (open squares).

Under turnover conditions (cyanide absent), the reduction of the cytochrome *a* haem also exceeds that of the cytochrome *c* haem, as shown in Fig. 7A. Half-reduction of the *a* haem requires only about 25 μM TMPD, whereas half-reduction of the *c* haem requires a TMPD level in excess of 80 μM (cf. the K_m value for TMPD measured at lower oxidase concentrations, Table I). In Fig. 7B, these data are replotted in the form of 'Γ' against $v/[c^{2+}][a^{3+}]$, according to the method of Nicholls and Chanady [27,28]. The results are consistent with the sequential electron transfer model of Eqn. 2:

$$\text{TMPD} \xrightarrow{k_1} c \underset{}{\overset{K_{eq}}{\rightleftharpoons}} \underset{a}{\text{Cu}_A} \xrightarrow{k_2} a_3 \cdot \text{Cu}_B \qquad (2)$$

PS3 cytochrome oxidase

in which k_1 is approx. $1 \cdot 10^6\ M^{-1} \cdot s^{-1}$, $K_{eq} = 10$ and $k_2 \approx 300\ s^{-1}$ (Table I and Fig. 7B). The redox potential of the cytochrome *a* is apparently some 30 to 60 mV higher than that of the cytochrome *c* haem at this pH value. Poole et al. [29] reported approximate equality for the redox potentials of *c* and *a* haems in intact PS3 membrane preparations, but this was in the absence of cyanide, and other conditions were also different. The bound cytochrome *c* is probably the primary electron acceptor. If haem *a* or Cu_A were the primary acceptor, then the Γ plot (Fig. 7B) would be expected to be horizontal. Moreover, it would then be hard to explain the much lower turnover of the

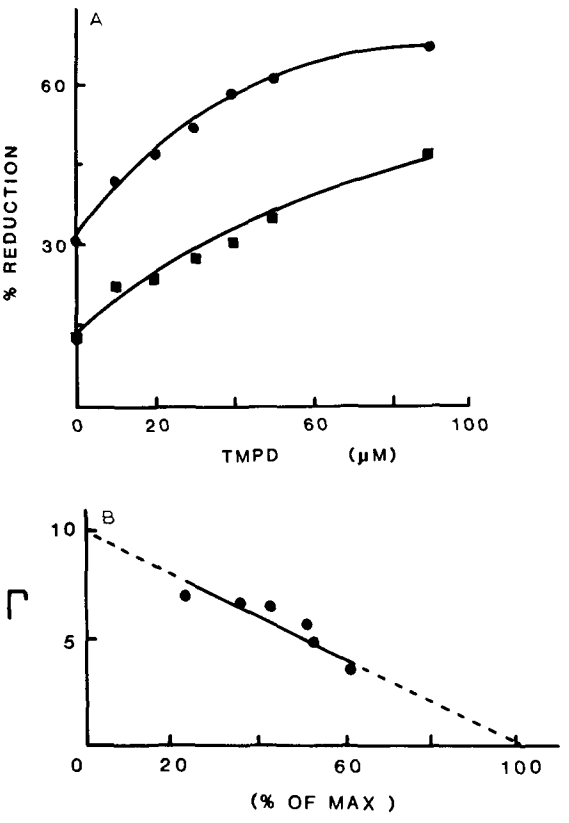

Fig. 7. Effect of TMPD concentration on the steady-state reduction level of cytochrome *c* and cytochrome *a* components of PS3 cytochrome oxidase. The enzyme (0.7 μM in aa_3) in 3 ml aerobic 50 mM sodium-phosphate buffer (pH 7.4) containing 0.25% Tween-80 was treated with TMPD at the indicated concentration in the presence of 8 mM ascorbate. (A) Percentage reduction plotted against TMPD concentration. (B) Plot of the flux ratio, Γ, for the cytochrome *c*/cytochrome *a* system against the apparent respiration rate constant. The ratio, $[a^{2+}][c^{3+}]/[a^{3+}][c^{2+}]$ or 'Γ', is plotted against $\bar{V}/[c^{2+}][a^{3+}]$ for the experiments shown in (A) after subtraction of the $[a^{2+}]$ and $[c^{2+}]$ values obtained in the absence of TMPD. ■, changes at 550–540 nm (cytochrome *c*); ●, changes at 604.5–630 nm (cytochrome *a*).

eucaryotic enzyme in the presence of TMPD alone [24]. As with the eucaryotic enzyme, however, it is not clear whether cytochrome *a* or Cu_A directly reduces the $a_3 \cdot Cu_B$ centre. It is possible that each is a specific reductant for only some of the redox states of $a_3 \cdot Cu_B$. The value of $1 \cdot 10^6\ M^- \cdot s^{-1}$ for *k* exceeds both that for free eucaryotic cytochrome *c* and that for the eucaryotic caa_3 complex [30]. It approximates the rate at which TMPD reduces the

TABLE III

RATES OF REACTION BETWEEN TMPD AND BOUND OR FREE CYTOCHROME *c*

All reactions were carried out a pH value between 7.0 and 7.4 and in media of comparatively low ionic strength.

Cytochrome *c*	k ($M^{-1} \cdot s^{-1}$)	Temp. (°C)	Ref.
1 Horse heart cyt *c* (in solution)	$3.5 \cdot 10^4$	20	30
2 Horse heart cyt *c* (in 'tight' complex with aa_3)	$1.3 \cdot 10^5$	20	30
3 The cyt c-c_1 centre in submitochondrial particles	$4.5 \cdot 10^5$	30	29
4 Cyt *c* haem in PS3 cyt caa_3	$1.1 \cdot 10^6$	32	this paper

bound *c* of submitochondrial particles (Table III). A somewhat similar conclusion has recently been reached for the reaction mechanism of *T. thermophilus* 'c_1aa_3' by Yoshida and Fee [31], whose paper appeared after submission of the present work for publication. The *T. thermophilus* enzyme also shows a high reactivity with TMPD and the absence of a high-affinity site for added cytochrome *c*.

Conclusions

Although the *c* haem in PS3 cytochrome caa_3 is located upon a 38 kDa subunit [14,18], its kinetic behaviour in the presence of ascorbate and TMPD resembles that of eucaryotic cytochrome *c* bound to the 'tight' (T) site on eucaryotic cytochrome aa_3 [2,25]. Full catalytic oxidase activity is exhibited by the c_1aa_3 enzyme in the presence of ascorbate and excess TMPD, and in the absence of other soluble cytochromes. No high-affinity ('T') site can be detected on PS3 cytochrome caa_3. Oxidation of added bacterial and eucaryotic cytochromes *c* seems to occur solely via a low-affinity ('L') site, as defined by Ferguson-Miller et al. [3] and analyzed by Brooks and Nicholls [25]. The enzyme is also fully active in energizing the membrane when incorporated into proteoliposomes, when challenged with TMPD alone, or with electron donor. We therefore regard this bound cytochrome *c* as analogous to tightly bound (T site) eucaryotic cytochrome *c* and not to eucaryotic cytochrome c_1 (cf. Refs. 15 and 31). The enzyme is therefore correctly named cytochrome caa_3, identifying it as a three-haem cytochrome complex. The natural electron donor to the complex is unknown; it is not necessary that it be another cytochrome *c* species.

Acknowledgements

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