OXIDATION OF VANADIUM IV BY CYTOCHROME c OXIDASE: EVIDENCE FOR A TERMINAL COPPER PATHWAY

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Summary: Both the positive vanadyl and negative vanadite ions are oxidized by mitochondria or purified cytochrome c oxidase. The oxidation does not require cytochrome c and is not inhibited by the cytochrome c antagonist polylysine. Oxidation rates for vanadyl compare well with rates for cytochrome c oxidation. Since the oxidation is not inhibited by azide, cyanide and carbon monoxide it does not appear to be catalyzed by the cytochrome a_3 pathway. Inhibition by diphenylthiocarbazone and salicylaldoxime indicate that one of the copper sites in cytochrome oxidase can be the site of oxidation.

Introduction

In addition to cytochrome c several inorganic and organic redox agents have been used as substrates for cytochrome c oxidase. For the most part these donors require cytochrome c as a mediator. Examples are ferrocyanide (1), silicomolybdate and nitroprussides (2). The tetrahalogen substituted 1,4benzoquinols and molybdooctacyanide (2) are exceptions in that they can react directly with the oxidase, but their oxidation is inhibited by the traditional inhibitors of cytochrome oxidase such as cyanide, carbon monoxide or hydroxylamine. This paper will describe unusual characteristics of the oxidation of vanadyl and vanadite which differ from those of previous artificial donors for the oxidase.

Methods

Mitochondria (3), electron transport particles, ETP, (4;alkaline procedure) and cytochrome oxidase (5) preparations have been described.

All oxidase assays were carried out polarographically in 1.8 ml final volume with a Clark electrode. Cytochrome c oxidase with ascorbate

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and N,N,N',N' tetramethylparaphenyldiamine (TMPD) at pH 7.0 was carried out as described (6). Tetrachloroquinol (TCHQ) oxidase was assayed in 0.03 M phosphate buffer pH 7.0, with 30 μg tetrachloroquinone, 0.4 mg dithiothreitol (DTT) and either 0.2 mg cytochrome c or 0.2 mg polylysine (2800 MW). Vanadyl oxidase was in 0.03 M phosphate buffer pH 7.0 with 1.2 μmoles vanadyl sulfate as substrate. Decavanadate was prepared by adding an excess of vanadium pentoxide to 0.1 M sodium hydroxide and decanting the yellow solution after standing for two days at pH 6.2. For assay 0.6 μmoles decavanadate were reduced with 0.4 mg dithiothreitol to the black vanadite ion in the assay cuvette in 0.03 M phosphate buffer pH 6.2. Enzyme was added after three minutes.

Cytochrome c Type IV and polylysine MW 2800 was obtained from Sigma; Vanadium sulfate and vanadium pentoxide from K and K laboratories. Dithizone, bathophenanthroline and salicylaldoxime were dissolved in ethanol. Equivalent levels of ethanol (up to 25 μl) were added to control assays.

Results

At pH 7.0 in phosphate buffer vanadyl (VO^{++}) ions are oxidized by cytochrome oxidase preparations about one-fourth the rate of TMPD, cytochrome c, ascorbate and at one-half the rate of the TCHQ, cytochrome c, DTT oxidation (Table I). The rate of vanadyl oxidation is greater than the rate of the TCHQ, polylysine, DTT system. Vanadite is oxidized at a much slower rate. Vanadite oxidation in phosphate or tris mes buffer shows an optimum at pH 6.0-6.2 with very little oxidation at alkaline pH. Vanadyl oxidation in phosphate buffer is maximum at pH 6.0 and decreases with increase of pH. In tris mes buffer vanadyl oxidase is maximum at pH 7.4 and there is no oxidation below pH 6.8. (Table II).

The effect of inhibitors on oxidase activity as assayed with various electron donor systems is shown in Table III. Chelator inhibition of

Table I

Oxidation of various electron donor systems by cytochrome oxidase

Substrate	Oxidation rate µatoms O/min x mg
Vanadyl sulfate	2.6
DTT + sodium decavanadate	0.1
ascorbate + cytochrome c	1.8
ascorbate + TMPD + cytochrome c	8.8
DTT + cytochrome c	0.2
DTT + TCHQ + Cytochrome c	4.4
DTT + TCHQ + polylysine	0.6

All assays in 0.03 M phosphate pH 7.0 at 37°.

vanadyl oxidase and lack of cyanide or carbon monoxide inhibition suggests oxidation at a copper site which bypasses cytochrome a_3.

Vanadyl and vanadite ions are oxidized very slowly by mitochondria or the inverted ETP vesicles (7). The rate of oxidation increases slowly on standing. If 5 µl 1% sodium deoxycholate is added to either preparation the maximum rate is revealed immediately (mitochondria 0.3, ETP 0.4 µatoms O/min x mg protein). When mitochondria are fractionated with deoxycholate (8) the vanadyl and vanadite oxidase activity is concentrated in the fractions which contain cytochrome c oxidase.

Discussion

Since free copper ions catalyse oxidation of vanadyl (but not vanadite) at a rate as fast or faster than the equivalent amount of copper contained in a cytochrome oxidase preparation it is possible that vanadyl oxidation by cytochrome oxidase is an artifact of adventitious copper in the preparation. The following considerations argue against that conclusion: (A) 10^{-3} M

Table 2

Effect of pH on oxidation of vanadyl and vanadite by cytochrome c oxidase

Substrate	Buffer	pH	Oxidation rate µatoms O/min/mg protein
Vanadyl	0.03 M PO_4	6.0	2.7
	0.03 M PO_4	8.0	0.7
	0.03 M Tris Mes	6.0	0.0
	0.03 M Tris Mes	7.4	2.8
Vanadite	0.03 M PO_4	6.0	0.5
	0.03 M PO_4	8.0	0.0
	0.03 M Tris Mes	6.2	1.0
	0.03 M Tris Mes	7.4	0.2

KCN completely inhibits oxidation by free Cu^{++}. KCl (0.05M) also inhibits oxidation by free Cu^{++} but not the oxidase. Oxidation of vanadyl by copper bathophenanthroline is also completely inhibited by cyanide. (B) Membranes washed with bathocuproine sulfonate to remove adventitious copper (9) loose less than 20% of their vanadyl oxidase activity. (C) Addition of Cu^{++} at levels found in cytochrome oxidase to oxidase or bathocuproine washed preparations inhibits vanadyl oxidase. (D) Boiled cytochrome oxidase (assayed without dilution) does not oxidize vanadyl ions. (E) Free Cu^{++} oxidizes vanadyl ions only at pH below 7.2 and shows higher activity with lower pH to pH 5.0 whereas the oxidase is active up to pH 9.0 and is inactive at pH 5.0. (F) The rate of oxidation by Cu^{++} at low concentrations declines very rapidly whereas low concentrations of enzyme maintain a constant rate for several minutes.

The reciprocal relation between inhibition of cytochrome c type oxidations by KCN, CO, NaN_3 and the copper chelator inhibitions of vanadyl

Table 3

Inhibition of oxidase activity using different electron donor systems with cytochrome c oxidase

Inhibitor	Concentration	Percent Inhibition				
		Vanadyl	Vanadate DTT	TCHQ DTT PL	cyt c TCHQ DTT	cyt c ascorbate
KCN	1×10^{-3} M	0	20-60	95	95	100
Carbon Monoxide	--	0	35	--	50	45
sodium azide	2×10^{-3} M	+40	+28	24	72	80
sodium fluoride	0.05 M	+15	--	50	30	0
dithizone	4×10^{-5}M	100	78	90	50	0
salicylaldoxime	3×10^{-4}M	100	--	25	20	0
hydroxylamine	2×10^{-3}M	50	100	--	50	45
bathophenanthroline	4×10^{-5}M	0	+40	50	60	0
orthophenanthroline	1×10^{-4}M	0	+1000	30	0	--
copper sulfate	2×10^{-5}M	100	100	80	30-50	8
tobacco smoke	--	100	100	93	75	45

Inhibitors preincubated 3 min with enzyme before adding substrates. CO and smoke were bubbled into the reaction vessel for 10 sec. DTT - Dithiothreitol, TCHQ - tetrachlorohydroquinone, PL - polylysine MW 2800. The stimulatory effects of phenanthrolines on vanadite oxidations are initial rates. The rates decline rapidly. A similar slow decline is seen with phenanthrolines and vanadyl. Control rates of oxidation are similar to Table 1, except vanadite which was run in tris mes pH 6.2 (Table 2). All other assays in phosphate buffer pH 7.0. + indicates stimulation.

oxidations are most interesting. The fact that the combined cytochrome c quinone rates show partial effects suggests that there are two terminal oxidases one of which is seen best with cytochrome c, and on the basis of CO sensitivity can be cytochrome a_3, and the other seen best in oxidation of vanadyl, can be proposed as a copper site on the basis of the chelator effects. The effects of smoke on vanadyl oxidation may be related to chelators in smoke which have been shown to inhibit tyrosinase (10). Other differences in the inhibitor effects with different donors can probably be related to the fact that cytochrome oxidase contains at least two heme types, a and a_3, and two copper atoms. For example, the effects of azide on cytochrome c oxidations suggest a specific effect on cytochrome a or a site before a_3 (11). It should be pointed out that the dithizone and bathophenanthroline inhibitions of cytochrome c, TCHQ, DTT oxidase are additive which suggests two different chelator sensitive sites in the oxidase.

The slow oxidation of vanadyl by right side out mitochondria or wrong side out ETP before detergent treatment suggests that the oxidation site is buried in the membrane. A hydrophilic site inside the membrane would be consistant with our previous proposal of binary membrane structure (7). A split pathway for cytochrome oxidase has previously been proposed by Jacobs _et al_. (2), and the stronger dithizone inhibition of the polylysine TCHQ oxidase as compared to ascorbate cytochrome c oxidase are consistant with the effect of nutritional copper deficiency on these two activities in mitochondria (12). Partial inhibition of succinoxidase by dithizone (13) suggests that the vanadyl oxidation site can play a role in overall mitochondrial oxidative activity. Strong inhibition of succinoxidase by cyanide and other complexing agents indicates that these agents may affect sites between the c cytochromes and the terminal oxidases.

A remarkable tendency for vanadium ions to be oxidized by copper proteins is further seen in the oxidation of vanadite by plastocyanin through the chloroplast photosystem I (14).

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