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Component of the alternative oxidase localized to the matrix surface of the inner membrane of plant mitochondria

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In mitoplasts from *Arum maculatum* spadices, succinate dehydrogenase (EC 1.3.99.1) and the alternative, cyanide-resistant oxidase activity (measured as *m*-chlorobenzhydroxamic acid-sensitive duroquinol oxidation) was unaffected by treatment with trypsin. In contrast, when 85% inside-out submitochondrial particles were treated with trypsin the alternative oxidase activity was inhibited by about 50% and succinate dehydrogenase activity by about 40%. Thus, a trypsin-sensitive component of the alternative pathway is located on the inner surface of the inner mitochondrial membrane. After trypsin treatment of the inside-out submitochondrial particles the inhibited alternative oxidase activity was partly restored by including 0.7 M citrate in the assay medium. This indicates that trypsin does not destroy the active site but merely causes a conformational change in the enzyme, thereby lowering its activity.

Alternative pathway; Inside-out submitochondrial particle; Mitoplast; Trypsin; (*Arum maculatum*, Plant, Mitochondria)

1. INTRODUCTION

Cyanide-insensitive oxidation of substrates, which is catalyzed by the so-called alternative oxidase, is found in mitochondria from many plant organs and tissues. However, nowhere is it as dominant as in the thermogenic spadix of some aroid species. Electron flow through the alternative oxidase pathway branches from the ordinary electron transport chain at ubiquinone and is not coupled to proton extrusion. The metabolic consequences of electron flow through the alternative pathway would be to keep the Krebs cycle running with a minimal ATP production (for a review see [1]. Whilst the end product of oxygen reduction is known to be water [2], the nature of the redox components involved in this reduction remains unknown; suggestions vary from a specific protein [3,4] to a complex autooxidation of ubiquinol without the specific involvement of a protein catalyst [5].

Alternative oxidase activity is measured as salicylhydroxamic acid- or *m*-Clam-sensitive, cyanide-resistant quinol oxidation. The quinol-O_2-oxidoreductase has been solubilised [6,7] and partially purified [3] from *Arum maculatum* and such preparations, although very unstable, have properties similar to the membrane-bound form. The solubilised oxidase from *Sauromatum guttatum* thermogenic tissue is sensitive to trypsin digestion [4], suggesting that a protein component is involved.

Virtually nothing is known about the molecular properties of the alternative oxidase including its location in the inner membrane. Indirect evidence suggests it may be located on the inner surface [8], whereas Lance et al. [1] placed it on the outer surface. In the present study, we have utilised the trypsin sensitivity of the enzyme associated with SMPs of well-defined polarity [9,10] to localise the trypsin-sensitive component of the alternative oxidase on the inner membrane. The results show it to be associated with the matrix face of the inner membrane of mitochondria from *Arum maculatum* spadices.

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Abbreviations: BSA, bovine serum albumin; DQH_2, durohydroquinol; *m*-Clam, *m*-chlorobenzhydroxamic acid; DCPIP, 2,6-dichlorophenolindophenol; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; IO-SMP, inside-out submitochondrial particles; SDH, succinate dehydrogenase; SDS, sodium dodecyl sulphate

2. MATERIALS AND METHODS

2.1. *Preparations*

Mitochondria were isolated from *Arum maculatum* spadices as in [11] and stored in liquid nitrogen until used. Mitochondria and mitochondria-derived material were kept on ice during all manipulations.

IO-SMP were produced from intact mitochondria by sonication in a high-salt medium without BSA essentially as in [10]. Mitoplasts were prepared as in [12]. Solubilised oxidase was prepared as described by [13] except that deoxycholate was used as detergent.

Protein was estimated according to [14], after solubilisation in 5% deoxycholate, using BSA (Sigma fraction V) as standard.

2.2. *Trypsin treatment*

Mitoplasts and IO-SMP were diluted to 6.8 mg protein/ml and incubated in the high-salt sonication medium with various amounts of

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trypsin (Sigma type III) for 30 min at 4°C. The reaction was stopped by addition of an equal weight trypsin inhibitor (Sigma type I-S). Controls were incubated in the same medium without trypsin, with or without trypsin inhibitor. Samples for the measurement of enzyme activities were taken out directly from the incubation mixture. Neither the trypsin nor the trypsin inhibitor interfered with any of the assays.

2.3. *Polarity assay*

Cytochrome *c* oxidase latency was measured by incubating the sample (15–35 μg mitochondrial protein) with reduced cytochrome *c* in a medium consisting of 0.3 M sucrose, 10 mM K-P_i (pH 7.2) and 100 mM KCl (B. Vigge and P. Gardeström, personal communication), with or without 0.024% (w/v) Triton X-100 (TX) and monitoring the oxidation of cytochrome *c* at 550 nm. Latency was calculated as [('rate + TX'-rate-TX')/'rate + TX' 1 × 100%.

2.4. *Enzyme assays*

DQH_2 oxidation was measured as O_2-consumption in an oxygen electrode (Rank Brothers, Cambridge, England) at 25°C in 0.3 M sucrose, 10 mM TES, 5 mM K-P_i (pH 7.2), 5 mM $MgCl_2$ and 0.1% (w/v) BSA (except in table 2). The oxygen concentration in these media was assumed to be 240 μM.

Succinate dehydrogenase activity was measured as the reduction of DCPIP in the presence of phenazine methosulphate as in [15] with 17–31 μg of mitochondrial protein per assay.

2.5. *SDS-PAGE*

SDS-polyacrylamide gel electrophoresis (10% resolving gel) was run essentially as in [16].

3. RESULTS AND DISCUSSION

Trypsin is too large (24 kDa) to pass through the outer mitochondrial membrane. To allow it to gain access to one or the other side of the inner mitochondrial membrane we therefore used (a) mitoplasts, where the outer membrane is removed and the outer surface of the inner membrane is exposed, or (b) IO-SMP, where the inner surface is exposed. As judged by the access of reduced cytochrome *c* to cytochrome *c* oxidase about half of the outer membranes were removed in our mitoplast preparations without loss of cytochrome *c* oxidase or alternative oxidase activity (table 1). The IO-SMP were more than 2-fold enriched in both cytochrome *c* oxidase and alternative oxidase activity and

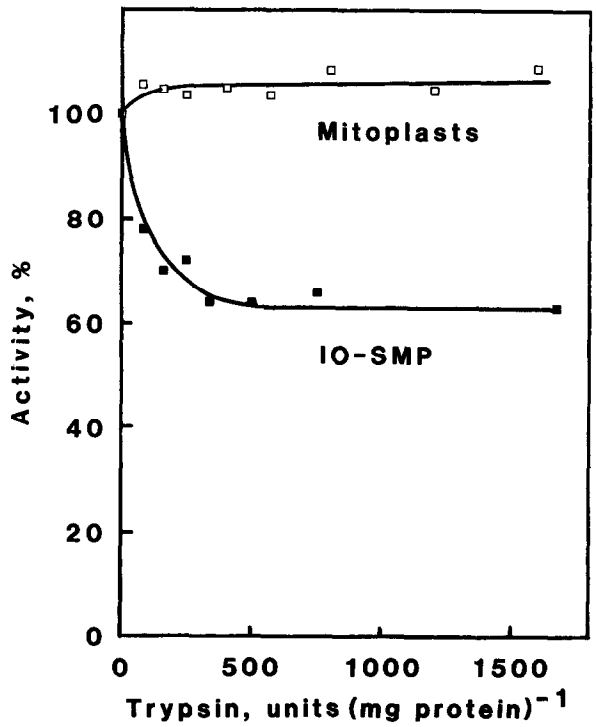

Fig.1. Trypsin inhibition of succinate dehydrogenase in IO-SMP and mitoplasts. 100% activity is 0.80 and 0.30 μmol DCPIP reduced min^{-1} mg^{-1} mitochondrial protein for IO-SMP and mitoplasts, respectively.

the latency of cytochrome *c* oxidase indicated that they were 85% inside-out (table 1) consistent with earlier reports [9].

The active site of SDH is known to be located on the inner matrix surface of the inner mitochondrial membrane [17]. Trypsin had no effect on SDH activity in the mitoplasts, but inhibited the activity by about 40% in the IO-SMP (fig.1) confirming both the polarity of the preparations and the efficiency of the trypsin treatment.

The effect of trypsin on alternative oxidase activity

Table 1

Characterization of preparations of mitoplasts and inside-out submitochondrial particles (IO-SMP)

	Experiment 1		Experiment 2	
Parameter	Mitochondria	Mitoplasts	Mitochondria	IO-SMP
Protein, mg	29	20	98	13
Cytochrome *c* oxidase				
Specific activity	1.5	2.2	1.4	3.6
Total activity	44	44	137	47
Latency, %	–	56	–	85
Alternative oxidase				
Specific activity	0.36	0.48	0.35	0.80
Total activity	10	9.6	34	10

Alternative oxidation was measured as *m*-Clam-sensitive oxidation of 1 mM DQH_2 in the presence of 2 mM KCN and 0.2 μM FCCP. Specific activities are given as μmol min^{-1} (mg protein)$^{-1}$, and total activities as μmol min^{-1}

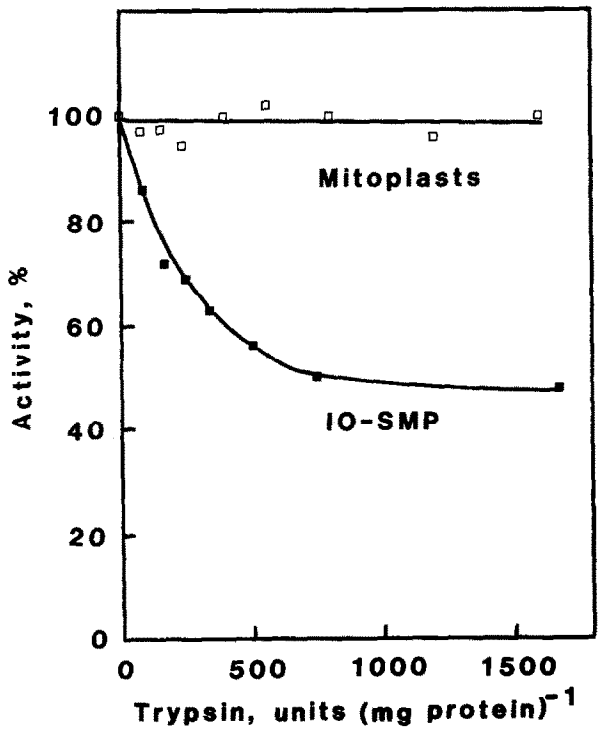

Fig.2. Trypsin inhibition of alternative oxidase activity in IO-SMP and mitoplasts. The activity was measured as described in the legend to table 1 and the 100% values are given there.

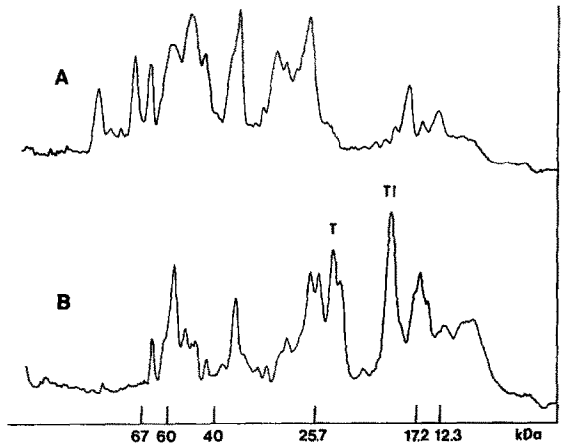

Fig.3. SDS-PAGE analysis of IO-SMP (150 μg mitochondrial protein) incubated without (A) or with (B) 700 units of trypsin mg^{-1} mitochondrial protein. The reaction was stopped by the addition of trypsin inhibitor. After electrophoresis the gel was stained with Coomassie brilliant blue and scanned at 600 nm. Molecular weight markers are, from left to right: BSA, catalase, horseradish peroxidase, chymotrypsinogen, myoglobin and cytochrome *c*. T, trypsin; TI, trypsin inhibitor.

showed the same pattern as for SDH: The activity was not affected in the mitoplasts, but it was inhibited by 50–55% in the IO-SMP (fig.2).

SDS-PAGE analysis of the IO-SMP showed that a number of polypeptides larger than 30 kDa disappeared upon trypsin treatment of the IO-SMP while more low-molecular-weight polypeptides (<20 kDa) appeared (fig.3). Specifically, several polypeptides of 30–40 kDa disappeared both after trypsin treatment of IO-SMP (fig.3) and after trypsin treatment of solubilized oxidase (results not shown). One or several of these could be the alternative oxidase which has been associated with three polypeptides of 35–37 kDa [18].

We conclude that a trypsin-sensitive component of the alternative oxidase pathway, likely the alternative oxidase itself, is located on the inner matrix surface of the inner membrane of *Arum* mitochondria.

After trypsin treatment of the inside-out submitochondrial particles (reaction stopped with trypsin inhibitor) the inhibited alternative oxidase activity was partly restored by including 0.7 M citrate in the assay medium (table 2). Stimulation by citrate of the alternative pathway in solubilised and intact mitochondria has been suggested to be caused by a stabilisation of the conformation of the oxidase [13]. If we assume that the same mechanism also reverses the trypsin inhibition our results indicate that trypsin does not attack the active site of the alternative oxidase but merely causes a conformational change of the enzyme, thereby decreasing its catalytic efficiency.

Table 2

Effect of a high citrate concentration in the assay medium on alternative oxidase activity

	Alternative oxidase activity, nmol		Effect of citrate, % O_2 min^{-1} mg^{-1}
	Treatment	Control	+0.7 M citrate
Control	986 ± 115	2217 ± 129	+ 125
Trypsin	453 ± 24	1605 ± 92	+ 254
Effect of trypsin, %	− 54	− 28	

IO-SMP were preincubated with or without 1000 units of trypsin mg^{-1} mitochondrial protein and the alternative oxidase activity was determined as described in the legend to table 1 in a medium containing 10 mM K-Pi with or without 0.7 M citrate. The values are means ± SD (n = 4). The oxygen concentration in the citrate buffer was assumed to be 130 μM [13]

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