CALCIUM UPTAKE BY RECONSTITUTED VESICLES IS MEDIATED BY A CHYMOTRYPSIN-SENSITIVE PEPTIDE ASSOCIATED WITH CYTOCHROME OXIDASE

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Received 22 July 1980

1. Introduction

Despite some controversy concerning the mechanism of Ca^{2+} uptake into mitochondria [1,2], it is generally agreed that the process is electrophoretic, driven by the respiratory activity of the inner mitochondrial membrane. A small polypeptide fulfilling the requirements of an ionophore has been isolated from calf heart mitochondria and it reportedly participates in the electrophoretic, ruthenium-red sensitive uptake of Ca^{2+} [3].

It was shown in [4,5] that submitochondrical particles prepared from beef heart and from rat liver mitochondria catalyze an energy-dependent inward movement of Ca^{2+}. Because the sonic particles have an 'inside-out' polarity, their ability to accumulate Ca^{2+} was at variance with the electrophoretic uptake of Ca^{2+} into mitochondria. But these data became reconcilable in view of the evidence for more than one mitochondrial Ca^{2+} transport system [6]. Thus, the process in the inverted particles represented a mechanism for the efflux of Ca^{2+} through a Ca^{2+}/H^+ antiporter which is described in [7]. This efflux system is an electrically neutral, ruthenium-red insensitive process regulated by the oxidation–reduction state of pyridine nucleotides [8]. However, the complexity of Ca^{2+} movements is further illustrated by the finding that efflux mechanisms in different mammalian tissues are clearly dissimilar [9], a fact which undoubtedly contributes to some of the diverse interpretations of and controversies over the transport of Ca^{2+}.

Abbreviations: COV, cytochrome oxidase vesicles; CTCOV, vesicles prepared with cytochrome oxidase after its exposure to chymotrypsin

We have investigated the uptake of Ca^{2+} by respiring reconstituted cytochrome oxidase vesicles and an independent study on this phenomenon has been reported [10]. Here we describe experiments on Ca^{2+} uptake in COV and on the extraction of a chymotrypsin-sensitive polypeptide of M_r 8800, an impurity in cytochrome oxidase preparations, which appears to be responsible for the observed Ca^{2+} movements. It is unlikely that this polypeptide is related to the peptide reportedly dissociable from subunit I of cytochrome oxidase [11] since chymotrypsin removes only the low molecular weight protein from the enzyme without affecting the level of subunit I.

2. Materials and methods

Cytochrome oxidase and proteolytically-digested cytochrome oxidase were prepared as in [12,13] except that chymotrypsin was added at a 40 μg/mg enzyme final conc. during digestion. Asolectin was purchased from Associated Concentrates, Woodside, NY; cytochrome *c* (type VI), valinomycin, ruthenium red and Dowex 50 X 8-100 were obtained from Sigma Chemical Co., St Louis, MO; ^{45}Ca from ICN Pharmaceuticals, Irvine, CA; ACS from Amersham, Arlington Heights, IL. Nigericin was a gift from Dr Hosley of Eli Lilly Co.

The cholate dialysis method [14] was used to reconstitute either the undigested or the digested enzyme (0.5 mg/ml) with sonicated asolectin (30 mg/ml) in 50 mM KP_i containing 1.2% cholate (pH 7.4). Following dialysis against 50 mM KP_i (pH 7.4) for 10 h, the vesicles were transferred to 50 mM KP_i (pH 6.0) and dialysed for an additional 12 h. Ca^{2+} uptake was measured as follows: vesicles were diluted

10-fold into 50 mM KP_i (pH 6.0) in the absence and presence of 23 mM Tris ascorbate (pH 6.8) plus 0.083% cytochrome *c* and incubated for 2 min at room temperature. $^{45}Ca^{2+}$ (10 mM Ca^{2+}, 1 mCi/ml) was then added to 100 μM final conc. Aliquots were removed and filtered through bovine serum albumin treated Dowex columns [15] at indicated times and eluted with 2.0 ml 2.5% glycerol. Eluates were counted with 10 ml ACS scintillation fluid in a liquid scintillation counter.

Ethanol extraction of cytochrome oxidase was done as follows: ice-cold absolute ethanol was added to cytochrome oxidase (1 mg/ml) in 50 mM KP_i containing 0.5% cholate (pH 7.4) to 60% final conc. within 30 s while vigorously stirring at 4°C. The stirring was stopped and the mixture was allowed to stand on ice for 5 min. The sample was centrifuged for 20 min at 10 000 rev./min (7800 × *g*) in a JA-20 Beckman rotor. The pellet (P_1) was suspended in 0.25 M sucrose. From the clear, light-green supernatant, ethanol was removed in a rotary evaporator at room temperature. The supernatant (S_1) was kept at 4°C for 12 h during which time a precipitate formed. The sample was centrifuged as above, the clear supernatant (S_2) was collected and the green pellet (P_2) was suspended in 0.25 M sucrose. Samples were stored at 4°C. Prior to testing, the fractions were dialyzed against 50 mM KP_i (pH 6.0) to remove any residual cholate. The activity of the fractions in the $^{45}Ca^{2+}$ uptake assay was measured by adding the fraction directly to CTCOV at the time of their 10-fold dilution into the reaction mixture and proceeding as above. Chymotrypsin treatment of the supernatant fraction was performed under the same conditions as those used for the proteolytic digestion of cytochrome oxidase.

Polyacrylamide slab-gel electrophoresis was done as in [16]. Protein was measured according to [17] in the presence of 1% sodium deoxycholate with bovine serum albumin as standard.

3. Results

3.1. $^{45}Ca^{2+}$ uptake in COV and in CTCOV

COV take up $^{45}Ca^{2+}$ in the presence of ascorbate plus cytochrome *c* (fig.1a); omitting either one or both greatly diminishes the rate of uptake. As seen in fig.1b, exposure of the enzyme to chymotrypsin markedly reduces the capability of the reconstituted

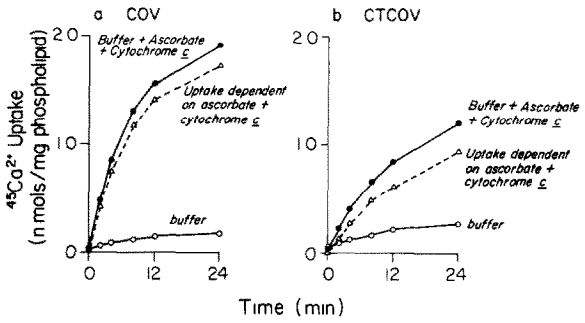

Fig.1. CTCOV show a marked reduction in their ability to take up Ca^{2+}: Vesicles prepared with cytochrome oxidase before and after exposure to chymotrypsin were assayed for Ca^{2+} uptake as in section 2. $^{45}Ca^{2+}$ was added at 0 time. The dashed line shows the uptake obtained in the presence of cytochrome *c* + ascorbate minus the uptake obtained in the absence of both.

CTCOV to take up $^{45}Ca^{2+}$. Although in later experiments $^{45}Ca^{2+}$ uptake was measured after 2 min, pronounced differences are noted over a 24 min time course.

It was shown in [13] that treatment of cytochrome oxidase with chymotrypsin did not impair its catalytic activity or the phenomenon of respiratory control after reconstitution. Analysis of the cytochrome

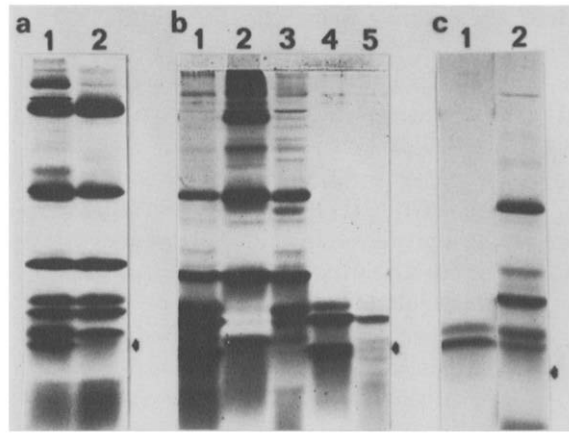

Fig.2. Polyacrylamide slab gel analysis of: (a) cytochrome oxidase before (1) and after (2) treatment with chymotrypsin, 42 μg in each slot. (b) fractions obtained from the ethanol extraction of cytochrome oxidase (1) 21 μg supernatant S_1; (2) 37 μg pellet P_1; (3) 18 μg pellet S_2; (4) 11 μg supernatant S_2; (5) 10 μg supernatant S_2 after proteolytic digestion. (c) fractions obtained from the ethanol extraction of chymotrypsin-treated cytochrome oxidase (1) 6 μg supernatant S_2; (2) 25 μg pellet P_2. Procedures for proteolytic digestion and for ethanol extraction are detailed in section 2.

oxidase preparation used in the experiment in fig.1 on polyacrylamide slab-gel electrophoresis revealed that the proteolytic enzyme degraded two polypeptides: a higher molecular weight peptide which migrates just below subunit II and a lower molecular weight peptide (fig.2a). However, it is the low molecular weight peptide which is involved with Ca^{2+} transport as will be shown below.

Addition of nigericin or nigericin plus valinomycin to COV inhibited $^{45}Ca^{2+}$ uptake as might be expected, but valinomycin added alone unexpectedly stimulated the uptake (table 1). This observation is not consistent with a role of this ionophore in the electrophoretic uptake of Ca^{2+} in mitochondria and will be discussed later.

3.2. *Extraction of an ionophore from cytochrome oxidase preparations*

After several attempts to isolate an active ionophoric polypeptide from cytochrome oxidase by fractionating subunits with detergents and gel filtration columns, a simple procedure was developed to extract the ionophore with 60% ethanol. This extraction yielded two fractions: (1) a supernatant fraction (S_2) which retained three low M_r polypeptides; (2) pellet fractions (P_1 + P_2) which retained all of the polypeptides except one of the low M_r peptides seen in the supernatant (fig.2b). As shown in fig.3, as little as 2 μg supernatant fraction markedly stimulated $^{45}Ca^{2+}$ uptake into CTCOV. Moreover, the supernatant was active either when added directly to CTCOV (fig.3) or when added before reconstitution; on the other hand, the pellet fractions (P_1 or P_2) were inactive in either case. To confirm that the ionophoric activity

Table 1
Effect of valinomycin and nigericin on the $^{45}Ca^{2+}$ uptake of COV and of CTCOV (nmol $^{45}Ca^{2+}$. mg phospholipid^{-1} . 2 min^{-1})

	COV	CTCOV
Complete system	0.34	0.18
+ valinomycin (0.2 μg/ml)	0.51	0.21
+ nigericin (0.2 μg/ml)	0.00	0.00
+valinomycin + nigericin (0.2 μg each/ml)	0.00	0.02

COV and CTCOV were assayed for $^{45}Ca^{2+}$ uptake as in section 2 except that valinomycin and nigericin were added as ethanolic solutions. The uptake values listed have been corrected for the uptake obtained in the absence of ascorbate plus cytochrome *c*

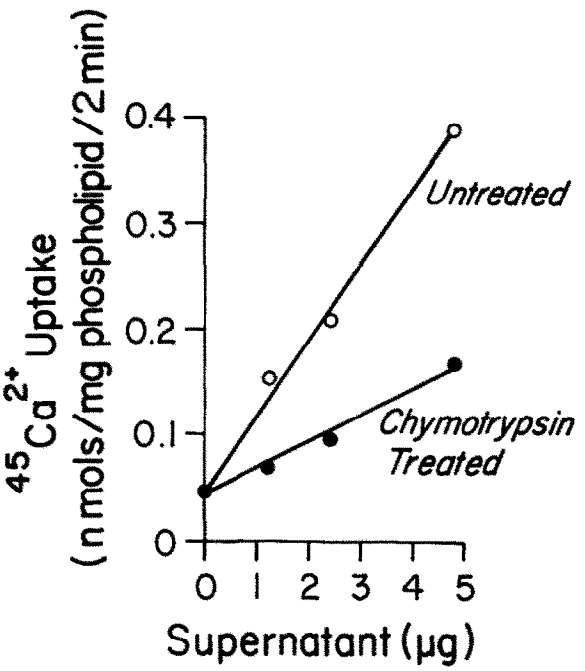

Fig.3. The stimulatory activity of the supernatant fraction on the Ca^{2+} uptake of CTCOV is diminished by exposure to chymotrypsin. The supernatant before and after treatment was added directly to CTCOV in the uptake assay. The values shown represent the Ca^{2+} uptake dependent on ascorbate + cytochrome *c*.

of the supernatant (S_2) stemmed from only one of its three peptides, ethanol extraction of the chymotrypsin-treated enzyme was also performed. As expected, this supernatant fraction (S_2) retained only the two higher M_r proteins observed in the S_2 fraction of the untreated enzyme (fig.2c). Furthermore, neither the supernatant nor the pellet stimulated Ca^{2+} uptake into CTCOV (table 2). The ionophoric pep-

Table 2
Effect of the fractions obtained from the ethanol extraction of untreated and of chymotrypsin-treated cytochrome oxidase on $^{45}Ca^{2+}$ uptake of CTCOV (nmol $^{45}Ca^{2+}$. mg phospholipid^{-1} . 2 min^{-1})

Complete system: 0.12	Fractions from enzyme	
	Untreated	Treated
+ 4 μg S_2	0.34	0.11
+ 6 μg P_2	0.12	0.13

The fractions were added directly to CTCOV in the uptake assay. The values listed represent uptake dependent on ascorbate plus cytochrome *c*

tide was relatively stable to heat (5 min at 85°C), but its exposure to chymotrypsin consistently led to diminution of activity (fig.3). Complete inactivation, however, was not achieved even with prolonged exposure to greater amounts of chymotrypsin. This chymotrypsin-sensitive polypeptide was estimated to have M_r ~8800 by polyacrylamide gel electrophoresis.

4. Discussion

At this time, we cannot assign a specific function to the ionophore present in cytochrome oxidase preparations. Several attempts to obtain a homogeneous preparation of the 8800 M_r polypeptide from the supernatant have thus far been unsuccessful. It is apparent, however, that it does not catalyze proton uptake in view of the high respiratory control of both COV and CTCOV as prepared by the cholate dialysis method [13].

The Ca^{2+} transport catalyzed by this ionophore is puzzling. The failure of valinomycin to inhibit Ca^{2+} uptake appears to eliminate it as a candidate for the electrophoretic uptake of Ca^{2+} into mitochondria. This verdict is further confirmed by the observation that ruthenium red at <100 μM did not inhibit the Ca^{2+} uptake. The ionophore also does not appear to be related to the Ca^{2+}/H^+ antiporter described in [7] since its activity was not stimulated by an artificially imposed pH gradient nor did its size coincide with that of the antiporter on polyacrylamide gels. (J. S.-M., W. P. Dubinsky, unpublished). Attempts to correlate the ionophoric activity to the reported proton pump activity of COV [18,19] with and without Ca^{2+} revealed that both COV and CTCOV pumped protons to about the same extent (J. T. Coin, J. S.-M., unpublished). Thus the role of the ionophore present in cytochrome oxidase preparations remains obscure. The curious stimulation of Ca^{2+} transport in COV by valinomycin suggests a complex mechanism perhaps involving transport of an anion [2,20].

Acknowledgement

This work was supported by grant CA08964 from the National Cancer Institute, DHEW.

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