Metabolite transport in isolated yeast mitochondria: fumarate/malate and succinate/malate antiports

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Received 20 August 1999; received in revised form 13 October 1999

Edited by Vladimir Skulachev

Abstract In this study, we investigated the metabolite permeability of isolated coupled *Saccharomyces cerevisiae* mitochondria. The occurrence of a fumarate/malate antiporter activity was shown. The activity differs from that of the dicarboxylate carrier (which catalyses the succinate/malate antiport) in (a) kinetics (K_m and V_{max} values are about 27 μM and 22 nmol min^{-1} mg protein^{-1} and 70 μM and 4 nmol min^{-1} mg protein^{-1}, respectively), (b) sensitivity to inhibitors, (c) K_i for the competitive inhibitor phenylsuccinate and (d) pH profiles.

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Key words: Yeast mitochondrion; Carrier; Fumarate; ADP; ATP; Transport

1. Introduction

Metabolite movement across the mitochondrial membrane is essential to both mitochondrial and cytosolic metabolism. Although many mitochondrial translocators have been identified, characterised and purified over the past 2 decades and their activity reconstituted in artificial liposomes [1,2], further investigations are still required to ascertain the occurrence of such translocators in mitochondria from different sources as well as to elucidate the role of transport processes in cell metabolism by the possible discovery of novel carriers.

In *Saccharomyces cerevisiae* mitochondria (SCM) the existence of certain carriers was reviewed [3] and their biochemistry investigated by swelling and isotopic techniques in isolated organelles. More recently, the existence of about 30 mitochondrial proteins belonging to the carrier family has been proposed in the light of genetic studies, with some of them studied in in vitro systems (for references see [4]). However, in both cases, transport was studied under conditions in which metabolism was either largely prevented or completely absent. This prompted us to apply already developed spectroscopic techniques [5–9] to study transport in yeast mitochondria in order to gain further insight into certain transport features as well as to find out the existence of novel translocators in mostly functional organelles. In particular, in the light of the similarities found between our SCM and type 1 mitochondria [10], we undertook experimental work to study the transport of citric cycle intermediates. In this regard, fumarate uptake by isolated yeast mitochondria has not been reported yet, though several fumarate translocators have already been shown in mammalian mitochondria [5–8]. In this paper we show that SCM contain the fumarate/malate antiporter separate from the dicarboxylate carrier [3,10].

2. Materials and methods

All reagents and enzymes were from Sigma (St. Louis, MO, USA) except zymoliase (ICN) and Bacto yeast extract (Difco). Substrate solution pH was adjusted to 7.20 by adding either Tris or HCl.

A strain (ATCC 18790) of *S. cerevisiae* was used. Cells were grown aerobically for 16 h at 28°C to a final A_{600nm} equal to 2 in a semi-synthetic medium containing 2% lactate as the carbon source and containing, per litre, 3 g Bacto yeast extract, 1 g KH_2PO_4, 1 g NH_4Cl, 0.5 g $CaCl_2 \cdot 2H_2O$, 0.5 g NaCl, 0.6 g $MgCl_2$, supplemented with 0.05% glucose. Medium pH was adjusted to 5.5 with NaOH.

2.1. Isolation of mitochondria

Mitochondria were isolated and checked for their intactness as in [11]. Mitochondrial proteins were measured as in [12]. In each experiment, mitochondrial respiration was measured at 25°C by a Clark oxygen electrode in a 1.5 ml closed cell containing 0.6 M mannitol, 20 mM HEPES–Tris (pH 7.4), 10 mM potassium phosphate, 2 mM $MgCl_2$, 1 mM EDTA, 5 mg/ml bovine serum albumin. When added with 0.5 mM ADP, SCM showed respiratory control index 2.05 ± 0.14 with 5 mM succinate used as a substrate. SCM showing a respiratory control index lower than 1.9 were discarded.

2.2. Transport assay

In the extramitochondrial phase, either ATP or malate appearance was revealed by using two $NADP^+$-linked detecting systems, namely the ATP detecting system (ATP D.S.) consisting of 2.5 mM glucose, 0.2 mM $NADP^+$, 0.5 enzymatic units (e.u.) hexokinase-glucose 6-phosphate dehydrogenase [9] and the malate detecting system (M.D.S.), consisting of 0.25 mM $NADP^+$, 0.2 e.u. malic enzyme (previously dialysed against 100 mM Tris–HCl pH 7.20) [8]. In both cases, the increase in NADPH concentration was followed fluorimetrically, using a Perkin-Elmer LS-50B luminescence spectrometer with excitation and emission wavelengths set at 334 and 456 nm, respectively. The fluorescence change rates were obtained as the tangent to the linear part of experimental curve and expressed as nmol $NADP^+$ reduced min^{-1} mg mitochondrial protein^{-1}. In order to obtain a quantitative measurement of the rate of $NADP^+$ reduction, the fluorimetric response was calibrated as in [13].

2.3. Computing

Where necessary, experimental plots were obtained by means of Grafit software (Erithacus).

3. Results

First, in some sets of experiments, SCM were checked with respect to their ability to synthesise ATP and to export it in the extramitochondrial phase. This was done essentially as already reported in rat liver mitochondria ([9] and Section 2). The control strength criterion [9] was applied by using atractyloside as an inhibitor, thus showing that the measured

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Abbreviations: Ap_5A, P_1P_5-diadenosine (-5′-pentaphosphate); SCM, *Saccharomyces cerevisiae* mitochondria

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PII: S0014-5793(99)01535-5

rate of ATP appearance is a measure of the ADP/ATP antiport. The dependence of the rate of ATP efflux on the ADP concentration was investigated in a Michaelis–Menten plot. Saturation kinetics were found; K_m and V_{max} values were about 20 μM and 56 nmol min^{-1} mg $protein^{-1}$, respectively.

The nature of the inhibition of the ADP/ATP antiport on the atractyloside concentration was studied as a Dixon plot. Competitive inhibition was found with a K_i value for atractyloside of about 0.04 μM. In order to distinguish between a purely and a partially competitive inhibition, the same data were plotted as the reciprocal of the fractional inhibition (*i*) versus the reciprocal of the atractyloside concentration. A partially competitive inhibition was found, as demonstrated by the ordinate intercept values that are greater than 1 [14].

Using these mitochondria, the occurrence of the fumarate/malate antiport, already reported in rat heat mitochondria, rat liver mitochondria, rat brain mitochondria and rat kidney mitochondria [5–8], was studied as reported in Section 2. Consistently, the capability of externally added fumarate to cause efflux of intramitochondrial malate from SCM was checked. The malate concentration in a SCM suspension is negligible, in fact no change in the fluorescence due to NADPH was observed in the presence of malic enzyme and $NADP^+$. As a result of fumarate addition to the mitochondria, an increase of fluorescence was observed showing the appearance of malate in the extramitochondrial phase. In agreement with [5–8], a possible explanation for this finding is that fumarate enters mitochondria in exchange with a small amount of endogenous malate via the putative fumarate/malate antiporter. Once inside the matrix, fumarate is hydrated to malate which in turn leaves mitochondria in exchange with further fumarate. Consistently, externally added thiocyanate (10 mM), a fumarase non-competitive inhibitor [15] able to enter mitochondria [16], was found to prevent malate efflux caused by fumarate addition (about 40% inhibition with 1 mM fumarate).

In order to ascertain whether the rate of NADPH fluorescence increase, caused by malate appearance, reflects the malate efflux outside mitochondria, rather than the intramitochondrial fumarase reaction, Triton X-100 (0.1%) was used. Externally added Triton was found to cause an increase in the rate of fluorescence due to fumarate addition, thus showing

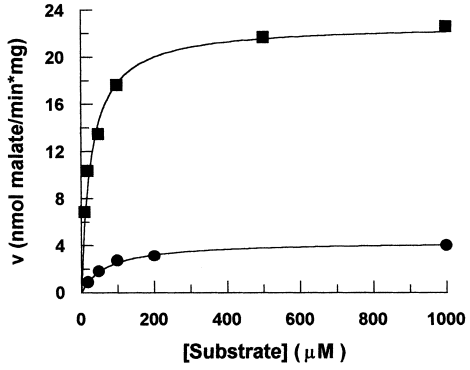

Fig. 1. Dependence of the malate efflux rate in the extramitochondrial phase on increasing substrate concentrations. Mitochondria (0.2 mg protein) were incubated at 25°C in 2 ml of standard medium containing 0.6 M mannitol, 10 mM KCl, 1 mM $MgCl_2$, 20 mM HEPES–Tris pH 7.20 and M.D.S. Fumarate (■) or succinate (●) was added at the indicated concentrations and the rate of malate appearance in extramitochondrial phase was monitored as reported in Section 2.

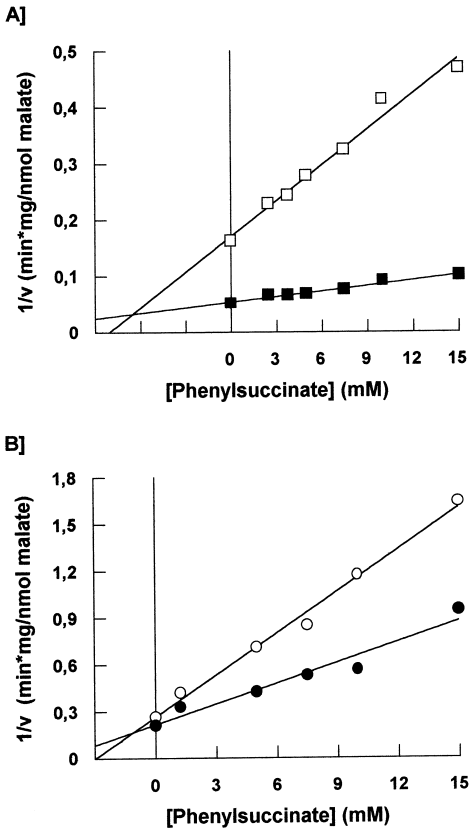

Fig. 2. Dixon plot of the inhibition by phenylsuccinate on fumarate/malate and succinate/malate antiports in SCM. Mitochondria (0.2 mg protein) were incubated in 2 ml of standard medium under the same conditions reported in Fig. 1. The rate of malate appearance in the extramitochondrial phase due to externally added fumarate (A) and succinate (B) was measured as reported in Section 2 by using 0.02 mM (□), 0.1 mM (■), 0.35 mM (○) and 1 mM (●) respectively, in either the absence or presence of phenylsuccinate, at the indicated concentrations.

that this is a measure of the transport occurring across the mitochondrial membrane.

The dependence of the rate of fumarate/malate exchange on increasing fumarate concentrations was investigated (Fig. 1). Saturation kinetics were found with K_m and V_{max} values about 27 μM and 22 nmol min^{-1} mg mitochondrial $protein^{-1}$, respectively. In the same experiment, the rate of malate efflux due to the addition of succinate was also investigated as a function of the substrate concentration. Saturation kinetics were found with K_m and V_{max} values of about 70 μM and 4 nmol min^{-1} mg mitochondrial $protein^{-1}$, respectively. The triton experiment showed that the rate of malate appearance due to externally added succinate reflects the rate of succinate/malate exchange due to the already reported dicarboxylate carrier [10].

The nature of the phenylsuccinate inhibition on both fumarate/malate and succinate/malate antiport was also investigated as a Dixon plot (Fig. 2). A competitive inhibition was found, with K_i values of about 6 and 1.3 mM, respectively. The sensitivity of both fumarate/malate and succinate/malate antiports to impermeable compounds, including 1 mM 1,2,3-benzenetricarboxylate, 1 mM benzylmalonate, 1 mM butylmalonate, 0.1 mM α-cyanocinnamate, 0.1 mM *N*-ethylmaleimide, 1 mM maleate, 10 mM methylfumarate and 10 mM

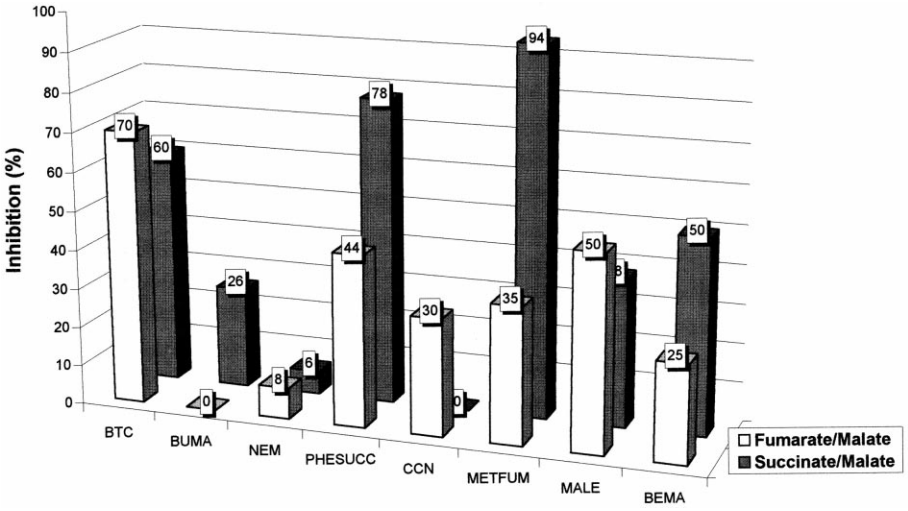

Fig. 3. Inhibition profile of the fumarate/malate and succinate/malate antiports in SCM. Mitochondria (0.2 mg protein) were incubated in 2 ml of standard medium under the same conditions reported in Fig. 1. The rate of malate appearance was measured as reported in Section 2 using a concentration of fumarate equal to 0.1 mM and of succinate equal to 0.35 mM in either the absence or presence of the following inhibitors: 1 mM 1,2,3-benzenetricarboxylate (BTC), 1 mM butylmalonate (BUMA), 0.1 mM *N*-ethylmaleimide (NEM), 10 mM phenylsuccinate (PHESUCC), 0.1 mM α-cyanocinnamate (CCN), 10 mM methylfumarate (METFUM), 1 mM maleate (MALE), 1 mM benzylmalonate (BEMA). The inhibition percentages are the means of three different experiments.

phenylsuccinate, was investigated with 0.1 mM fumarate and 0.35 mM succinate used as substrates, in three different experiments. As reported in Fig. 3, each translocator shows a specific inhibition profile. In particular, externally added α-cyanocinnamate, an inhibitor of the pyruvate carrier in mammalian mitochondria [17], proved to give $32 \pm 3\%$ inhibition on fumarate/malate antiport without affecting succinate/malate antiport. Surprisingly, no inhibition was observed on fumarate/malate exchange in the presence of butylmalonate, which inhibits the dicarboxylate carrier by $27 \pm 3\%$. Moreover, the thiol reagent mersalyl (up to 1 mM), which cannot be used under the experimental conditions of Fig. 3, since it can inhibit malic enzyme reaction, was found to inhibit fumarate uptake showing no effect on succinate uptake, as measured by swelling techniques (data not shown).

In another set of experiments, the pH dependence of the two transport processes was investigated using substrate concentrations equal to 1 mM. pH profiles between pH 6.6 and 8

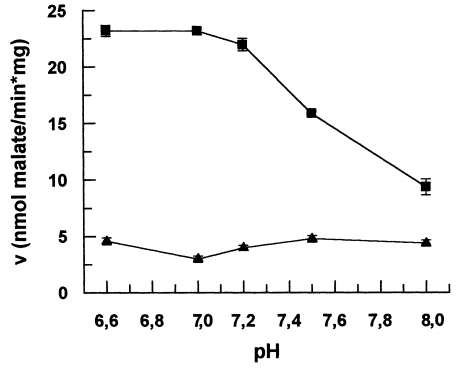

Fig. 4. pH dependence of the fumarate/malate and succinate/malate exchange rate in SCM. Mitochondria (0.2 mg protein) were incubated, under the same conditions reported in Fig. 1, in 2 ml of the standard medium whose pH was adjusted to the indicated values with either Tris or HCl. Fumarate (■) and succinate (▲) concentrations were 1 mM. The standard deviation of the data was determined using the mean of three experiments.

were found to differ from each other (Fig. 4): the rate of succinate/malate was rather constant in the investigated pH range, whereas the rate of fumarate/malate exchange was about 23 nmol min^{-1} mg $protein^{-1}$ in the range 6.6–7.2 and decreased up to 9 for higher pH values.

4. Discussion

In this paper, we have studied the permeability properties of isolated coupled SCM by means of a spectroscopic technique already used to investigate mammalian mitochondrial translocators [5–9]. We have chosen this technique because it allows us to monitor mitochondrial transport in SCM with active metabolism. It should be noted that the use of isolated coupled mitochondria in transport studies leads to a better investigation of both the role of transport in metabolism and the occurrence of certain antiports which catalyse the transport of extramitochondrial metabolites in exchange with others which are synthesised in the matrix, following counteranion uptake.

In this paper we show that, as reported for other mitochondria [9,18], the rate of ATP production outside mitochondria depends on the activity of the ADP/ATP translocator. Moreover, the V_{max} of the ADP/ATP antiport, about 56 nmol min^{-1} mg $protein^{-1}$, is different from those measured in mammalian and plant mitochondria under the same experimental conditions: they were 20 nmol min^{-1} mg $protein^{-1}$, 40 nmol min^{-1} mg $protein^{-1}$ and 110 nmol min^{-1} mg $protein^{-1}$ in rat liver mitochondria [9], in durum wheat mitochondria [18] and in *Solanum tuberosum* cell mitochondria (Fratianni et al, in preparation), respectively. Interestingly, the nature of atractyloside inhibition was found to be partially competitive, thus showing that atractyloside can bind to a saturable site of the translocator, different from the ADP binding site.

Here we show that the fumarate/malate antiport takes place in a carrier-mediated manner in isolated SCM. Widely used criteria demonstrating the occurrence of carrier-mediated transport were successfully applied, i.e. the hyperbolic de-

pendence on the substrate concentration and the inhibition by compounds that cannot enter the mitochondrial matrix. Since our SCM are rather similar to type 1 mitochondria [10], a detailed investigation was carried out aimed at ascertaining whether the fumarate/malate exchange is mediated by the dicarboxylate translocator. The observed differences in the V_{max} values, in the inhibitor and in the pH profiles and in the K_i values for the competitive inhibitor phenylsuccinate (in this case K_i reflects a peculiar feature of the inhibitor–carrier interaction), clearly show that the fumarate/malate and the succinate/malate antiporters are two separate yeast translocators. Interestingly, externally added fumarate failed to cause efflux of phosphate outside SCM, thus excluding the possibility that malate efflux is the result of the combined actions of the fumarate/phosphate and the phosphate/malate antiporters as considered in [6].

A proposal about the existence of the succinate/fumarate translocator has been made recently [19,20]. However, its sensitivity towards inhibitor, even though measured under completely different conditions, appears to exclude that the proposed succinate/fumarate carrier is the fumarate/malate antiporter. Consistently, we exclude that succinate/malate could be a result of the combined action of the proposed succinate/fumarate and the fumarate/malate translocators.

The physiological role of the novel fumarate/malate translocator can be only a matter of speculation: we could assume that this carrier could be involved in the cytosolic purine biosynthesis and in the arginine metabolism [21,22]. However, this point is still to be investigated.

Acknowledgements: The helpful co-operation of Michelina Iacovino who participated as a student in this work is gratefully acknowledged. This work was partially financed by a PRIN Bioenergetics and Membrane Transport grant to S.P.

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