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The role of metal ions in the uptake of aspartate aminotransferase and malate dehydrogenase into isolated rat liver mitochondria in vitro

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To gain further insight into the mitochondrial receptor area which allows selective uptake of both purified aspartate aminotransferase and malate dehydrogenase into mitochondria, the inhibition of metal complexing agents such as bathophenanthroline and tiron on the uptake of both enzymes has been investigated. In view of the nature of the inhibition found, we propose the existence of metal ion(s) at or near the aspartate aminotransferase, but far from the malate dehydrogenase binding site.

Mitochondria *Transport* *Asparate aminotransferase* *Malate dehydrogenase* *Metal-complexing agent*

1. INTRODUCTION

The existence of membrane receptors able to selectively recognize mitochondrial proteins synthesized in the cytosol has been proposed for ADP/ATP carrier [1], apocytochrome *c* [2] and ornithine transcarbamyltransferase [3]. Using a model system developed to investigate uptake of mature proteins into mitochondria (see [4]) we have recently shown the existence of a common receptor area in the mitochondrial membrane which allows for selective recognition and internalization of both purified AAT and MDH into isolated mitochondria in vitro [5]. Mutual inhibition of these enzymes for their uptake has been also found [5]. Features of this receptor area were investigated here by testing the effect of impermeable specific compounds such as metal-complexing agents on both AAT and MDH uptake. The presence of metal ion(s) in the AAT and MDH receptor is demonstrated.

Abbreviations: AAT, aspartate aminotransferase; MDH, malate dehydrogenase

2. EXPERIMENTAL

All reagents used were from Sigma. Mitochondrial AAT and MDH from rat liver were purified according to [6,7]. Rat liver mitochondria and sulphite-loaded mitochondria were prepared as reported [7]. The treatment of mitochondria with tiron (final concentration was 30 and 20 μM in the AAT and MDH experiments, respectively) was carried out essentially according to [5]. Mitochondrial protein was determined according to [8].

Measurement of the uptake rate of isoenzymes into mitochondria was carried out by using fluorimetric assays of the endogenous enzyme activity after incubation of the mitochondria with purified isoenzymes, essentially as described [5]. We were able to measure the initial rate of fluorescence change owing to modification made of the cuvette holder to allow for continuous stirring of each sample and addition of substrates and enzymes without opening the cover (mixing time was 0.5–1 s).

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3. RESULTS

Fresh mitochondria (table 1, exp.1) were incubated in the cell of a spectrofluorimeter set to record the rate of fluorescence decrease of intramitochondrial NAD(P)H resulting from addition of aspartate followed by 2-oxoglutarate (the substrate pair for AAT) to the mitochondrial suspension (line 1). The initial rate of fluorescence change is proportional to intramitochondrial AAT activity [9]. Line 2 shows the effect of adding AAT to the suspension 30 s before 2-oxoglutarate. The increase in the rate of fluorescence change, compared with line 1, 47%, in this experiment, is due to the uptake of the enzyme into mitochondria [9]. Addition of bathophenanthroline 30 s before the enzyme almost completely abolished the increase in rate of fluorescence change (line 4); i.e. it prevented uptake of mAAT into the organelles. Bathophenanthroline added alone did not change the observed rate of change of fluorescence (line 3) and neither did the inhibitor when added after addition of AAT (line 5).

To attribute the inhibition found to the chelating properties of the bathophenanthroline, tests were made of the prevention and reversal of this inhibitory effect by metal ions, such as Co^{2+}, able to react with this complexing agent [10]. Lines 8 and 9 show that Co^{2+}, which has no effect when added alone (lines 6 and 7), was effective both in the par-

Table 1

Prevention and reversal by Co^{2+} of the inhibition by metal-complexing agents of the mAAT and mMDH uptake into mitochondria

	Line		Additions					Relative rate of change of fluorescence
			$t=0$	$t+30$ s	$t+60$ s	$t+75$ s	$t+90$ s	
Exp.1	1	MIT	ASP	–	–	–	OG	100
	2	MIT	ASP	–	mAAT	–	OG	147
	3	MIT	ASP	BPHEN	–	–	OG	100
	4	MIT	ASP	BPHEN	mAAT	–	OG	100
	5	MIT	ASP	–	mAAT	BPHEN	OG	142
	6	MIT	ASP	Co^{2+}	–	–	OG	100
	7	MIT	ASP	Co^{2+}	mAAT	–	OG	147
	8	MIT	ASP	BPHEN + Co^{2+}	mAAT	–	OG	128
	9	MIT	ASP	BPHEN	mAAT	Co^{2+}	OG	124
Exp.2	1	MIT	–	–	–	–	OAA	100
	2	MIT	–	–	mMDH	–	OAA	148
	3	MIT	–	TIR	–	–	OAA	100
	4	MIT	–	TIR	mMDH	–	OAA	100
	5	MIT	–	–	mMDH	TIR	OAA	144
	6	MIT	–	Co^{2+}	–	–	OAA	100
	7	MIT	–	Co^{2+}	mMDH	–	OAA	144
	8	MIT	–	TIR + Co^{2+}	mMDH	–	OAA	135
	9	MIT	–	TIR	mMDH	Co^{2+}	OAA	131

Fresh mitochondria (2 mg protein) (exp.1) and sulphite-loaded mitochondria (1.3 mg protein) (exp.2) were incubated in 2.0 ml of a standard medium consisting of 0.25 M sucrose, 1 mM EDTA, 20 mM Tris-HCl, pH 7.25, plus 1 mM sodium-arsenite (in exp.1) and 2 μg rotenone, at 20°C. Additions, at times indicated, were made at the following concentrations: 12 mM aspartate (ASP), 3 mM 2-oxoglutarate (OG), 17.12 nM mAAT, 25 μM bathophenanthroline (BPHEN), 2 mM oxaloacetate (OAA), 15.15 nM mMDH, 20 μM tiron (TIR), 10 μM $CoCl_2$, (Co^{2+}). The rate of fluorescence decrease value of the intramitochondrial NAD(P)H was expressed as a percentage of the rate of the control reaction

tial prevention (line 8) and reversal (line 9) of the bathophenanthroline inhibition on enzyme uptake. The same results were obtained when use was made of another metal-complexing agent tiron (not shown).

A similar experiment using MDH is shown in exp.2. In this case it was necessary to use sulphite-loaded mitochondria to partially inhibit endogenous MDH activity, as uptake of oxaloacetate into mitochondria would otherwise become rate limiting [7]. Tiron was used as a possible inhibitor instead of bathophenanthroline, whose addition in the presence of 2 mM oxaloacetate (the substrate which starts the reaction) causes a very high fluorescence increase which makes the assay impossible. Essentially the same results have been obtained, i.e. tiron inhibits the uptake of MDH being the inhibition absent when tiron was added after MDH and prevented or reversed by metal ions.

Prevention and reversal experiments (line 6–9) demonstrate that tiron inhibits owing to its ability in chelating metal ion(s).

Control was made that metal-complexing agent did not collapse the pH gradient which is known to be essential for protein uptake [11,12].

The inhibition of protein uptake observed by metal-complexing agents could be due to reaction of the reagent with metal ion(s) in mitochondria, protein, or both. The fact that AAT and MDH incubation with bathophenanthroline or tiron (each at an inhibiting concentration) prior to addition to the mitochondrial suspension caused no uptake inhibition (not shown) suggests that the effect of the inhibitor is exerted on the mitochondria rather than on the isoenzymes. Consistently, when tiron-treated mitochondria were used, the uptake of both AAT and MDH into mitochondria in the absence of externally added inhibitor was com-

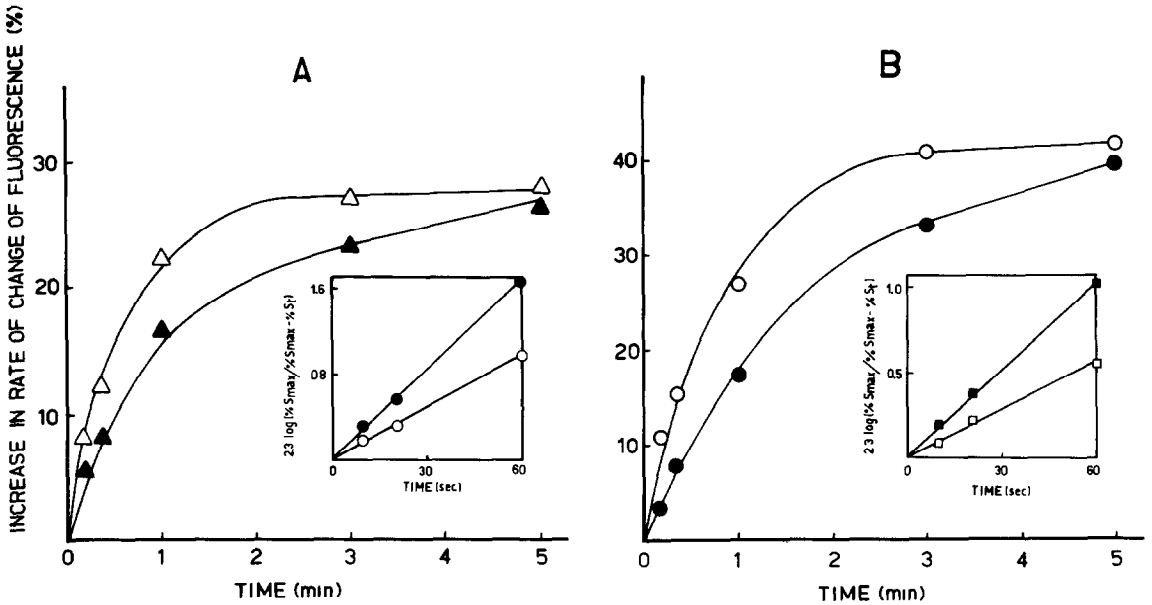

Fig.1. Time-course of mAAT and mMDH uptake into mitochondria in the presence or absence of metal-complexing agent. Fresh mitochondria (1.8 mg protein) (A) and sulphite-loaded mitochondria (1.3 mg protein) (B) were incubated at 20°C as reported in table 1 (exp.1 and 2, respectively). (A) 17.12 nM AAT was added in the presence (▲) or absence (△) of 25 μM bathophenanthroline, 1 min after 12.5 mM aspartate, and preincubated for the times shown prior to addition of 3 mM 2-oxoglutarate, which starts the assay of intramitochondrial AAT activity. (B) 15.15 nM MDH was added, in the presence (●) or absence (○) of 25 μM tiron, and after the time intervals shown 2 mM oxaloacetate was added to start the assay of intramitochondrial MDH activity. Results are expressed as the percentage increase in rate of change of fluorescence, measured by the slopes of tangents drawn to the initial parts of the experimental curve as compared with that of the control reaction without added enzyme. In the insets the same results are replotted in terms of 2.3log [% S_{max}/(% S_{max} − % S_t)] against time, where S_{max} and S_t are the percentage stimulation at completion of the uptake and at time t respectively, in the presence (○, □) or absence (●, ■) of the metal-complexing agent.

pletely inhibited. In both cases, reversal of the inhibition by added Co^{2+} was found, showing that integrity of mitochondria was obtained in the presence of the metal-complexing agent. It should be noted that none of the compounds tested were found to effect enzyme activity.

To obtain information about the mechanism of the isoenzyme uptake inhibition by metal-complexing agents, kinetics of the uptake (fig.1) of both mAAT (A) and mMDH (B) were carried out in the absence or presence of either bathophenanthroline (A) or tiron (B), essentially as reported [5]. The uptake of isoenzymes is shown as a percentage of stimulation of rate of fluorescence change compared with the control (in the absence of external enzyme) as a function of preincubation time of the mitochondria with isoenzymes before addition of the substrate which starts the reaction. Inhibition decreasing with time was found in both cases; i.e. the rate of uptake rather than the capacity of the mitochondria for protein uptake was inhibited. In the case of AAT the first-order rate constant was found to be 1.7 and 1 min^{-1}, respectively, in the absence or presence of bathophenanthroline in good agreement with values reported [5] (inset fig.1A), whereas in the case of MDH it was 1 and 0.6 min^{-1}, respectively, in the absence or presence of tiron (inset fig.1B).

The nature of tiron inhibition on both AAT and MDH uptake was kinetically investigated (fig.2) in the presence or absence of inhibitor and shown as both Dixon plot (A) and double reciprocal plot (B), respectively. Tiron was found to inhibit competitively AAT uptake into mitochondria (K_i value is 40 μM) (fig.2A), whereas MDH uptake was inhibited in a non-competitive way (K_i value is 9 μM) (fig.2B).

According to the different K_i values obtained, tiron can largely inhibit the rate of MDH uptake without significantly influencing AAT uptake. Thus, prevention by tiron of the inhibition shown by MDH on the rate of AAT uptake [4] has been tested (table 2). Tiron (10 μM), which strongly inhibits the MDH uptake (not shown), does not significantly change the rate of fluorescence decrease either in the absence (line 3) or presence

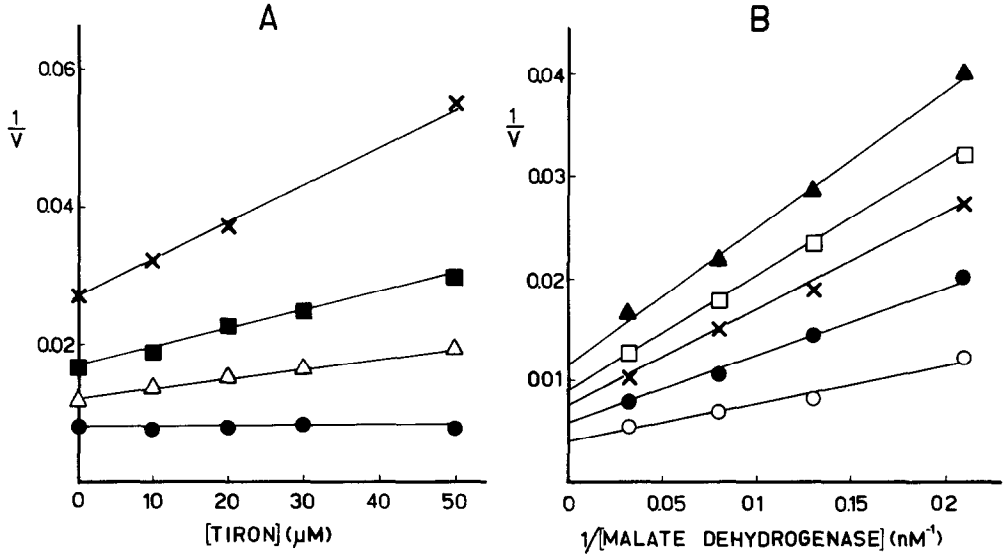

Fig.2. Kinetic analysis of the inhibition of the AAT and MDH uptake rate by tiron. The uptake rates of AAT (A) and MDH (B) were measured as reported in table 1 (exp.1 and 2, respectively). 20 s incubation of enzymes was allowed with mitochondria, either fresh (1.5 mg protein) (A) or sulphite-loaded (1 mg protein) (B). The rate of uptake (V) is expressed as % stimulation/min. Tiron was added 30 s before the isoenzymes. (A) Dixon plot of results obtained with 3.3 (×), 6.25 (■), 12.5 (△) and 25 nM (●) AAT in the presence of tiron at the indicated concentrations. (B) Double reciprocal plot obtained with MDH, at the indicated concentrations, in the absence (○) or presence of 5 (●), 10 (×), 15(□) and 20 μM (▲) tiron.

Table 2

Prevention by tiron of the inhibition by malate dehydrogenase on the uptake of aspartate aminotransferase

Line	Additions				Relative rate of change of fluorescence
	$t=0$	$t=30$ s	$t=60$ s	$t=80$ s	
1 MIT	ASP	–	–	OG	100
2 MIT	ASP	–	mAAT	OG	155
3 MIT	ASP	TIR	–	OG	107
4 MIT	ASP	TIR	mAAT	OG	155
5 MIT	ASP	–	mMDH	OG	100
6 MIT	ASP	–	mMDH + mAAT	OG	116
7 MIT	ASP	TIR	mMDH	OG	107
8 MIT	ASP	TIR	mMDH + mAAT	OG	155

Fresh mitochondria (2 mg protein) were incubated as reported in table 1 (exp.1). Additions at times indicated were as follows: 10 μM tiron (TIR), 15.15 nM mMDH, 12.5 nM mAAT

(line 4) of added AAT. On the other hand, inhibition of AAT uptake by MDH is completely prevented by tiron (compare line 8 with 6). Control was made that no significant effect due to the presence of MDH and tiron on the control reaction was found (lines 1,3,5,7).

4. DISCUSSION

The existence of a common receptor with two separate binding sites for AAT and MDH previously shown [5] has been confirmed here in view of the inhibition shown by the metal complexing agents. In this case our approach to the problem was suggested both by the observation that blockage of metal ion(s) present in the translocators of several metabolites [13,14] prevents their uptake into mitochondria and by the reported inhibition by phenanthroline on processing of the β-subunit of F_1-ATPase and subunit 9 of the F_0F_1-ATPase precursors [15]. In this latter case, however, no control of either the integrity of mitochondria during the course of the experiment or the specificity of the inhibition was produced. In contrast, our model system allowed us to check that insignificant damage of mitochondria occurs following metal-complexing agent addition, as revealed by prevention and reversal experiments which per se show that inhibition is strictly dependent on the ability of inhibitor in chelating metal ion(s). The partial reversal of inhibition could be due to possible conformational change of the receptor area.

Tiron was found to inhibit uptake of both AAT and MDH. The nature of this inhibition differs in each case: competitive in the case of AAT and non-competitive in the case of MDH, thus suggesting localization of metal ion(s) at or near the AAT but far from the MDH binding sites. Although the different nature of tiron inhibition could also be due to the existence of two distinct receptors, the prevention shown by tiron on MDH inhibition of AAT uptake demonstrates that this is not the case (see also [5]).

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