Reconstitution of ornithine transport in liposomes with Lubrol extracts of mitochondria

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The ornithine translocase of beef liver mitochondria was extracted with Lubrol WX and reconstituted in liposomes. The uptake of ornithine by the reconstituted vesicles followed Michaelis-Menten kinetics, and was dependent on the intraliposomal pH, the time of sonication of the reconstituted liposomes and the phospholipid to detergent ratio. It is concluded that the ornithine translocator can be reconstituted, which makes the purification of this translocator feasible.

Ornithine uptake *Beef liver mitochondria* *Liposome* *Reconstitution* *HHH-syndrome* *Ornithine translocator*

1. INTRODUCTION

A number of distinct translocator systems have been identified in mitochondria [1,2] and at least four of these have been purified to various degrees and reconstituted into liposomes: the phosphate translocator [3], the ADP-ATP translocator [4,5], the citrate translocator [6] and the dicarboxylate translocator [7]. A preliminary report on the reconstitution of the glutamate translocator has been given as well [8]. Reconstitution of these translocators into liposomes is the only means of assay for functional activity of these proteins. The uptake of ornithine by liver mitochondria proceeds via a specific translocator [9], where ornithine$^+$ is exchanged for a hydrogen ion [10]. Evidence for an additional mechanism of uptake of ornithine by liver mitochondria, via an ornithine/citrulline antiporter [11] could not be confirmed [12].

This communication describes experiments showing that the ornithine translocator can be solubilized and reconstituted into liposomes, which should make the purification of this translocator feasible. The physiological significance of this transport system is evident from the description of patients with hyperammonemia, hyperornithinemia and homocitrullinuria [13–16], the HHH syndrome, due to an inherited defect in the transport of ornithine across the inner mitochondrial membrane [14,15,17–19].

2. MATERIALS AND METHODS

Beef liver mitochondria were isolated as in [20]. Extracts of these mitochondria were prepared by incubating the mitochondria at a protein concentration of 10 mg/ml in 10 mM Mops (pH 7.6) with 0.3 mg Lubrol WX/mg protein for 10 min at 0°C, followed by centrifugation at 100000 × *g* for 60 min. The supernatant was used for further experiments.

Liposomes were prepared by resuspending a mixture of 300 mg phosphatidylcholine and 15 mg cardiolipin, dissolved in chloroform–methanol and evaporated to dryness in a rotary evaporator, in 10 ml of a buffer containing 100 mM sucrose, 50 mM Mops and 2 mM $MgCl_2$ (pH 7.0). The mixture was subjected to sonication at 0°C for 5 min. One ml of the beef liver mitochondrial extract was then added and the mixture was allowed to stand at 0°C for 10 min. It was then quickly frozen and

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thawed and sonicated at 0°C by brief pulses of 10 s, interspaced by 20 s of further cooling. The sonications were carried out with a Branson Sonifier, equipped with a microtip, at 40 W.

To measure the uptake of ornithine by the reconstituted liposomes, 1.8 ml of these liposomes were incubated at 37°C with 3.0 ml of a buffer containing 100 mM sucrose, 50 mM Mops, 2 mM $MgCl_2$ and 0.5 mM ornithine (pH 7.0). [1-^{14}C]Ornithine was added to a final concentration of 0.5 μCi/ml. Samples of 0.8 ml were taken at timed intervals and immediately applied to columns (1.5 × 0.7 cm) of Dowex 50 (200–400 mesh), equilibrated with 100 mM sucrose, 50 mM Mops, 2 mM $MgCl_2$ (pH 7.0) and eluted with 1.0 ml of the same buffer. An aliquot of the eluate was taken and counted by liquid scintillation.

Protein was determined as in [21]. Phosphatidylcholine from egg yolk and cardiolipin from bovine heart were obtained from Sigma (St. Louis, MO). [1-^{14}C]Ornithine (spec. act. 56 mCi/mmol) was purchased from Amersham (Arlington Heights, IL).

3. RESULTS

Liposomes not reconstituted with mitochondrial extracts do not take up ornithine as could be demonstrated by passage of such liposomes over the Dowex columns, after incubation with [1-^{14}C]ornithine for various amounts of time. No radioactivity could be detected in the eluate, except for a small amount (0.09% of the total radioactivity applied to the column), which was also observed when the non-reconstituted liposomes were omitted from the incubation mixture. This blanc which is apparently a contaminant of the [1-^{14}C]ornithine not absorbed by the Dowex, was therefore substracted.

Fig.1 shows the time course of ornithine uptake in reconstituted liposomes at 37°C. Such curves permit the estimation of initial rates of uptake, which in this case was 470 pmol·min^{-1}·mg protein^{-1}. The rate of uptake of ornithine is strongly dependent on sonication of the reconstituted liposomes as illustrated in fig.2. The optimal time of sonication is apparently less than 30 s. It is known that this optimal time of sonication varies widely between reconstituted systems (cf. [22]).

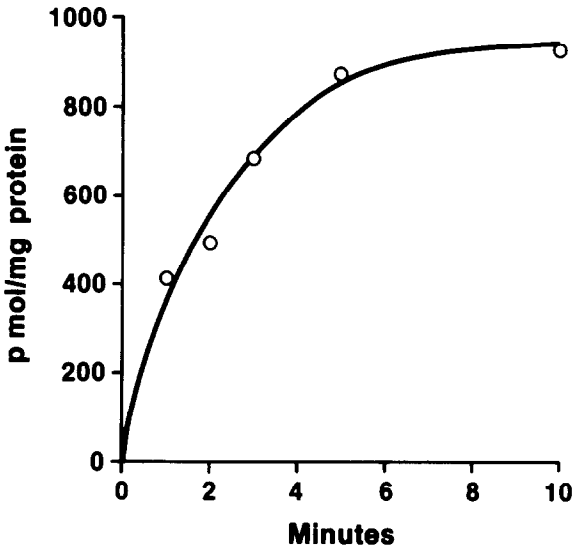

Fig.1. Time course of ornithine uptake in reconstituted liposomes. For experimental details see section 2.

Although a detergent is necessary to solubilize the translocator, the presence of the detergent may interfere with the uptake process. This proved indeed to be the case as is illustrated in fig.3. No uptake of ornithine was observed when liposomes were carried through the reconstitution procedure in the presence of the same amounts of Lubrol, but without mitochondrial extract. It is clear that an even higher phospholipid to detergent ratio may result in higher rates of uptake. This would require

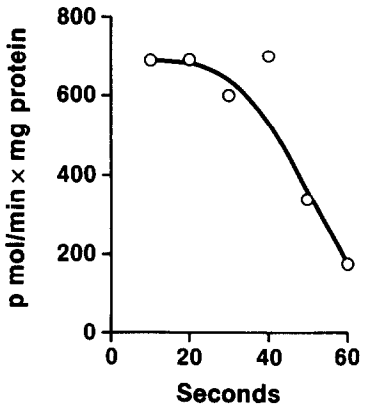

Fig.2. Effect of sonication of reconstituted liposomes on ornithine uptake. For experimental details see section 2. Initial rates were determined from curves similar to that depicted in fig.1.

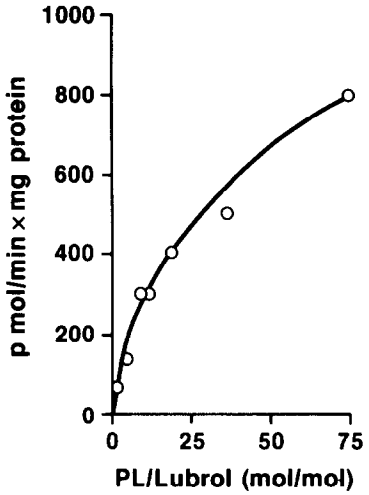

Fig.3. Effect of the phospholipid (PL) to detergent (Lubrol) ratio on the uptake of ornithine in reconstituted liposomes. For experimental details see section 2. Initial rates were determined from curves similar to those depicted in fig.1.

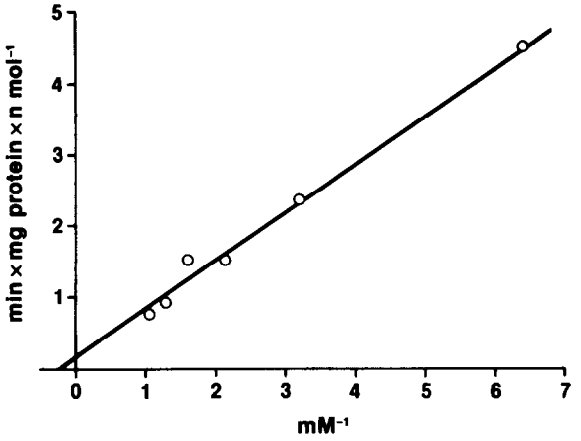

Fig.4. Lineweaver-Burk plot for the uptake of ornithine by reconstituted liposomes. The straight line was drawn by the least squares method, yielding a correlation coefficient of 0.994.

further purification of the ornithine translocator which is beyond the scope of the present communication.

It has been shown that liver mitochondria take up ornithine$^+$ in exchange for a hydrogen ion [10]. It can then be expected that a lower internal liposomal pH will result in a higher rate of uptake of ornithine. Liposomes were therefore prepared in buffers of pH 6.5, 7.0 and 7.5, respectively (the composition of the buffer did otherwise not change from that given in section 2). After reconstitution, the suspensions were readjusted to pH 7.0 and the rates of ornithine uptake were then determined at pH 7.0 and at an ornithine concentration of 2 mM. Rates of uptake of 8.2, 4.7, and 1.2 nmol·min^{-1}·mg protein were observed in reconstituted liposomes, prepared at pH 6.5, 7.0 and 7.5, respectively. The rate of uptake is thus indeed dependent on the internal pH of the liposomes, as in the intact mitochondria.

Ammonium sulfate fractionation of the mitochondrial extract yielded 2 fractions with translocator activity, precipitating between 0–20% saturation and between 60–90% saturation, respectively. An 11-fold purification was achieved for both fractions in 83% yield. None of the proteins precipitating between 20 and 60% saturation showed ornithine translocase activity.

The apparent K_m value for the uptake of ornithine by the reconstituted liposomes was found to be 4.9 mM, with a maximum rate of uptake of 7.1 nmol·min^{-1}·mg protein^{-1} (fig.4). This K_m value is slightly higher than that observed in intact mitochondria [10,12], while the maximum rate of uptake is 18% of that found in intact mitochondria [12].

4. DISCUSSION

In all previous studies on the reconstitution into lipid vesicles of mitochondrial translocators the assay system has made use of specific inhibitors of the translocator to stop the uptake reaction. Unfortunately, such an inhibitor is not known for ornithine uptake. The uptake is, however, sufficiently slow as compared to the time required to separate the liposomes from the labelled ornithine by the small Dowex columns to permit the determination of the time course of uptake (fig.1). The initial rates are likely to be overestimated because of the unavailability of an instantaneous and complete inhibitor of the uptake. The lack of such an inhibitor also poses the problem that the reconstituted system cannot be tested for specificity. The rate of uptake is, however, dependent on the phospholipid to detergent ratio, the time of sonication of the reconstituted liposomes and on the internal pH of the liposomes. This would not

be expected to be the case if the uptake was due to non-specific absorption to the reconstituted liposomes. Moreover, non-specific absorption is a rapid process and would not be expected to follow a time course as shown in fig.1.

The uptake followed Michaelis-Menten kinetics (fig.4), which again would not be expected for non-specific absorption of ornithine to the proteins of the mitochondrial extract. Ammonium sulfate fractionation of the crude mitochondrial extract showed furthermore the uptake activity to be limited to two discrete fractions, while no translocator activity could be observed in the other fractions. It remains to be established what the relationship between these two fractions is. Formation of aggregates may be the reason for these fractions. HPLC over Bio-Sil TSK-20 showed an M_r distribution in agreement with such an explanation, but further work is needed to clarify this.

The kinetic parameters of the reconstituted system are less favorable than those observed for the intact mitochondria. The phospholipid composition of the reconstituted vesicles, which has not been investigated here, is known to influence the rate of uptake in other reconstituted mitochondrial systems [4,22].

In summary, these studies have demonstrated the possibility to reconstitute the ornithine translocator in liposomes. An assay system for this translocator has thus become available which is a prerequisite to the purification of the carrier protein.

ACKNOWLEDGEMENT

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