RECONSTITUTION OF ADENINE NUCLEOTIDE TRANSPORT WITH PURIFIED ADP, ATP-CARRIER PROTEIN

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1. Introduction

Starting with our first report in 1974 [1], the ADP, ATP carrier of beef heart mitochondria was solubilized and purified in an undenatured form [2,3]. The protein was stabilized in the native state by complexing with high affinity ligands carboxyatractylate (CAT) or bongkrekate (BKA) enabling easy purification and characterization of chemical and physical parameters [4,5]. It seems to be very important to incorporate the isolated and purified protein into artificial membranes in order to study the catalytic activity of this protein, separate from the complexities in the mitochondrial membrane. For this purpose we isolated the unliganded protein with sufficient purity with a special rapid procedure, described elsewhere [6,7], in order to overcome the denaturation of the unprotected protein. By applying a simple method for reincorporating the protein into the phospholipid vesicles, the original binding activity towards BKA and CAT was reconstituted [7]. Extending this procedure further to a three step reincorporation method, we have now been able to reconstitute transport activity with the purified protein. This reconstitution with the purified carrier protein permits to study parameters pertinent to the catalytic activity of the carrier and to relate these parameters to the molecular level of the pure protein incorporated into the artificial membrane.

Recently also a reconstitution of ADP, ATP transport has been published by Shertzer and Racker [8,9] using crude cholate extracts from mitochondrial membrane preparations and reporting relatively low and widely varying activities. In such a system the correlation of the molecular parameters with the reconstituted function is not possible.

Abbreviations: BKA, bongkrekate; CAT, carboxyatractylate; LAPAO, 3-lauramido-*N,N*-dimethylpropylaminoxide

2. Materials and methods

3-Lauramido-*N,N*-dimethylpropylaminoxide (LAPAO) (commercial name: Aminoxide WS-35) was a gift from the Theo Goldschmidt AG. Phospholipids were obtained from E. Merck, Darmstadt (egg yolk, phospholipids), Koch-Light Laboratories (cardiolipin), CAT was purchased from Boehringer, Mannheim, and BKA was a gift from Professor W. Berends (Delft). All radioactive nucleotides were from NEN Chemicals GmbH. Pure lecithin was prepared from egg yolk phospholipids according to [10], and mitochondrial phospholipids were isolated as described in [11]. Hydroxylapatite was prepared as described by Bernardi [12]. Polyacrylamide gel electrophoresis (SDS–PAGE) was carried out according to Neville and Glossmann [13]. LAPAO was assayed by a redox titration method [14]. Isolation of the binding protein with LAPAO was basically similar to that previously described for the CAT-binding protein with Triton X-100 [2,3]. Modifications of this method for isolating the unliganded binding protein were described elsewhere [7].

Reconstitution of the carrier protein was done in a three step procedure. Firstly, phospholipids (about

40 mg/ml) were sonicated in the presence of 150 mM mannitol, 20 mM ATP, 10 mM Tricine/KOH, pH 7.5, 0.1 mM EDTA until the liposome suspension was clear, (10–15 min) using a Branson sonifier with microtip. Secondly, the isolated carrier protein was mixed with the liposomes, total incorporation is achieved in 5–10 min [7]. Thirdly, the suspension was sonicated once more for short times (3–5 min). External nucleotides were removed by a passage over Dowex–formate columns [15], which were equilibrated with 150 mM mannitol. To the liposomes, the indicated nucleotides (250 μM) and 1 mM $MgCl_2$ were added. The reaction was stopped with 10 μM CAT after the indicated times. Adding the inhibitor before the radioactive nucleotide gives the corresponding blank. The reaction mixture was passed through small Dowex–formate columns; all Dowex columns were equilibrated with 150 mM mannitol. Aliquots of the eluted liposomes with internal radioactive adenine nucleotides were counted in a liquid scintillation counter.

3. Results

The unliganded adenine nucleotide carrier has been solubilized using the nonionic detergent LAPAO and by treatment of the extract with hydroxylapatite according to the previously described procedure [7]. As judged by the polypeptide composition shown in fig.1A, the protein is relatively pure and contains about 70% carrier protein as calculated from parallel experiments with [^{3}H]CAT protein. This preparation differs from the protein extracts used by Shertzer et al. [8,9], originating from submitochondrial particles or 'ASUA-particles' with cholate extraction and ammonium sulfate fractionation, containing only 10% 30 000 mol. wt proteins in the extracts from submitochondrial particles and some enrichment in the case of 'ASUA-particles', reaching 40% 30 000 mol. wt protein (cf. fig.1A, B with 1C), and makes the protein solubilized and purified according to our procedure especially suited for defined reconstitution. Evidence for the chemical purity of the isolated protein as based on chromatography and immunological methods were given previously [7].

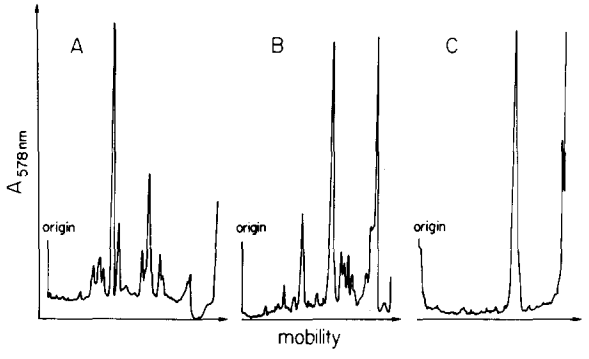

Fig.1. Polypeptide composition of various adenine nucleotide carrier preparations. Preparation according to Shertzer et al. [8,9] from submitochondrial particles (A), 'ASUA-particles' (B), and our preparation (C). Densitometric traces of SDS–polyacrylamide gel electrophoresis. The peak near the front of gel (C) is caused by staining the detergent LAPAO. For molecular weight determinations see ref. [3].

3.1. *Exchange kinetics*

The time course of the nucleotide exchange is shown in fig.2, measured according to the method

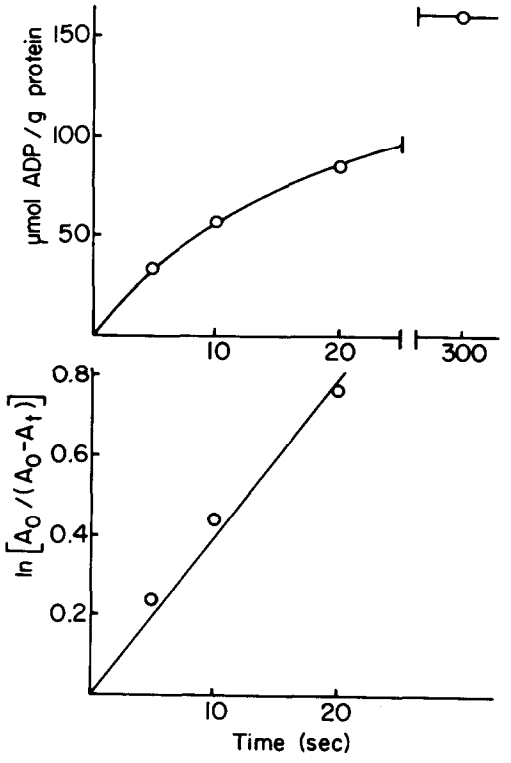

Fig.2. Time course of adenine nucleotide exchange (internal ATP, external ADP, 21°C). Exchange kinetics (A) and semi-logarithmic plot (B) of $\ln A_\circ/(A_\circ - A_t)$ ($A_\circ$, maximum exchange 160 µmol ADP/g; A_t, exchange at time t) according to the relation $\ln A_\circ/(A_\circ - A_t) = (v/A_\circ)t$ (cf. ref. [17]).

described above. The kinetics can be described in terms of the first order rate law, when the exchange is related to the maximum level shown in fig.2a. From the slope, the reaction velocity can be calculated. Often in other experimental series especially with higher time resolution (not shown) deviation from straight lines is observed indicating some heterogeneity of the employed carrier molecules and vesicles. The translocation rate derived from the first order kinetics taken in all further experiments represents the activity of the ADP, ATP carrier. Another parameter studied separately from the kinetics is the extent of the exchange as compared to the total nucleotide offered in the vesicles. Although this may seem to be an obvious requirement, adenine nucleotide transport activity in the literature has often been erroneously evaluated from extent of exchange in the native and reconstituted systems.

3.2. *Effect of protein, detergent and phospholipid concentration*

The first question in reconstitution concerned the dependence of activity and the exchanged amount of nucleotide on the amount of protein added to a constant amount of vesicles (fig.3). The maximum amount of nucleotides exchangeable in the vesicles increases with the amount of protein added, reaching a maximum at about 2.5 μg protein/mg phospholipid.

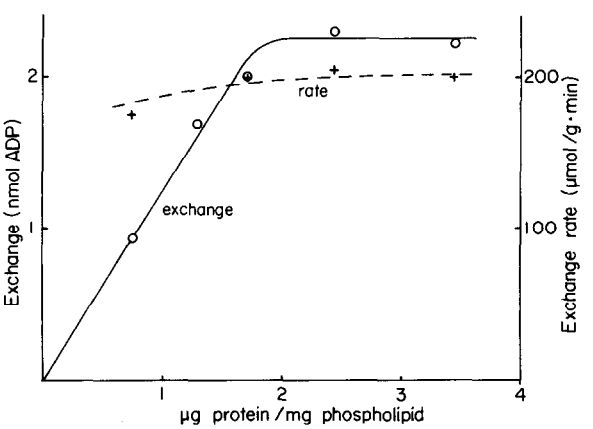

Fig.3. Dependence of exchange (——o——) and exchange rate (– – + – –) on the protein/phospholipid ratio. Increasing amounts of solubilized carrier protein is added to a constant amount of liposomes (internal ATP, external ADP), prepared from a mixture of lecithin with 1% of cardiolipin. The amount of detergent was kept constant by supplementing LAPAO.

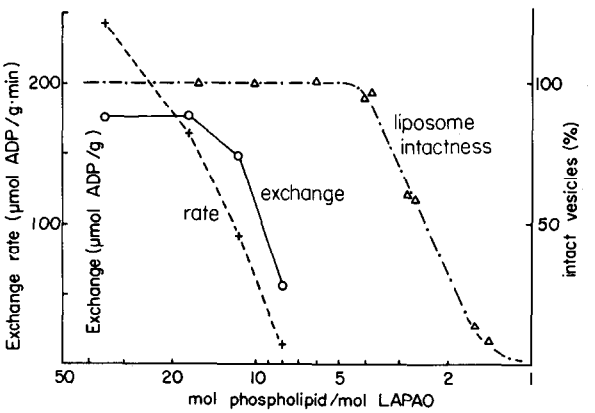

Fig.4. Effects of detergent or exchange activity. Increasing amounts of detergent are added to the reconstituted system (0.8 μg protein/mg phospholipid). Both maximum exchange (——o——) and exchange rate (– – + – –) are measured. The intactness of the vesicles is determined as described in ref. [7].

Together with the protein, detergent is added to the vesicles which has an inhibitory effect on the carrier activity as shown in fig.4. Here in parallel experimental series the amount of detergent was varied, keeping the protein/phospholipid ratio constant. Vesicles tolerate a molar ratio of 0.2 LAPAO to lecithin before breaking down, as previously shown [7]. The specific activity of the carrier, however, decreases at much lower amounts of detergent, reaching zero before the membranes are broken down. For practical purpose these results stress the importance to maintain the total detergent added at a constant level when comparing various conditions. Therefore in the experiments of fig.3, the detergent concentration was held at a common constant level in each sample.

3.3. *Phospholipid specificity*

Both the maximum exchange and initial velocity depend to some extent on the type of phospholipids used for the reconstitution (table 1). The bulk phospholipid used for the reconstitution is purified egg lecithin. Addition of acidic phospholipids increase the transport activity several fold. The maximum velocity is obtained with a mixture of lecithin and 20% of mitochondrial phospholipids. Pure mitochondrial phospholipids give liposomes with a very small internal volume unsuited for measuring exchange.

Table 1
Phospholipid specificity

Phospholipids (weight ratio)	Exchange (μmol ADP/g)	Exchange rate (μmol ADP/g × min)
PC	44	82
DPG/PC = 1/99	106	258
DPG/PC = 1/19	134	330
PA/PC = 1/99	96	167
MPL/PC = 1/13	188	492
MPL/PL = 1/4	91	508
EYPL	126	285

Exchange and exchange rate are measured at 21°C in the presence of 1 mM $MgCl_2$ with internal ATP and external ADP
Abbreviations: PC, lecithin; DPG, cardiolipin; MPL mitochondrial phospholipids; EYPL, egg yolk phospholipids; PA, phosphatidic acid

3.4. *Dependence on divalent cations*

Divalent cations are also important for exchange activity. Maximum activity was obtained with 1 mM Mg^{2+}, but also Ca^{2+} and Mn^{2+} are effective. This relative unspecificity indicates that cations do not interact with the nucleotide and with the carrier but rather with the negatively charged phospholipids. Combination of Mn^{2+} and Mg^{2+} does not enhance the activity further, which differs from the system of Shertzer and Racker [8].

3.5. *Substrate specificity*

The high specificity of the nucleotide transport for ADP and ATP since the beginning has been a major argument for defining an ADP, ATP carrier in mitochondria. In the reincorporated artificial system also a high specificity for ADP was ATP is found (table 2). The exchange is inactive with AMP, whether present internally or externally and is also inactive with external guanine nucleotides.

3.6. *Effect of inhibitors*

Another essential criterion in defining the specific ADP, ATP-transport system in mitochondria has been the high sensitivity to inhibitors such as CAT and BKA. Both these agents inhibit to more than 95% the transport activity in the reconstituted system (results not shown). If the same protein is isolated as the CAT complex and incorporated under the same conditions into the nucleotide-loaded vesicles, the exchange activity is less than 2%. This experiment also excludes

Table 2
Nucleotide specificity

Internal nucleotide	External nucleotide	Exchange (μmol ADP/g)
–	ATP, ADP	< 5
AMP (10 mM)	ATP, ADP, AMP	< 5
ADP (10 mM)	ATP	135
	ADP	118
	AMP	< 5
	GDP	< 10
ATP (20 mM)	ATP	250
	ADP	173
	AMP	< 5
	GDP	< 10

Exchange of the indicated external and internal nucleotides with liposomes consisting of lecithin with 20% of mitochondrial phospholipids at 21°C. All external nucleotides are added at a concentration of 250 μM

that the transport activity depends on any component of the 20–30% contaminating proteins, present after the short-cut isolation of the unliganded protein.

4. Discussion

The reported results clearly correlate the adenine nucleotide-transport activity of the inner mitochondrial membrane with an isolated and purified 30 000 mol. wt protein for the first time. Although this protein is largely pure, objections raised on the basis of small contaminations by other proteins were ruled out by experiments in which the protein was isolated as CAT–protein complex and transport activity after incorporation was completely abolished. CAT was previously shown to be bound exclusively to the 30 000 mol. wt protein [2,3]. The reconstituted activity can be compared to that in beef heart mitochondria by relating the exchange activity to the amount of carrier molecules present in both systems. In beef heart mitochondria a turnover rate of the carrier of 550 min^{-1} was determined [16]. The content of carrier protein, which is a dimer of two 30 000 mol. wt polypeptides [3] is defined by the CAT-binding site. In the reconstituted system the

turnover rate is calculated as 45 min^{-1} (cf. table 1) which amounts to 8% of the original mitochondrial activity.

Acknowledgements

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