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CHARACTERIZATION OF PYROPHOSPHATE EXCHANGE BY THE RECONSTITUTED ADENINE NUCLEOTIDE TRANSLOCATOR FROM MITOCHONDRIA

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SUMMARY : The transport of inorganic pyrophosphate (PP_i) by the adenine nucleotide translocator from beef heart mitochondria was studied in a reconstituted system. The transport of PP_i is dependent on appropriate transmembrane substrates. The activity of PP_i exchange is about one tenth as compared to the ADP/ATP exchange, whereas the transport affinity for PP_i is very low (2-5 mM). The adenine nucleotide carrier catalyzes a strict counterexchange of PP_i and nucleotides with an exchange stoichiometry close to 1. The inhibitor specificity of PP_i exchange is comparable to that of ADP/ATP exchange.

It has been shown that inorganic pyrophosphate (PP_i) can enter mitochondria in exchange for matrix adenine nucleotides (AdN). The transport reaction is slow in rat liver mitochondria (1,2), but considerable activity and high affinity for PP_i (K_m of about 20 µM) have been observed in rat heart mitochondria (3). However, important questions have remained open, for instance regarding the true exchange activity as compared to ADP/ATP exchange, the exact transport stoichiometry and the inability of PP_i to compete with AdN, which is not readily understandable considering the reported high affinity (3).

In order to elucidate the significance of this side reaction of the adenine nucleotide translocator (AdN-T) and to characterize kinetically this process, PP_i transport was studied in a reconstituted system of the AdN-T from beef heart mitochondria incorporated into phospholipid vesicles. Thereby possible artefacts caused by PP_i binding to other proteins or due to cleavage by pyrophosphatases can be avoided. The results obtained here show that PP_i exchanges

Abbreviations: AdN, adenine nucleotides; AdN-T, adenine nucleotide translocator; BKA, bongkrekate; CAT, carboxyatractylate; P_i, orthophosphate; PP_i, inorganic pyrophosphate.

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at a 1:1 stoichiometry against AdN. The exchange reaction is further characterized by a very low affinity for PP_i although it shows quite high transport rates.

Experimental

Isolation of egg yolk phospholipids for the preparation of liposomes (4) and isolation, purification and reconstitution of the AdN-T from beef heart mitochondria (5) have been described previously. Reconstituted exchange was measured both in the forward (6) and backward direction (5). After the exchange experiment the vesicle suspension was analyzed for labelled substrates according to ref. (7); the true transport rates were extrapolated as described earlier (6). In general, only relative exchange rates can be determined in back exchange experiments, i.e. [^{14}C]ATP - dpm/min, since the actual internal AdN pool size of the functionally active phospholipid vesicles would have to be known in order to calculate true efflux rates in this case (8). Nevertheless, within one experiment, i.e. working with one set of liposomes loaded with labelled internal substrate (5), these relative exchange rates can be directly compared.

Results and Discussion

The reconstituted ADP/ATP carrier catalyzes an exchange of pyrophosphate

In order to investigate whether transport of PP_i is in fact an exchange reaction catalyzed by the AdN-T, the dependence of PP_i transport on various substrates on both sides of the liposomal membrane is tested. Table I shows the uptake rates of [^{14}C]ATP and [^{32}P]PP_i in the presence of various internal ions. Transport of both ATP and PP_i is equally dependent on the presence of a trans-

Table I

Dependence of carrier activity on internal substrates

Internal substrate	External substrate	Uptake rate (μmol x g^{-1} x min^{-1})
Na_2SO_4	[^{14}C]ATP (0.1 mM)	< 5
Na_2HPO_4	"	< 5
PP_i	"	215
ATP	"	2400
Na_2SO_4	[^{32}P]PP_i (4 mM)	< 3
Na_2HPO_4	"	< 3
PP_i	"	118
ATP	"	170

For all internal substrates the concentration was 30 mM together with 20 mM Tricine, pH 7.5. Uptake rates were determined according to (6).

membrane substrate, i.e. PP_i uptake is possible in exchange against internal ATP or PP_i, whereas no transport activity is observed in the presence of internal sulfate or P_i. The same holds true for back exchange experiments (not shown). On the basis of these results it can be concluded that the transport reaction in the reconstituted system is in fact an exchange process.

The ADP/ATP carrier catalyzes a strict counterexchange of pyrophosphate

The reconstituted system offers the possibility of measuring quantitatively the stoichiometry of the exchange of PP_i against other substrates. Since the size of the internal AdN pool of the liposomes containing functionally active carrier protein is not known, the true exchange rates in a back exchange experiment cannot be determined directly (5,8). An experiment for establishing the exchange stoichiometry starts with the labelling of the internal ATP-pool (5 mM) of the reconstituted liposomes by exchange with [^{3}H]-labelled ATP from the outside. This reaction is continued until complete equilibration between internal and external pool is achieved which usually takes 20-30 min at room temperature. The external nucleotides are now removed by passage over Sephadex G-75 and the reconstituted liposomes containing [^{3}H]ATP in the internal volume are divided into two portions. With one part a counterexchange experiment involving the addition of [^{14}C]ATP (100 μM) is carried out (experiment a in Table II) the other part is used for a corresponding experiment with [^{32}P]PP_i (4 mM) (experiment b in Table II). Since the strict 1:1 stoichiometry of ATP/ATP exchange has been established (9) and since the specific radioactivity of the externally added [^{14}C]ATP is known, the specific activity of the [^{3}H]ATP which exchanges against [^{14}C]ATP in the first part of the experiment can be calculated.

In the second part of the experiment again the specific radioactivity of the external substrate, in this case [^{32}P]PP_i, is known. The [^{3}H]ATP/[^{32}P]PP_i exchange experiment can now be used to calculate the stoichiometry of the ATP/PP_i counterexchange. The data shown in Table II demonstrate that this combined forward and back exchange experiment leads to an exchange stoichiometry close to 1 over the whole course of the exchange reaction.

Table II

Stoichiometry of ATP/PP_i counterexchange

Experiment a : internal [^{3}H]ATP (5mM) / external [^{14}C]ATP (0.1mM)	exchange time (min) 0.5	2	5
uptake of [^{14}C]ATP (μmol x g^{-1})	565	865	1025
efflux of [^{3}H]ATP (dpm x μg^{-1})	1450	2320	2790
[^{3}H]ATP efflux / [^{14}C]ATP uptake (dpm/nmol) i.e. specific activity of [^{3}H]ATP	2565	2680	2720

Experiment b : internal [^{3}H]ATP (5mM) / external [^{32}P]PP_i	exchange time (min) 5	15	25
uptake of [^{32}P]PP_i (μmol x g^{-1})	395	675	885
efflux of [^{3}H]ATP (μmol x g^{-1})	490	875	1080
efflux of [^{3}H]ATP / uptake of [^{32}P]PP_i i.e. stoichiometry of ATP/PP_i exchange	1.24	1.30	1.22

The specific radioactivity of the externally added [^{14}C]ATP and [^{32}P]PP_i is known. Experiment a is designed to establish the specific radioactivity of the internal [^{3}H]ATP. This value (2655 dpm/nmol) is then used in experiment b for calculating the stoichiometry of [^{3}H]ATP/[^{32}P]PP_i counterexchange. For further details see text.

Inhibitor specificity

Table III shows the inhibitor specificity of the back exchange reactions of labelled internal ATP against external PP_i. The specific inhibitors CAT and BKA block the carrier activity, the residual activity left after addition of CAT being fully inhibited by BKA. This observation, i.e. partial inhibition by the membrane-impermeable ligand CAT, reflects the random orientation of carrier protein in the reconstituted system, namely both right-side out and inside-out, as described previously (10,11). Palmitoyl-CoA, another inhibitor of adenine nucleotide exchange (12), also blocks the PP_i transport. Using the transport affinity K_m for PP_i (cf. Table IV) in this reconstituted system, the K_i value for competitive inhibition by palmitoyl-CoA can be determined. It amounts to about 2 μM, which closely resembles the value reported for inhibition of ADP/ATP exchange in mitochondria (12).

Table III

Inhibition of PP_i transport

Inhibitor	relative exchange rate
-	100
carboxyatractylate 20 µM	9
bongkrekate 2 µM	< 1
CAT 20 µM and BKA 2 µM	< 1
Palmitoyl CoA 20 µM	29
Palmitoyl CoA 100 µM	5

Exchange of internal [^{14}C]ATP (30 mM) against external PP_i (10 mM) was measured in the presence of various inhibitors. Since backward exchange was measured, only relative exchange rates can be given (see text).

Kinetic data of PP_i transport

The basic kinetic data for pyrophosphate exchange, i.e. maximum exchange velocity (V_{max}) and transport affinity (K_m), are determined in the reconstituted system as described for the ADP/ATP exchange (6). Exchange with PP_i has been analyzed in the cases of homologous PP_i exchange (PP_i internal and external) and in those of heterologous exchange using internal AdN and external PP_i. The heterologous exchange of internal PP_i against external AdN is difficult to measure exactly, since the internal substrate binding sites of the ADP/ATP carrier are

Table IV

Kinetic data of PP_i exchange

Substrate		Exchange rate (V_{max})		Transport affinity (K_m)
internal	external	($\mu mol \cdot g^{-1} \cdot min^{-1}$)	(relative)	(mM)
ATP 20 mM	[^{14}C]ATP 5 - 100 µM	2650	100	0.015
PP_i 30 mM	[^{32}P]PP_i 0.5 - 8 mM	235	9	2.1
[^{14}C]ATP 20 mM	ATP 2 mM	n.d.[1)]	100[1)]	n.d.
^{14}C ATP 20 mM	PP_i 0.5 - 8 mM	n.d.[1)]	16[1)]	4.7

The first two experiments (label outside) were measured as forward exchange, the last two (label inside) as back exchange in the reconstituted system.

1) in back exchange experiments only relative exchange rates can be determined, for details see text.

saturated with nucleotides taken up from the outside already within a few seconds after starting the exchange, due to the extremely large difference in the K_m's of ATP and PP_i. The data in Table IV demonstrate that exchange against PP_i is quite active as compared to nucleotide exchange, whereas the apparent K_m of PP_i is two or three orders of magnitude lower than that of ATP. These results are in disagreement with the reported high affinity for PP_i (3), however, they would easily explain the observed inability of PP_i to compete with AdN described in the same paper. The observed difference in exchange rates between homologous PP_i/PP_i exchange and heterologous ATP/PP_i exchange can be explained fully by the kinetic analysis derived for ADP/ATP transport (6). This will be presented in detail elsewhere.

Conclusions

The experiments reported here indicate that the reconstituted AdN-T from beef heart mitochondria translocates PP_i at a considerable rate in an exchange reaction characterized by a strict 1:1 stoichiometry. The most interesting aspects of these results (for future experiments) are based on two facts :
(a) PP_i can be used as an alternative substrate showing quite high exchange activity, but extremely low binding affinity. This fact may be used to elucidate influences on the binding step of the exchange reaction catalyzed by the AdN-T.
(b) In heterologous exchange experiments (PP_i against AdN) an extremely asymmetric distribution of substrate binding sites on the two sides of the membrane can be achieved, due to the different transport rates. This situation resembles ADP/ATP exchange in energized mitochondria, i.e. in the presence of high membrane potentials (5,6). These aspects are at present under investigation.

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References

1. Asimakis, G.K. and Aprille, J.R. (1980) Arch.Biochem.Biophys. 203, 307-316.
2. D'Souza, M.P. and Wilson,D.F. (1982) Biochim.Biophys.Acta 680, 28-32.

3. Vercesi,A. and Lehninger,A.L. (1984) Biochem.Biophys.Res.Commun. 118, 147-153.
4. Krämer,R. (1982) Biochim.Biophys.Acta 693, 296-304.
5. Krämer,R. and Klingenberg,M. (1980) Biochemistry 19, 556-560.
6. Krämer,R. and Klingenberg,M. (1982) Biochemistry 21, 1082-1089.
7. Krämer,R. (1984) Biochim.Biophys.Acta 765, 353-362.
8. Pfaff,E., Heldt,H.W. and Klingenberg,M. (1969) Eur.J.Biochem. 10, 484-493.
9. Klingenberg,M. (1976) in The Enzymes of Biological Membranes (Martonosi,A.N.,ed.) Vol.3, pp.383-438, Plenum Press, New York.
10. Krämer,R. and Klingenberg,M. (1979) Biochemistry 18, 4209-4215.
11. Krämer,R. (1983) Biochim.Biophys.Acta 735, 145-159.
12. Chua,B.H. and Shrago,E. (1977) J.Biol.Chem.252, 6711-6714.