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INFLUENCE OF DIVALENT CATIONS ON THE RECONSTITUTED ADP, ATP EXCHANGE

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Summary

1. Divalent cations cause a decrease in the exchange activity of the reconstituted ADP,ATP translocator from beef heart mitochondria. This effect is due to complex formation with the adenine nucleotides.

2. It is confirmed that only the free nucleotides are transported. A possible competition of free adenine nucleotides and the Mg^{2+}-complexes for the binding site at the carrier protein is excluded.

3. The stability constants (K_n) for the cation-nucleotide complexes are derived from these experiments. For Mg^{2+}-ATP, $K_n = 0.8 \cdot 10^4\ M^{-1}$ and for Mg^{2+}-ADP, $K_n = 0.8 \cdot 10^3\ M^{-1}$ is obtained.

4. The carrier system was reconstituted with the neutral phospholipids phosphatidylcholine and phosphatidylethanolamine. Interaction of the divalent cations with these phospholipids seem not to be important for the exchange suppression.

Introduction

There is convincing evidence that only free ADP and ATP, and not the magnesium complexes, are transported by the adenine nucleotide carrier from beef heart mitochondria across the inner mitochondrial membrane [1—3]. A considerable part of nucleotides present in the cytosol and mitochondria at a concentration of 7—15 mM [4—6] are complexed by Mg^{2+} which, for example in rat liver, is present both in the cytosol (about 10 mM total Mg^{2+} concentration) and in the matrix space (about 30 mM) of mitochondria [7,8]. The complex formation of Mg^{2+} with nucleotides, especially in the intramitochondrial space [9,10], seems to be an important factor changing the shares of ADP versus ATP

translocation. When working with mitochondria, however, it proves to be difficult to define the concentrations of internal ADP and ATP whether free or complexed with Mg^{2+}, and also the concentration of Mg^{2+} due to binding to various internal compounds [11,12]. The presence of adenylate kinase and ATPase activity causes further complications.

The reconstituted ADP,ATP carrier [13–15] offers the possibility to use a defined system, consisting only of purified carrier protein and phospholipids. In the liposomal system the ADP,ATP carrier has the original transport specificity [14], specific activity [15] and regulation phenomena [16], and thereby represents a suitable model for elucidating the influence of Mg^{2+} on the translocation of ADP and ATP by the adenine nucleotide carrier.

Materials and Methods

Egg yolk phospholipids were obtained from E. Merck, Darmstadt, Triton X-100 from Sigma Chemical Company. Pure lecithin was prepared from egg yolk phospholipids according to Beyer and Klingenberg [17]. Carboxyatractylate and nucleotides were purchased from Boehringer, Mannheim, and radioactive nucleotides from New England Nuclear Chemicals GmbH. Hydroxylapatite was prepared as described by Bernardi [18].

Isolation, purification, reconstitution of the adenine nucleotide carrier protein from beef heart mitochondria and the exchange assay were performed as previously described [15]. For one experiment. comprising 48–60 inhibitor stop assays, 500 mg of egg yolk phospholipid with 0.6–1 mg of inserted carrier protein was used. The divalent cations were added 1–2 min before the exchange assay. Mg^{2+} was incorporated into the liposomes during their preparation by sonication; excess Mg^{2+} was consecutively removed together with the external nucleotides by passage over Sephadex G-75. All exchange velocities given in the text are initial velocities calculated from the kinetics with inhibitor stop before adding labeled nucleotides (blank value) and stops after 30, 60 and 90 s exchange. It was not possible to use external concentrations of divalent cations higher than 10 mM due to unsatisfactory separation of the external nucleotides by Dowex 1-X8 columns under these conditions.

Results and Discussion

Adenine nucleotide exchange activity in the reconstituted system is decreased by added Mg^{2+} as shown in Fig. 1A. This is in agreement with the fact, that only the free nucleotides are transported by the ADP,ATP carrier [1–3] and not the complexes with Mg^{2+}. Because of the tighter binding of Mg^{2+} to ATP than to ADP [19] exchange with external ATP is inhibited already at lower concentrations of Mg^{2+} as compared to ADP. When both nucleotides are present simultaneously in the external space (Fig. 1B), the uptake of ADP is not diminished by low concentrations of Mg^{2+}, as it was the case in Fig. 1A, but it is even increased to some extent. This observation can be rationalized by competition of ADP for the exchange capacity.

The results shown in Fig. 1A offer the possibility to calculate the corresponding stability constants for the cation-nucleotide complexes. The apparent

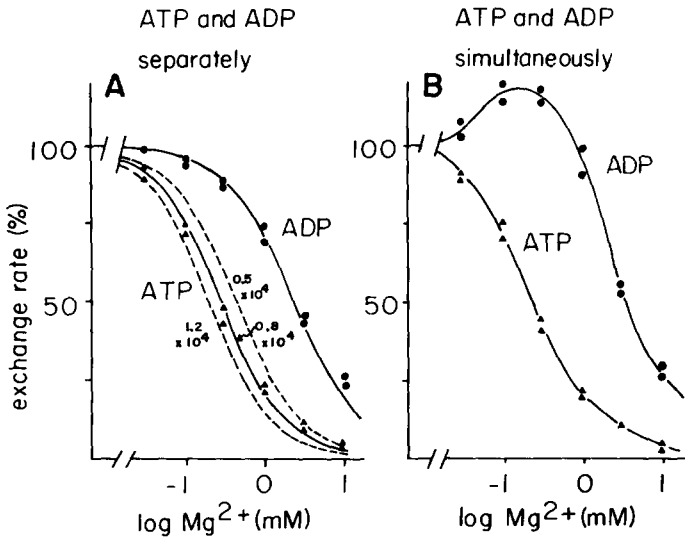

Fig. 1. Effect of external Mg^{2+} on the uptake of ADP and ATP. The exchange velocities are normalized to the exchange without added Mg^{2+} = 100%. The absolute values are in the range of 250—400 μmol · g^{-1} · min^{-1}. A. ADP (50 μM, •) and ATP (50 μM, △) exchange was assayed in different experiments. The lines are computer fitted curves (see in the text) for the indicated stability constants. B. Both ADP (•) and ATP (△) were present simultaneously, curves are not calculated.

K_m values for ADP and ATP with respect to the exchange activity in the egg yolk phospholipid liposomes amount to 55—60 μM (Krämer, R. and Klingenberg, M., unpublished result). In Eqns. 1 and 2 for the stability constants (K_n) of the cation-nucleotide complexes

$$K_N = \frac{[MeN]}{[Me_f] \cdot [N_f]} \tag{1}$$

$$[N_f] = \frac{[MeN]}{[Me_f] \cdot K_N} \tag{2}$$

one can substitute the unknown concentrations of the cation-nucleotide complex (MeN) and of the free cation (Me_f) with the conservation relations Eqns. 3 to 5 by the known total concentration of divalent cations (Me_o) and nucleotides (N_o).

$$[MeN] = [N_o] - [N_f] \tag{3}$$

$$[Me_f] = [Me_o] - [MeN] \tag{4}$$

$$= [Me_o] - [N_o] + [N_f] \tag{5}$$

After rearrangement for the free nucleotide concentration $[N_f]$ the following relation is obtained.

$$[N_f]^2 + \left([Me_o] - [N_o] + \frac{1}{K_n}\right)[N_f] - \frac{[N_o]}{K_N} = 0\,, \tag{6}$$

By insertion into the 'Michaelis-Menten' Eqn. 7 the relative exchange

$$\frac{V}{V_m} = \frac{[N_f]}{K_m + [N_f]} \tag{7}$$

velocities for a given value of K_n can be calculated since all other parameters are known. This was done, based on the experimental data for Fig. 1A for Mg^{2+}. The continuous lines are computed according to Eqns. 6 and 7 and give the values for K_n in Table I. The stability constants for the Mg^2-ADP and Mg^{2+}-ATP complexes as derived from these experiments, come close to the values obtained by Alberty [19] or Taqui Khan and Martell [20] and are different from other measurements [21,22]. The dashed lines in Fig. 1A besides the data for ATP exchange represent computed curves for assumed K_n values of $1.2 \cdot 10^4\ M^{-1}$ and $0.5 \cdot 10^4\ M^{-1}$, respectively, demonstrating the relative accuracy of the applied method.

So far only interaction of the added divalent cations with nucleotides has been considered; there may be also interaction with the phospholipid head groups of the liposomal membrane [11,12]. The effects on the exchange of ADP and ATP, however, are very similar and independent of the different absolute exchange activity [14,15] whether, instead of egg yolk phospholipids, a mixture of pure phosphatidylcholine and phosphatidylethanolamine are used (data now shown). Stimulating effects of low Mg^{2+} and Mn^{2+} concentrations on the nucleotide exchange, as observed earlier [14,23], might be due to interaction with negatively charged phospholipids. This should be the case especially with soybean phospholipids [23], where electrostatic repulsion between the high amounts of negatively charged phospholipids and nucleotides can be reduced by divalent cations [24].

A definite correlation between cation binding to the nucleotides and decrease of exchange can also be demonstrated by different divalent cations (Fig. 2 and Table I). Based on the known difference of binding affinity (Mn > Mg > Ca) a corresponding sequence of exchange inhibition due to the formation of cation-nucleotide complexes is seen.

An important advantage of the reconstituted system as compared to mitochondria is the possibility to establish definite internal concentrations of nucleotide and — in this case — of free Mg^{2+}. The inhibition of nucleotide efflux by increasing internal Mg^{2+} concentration obviously causes a 'transmembrane effect' on the uptake of nucleotides as shown in Table II. This can be explained by taking into account that the adenine nucleotide transport is a strict counterexchange also in the reconstituted system. Thereby the diminished efflux activity limits the basically uninhibited uptake of nucleotides.

TABLE I

STABILITY CONSTANTS OF THE CATION-NUCLEOTIDE COMPLEXES

The data for the Mn^{2+} and Ca^{2+} complexes are obtained by experiments similar to that in Fig. 1A for Mg^{2+} (not shown). The stability constant for the Ca^{2+}-ADP complex could not be determined with sufficient accuracy due to unsatisfactory adsorption of nucleotides to Dowex 1-X8 with high Ca^{2+}-concentrations (see Methods).

Me^{2+}	K_{ATP} (M^{-1})	K_{ADP} (M^{-1})
Mn	$1.7 \cdot 10^4$	$1.9 \cdot 10^3$
Mg	$0.8 \cdot 10^4$	$0.8 \cdot 10^3$
Ca	$0.3 \cdot 10^4$	—

TABLE II

EFFECT OF INTERNAL Mg^{2+} ON THE UPTAKE OF ATP AND ADP

The free internal concentrations of nucleotides were calculated according to Eqn. 6 on the basis of the determined stability constants of Table I. The measured uptake velocities and the calculated efflux velocities (Eqn. 7) are related to experiments without internal Mg^{2+}, designated as 'control'.

$Mg^{2+}_{internal}$ (mM)	Total ADP, ATP (internal) (mM)	Free ADP, ATP (internal) (μM)	Uptake velocity (% of control)	Efflux velocity (% of control)
15	5, 5	865, 100	74	78
30	5, 5	290, 30	62	59
50	5, 5	150, 15	33	47
50	2, 2	50, 5	18	27

These experiments demonstrate also for the inner side of the phospholipid membrane that Mg^{2+} suppresses the exchange by complexation of the free nucleotides to the same extent.

Though it is clearly shown that only the free nucleotides are accepted by the carrier molecule, the question remains, whether the Mg^{2+}-nucleotide complexes have an inhibitory effect on the carrier function; in other words: is there any kind of competition between free nucleotides and nucleotide-Mg^{2+} complexes for the nucleotide binding site on the ADP,ATP-carrier protein?

Under the xperimental conditions of Fig. 1 (50 μM ATP external), the ratio Mg^{2+}-nucleotide/free nucleotide $\simeq 1$ for half maximum exchange velocity. A possible inhibition by the Mg^{2+} complex should be more clearly discerned on raising the external nucleotide concentration 10-fold as shown in Fig. 3. This is not convenient in normal experiments because it renders the determination of the initial exchange velocity more difficult, due to the small differences at short exchange times. In this case the concentration of Mg^{2+}-nucleotide com-

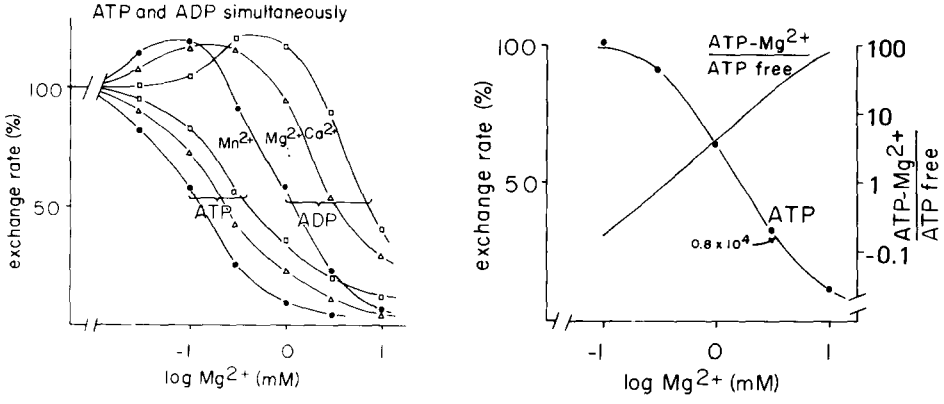

Fig. 2. Effect of external Mn^{2+}, Mg^{2+} and Ca^{2+} on the uptake of ADP and ATP, all other conditions as in Fig. 1B.

Fig. 3. Effect of external Mg^{2+} on the uptake of ATP (0.5 mM), all other conditions as in Fig. 1A. The ratio ADP-Mg^{2+}-complex/free ADP is calculated by Eqn. 6 and 3, the curve for exchange decrease is the computer fitted line. The absolute value was 100% = 470 μmol $\cdot$ g^{-1} $\cdot$ min^{-1}.

plex exceeds that of the free nucleotide by more than ten times for half maximum exchange velocity. If the Mg^{2+}-nucleotide complex would inhibit, the measured K_n should be clearly decreased as compared to that calculated in Fig. 1. The extrapolated stability constant (K_n) of the Mg^{2+}-ATP complex, however, remains unchanged. Thus it is concluded that the Mg^{2+}-nucleotide complex does not compete with free nucleotides in the case of ADP,ATP translocation.

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