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THE RECONSTITUTED ADP/ATP CARRIER FROM MITOCHONDRIA IS BOTH INHIBITED AND ACTIVATED BY ANIONS

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Anions were found to have a number of different effects on the reconstituted ADP/ATP carrier from mitochondria. (1) Binding of adenine nucleotides to the active site of the translocator is competitively inhibited by various anions. These anions can be arranged in a sequence of increasing competitive effect due to their order in a lyotropic series, and also due to increasing charge. (2) Apart from this competition effect, the presence of a sufficiently high concentration of anions turned out to be absolutely essential for functional ADP/ATP exchange in the reconstituted system. The activating anions too can be arranged in sequence, similar to that of the competition effect. The adenine nucleotide transport shows sigmoidal dependence on the stimulating anions with a Hill coefficient of $n = 2$. Addition of anions does not change the basic amount of functionally active translocator molecules. (3) The different effects of anions, i.e., inhibition and activation, were shown to take place at different sites and to be due to different mechanisms. Anions compete with substrates both at the outer (cytosolic) and at the inner (matrix) active site, whereas anion activation is observed solely by interaction with the cytosolic side of the translocator protein. (4) Activation of the reconstituted ADP/ATP exchange by anions could be discriminated from an activating influence of anionic phospholipids in the surroundings of the carrier protein.

Introduction

The adenine nucleotide carrier from the inner mitochondrial membrane is influenced by various factors pertaining to the surrounding hydrophilic and hydrophobic phases, as well as by transmembrane parameters. Several of these factors have been studied in great detail in the reconstituted system [1–5]. One of the important parameters is the ionic composition of the surrounding water phase. Two aspects of this field have already been covered in earlier publications, namely the influence of divalent cations on the ADP/ATP exchange activity [3] and the effect of ionic strength on the transport function, mediated by changes in surface potential of the phospholipid membrane in which the carrier protein is embedded [5].

When we investigated the influence of surface charges on the nucleotide transport, we discovered – apart from those effects which could be attributed to pure surface potential modulation – an additional influence of anions on both the affinity and the velocity of the reconstituted ADP/ATP exchange [5]. Functional interaction with anions is known for a variety of proteins [6], including soluble enzymes, such as alcohol dehydrogenase [7,8] as well as membrane proteins, e.g., ATPase [9]. The functional stimulation observed in these cases is usually restricted to special anions, and is interpreted to be a consequence of anion binding

Abbreviation: Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]glycine.

to specific binding sites at the respective enzyme molecule.

It will be shown in the following that anions have a twofold effect on the functionally reconstituted nucleotide translocation. On the one hand they inhibit ADP/ATP exchange by competition with substrates at the binding site, whereas on the other hand they strongly activate nucleotide transport due to cooperative binding at different sites of the protein.

Materials and Methods

Chemicals

The chemicals and their sources were as follows: Triton X-100 (Sigma); carboxyatractylate, valinomycin and nucleotides (Boehringer-Mannheim); radioactive nucleotides (Amersham-Buchler); Dowex 1-X8 (Bio-Rad); Sephadex (Pharmacia); dicetyl-phosphate and 2-*p*-toluidinylnaphthalene-6-sulfonate (Sigma). Pentadecylumbelliferone was a gift from Professor Fromherz (Ulm) and bongkrekate was a gift from Professor Berends (Delft). Hydroxyapatite was prepared as described previously [10]. All other chemicals were of analytical grade. [^{3}H]carboxyatractylate was prepared according to Ref. 11.

Determinations

Protein concentration was determined by the method of Lowry et al. in the presence of 1% sodium dodedyl sulphate [12] and phosphorus was estimated by the method of Chen et al. [13]. The surface potential was determined as described previously [5] by fluorescence of the dye 2-*p*-toluidinylnaphthalene-6-sulfonate [14] or pentadecylumbelliferone [15].

Lipids and liposomes

Egg yolk phospholipids were prepared according to Ref. 16. Purification and separation of single phospholipid species were performed as described previously [17]. In all experiments, with the exception of those described in Table IV, purified egg yolk phospholipids with addition of 5% cholesterol were used in the reconstitution. Pure phosphatidylserine was bought from Sigma. For the preparation of liposomes with added negatively charged lipids, the individual lipids were mixed in chloroform, evaporated to dryness and sonicated under nitrogen atmosphere in a Branson sonifier.

Isolation, reconstitution and assay of ADP/ATP carrier protein

The adenine nucleotide carrier was isolated from beef-heart mitochondria by hydroxyapatite chromatography in a batch procedure using Triton X-100 as described previously [10]. The carrier protein was incorporated into preformed liposomes and the ADP/ATP translocation activity was reconstituted by a freeze-thaw procedure [18] and a second sonication [19]. The sonication buffer included all ions and nucleotides which had to be present afterwards in the internal liposomal volume. For most experiments, the internal space contained 20 mM Na_4ATP/30 mM Na_2SO_4/10 mM Tricine-NaOH (pH 7.5). The external medium was exchanged by chromatography on Sephadex G-75 columns in order to obtain the desired external ionic conditions. Reconstituted adenine nucleotide exchange in the forward [2,19] and backward direction [10] has been described previously. After stopping the exchange reactions with carboxyatractylate and/or bongkrekate, as indicated in the corresponding experiments, radioactive external nucleotides were removed by ion-exchange chromatography on Dowex 1-X8 columns (Cl^- form). 100–200 μl of liposomal suspension was subjected to columns 5 × 30 mm. In order to minimize loss of lipid and protein, the columns were preequilibrated with egg-yolk phospholipid liposomes (3–5 mg phospholipid/column) and bovine serum albumin (2 mg/column). The liposomes of the exchange assay were eluted with a defined volume of 50 mM NaCl. Aliquots of the eluate were analyzed by liquid scintillation counting of the amount of internal radioactively labeled nucleotides.

Extrapolation of true exchange velocities and of individual kinetic parameters was carried out according to Ref. 2.

Further experiments served to differentiate between the two populations of reconstituted nucleotide carriers (right-side-out and inside-out) by alternate titration of the exchange with carboxyatractylate and/or bongkrekate, respectively. The procedure has been described in detail

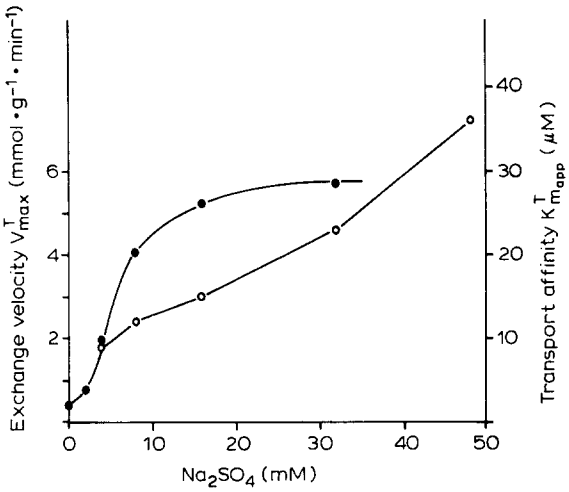

Fig. 1. Influence of anions on transport affinity K_m and exchange velocity V_{max}. The external ions of reconstituted liposomes were substituted by sucrose of the same osmolarity by gel filtration. Residual buffer was 1 mM Tricine-NaOH (pH 7.5). Na_2SO_4 was then added prior to the exchange assay under constant osmotic pressure conditions. The apparent transport affinity K^T_{mapp} (O———O) and the maximum exchange velocity V^T_{max} (●———●), both for ATP uptake (index T), were determined as described.

previously [10].

Binding of ^{3}H-labeled carboxyatractylate to the reconstituted ADP/ATP translocator was assayed by a batch type procedure with Dowex-Cl^- as adsorbant [5].

Results

In experiments with membrane-bound enzymes and carriers, variation of the ionic strength of the surrounding water phases may give rise to several possible complications. Ions in the hydrophilic environment may influence the activity of these systems either directly by binding to the protein itself or by adsorption to the membrane or indirectly by changing the actual surface potential of the phospholipid membrane in the neighbourhood of the protein [14,20]. Especially surface potential effects can be of importance in biological systems with negative surface charges. These complications have largely been avoided in the experiments reported here by use of a reconstituted system. In this system, purified egg yolk phospholipids were used, the surface charge of which was determined by fluorescent dyes [5,14,15] to be less than 0.5% negatively charged phospholipids. Thus, variation of surface potential, the most serious artefact to be considered in this type of experiment in biological systems, can be neglected.

Lowering the ionic strength in the external phase of liposomes which carry reconstitutively active adenine nucleotide carrier molecules has a twofold effect on the ADP/ATP exchange activity (Fig. 1): (a) the apparent transport affinity increases (i.e., the K_m decreases) and (b) the maximum exchange velocity decreases. Both these observations cannot be due to the small amount of residual negative surface charges, because a negative surface potential should definitely modulate the K_m of negatively charged substrates in the opposite direction and should have no effect at all on V_{max}. The changes both in K_m and in V_{max} are completely reversible by adding ions back to the reconstituted protein (experiments not shown).

In principle, both cations and anions could be responsible for these observations. It has been shown previously, for example, that the presence

TABLE I

VARIATION OF CATIONS AND ANIONS INFLUENCE ON THE RECONSTITUTED ADENINE NUCLEOTIDE EXCHANGE

External ATP (5–100 μM) was exchanged against internal ATP (15 mM). Addition of ions as described in Fig. 1. The concentration of the salts was 32 mM (determination of the apparent transport affinity K^T_{mapp} for ATP) and 16 mM (measurement of exchange velocity V^T_{max} for ATP).

Ions	K^T_{mapp} (μM)	V^T_{max} (mmol/g per min)
Variation of cations		
LiCl	12	0.96
NaCl	10	1.17
KCl	10.5	1.23
RbCl		1.14
CsCl	10	
NH_4Cl		1.07
Choline chloride	12.5	
Variation of anions		
NaF	8.5	0.58
NaCl	10	1.17
NaBr		1.82
NaJ		2.45
$NaClO_4$	31	3.20
Na_2SO_4	28	

of divalent cations strongly modulates the adenine nucleotide carrier activity [3] and it is known that monovalent cations too bind to nucleotides [21]. The influence of cations and anions can be distinguished by individual variation of the two ion components as shown in Table I. Since the various cations do not significantly modulate the reconstituted ADP/ATP exchange, the effects observed both on the K_m and on the V_{max} can be unequivocally attributed to interactions of anions with the nucleotide carrier from the outer hydrophilic space.

A possible objection to this interpretation would be the concept of a specific adsorption of these anions to the phospholipid membrane and/or the protein, which would create a local surface potential and would then possibly be responsible for the observed changes. This objection can be ruled out in the reconstituted system. In a previous publication [5], the binding of the negatively charged inhibitor carboxyatractylate to the ADP/ATP carrier was quantitatively analyzed and found to be sensitive to variation of the local surface potential. However, when measuring carboxyatractylate binding in the presence of the various ions listed in Table I, the apparent dissociation constant of carboxyatractylate, which should monitor an influence of surface potential [5], was found not to depend on the type of anion (experiments not shown). Thus the change in transport affinity K_m induced by variation of the ionic strength was, on the one hand, not due to interaction of cations with the anionic substrates or with the protein itself and proved, on the other hand, not to be dependent on adsorption of anions to the surroundings of the protein. It is therefore reasonable to assume that a direct competition of the various anions with the anionic substrates ADP and ATP at the active site is responsible for the observed changes in K_m. Usually an inhibition of this type is analyzed by variation of inhibitor concentration in the presence of fixed substrate concentrations [22]. This method cannot be applied here, since addition of anions, besides the competitive effect to be considered here, also gives rise to activation of the reconstituted adenine nucleotide exchange (Fig. 1). The determination of inhibition constants is possible, however, by a replot of the apparent affinities determined at different concentrations of the competitive inhibitor according to:

$$K_{m\,app} = K_m + (K_m/K_i)\,I$$

where I represents the concentration of inhibiting anions. Fig. 2 shows the apparent transport affinity for ATP, $K^T_{m\,app}$, in dependence on added phosphate ions (I). The intercept represents the true transport affinity K^T_m and the value for K_i can be derived from the slope, K^T_m/K_i.

This procedure has been applied for various anions and the observed K_i-values are given in Table II. The inhibition by the strong competitor GTP cannot be determined very accurately by this type of graphic extrapolation. In order to obtain K_i-values for the 'competitive' effect of ATP (lowest line in Table II), the apparent K_m values for ADP in exchange experiments with external and internal ADP in reconstituted liposomes were measured under varying additions of unlabeled ATP in the external space as previously described in Fig. 1 of Ref. 2. The K_i value for ATP was then calculated in exactly the same way as for the other anions, disregarding the fact that in this case the competing anion (ATP) is also transported. Apparently, the competitive effect of the various anions, i.e., the affinity towards the nucleotide binding site varies over a wide concentration range. The sequence of K_i-values given in Table II will be discussed later on in connection with the activating power of these anions (cf. Table V).

All these observations can be further elucidated in the reconstituted system. It has already been

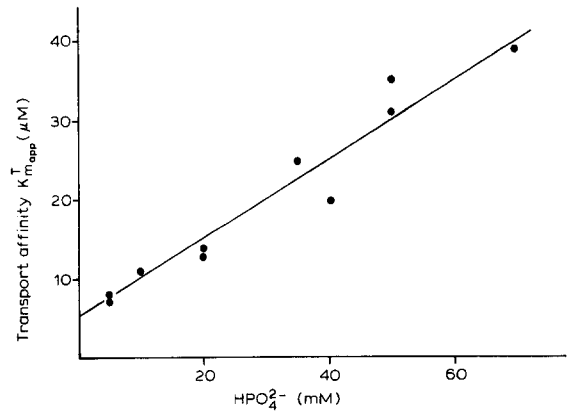

Fig. 2. Extrapolation of the inhibition constant K_i for anions and of the true transport affinity K_m for ATP uptake; the calculation is given in the text.

TABLE II

INHIBITION CONSTANTS K_i FOR SEVERAL ANIONS

Experimental conditions as described in Table I.

Anion	K_i (mM)
F^-	250
Cl^-	85
HCO_3^-	21
ClO_4^-	11
HPO_4^{2-}	14
SO_4^{2-}	12.5
Aconitate^{3-}	5.2
$P_2O_7^{4-}$	0.9
GTP^{4-}	0.25
ATP^{4-}	0.015 [a]

[a] For derivation of the 'K_i-value' of ATP, see text.

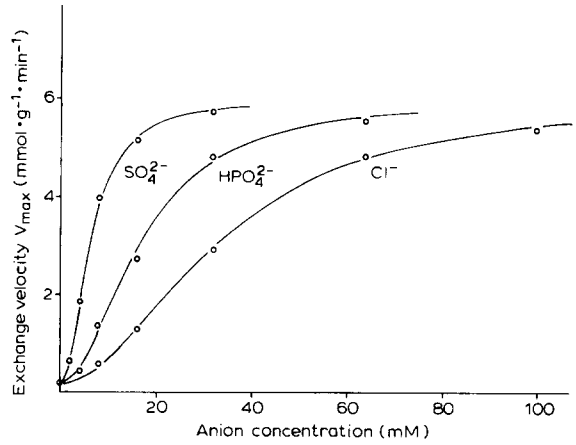

Fig. 3. Influence of anions on the exchange velocity V_{max}^T of ATP. External ATP (200 μM) was exchanged against internal ATP (15 mM) in the presence of 0.25 μM bongkrekic acid. Addition of anions as described for Fig. 1.

shown that the adenine nucleotide carrier protein is inserted into the liposomal membrane in the two different orientations and that these two carrier populations can be functionally distinguished by the use of the side-specific inhibitors carboxyatractylate and bongkrekic acid [23]. By this method, the interaction of anions can be investigated also at the internal side of the protein which is located at the outside in the case of carrier proteins oriented inside-out. Although the true K_m of nucleotides at this side is shifted to somewhat higher values [5], the observed K_i-values for various anions turned out to be very similar to the data of Table II, both in magnitude and in sequence (not shown).

Whereas the interpretation of the influence of surrounding ions on the apparent nucleotide transport affinity as a form of competitive inhibition seems to be relatively simple, the stimulating influence on the exchange activity is much more difficult to understand. Fig. 3 shows the pronounced effect of selected anions on the V_{max} of the reconstituted ADP/ATP exchange. The low residual activity observed at low ionic strength is, on the one hand, due to the small amount of buffer anions present, but can, on the other hand, also be attributed to a residual activity of inside-out oriented carrier proteins (see below) which are not fully inhibited by the added amount of bongkrekic acid. The exchange activity is dependent on the kind of anion added and the activation curves are of sigmoidal shape. Although very different amounts of the various anions are needed for stimulation, the obtained maximum exchange activity is identical for all activators. It should be mentioned here again that an influence of cations on the activation process has been ruled out (Table I).

Before we try to interpret these data further, the following basic question has to be solved: is the observed activation due simply to an increase in the number of active carrier molecules? In previous experiments the same question was solved by titration of the active carrier proteins with the inhibitor carboxyatractylate [4,10]. This method does not seem not to be sufficiently reliable in the present case, since nucleotide binding and carboxyatractylate-binding may be affected differently by activating anions. Fig. 4 shows an experiment on the basis of which the question described above can be answered. In contrast to the data of the preceding figures and tables, it is not the initial velocity that is investigated here, but the equilibration of external and internal nucleotides during long reaction times. Since the reconstitution procedure applied here leads to the incorporation of not more than one protein per liposome, the amount of labeled nucleotides in the internal volume after exchange equilibration is linearily dependent on the amount of active carrier molecules. Although the exchange activity with suboptimal concentra-

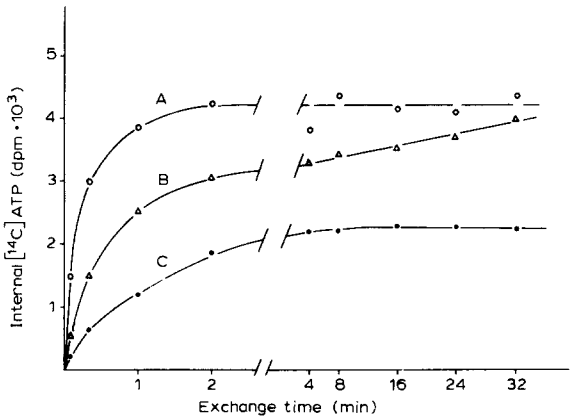

Fig. 4. Influence of anions on nucleotide exchange equilibration. Conditions as described for Figs. 1 and 3. The amount internal labeled [^{14}C]ATP was measured in the presence of 40 mM Na_2SO_4 (A), 4 mM Na_2SO_4 (B) and 4 mM Na_2SO_4 plus 50 μM carboxyatractylate (C).

tions of activating anions (4 mM SO_4^{2-}) is much lower than that under optimal conditions (40 mM SO_4^{2-}), an equivalent endpoint value is achieved after an appropriate reaction time. Thus, the total amount of functionally active carrier molecules remains constant.

The ADP/ATP exchange in the presence of carboxyatractylate reflects the activity of the inside-out oriented carrier protein [4,10].

This value (line C in Fig. 4) may be subtracted from the two other curves (A and B), thereby resulting in the true activities of the right-side-out oriented ADP/ATP carriers. However, the conclusions drawn above are not changed by this correction.

On the basis of the results described above the activation of the reconstituted adenine nucleotide transport by anions can be interpreted by the following hypothesis, comprising the different effects of anions on the exchange rates which lead to a sigmoidal dependence on the activating ions. The adenine nucleotide carrier binds a number of n anions (A), thereby changing its functional state. Without bound anions the translocator (C) has only a very low exchange activity ($V_{\rightarrow}^{0}$); with anions bound to the carrier protein (CA_n), the transport activity becomes drastically enhanced ($V_{\rightarrow}^{*}$). Partial saturation with activating anions leads to an activation of only part of the carrier molecules. This correlates with an exchange velocity ($V_{\rightarrow}$) which is inbetween the two extreme values. The derivation of the kinetic equations of this model is given in the Appendix, the formalism is similar to the treatment of cooperative binding and the modulation of enzyme activity. Accordingly, the graphic extrapolation of the stoichiometry n and the activation constant K_a resembles a Hill plot [24].

In Fig. 5 the dependence of activating anions (cf. Fig. 3) is replotted in the form of a Hill diagram. The data for the various anions, measured and calculated in the same way, are summarized in Table III. As seen in this Table and in Fig. 5, straight lines with slopes of about 2 are obtained. The concentration of anions necessary for half-activation of the reconstituted ADP/ATP exchange ($K_{0.5}$) varies over more than one order of magnitude in the millimolar range. The sequence of the $K_{0.5}$-values will be discussed later in connection with the competition effect of these anions (Table V).

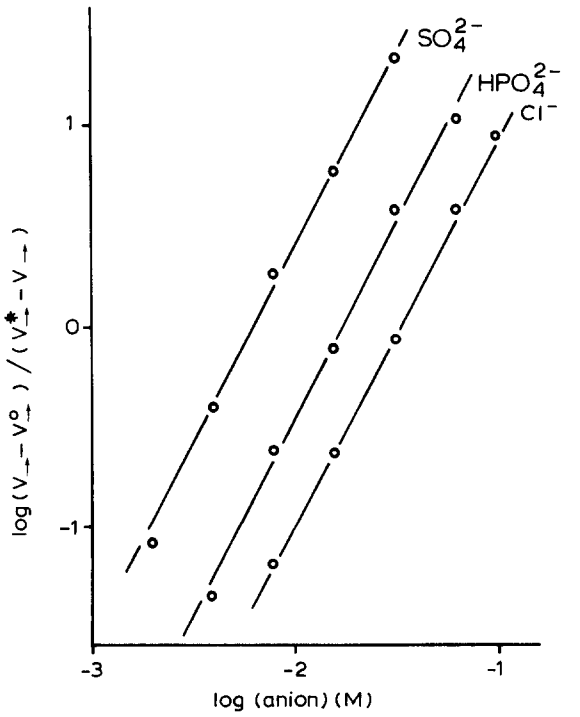

Fig. 5. Activation of the reconstituted ATP/ADP exchange by anions. The data are taken from Fig. 3, calculation as described in the Appendix.

TABLE III

ACTIVATION OF ADP/ATP EXCHANGE BY ANIONS: STOICHIOMETRY FACTOR n AND ACTIVATION CONSTANT K_a

Exchange conditions as described in Fig. 3. Derivation of n, K_a and $K_{0.5}$, see the appendix.

Anion	n	K_a (M^{-2})	$K_{0.5}$ (mM)
F^-	1.7	150	82
Cl^-		650	39
Br^-		2200	21
I^-		6000	13
ClO_4^-		23000	6.5
HCO_3^-		1050	31
SO_4^{2-}	1.9 ± 0.2	31000	5.5
HPO_4^{2-}		4400	15
$P_2O_7^{4-}$		20000	7
Aconitate^{3-}		3700	16.5
GTP^{4-}		30000	6
ATP^{4-}		60000 [a]	4 [a]

[a] Activation of exchange by ATP has to be measured with a back-exchange technique (see Methods).

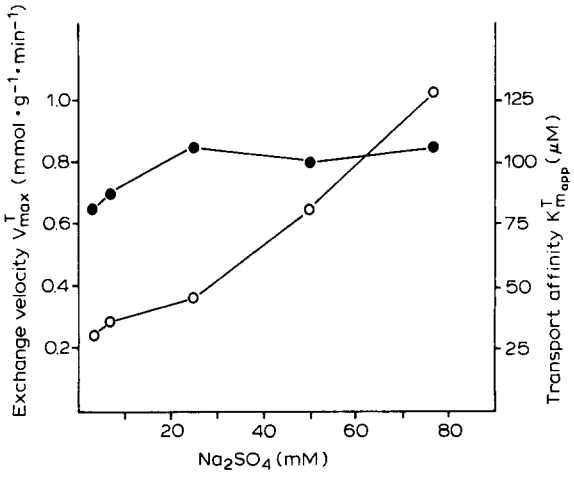

Fig. 6. Influence of anions on transport affinity K_m and exchange velocity V_{max} of ADP/ATP transport by inside-out oriented carrier protein. Conditions as described for Fig. 1; in addition 50 μM carboxyatractylate were added to the exchange assay in order to block the right-side-out oriented nucleotide carrier protein.

The activating influence of anions has been further investigated in the reconstituted system with respect to its specificity for the two alternative types of orientation of the inserted adenine nucleotide carrier. This was done in the same way as described above with respect to the competition by these anions. In contrast to the results concerning the inhibitory effects, a definite side specificity was found in the case of anion activation. Fig. 6 gives an example of the influence of SO_4^{2-} on the exchange in the case of inside-out oriented carrier proteins. When comparing the results with the data from Fig. 3, the difference becomes obvious: no significant stimulation by anions on the matrix side of the translocator can be detected. Due to the lower affinity of this binding site for nucleotides and the extreme sensitivity to negative surface charges [5], the influence of ions on the nucleotide transport by inside-out oriented carrier proteins is much more difficult to measure than in the experiments reported above with the right-side-out oriented translocator. Nevertheless, the difference in respect to interaction with anions is very clear: activation by anions is a basic requirement for carrier function at the cytosolic side of the protein, whereas it is virtually absent at the matrix side.

There is an important objection to this interpretation which should be taken into consideration. In previous publications we described the specific activation of the reconstituted adenine nucleotide carrier by negatively charged phospholipids [4,5]. It seems to be possible that negatively charged phospholipid headgroups interact with the putative anion binding sites and that the two stimulating effects, i.e., activation by anions and by negatively charged phospholipids, are in fact based on the same mechanism. In order to elucidate this situation, activation of the reconstituted adenine nucleotide exchange by anions was measured in liposomes with various lipid composition (Table IV). Experiments with negatively charged liposomes under conditions of low ionic strength are rather difficult, since negative surface potential leads to a strong increase in the apparent K_m [5]. The values obtained for $K_{0.5}$ with these types of liposomes are thus not as accurate as those in Table III. In order to compare the extent of activation measured in liposomes with different membrane compositions, an activation factor (f_a) is introduced which represents the ratio of fully activated transport ($V^*_{\rightarrow}$, see Appendix) and residual transport activity without anion activation ($V_{0\rightarrow}$), i.e., $f_a = V^*_{\rightarrow}/V^0_{\rightarrow}$. The observed increase in

TABLE IV

ACTIVATION OF RECONSTITUTED ADP/ATP EXCHANGE BY ANIONS IN NEGATIVELY CHARGED LIPOSOMES

Liposomes with different composition were prepared as described in Methods (PC = phosphatidylcholine, PE = phosphatidylethanolamine, PS = phosphatidylserine, DCP = dicetylphosphate, Chol = cholesterol). The exchange conditions were the same as described in Fig. 1, the transport was stimulated by Na_2SO_4. For derivation of the transport activation constant $K_{0.5}$ and activation factor f_a, see Appendix and text.

Membrane composition	f_a	V^T_{max} (mmol/g per min)	n	$K_{0.5\,app}$ (mM)
Egg-yolk phospholipids	12	3.2	1.92	6.5
PC	–	0.035		
PC/Chol (90:10)	–	0.27		
PC/PE/Chol (70:20:10)	9.5	2.85	2.1	4.9
PC/PE/PS/Chol (60:20:10:10)	14	2.6	1.88	18.0
Egg-yolk phospholipids/DCP/Chol (85:5:10)	11	3.8	1.96	16.0

the apparent $K_{0.5}$ values, i.e., decrease in apparent affinity, for negatively charged liposomes is easily explained on the grounds of electrostatic repulsion of the activating anions by the negative surface potential [5]. However, the data of Table IV show clearly that activation by anions can also be observed in the presence of negatively charged phospholipids. The activation is identical to that in uncharged liposomes, both with respect to the Hill coefficient n and to the activation constant $K_{0.5}$, when the electrostatic repulsion by negative surface charges is taken into account.

TABLE V

COMPARISON OF INHIBITION (K_i) AND ACTIVATION ($K_{0.5}$)OF ADP/ATP EXCHANGE BY ANIONS

The values of inhibition constants K_i and activation constants $K_{0.5}$ for the listed anions are taken from Tables II and III.

Anion	K_i (mM)	$K_{0.5}$ (mM)	$K_{0.5}/K_i$
F^-	250	82	0.27
Cl^-	85	39	0.46
HCO_3^-	21	31	1.48
HPO_4^{2-}	14	15	1.07
SO_4^{2-}	12.5	5.5	0.44
ClO_4^-	11	6.5	0.59
Aconitate^{3-}	5.2	16.5	3.2
$P_2O_7^{4-}$	0.9	7	7.8
GTP^{4-}	0.25	6	24
ATP^{4-}	0.015	4	265

Discussion

There are at least three different modes of interaction of anions with the ADP/ATP translocator inserted in the membrane: nucleotide anions as transport substrates, anions as competitive inhibitors and anions as transport activators. The two latter aspects have been investigated here in detail.

When comparing these aspects, in particular the competition on the one hand (Table II) and the activation on the other (Table III), a considerable similarity becomes apparent. Both modulating influences increase with increasing charge of the respective anion. With monovalent anions as effectors, the sequence in both cases resembles a lyotropic series and the influence increases with increasing chaotropicity [25,26]. In spite of this striking similarity, it is difficult to rationalize a simultaneous event of competitive inhibition (alternative binding of nucleotide and effector-anion) and activation (synergistic binding of nucleotide and effector-anion). However, when considering the two processes in detail, such an interpretation is not necessary or plausible at all, since important differences exist between the two modulating influences.

1. In Table V the data for K_i (competitive inhibition) and $K_{0.5}$ (cooperative activation) are compared. The ratio $K_{0.5}/K_i$ has no direct kinetic meaning, it is only used to compare the two effects. Nevertheless, if inhibition and activation were the consequences of a binding event to the same side, then the ratio $K_{0.5}/K_i$ would have to be similar for all bound effectors. Table V shows that this is not the case. Not only do the anion sequences of the two effects differ, but also – and to a large extent – the respective $K_{0.5}/K_i$ ratios. Aconitate and pyrophosphate, for instance, are strong inhibitors, but relatively weak activators ($K_{0.5}/K_i > 1$), whereas the situation is reversed in the case of chloride and sulfate. This discrepancy

becomes even more obvious when nucleotides are considered. GTP is a strong inhibitor, presumably due to its structural similarity to the substrate, but its activation efficiency is not better than, for instance, that of SO_4^{2-}. Activation by ATP can only be measured in relatively complicated back-exchange experiments (not shown). Particularly in this case the two constants differ, in fact by about three orders of magnitude.

2. The process of interaction of anions with the adenine nucleotide carrier is different depending on whether it is an inhibition or an activation process. The interaction of substrates and other anions apparently takes place on the basis of simple competition for one single binding site, i.e., the adenine nucleotide binding site of the carrier dimer [23]. In contrast, activation was shown to occur on the basis of cooperative binding of anions to at least two binding sites.

3. The sidedness or symmetry of the two effects differs. Whereas competition takes place both at the cytosolic side and at the matrix side of the carrier protein with similar specificity and affinity, activation by added anions can only be detected at the cytosolic face of the adenine nucleotide carrier.

Thus, the two types of influence of anions on the nucleotide transport in the reconstituted system can clearly be distinguished. When summarizing the different aspects mentioned above, inhibition by anions can obviously be attributed to direct competition at the nucleotide binding site, whereas activation must be caused by binding of anions to sites different from the active site. The stoichiometry of at least two activating binding sites per protein dimer can be derived from the activation kinetics (see Appendix and Fig. 4). This is further corroborated by the important fact that the stoichiometry of $n = 2$ is completely independent of the number of charges of the activating anion (Table III). An interpretation of these data to the effect that one anion binds per carrier monomer seems to be logical, but is nevertheless speculative at the present stage of investigation. This interpretation would in fact be consistent with the observed stoichiometry of two cooperative interacting binding sites per active carrier dimer.

The nature of the activating anion binding sites has not yet been clarified. The direct effect observed on the rate constants $k_{\rightarrow}$ strongly favours the interpretation that binding of anions would lower the energy barrier for the conformational change of the carrier protein which is necessary in the translocation event during its reaction cycle [23]. The increase of conformational mobility of the protein can occur by neutralization of an interfering positive charge or on the level of amino-acid–amino-acid interaction, and amino-acid–phospholipid interaction, respectively. The latter interpretation would be in agreement with the sequence of the activating effect, which parallels the chaotropicity [25,26] of the respective monovalent anions (Table III). This, however, is not valid for the divalent anions, where the 'antichaotropic' anion SO_4^{2-} is the best activator.

Correlation of these results with the physiological situation in mitochondria, in mitoplasts and also in inverted sonic particles, is beyond the scope of this paper. It should, however, be mentioned here that, in principle, these effects can also be observed when the adenine nucleotide carrier is in its physiological surroundings. This will be discussed in detail in a further publication.

Appendix

The calculations for the activation of the reconstituted adenine nucleotide transport by anions are based on a model in which the translocator changes between two functional states in dependence on bound anions (cf. Results). The carrier protein (C) binds anions (A), the equilibrium is described by K_a:

$$[C] + n[A] \rightleftharpoons [CA_n]$$

$$K_a = \frac{[CA_n]}{[C][A]^n} \tag{1}$$

Without bound anions both the exchange activity ($V^0_{\rightarrow}$) and the rate constant ($k^0_{\rightarrow}$) are low; they are high in the presence of bound activators ($V^*_{\rightarrow}$, $k^*_{\rightarrow}$). Without membrane potential and under saturating substrate concentrations for the exchange of external and internal nucleotides by the total number of carrier proteins ($[C]_0$) we obtain:

without activation	$V^0_{\rightarrow} = k^0_{\rightarrow}[C]_0$	(2)
with full activation	$V^*_{\rightarrow} = k^*_{\rightarrow}[C]_0$	(3)
with partial activation	$V_{\rightarrow} = k^0_{\rightarrow}[C] + k^*_{\rightarrow}[CA_n]$	(4)

In addition we have a valid conservation equation of:

$$[C]_0 = [C] + [CA_n] \quad (5)$$

Insertion of Eqns. 2, 3 and 5 in Eqn. 4 leads to:

$$V_{\rightarrow} = V_{\rightarrow}^0 \frac{[C]_0 - [CA_n]}{[C]_0} + V_{\rightarrow}^* \frac{[CA_n]}{[C]_0}$$

This equation is solved for $[CA_n]$:

$$[CA_n] = \frac{V_{\rightarrow} - V_{\rightarrow}^0}{V_{\rightarrow}^* - V_{\rightarrow}^0}[C]_0 \quad (6)$$

Eqn. 5 can be inserted into Eqn. 1:

$$K_a = \frac{[CA_n]}{([C]_0 - [CA_n])[A]^n}$$

When $[C][A]_n$ is replaced according to Eqn. 6, we obtain in logarithmic form:

$$\log \frac{\frac{V_{\rightarrow} - V_{\rightarrow}^0}{V_{\rightarrow}^* - V_{\rightarrow}^0}[C]_0}{[C]_0 - \frac{V_{\rightarrow} - V_{\rightarrow}^0}{V_{\rightarrow}^* - V_{\rightarrow}^0}[C]_0} = n \log[A] + \log K_a$$

The left side of this equation can be simplified by cancelling out $[C]_0$ and multiplying both numerator and denominator by $(V_{\rightarrow}^* - V_{\rightarrow}^0)$, arriving at:

$$\log \frac{V_{\rightarrow} - V_{\rightarrow}^0}{V_{\rightarrow}^* - V_{\rightarrow}} = n \log[A] + \log K_a \quad (7)$$

The values for $V_{\rightarrow}^0$ (without anions), $V_{\rightarrow}^*$ (maximum activation), and $V_{\rightarrow}$ (activity for a definite concentration of activating anions) can be kinetically determined. The logarithmic velocity ratio of Eqn. 7 is then plotted against the logarithm of the anion concentration. This evaluation formally resembles a Hill diagram [24]. Thereby the stoichiometry factor n and the equilibration or activation constant K_a can be extrapolated.

In order to compare the power of activation of these anions with their competition effect, the activation constant K_a (dimension M^{-n}!) is converted into a constant $K_{0.5}$, which equals the concentration of anions leading to half maximum activation. $K_{0.5}$ can be calculated from K_a according to $K_{0.5} = 1/K_a^{1/n}$.

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