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ANALYSIS OF PHOSPHORYL TRANSFER MECHANISM AND CATALYTIC CENTRE GEOMETRIES OF TRANSPORT ATPase BY MEANS OF SPIN-LABELLED ATP

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Summary

Spin-labelled ATP [3′-*O*-(1-oxyl-2,2,5,5-tetramethyl-3-carbonyl pyrrolidine)-adenosine 5′-triphosphate], abbreviated SL-ATP, is used to study firstly the occurrence of an associative phosphorane mechanism for the phosphoryl transfer from ATP to the transport-ATPase protein, and secondly the presence of two geometrically unequal catalytic centres in the two catalytic peptide chains deduced to explain the existence of two K'_D(ATP) values under equilibrium conditions and two K_m(ATP) values under turnover conditions.

1. In the presence of Na^+, K^+ and Mg^{2+}, SL-ATP is not hydrolysed by transport-ATPase from three different sources. In the presence of Na^+ and Mg^{2+}, SL-ATP reacts initially like ATP with the enzyme, as indicated by the production of a similar ouabain-binding protein conformation. With both nucleotides, this initial reaction includes the formation of the covalent enzyme-nucleotide complex through nucleophilic attack of the aspartate carboxyanion of the catalytic centre on the terminal phosphorus atom of the triphosphate chain. This produces the ouabain-binding conformation of the enzyme. Unlike ATP, the covalent enzyme-SL-ATP complex resists further transformation.

2. In the presence of Na^+, K^+ and Mg^{2+}, the influence of SL-ATP on ATP hydrolysis by transport-ATPase depends on the ATP concentration chosen. At low ATP concentration, when the enzyme works as Na^+-ATPase, SL-ATP does not affect the rate of ATP cleavage. At high ATP concentration, however,

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Abbreviations: transport-ATPase, comprising Na^+-transporting ATPase (Na^+-ATPase) and Na^+- plus K^+-transporting ATPase ($Na^+ + K^+$)-ATPase; SL-ATP, spin-labelled ATP.

when the enzyme works as ($Na^+ + K^+$)-ATPase, SL-ATP reduces the rate of ATP hydrolysis to the level of Na^+-ATPase activity, apparently due to the formation of the covalent enzyme-SL-ATP complex.

3. SL-ATP in the covalent enzyme-SL-ATP complex shows an ESR spectrum which is indistinguishable regarding the overall shape, the rotational correlation time, τ, and the hyperfine coupling constant, a_N, from the ESR spectrum of free SL-ATP. Consequently, the dimensions of the catalytic centre cleft of transport-ATPase provide the labelled group of SL-ATP, opposite to its 3′-O-esterification site at the ribose moiety, in a wide-cleft groove, enough free space for an essentially unhindered rotational mobility within an aqueous environment like that of the bulk medium. Judged from literature data, similarly wide grooves exist in the catalytic centre clefts of mitochondrial and myosin ATPases.

4. In the framework of present knowledge, the idea is put forward that the structural unit forming the binding site for the AMP moiety of ATP in ATPases is similar to the structural unit forming the binding site for the AMP moiety of NAD and ADP in several dehydrogenases and kinases.

Introduction

The fundamental question concerning the molecular mechanism of the interconversion of Gibbs energy of ATP and Gibbs energy of Na^+ and K^+ electrochemical gradients by transport-ATPase is but scarcely treated in literature (for a recent thermodynamic consideration see Ref. 1). The present study is primarily intended to scrutinize two elemental postulates of the flip-flop concept of transport-ATPase function concerning the phosphoryl transfer mechanism and the catalytic centre geometries [2—7]. Spin-labelled ATP (SL-ATP) was chosen as an analytical tool because of the anticipation that the bulky reporter group, due to the restriction of the stereochemical flexibility and the local enlargement of the space-filling requirements of the nucleotide, should firstly indicate conformational rearrangements of both nucleotide and enzyme protein if occurring during the catalytic reaction, and secondly should reflect differences in the geometries of the two catalytic centres of the enzyme if present. To further the understanding for the design of the experiments and the interpretation of the data, the following overview presents the pertinent postulates of the concept underlying the study, and the major results rationalized in the framework of the conceptual postulates.

Firstly, the concept postulates that an associative phosphorane mechanism of the phosphoryl transfer reactions underlies the Gibbs energy-releasing chemical reactions during ATP cleavage utilized in transport work [2,4,5]. More specifically, the phosphoryl transfer from ATP to the enzyme protein is proposed to proceed through the reactions, I → II → III → IV, as represented in Fig. 1. SL-ATP will be shown to be a novel type of a mechanism-based enzyme inhibitor (k_{cat} inhibitor, cf. Ref. 8). It arrests the catalytic action of transport-ATPase after the formation of the covalent enzyme-SL-ATP complex (analogous to I → II) because the ligands of the terminal phosphorus atom, owing to steric hindrance by the bulky reporter group at the ribose moiety of

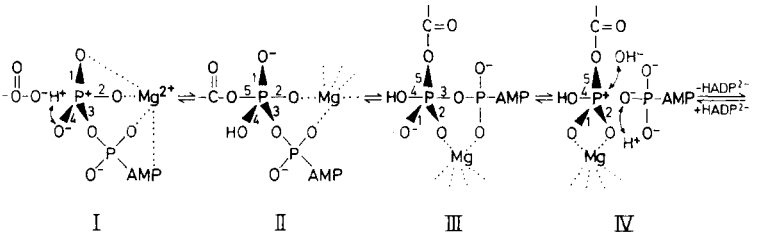


Fig. 1. Associative phosphorane mechanism of the phosphoryl transfer from ATP to enzyme protein proposed to underlie the first Gibbs energy-releasing chemical reaction in ATP cleavage by transport-ATPase. The reaction starts by the formation of the covalent enzyme-ATP complex (II) through nucleophilic attack of the carboxylate anion, provided by the aspartate residue in the catalytic centre, on the terminal phosphorus atom of the triphosphate chain (I). The phosphorane intermediate formed (II), owing to its stereochemical nonrigidity, allows the non-dissociative pair-wise exchange of equatorial and axial bonds of the ligands of the ATP terminal phosphorus atom (II → III). This pseudorotation includes the shift of the ADPO-ligand (bond 3) from the equatorial to the apical position, and the shift of the carboxyl ligand (bond 5) from the apical to the equatorial position, thus allowing the cleavage of the bond between the phosphorus atom and the bridge oxygen atom (III → IV), the removal of ADP, and the formation of the phosphorylated enzyme intermediate. Appropriately modified version of earlier published reaction schemes [2,4,5].

SL-ATP, cannot undergo the pseudorotation requiring conformational rearrangements of both enzyme protein and SL-ADPO-residue (analogous to II → III). Hence, in line with the postulate, the circumstantial evidence obtained with SL-ATP indicates the presence of both the formation of the covalent enzyme-ATP complex (I → II) and the pseudorotation of the ligands of the ATP terminal phosphorus atom (II → III), these being the prerequisites for cleavage of the bond between the terminal phosphorus atom and the bridge oxygen atom (III → IV), the removal of ADP, and the formation of the phosphorylated enzyme intermediate, i.e., for the function of transport-ATPase.

Secondly, the concept postulates that the presence of two K'_D(ATP) values under equilibrium conditions, as well as of two K_m(ATP) values under turnover conditions of transport-ATPase, which differ by almost three orders of magnitude, reflects large differences in the geometries of the two catalytic centres in the two catalytic peptide chains favouring or disfavouring binding of ATP to the first and the second centre, respectively [6,7]. In line with this postulate, SL-ATP and ATP, differing in the space-filling requirements for binding by the presence or absence of the bulky reporter group, will be shown to exhibit reverse affinity ratios to the first and second catalytic centre.

In the covalent enzyme-SL-ATP complex, the reporter group attached to the ribose moiety of SL-ATP will be shown to have unhindered rotational mobility, indicating the presence of a groove in the catalytic centre cleft opposite to the 3′-O of the ribose at which the reporter group is positioned. Since a similar groove appears to exist in the binding clefts of other ATPases as well as in kinases and dehydrogenases, it will be proposed that these enzymes are members of a family in which the binding site for the AMP moiety of ATP, ADP or NAD is formed by a similar structural unit.

Experimental Procedure

Materials

Na_2ATP and pyruvate kinase (from rabbit muscle, suspended in 2.1 M

$(NH_4)_2SO_4$, 90 units activity/mg protein) were supplied by Reanal (Budapest), lactate dehydrogenase (from rabbit muscle, 550 units activity/mg protein) as well as phospho*enol*pyruvic acid (tricyclohexylamine salt) by Boehringer (Mannheim), and Na_2NADH by VEB Arzneimittelwerk Dresden (Dresden). [21-^{3}H(n)]Ouabain (170 mCi/mmol) was obtained from the Central Institute of Isotope and Radiation Research/Isocommerz GmbH (Berlin-Buch, G.D.R.).

Synthesis of spin-labelled ATP (SL-ATP)

The procedure followed in principle the method described in Ref. 9. 3′-*O*-(1-Oxyl-2,2,5,5-tetramethyl-3-carbonyl pyrrolidine)adenosine 5′-triphosphate (1) of which the structure is represented in Fig. 2, was synthesized by the esterification of the ribose moiety of ATP with 3-carboxy-1-oxyl-2,2,5,5-tetramethyl pyrrolidine (2) prepared according to the method of Rosantsev [10]. *N,N′*-Carbonyl diimidazole (3) was used to facilitate the formation of the activated carboxylic acid (cf. Ref. 9). 4 mmol each of 2 and 3 were mixed in dry form, dissolved in 1.5 ml dimethylformamide and allowed to react at room temperature for 30 min to form the imidazolide. 1 mmol ATP dissolved in 1.5 ml water was added, and the mixture stirred at room temperature for 45 min. The precipitate formed after addition of 5 ml acetone was collected by centrifugation. The solution of the sediment in 2.0 ml water was passed through 170 g silica gel (Merck, 0.05—0.2 mm) which had been washed with 2 N HCl and water. SL-ATP was separated from unreacted ATP by elution of the column with mixtures of *n*-butanol/water/glacial acetic acid in ratios changing step-wise from 7 : 2 : 1 to 6 : 2 : 2 to 5 : 3 : 2 (v/v/v). The fractions containing SL-ATP were collected and extracted with diethyl ether to remove residual starting material. After evaporation of water from the aqueous phase, the residue was taken to dryness by lyophilization. 0.32 mmol SL-ATP (32% of theoretical yield) was obtained as a pale-yellow substance.

The ESR spectrum of the product, shown in Fig. 3 and evaluated in Results, proved the presence of the spin label. The esterification could result in the formation of either the 2′-*O* or the 3′-*O* isomer, but the accumulation of data on the course of reactions carried out under similar conditions (cf. Ref. 9) indicates that the 3′-*O* isomer is the final reaction product. Indeed, the 2′-hydroxyl group is more open to electrophilic attack than the 3′-hydroxyl group, so that the 2′-hydroxyl group is kinetically more reactive for substitution. However, such substitution is relatively less stable in comparison to the esterification at the 3′-hydroxyl group. Hence, in Fig. 2, the spin label is shown to be attached to the 3′-0 position.

The SL-ATP preparation appeared to be free from ATP in thin-layer chromatography on polyethyleneimine-impregnated cellulose F (Merck) developed with 0.4 M aqueous KH_2PO_4, pH 3.5. In this chromatographic system, ATP (R_f 0.20) and SL-ATP (R_f 0.35) are clearly separated. As judged from the sensitivity limit of the fluorescence indicator, SL-ATP contained, if any, less than 2% ATP contamination. In the near-ultraviolet region, SL-ATP in aqueous solution (0.1 mM, pH 4.5) showed an absorption maximum near 257 nm with a molar absorption coefficient of 14 900 $M^{-1} \cdot cm^{-1}$ which is very similar to that of ATP (λ_{max} = 257 nm, ϵ = 14 700 $M^{-1} \cdot cm^{-1}$ [11]). As judged from determinations of orthophosphate, the SL-ATP preparation contained 10—15

mol% P_i. With this ATP : P_i ratio, P_i does not inhibit $(Na^+ + K^+)$-ATPase activity [12] so that the resistance of SL-ATP to hydrolysis by the enzyme described in Results could not be caused by the P_i contamination of SL-ATP. After hydrolysis of SL-ATP with 60% $HClO_4$, the adenine : P_i ratio was found to be 1 : 2.86 (mean value of three independent determinations after correction for the P_i contamination), i.e., the phosphorus contents of the SL-ATP preparation amounted to 95.3% of the theoretical value. As indicated by this result and corroborated qualitatively by thin-layer chromatographic analysis, the SL-ATP preparation contained about 5% SL-ADP. This contamination is too low to interfere with the studies reported in the results section.

Spectroscopic measurements

The ESR spectra were measured using a Varian E-3 spectrometer operating at the X-band, the ultraviolet spectra were recorded using a Beckman DK-2A spectrophotometer, and the circular dichroism measurements were made on a Roussel-Jouan 185 dichrographe.

Transport-ATPase studies

To characterize the properties of SL-ATP, transport-ATPase preparations from cardiac muscle [13], brain [14] and kidney [15] of the pig were used, the specific activities of which varied between 10—20, 40—70 and 900—1200 μmol P_i/h per mg protein, respectively. The assay medium contained 2 mM imidazole-ATP, 4 mM $MgCl_2$, 80 mM NaCl, 5 mM KCl and 80 mM imidazole-HCl buffer (pH 7.4, 20°C). The incubations were performed at 37°C for 15 or 30 min. The rate of ATP hydrolysis in the presence of 0.5 mM ouabain, which gave an average of 5 and 2% of $(Na^+ + K^+)$-ATPase activity in the preparations from brain and cardiac muscle, respectively, was measured concurrently, and was subtracted from the activity in the absence of ouabain to give the $(Na^+ + K^+)$-ATPase activity. The molar concentration of transport-ATPase protein in the assay media described in the legends to Tables I—IV and Fig. 3 was calculated on the basis of the assumptions that the molecular weight of the enzyme protein is 250 000 [16,17] and the activity of the enzyme entity amounts to 2400 μmol P_i/h per mg protein (cf. Refs. 15 and 17). The release of P_i from ATP was followed by either the direct determination of P_i [18] or by indirect determination of P_i by means of an optical test [19]. The binding of ouabain to transport-ATPase was measured by the help of a filtration procedure [20]. The protein contents of the enzyme preparations were determined by using the method of Lowry et al. [21] using bovine serum albumin (Boehringer, Mannheim) as standard protein. The representative data presented in Tables I—IV and in Fig. 3 are averages of three or more independent experiments, each performed in triplicate.

Results and Discussion

1. SL-ATP — a mechanism-based inhibitor of transport-ATPase

Although SL-ATP becomes slowly hydrolysed by the ouabain-insensitive ATPase present in the brain enzyme preparation at about 5% of transport-ATPase activity (not demonstrated), SL-ATP in the presence of Na^+, K^+ and

TABLE I

LACK OF CLEAVAGE OF SL-ATP BY TRANSPORT-ATPase, BUT INHIBITORY EFFECT OF SL-ATP ON ($Na^+ + K^+$)-ATPase ACTIVITY

The activity of the enzyme preparations from porcine cardiac muscle [13] and porcine brain [14] was measured by orthophosphate determinations [18] in terms of the liberation of P_i from ATP and/or SL-ATP during incubations at 37°C for 30 min in the presence of 80 mM imidazole-HCl buffer (pH 7.4, 20°C), 80 mM NaCl, 5 mM KCl, 4 mM $MgCl_2$, and 0.7 nM transport-ATPase protein, all contained in a volume of 3.0 ml. The reaction was started by the addition of the enzyme

ATP (mM)	SL-ATP (mM)	Hydrolysis rate			
		Muscle preparation		Brain preparation	
		μmol P_i/h per mg protein	%	μmol P_i/h per mg protein	%
1.0	—	10.5	100	53.6	100
—	2.0	0	0	0	0
1.0	2.0	3.66	35	21.6	40

Mg^{2+}, does not undergo hydrolysis even at prolonged incubation periods with the enzyme preparations from cardiac muscle and from brain (Table I) as well as from kidney (not shown).

If not hydrolysed, nucleoside 5′-triphosphates can be examined as to their reactivity with transport-ATPase by probing their capability to initiate an enzyme conformation attracting the negative effector, ouabain. Nucleotides behave like ATP when their reaction with the enzyme produces an ouabain binding protein conformation. For instance, both β,γ-methylene-ATP and ATP-dialdehyde are not hydrolysed; although both show high affinity to the enzyme, only the latter nucleotide initiates the change of enzyme conformation exhibiting high ouabain affinity [20].

In the presence of Na^+ and Mg^{2+}, SL-ATP like ATP reacts with the enzyme and promotes ouabain binding to the enzyme *. However, as judged by the concentration required to produce maximum ouabain binding, the effectivity of SL-ATP is about 10 times lower than that of ATP (Tables II and III). From the whole set of similar data, the $K_{0.5}$(SL-ATP) value was computed to average 0.017 mM. Since the SL-ATP preparation could eventually be contaminated by less than 2% with ATP, it was important to establish, as demonstrated in Table III, that the preparation does not lose any effectivity in promoting ouabain binding to the enzyme after exhaustion of all ATP present, i.e., it was SL-ATP which through reaction with the enzyme produced the ouabain binding conformation. In terms of the associative phosphorane mechanism of the phosphoryl transfer reactions catalysed by transport-ATPase (cf. Fig. 1 and Refs. 2, 4 and 5), SL-ATP may be assumed to form, like ATP initially, the covalent enzyme-nucleotide complex through nucleophilic attack of the

* The high NaCl concentration applied is known to annul orthophosphate plus Mg^{2+}-supported ouabain binding (see for instance, Table 4 in Ref. 34) so that the P_i contamination of SL-ATP cannot be involved in SL-ATP, Mg^{2+} plus Na^+-supported ouabain binding.

TABLE II

COMPARISON OF ATP AND SL-ATP AS TO THEIR EFFECTIVITY IN PROMOTING OUABAIN BINDING TO TRANSPORT-ATPase

Ouabain binding to the enzyme from porcine cardiac muscle [13] was measured during incubations at 37°C in the presence of 30 mM imidazole-HCl buffer (pH 7.2, 22°C), 5 μM Tris-EDTA, 80 mM NaCl, 1 mM $MgCl_2$, 0.9 nM transport-ATPase protein, and 0.2 μM [^{3}H]ouabain (175 nCi ^{3}H/nmol ouabain), all contained in a total volume of 5.0 ml. The reaction was started by addition of [^{3}H]ouabain

Promotion by		pmol ouabain bound per unit enzyme activity * within various time periods (min)				
ATP (μM)	SL-ATP (μM)	2	4	6	20	40
5	—	2.1	3.1	3.7	4.0	3.6
—	5	0.5	0.5	0.5	0.7	0.8
10	—	2.2	3.0	3.7	3.7	3.5
—	10	0.8	0.8	1.1	1.1	1.1
100	—	2.2	3.1	3.9	3.8	3.8
—	100	2.1	3.0	3.2	4.0	3.7

* One unit of activity is defined as that amount of enzyme which under the assay conditions liberates 1 μmol of phosphate from ATP within 1 h at 37°C.

carboxylate oxyanion, provided by the aspartate residue in the catalytic centre, on the terminal phosphorus atom of the triphosphate chain. With both nucleotides, this reaction appears to initiate the ouabain binding conformation of the enzyme. In the covalent enzyme-SL-ATP complex, however, the ligands of the terminal pentacoordinated phosphorus atom cannot undergo the concerted pair-wise exchange of their equatorial and apical bonds (pseudorotation) because the bulky reporter group in the SL-ADPO ligand sterically hinders the conformational rearrangements of both the nucleotide and the enzyme protein pertinent to the pseudorotation. More specifically, the absence

TABLE III

PERSISTENCE OF THE PROMOTING EFFECT OF SL-ATP ON OUABAIN BINDING TO TRANSPORT-ATPase AFTER ITS PREINCUBATION WITH THE ENZYME TO REMOVE EVENTUAL TRACES OF ATP CONTAMINATION IN THE SL-ATP PREPARATION

The preincubation with enzyme from porcine cardiac muscle [13] was carried out at 37°C for 20 min in the presence of 30 mM imidazole-HCl buffer (pH 7.2, 22°C), 0.12 mM Tris-EDTA, 80 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0.1 mM imidazole-ATP or 1 mM SL-ATP, and 11 nM transport-ATPase protein, all contained in a volume of 5.0 ml. K^+ which was found to prevent ouabain binding to the enzyme due to hindering of formation of enzyme-SL-ATP complex was removed from the preincubation medium by its passage through a column of CM-Sephadex C-25 (particle size 40—120 μm; Pharmacia, Uppsala) in the imidazole form using water as eluant. The eluates were appropriately diluted to give the assay conditions for ouabain binding as specified in Table II except that the concentration of SL-ATP was 50 μM and that of ATP would have been 5 μM if no breakdown had occurred

Nucleotide	Preincubation with enzyme	pmol ouabain bound per unit enzyme activity within various time periods (min)	
		10	20
ATP	no	3.3	3.5
SL-ATP	no	2.9	3.3
ATP	yes	0	0
SL-ATP	yes	3.1	3.3

of the shift of the ADPO ligand from the equatorial to the apical position and the absence of the shift of the carboxyl ligand from the apical to the equatorial position (cf. Fig. 1) render the cleavage of the bond between the terminal phosphorus atom and the bridge oxygen atom followed by the removal of SL-ADP impossible, as well as the formation of the phosphorylated enzyme intermediate, thus accounting for the resistance of SL-ATP to hydrolysis by transport-ATPase. This implication is underlined by the following consideration. If the above-outlined ligand permutation could occur, the denoted bond could be cleaved and SL-ADP released so that the same phosphorylated enzyme intermediate, as formed with ATP, would be formed, i.e., SL-ATP should also become hydrolysed. The alternative interpretation that the reporter group directly produces the hydrolytic resistance of the bond, e.g., by changing the electron distribution in the triphosphate chain, appears to be unlikely because the reporter group is distantly located from the scissile bond.

SL-ATP works like a mechanism-based enzyme inhibitor ('suicide reagent', cf. Ref. 8), since SL-ATP, itself as chemically unreactive as ATP, forms the same covalent enzyme-nucleotide complex with transport-ATPase as does ATP. In the case of SL-ATP, however, the complex resists further transformation.

2. Detection of differences in the geometries of the two catalytic centres

Transport-ATPase can work in two major operational modes determined by the presence of low or high concentrations of ATP in the incubation medium also containing Na^+, K^+ and Mg^{2+} [3,22]. At low ATP concentration, sufficient to saturate only the high-affinity catalytic centre ('first centre'), the enzyme operates as Na^+-ATPase involving only one of the two catalytic chains (monomeric operational mode). At high ATP concentration, also saturating the low-affinity catalytic centre ('second centre'), the enzyme works as ($Na^+ + K^+$)-ATPase involving both catalytic chains (dimeric operational mode). In order to account for the occurrence of two K_m(ATP) values in the turnover systems and two K'_D(ATP) values in the equilibrium system both differing by almost three orders of magnitude, it was proposed that the two catalytic chains of transport-ATPase carry two catalytic centres of highly different geometries favouring or disfavouring ATP binding [3,6,7]. Since SL-ATP and ATP differ only in the space-filling requirements for binding to the catalytic centres, eventual different influences of SL-ATP on Na^+- and ($Na^+ + K^+$)-ATPase activity should reflect differences in the geometries of the first and second catalytic centre.

At low concentrations of ATP moderately surpassing the K_m(ATP) value of Na^+-ATPase (0.001 mM [23]), SL-ATP, even at SL-ATP : ATP concentration ratios as high as 40 : 1, does not inhibit ATP hydrolysis (Table IV). The absence of a competitive inhibitory action of SL-ATP on Na^+-ATPase activity indicates that SL-ATP, due to steric hindrance by the bulky spin-label group, does not fit into the first catalytic centre. Ligation of the enzyme with K^+ appears to be involved in shaping that dimension of the catalytic centre cleft that prevents SL-ATP from binding, since, in the absence of K^+ ligation, SL-ATP binds without difficulty to the first catalytic centre as shown by the SL-ATP-effected promotion of ouabain binding to the enzyme after K^+ removal

TABLE IV

LACK OF INHIBITORY EFFECT OF SL-ATP ON Na^+-ATPase ACTIVITY OCCURRING AT LOW ATP CONCENTRATIONS, BUT PRESENCE OF INHIBITORY EFFECT OF SL-ATP ON ($Na^+ + K^+$)-ATPase ACTIVITY OCCURRING AT HIGHER ATP CONCENTRATIONS

The activity of the enzyme preparation from porcine cardiac muscle [13] was measured by the optical method [19] in terms of the liberation of P_i from ATP during incubations at 37°C for 15 min in the presence of 25 mM imidazole-HCl buffer (pH 7.65, 20°C), 130 mM NaCl, 5 mM KCl, 4 mM $MgCl_2$, 0.4 mM phospho*enol*pyruvate, 0.14 mM NADH, 3.8 units of pyruvate kinase, 3.4 units of lactate dehydrogenase, and 0.4 nM transport-ATPase protein, all contained in a volume of 0.5 ml. The reaction was started by the addition of the enzyme

Presence of SL-ATP (mM)	Hydrolysis rate (μmol P_i/h per mg protein) of ATP (mM)							
	0.03	0.04	0.05	0.10	0.30	0.50	1.00	2.00
0	1.2	1.1	1.5	3.2	5.4	5.9	5.3	5.3
0.1	0.8	0.9	1.3	2.7	—	3.9	3.8	2.1
0.4	0.8	1.0	1.5	2.5	4.4	2.4	3.0	2.0
0.8	1.2	1.0	1.4	2.0	1.5	2.5	2.2	1.4
1.2	1.2	1.1	1.3	—	—	1.1	1.7	1.0
1.6	—	—	1.4	1.3	—	1.0	1.6	0.9

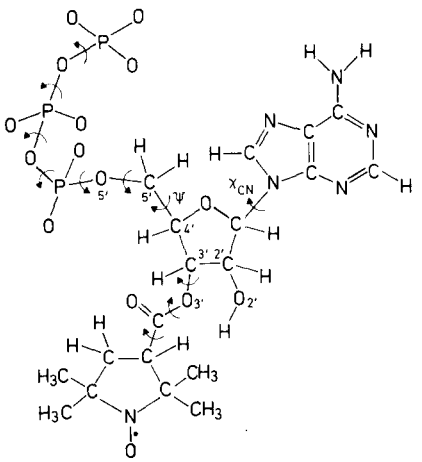
from the incubation medium (cf. Table III). Apparently, K^+ ligation occludes the groove of the catalytic centre cleft in which the reporter group of SL-ATP otherwise becomes buried.

At high ATP concentrations approaching or surpassing the K_m(ATP) value of ($Na^+ + K^+$)-ATPase (0.5 mM [23]), SL-ATP even at SL-ATP : ATP concentration ratios as low as 1 : 20 does inhibit ATP hydrolysis (Table IV), apparently due to the formation of the covalent enzyme-SL-ATP complex. Even at the highest concentration, SL-ATP cuts down ($Na^+ + K^+$)-ATPase activity to the level of Na^+-ATPase activity only, indicating the monomeric character of this operational mode (cf. Ref. 24).

Taken together, the results show that the affinity of the first catalytic centre to SL-ATP is much lower than to ATP, and conversely that the affinity of the second catalytic centre to SL-ATP is much higher than to ATP. This variation of the affinity ratios due to change of the space-filling requirements of the two nucleotides reflects significant differences in the geometries of the first and second catalytic centres.

3. Probable likeness of the solution conformations of SL-ATP and ATP

The CD spectra of SL-ATP and ATP taken in aqueous 0.1 mM solution (pH 4.5) are very similar showing negative Cotton effects at the positions of their ultraviolet absorption bands (not demonstrated). This finding favours the supposition that SL-ATP, like ATP, exists in aqueous solution in a conformation in which the base shows an *anti* conformation with respect to the ribose (cf. Ref. 25). Moreover, in the ATP solution conformation [26], the phosphate chain is folded back towards the adenine base with torsional angles approximating to a right-handed helix. The described overall conformation of ATP appears to be maintained in SL-ATP as visualized in Fig. 2, since SL-ATP reacts with the first catalytic centre, like ATP, to produce an ouabain-binding conformation of the enzyme, and since SL-ATP also reacts with



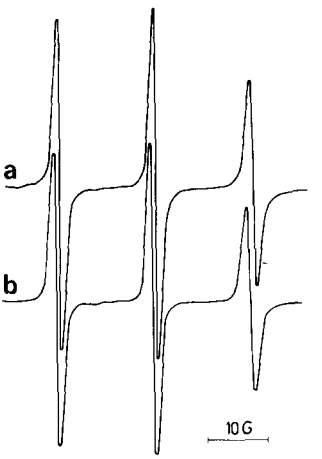


Fig. 2. Structural and conformational formula of 3′-*O*-(1-oxyl-2,2,5,5-tetramethyl-3-carbonyl pyrrolidine)adenosine 5′-triphosphate (1) (abbreviated spin-labelled ATP or SL-ATP). As visualized by the figure, the conformation of SL-ATP is assumed to be essentially similar to that of the solution conformation of ATP [26], i.e., the phosphate chain is folded back towards the adenine base with torsional angles approximating to a right-handed helix and the base shows an *anti* conformation with respect to the ribose.

Fig. 3. Similarity of the ESR spectra of SL-ATP measured in the absence (a) of transport-ATPase (hyperfine coupling constant $a_N = 16.1 \pm 0.2$ G, rotational correlation time $\tau = 10 \cdot 10^{-11}$ s) or in the presence (b) of enzyme ($a_N = 16.1 \pm 0.2$ G, $\tau = 13 \cdot 10^{-11}$ s). 0.5 mM SL-ATP was incubated at 37°C for 5 min in a medium (total volume 0.25 ml) containing 30 mM imidazole-HCl buffer (pH 7.4, 20°C), 80 mM NaCl, 3 mM $MgCl_2$, and in the case of b, additionally 10 μM transport-ATPase protein (highly purified preparation from porcine kidney [15]). The spectra were recorded at room temperature using a modulation amplitude of 1.0 G with a time constant of 0.05 s, and a scan rate of 100 G/2 min. Incubation at 0°C and recording at 7.5°C gave similar spectra.

the second catalytic centre preventing ATP from binding to it and thus blocking the dimeric ($Na^+ + K^+$)-ATPase activity (cf. sections 1 and 2).

As judged from the behaviour of the Dreiding stereo model of SL-ATP put in the solution conformation of ATP, and from the similarity of the ESR spectra of free spin label and of spin-labelled ATP (see section 4), the rotational mobility of the reporter group attached at 3′-O of the ribose moiety of ATP is scarcely restricted. The reporter group could alter the conformational flexibility of the ribofuranose itself (i.e., the poise of the equilibrium between the C-3′ *endo* and the C-2′ *endo* conformation), and of the exocyclic bonds between C-5′ and O-5′ as well as between C-4′ and C-5′ (cf. Ref. 27), possibly, therefore, influencing the rotational freedom of the adenine ring and of the triphosphate chain about the torsion angles X_{CN} and ψ, respectively (see Fig. 2). However, the peculiar biochemical behaviour of SL-ATP as compared to ATP, i.e., the relatively low affinity to the first catalytic centre, the relatively high affinity to the second catalytic centre, and the suppression of the pseudorotation of the ligands of the SL-ATP terminal phosphorus atom, are best rationalized in terms of steric hindrance by the bulky reporter group.

4. Probe of dimension of the catalytic centre cleft by using SL-ATP

Recorded in pure aqueous solution, the ESR spectra of the nitroxide

reporter group in the free spin label and in the spin-labelled ATP (not shown) are similar and consist of three sharp lines resulting from the hyperfine interaction with the ^{15}N nucleus. The rotational correlation time, τ, is $3 \cdot 10^{-11}$ s with the free spin label, and $10 \cdot 10^{-11}$ s with the spin-labelled ATP. This difference between the two τ values may be due to reduced rotational mobility of spin-labelled ATP compared to free spin label. Thus, the internal rotational flexibility of the reporter group about the bonds connecting it with the ribose moiety of ATP does not appear to be greatly restricted.

If a spin label is rigidly bound to a protein, such that τ is $1 \cdot 10^{-8}$ s or longer, the anisotropic electron-nuclear interactions broaden and displace the sharp lines to give a spectrum characteristic of an immobilized radical. However, SL-ATP, incubated with transport-ATPase under conditions which form the covalent enzyme-SL-ATP complex, shows an ESR spectrum which is indistinguishable as to the overall shape, the rotational correlation time, and the hyperfine coupling constant from the ESR spectrum of SL-ATP incubated under otherwise similar conditions in the absence of the enzyme (Fig. 3). Calculated on the basis of the $K_{0.5}$ SL-ATP value (cf. section 1), the concentration of the enzyme-SL-ATP complex was about 9 μM (i.e., 2% of added SL-ATP was bound). This concentration is high enough to detect distortions of the ESR spectrum if they occur due to restriction of the rotational freedom of the reporter group in SL-ATP by tight interaction with the peptide chain segments lining the catalytic centre cleft.

To produce the required complex concentration, an unusually high concentration of the enzyme had to be used, resulting in a rather high viscosity of the incubation medium. The possibility that the viscosity could have prevented SL-ATP from forming a covalent complex with the enzyme can be excluded since the tumbling frequencies were not reduced to the level of a strongly immobilized nitroxide radical as found in glycerol/water mixtures of high viscosity (cf. Ref. 25).

Thus, the finding that the rotational correlation time of SL-ATP is not reduced in its covalent complex with the enzyme indicates that the catalytic centre cleft provides, in a wide groove, enough free space for essentially unhindered mobility of the reporter group in SL-ATP opposite to 3′-O of the ribose moiety. Moreover, the hyperfine coupling constant, indicating the polarity of the reporter group environment, was also not changed by the formation of the enzyme-SL-ATP complex. Hence, that groove of the catalytic centre cleft in which the reporter group of SL-ATP is free to rotate is filled with water of properties like that of the bulk medium.

Similarly wide grooves appear to occur in the catalytic centre clefts of mitochondrial and myosin ATPases. This idea is suggested by the findings that derivatives of ATP or ADP carrying at 3′-O of the ribose moiety bulky substituents such as the 3′-*O*-{3-[*N*-(4-azido-2-nitrophenyl)amino]propionyl}, or the analogous butyryl residue, show rather high affinities to the ATPases and partially also substrate-like properties [9,28—30]. As will be discussed under section 5, the structural unit forming the binding site for the AMP moiety of ATP, ADP or NAD appears to be similar in ATPases, kinases and dehydrogenases, respectively. If so, it is most interesting to note that the three-dimensional model of glyceraldehyde-3-phosphate dehydrogenase shows

opposite to 3′-O of the adenosine 5′-monophosphate moiety of NAD a groove which appears to provide much free space (cf. Ref. 31).

5. Possible structure of the binding site for the AMP moiety of ATP in the catalytic centre cleft

Transport-ATPase seems to be member of a family of enzymes with a similar structure of the AMP binding site. As a matter of fact, the same basic structural unit for binding of the AMP moiety was shown to be present in the NAD binding domain of dehydrogenases, was made probable to be incorporated in the ADP binding domain of kinases, and was expected to occur in the ATP binding domain of energy transfer systems [31,32]. In dehydrogenases, the main structural elements of the binding site for the AMP moiety of NAD are three strands of parallel β-pleated sheets and two α-helices arranged alternately in the sequence, βA, αB, βB, αC, βC [31]. The same structural unit may form the binding site for the AMP moiety of ATP and ADP in the catalytic centre cleft of transport-ATPase the structure of which is composed of 13% β-pleated sheets and 35% α-helices (Malur, J. and Grosse, R., unpublished data). If so, the electric field originating from the axial dipole moment vectors of the α-helices (cf. Ref. 33) may assist in attracting and binding of ATP by its positively charged adenine moiety to the negatively charged carboxyl termini of α-helices, thus accelerating the formation of the enzyme-ATP complex.

Addendum

The more general significance of the conclusions reached in the present paper is shown by the fact that analogous conclusions were recently reached by Schäfer and Onur in studies on 3′ esters of ADP as energy-transfer inhibitors and probes of the catalytic site of oxidative phosphorylation (Schäfer, G. and Onur, G. (1979) Eur. J. Biochem. 97, 415—424). The analogies follow from the following verbal quotation. "The results are interpreted in terms of a model suggesting that, in the process of ATP synthesis, a hydrophobic cavity on the enzyme is exposed only in the energized state, accepting the large 3′ substituent. The substituent is assumed to inhibit phosphoryl transfer and/or conformational transitions inherent in the process of ADP phosphorylation by steric hindrance. In accordance with this, the corresponding ATP derivatives were found unable to drive energy-linked processes and are not hydrolysed by F_1 ATPase."

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