Comparative Studies on the ATP-Binding Sites in Ca^{2+}-ATPase and $(Na^+ + K^+)$-ATPase by the Use of ATP-Analogues *

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ATP-Analogues, Ca^{2+}-ATPase, $(Na^+ + K^+)$-ATPase, ATP-Binding Site

The effects of ATP-analogues on Ca^{2+}-ATPase and $(Na^+ + K^+)$-ATPase have been studied. The participation of sulfhydryl groups in the recognition of ATP by both transport ATPases is indicated by the fact, that the disulfide of thioinosine triphosphate inactivates both enzymes. The reactivity of rapidly and slowly reacting sulfhydryl groups in the ATP binding sites of both enzymes is altered by the presence of transport substrates. At least in $(Na^+ + K^+)$-ATPase Na^+ and Mg^{2+} appear to alter the structure of the ATP binding site, which conclusion is fortified by the fact, that the photoinactivation of the enzyme by 3′-O-[3-(2-nitro-4-azidophenyl)-propionyl]-ATP needs Mg^{2+}. Chromium(III)ATP, a MgATP analogue, inactivated both transport ATPases by the formation of a stable chromo-phosphointermediate. In the case of Ca^{2+}-ATPase this was concomited by the occlusion of Ca^{2+} in a stable form. No occlusion of Na^+ was observable so far in the $(Na^+ + K^+)$-ATPase. Contrary to the expectation of the Albers-Post-scheme the hydrolysis of the phosphointermediate formed from chromium(III)ATP was protected by K^+, but activated by high concentrations of Na^+. Consequently, despite of the inhibition of $(Na^+ + K^+)$-ATPase activity, chromium(III)-ATP supported the $Na^+ - Na^+$-exchange reaction in everted red bood cells.

Introduction

ATP hydrolysis by all transport ATPases known so far proceeds via the formation of an acylphosphoenzyme, whose synthesis and hydrolysis is linked to cation transport processes through cellular membranes (Table 1). The apparent similiarities in the ATP hydrolytic mechanisms of these cation pumps may lead to the hypothesis, that the motor driving these pumps is similar in all transport ATPases. If this assumption is right, one has to postulate that the differing cation specificities of the various transport ATPases are caused by the existance of a specific ionophoric part in each pump. In fact, tryptic fragments with ionophoric properties have been isolated from $(Na^+ + K^+)$-ATPase and Ca^{2+}-ATPase of sarcoplasmic reticulum [19]. But no data on the molecular structure of these peptides are available. Although a similar structure of the phosphate acceptor peptide has been reported to exist [20 – 22], it has not been studied so far whether the ATP recognition site is similar in these both enzymes. Information to that question should easily be obtained from a comparative study on the properties of ATP-analogues in both enzymes. In addition such a study might give further inside into the transport mechanism, because Grisham showed for the $(Na^+ + K^+)$-ATPase, that the transport substrates Na^+ and K^+ can be located in the neighbourhood of ATP within the ATP binding site [23] or at the phosphointermediate [24]. Consequently Grisham and Mildvan suggest, that the Na^+-transport out of the cell starts in the ATP binding site and that the K^+ transport ends there [24]. For the Ca^{2+}-ATPase of sarcoplasmic reticulum no such studies have been reported so far.

To get more information on the relatedness of the ATP binding sites in $(Na^+ + K^+)$-ATPase and Ca^{2+}-ATPase of sarcoplasmic reticulum, protein-reactive and unreactive ATP analogues have been synthesized and their action on both enzymes was compared [25 – 35]. As will be shown in this article, ATP analogues can be helpful to

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Abbreviations: CrATP, chromium(III)complex of ATP; $(SnoPPP)_2$, disulfide of thioinosine 5′-triphosphate; $(SnoPP[NH]P)_2$, disulfide of thioinosine-[β,γ-imido] triphosphate; $(SnoPP[CH_2]P)_2$, disulfide of thioinosine [β,γ-methylene] triphosphate; N_3-ATP, 3′-O-[3-(2-nitro-4-azidophenyl-)propionyl] adenosine triphosphate; EGTA, [ethylenebis(oxoethylenenitrilo)] tetraacetic acid.

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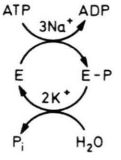
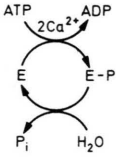
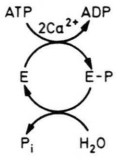
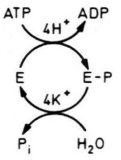
Table I. Synopsis of molecular and catalytic properties of transport ATPases.

	$(Na^+ + K^+)$-ATPase [1–5]	Ca^{2+}-ATPase of sarcoplasmic reticulum [1, 5–12]	Ca^{2+}-ATPase of plasma membranes [13–18]	$(H^+ + K^+)$-ATPase [1]
Catalytic protein α (M_r)	100 000	100 000	130 000	100 000
suggested molecular structure	$\alpha\beta$, $(\alpha\beta)_2$, $(\alpha\beta)_4$	α, α_2, α_4		
Phosphointermediate	Aspartylphosphate	Aspartylphosphate	Acylphosphate	Acylphosphate
High affinity ATP-Binding Site	$K_D = 0.12-0.22$ μM	$K_D = 2-3$ μM		
Minimal reaction sequence				
Amino Acids participating in ATP-recognition and catalysis	Cys, Arg Tyr, Asp	Cys, Arg, Lys Asp	Cys	Arg
Ions transported per ATP hydrolyzed	3 Na^+ out 2 K^+ in	2 Ca^{2+} out	2 Ca^{2+} out	4 H^+ out 3.5 K^+ in
Specific Inhibitor	Cardiac Glycosides	–	–	–
Specific Activator	–	–	Calmodulin	–

a) determine the nature of the amino acids participating in the recognition of ATP [25–28, 32];
b) quantitate the number of ATP-binding sites per pump [32];
c) characterize the affinity of the transported ions for the ATP binding sites [34];
d) monitor structural changes at the ATP binding sites [33, 34];
e) decelerate the turnover of transport ATPase [29–31, 35].

Trials to slow down the turnover of the Na^+-pump and the Ca^{2+}-pump of sarcoplasmic reticulum by the use of MgATP complex analogues

The processes occuring during the active cation transport of Na^+, K^+ and Ca^{2+} through cellular membranes by $(Na^+ + K^+)$-ATPase and Ca^{2+}-ATPase could probably be studied in a much easier and more detailed way, if it would be possible to decelerate the turnover of both transport ATPases. In studying the assumed inert Mg^{2+} substitution complexes of ATP, namely Chromium(III)ATP (CrATP) and Cobalt(III)ATP (CoATP) were became aware that these MgATP complex analogues inactivate $(Na^+ + K^+)$-ATPase slowly [29–31, 36]. Consequently the mechanism of inactivation was studied and it was tested furthermore, if Ca^{2+}-ATPase of sarcoplasmic reticulum behaved similarly [35].

Studies with $(Na^+ + K^+)$-ATPase

Inactivation of $(Na^+ + K^+)$-ATPase by CrATP is enhanced by low concentrations of sodium [31, 36] but inhibited by potassium [29] (Fig. 1). This finding suggests that CrATP binds to the high affinity ATP binding site. In agreement with this assumption ATP protects the enzyme against the inactivation [29]. From the kinetics of the inactivation a dissociation constant of 43 μM can be evaluated at 37 °C for the α, β, γ tridentate of CrATP, whereas a dissociation constant of 8 μM is found for the β, γ bidentate [29–31]. This finding suggests that Mg is bound in the MgATP-substrate between the β and γ phosphorus and that $(Na^+ + K^+)$-ATPase needs a straight triphosphate chain in the ATP binding site and not a curved one (which is formed by the α, β, γ tridentate of CrATP). The inactivation of $(Na^+ + K^+)$-ATPase is at least in part caused by the

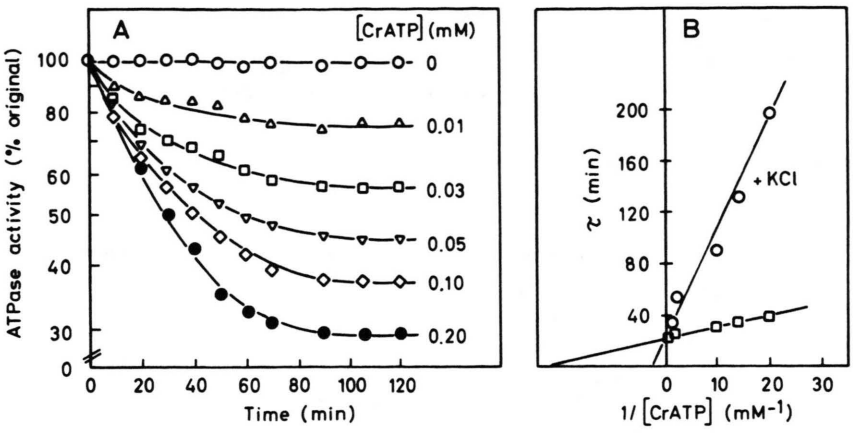


Fig. 1. Study of the protective effect of K^+ against the inactivation of $(Na^+ + K^+)$-ATPase by CrATP (from ref. [29]). A) Time course of inactivation at different CrATP concentrations. B) Demonstration of an apparent competitive effect of K^+ on the binding of CrATP.

formation of a stable phosphointermediate. This conclusion can be drawn from the fact that radioactivity is incorporated into the catalytic protein, when [γ-^{32}P]CrATP and [^{51}Cr]ATP are used for the inactivation, whilst [α-^{32}P]ADP is released from the enzyme using [α-^{32}P]CrATP as substrate (Fig. 2) [29]. As may be suggested from the intermediary binding of [α-^{32}P]CrATP to the enzyme leading to an inactivation (Fig. 2), also the chromium complex of the non-hydrolyzable adenosine 5'-[β, γ-imido] triphosphate inactivates the enzyme. Such a finding leads to the conclusion of a tight binding of the MgATP analogues at the ATP binding site of $(Na^+ + K^+)$-ATPase [29, 36].

According to the work of Albers and Post ATP is used in $(Na^+ + K^+)$-ATPase to phosphorylate the catalytic protein depending on Na^+. This phosphointermediate is cleaved K^+-dependent [1–4]. According to this reaction sequence K^+ ions should enhance the hydrolysis of the phosphoenzyme formed from CrATP and reactivate thereby the inactive enzyme. However, contrary to the expectations, this was not the case, but Na^+ ions in high concentrations enhanced the reactivation, which was inhibited by K^+ (Fig. 3). The data obtained from these kinetic studies [29–31, 36, 37] are summarized in Fig. 4. The peculiar finding of a Na^+-activated dephosphorylation and reactivation of the by CrATP inactivated and phosphorylated $(Na^+ + K^+)$-ATPase, which is inhibited by K^+, led us to look for the properties of sidereactions of the Na^+-pump in red blood cells: In addition to a $Na^+ - K^+$ exchange, uncoupled Na^+ efflux, $Na^+ - Na^+$ exchange and $K^+ - K^+$ exchange can be measured [2] (Fig. 4). The by ouabain inhibitable $Na^+ - Na^+$ exchange is inhibited by low concentrations of K^+, too. Therefore, it was of interest to look for the possibility that CrATP may support the

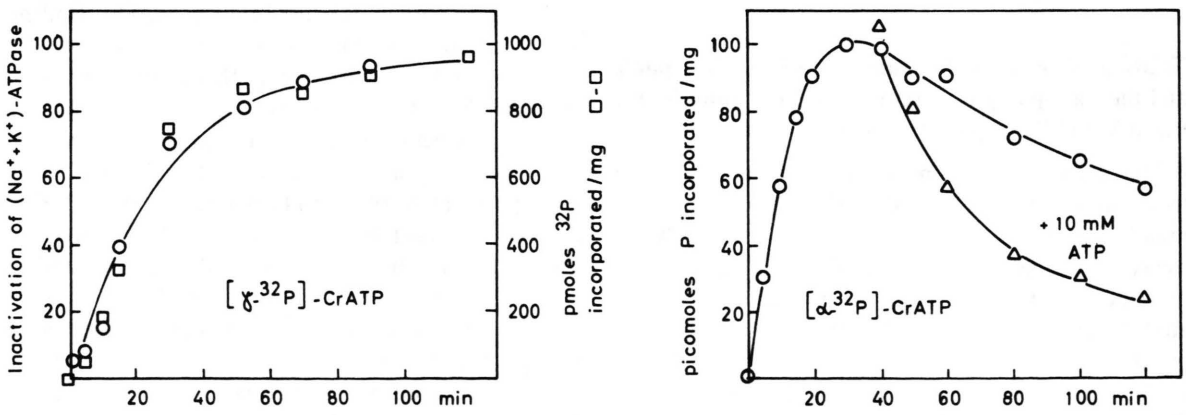


Fig. 2. Comparison of the incorporation of radioactivity from [α-^{32}P] and [γ-^{32}P]CrATP into the protein of $(Na^+ + K^+)$-ATPase and demonstration of the inactivating effect. 0.1 mM CrATP was used (from ref. [29]).

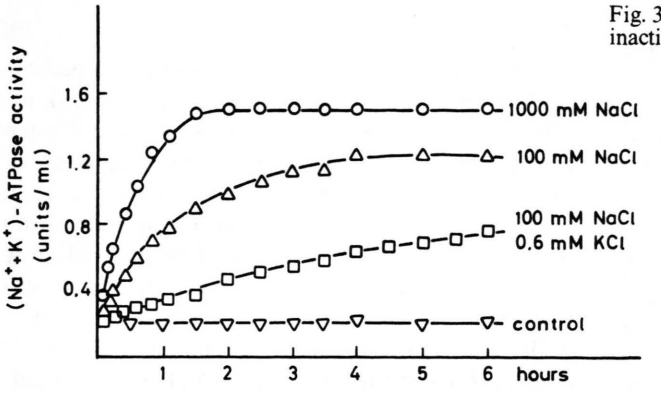


Fig. 3. Study of the effect of Na^+ and K^+ on the by CrATP inactivated $(Na^+ + K^+)$-ATPase.

$Na^+ - Na^+$ exchange in everted red blood cells. Indeed, CrATP supported the Na^+ uptake in Na^+ containing everted red blood cell vesicles, but was unable to do so in K^+ containing everted red blood cells (Table II) [36]. When CrATP is able to fuel the $Na^+ - Na^+$ exchange it seems possible that the by CrATP inactivated enzyme may lock Na^+ in an occluded form within the membrane (Fig. 4). However, despite of intensive trials, we were unable so far to find a postulated occluded Na^+-form [38].

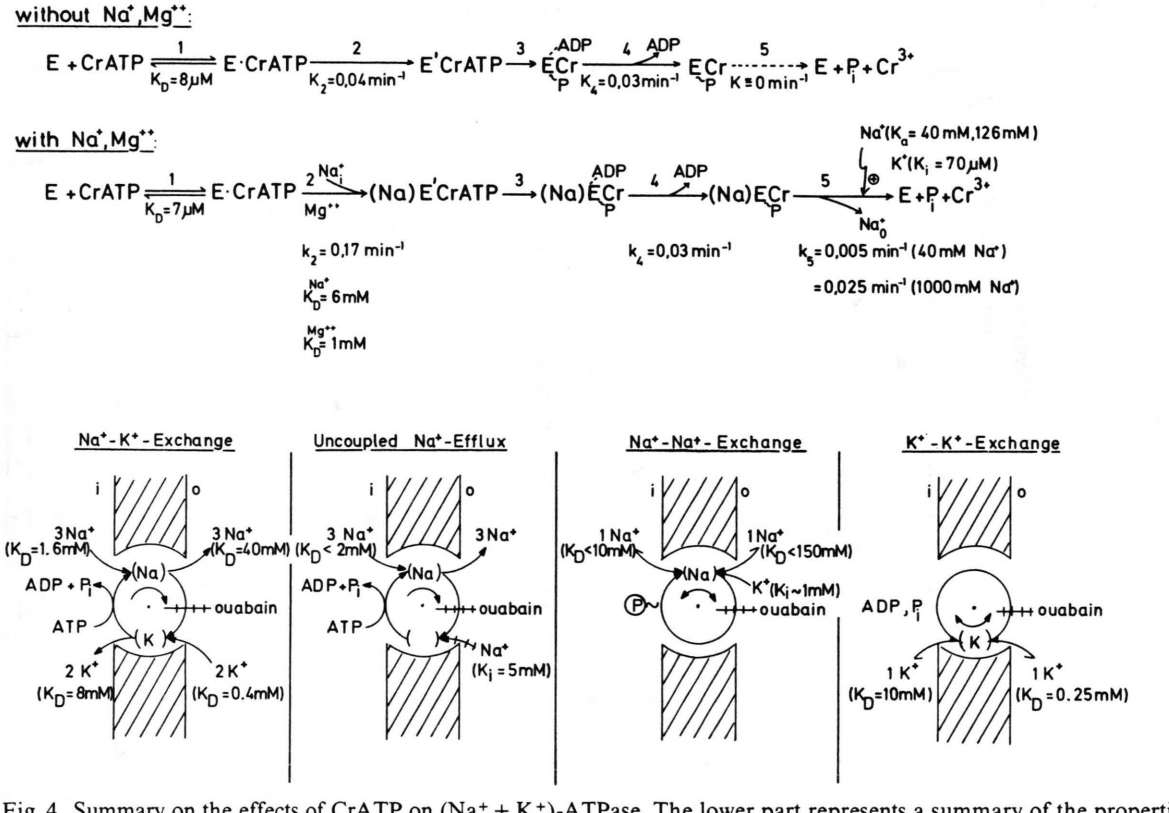


Fig. 4. Summary on the effects of CrATP on $(Na^+ + K^+)$-ATPase. The lower part represents a summary of the properties of the sodium pump and their side-reactions found in red blood cells.

Table II. Uptake of $^{22}Na^+$ into everted human red blood cells loaded with 100 mM Na^+ or 20 mM Na^+ + 5 mM K^+ with ATP or CrATP as substrates (from [37]). Everted red blood cells were equilibrated with 25 mM Tris-HCl pH 7.4, 1 mM $MgCl_2$, 20 mM NaCl and 5 mM KCl ($Na^+ + K^+$-containing vesicles) or with 25 mM TrisHCl pH 7.4, 100 mM NaCl, 1 mM $MgCl_2$ (Na^+-containing vesicles). The vesicles were incubated at 37 °C either in 20 mM Tris-HCl pH 7.4, 5 mM $^{22}NaCl$, 1 mM $MgCl_2$ (Na^+-containing vesicles) or in 15 mM Tris-HCl pH 7.4, 0.6 mM $MgCl_2$, 1.2 mM KCl, 6.1 mM NaCl, 6.1 mM choline chloride ($Na^+ + K^+$-containing vesicles) and the $^{22}Na^+$ taken up was measured in the vesicles after sedimentation for 10 min in a rotor 50 Ti at 20 000 rpm in the Beckman ultracentrifuge and washing of the vesicles with the same medium without $^{22}Na^+$.

	$^{22}Na^+$ uptake in 10 min (nmol/mg protein)
$Na^+ + K^+$-containing everted red blood cells:	
40 μM ATP	26.50
40 μM ATP + 0.2 mM vanadate	10.63
0.1 mM CrATP	10.63
Na^+-containing everted red blood cells:	
0.5 mM ATP + 0.2 mM ADP	0.70
0.5 mM ATP + 0.2 mM ADP + 0.2 mM ouabain	0.20
0.12 mM CrATP	0.73
0.12 mM CrATP + 0.2 mM ouabain	0.23

Studies with Ca^{2+}-ATPase

A reason for our failure to demonstrate on occluded Na^+-form in $(Na^+ + K^+)$-ATPase is the weak affinity of Na^+ for this enzyme. Ca^{2+}-ions have a much higher affinity for the Ca^{2+}-pump than Na^+ ions for the Na^+-pump [5]. If the argumentation is justified, occluded Ca^{2+} ions should be demonstrable in the membranes of the Ca^{2+}-ATPase of the sarcoplasmic reticulum, provided this enzyme can be inactivated by CrATP too. In fact, CrATP inactivates the Ca^{2+}-ATPase and this process is enhanced by Ca^{2+} (Fig. 5). Like in the $(Na^+ + K^+)$-ATPase the inactivation of Ca^{2+}-ATPase is due to the formation of a stable phosphointermediate [35]. In studies with crude sarcoplasmic reticulum vesicles, CrATP fuelled the uptake of $^{45}Ca^{2+}$ like ATP, but to a much smaller extent (Fig. 6). When the stoichiometry of Ca^{2+} uptake to phosphorylation of the catalytic protein was evaluated, a molar ratio of almost 2 Ca^{2+} incorporated per phosphate incorporated was found [35]. The by CrATP accumulated Ca^{2+} behaved different than the by ATP transported Ca^{2+} (Fig. 6): Whereas the addition of the Ca^{2+} ionophore X-537 A led to a

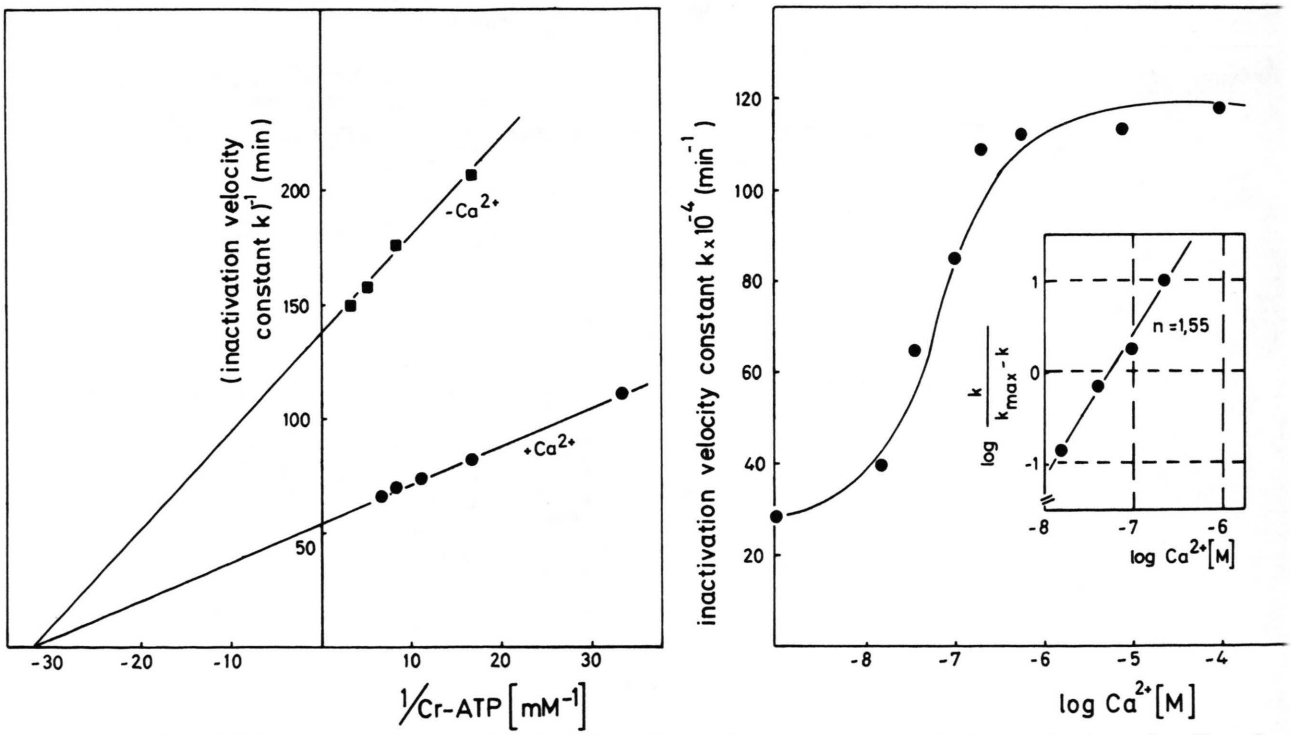


Fig. 5. Effect of Ca^{2+} on the inactivation of Ca^{2+}-ATPase of sarcoplasmic reticulum by CrATP. A) Study on the effect of Ca^{2+} on the affinity of CrATP. B) Evaluation of the Ca^{2+}-affinity. The half maximal stimulation is observed at 80 nM (from ref. [35]).

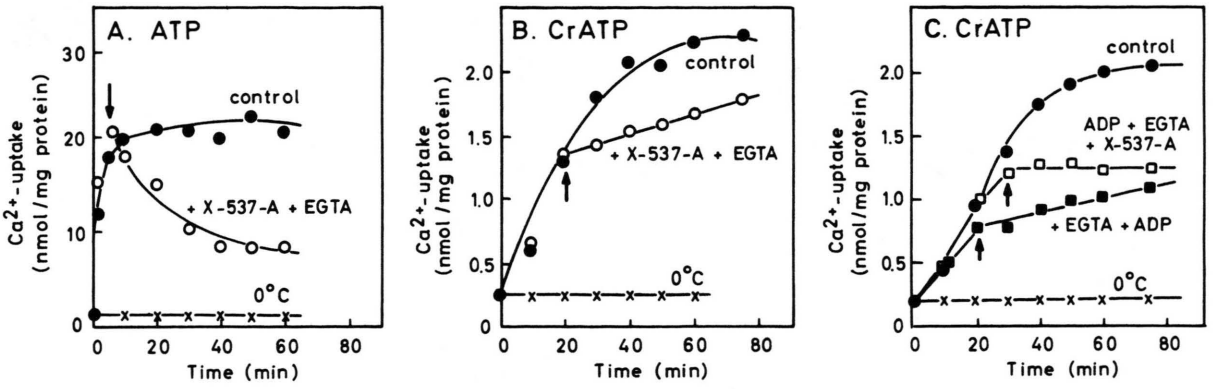


Fig. 6. Comparison of the effects of the Ca^{2+}-ionophore X-537 A on the Ca^{2+}-uptake by sarcoplasmic reticulum vesicles driven by the hydrolysis of ATP or CrATP. Demonstration of the lack of reversibility of the Ca^{2+}-pump by ADP (from ref. [35]).

release of the by hydrolysis of ATP accumulated $^{45}Ca^{2+}$, such an effect was not seen with vesicles, which had taken up their Ca^{2+} in the presence of CrATP. Apparently an occluded Ca^{2+}-form [39–41] was formed using CrATP as fuelling substrate. To strengthen this assumption a dodecylsulfate electrophoresis of purified Ca^{2+}-ATPase was done, which had been incubated with $^{45}Ca^{2+}$ and CrATP. In fact in such a system most of the $^{45}Ca^{2+}$ migrated with the catalytic protein bearing the phosphorus accepted from [γ-^{32}P]CrATP (Fig. 7). Apparently the formation of a stable chromo-phosphointermediate occludes Ca^{2+} in a stable form. Presumably this phenomenon might be helpful to get further insights into the mechanism of Ca^{2+} translocation by the Ca^{2+}-ATPase. But in addition, this finding makes it more likely, that an occluded Na^+-form can be detected in $(Na^+ + K^+)$-ATPase under appropriate conditions.

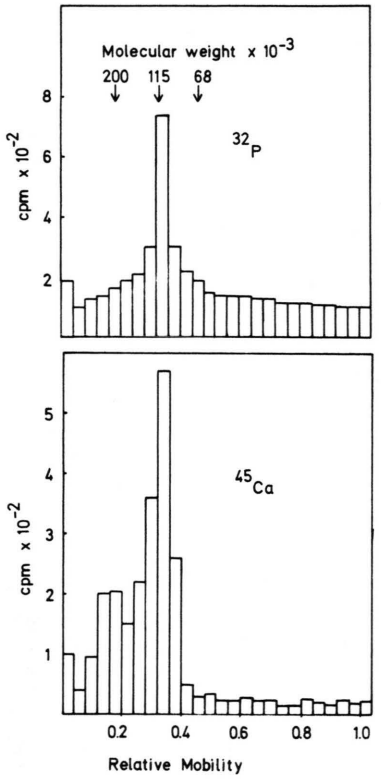


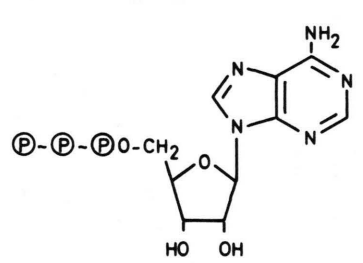
Fig. 7. Dodecylsulfate electrophoresis of Ca^{2+}-ATPase that had taken up $^{45}Ca^{2+}$ and that had been phosphorylated from [γ-^{32}P]CrATP (from ref. [35]).

Studies on $(Na^+ + K^+)$-ATPase and on Ca^{2+}-ATPase with proteinreactive ATP-analogues

The studies reported so far indicate, that CrATP can be used in both transport ATPases to study partial reactions of the transport process and eventually to detect occluded ions. If the ion transport starts at the ATP binding site [23, 24], it seems possible that the conformational changes reported to occur during the cation transport in both ATPases [2–5, 9, 10] are visible at the ATP binding sites. Such conformational changes should then be demonstrable by the use of protein-reactive ATP-analogues.

Studies with $(Na^+ + K^+)$-ATPase

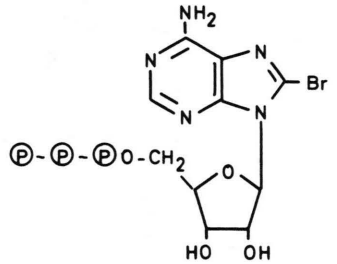
Because the sodium pump of human red blood cells is believed to be rather ATP specific [42], it seemed necessary to look for the effects of the substitution of the ATP molecule on the affinity of



ATP

(K_D = 1.8 x 10^{-7} M)

anti-conformation



8-Br = ATP

(K_D = 4.2 x 10^{-6} M)

syn-conformation

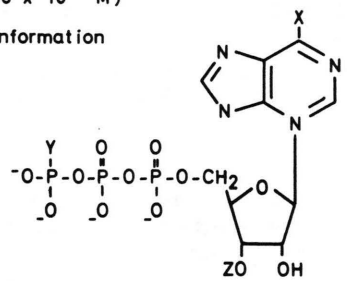
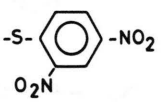
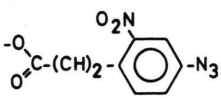


Fig. 8. Structure-affinity relationship of modified ATP on $(Na^+ + K^+)$-ATPase (from ref. [27, 32, 33]).

	K_D [M]	Temp. [°C]	X	Y	Z	tissue source
ATP	1.8 x 10^{-7}	0	$-NH_2$	O	H	beef brain
	3 - 6.5 x 10^{-6}	37	$-NH_2$	O	H	pig kidney and beef brain
CrATP	4.3 x 10^{-5}	37	$-NH_2$	O	H	pig kidney
ATPγS	2.2 x 10^{-7}	0	$-NH_2$	S	H	beef brain
ATPγF	2.4 x 10^{-6}	0	$-NH_2$	F	H	beef brain
dnp-sITP	5.4 x 10^{-6}	0		O	H	beef brain
Cl-ITP	9.2 x 10^{-6}	0	-Cl	O	H	beef brain
sITP	1.8 x 10^{-5}	0	-SH	O	H	beef brain
ITP	5.8 x 10^{-5}	0	-OH	O	H	beef brain
$(sITP)_2$	1.8 x 10^{-6}	37	-S-	O	H	pig kidney
N_3-ATP	1.9 x 10^{-5}	21	$-NH_2$	O		pig kidney

$(Na^+ + K^+)$-ATPase (Fig 8). We couldn't only learn that isolated $(Na^+ + K^+)$-ATPase like Ca^{2+}-ATPase from sarcoplasmic reticulum has no ATP specificity [1–3, 11, 43], but we also learned that modifications of the molecule are possible without severe disturbance of the affinity at the 6-amino group of the purine ring, at the ribose moiety and the terminal phosphate (Fig. 8).

Sulfhydryl groups play an essential role in both transport ATPases [1–3, 9–11]. There is indirect evidence that SH-groups are located in the ATP binding site [44–47]. To prove this assumption we incubated both enzymes with the disulfide of thioinosine triphosphate $(SnoPPP)_2$ [26, 28, 32, 34]. This ATP-analogue reacts with sulfhydryl groups in forming a mixed disulfide (Fig. 9).

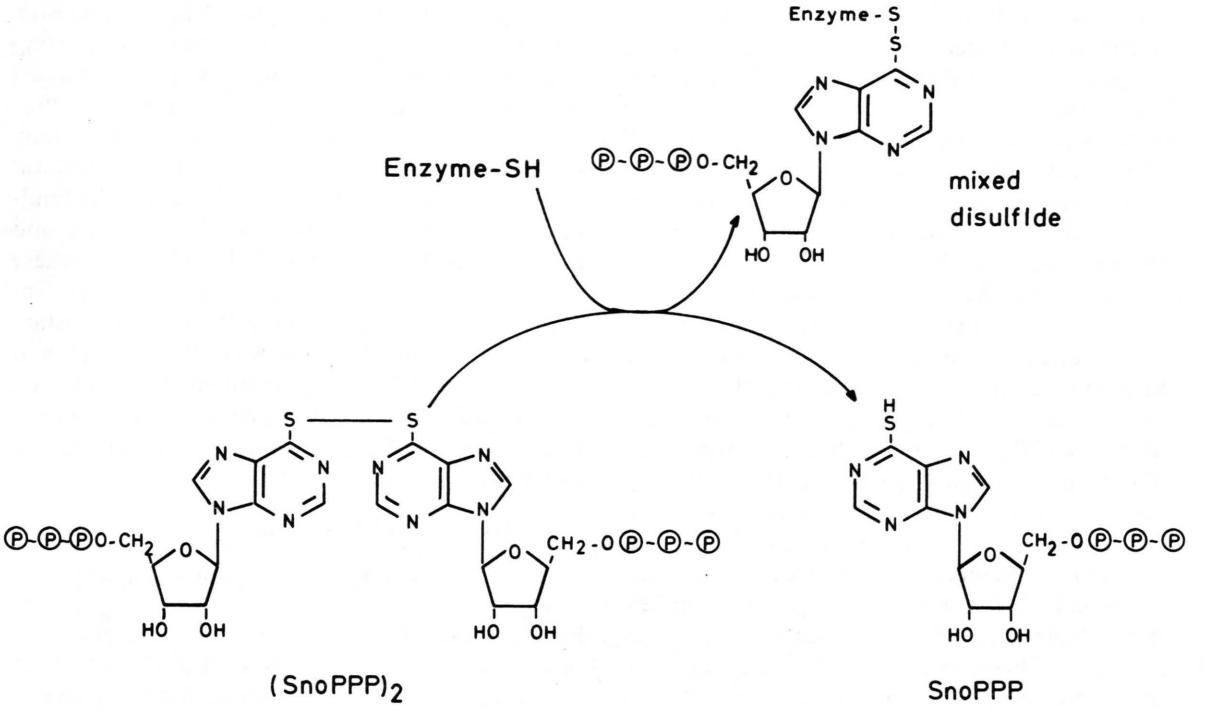


Fig. 9. The disulfide of thioinosine triphosphate and its reaction mechanism.

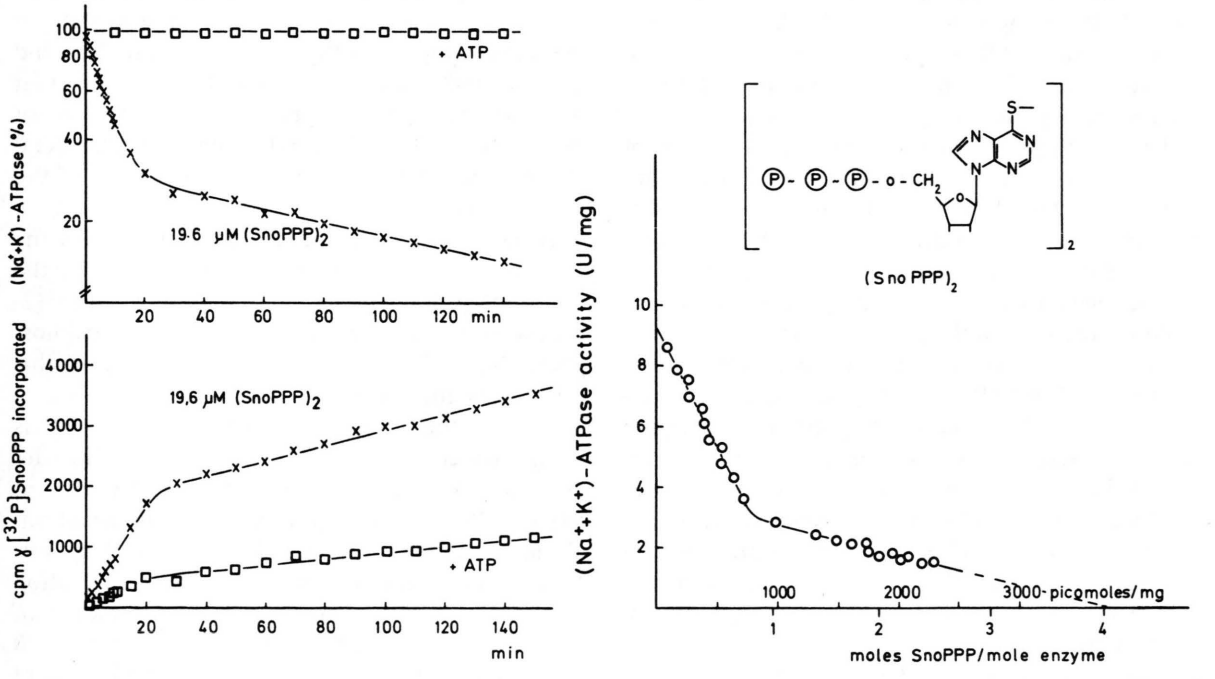


Fig. 10. Titration of the capacity of the sites reacting with 21 µM ([γ-^{32}P]SnoPPP)$_2$ in $(Na^+ + K^+)$-ATPase. A) Alteration of the activity; B) Incorporation of radioactivity; C) Plot of the activity against incorporation of SnoPPP (from ref. [32]).

$(Na^+ + K^+)$-ATPase is inactivated by this ATP analogue and the inactivation is hindered by the presence of an excess ATP (Fig. 10) [32]. Unexpectedly the inactivation proceeded with 2 inactivation velocity constants. From the protective effect of ATP on the inactivation it could be evaluated, that the rapidly reacting sulfhydryl group belonged to a high affinity ATP binding site ($K_D = 2.95$ μM) and the slowly reacting sulfhydryl group to a low affinity ATP binding site ($K_D = 77$ μM). A dissociation constant of 18.6 μM of the enzyme-$(SnoPPP)_2$ complex was unexpectedly found for each sulfhydryl group containing site. One may speculate that this finding is due to an altered interaction of the disulfide bond in $(SnoPPP)_2$ with the sulfhydryl group in the ATP binding site as compared to ATP as substrate. If Na^+ ions up to 10 mM were added to the incubation, the reactivity of both SH-groups was enhanced, but the presence of K^+ protected against the inactivation [34]. This finding is consistent with the expected behaviour of an ATP-affinity label of $(Na^+ + K^+)$-ATPase, since ATP binding has been shown in this enzyme to be hindered by the additional presence of K^+ [48, 49]. The protective effect of K^+ against the inactivation of $(Na^+ + K^+)$-ATPase by $(SnoPPP)_2$ could be used to determine the apparent affinity of K^+ for the high and the low affinity ATP binding sites: K^+ binds with the high protection constant of 0.26 mM at the high affinity ATP site but with the low protection constant of 4 mM at the low affinity site [34].

To get more information on the structure of $(Na^+ + K^+)$-ATPase, the enzyme was titrated with radioactive $(SnoPPP)_2$ (Fig. 10). It turned out that the capacity of the rapidly reacting sulfhydryl group was identical to the capacity of the phosphointermediate and that approximately 3 other sites with a slowly reacting sulfhydryl group exist in the enzyme. It seems possible from these experiments that the $(Na^+ + K^+)$-ATPase exists as a tetramer of catalytic subunits. The finding of a tetrameric enzyme does not mean that the subunits interact in a reciprocating way in the form of a half-of-the sites reactivity [50 – 52]. Difficult to reconcile with that assumption is the finding that no complete inactivation is found after the complete blockade of the rapidly reacting sulfhydryl group in the high affinity ATP binding site.

The above reported data on the action of $(SnoPPP)_2$ on $(Na^+ + K^+)$-ATPase clearly show that a sulfhydryl group exists in the ATP binding site. This SH-group may interact with the 6-amino group of the purine ring of ATP and may thereby participate in the recognition of the substrate. Proteinreactive ATP analogues, like $(SnoPPP)_2$, may possibly be used as a probe to study structural changes within the ATP binding site due to the binding of the transport substrates Na^+ and K^+, and eventually of Mg^{2+}-provided the affintiy of these ATP analogues is not altered thereby. Indeed, intensive studies showed, that the dissociation constant of the enzyme complex with $(SnoPPP)_2$ is not altered by Na^+ or K^+ [34]. The enhancement of inactivation by Na^+ and the protective effect of K^+ must therefore be contributed to an alteration of the inactivation velocity constant k_2 (Eqn. 1):

$$E_{SH} + (SnoPPP)_2 \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E_{SH} \cdot (SnoPPP)_2 \xrightarrow{k_2} ES\text{-}snoPPP + SnoPPP.$$

Fig. 11 shows, that also Mg^{2+} ions enhance the inactivation velocity constant k_2, but do not affect the affinity of the ATP-analogue for the enzyme. The Mg^{2+}-concentrations necessary to achieve this effect are in the range of the low affinity Mg^{2+} binding site [53]. The occupation of such a low affinity Mg^{2+} binding site has been discussed to induce conformational changes [54 – 58]. Since the affinity of the enzyme for $(SnoPPP)_2$ is not changed by Na^+ and Mg^{2+} but the reactivity, it is plausible to assume that the distance between the disulfide bridge of $(SnoPPP)_2$ and the sulfhydryl group within the ATP binding site is decreased due to an alteration of the enzyme conformation

To assure our assumption that $MgCl_2$ alters the structure of the ATP binding site, we looked for the effect of Mg^{2+} on the photoinactivation by 3′-O-[3(2-nitro-4-azidophenyl)-propionyl] adenosine triphosphate, N_3-ATP [33]. As you see from Fig. 12, this ATP analogue does not inactivate $(Na^+ + K^+)$-ATPase in the absence of Mg^{2+}, although ATP is bound under these conditions [48, 49]. The dependence on $MgCl_2$ of the photoinactivation at 50 μM N_3-ATP probably means that a conformational change at the ribose subsite is induced by mM $MgCl_2$-concentrations. Since the photoinactivation by the azido-ATP is hindered by the presence of ATP or K^+, and since the α-subunit of $M_r = 100\,000$ is labelled by the radioactive azido-ATP it is evident that the analogue reacts at the ATP binding site [33].

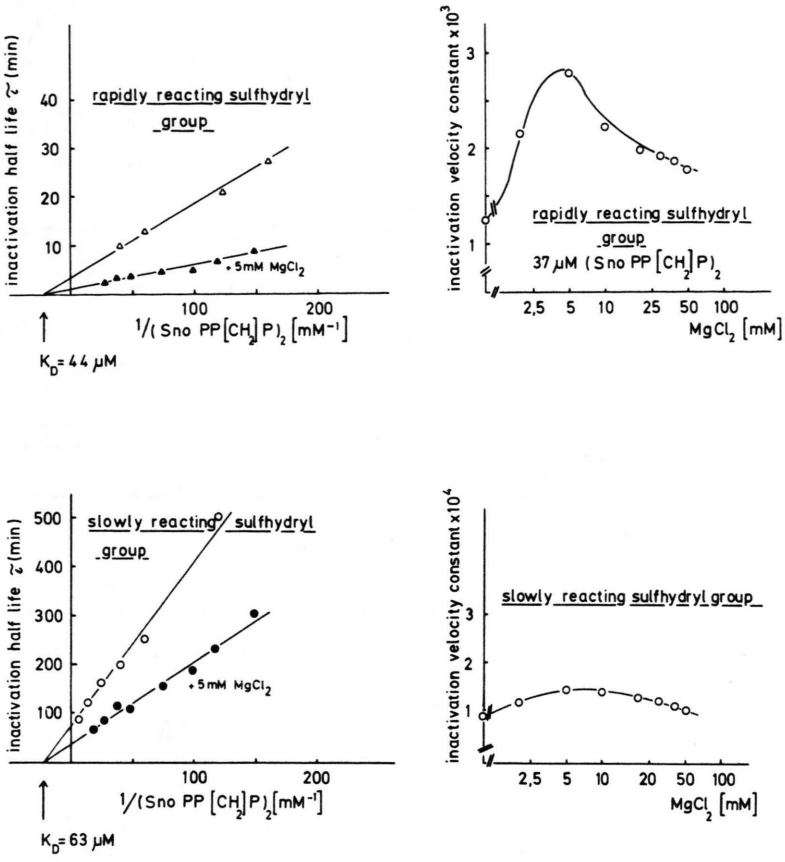


Fig. 11. Effect of Mg^{2+} on the reactivity of slowly and fast reacting sulfhydryl groups of the ATP binding sites of $(Na^+ + K^+)$-ATPase with $(SnoPPP)_2$ (from ref. [34]).

Studies with Ca^{2+}-ATPase

Since ATP affinity labels were helpful to localize an essential SH-group in the ATP binding site and to detect conformational changes upon ligand binding in the ATP binding site of $(Na^+ + K^+)$-ATPase, we were interested to see, if similar results could be obtained for the Ca^{2+}-ATPase of the sarcoplasmic reticulum.

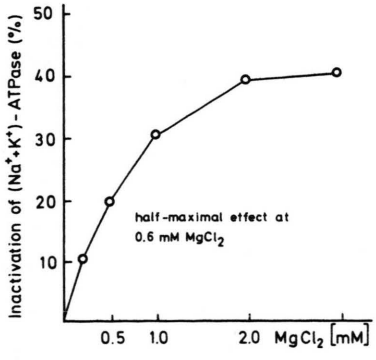


Fig. 12. Mg^{2+}-dependence of the photoinactivation by N_3-ATP of $(Na^+ + K^+)$-ATPase (from ref. [33]).

Since the disulfide of thioinosine triphosphate was a substrate of Ca^{2+}-ATPase and fuelled the Ca^{2+}-uptake into sarcoplasmic reticulum vesicles, the β,γ methylene derivative of $(SnoPPP)_2$ was synthesized to study the role of sulfhydryl groups in the recognition of ATP by the Ca^{2+}-ATPase [28]. Consistent with the earlier assumption of Hasselbach [46, 47], incubation of the purified enzyme with this ATP-analogue led to an inactivation of Ca^{2+}-ATPase (Fig. 13) [28]. Since ATP in excess protected the enzyme against the inactivation, one has to assume, that also in this enzyme a SH-group is located in the ATP binding site and interacts with the 6-amino group of the purine ring of ATP.

Like in the $(Na^+ + K^+)$-ATPase two different reactive sulfhydryl groups are seen in the absence of $MgCl_2$ and the transport substrate Ca^{2+} (Fig. 13 A). The rapidly reacting sulfhydryl group can be as-

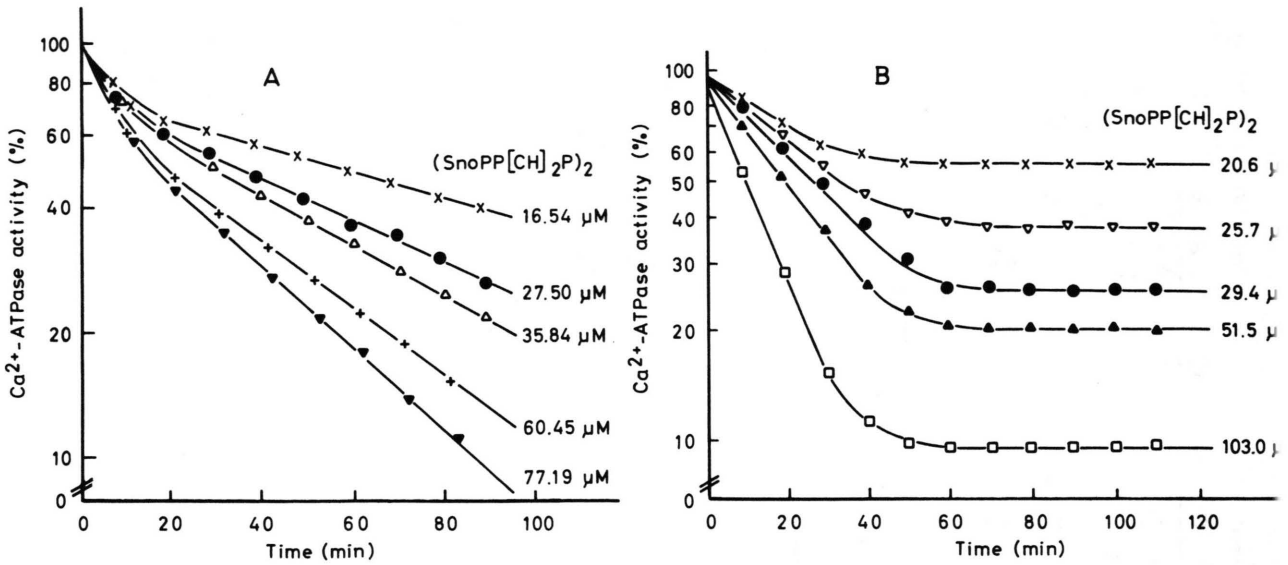


Fig. 13. Inactivation of Ca^{2+}-ATPase by the disulfide of thioinosine triphosphate. Left diagramm: Inactivation in the presence of 100 mM KCl + 3.6 mM EGTA; Right diagramm: Inactivation in the presence of 100 mM KCl + 5 mM $MgCl_2$ + 0.82 mM $CaCl_2$ (from ref. [28]).

cribed to a high affinity ATP binding site and the slowly reacting group to a low affinity site. Both sites bind the ATP analogue with the same affinity (Table III) [28]. A considerable difference in the reactivity of the sulfhydryl group can be detected, however, when the kinetics of inactivation are studied in the presence of Mg^{2+} and the transport substrate Ca^{2+}: There exists now only one type of SH-groups (Fig. 13 B). The presence of Mg^{2+} and the transport substrate appears to induce a conformational change in one ATP binding site, which makes the low affinity ATP site accessable only for the bulky $(SnoPPP)_2$ (Table III). However, studies with [γ-^{32}P] $(SnoPPP)_2$ show an early burst in the phosphorylation of the enzyme protein under these conditions (Fig. 14 B). Since this incorporated radioactivity is sensitive to hydroxylamine, one has to conclude that the enzyme is phosphorylated from [γ-^{32}P] $(SnoPPP)_2$. If the assumption is justified that the phosphorylation of the enzyme starts from the high affinity ATP binding

Table III. Comparison of the dissociation constants and the inactivation velocity constants of the complexes of Ca^{2+}-ATPase with disulfides of thioinosine triphosphate and evaluation of the ATP affinity from its protective effect on the inactivation (from ref. 28).

Nucleotide	Dissociation constant at 20 °C		Inactivation velocity constant at 20 °C	
	Rapid part K'_d [µM]	Slow part K''_d [µM]	Rapid part $10^2 \cdot k'_2$ [s^{-1}]	Slow part $10^3 \cdot k''_2$ [s^{-1}]
Presence of 100 mM KCl + 3.6 M EGTA:				
$(SnoPPP)_2$	111	117	1.7	1.1
$(SnoPP[CH_2]P)_2$	143	153	1.4	1.1
ATP	49	114	–	–
Presence of 5 mM $MgCl_2$ + 100 mM KCl + 0.82 mM $CaCl_2$:				
$(SnoPP[CH_2]P)_2$	–	113	–	–
ATP	–	140	–	–

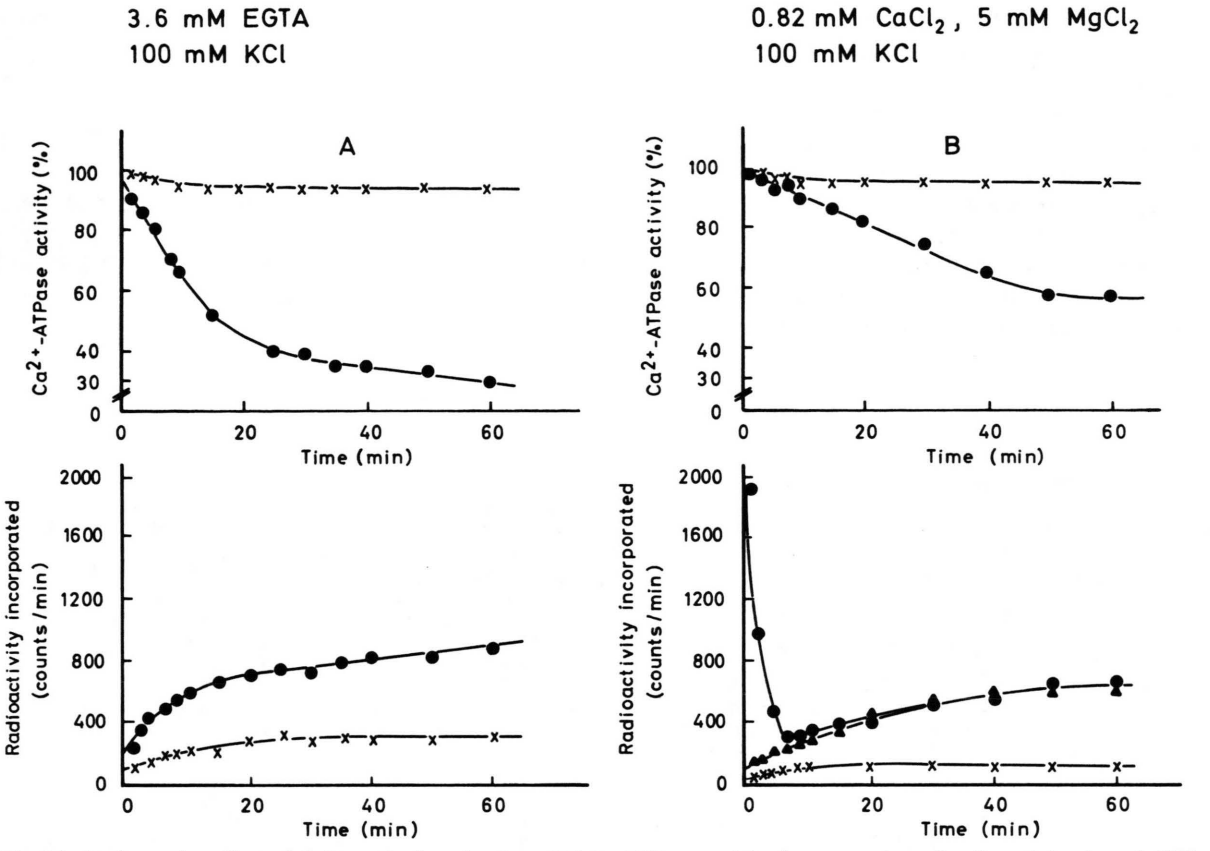


Fig. 14. Study on the effect of Ca^{2+} on the inactivation of Ca^{2+}-ATPase and the incorporation of radioactivity from [γ-^{32}P] $(SnoPPP)_2$. A) Inactivation and incorporation of radioactivity in the absence of bivalent cations. B) Inactivation and incorporation of radioactivity in the presence of Ca^{2+} and Mg^{2+}. Triangles represent the data after treatment of the inactivated enzyme with hydroxylamine (from ref. [28]).

site, one has to conclude that the low affintiy ATP binding site can be converted to the high affinity site. Fig. 14 furthermore shows that the inactivation of Ca^{2+}-ATPase is due to the covalent fixation of the sITP to the enzyme. It has been shown furthermore, that the inactivated enzyme can be reactivated by dithiothreitol [28]. A titration of the ATP binding sites in Ca^{2+}-ATPase has not been done so far. Preliminary data show that 3'-O-[3-(2-nitro-4-azidophenyl)-propionyl] adenosine triphosphate also acts as a photoaffinity label on Ca^{2+}-ATPase. However, the extent of inactivation is considerably smaller than in $(Na^+ + K^+)$-ATPase and the inactivation appears to be independent of $MgCl_2$ and Ca^{2+} (Rempeters, unpublished).

Conclusions

The data known so far seem to indicate that the ATP binding sites of $(Na^+ + K^+)$-ATPase and of Ca^{2+}-ATPase seem to be constructed in a similar way. In both ATP binding sites a sulfhydryl group is essential for the recognition of ATP. In the absence of transport substrates both transport ATPases exhibit high and low affinity ATP sites which contain rapidly and slowly reacting sulfhydryl groups. Both ATP binding sites appear to be changed in their structure upon binding of the transport substrates. But the kind of alterations differs in both ATPases: Whereas in Ca^{2+}-ATPase a rapidly reacting sulfhydryl group is lost upon binding of Ca^{2+},

binding of the transport substrates Na^+ and K^+ to $(Na^+ + K^+)$- ATPase alters only the reactivity of the sulfhydryl groups within the high and low affinity sites. One may suggest therefore, despite of many similiarities in the ATP recognition and the ATP hydrolytic mechanism, that the mechanism of the conversion of chemical energy into transport work is varied in the different pumps according to the needs of a specific ionophoric part. It seems possible that the ionophoric part of both transport ATPases can easier be studied by deceleration of the turnover of both cation pumps by the use of the MgATP analogue, chromium (III)ATP.

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