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THE PRE-STEADY-STATE HYDROLYSIS OF ATP BY PORCINE BRAIN (Na^++K^+)-DEPENDENT ATPase

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Summary

The hydrolysis of [γ-^{32}P]ATP by porcine brain (Na^++K^+)-stimulated ATP phosphohydrolase (EC 3.6.1.3) has been studied at 28°C in a rapid mixing quenched-flow apparatus. An "early burst" in the release of P_i from ATP has been observed when the enzyme is mixed with ATP, Na^+ and a relatively high concentration of K^+ (10 mM) but the burst is less pronounced with 0.5 mM K^+. This "early burst" of P_i release is suppressed when the enzyme is pre-mixed with 10 mM K^+ or 20% (v/v) dimethylsulphoxide before mixing with ATP and Na^+, and premixing of enzyme with Na^+ antagonizes this effect of dimethylsulphoxide. The results have been analysed by a non-linear least squares regression treatment and are consistent with a mechanism involving three steps, one of which may be a relatively slow change in enzyme conformation following release of P_i from its covalent linkage with the enzyme, in addition to formation of the enzyme-substrate complex. Rate constants (and S.E.) for these steps have been calculated and the roles of phospho-enzyme and other intermediates in the reaction mechanism of the transport ATPase are discussed.

Introduction

Evidence that has been accumulated from studies of the activation of the (Mg^{2+}+Na^++K^+)-dependent transport ATPase (EC 3.6.1.3) by Na^+ and K^+, the phosphorylation of the enzyme by ATP, and the modifying effects of Na^+ and K^+ on the inhibition of the enzyme by trypsin, beryllium, dicyclocarbodiimide and fluoride and the binding of ouabain [1—8] suggests that the reaction mechanism of this enzyme involves a number of phosphorylated and non-phosphorylated enzyme intermediates. This raises the possibility that the rate of hydrolysis of ATP by the transport ATPase may be limited by a step, such as a change in conformation of the enzyme, that does not involve direct release of orthophosphate from a covalent linkage. Studies of the early time course of

release of orthophosphate have provided evidence that a rate-limiting step of this kind does occur during hydrolysis of ATP by both actomyosin ATPase [9] and the Ca^{2+}-ATPase of the sarcoplasmic reticulum [10], while recently Froehlich et al. [11] have suggested that dissociation of non-covalently linked product orthophosphate limits the rate of the transport ATPase of the electric eel. In this paper we show that the time course for release of orthophosphate from ATP by a transport ATPase preparation from pig brain can be modified by agents such as K^+ and dimethylsulphoxide (Me_2SO) and analysis of the results suggests that the enzyme can exist in at least two conformations in the absence of ATP. These conformations may well be significant for the transport function of the enzyme.

Methods

Preparation of transport ATPase. Pig brain (50 g) was homogenized in four volumes of 0.25 M sucrose containing 25 mM imidazole · HCl (pH 7.4) and 5 mM EDTA/Tris (pH 7.4) for four times 10 s at 4° C in a Waring blender. The homogenate was centrifuged at 12 000 × *g* for 20 min to give a loosely packed sediment which was resuspended in 500 ml 0.8 M sucrose containing 25 mM imidazole · HCl (pH 7.4) and 5 mM EDTA/Tris (pH 7.4) and centrifuged at 70 000 × *g* for 90 min. This yielded a pellet containing mainly mitochondria and synaptosomes [12] and a supernatant with a floating layer of myelin. The pellet was homogenized in 350 ml 10 mM imidazole · HCl, 5 mM EDTA/Tris (pH 7.4) and stirred for 90 min at 4° to disrupt synaptosomes osmotically. The suspension was then made 1 M in sucrose (by addition of 2.5 M sucrose solution) and centrifuged at 70 000 × *g* for 60 min to sediment mitochondria. Dilution of the supernatant to 0.9 M sucrose with water and centrifugation at 70 000 × *g* for 60 min then yielded a pellet of synaptosomal membranes which were suspended in distilled water before treatment with NaI as described by Atkinson and Lowe [13]. The resultant NaI-treated enzyme was used within 3 days of storage at 0° C without freezing and thawing. Preparations had specific ouabain-inhibitable transport ATPase activity of 1—2 μmol/mg protein per min measured at 37° C in a medium containing 100 mM NaCl, 30 mM ATP/Tris (pH 7.8), 3 mM $MgCl_2$ and 50 mM Tris · HCl (pH 7.8) [13].

The early time course of hydrolysis of [γ-^{32}P]ATP. Reactions were carried out at 28° C in an Aminco-Morrow stopped-flow apparatus adapted for quenched-flow experiments. The two loading syringes were charged with (A) 2.5 ml of a suspension of enzyme (approx. 2 mg protein/ml) in a solution of 50 mM Tris · HCl (pH 7.8) and 0.2 mM $MgCl_2$, and (B) 2.5 ml of a solution containing [γ-^{32}P]ATP (10—100 μM), 50 mM Tris · HCl (pH 7.8) and selected concentrations of NaCl, KCl, RbCl and Me_2SO. The enzyme-syringe (A) also contained selected additives in some experiments. After mixing, the reaction mixture was carried by tubing of variable length to the stopping solution which consisted of 5 ml 8.4% (v/v) $HClO_4$ containing 1.5 mM phosphate/Tris and 0.8 mM ATP/Tris, at 0° C. 1 ml samples of the quenched reaction mixture were added to a mixture of 0.4 ml 1 M Tris/maleate (pH 6.0) and 0.63 ml 1 M NaOH to give a final pH of 6.0 and thereby arrest hydrolysis of ATP, before addition of activated charcoal (20 mg) to absorb ATP. The charcoal was sedimented by

centrifugation at 2000 × *g* for 5 min and washed with 1 ml Tris/maleate buffer. The combined supernatant and charcoal washings (2.7—2.8 ml) were then mixed with 0.04 ml 40 mM KH_2PO_4 and 0.5 ml 2.97 M H_2SO_4 containing 5.1% ammonium molybdate before extraction with 2.5 ml isobutanol/benzene (1 : 1, v/v). 2 ml of the resultant organic phase were then added to 10 ml 1 M NaOH which extracted the phosphomolybdate into the aqueous phase and decolorized the solution. Radioactivity was then assayed by measuring Cerenkov radiation in a scintillation counter. In control runs, $MgCl_2$ was omitted from, and 10 mM EDTA/Tris (pH 7.8) was added to, the enzyme-syringe (A) to inhibit transport ATPase activity. In measurements of phosphorylation of the enzyme by [γ-^{32}P]ATP experiments were carried out similarly but after quenching the precipitated enzyme was washed three times by homogenization in 5% (v/v) $HClO_4$ containing 0.6 mM KH_2PO_4 and 0.6 mM ATP/Tris, then dissolved in 5 ml 0.1 M NaOH containing 2% (w/v) Na_2CO_3 before measurement of Cerenkov radiation. Maximum incorporation of ^{32}P into the enzyme from [γ-^{32}P]ATP in the presende of 100 mM NaCl, 0.1 mM $MgCl_2$, 50 mM Tris · HCl (pH 7.8) and 0.1 mM [γ-^{32}P]ATP/Tris (pH 7.8) was 0.1—0.15 nmol/mg protein.

Preparation of [γ-^{32}P]ATP. [γ-^{32}P]ATP was made from disodium ATP (Boehringer) by the method of Glynn and Chappell [14]. [^{32}P]Orthophosphate contaminating the ATP was then reduced to 0.1—0.5% by chromatography of DEAE-cellulose as described by Mardh [15].

Results

The time course of ATP hydrolysis

The early time course for the release of orthophosphate from [γ-^{32}P]ATP by the pig brain transport ATPase preparation is shown in Fig. 1. Release of orthophosphate is relatively slow in the absence of potassium and is substantially

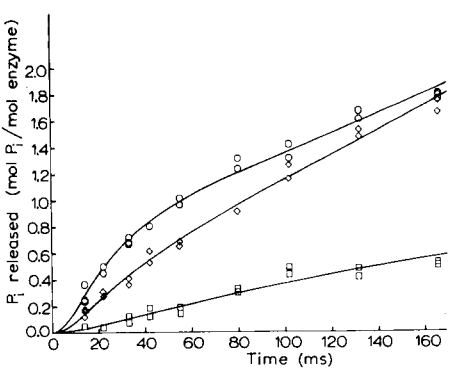


Fig. 1. The effect of K^+ on P_i liberation by the transport ATPase in the presence of 50 mM NaCl at 28° C. The enzyme-syringe contained enzyme (2 mg protein/ml), 50 mM Tris · HCl (pH 7.8) and 0.2 mM $MgCl_2$, and the substrate-syringe contained 50 mM Tris · HCl (pH 7.8), 20 μM [γ-^{32}P]ATP/Tris (pH 7.8) and 100 mM NaCl with (A) no KCl (□), (B) 1 mM KCl (◇), and (C) 20 mM KCl (○). Lines were calculated from the rate constants for experiments 1—3 in Table I. Turnovers are based on the maximum amount of enzyme phosphorylated by 100 μM [γ-^{32}P]ATP in the presence of 100 mM NaCl and absence of KCl, with other conditions as above.

accelerated by addition of 0.5 mM KCl, while with 10 mM KCl there is an initial very rapid release of orthophosphate followed by a slower phase. In the experiments shown, this steady-state rate with 10 mM KCl was slightly slower than that found with 0.5 mM KCl, but in similar experiments steady-state rates at these two potassium concentrations were similar. This failure of 10 mM KCl to stimulate transport ATPase activity above the rate found with 0.5 mM KCl is consistent with results of Post et al. [16] who showed that high potassium concentrations inhibited transport ATPase activity at ATP concentrations of 13 μM and less. The observed "early burst" of phosphate release is clearly significant for the mechanism of action of the transport ATPase and can be explained qualitatively by a reaction cycle containing a step in which phosphate is released followed by a slower (rate-limiting) step before completion of the cycle. Since it is accepted by most workers that at least one phosphorylated intermediate is involved in the activity cycle of the transport ATPase, one of the simplest schemes capable of accounting for the early burst of phosphate release is

$$E_1 + ATP \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E_1(ATP) \underset{k_{-2}\ ADP}{\overset{k_2\ ADP}{\rightleftharpoons}} E_2P \underset{k_{-3}\ P_i}{\overset{k_3\ P_i}{\rightleftharpoons}} E_2 \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E_1$$

where E_2P is a phosphorylated enzyme derivative and E_1 and E_2 are two conformations of the enzyme. This scheme leaves out the intermediate, E_1P, in order to make kinetic analysis manageable and this seems justified since Siegel and Albers [17] and Post et al. [18] found little Na^+-dependent ADP-ATP exchange activity or ADP-sensitive phospho-enzyme under the usual conditions of reaction and therefore conversion of E_1P to E_2P is unlikely to be rate-limiting. Using this scheme equations describing the time course of orthophosphate release and the proportion of enzyme in the form of E_2P can be derived, as outlined in Appendix. These equations make it possible to obtain best fits to the experimental data for the rate constants, k_2, k_3 and k_4 together with the standard errors of these constants using the non-linear least squares fitting procedure of De Weer and Lowe [19]. The lines in Fig. 1 and all subsequent figures have been drawn using the "best fit" computed rate constants and the Calcomp plotter at the University of Manchester Regional Computer Centre, and Table I lists the values of the constants used and their standard errors. The results show that, in the presence of 10 μM ATP, addition of 0.5 mM KCl to the reaction mixture (containing 50 mM NaCl) increased the rate constant for dephosphorylation of the phospho-enzyme (k_3) from 5.2 ($\pm$0.8) to 23.0 ($\pm$4.2) s^{-1} and established k_4 (the rate on conversion of E_2 to E_1) at 17.8 ($\pm$2.8) s^{-1}. Increasing the potassium concentration further to 10 mM led to a further increase in k_3 to 50.1 ($\pm$12.5) s^{-1}, but significantly reduced k_4 to 9.3 ($\pm$0.6) s^{-1}, and it is this dual effect of high potassium concentration on k_3 and k_4 that explains the fact that with 10 mM KCl the rate of phosphate release is initially much greater than but later, similar to that with 0.5 mM KCl. This result is consistent with the suggestion of Post et al. [16] that conversion of E_2 to E_1 is inhibited by potassium (although it is not necessary to assume an occluded E_2-K^+ complex) and in view of the fact that these workers also found that rubidium is a more effective inhibitor of this conversion than potassium, the early stages of ATP hydrolysis were studied with 10 mM RbCl replacing 10 mM KCl at 10 μM

TABLE I

RATE CONSTANTS COMPUTED FOR THE TRANSPORT ATPase

Experiments were carried out as described in Methods with additions to the enzyme-syringe as indicated below. The final concentration of NaCl was 50 mM in all experiments. Rate constants were computed from the experimental data (P_i release in experiments 1—10 and phospho-enzyme formation in experiments 11 (P)—13(P)) by non-linear least squares regression using the rate equations in Appendix.

Expt. No.	Additions to the enzyme-syringe	Final ATP concentration (μM)	Final K^+ concentration (mM)	Rate constants (s^{-1} ± S.E.)		
				k_2	k_3	k_4
1	None	10	0	79.9 ± 55.9	5.2 ± 0.8	1.6 ± 3.2
2	None	10	0.5	110.4 ± 53.4	23.0 ± 4.2	17.8 ± 2.8
3	None	10	10	119.6 ± 55.9	50.1 ± 12.5	9.3 ± 0.6
4	None	10	10 *	227.9 ± 112.7	34.8 ± 3.9	7.1 ± 0.4
5	None	5	10	70.6 ± 41.0	47.3 ± 20.6	9.3 ± 0.5
6	None	50	10	180	179	33.5
7	20 mM KCl	10	10	12.8	>300	24.1
8	20% (v/v) Me_2SO	10	0.5	12.1	>300	6.2
9	20% (v/v) Me_2SO, 50 mM NaCl	10	10	62.5 ± 71.0	70.7 ± 87.7	14.5 ± 0.4
10	20% (v/v) Me_2SO, 50 mM NaCl, 20 mM KCl	10	10	17.7 ± 1.7	163.7 ± 75.0	13.6 ± 1.3
11 (P)	None	10	10	20.4 ± 1.9	78.1 ± 8.4	29.6 ± 6.2
12 (P)	None	10	0.5	29.4 ± 2.7	21.1 ± 6.3	88.9 ± 9.3
13 (P)	20% (v/v) Me_2SO	10	0.5	43.5 ± 2.0	10.8 ± 1.2	24.7 ± 5.1

* 10 mM RbCl replaced 10 mM KCl.

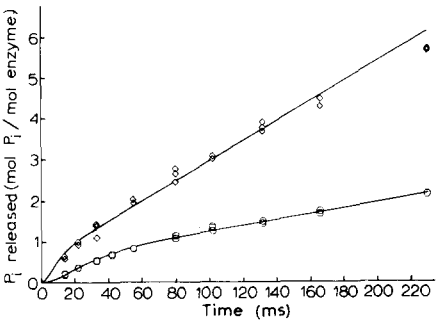


Fig. 2. The effect of the concentration of ATP on P_i liberation by the transport ATPase in the presence of Na^+ and K^+. The enzyme-syringe contained enzyme (2 mg/ml (○) or 1 mg/ml (◇)), 50 mM Tris · HCl (pH 7.8), and 0.2 mM $MgCl_2$. The substrate-syringe contained 50 mM Tris · HCl (pH 7.8), 100 mM NaCl, 20 mM KCl and either (A) 10 μM (○) or (B) 100 μM [γ-^{32}P]ATP/Tris (pH 7.8) (◇). Lines were calculated from the rate constants for experiments 5 and 6 in Table I.

[γ-^{32}P]ATP. The overall shapes of the time courses of phosphate release were similar with rubidium and potassium but both k_4 (Table I) and the steady-state rate of ATP hydrolysis were slower with rubidium indicating that rubidium is more effective than potassium in inhibiting conversion of E_2 to E_1.

As expected from the work of Post et al. [16] and Kanazawa et al. [20] the rate of hydrolysis of ATP was found to be strongly dependent on the concentration of ATP in the presence of 10 mM KCl (Fig. 2) and although a complete least squares fit was not obtained in the presence of 50 μM [γ-^{32}P]ATP, high ATP concentration appeared to increase the rate of conversion of E_2 to E_1 as well as apparently increasing k_2 and k_3.

Effects of pre-mixing enzyme with potassium and Me_2SO

It has been suggested that the apparently paradoxical observation that Me_2SO inhibits the (Na^++K^+)-dependent ATPase activity but stimulates the K^+-dependent *p*-nitrophenyl phosphate (pNPPase) activity of the transport ATPase [21] is due to the fact that the E_2 conformation of the enzyme is mainly responsible for pNPPase activity, and that E_2 is stabilized in the presence of Me_2SO [16]. Similarly, stimulation of pNPPase activity by ATP (or CTP) plus Na^+ can be explained if E_2, generated by dephosphorylation of the phosphoenzyme, is responsible for pNPPase activity [16]. If these propositions are correct then the "early burst" of phosphate release during hydrolysis of ATP by the transport ATPase should be abolished if the enzyme is converted to the E_2 conformation by pre-exposure to potassium or Me_2SO before addition of [γ-^{32}P]-ATP. Fig. 3a shows that pre-treatment of the enzyme with 20 mM KCl did in fact greatly reduce the "early burst" of phosphate causing substantial changes in the calculated rate constants (Table I). In fact only an imperfect fit to the rate equations was possible at realistic values of k_3 and this may reflect the fact that when the enzyme is in the E_2 conformation before mixing with ATP the reaction sequence is effectively a two-step pathway making the model used inapplicable. Pre-exposure of the enzyme to Me_2SO caused similar changes in the calculated rate constants (Table I) but this effect of Me_2SO was antagonized by sodium indicating that the E_1 conformation probably predominates in the pres-

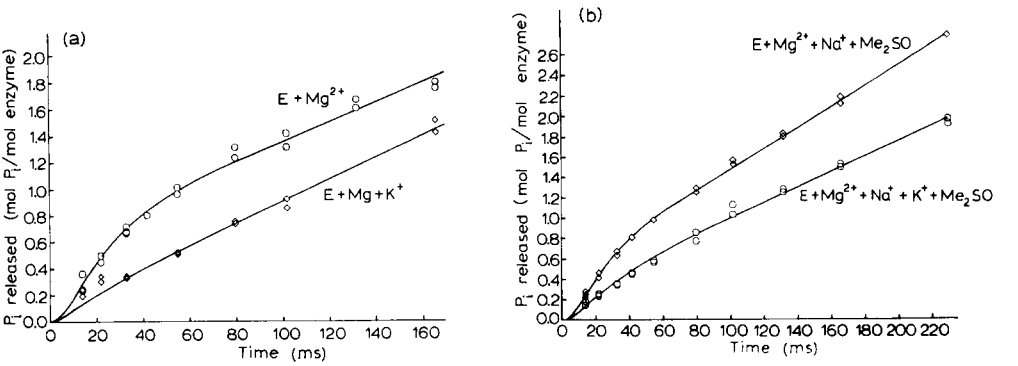


Fig. 3. Effects of pre-exposure of enzyme to K^+, Na^+ and Me_2SO on liberation of P_i by the transport ATPase. Experiments were carried out as described in Methods with the enzyme pre-mixed with 50 mM Tris · HCl (pH 7.8) and 0.2 mM $MgCl_2$ with (A) no further addition (○, Fig. 3a), (B) 20 mM KCl (◇, Fig. 3a), (C) 20% (v/v) Me_2SO and 100 mM NaCl (◇, Fig. 3b) or (D) 20% (v/v Me_2SO, 100 mM NaCl and 20 mM KCl (○, Fig. 3b). Final concentrations in all these experiments were 50 mM Tris · HCl (pH 7.8), 0.1 mM $MgCl_2$, 50 mM NaCl, 10 mM KCl, 10 μM [γ-^{32}P]ATP/Tris (pH 7.8), and (when present) 20% (v/v) Me_2SO. Lines were calculated from the rate constants for experiments 1, 7, 9 and 10 in Table I.

ence of sodium plus Me_2SO. However, in the presence of sodium, potassium and Me_2SO (Fig. 3b) the "early burst" of phosphate liberation was again suppressed indicating the presence of the E_2 conformation. Such antagonistic effects of sodium and potassium were not unexpected in view of the previously reported modifying effects of these ions on inactivation of the transport ATPase by agents such as trypsin [4], beryllium [5] and dicyclocarbodiimide [6].

The early time course of phosphorylation of the transport ATPase

The rate equations in Appendix make it possible to calculate the proportion of enzyme expected to phosphorylated during the "early burst" of phosphate release as well as during the steady state. Fig. 4 compares the expected time

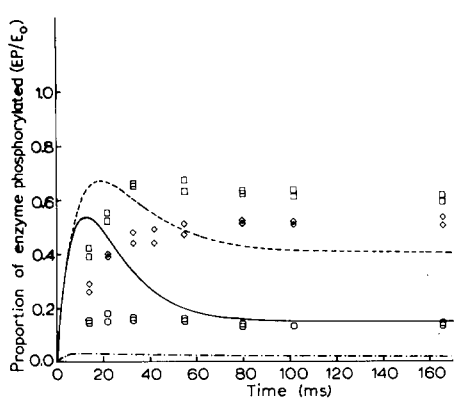
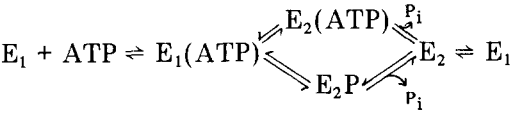


Fig. 4. Time courses for the phosphorylation of the transport ATPase by ATP under various conditions. Phospho-enzyme was measured as described in Methods in the presence of 0.5 mM KCl (◇) and 10 mM KCl (○), under the conditions described for experiments 2 and 3 (Table I and Fig. 1). Also in one experiment (□) enzyme (2 mg/ml) was premixed with 20% (v/v) Me_2SO in addition to buffer and $MgCl_2$ before starting phosphorylation in the presence of 50 mM NaCl and 0.5 mM KCl. Lines were calculated from the rate constants for experiment 2 (· · · · · ·), 3 (———) and 8 (· — · — ·) in Table I.

course for formation of E_2P (calculated using rate constants computed from the rate of release of phosphate) with the measured amounts of phospho-enzyme found under appropriate experimental conditions. In the presence of both 0.5 and 10 mM KCl the measured steady-state concentrations of phospho-enzyme agree reasonably well with values predicted using the rate constants in Table I, but during the early stages of reaction the measured amounts of phospho-enzyme were much less than those calculated and the predicted "overshoot" in phospho-enzyme was not observed. In keeping with this, values for the rate constants computed from the measured amounts of phospho-enzyme (Table I, experiments 11, 12 and 13) differ from those computed from the time course of phosphate release (experiments 2, 3 and 8). This result implies either that the phospho-enzyme is not a true intermediate of the transport ATPase (which seems unlikely in view of the wealth of supportive evidence for its existence) or that the kinetic model used is too simple. Introduction of a second phospho-enzyme species (E_1P) into the scheme is not helpful, but the secondary $E_2(ATP)$ complex suggested by Kanazawa et al. [20] and Fukushima and Tonomura [22] might explain the discrepancy if this complex were a precursor of E_2P or could be hydrolysed directly providing an alternative pathway for the release of phosphate, as shown below.



However, preliminary attempts to detect such an intermediate, by comparing the amounts of phosphate released after stopping transport ATPase activity with $HClO_4$ and with EDTA, have not so far been successful under our conditions of study. The rate constants in Table I predict very low steady-state amounts of phospho-enzyme in the presence of Me_2SO, yet in practice more phospho-enzyme was found in the presence than in the absence of this solvent (Fig. 4). As discussed above, this may indicate that the reaction pathway of the enzyme is modified by pre-exposure to Me_2SO or potassium.

Discussion

The results presented in this paper show, like the studies of Froehlich et al. [11] with the transport ATPase from electric eel, that there is an "early burst" of orthophosphate release following mixing of pig brain synaptosomal transport ATPase with ATP and 10 mM KCl. The kinetic analysis also shows that this result is fully consistent with the mechanism proposed by Post et al. [16] in which the overall rate of ATP hydrolysis in the presence of relatively high concentrations of potassium is largely determined by a rate-limiting step involving conversion of the E_2 conformation of the enzyme to E_1. The "early burst" of phosphate does not therefore demand postulation of a new relatively stable intermediate such as $E(P_i)$ (as has been suggested to explain the kinetics of both the transport ATPase [11] and the Ca^{2+}-ATPase of the sarcoplasmic reticulum [10]) and indeed prevention of the "early burst" by pre-exposure of the enzyme to potassium or Me_2SO seems much easier to explain in terms of a stabilized E_2 conformation. However, further experiments are necessary to

finally distinguish between these two possibilities.

The uses of different enzyme preparations, temperatures and concentrations of ATP make it difficult to compare the rate constants measured in this paper with those reported by Froehlich and Taylor [10] and Mardh [15,24]. However, both our results and those of Mardh indicate a dual effect of potassium on dephosphorylation of the enzyme with, at 10 mM potassium, a rapid rate of dephosphorylation followed by a relatively slow process. In the presence of 100 μM [γ-^{32}P]ATP Mardh found a substantial transient overshoot in the amount of phosphorylated enzyme present after mixing enzyme with fairly high concentrations of Na^+ and K^+ and he interpreted the later fall in phospho-enzyme concentration as being due to a rapid dephosphorylation of E_2P associated with a slower conversion of E_1P to E_2P (a conformational change in the phospho-enzyme). However, only a small overshoot in phospho-enzyme has been observed at ATP concentrations of 5—12 μM (Mardh [24], Froehlich et al. [11], this paper), yet the early pronounced burst of phosphate release persists at these lower ATP concentrations. This seems to necessitate postulation of another intermediate in the enzyme cycle, such as the E_2(ATP) suggested by Kanazawa et al. [20], and further work is needed to resolve this question.

The results reported here are consistent with the view that the transport ATPase exists mainly in one conformation (E_1) in the absence of ligands or the presence of Na^+, whereas another conformation (E_2) predominates in the presence of 20 mM K^+ or Me_2SO in the absence of Na^+: furthermore, increasing the concentration of potassium from 0.5 to 10 mM slows down the rate of conversion of E_2 to E_1. These findings are significant not only for the well-established fact that the apparent affinity of the pNPPase activity of the enzyme for potassium varies according to whether Na^+ (and CTP or ATP) or Me_2SO are present, but also for inhibition of transport ATPase by potassium at low concentrations of ATP [16]. This inhibition can be relieved by increasing ATP to high (physiological) concentrations and this is presumably either because ATP at high concentration binds to the substrate site on E_2 and this leads to a relatively rapid conversion of E_2(ATP) to E_1(ATP) or perhaps of E_2(ATP) to E_2P, or because there is a low affinity modifier site for ATP which, when occupied, accelerates the rate of conversion to E_2 to E_1. The effect of K^+ on the rate of conversion of E_2 to E_1 may also be important physiologically since if intra-cellular K^+ is responsible, this would effectively gear the rates of transport of Na^+ and K^+ to the cellular K^+ concentration.

Appendix

The early time course of release of P_i from ATP by the transport ATPase can be analysed using the following model

$$E_1 + ATP \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E_1(ATP) \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E_2P \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E_2 \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E_1$$

(ATP above $E_1(ATP)$; ADP below $E_1(ATP)$; P_i above E_2P; P_i below E_2P)

so that, initially

$$dE_2/dt = k_3E_2P - k_4E_2 + k_{-4}E_1$$

and

$$dE_2P/dt = k_2(E_0 - E_1 - E_2P - E_2) - k_3E_2P$$

where E_o is the total concentration of enzyme.

These simultaneous rate equations can be integrated using the Laplace transformation and when $[ATP] >> k_{-1}/k_1$ (K_s) and $k_3 > k_{-4}K_s/[ATP] < k_4$, the following equations can be derived.

(a) When $b > a^2$

$$E_2P = (k_2k_4E_0/b)(1 - \exp(-at)[\cos(ct) + g \cdot \sin(ct)])$$

and

$$P_i = \frac{k_2k_3k_4E_0}{b}\left[t - \exp(-at)\left\{\frac{e}{b} \cdot \sin(ct) + \frac{f}{b} \cdot \cos(ct)\right\} + \frac{f}{b}\right]$$

(b) When $b < a^2$

$$E_2P = (k_2k_4E_0/b)[1 - \exp(-at)(\cosh(ct) + g \cdot \sinh(ct))]$$

and

$$P_i = \frac{k_2k_3k_4E_0}{b}\left[t - \exp(-at)\left\{\frac{j}{h} \cdot \sinh(ct) - \frac{f}{h} \cdot \cosh(ct)\right\} - \frac{f}{h}\right]$$

(c) When $b = a^2$

$$E_2P = (k_2k_4E_0/b)[1 - \exp(-at)(1 + (a - d)\,t))]$$

and

$$P_i = \frac{k_2k_3k_4E_0}{b}\left[t - \exp(-at)\left\{\frac{f}{a^2} + \frac{(d-a)}{a} \cdot t\right\} + \frac{f}{h^2}\right]$$

In these equations

$$a = (k_2 + k_3 + k_4)/2 \qquad b = k_2k_3 + k_2k_4 + k_3k_4 \qquad c = \sqrt{|b - a^2|} \qquad d = d/k_4$$

$$e = c - ga \qquad f = d - 2a \qquad g = (a - d)/c \qquad h = c^2 - a^2 \qquad j = ga + c$$

In all the experiments described in the preceding paper ATP was 5—50 μM and since K_s (measured in the absence of Mg^{2+}) is 0.12 μM [25] the above equations for the time courses of formation of E_2P and P_i will be applicable if the model presented is correct and k_{-4} is not substantially greater than k_3 and k_4.

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