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$(Na^+ + K^+)$-ATPase: confirmation of the three-pool model for the phosphointermediates of Na^+-ATPase activity. Estimation of the enzyme-ATP dissociation rate constant

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(1) The dephosphorylation kinetics of acid-stable phosphointermediates of $(Na^+ + K^+)$-ATPase from ox brain, ox kidney and pig kidney was studied at 0°C. (2) Experiments performed on brain enzyme phosphorylated at 0°C in the presence of 20–600 mM Na^+, 1 mM Mg^{2+} and 25 μM [γ-^{32}P]ATP show that irrespectively of the EP-pool composition, which is determined by Na^+ concentration, all phosphoenzyme is either ADP- or K^+-sensitive. (3) After phosphorylation of kidney enzymes at 0°C with 1 mM Mg^{2+}, 25 μM [γ-^{32}P]ATP and 150–1000 mM Na^+ the amounts of ADP- and K^+-sensitive phosphoenzymes were determined by addition of 1 mM ATP + 2.5 mM ADP or 1 mM ATP + 20 mM K^+. Similarly to the previously reported results on brain enzyme, both types of dephosphorylation curves have a fast and a slow phase, so that also for kidney enzymes a slow decay of a part of the phosphoenzyme, up to 80% at 1000 mM Na^+, after addition of 1 mM ATP + 20 mM K^+ is observed. The results obtained with the kidney enzymes seem therefore to reinforce previous doubts about the role played by $E_1 \sim P(Na_3)$ as intermediate of $(Na^+ + K^+)$-ATPase activity. Furthermore, for both kidney enzymes the sum of ADP- and K^+-sensitive phosphoenzymes is greater than E_{tot}. (4) In experiments on brain enzyme an estimate of dissociation rate constant for the enzyme-ATP complex, k_{-1}, is obtained. k_{-1} varies between 1 and 4 s^{-1} and seems to depend on the ligands present during formation of the complex. The highest values are found for enzyme-ATP complex formed in the presence of Na^+ or $Tris^+$. (5) The results confirm the validity of the three-pool model in describing dephosphorylation kinetics of phosphointermediates of Na^+-ATPase activity.

Introduction

The energy for ion transport by the various ion translocating ATPases like the $(H^+ + K^+)$-ATPase (EC 3.6.1.36) from gastric mucosa [1], $(Na^+ + K^+)$-ATPase (EC 3.6.1.37) which is found in almost all cell membranes [2], and the Ca^{2+}-ATPases (EC 2.6.1.38) of plasma membranes or from sarcoplasmic reticulum [3,4], is ultimately derived from the hydrolysis of ATP to ADP and inorganic phosphate, P_i. Studies of the enzymatic mechanism of these ATPases are therefore a useful tool in the elucidation of the ion transport process.

It is generally agreed that the ATP hydrolysis by all these ATPases proceeds through the formation of one or more phosphoenzyme forms where the phosphate is bound covalently with an acid-stable bond to an aspartic acid residue at the substrate site (for a review, see Ref. 5). In the case of $(Na^+ + K^+)$-ATPase, with which we are concerned here, studies of phosphorylation and de-

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phosphorylation of such intermediates has led to a scheme for ATP hydrolysis, the Albers-Post scheme [6,7] (for expanded versions, see Refs. 2 and 8), which has been accepted for a number of years as a frame of reference both for enzymatic and transport studies.

$(Na^+ + K^+)$-ATPase can hydrolyze ATP and transport Na^+ not only in the presence of $Na^+ + K^+$ but also in the presence of Na^+ alone. According to the Albers-Post scheme, the steps involving phosphorylation, several of the phosphorylated intermediates, and Na^+-transport are the same whether $Na^+ + K^+$ or Na^+ alone is present. This hypothesis has, however, been disputed [9–12] and recently we have obtained further experimental evidence against the common involvement of some of the phosphorylated intermediates [13–15]. A detailed study of the dephosphorylation kinetics of the phosphoenzyme species produced in the presence of $[^{32}P]ATP$, Na^+ and Mg^{2+} (but in the absence of K^+) led to the following conclusions [15]:

(a) The minimal scheme for ATP-hydrolysis with Na^+ alone contains at least three different EP-pools, A, B and C, in succession (Fig. 1).

(b) A can react directly with ADP (commonly described as 'ADP-sensitive phosphoenzyme'). Addition of ADP also leads to fairly rapid disappearance of part of pool B, which, especially at higher (extracellular) Na^+-concentrations is converted rapidly to A.

(c) K^+ reacts with B and C and this leads to rapid dephosphorylation of these two pools. After addition of K^+ and the rapid disappearance of B and C, only A ('ADP-sensitive EP') is left and under these conditions pool A disappears slowly since

(d) K^+ blocks the conversion of A to B, i.e. blocks, or drastically slows down, the conversion of 'ADP-sensitive EP' to 'K^+-sensitive EP'. This, of course, makes the ADP-sensitive EP, formed in the presence of Na^+ alone, incompetent as an intermediate in the $(Na^+ + K^+)$-ATPase cycle [15].

The present report investigates further some of the properties of the different EP-species. Firstly it is demonstrated, in accordance with conclusion (b) and (c) above, that the EP remaining after K^+ provoked dephosphorylation is all ADP sensitive, and that, conversely, after primary dephosphorylation with ADP the EP is all K^+ sensitive.

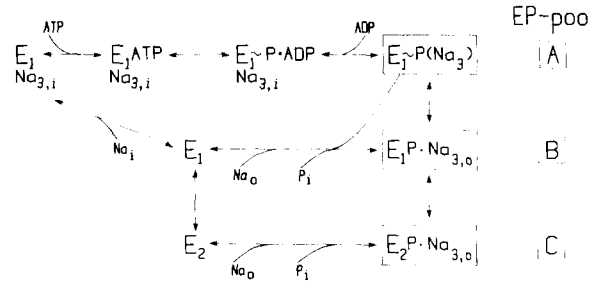


Fig. 1. Minimal scheme for Na^+-ATPase activity and Na^+-transport by $(Na^+ + K^+)$-ATPase (K^+ absent). Subscript i and o signifies 'intracellular' and 'extracellular' respectively. (Na) in pool A is probably occluded in the membrane [2]. The EP-pools A, B and C are equilibrium pools in which the Na occupancy may vary as detailed in Fig. 8 Ref. 15. It is not known whether the dissociation of Na^+ from and the dephosphorylation of $E_1 \sim P$, E_1P and E_2P (to E_1 and E_2) is ordered or random.

Secondly it is shown that the characteristic features of dephosphorylation kinetics are common for the brain enzyme and two types of kidney enzyme. The former is about 10% pure and contains two forms of the catalytic subunit, α and α^+, whereas the kidney enzymes are purer and contain only α [16,17]. And thirdly it is confirmed by direct measurements that the slow phase of ADP dephosphorylation, following the removal of pool A and part of pool B (Fig. 1 and Ref. 15), represents the dissipation of what is left of the K^+-sensitive EP (B and C, Fig. 1) and not, as has been suggested by Pickart and Jencks [18] for Ca^{2+}-ATPase, a slow disappearance of acid-stable $E_1 \sim P + E_1 \sim P \cdot ADP$ due to a slow dissociation of ATP from EATP.

Materials and Methods

Enzyme preparations. $(Na^+ + K^+)$-ATPase was prepared from ox brain as described by Klodos et al. [19], from ox kidney by the SDS procedure of Jørgensen [20] and from pig kidney by the SDS procedure of Jørgensen [20] with the modifications given by Jensen et al. [21]. The preparations had the following properties (ox brain, ox kidney, pig kidney): $(Na^+ + K^+)$-ATPase activity (U/mg protein): 4–5, 15, 12; K^+-dependent *p*-nitrophenylphosphatase activity (U/mg protein): 0.75,

2.4, 2.1. One unit (U) corresponds to 1 μmol substrate split per minute. The phosphorylation site concentration (nmol/mg protein) was 0.4–0.5, 2.1, 1.6, respectively.

Formation and determination of acid-stable phosphorylated intermediates. Generally, the radioactive phosphorylated intermediates, $E^{32}P$, were formed by incubation of the enzyme preparation (0.1–0.3 mg protein per ml) for 30–60 s at 0°C with 25 μM [γ-^{32}P]ATP, 1 mM $MgCl_2$ and varying concentrations of Na^+, in 30 mM imidazole buffer (pH 7.4 at 0°C). Specific conditions regarding preincubation procedures and dephosphorylation experiments are given under Results and in the legends to figures and tables.

Determination of the acid-stable $E^{32}P$ was performed as described earlier [13], except that the EP was precipitated with 5% trichloroacetic acid in the presence of 1 mM sodium pyrophosphate. Phosphorylation blanks, socalled K^+-blanks, accounting for unspecific phosphoprotein formation, were obtained by substituting all Na^+ with K^+ under otherwise identical conditions. They comprised about 3% of maximal phosphorylation. For a discussion of blanks, see Ref. 13.

SDS-gel electrophoresis. The electrophoresis was carried out on 6% acrylamide slab gels using the buffer system of Laemmli [22].

Reagents. All reagents were reagent grade. ATP and ADP were obtained as sodium salts from Boehringer Mannheim Biochemicals, F.R.G., and [γ-^{32}P]ATP was obtained from New England Nuclear, Boston, MA and Amersham International, U.K. ATP was converted to its Tris salt by chromatography on a Dowex I column (Sigma Chemical Co., ST. Louis, MO), and [γ-^{32}P]ATP was purified on DEAE-Sephadex G-25 [23]. Purified ATP contained < 0.5 mol% ADP. Since the K^+ contamination in commercial ADP batches can be rather high (corresponding to 100 μM K^+ in a 2.5 mM ADP solution), ADP was purified by chromatography on a Dowex 50 WH^+ column. The acid eluate was neutralized immediately by propanediol.

Results

The EP-pool consists entirely of ADP- or K^+ sensitive phosphoenzymes

When ($Na^+ + K^+$)-ATPase is incubated with ATP, Na^+ and Mg^{2+} at 0°C as described in Methods (and in the figure legends) the steady-state concentration of phosphointermediates, EP, corresponds to 90–100% of the ATP-binding site concentration [13]. Classically, EP is considered to consist of ADP-sensitive, $E_1 \sim P$, and K^+-sensitive phosphoenzyme, E_2P, and it has been customary [15,24,25] to determine these two types of EP in dephosphorylation experiments with ADP and K^+, respectively. In this case the percentage proportion of $E_1 \sim P$ and E_2P by definition will be 100% − *i*ADP and 100% − *i*K, respectively, where 100% is the level of EP before addition of ADP or K^+ and *i*ADP and *i*K are ordinate intercepts of the linear extrapolations, in semilog plots, of the slow phases of the dephosphorylation curves (e.g. Fig. 1 Ref. 15 and Fig. 3, this paper).

When $E_1 \sim P$ and E_2P were thus defined and determined it appeared that in several experiments with ($Na^+ + K^+$)-ATPase from the electric organ of *Electrophorus electricus* [24,26–29], from the rectal gland of the shark [26] and from ox brain [15] the sum $E_1 \sim P + E_2P$ was larger than 100%. This showed that the two-pool model for EP composition was insufficient and lead us to propose a three-pool model as the minimum requirement, see Fig. 1 and Ref. 15. Also this model requires, however, that all the acid-stable phosphointermediates are ADP-sensitive and/or K^+-sensitive.

The validity of this part of the hypothesis is confirmed by the experiments shown in Fig. 2. Ox brain ($Na^+ + K^+$)-ATPase was phosphorylated with 25 μM [γ-^{32}P]ATP, 1 mM Mg^{2+} and 20, 300 or 600 mM Na^+, respectively. After 60 s, phosphorylation by radioactive ATP was stopped by addition of 1 mM unlabelled ATP. Dephosphorylation of $E^{32}P$ was monitored after simultaneous or sequential addition of 2.5 mM ADP and 20 mM K^+. Irrespective of the composition of the EP-pool, which is determined by the Na^+ concentration, the following results were obtained: More than 97% of the $E^{32}P$ disappears rapidly (within 2 s) when 1 mM ATP, 2.5 mM ADP and 20 mM K^+ are added simultaneously (data not shown). If ADP or K^+ are added alone with the 1 mM ATP the curves are biphasic as usual, and when the 'missing' ligand (K^+ or ADP) subsequently is added during the slow phase of dephosphorylation, virtually all remaining $E^{32}P$ disap-

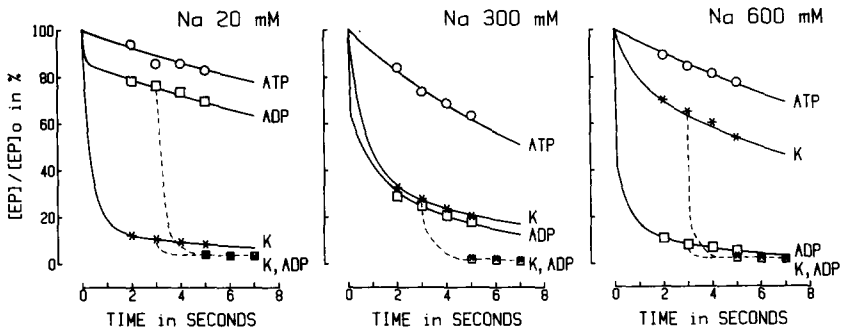


Fig. 2. Dephosphorylation characteristics of the phosphointermediates, EP, of ox-brain Na^+-ATPase. $E^{32}P$ was formed (and dephosphorylated) at 0°C in the presence of 20, 300 and 600 mM Na^+ as described in Methods and the text. After 60 s, dephosphorylation of $E^{32}P$ was initiated ($t = 0$) by addition of 1 mM ATP (○), 1 mM ATP + 2.5 mM ADP (□) or 1 mM ATP + 20 mM K^+ (*). A further addition after 3 s of 20 mM K^+ to the 'ADP experiment' or 2.5 mM ADP to the 'K^+ experiment' led to virtually complete disappearance of $E^{32}P$ (dotted lines (⊡)). The points show the average (± S.D., covered by the points) of three or four experiments. The full lines are calculated according to the three-pool model for phosphorylated intermediates (Fig. 1) as described in detail in Ref. 15. The rate constants used were similar to those in Ref. 15.

pears rapidly (within 2 s, Fig. 2). There is not more than 3% of 'insensitive' EP [30,31] in steady state and no such form is produced during dephosphorylation with ADP or K^+.

ADP- and K^+-sensitive phosphointermediates of ($Na^+ + K^+$)-ATPase from different sources

In the above we confirmed the validity of three-pool model in description of dephosphorylation kinetics of phosphoenzymes from ox brain.

In the present paper we also compare the properties of phosphoenzymes from brain with those of kidney enzymes. It has been previously shown that the phosphoenzyme formed by pig kidney enzyme, in the presence of 150 mM Na^+, is, contrary to brain enzyme, almost 'ADP-insensitive' [32]. Furthermore, enzyme preparations from kidney differ from our brain enzyme in certain other ways: The specific activity is higher (Methods), and they contain only one type of α-peptide (namely α), whereas in the brain enzyme two closely positioned 100 kDa bands (α and α^+) could be demonstrated by gel electrophoresis (Refs. 16 and 17, and experiment in cooperation with dr. P. Ottolenghi, data not shown) *.

We now show that biphasic dephosphorylation curves, after addition of either ADP or K^+, are also characteristic of the phosphoenzyme from ox kidney and pig kidney ($Na^+ + K^+$)-ATPase (Fig. 3). It is in line with earlier studies, that the ratio ADP-sensitive/K^+-sensitive EP increases with [Na^+] and that the quantitative relationship between these parameters depends on, among other things, the source of the enzyme (for references, see Ref. 13).

Validity of the three-pool model for kidney enzymes is shown by the fact that for both of them the sum $E_1 \sim P + E_2P$ is greater than 100%, see Table I. This is especially evident for the pig kidney enzyme when the ratio between these two EP-classes approaches 1. For comparison Table I contains corresponding data for the ox brain enzyme obtained in the authors laboratory [15]. Furthermore, similarly to data on brain enzyme, the slow disappearance of a part of the phosphoenzyme, up to 80% at 1000 mM Na^+, after addition of K^+, seems to indicate a slow conversion (if any) of pool A to pool B and C in the presence of K^+, see (c) and (d) in Introduction.

The slow phase in dephosphorylation caused by ADP is not influenced by the enzyme-ATP dissociation rate constant

Our usual interpretation of the biphasic 'ADP-dephosphorylation' curve (e.g., Fig. 2 and 3) is that the fast phase represents dephosphorylation of pool A ($E_1 \sim P$) and that the slow phase characterizes the amount and the rate coefficients for

* Our earlier inability to demonstrate α^+ in brain enzyme [36] was due to the use of a less selective gel electrophoresis system.

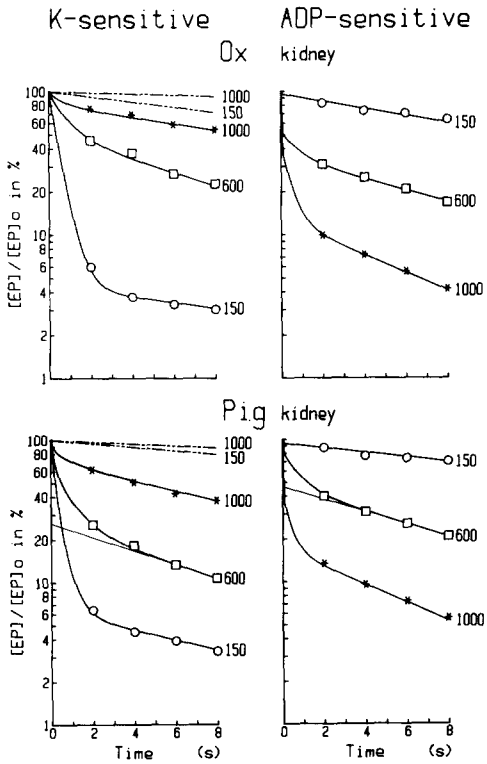
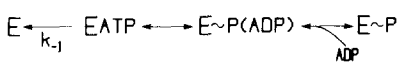


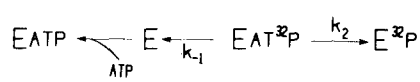
Fig. 3. Dephosphorylation of the phosphoenzymes (EP) from pig and ox kidney ($Na^+ + K^+$)-ATPase. The enzymes were phosphorylated with 25 μM [^{32}P]ATP and the Na^+ concentrations (mM) indicated for each curve (Methods). After 60 s ($t = 0$) either 1 mM ATP (dotted lines), 1 mM ATP + 20 mM K^+ (left-hand panel) or 1 mM ATP + 2.5 mM ADP (right-hand panel) was added and $E^{32}P$ was determined (Methods) at the time points shown. For the sake of clarity the points are left out from the 'ATP curves' and only curves (dotted lines) for $Na^+ = 150$ mM and 1000 mM are shown. All lines (except those extrapolating the pig-kidney 600 mM Na^+ curves back to the ordinate) are calculated on the basis of the three-pool model (Fig. 1) as described in principle in Ref. 15. As exemplified for pig kidney, $Na^+ = 600$ mM, the amount of K^+-sensitive phosphoenzyme is determined as rapidly disappearing EP after K^+ addition (left-hand panel), here equal to 100%–25% = 75%, likewise the ADP-sensitive EP in this case is 100% – 45% = 55%. For further explanation see the first paragraph in Results.

(spontaneous) dephosphorylation of the remaining $E^{32}P$, i.e. part of pool B and pool C (c.f. Fig. 1 and Ref. 15). It has justifiably been argued, however (Reynolds, J., Tanford, C. and Johnson, E., personal communication), that the biphasic curve obtained upon addition of 'saturating' [ADP] might reflect a primary rapid conversion of $E_1 \sim P$ to $E_1 \sim P(ADP)$, a secondary rapid equilibration between $E_1 \sim P(ADP)$ and E_1ATP and a subsequent slow dissociation of EATP. These processes are illustrated in Scheme I. This is the interpretation that Pickart and Jencks offer for the dephosphorylation kinetics of the sarcoplasmic reticulum Ca^{2+}-ATPase [18]. Here the $\sim P$ bond in both $E \sim P$ and $E \sim P(ADP)$ is presumed to be acid-stable and the sum of both species is therefore determined by the usual acid precipitation of EP. $E \sim P(ADP)$ and EATP are assumed to be in rapid equilibrium. EATP → E is pictured as a 'one-way' process because E is captured in a dead-end complex, EADP (not shown).



Scheme I.

To distinguish between our usual model and that proposed by Pickart and Jencks, it is necessary to obtain an estimate of the rate constant k_{-1} for the dissociation of EATP under the conditions of our dephosphorylation experiments. The rationale behind the experiments described below (and in Table II and Fig. 4) is illustrated in Scheme II.



Scheme II.

In this scheme ATP is unlabelled nucleotide, $AT^{32}P$ is radioactive nucleotide ([^{32}P]ATP) and the parameter measured is acid-stable $E^{32}P$, which has the same specific radioactivity as [^{32}P]ATP. The two left-going processes are essentially irreversible since [ATP]/[[^{32}P]ATP] = 40. The assumption that $E^{32}P$ is not dephosphorylated and that its formation may be described by one rate constant, k_2, is discussed below.

The $EAT^{32}P$ complex is formed with 25 μM [^{32}P]ATP under conditions where one or more of the ligands necessary for phosphorylation are missing (i.e. $k_2 = 0$, 'preincubation' conditions Table II). On simultaneous addition of the missing ligands and 1 mM unlabelled ATP (40-times [^{32}P]ATP), $EAT^{32}P$ will disappear by dissociation and by formation of $E^{32}P$. As shown below, determination of the 'rate of formation' of $E^{32}P$

TABLE I

PROPORTION OF 'ADP-SENSITIVE' AND 'K^+-SENSITIVE' EP AS A FUNCTION OF ENZYME SOURCE AND Na^+

Determination of steady-state values for '$E_1 \sim P$' and 'E_2P' (in the absence of K^+) was carried out as described in the text. The kidney enzyme data are taken from the duplicate experiments in Fig. 2.

Enzyme source	$[Na^+]$ (mM)	'$E_1 \sim P$' (%) (100 − *i*ADP)	'E_2P' (%) (100 − *i*K)	'E_1P' + 'E_2P' (%)
Ox brain *	20	15	88	103
	300	67	68	135
	600	83	38	121
Ox kidney	150	9	96	105
	600	63	45	108
	1000	87	18	105
Pig kidney	150	10	94	104
	600	55	75	130
	1000	84	32	116

* The values for ox brain enzyme are taken from Nørby, Klodos, Christiansen [15].

TABLE II

APPARENT RATE CONSTANTS FOR PHOSPHORYLATION, k_2, AND EATP DISSOCIATION, k_{-1}

Ox brain ($Na^+ + K^+$)-ATPase, 0.4 mg protein/ml, was equilibrated 10 min at 0°C with 30 mM imidazole-HCl buffer (pH 7.4), 1 mM EDTA (expts. 1–3, 5–9), 1 mM Mg^{2+} (Expt. 4), 150 mM Na^+ (Expts. 5, 9) or 60 mM $Tris^+$ (Expts. 3, 8). This was followed by 'Preincubation, 30 s with' the ligands indicated. In the first series of experiments the missing ligands were added and $E^{32}P$ after 1 s determined ($E^{32}P_1/E_{tot}$). In the second series, 1 mM ATP was added with the missing ligands and $E^{32}P$ after 1 s determined ($R_0 = E^{32}P_{1,ATP}/E^{32}P_1$, see Eqn. 3). The rate constants were calculated as described in the text (Eqns. 2, 3, 5 and Fig. 4 A, B). In all experiments, $E^{32}P_1$, E_{tot} and R_0 were corrected for the $E^{32}P$ formed during "Preincubation". This was about 0% in Expts. 1–3 and 6–8, 5% in Expts. 5 and 9, and 15% in Expt. 4 (contaminating Mg^{2+} and Na^+, respectively).

Expt. No.	Preincubation 30 s with $[^{32}P]$ATP, 25 μM and	Missing ligands, mM		$\frac{E^{32}P_1}{E_{tot}}$ (%) mean ± S.D.	(n)	Phosphorylation 'rate constant' k_2 (s^{-1})	R_0, see Eqn. 3 mean ± S.D.	Dissociation 'rate constant', k_{-1} (s^{-1}) from k_2 and R_0 (Fig. 4A)	see Fig. 4B
1	–	Mg Na	1 150	57 ± 3	(3)	0.8–0.9	46 ± 2	2.1–2.4	1.4
2	–	Mg Na	1 300	51 ± 6	(2)	0.6–0.8	37 ± 2	2.7–3.4	1.4–2.1
3	Tris 60 mM	Mg Na	1 150	82 ± 8	(3)	1.3–2.2	42 ± 8	2.1–5	> 3.5
4	Mg 1 mM	Na	150	76 ± 3	(3)	1.3–1.6	61 ± 3	1.3–1.7	1
5	Na 150 mM	Mg	1	100 ± 3	(3)	> 3.5	52 ± 1	3.3–3.7	> 3.5
6	–	Mg [a] Na	1 150	34 ± 4	(3)	0.4–0.5	31 ± 4	3.0–4.0	> 3.5
7	–	Mg [a] Na	1 300	43 ± 6	(3)	0.5–0.7	27 ± 3	3.7–5.1	> 3.5
8	Tris 60 mM	Mg [a] Na	1 150	58 ± 4	(4)	0.8–1.0	32 ± 3	3.3–4.4	> 3.5
9	Na 150 mM	Mg [a]	1	47 ± 4	(3)	0.6–0.7	36 ± 2	2.8–3.3	> 3.5

[a] 20 mM KCl added with the missing ligands.

in such experiments and in the parallel experiments where no unlabelled ATP is added (i.e. $k_{-1} = 0$) allows one to obtain an estimate of k_{-1}.

With these assumptions, the amount of $E^{32}P$ formed in 1 second after addition of the missing ligands and 1 mM unlabelled ATP, $E^{32}P_{1,ATP}$, is (Scheme II)

$$E^{32}P_{1,ATP} = E_{tot} \cdot \frac{k_2}{k_{-1} + k_2}(1 - e^{-(k_{-1}+k_2)}) \quad (1)$$

An estimate of k_2 is obtained in the parallel experiment where no ATP is added (k_{-1} effectively equal to zero)

$$E^{32}P_1 = E_{tot}(1 - e^{-k_2}) \quad (2)$$

It is obvious that $E^{32}P_{1,ATP} \leqq E^{32}P_1$. From the ratio R_0 between these parameters

$$R_0 = \frac{E^{32}P_{1,ATP}}{E^{32}P_1} = \frac{k_2}{k_{-1} + k_2} \frac{1 - e^{-(k_{-1}+k_2)}}{1 - e^{-k_2}} \quad (3)$$

one can now estimate k_{-1} when k_2 is known.

The conditions of these experiments and the results ($E^{32}P_1/E_{tot}$, R_0, k_2 and k_{-1}) are given in Table II. The method of estimating k_{-1} from R_0 and k_2 is illustrated in Fig. 4A for some typical experiments (Expts. 1, 4, 5 and 6, Table II).

A second way of determining k_{-1} was also attempted. Following the 30 s preincubation (Table II), 1 mM unlabelled ATP (and 20 mM K^+ in Expts. 6–9) was added. After 1, 2 or 3 s the medium was supplemented with the missing ligands and phosphorylation allowed to proceed for 1 s. According to Scheme II the amount of $E^{32}P$ formed is expressed by

$$E^{32}P_{1,ATP(t)} = E_{tot} \cdot \frac{k_2}{k_{-1} + k_2} \cdot (1 - e^{-(k_{-1}+k_2)}) \cdot e^{-k_{-1}t} \quad (4)$$

Defining the ratio $R_t = E^{32}P_{1,ATP(t)}/E^{32}P_1$, division of Eqn. 4 with Eqn. 2 gives

$$R_t = R_0 \cdot e^{-k_{-1}t} \quad (5)$$

In Fig. 4B values for R_t are plotted against time for four typical experiments. As R_t after 1 s is

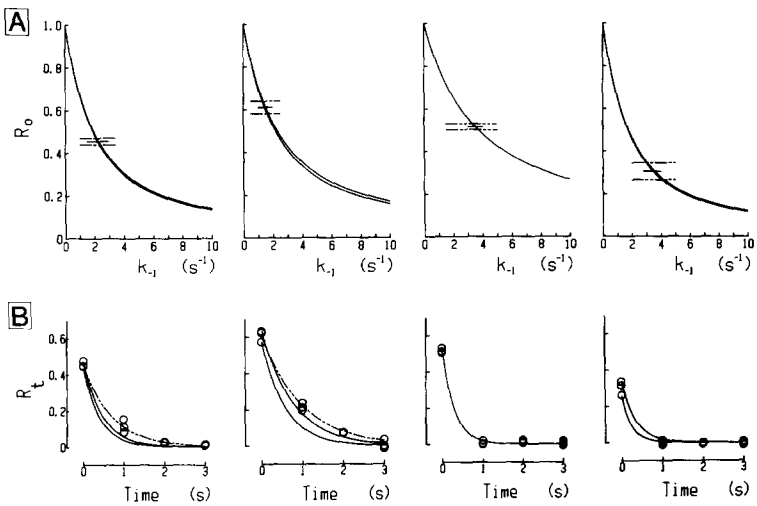


Fig. 4. This figure illustrates two methods for estimation of the $EAT^{32}P$ dissociation rate constant k_{-1}. Experiments No. 1, 4, 5 and 6, see Table II, are shown from left to right.

(A) The relationship between R_0, the ratio between $E^{32}P$ formed in 1 s in the presence and absence of 1 mM unlabelled ATP (Eqn. 3 and Table II), and k_{-1}. The curves are drawn using the phosphorylation 'rate constants', k_2, (Eqn. 2 and Table II). The horizontal lines show R_0 taken from Table II.

(B) R_t, defined in Eqn. 5 and signifying the relative amount of $E^{32}P$ formed in 1 s, as a function of the time between addition of 1 mM ATP to preformed $EAT^{32}P$ and completion of the medium with the missing ligands. Expt. 1, ($Mg^{2+} + Na^+$); expt. 4, (Na^+); expt. 5, (Mg^{2+}) and expt. 6, ($Mg^{2+} + Na^+ + K^+$). For details see Table II. Full lines are calculated with the k_{-1} values from panel A, dotted lines (expts. 1 and 4) represent a better fit of $R_t = R_0 \cdot e^{-k_{-1}t}$ (Eqn. 5).

close to zero in several cases (Nos. 5–9, Table II) one can under these conditions only obtain a lower estimate of k_{-1}. The estimates of k_{-1} from Eqn. 5 and Fig. 4B are also listed in Table II. As can be seen from Eqn. 5, this method is independent of the exact value of k_2. The two methods for k_{-1} determination give comparable values. There is thus good reason to believe that the value of k_2 determined in the presence of 25 μM ATP (equation (2)) is valid at the 1 mM concentration of ATP present in the determination of R_0 and thereby k_{-1} by the former method.

Before we evaluate the results, a word of caution should be said about the application of the simple model in Scheme II and Eqns. 1–5 when K^+ is added together with the missing ligands as in Expts. 6–9. The assumption, that $E^{32}P$ is not dephosphorylated during the 1-s phosphorylation period does not hold with K^+ in the medium since K^+ will rapidly dephosphorylate all the 'E_2P' (pool B and C, Fig. 1) that is formed. $E^{32}P_i$ is therefore smaller than predicted by the model and k_2 in Expts. 6–9 is therefore definitely a minimal estimate. As regards k_{-1} in these cases, the fact that $E^{32}P_{i,ATP}$ (1 mM unlabelled ATP added with the missing ligands and K^+) is only about 1/3 of $E^{32}P_i$ (no unlabelled ATP) shows that k_{-1} is somewhat larger than k_2, so that the values obtained $k_{-1} = 3$–$5\ s^{-1}$, appear quite realistic.

Since we, however, were mainly concerned with the interpretation of biphasic 'ADP-dephosphorylation curves', see first paragraph in this section, Expts. 1–5 are of prime importance. Here k_{-1} is always larger than $1\ s^{-1}$ and in four out of five experiments k_{-1} is $> 2\ s^{-1}$. There may be a tendency for k_{-1} to be smallest when Mg^{2+} is present in the preincubation medium (the preformed complex is then $EAT^{32}P \cdot Mg$, Expt. 4) and largest when the enzyme is preincubated with Na^+ (or $Tris^+$).

Although our first priority was to measure k_{-1}, the experiments also give us some information regarding the 'phosphorylation rate constant' k_2. From the experiments without added K^+, Expts. 1–5 in Table II, it is obvious that k_2 is dependent on the ligands in the preincubation medium. If we assume that the binding of the ligands Na^+, Mg^{2+} and $Tris^+$ is diffusion controlled and therefore very fast ($k_{on} = 10^9\ M^{-1} \cdot s^{-1}$, see Discussion), it is concievable that the $EAT^{32}P$(cation) complex

TABLE III

PUBLISHED RATE CONSTANTS FOR $E + L \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} EL$ WHERE E IS $(Na^+ + K^+)$-ATPase AND L IS THE LIGAND SHOWN IN COLUMN 1

Ligand (L)	k_1 ($M^{-1} \cdot s^{-1}$)	k_{-1} (s^{-1})	Ligands	Conditions	Ref.
ATP		9	2 mM Mg	Guinea pig kidney	33
		20	no ligands	directly (from exchange	
		30	120 mM Na	with unlabelled ATP, 25°C)	
FTP	$(1–3) \cdot 10^7$	33	40 mM Na	pig kidney, directly	42
FDP	$3 \cdot 10^7$	112		(fluorescence, 21°C)	
Eosin	$4 \cdot 10^7$	11	20 mM Na	Shark rectal gland, directly (fluorescence, 6°C)	35
ATP	10^7	35	Mg, Na, K	Electroplax, simulation of transient kinetics, 20°C	43
ATP	$3 \cdot 10^7$	20	150 mM Na	Ox brain, simulation of steady-state kinetics	44, 45
MgATP	10^7	6		37°C	
ATP		1.5	Mg	Ox brain, directly	Table II,
		2.5	No ligands	(from exchange with	present
		3.5	Na	unlabelled ATP, 0°C)	paper

during preincubation is slowly transformed from a non-phosphorylating form (E_2) to a phosphorylating form (E_1). These observations and reasoning is in accordance with previously published results [33–35].

Discussion

Interpretation of the biphasic dephosphorylation curves with ADP and K^+

For $(Na^+ + K^+)$-ATPase it is universally accepted that at least two types of EP are formed when the enzyme is phosphorylated in the presence of ATP, Mg^{2+} and Na^+ (but in the absence of K^+). '$E_1 \sim P$' is formed first and reacts readily with ADP to form ATP. The subsequent 'E_2P' is produced from '$E_1 \sim P$', and 'E_2P' does not react directly with ADP, but it is 'K^+-sensitive' in the sense that K^+ catalyzes its rapid dephosphorylation (for a discussion and reference to earlier work consult Refs. 2, 13, 15 and 36).

It is expected – it actually is the basis of the above view on the phosphorenzymes – that upon addition of ADP a portion of EP will disappear rapidly and a similar picture is seen when K^+ is added.

We have only been able to find two studies in which the simple experiment to add K^+ together with ADP and watch all EP disappear rapidly or to add K^+ and ADP (or ADP and K^+) sequentially to ensure, that what is left of EP after addition of one of the ligands is really dephosphorylated rapidly by the other, has been performed. This has been done by Fukushima and Post [37] with a Ca^{2+}-phosphoenzyme from guinea pig kidney $(Na^+ + K^+)$-ATPase and by Taniguchi et al. [38] with a pig kidney phosphoenzyme. In the present paper we have found for the ox brain enzyme that virtually all EP disappears upon simultaneous addition of ADP and K^+ (data not shown) and the components (EP complexes) dephosphorylating slowly in the 'ADP experiment' or 'K^+-experiment' are rapidly dephosphorylated when K^+ or ADP is added, see Fig. 2. (In the experiments in this section there was 1–3% of the phosphoenzyme, after subtraction of the blanks, that was 'insensitive' to both ADP and K^+. The question whether this EP-species really belongs in the blank-pool, or whether it represents a type of 'insensitive EP' described by Post et al. [30] and Askari and Huang [31] is not resolved).

ADP- and K^+-sensitive phosphointermediates of $(Na^+ + K^+)$-ATPase from different sources. In a recent paper [36] we discussed (and rejected) the suggestion that biphasic dephosphorylation curves might reflect inhomogeneity in the brain enzyme preparation used in our investigations (Refs. 13 and 15, and the present paper). Here we now confirm the common observation that the shape of these dephosphorylation curves, whether obtained by ADP or by ATP + K^+ additions, is 'bi-' or 'multi-phasic' for phosphointermediates of Na^+-ATPase from various sources.

We also show that the sum of the ADP-sensitive and the K^+-sensitive amounts of EP is larger than 100% of E_{tot}, not only for brain enzyme (present paper and Ref. 15), but also for kidney enzyme from ox and pig. Note that the dephosphorylation curves in Fig. 3 were all simulated by the three-pool model [15] although, because of obvious species (source) differences in Na^+ sensitivity, the same rate coefficients could not be used for brain and kidney enzyme. That ['$E_1 \sim P$'] + ['E_2P'] > 100% has now been demonstrated for $(Na^+ + K^+)$-ATPase from ox brain [15], rat brain [39], the electric organ of *Electrophorus electricus* [24,26–29], shark rectal gland [26] and kidney from the rat [39], ox and pig (present paper). In this connection it might be of interest that only the preparations from brain tissue have the two types of catalytic peptide α and α^+, whereas the kidney preparations (Refs. 16 and 17, and the present paper) and those from the electric eel have only the α-peptide form [16]. The kinetic differences between α and α^+ are small or absent especially within brain preparations (Ref. 40, and Sweadner, K.J., personal communication) and it should be clear that neither the biphasic dephosphorylation curves, nor the fact that ['$E_1 \sim P$'] + ['E_2P'] > 100% can be ascribed to an inhomogeneity of the enzyme preparation.

The slow phase of 'K^+-dephosphorylation curves'

The rapid disappearance of K^+-sensitive EP in these experiments is followed by a slow dephosphorylation. The amount of EP in this slow phase increases with Na^+. The slope (rate coefficient) is approx. 0.1 s^{-1}, independently of the Na^+ con-

centration, as can be seen from Figs. 2 and 3 in the present paper as well as Figs. 2 and 3 in Ref. 15. It should also be noted that the rate coefficient for this slow dephosphorylation is obtained with 1 mM ATP in the medium, a concentration which is close to optimal for $(Na^+ + K^+)$-ATPase activity. ATP in the range 25 μM to 1 mM seems, however, to be without influence on this process since the rate coefficient is 0.1 s^{-1} whether the chase for stopping phosphorylation with [^{32}P]ATP is 1 mM ATP or 10 mM EDTA (not shown). A rate coefficient of 0.1 s^{-1} is about one order of magnitude smaller than expected if the slow component was unmodified ADP-sensitive $E_1 \sim P$ (pool A). In that case the rate coefficient should be around 1 s^{-1}, namely the sum of $k_A = 1\ s^{-1}$ (conversion of A to B, Fig. 1) and $k_a = 0.1\ s^{-1}$ (spontaneous dephosphorylation of A, Fig. 1), as is apparent from our analysis in Ref. 15. This discrepancy led us to conclude that the presence of K^+ prevents the conversion of A to B (prevents the deocclusion of Na^+ to the external phase) so that the slow phase reflects the spontaneous dephosphorylation of $E_1 \sim P(Na_3)$, $k_a = 0.1\ s^{-1}$. The fact that the component in the slow phase disappears rapidly when ADP is added (Fig. 2) confirms our conclusion that it represents pool A (with bound K^+!) and not some K^+- and ADP-insensitive 'dead end' form of EP. The inhibitory effect of K^+ on the $A \rightarrow B$ conversion was shown to make $E_1 \sim P(Na_3)$ kinetically incompetent (at least at 0°C) as an intermediate in the $(Na^+ + K^+)$-ATPase reaction, although it was a perfectly competent intermediate in the Na^+-ATPase cycle [14,15].

The 'ADP-dephosphorylation curves'

The rapid phase in 'ADP-experiments' according to our analysis represents disappearance of $E_1 \sim P(Na_3)$ and part of $E_1\text{-}P \cdot Na_{3,o}$ (pool A and B, respectively, Fig. 1, and Ref. 15). The slow phase then characterises the dissipation of the rest of pool B and $E_2P \cdot Na_{3,o}$, pool C. These species should both be K^+-sensitive and Fig. 2 confirms that this is so.

However, proponents of Pickart's and Jencks' model for 'ADP-dephosphorylation curves', Scheme I and Ref. 18, might argue that the effect of K^+ on the slow phase is simply to increase the rate constant, k_{-1}, for EATP dissociation, the step, that according to their analysis is rate limiting (at least in the case of Ca^{2+}-ATPase). It has actually been reported by Shigekawa and Kanazawa [41] that K^+ under certain conditions increases k_{-1} for Ca^{2+}-ATPase, and for $(Na^+ + K^+)$-ATPase it is well known that K_{diss} for EATP increases drastically when K^+ is added in equilibrium binding experiments [23]. We have specifically adressed the problem concerning the interpretation of the slow phase in 'ADP-experiments' (third section in Results) which we shall discuss in the following.

The rate constant, k_{obs}, for the slow phase of 'ADP-dephosphorylation curves' is dependent upon $[Na^+]$. In our hands it increases from 0.05 s^{-1} for $[Na^+] = 20$ mM to about 0.25 s^{-1} for $[Na^+] = 600$ mM (see Fig. 2 in Ref. 15). Under comparable conditions we have now obtained estimates of the EATP dissociation rate constant and it appears from Table II, that it is in all cases 5–10 times larger than k_{obs} for the slow phase in 'ADP-dephosphorylation' experiments. So, firstly the slow phase cannot be explained by a rate limiting EATP dissociation step and (therefore) secondly, even if K^+ might increase EATP dissociation (Table II) this cannot be the explanation for the K^+ effect. Thus there is no support for the model of Pickart and Jencks [18] in our study. The off-rate constant for EATP, k_{-1}, estimated in the present study may be evaluated by comparison with similar constants determined by others in various ways. Table III gives some more or less directly determined values for k_{-1} for the dissociation of ATP or analogs from $(Na^+ + K^+)$-ATPase. They are all larger than those (minimal values) determined here at 0°C probably because of the differences in temperature, but enzyme source may also play a role. It is interesting that the general influence of ligands observed by Mårdh and Post [33] for the guinea pig kidney enzyme at 25°C is reproduced by us for ox brain enzyme at 0°C, in that k_{-1} (with Mg^{2+}) $< k_{-1}$(no ligands) $< k_{-1}(Na^+)$, see Table III.

The value for k_{-1} might also be estimated from $K_{diss} = k_{-1}/k_1$ and k_1, which is the association rate constant for E + ATP. Generally, k_1 can be expected to show little variation with temperature, since it is roughly proportional to the temperature in Kelvin [46], and to fall in the range 10^7–10^8

$M^{-1} \cdot s^{-1}$ for physiological substrates [46–48]. (This is a slightly smaller value than $k_{encounter}$ for a strictly diffusion controlled process [48]). The k_1 values in Table III are thus in the expected range and k_1 for other nucleotide binding enzymes are quite comparable: $(0.5–1.5) \cdot 10^7$ $M \cdot s^{-1}$ for myosin [49–51] and $(0.7–2.3) \cdot 10^7$ for ADP binding to creatine kinase [52]. If we use a value for $K_{diss} = 1.2 \cdot 10^{-7}$ M determined in our laboratory for the ox brain enzyme at 0°C with or without Na^+ [23] and $k_1 = 10^7$ $M^{-1} \cdot s^{-1}$, we obtain $k_{-1} = 1.2$ s^{-1}. This is close to the value determined directly under comparable conditions (Table II).

Concluding remarks

The three-pool model for the role and properties of the phosphointermediates of Na^+-ATPase proposed by us in 1983 [15] (see also Fig. 1 in the present paper and Ref. 36) is in principle confirmed by the present study. Very recently three other laboratories have reached similar conclusions. Lee and Fortes [53] studied the kinetics of anthroylouabain binding to dog kidney ($Na^+ + K^+$)-ATPase and found that at least three kinetically distinct EP-forms are required to explain the dependence of anthroylouabain binding on [Na^+]: E_1P with three occluded Na^+ ions, E_xP with 1 or 2 externally bound Na^+, and E_2P. Likewise Yoda and Yoda [29] confirm the necessity of the three-pool model in explaining phosphorylation and dephosphorylation kinetics of ($Na^+ + K^+$)-ATPase from the electric organ of *Electrophorus electricus*. Taniguchi et al. [38] have studied the ligand-dependent fluorescence and light scattering behaviour of pig kidney ($Na^+ + K^+$)-ATPase labelled with *N*-(*p*-(2-benzimidazolyl)phenylmaleimide, especially under conditions where the enzyme is phosphorylated by ATP in the presence of 2 M Na^+. From the effect of ADP, K^+ and oligomycin they suggest a scheme with three forms of E_1P and one E_2P in succession

$$Na \cdot E\text{-}ATPMg \rightarrow E_1P_l \rightarrow E_1P_h \rightarrow E_1P_{l'} \rightarrow E_2P$$

where the subscripts l (l′) and h signify components with low and high light scattering, respectively. Without going into detail it seems as if their ($E_1P_l + E_1P_h$), $E_1P_{l'}$, and E_2P are analogous to pool A, B and C in our previous study (Fig. 8 in Ref. 15).

For comparative reasons it is important that several authors have proposed the existence of more than two phosphointermediates in the reaction sequence of Ca^{2+}-ATPase from sarcoplasmic reticulum. Studies on the effect of Ca^{2+} and Mg^{2+} (and K^+) on the kinetics of phosphorylation, dephosphorylation and ATP synthesis from ADP-sensitive EP led Takisawa and Tonomura [54] to propose a scheme with two ADP-sensitive and two ADP-insensitive EP complexes. The similar kind of experiments at 0°C by Yamada and Ikemoto [55] and at 21°C by Froehlich and Heller [56] suggested the consecutive formation of an ADP-sensitive and two insensitive EP complexes. Also, a striking similarity is apparent between the reaction mechanism underlying the Na^+-ATPase and Na^+-Na^+ exchange activities of ($Na^+ + K^+$)-ATPase [15] and the Ca^{2+}-Ca^{2+} exchange catalyzed by Ca^{2+}-ATPase in sarcoplasmic reticulum vesicles [57]. The reaction sequence proposed by Inao and Kanazawa in the latter paper contains three EP-species in succession the properties of which (with Ca^{2+} substituted for Na^+) in principle are very much like those of EP in pool A, B and C in our model [15].

The three-pool model for the phosphoenzymes of the Na^+-ATPase activity (and Ca^{2+}-ATPase activity?) has provided us with a more detailed understanding of the steps involved in these processes. One conclusion from the results of the analysis is that $E_1 \sim P(Na_3)$ when K^+ is added apparently binds K^+ and becomes $E_1 \sim P(Na_3)K$. This species is only slowly (if at all) converted to $E_1PNa_{3,o}$ or $E_2PNa_{3,o}$, i.e., the deocclusion of Na^+ to the external phase is significantly slowed down by K^+. On the other hand, $E_1 \sim P(Na_3)K$ has retained its ADP sensitivity and Na^+ may be deoccluded to the inside upon addition of ADP. A logical consequence is that $E_1 \sim P(Na_3)$ formed in Na^+ medium alone is incompetent in the ($Na^+ + K^+$)-ATPase reaction and that therefore the Albers-Post scheme does not describe this reaction properly. Further studies should concentrate in isolating and characterizing intermediates in the (Na^+K^+)-ATPase cycle, i.e. those EP complexes that are formed in the presence of Na^+ together with K^+ and which are turning over rapidly enough to be intermediates in that cycle.

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