Biochimica et Biophysica Acta, 643 (1981) 463—482
Elsevier/North-Holland Biomedical Press

BBA 79222

THE STEADY-STATE KINETIC MECHANISM OF ATP HYDROLYSIS CATALYZED BY MEMBRANE-BOUND ($Na^+ + K^+$)-ATPase FROM OX BRAIN

II. KINETIC CHARACTERIZATION OF PHOSPHOINTERMEDIATES

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(Received November 25th, 1980)

Key words *($Na^+ + K^+$)-ATPase; Steady-state kinetics, Phosphointermediate; (Ox brain)*

Summary

(1) The kinetics of the phosphorylated enzymic intermediates of ($Na^+ + K^+$)-ATPase from ox brain, which are formed by incubation of the enzyme with 25 μM $AT^{32}P$, 150 mM Na^+ and 1 mM Mg^{2+}, have been studied in dephosphorylation experiments at 1°C. The dephosphorylation of the ^{32}P-labelled enzyme was initiated by addition of either 1 mM unlabelled ATP, 2.5 mM ADP or 1 mM unlabelled ATP + ADP in concentrations from 25 to 1000 μM.

(2) In the absence of ADP the dephosphorylation curve was linear in a semilogarithmic plot almost from $t = 0$, whereas by addition of ADP a biphasic behaviour was obtained. The slope of the slow phase of dephosphorylation was virtually independent of the ADP concentration.

(3) The results were analysed by the mathematical equation corresponding to the simplest possible model for the interconversion and breakdown of the phosphointermediates:

$$\xleftarrow[k_{-1}]{} E_1P \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E_2P \xrightarrow{k_3}$$

the equation being:

$$[E_1P] + [E_2P] = H \cdot e^{-\alpha t} + G \cdot e^{-\beta t}$$

where α, β, H and G are functions of all the rate constants and H and G furthermore are functions of the initial values for $[E_1P]$ and $[E_2P]$.

(4) The analysis confirmed the model and enabled the determination of all the rate constants.

(5) k_{-1} was found to be equal to $k'_{-1} + k''_{-1} \cdot [ADP]$ indicating an ADP-

independent 'spontaneous' dephosphorylation of E_1P. The rate constant for this process was close to that for dephosphorylation of E_2P, i.e., $k'_{-1} \simeq k_3$. Also the value of k''_{-1} was determined.

(6) k_3 was found to be at least $10 \cdot k_{-2}$. The implication of this for the role of the E_1P to E_2P transition in the $(Na^+ + K^+)$-stimulated ATP hydrolysis will be discussed in detail in the following paper (Plesner, I.W., Plesner, L., Nørby, J.G. and Klodos, I. (1981) Biochim. Biophys. Acta 643, 483–494).

(7) A refinement of the model, accounting for the effect of Na^+ on the steady-state ratio between $[E_1P]$ and $[E_2P]$ is proposed:

$$\underset{k_{-1}}{\overset{}{\leftarrow}} E_1P(Na) \underset{b[Na^+]}{\overset{a}{\rightleftharpoons}} E'_1P \overset{c}{\underset{k_{-2}}{\rightleftharpoons}} E_2P \overset{k_3}{\rightarrow}$$

At $[Na^+]$ = 150 mM as used here, $E_1P(Na)$ and E'_1P are assumed to be in rapid equilibrium.

(8) Comparison of our results with those of others underlines the general validity of the conclusions of the present paper.

Introduction

A few years after the discovery by Skou [1] of the $(Na^+ + K^+)$-activated ATPase ($(Na^+ + K^+)$-ATPase, EC 3.6.1.3), it was demonstrated independently in a number of laboratories (with enzyme preparations from a variety of sources) that $(Na^+ + K^+)$-ATPase in the presence of Mg^{2+} and Na^+ incorporated the γ-phosphate from ATP into an acid-stable phosphoenzyme bond [2–10]. The breakdown (dephosphorylation) of the phosphoenzyme was found to be stimulated by K^+ [5,8–10] and at least part of the phosphoenzyme could react with ADP to form ATP in an Na^+-stimulated ADP-ATP exchange. This was reflected by a rapid decomposition of part of the phosphoenzyme when ADP was added [11–13].

On the basis of these and related observations it was suggested that the phosphorylated intermediate might exist in two different conformations: E_1P which is 'ADP-sensitive' and E_2P which is 'K^+-sensitive' [12,14]. For a more extensive discussion of this early work on $(Na^+ + K^+)$-ATPase the reader is referred to reviews by Skou [6], Heinz [15], Albers [16], Whittam and Wheeler [17], Bonting [18] and Schwartz et al. [19].

One of the original proposals for the reaction sequence for $(Na^+ + K^+)$-ATPase, the Post-Albers scheme [16,20], includes E_1P and E_2P as intermediates in the hydrolytic cycle, and there is indeed abundant evidence that the phosphoenzymes are intermediates in at least some of the reactions catalyzed by $(Na^+ + K^+)$-ATPase. First of all they are formed with $(Na^+ + K^+)$-ATPase preparations from all species and tissues tested (e.g., see Ref. 21). Secondly, EP may be formed in the presence of P_i [22–24] and under suitable ionic conditions ATP can be synthesized from ADP and P_i by $(Na^+ + K^+)$-ATPase in a stepwise procedure [24,25]. This might reflect the biochemical events accompanying the backwards running of the pump [26–29]. The phosphate is bound to an aspartyl group in an acid-stable acyl phosphate bond [30,31] and it has been amply demonstrated that the phosphoenzymes formed from P_i and from

ATP under a variety of conditions (E_1P, E_2P) are chemically identical with regard to the group being phosphorylated [32—35].

The Post-Albers scheme and more or less expanded versions thereof [36—39] have been extensively used as a frame of reference for the large number of observations on the properties of ($Na^+ + K^+$)-ATPase. Although widely accepted, the sequence, especially with regard to the role of the phosphorylated intermediates in ($Na^+ + K^+$)-stimulated ATP hydrolysis and cation translocation when K^+ is present, has been disputed from time to time [6,40—47].

In the present paper we have studied the transient kinetics of the phosphorylated intermediates with the purpose of determining the rate constants for their interconversion and decomposition (dephosphorylation).

A mathematical model has been developed, which describes the time dependence of E_1P and E_2P in accordance with the following scheme (which may be considered as part of the hydrolytic cycle for Na^+-ATPase [37]):

$$E^{P}_{ADP} \underset{E_1 + P_i}{\overset{ADP}{\leftarrow}} E_1P \rightleftharpoons E_2P \rightarrow E_2 + P_i \qquad \text{(S1)}$$

The results obtained confirm that E_1P and E_2P are formed consecutively at least in the presence of Mg^{2+}, ATP and Na^+ (without K^+). Furthermore, a quantitive evaluation of the various reactions (with ADP, with K^+, 'spontaneous dephosphorylation') in which the phosphoenzymes may engage is made possible by combining the model and the results obtained. Some of the conclusions reached are incorporated into the formulation of the combined model for ($Na^+ + K^+$)-ATPase and Na^+-ATPase described in paper III in this series [48].

Materials and Methods

Enzyme. The enzyme was prepared from ox brain by using the method of Klodos et al. [49].

The specific activity [50] of preparations I—IV was 2.68, 3.33, 3.26 and 3.29 units · (mg protein)$^{-1}$, respectively. The steady-state level of phosphorylation with 25 μM ATP is given in Table I. This level was unaffected by ATP up to 250 μM and addition of oligomycin (20 $\mu g \cdot ml^{-1}$ = 50 $\mu g \cdot$ (mg protein)$^{-1}$) increased the level by only 5%. The turnover number, based on ^{32}P sites, was thus 9690 ± 300 min^{-1}.

Reagents. ATP and ADP were obtained as sodium salts from Boehringer, F.R.G. and [γ-^{32}P]ATP from the Radiochemical Centre, Amersham, U.K. ATP was converted to its Tris salt by chromatography on a Dowex I column and [γ-^{32}P]ATP was purified on DEAE-Sephadex G-25 as described earlier [51]. ATP thus purified contained less than 0.5 mol % ADP. All other reagents were reagent grade.

Formation and determination of phosphorylated intermediates. The phosphorylated intermediates, EP, were formed by incubation at 1°C of the enzyme preparation (0.4 mg protein · ml^{-1}) with 1 mM $MgCl_2$, 150 mM NaCl, 25 μM [γ-^{32}P]ATP (spec. act. ≈2.5 · 10^4 cpm/nmol) and 30 mM imidazole buffer (pH 7.4 at 1°C). The total volume was 3 ml. Under these conditions, a steady-state level of EP, constant for several minutes, is reached within less than 2 s [47].

In the dephosphorylation experiments (see Results), 0.1 ml of 30 mM ATP, 0.1 ml of 30 mM ATP + the appropriate ADP concentration or 0.1 ml of 75 mM ADP was added to 2.9 ml of the phosphorylation medium after 60 s of prephosphorylation (see above). The complete reaction mixture contained less than 10 μM K^+ as measured by flame photometry.

Determination of EP was performed as follows. To 3 ml sample were added 3 ml ice-cold $HClO_4$ + $Na_4P_2O_7$ (final concentrations 4% (w/v) and 1 mM, respectively). After 30 min at 0°C the precipitated protein was isolated and washed by four successive centrifugations at 6000 × *g* (40 min + 3 × 20 min) and resuspensions in 5 ml ice-cold 0.1% CCl_3CO_2H + 10 mM $Na_4P_2O_7$ + 10 mM KH_2PO_4. Further and longer washings reduce the yield of EP (which after all seems to have a limited acid stability) without reducing the blanks. After the final wash and centrifugation the pellet was dissolved in 0.55 ml of 1 M NaOH by heating for 30 min at 55°C. Protein was measured in 50-μl duplicates by the using the method of Lowry et al. [52] with bovine serum albumin as standard and another 400 μl of the hydrolysate was used for determination of ^{32}P as described earlier [53].

Phosphorylation blanks. The intention of this study was to correlate the experimental data with a mathematical model in a rather stringent way. Special consideration was therefore given to the various possible forms of 'non-specific phosphorylation' and to contamination of the protein with ^{32}P, the phosphorylation blanks. We shall briefly mention several possibilities and discuss how they may be controlled or eliminated.

1. The so-called 'stable phosphorylation', an apparently irreversible labelling of the enzyme preparation, seen by Skou and Hilberg [54], is not observed with the reagents and procedures described in the present paper. It was suggested (Skou, J.C., personal communitation) that it was due to polyphosphates present in their batches of [γ-^{32}P]ATP.

2. Monovalent cation-independent formation of organic phosphate compounds (^{32}P-labelled lipids, phosphoproteins other than (Na^+ + K^+)-ATPase) is usually not encountered to any appreciable extent in our enzyme preparations. A suitable blank (K^+ blank) is here the phosphorylation level obtained when all Na^+ is substituted with 150 mM K^+ (Refs. 53, 55 and 56, but see below). A blank prepared by mixing the acid-denatured enzyme with the appropriate substrates [57—59] will not correct for the side products formed during phosphorylation and in our hands it is always lower than the K^+ blank. The K^+ blank is independent of the presence of Mg^{2+} and a 'phosphorylation mixture' without added Mg^{2+} containing 10 mM EDTA with or without 150 mM Na^+ gives the same value as the K^+ blank.

3. We have observed that the K^+ blank, when precipitated and washed as described by Klodos and Skou [53] and Skou and Hilberg [54] (without pyrophosphate), may vary considerably with the [γ-^{32}P]ATP batch even if the latter is purified. Usually it is about 10% but sometimes values up to 100—150% of the maximal specific phosphorylation are encountered.

This unidentified 'contamination' (part of it, but by no means all of it in the high blank cases, is apparently caused by unspecific absorption of [γ-^{32}P]ATP to the protein [47,53,60]) can be prevented or eliminated by inclusion of pyrophosphate in the precipitating and washing solutions as described above. The

average value for the specific phosphorylation is not influenced, but the accuracy is increased by the pyrophosphate procedure. The use of pyrophosphate was suggested to us by Dr. Paul Ottolenghi.

Conclusion. The blanks are prepared as are the samples but with 150 mM KCl instead of 150 mM NaCl. Care is taken that the specific activity and the concentration of the nucleotides are the same as in the samples. The blank values were independent of time from 5 to 300 s. They were usually precipitated after 10 s of incubation.

Nomenclature. The results to be reported are all measurements of ^{32}P bound to $(Na^+ + K^+)$-ATPase in an acid-stable manner ($E^{32}P$). $[EP]'$ denotes the concentration of $E^{32}P$ corrected for blank. Subscripts 0 and ∞ refer to the point in the experiment: just before the start of the dephosphorylation experiment, and 1—2 min later where the new steady-state level has been obtained, respectively. [EP] is $[EP]' - [EP]'_\infty$ and as discussed in Appendix A, [EP] is the quantity that will apply to the model described in the following section. $[EP]_{rel}$ is $[EP]/[EP]_0$.

Mathematical model for dephosphorylation of $E_1P + E_2P$

As mentioned in the Introduction and documented in the Results, we have been able to establish experimental conditions allowing us to study quantitatively the dephosphorylation of E_1P and E_2P. The simplest model that can explain the observations is:

$$\overset{k_{-1}}{\longleftarrow} E_1P \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E_2P \overset{k_3}{\longrightarrow} \qquad \text{(S2)}$$

The letters P symbolize ^{32}P bound to $(Na^+ + K^+)$-ATPase in an acid-stable 'ADP-sensitive' form, E_1P, and a 'K^+-sensitive' form, E_2P. The scheme assumes that there is no production of EP from P_i (the concentration of P_i is less than 10 μM and the affinity for P_i is low under the conditions of our experiments, e.g. see Refs. 23 and 61 *). The rate of formation of E_1P from $[\gamma\text{-}^{32}P]ATP$ is likewise set equal to zero. Although this is not exactly true experimentally, we show in Appendix A that incorporating this (constant) rate, as well as the resulting (non-zero) steady-state concentrations at infinite time, allows the definition of new experimental variables satisfying a set of different equations identical in form to those describing scheme S 2 (Eqns. 1 and 2).

The equations describing the time course for $[E_1P]$ and $[E_2P]$ are

$$\frac{d[E_1P]}{dt} = k_{-2}[E_2P] - (k_{-1} + k_2)[E_1P] \qquad (1)$$

$$\frac{d[E_2P]}{dt} = k_2[E_1P] - (k_{-2} + k_3)[E_2P] \qquad (2)$$

The mathematical model corresponds to that for an open two-compartment system without input [62,63]. The solutions of Eqns. 1 and 2 are:

$$[E_1P] = C_1 \cdot e^{-\alpha t} + C_2 \cdot e^{-\beta t} \qquad (3)$$

* In our experience, up to 0.5 mM P_i is without effect on the phosphorylation level obtained.

$$[E_2P] = C_3 \cdot e^{-\alpha t} + C_4 \cdot e^{-\beta t} \tag{4}$$

and the equation for $[EP] = [E_1P] + [E_2P]$ is:

$$[EP] = H \cdot e^{-\alpha t} + G \cdot e^{-\beta t} \tag{5}$$

where $H = C_1 + C_3$, $G = C_2 + C_4$ and $H + G = [EP]_o$.

In this section we shall emphasize some basic properties of the model whereas a more thorough analysis relevant to our own results will be found under Results and Discussion.

As detailed below, α and β are functions of the rate constants in scheme S2, and H and G are functions of the rate constants and $[E_1P]_o$ and $[E_2P]_o$.
We define the following quantities:

$$u \equiv (k_{-1} + k_2) - (k_{-2} + k_3) \tag{6}$$

$$h \equiv \sqrt{u^2 + 4k_2k_{-2}} \qquad \text{(so that always } h > u\text{)} \tag{7}$$

with this notation:

$$\alpha = \tfrac{1}{2}(k_{-1} + k_2 + k_{-2} + k_2 - h) \tag{8}$$

$$\beta = \tfrac{1}{2}(k_{-1} + k_2 + k_{-2} + k_3 + h) \quad \Rightarrow \beta > \alpha \tag{9}$$

$$C_1 = \frac{2k_{-2}[E_2P]_o + (h - u) \cdot [E_1P]_o}{2h} \tag{10}$$

$$C_2 = [E_1P]_o - C_1 \tag{11}$$

$$C_3 = C_1 \cdot \left(\frac{h + u}{2k_{-2}}\right) \tag{12}$$

$$C_4 = [E_2P]_o - C_3 \tag{13}$$

The expressions for H and G (Eqn. 5) are now obtained from Eqns. 10–13:

$$H = \frac{(h - u + 2k_2) \cdot [E_1P]_o + (h + u + 2k_{-2}) \cdot [E_2P]_o}{2h} \tag{14}$$

$$G = \frac{(h + u - 2k_2) \cdot [E_1P]_o + (h - u - 2k_{-2}) \cdot [E_2P]_o}{2h} \tag{15}$$

since $h > u$, it is clear that $H > 0$.

Furthermore, we want to point out that at steady state we have the following relationship:

$$\frac{d[E_2P]_{st}}{dt} = 0 = k_2[E_1P]_{st} - (k_{-2} + k_3)[E_2P]_{st}$$

The steady-state situation corresponds to the zero-time situation in the dephosphorylation experiments so that:

$$\frac{[E_1P]_{st}}{[E_2P]_{st}} = \frac{[E_1P]_o}{[E_2P]_o} = \frac{k_{-2} + k_3}{k_2} \tag{16}$$

In Results the concentration of E_1P and E_2P (and their sum) is often expressed relative to $[EP]_o$, e.g.

$[E_1P]_{rel} = [E_1P]/[EP]_o$,

which gives us:

$$[E_1P]_{o,rel} + [E_2P]_{o,rel} = 1 \tag{17}$$

$$\frac{[E_1P]_{o,rel}}{[E_2P]_{o,rel}} = \frac{k_{-2} + k_3}{k_2} \quad \text{(like Eqn. 16)} \tag{18}$$

Correspondingly, Eqn. 5, by division with $[EP]_o$ on both sides becomes:

$$[EP]_{rel} = H_{rel}\ e^{-\alpha t} + G_{rel} \cdot e^{-\beta t} \tag{19}$$

where H_{rel} and G_{rel} are functions of the rate constants only. By definition: $H_{rel} + G_{rel} = 1$.

Results

When the phosphorylation of the enzyme by [γ-^{32}P]ATP is stopped or drastically slowed down by the addition of unlabelled ATP, of ADP or of an Mg^{2+} chelator [20,42,53,64], the level of $E^{32}P$ declines and approaches a new, much lower steady-state value. In the experiments to be described we have characterized quantitatively the dephosphorylation after addition of 1 mM ATP, 2.5 mM ADP or 1 mM ATP + up to 1 mM ADP.

Dephosphorylation with 1 mM ATP

When the phosphorylating medium, 60 s after the addition of 25 μM [γ-^{32}P]-ATP, is made 1 mM with respect to ATP, the specific radioactivity is decreased by about a factor of 40 and a new steady-state level of EP, $[EP]'_\infty$, approx. 2.5% of that before ATP addition should be approached. The value for $[EP]'_{\infty,rel}$ observed is 2.4 ± 0.6% (n = 7). A representative experiment of this type is shown on Fig. 1. After a small, but significant, initial rapid decrease, $[EP]_{rel}$ declines

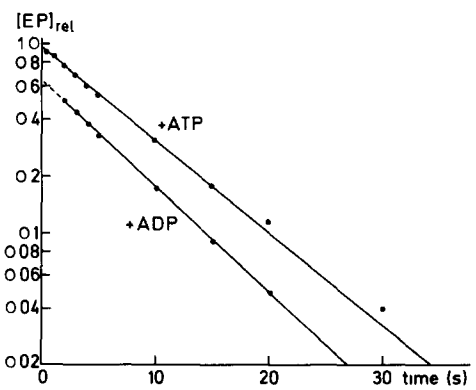

Fig. 1. Dephosphorylation of $E^{32}P$ after addition of 1 mM ATP or 2.5 mM ADP at time 0. The enzyme was phosphorylated and dephosphorylation initiated as described under Materials and Methods. The ordinate, $[EP]_{rel}$, is the relative concentration of $[E^{32}P]$ corrected for the new steady-state level (see Nomenclature). The lines drawn are calculated by non-linear least-square regression according to Eqn. 20. See also Table I.

TABLE I

RATE CONSTANT, α, AND ORDINATE INTERCEPT, H_{rel}, OF THE SLOW MONOEXPONENTIAL PART OF DEPHOSPHORYLATION CURVES (cf. Fig. 1 and Eqn. 20)

The enzyme was phosphorylated as described in Materials and Methods with 25 μM $AT^{32}P$ for 60 s and dephosphorylation initiated by addition of 1 mM ATP or 2.5 mM ADP. The results are ± S.E.

Enzyme preparation	EP level (pmol · mg^{-1})	Dephosphorylation in the presence of					
		1 mM unlabelled ATP			2.5 mM ADP		
		α (s^{-1}) ($X10^3$)	H_{rel} ($X10^2$)	n	α (s^{-1}) ($X10^3$)	H_{rel} ($X10^2$)	n
I	302	124, 133	91.9, 95.6	2	125, 148	59.5, 61.3	2
II	321 ± 5	130 ± 10	99.2 ± 2.0	4	134 ± 5	61.1 ± 2.1	4
III	331 ± 7	134 ± 7	93.8 ± 2.6	3	151 ± 3	59.2 ± 1.3	3
IV	343 ± 3	125 ± 3	98.8 ± 0.8	6	137 ± 6	62.1 ± 1.3	6

in a monoexponential way which according to Eqn. 19 is described by:

$$[EP]_{rel} = H_{rel}\ e^{-\alpha t} \tag{20}$$

(remember that $\alpha < \beta$, Eqns. 8 and 9). H_{rel} is thus the extrapolated ordinate intercept of the line.

At first sight it may be surprising that the time course of $[EP]_{rel}$ is one exponential function almost from $t = 0$, and one may ask whether this in any way is compatible with the model in scheme S2 and Eqn. 19. However, it for instance $k_{-1} = k_3$, the model is obviously equivalent to a one-pool model (irrespective of the proportions of E_1P and E_2P present and here we have appreciable concentrations of both, see later and Ref. 47) with one output rate constant k_3. $[EP]_{rel}$ vs. time should then be a single exponential function. It can accordingly easily be shown that for $k_{-1} = k_3$, $G_{rel} = 0$, $H_{rel} = 1$ and $\alpha = k_3$ in Eqn. 19. The exact values obtained for H_{rel} and α, given in Table I, will be used in the estimation of rate constants later in this section.

Dephosphorylation with ADP alone

A rough estimate based on the published binding constants for ATP and ADP under 'high-affinity' conditions as used here [65,66] justifies the assumption that 2.5 mM ADP will be as effective as 1 mM ATP in stopping phosphorylation from $AT^{32}P$. Mg^{2+}, which is absent in the binding studies cited, probably only slightly decreases the affinity for ATP [67] and ADP (Jensen, J., personal communication). According to schemes S1 and S2, ADP is furthermore expected to increase k_{-1} so as to remove E_1P rapidly. The steady-state value of $[EP]'_{rel}$ in the presence of 25 μM $AT^{32}P$ and 2.5 mM ADP was accordingly found to be low: 2.7 ± 0.6% (n = 7).

The dephosphorylation curve with 2.5 mM ADP is clearly biphasic (Fig. 1) and again the 'slow' exponential curve is described by Eqn. 20. The values obtained (and used later) for H_{rel} and α are given in Table I. It should be noted that the 'ADP-curve' in Fig. 1 is qualitatively and quantitatively similar to that shown in Fig. 2 for 1 mM ATP + 1 mM ADP, indicating that in this respect 2.5 mM ADP may be denoted as 'saturating'.

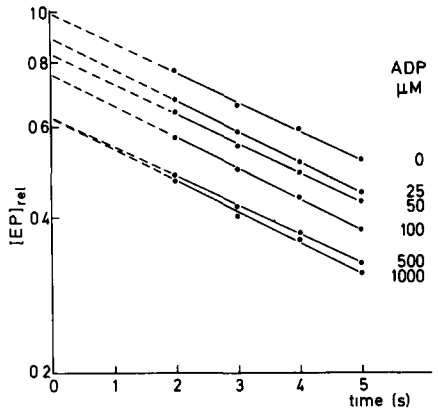

Fig. 2. Dephosphorylation of $E^{32}P$ after addition at time 0 of 1 mM ATP + the ADP concentration indicated. See legend to Fig. 1 and Table II.

Dephosphorylation with 1 mM ATP + ADP

In the experiments where 1 mM ATP and no ADP is added (see above), the ADP concentration is not exactly zero. The estimated concentration is 1—6 μM, namely less than 5 μM contamination from unlabelled ATP and about 1 μM from hydrolysis of [γ-^{32}P]ATP during phosphorylation. In the experiments reported in this section we have studied the dephosphorylation by adding 1 mM ATP, and we have varied k_{-1} by adding ADP simultaneously with the unlabelled ATP.

The results reveal that the dephosphorylation curves (Fig. 2) are biphasic. The initial rapid drop in $[EP]_{rel}$, calculated as $G_{rel} = 1 - H_{rel}$, is clearly a function of [ADP] for [ADP] < 1 mM whereas α, the rate constant for the slower

TABLE II

RATE CONSTANT, α, AND ORDINATE INTERCEPT, H_{rel}, OF THE SLOW MONOEXPONENTIAL PART OF DEPHOSPHORYLATION CURVES (cf. Figs. 1 and 2, and Eqn. 20)

The enzyme was phosphorylated as described in Materials and Methods with 25 μM AT^{32}P for 60 s and dephosphorylation initiated by addition of 1 mM ATP + the ADP concentration indicated. The results are ± S.E.

ADP added (μM)	Enzyme II			Enzyme III		
	α (s^{-1}) ($\times 10^3$)	H_{rel} ($\times 10^2$)	n	α (s^{-1}) ($\times 10^3$)	H_{rel} ($\times 10^2$)	n
0	130 ± 10	99.2 ± 2.0	4	134 ± 7	93.8 ± 2.6	3
25	135 ± 6	88.2 ± 0.8	3			
33	129 ± 6	87.1 ± 3.2	4			
50	131 ± 4	82.6 ± 2.0	3	152 ± 4	82.1 ± 1.0	3
66	136 ± 7	81.3 ± 2.9	4			
100	138 ± 3	75.3 ± 1.7	5			
250	126 ± 10	67.6 ± 2.0	4			
500	130, 132	61,8, 63.7	2			
1000	132, 145	61,0, 61,2	2			
2500 *	134 ± 5	61.1 ± 2.1	4	151 ± 3	59.2 ± 1.3	3

* No ATP added.

monoexponential part (see Eqn. 20) apparently is independent of [ADP] over the whole range of ADP concentrations (Table II).

Estimations of the rate constants

Before we evaluate the results according to the mathematical model we shall emphasize the three important observations that form the basis for this evaluation.

(1) In experiments where ADP is present during dephosphorylation the time course is clearly biphasic. H_{rel} decreases with increasing [ADP] and approaches a minimum value (Table II).

(2) $[EP]_{rel}$ is a monoexponential function of time virtually from $t = 0$ in the experiments of Figs. 1 and 2 where no ADP is added, i.e., H_{rel} is close to 1 (Tables I and II).

(3) The rate constant, α, for the slow phase of dephosphorylation is almost independent of the ADP concentration.

ad.1. An analysis of the expression for H (Eqn. 14) shows that H_{rel} always decreases with increasing k_{-1} when $k_{-1} \geqslant k_3$ (which is the case here, see below) and that $dH_{rel}/dk_{-1} \to 0$ when $k_{-1} \to \infty$. H_{rel} thus approaches a plateau with increasing [ADP]: For $k_{-1} \to \infty$ we obtain from Eqn. 6 that $u \to \infty$, and from Eqn. 7 that $h \to u$, leading to:

$$H_{rel} \to \frac{2k_2[E_1P]_{o,rel} + (2u + 2k_{-2})[E_2P]_{o,rel}}{2u} \to [E_2P]_{o,rel}$$

Furthermore, $[E_1P]_{o,rel} = 1 - [E_2P]_{o,rel}$. From Table II we then obtain the steady-state concentrations of E_1P and E_2P:

$$[E_1P]_{o,rel} \approx 0.4 \quad \text{and} \quad [E_2P]_{o,rel} \approx 0.6 \tag{21}$$

This gives us the value for the ratio (cf. Eqn. 16):

$$\frac{k_{-2} + k_3}{k_2} = \frac{0.4}{0.6} = 0.67 \tag{22}$$

ad.2. As mentioned earlier, $H_{rel} = 1$ when $k_{-1} = k_3$ or $k_{-1}/k_3 = 1$. Since the average value for H_{rel} for the four preparations in Table I is not exactly 1 but about 0.97, we must examine the consequence for k_{-1}/k_3 of this deviation of H_{rel} from 1. This analysis is given in Fig. 3 which shows H_{rel} as a function of k_{-1}/k_3 for different k_3/k_{-2} ratios, taking the relationship between k_{-2}, k_3 and k_2 given above in Eqn. 22 into account. The analysis reveals, that for $k_3/k_{-2} > 5$ (see ad.3.) the observed H_{rel} corresponds to $1 < k_{-1}/k_3 < 1.5$.

ad.3. When [ADP], and thereby k_{-1}, is increased (see Table II) H_{rel} falls from about 1 to 0.6 whereas α changes very little. From the expression for α (Eqn. 8) it is easy to show that for $k_{-1} >> k_2$, k_{-2}, k_3, which corresponds to the minimum value for H_{rel} (see ad.1.):

$$\alpha \to \alpha_{max} = k_{-2} + k_3 \tag{23}$$

and that α, for $H_{rel} = 1$ ($k_{-1} = k_3$, see Fig. 3), is:

$$\alpha_1 = k_3 \, . \tag{24}$$

The analysis given in Fig. 4, shows, furthermore, that relative independence of

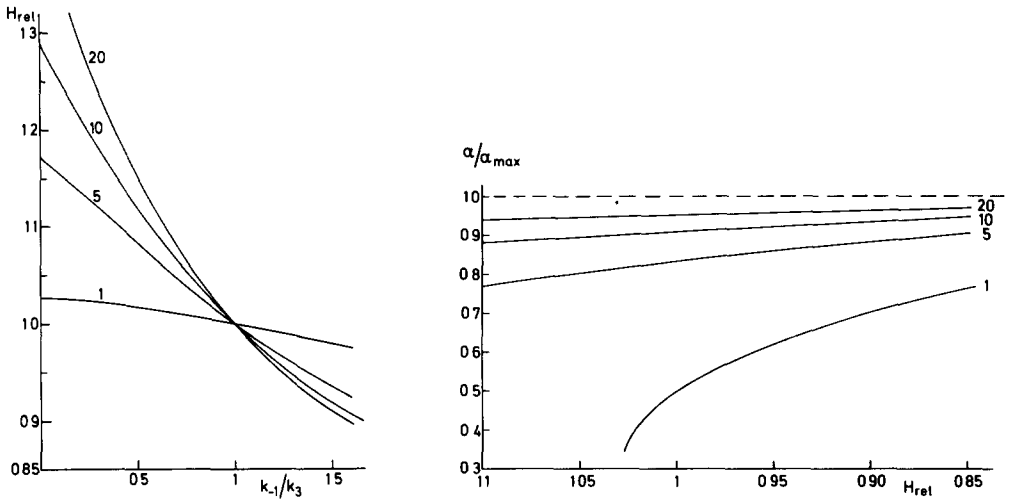

Fig. 3. Computer simulation of H_{rel}, the extrapolated ordinate intercept of the slow phase of dephosphorylation (Eqn. 20), as a function of k_{-1}/k_3 at four different values for k_3/k_{-2} (indicated on the curves). In the calculation, k_3 was 1 and for each curve k_2 was obtained from Eqn. 16, $k_2 = (k_3 + k_{-2}) \cdot [E_2P]_{o,rel}/[E_1P]_{o,rel}$, where $[E_2P]_{o,rel}$ and $[E_1P]_{o,rel}$ are 0.6 and 0.4, respectively (see Eqn. 21).

Fig. 4. Computer simulation of the relationship between α/α_{max} and H_{rel} (where α and H_{rel} are the rate constant and the ordinate intercept of the slow phase of dephosphorylation (see Eqns. 20 and 23). The relationship was computed for the four different values of k_3/k_{-2} shown. The value for k_2 used was calculated as described for Fig. 3.

α upon H_{rel} only obtains if:

$$k_3/k_{-2} > 10\text{—}20 \qquad (25)$$

These considerations lead to the following conclusions:

(a) $\alpha \simeq 0.13\ s^{-1}$ in the ATP-dephosphorylation experiments of Table I must be very close to $\alpha_1 = k_3$ (Eqn. 24).

(b) An approximate value for k_{-2} may be obtained from the (paired) difference between $\alpha_1 = k_3$ and $\alpha_{max} = k_{-2} + k_3$ (from the ADP-dephosphorylation, Table II).

(c) k_2 can be calculated from Eqns. 22 and 23.

The values obtained are given in Table III. Note that $k_3 > 10 \cdot k_{-2}$.

TABLE III

RATE CONSTANTS OBTAINED IN THE PRESENT PAPER

The methods used in the calculation of the constants are described in detail in the text.

Kinetic model	Constant	Value
$E^{P}_{ADP} \overset{k''_{-1}}{\leftarrow} E_1P \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E_2P \overset{k_3}{\rightarrow}$ $\downarrow k'_{-1}$ E_1	k''_{-1} k'_{-1} k_2 k_{-2} k_3	$2.5 \cdot 10^3\ M^{-1} \cdot s^{-1}$ $0.13\ s^{-1}$ $0.21\ s^{-1}$ $0.01\ s^{-1}$ $0.13\ s^{-1}$

Characterization of k_{-1}

We have concluded above that $k_{-1} \geqslant k_3$, even with [ADP] as low as approx. 5 μM. This leads us to assume that there is a spontaneous dephosphorylation of E_1P to E_1 and P_i as well as an ADP-dependent dephosphorylation to ATP (see scheme S1):

$$k_{-1} = k'_{-1} + k''_{-1} \cdot [\mathrm{ADP}] \tag{26}$$

Preliminary efforts to extract information about k_{-1} from the observed variation of H_{rel} with [ADP] shown on Fig. 2 and in Table II disclosed that $(1 - H_{rel})^{-1} = G_{rel}^{-1}$ was close to being a linear function of $[\mathrm{ADP}]^{-1}$. That this should be so is by no means obvious from the theoretical expression for G_{rel}:

$$G_{rel} = \frac{(h + u - 2k_2)[E_1P]_{o,rel} + (h - u - 2k_{-2})[E_2P]_{o,rel}}{2h} \tag{27}$$

In Appendix B a relationship between G_{rel} and [ADP] is derived. This relationship (Eqn. B5 or B6) is an approximation for G_{rel} valid under the conditions that $k_{-2} << k_3 < k_2$ which is the case in our experiments.

In Fig. 5 we have plotted $G_{rel}/[\mathrm{ADP}]$ vs. G_{rel}, following Eqn. B6 in Appendix B. The resulting linear relationship supports the validity of the necessary assumption $k'_{-1} \approx k_3$ and enables us to obtain a value for k''_{-1} from the slope of the line:

$$\frac{k''_{-1}}{k_2 + k_{-2}} \approx 12\ \mathrm{mM}^{-1} \Rightarrow k''_{-1} = 2.5 \cdot 10^3\ \mathrm{M}^{-1} \cdot \mathrm{s}^{-1}$$

The intercept on the abscissa of Fig. 5 is $[E_1P]_{o,rel} = 0.42$. It is not surprising

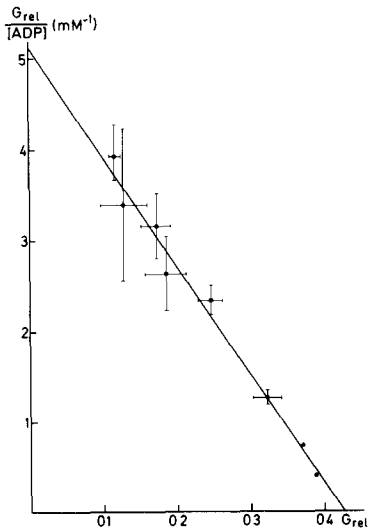

Fig. 5. Eadie-Scatchard plot of the dependence of G_{rel} on the concentration of ADP present during dephosphorylation (see Eqn. B6 in Appendix B). The data are taken from Table II ($G_{rel} = 1 - H_{rel}$), with the exception that the ADP concentrations given in Table II are corrected for ADP contamination in the unlabelled ATP plus that arising from hydrolysis of $AT^{32}P$ in the 60 s phosphorylation period (see text). [ADP] in this figure is thus $[\mathrm{ADP}]_{added}$ + 5 μM. The bars indicate ± S.E. and the line is calculated by unweighted linear regression.

that this value is slightly higher than that obtained from Table I, since it is an extrapolated value for [ADP] → ∞.

The rate constants obtained in the present paper are assembled in Table III.

Discussion

It appears from the results and arguments presented above that the mathematical approach used in this paper is necessary for the concise elucidation of the kinetics of phosphointermediates in the Na^+-ATPase reaction. In the following we shall therefore use this line of reasoning not only to compare the rate constants obtained by us with those reported by others (or those that may be deduced from their work), but also in evaluating different proposals for the metabolism and properties of the phosphointermediates. The purpose of this discussion is thus to see whether our observations represent features of Na^+-ATPase kinetics valid also at higher temperatures and for (Na^+ + K^+)-ATPase preparations from other sources.

Spontaneous dephosphorylation of E_1P (to $E_1 + P_i$) and Na^+-ATPase activity. The effect of Na^+ on the $E_1P \rightleftharpoons E_2P$ intercoversion

When phosphorylation with $AT^{32}P$ is stopped by addition of unlabelled ATP or EDTA (not shown) the dephosphorylation curve is monoexponential (Fig. 1). We have concluded that this 'one-pool' behaviour reflects a spontaneous dephosphorylation of E_1P with the rate constant k'_{-1} being approximately equal to the dephosphorylation constant, k_3, for E_2P *.

Models for (Na^+ + K^+)-ATPase in general do not contain a dephosphorylation step for E_1P (e.g., see Refs. 37 and 39), but the existence of such a reaction has been suggested both by Blostein [38] and Garrahan et al. [68] in connection with their studies of the Na^+ activation of Na^+-ATPase activity. Support for this alternative route for Na^+-ATPase is found when we compare the effect of Na^+ on phosphorylation, on the $E_1P \rightleftharpoons E_2P$ interconversion and on Na^+-ATPase activity. $K_{0.5}$ for Na^+ for phosphorylation is about 1 mM [9,24,69] and this probably reflects binding of Na^+ to one to threee high-affinity inner sites on the ATPase [38,68]. With sufficient ATP and Mg^{2+}, the phosphorylation level (E_1P + E_2P) is thus maximal with [Na^+] > 10 mM but the proportion of E_1P to E_2P can be drastrically increased by increasing the concentration of Na^+ further. $K_{0.5}$ for this Na^+ effect is in the range of 100—200 mM depending on temperature and on the source of enzyme [69—71], and the low affinity might suggest that Na^+ here binds to external sites. The observation that ADP-ATP exchange is activated in the same range of Na^+ concentrations [72] likewise shows that Na^+ promotes E_1P formation from E_2P. In the case of Na^+-ATPase activity we note that here also Na^+ has a dual effect, resulting in peculiar biphasic activation curves disclosing the presence of high-affinity and low-affinity sites with $K_{0.5} < 1$ mM and $K_{0.5} > 50$—100 mM, respectively [20,59,68,72]. All the studies on Na^+-ATPase cited were done at 37°C. A logical explanation

* A monoexponential dephosphorylation curve would also obtain if there was rapid equilibrium between E_1P and E_2P. In that case addition of saturating concentrations of ADP would dephosphorylate all EP immediately and this is clearly not the case here.

of these effects of Na^+ on the E_1P level and the Na^+-ATPase is that E_1P can dephosphorylate with P_i production, and as discussed by Blostein [38] this 'alternative' route of Na^+-ATPase might under some conditions be faster than the 'ordinary' one due to bypassing of the step $E_2 \rightarrow E_1$.

At this point we may ask whether the effect of high $[Na^+]$ on $[E_1P]/[E_2P]$ discussed above can be explained by our mathematical model, since we find $k_{-2} << k_3$ at 150 mM Na^+ and $[E_1P]/[E_2P] = (k_{-2} + k_3)/k_2$ *. The ideas of Garrahan et al. [68] that there are two parallel routes for Na^+-ATPase offer one possible explanation if it is assumed that E_1P with both 'inner' and 'outer' sites occupied by Na^+ is converted to E_2P with a lower rate constant than E_1P with only Na^+ on the inner sites. In this case k_2 would be a function of $[Na^+]$.

Another explanation would be that E_1P on the way to E_2P looses one Na^+ (see also Ref. 25):

$$\overset{k_{-1}}{\leftarrow} \underset{Na}{E_1P} \underset{b[Na^+]}{\overset{a}{\rightleftharpoons}} E_1'P \underset{k_{-2}}{\overset{c}{\rightleftharpoons}} E_2P \overset{}{\underset{k_3}{\rightarrow}} \qquad \text{(S3)}$$

(For the sake of clarity, only one Na^+ is considered in this scheme.)

If we assume that under our conditions both a and $b \cdot [Na^+]$ are much greater than c, ADP-sensitive EP will be $[E_1P] = [E_1P(Na)] + [E_1'P]$ and we get:

$$\frac{[E_1P]_o}{[E_2P]_o} = (k_3 + k_{-2}) \Big/ \frac{a \cdot c}{a + b[Na^+] + c}$$

k_2 in our scheme is thus $a \cdot c/(a + b[Na^+] + c)$ and this refinement of our model will then explain the effect of Na^+ upon the $[E_1P]/[E_2P]$ ratio. It might be noted that this model opens the possibility that Na : Na exchange does not involve the E_2P form.

Before concluding this part of the discussion we want to emphasize one point. The proportion of $[E_1P]$ to $[E_2P]$ in the native enzyme is not only a function of Na^+ as discussed above, but also depends on the source of enzyme. Generally, it is comparatively easy to demonstrate E_1P in enzyme from brain (this paper and Refs. 47, 57 and 69) whereas enzyme from kidney or the electric organ of *Electrophorus* predominantly forms $[E_2P]$, indicating that $k_2 >> k_3 + k_{-2}$ except at very high $[Na^+]$ for these preparations [12,25,70,73,74]. The latter are therefore not suited to test our model as regards the one-pool behaviour of the $E_1P + E_2P$ system. The monoexponential dephosphorylation curves published for rat brain at 0°C [69] and ox brain at 21°C [57], however, support our conclusion that E_1P dephosphorylates with about the same rate constant as does E_2P.

The 'forward' and 'backward' reaction of E_2P. The implications of the inequality $k_3 > k_{-2}$

The conclusion that $k_3 > k_{-2}$, i.e., that the rate of dephosphorylation of E_2P is considerably larger than the rate of the backward reaction $E_2P \rightarrow E_1P$, stems from the observation that the rate constant for the slow phase of the dephosphorylation curve is independent of ADP. The relatively few quantitative studies

* Preliminary results in our laboratory show that doubling the Na^+ concentration to 300 mM increases the $[E_1P]/[E_2P]$ ratio from about 0.7 to about 2 without changing k_3.

TABLE IV

PUBLISHED RATE CONSTANTS (OR CONSTANTS DEDUCED FROM PUBLISHED CURVES) FOR THE DEPHOSPHORYLATION OF $E^{32}P$ INITIATED BY ADDITION OF EXCESS OF UNLABELLED ATP (α_{ATP}) OR ADP (α_{ADP}, THE RATE CONSTANT FOR THE SLOW PHASE)

Our interpretation that $\alpha_{ATP} = k_3$ and $\alpha_{ADP} = k_3 + k_{-2}$ is indicated (cf. Eqns. 23 and 24).

Enzyme	Temperature (°C)	k_3 (α_{ATP}) (s^{-1})	$k_3 + k_2$ (α_{ADP}) (s^{-1})	Reference
Brain				
Rat	0	0.17	0.20—0.23	[69]
Ox	1	0.13	0.14	this paper
Ox	21	≈3—4	$\simeq \alpha_{ATP}$ *	[57]
Kidney				
Guinea-pig	0	0.06	0.07	[25]
	0	0.09		[76]
	8.5	0.07		[9]
	15	0.2		[20]
Rabbit	0	0.09	0.09	[75]
	2	0.05	0.05	[74]
Electric organ				
Electric eel	21	4	$\simeq \alpha$ATP *	[73]

* The authors state that the slopes of the ATP and ADP curves are the same.

of this phenomenon reveal that this feature is general rather than being unique for the experimental conditions of this paper (Table IV, which also gives available data for k_3, the slope of ATP-dephosphorylation curves).

It is of special interest to compare our results with those or Mårdh [57] obtained at 21°C with an ox brain ($Na^+ + K^+$)-ATPase preparation of about the same purity as ours (judged from the maximal phosphorylation level). First of all, it appears from Table IV that $k_3 + k_{-2} \approx k_3$ and that therefore $k_3 > k_{-2}$ also at temperatures higher than 1°C. The implication of this for the role of the sequence $E_1P \rightarrow E_2P$ in the ($Na^+ + K^+$)-stimulated ATP hydrolysis is discussed in detail in paper III of this series [48]. Secondly, it is possible from the value of k_3 in Table IV and the values for $[E_1P]_o$ and $[E_2P]_o$ obtained by Mårdh to calculate $k_2 \simeq 7$—$8\ s^{-1}$ at 21°C by means of Eqn. 16. These considerations reveal that an increase in temperature of 20°C increases both k_3 and k_2 (and the Na^+-ATPase activity, cf. Refs. 47 and 57) by a factor of about 30.

Although k_{-2} is small, the conversion of E_1P to E_2P cannot be completely irreversible if synthesis of ATP from ADP and P_i occurs through this pathway. Taniguchi and Post [24] have demonstrated that this synthesis requires very high concentrations of Na^+ ($K_{0.5}$ is of the order of 1 M) to proceed. This might be explained by scheme S3 proposed above: to get a net flux from E_2P to $E_1'P$, we must reduce $[E_1'P]$ sot that $[E_1'P] \cdot c < k_{-2}[E_2P]$ and this could be attained by very high $[Na^+]$.

The reaction of E_1P with ADP

In a computer simulation study of a simplified model for ($Na^+ + K^+$)-ATPase, Mårdh and Linddahl [77] have assumed a rate constant of 2400 $min^{-1} = 40\ s^{-1}$ for the reaction $E_1P \rightarrow E_1$ in order to get a good fit of their model to their data.

They note that this signifies a high value for the second-order rate constant (denoted by us as k''_{-1}) for the reaction of E_1P with ADP, since their ADP concentration is of the order of 1 μM. If we assume that the factor of about 30, which represents the effect of a 20°C increase in temperature for k_3 and k_2 (see Table IV and the above calculation of k_2), can be used here, 40 s^{-1} at 21°C is about 1.3 s^{-1} at 1°C. With our value of $k''_{-1} = 2.4 \cdot 10^3\ M^{-1} \cdot s^{-1}$ this corresponds to an ADP concentration of about 0.5 mM. This improbably high value indicates a profound disagreement between our determination of k''_{-1} and this particular assumption in the simulation study.

With certain assumptions, it is possible to calculate a minimum value for the dissociation constant K_d for:

$$E^P_{ADP} \underset{k''_{-1}\,[ADP]}{\overset{k_1}{\rightleftharpoons}} E_1P$$

when the value of the apparent rate constant for phosphorylation, approx. 10 000 min^{-1} at saturated [ATP] determined by Mårdh and Zetterquist at 21°C [60], is assigned to k_1. Using the temperature correction factor of approx. 30 (see preceding section), this gives $k_1 \simeq 5\ s^{-1}$ at 1°C and hence $K_d = k_1/k''_{-1} = 5/2.5 \cdot 10^3 \approx 2$ mM. The finding of Beaugé and Glynn [72] that K_m for ADP in the ATP-ADP exchange is in the millimolar range seems to support the above somewhat speculative considerations and underline the difference of several orders of magnitude between the affinity of E_1P and of E_1 for ADP, the dissociation constant of E_1ADP being about 0.5 μM [66].

Alternative kinetic mechanisms

In this section we shall discuss briefly some alternative proposals for the kinetic mechanisms of dephosphorylation and interconversion of the phosphointermediates.

Skou [78] has suggested that the ADP-sensitive and K^+-sensitive phosphointermediates were formed in parallel and not in succession. With only Na^+ in the medium this is clearly not so, since it would give an 'all or none' response for H_{rel} when ADP was added. In this case, the dephosphorylation would be described by the sum of two independent exponential functions:

$$[EP]_t = [E_1P]_o \cdot e^{-\gamma t} + [E_2P]_o \cdot e^{-\delta t}$$

Extrapolation of the slow phase to zero time would thus always yield $H_{rel} = [E_2P]_{o,rel}$ independent of [ADP].

The studies of Saito [79] and Kanazawa et al. [56] have led them to propose a model quite different from ours:

$$E_2S \overset{K_3}{\rightleftharpoons} EP \overset{k_{+4}}{\longrightarrow} E + P_i + ADP$$

where only EP is precipitated by acid and where K_3 and k_{+4} are both K^+ dependent. In later models proposed by Fukushima and Tonomura [42,80], a K^+-sensitive EP (ADP) precedes an ADP-sensitive EP in the ATP-hydrolysis cycle:

$$\underset{1}{E} \rightleftharpoons \underset{2}{E_1ATP} \rightleftharpoons \underset{3}{E_2ATP} \rightleftharpoons \underset{4}{EP(ADP)} \rightleftharpoons \underset{5}{EP} \rightarrow E$$

(This is the sequence proposed in Ref. 42. The later version [80] shows reac-

tions 2 and 4 to be irreversible). These suggestions are in direct contrast to the observations of Post et al. [13,25] and to studies on the reversal of the ATPase reaction [24]. Furthermore, the biphasic dephosphorylation curves obtained with 1 mM ATP + ADP (Fig. 2) are incompatible with this scheme.

Karlish et al. [37] have made an interesting suggestion to explain the effect of ADP on Na : Na exchange. They propose as a possibility that ADP may react with E_2P and accelerate its conversion to E_1P in analogy with the effect of ATP on the $E_2 \rightarrow E_1$ conversion. We can, however, rule out that mechanism at least for the brain enzyme at 1°C, since we find the rate constant k_{-2} to be small even in the presence of 2.5 mM ADP. Perhaps scheme S3 offers a more likely explanation. It contains a rapidly reversible interconversion between E_1P (Na) and $E_1'P$ involving the off and on reaction of Na^+ and leave out the presumably slow step between E_2P and E_1P as part of Na : Na exchange.

Conclusion

In this paper we have presented an experimental and theoretical analysis that strongly supports the validity of scheme S1 (see Introduction). Application of the mathematical model corresponding to scheme S1 on our own results, and on those reported by others, has enabled us to extend the previous description of the phosphointermediates to a quantitative one in terms of rate constants for the processes involved. This analysis indicates that our conclusions are valid in general for Na^+-ATPase and are not specific for our particular experimental conditions. A proposed refinement of the model, scheme S3, provides a framework for the explanation of the dual effects of Na^+ on phosphorylation and Na^+-ATPase activity, a framework which may also be used in the evaluation of the Na : Na exchange mechanism.

Among the results we want to emphasize are

(a) that there seems to be a dephosphorylation of E_1P to E_1 and P_i with the same rate constant as the dephosphorylation of E_2P, and

(b) that the $E_2P \rightarrow E_1P$ reaction (the backward reaction) is slow compared to the $E_2P \rightarrow E_2 + P_i$ process.

These properties turn out to be important elements in the analysis of the steady-state kinetics of the $(Na^+ + K^+)$-ATPase as described in the following paper [48].

Appendix A

The model describing the time course of dephosphorylation according to scheme S2 assumes that the input to the EP pool is zero and that $[EP]'_\infty$ therefore is zero.

We shall show here that if there is an input with the constant rate γ, meaning that $[EP]'_\infty > 0$, the same model applies if $[EP] = [EP]' - [EP]'_\infty$ is used as the variable instead of $[EP]'$.

$$\text{rate of input} = \gamma \rightarrow E_1P \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E_2P \xrightarrow{k_3}$$

The time course of EP is described by:

$$\frac{d[E_1P]'}{dt} = k_{-2}[E_2P]' - (k_{-1} + k_2)[E_1P]' + \gamma \qquad \text{(A1)}$$

$$\frac{d[E_2P]'}{dt} = k_2[E_1P]' - (k_{-2} + k_3)[E_2P]' \tag{A2}$$

At $t = \infty$ the new steady state is reached:

$$0 = k_{-2}[E_2P]'_\infty - (k_{-1} + k_2)[E_1P]'_\infty + \gamma \tag{A3}$$

$$0 = k_2[E_1P]'_\infty - (k_{-2} + k_3)[E_2P]'_\infty \tag{A4}$$

Furthermore, since $[E_1P]'_\infty$ and $[E_2P]'_\infty$ are constant we have:

$$\frac{d[E_1P]'}{dt} = \frac{d([E_1P]' - [E_1P]'_\infty)}{dt} \quad \text{and likewise for } [E_2P]' \tag{A5}$$

combination of Eqns. A1—A3 and Eqns. A2—A4 and Eqn. A5 leads to:

$$\frac{d[E_1P]}{dt} = k_{-2}[E_2P] - (k_{-1} + k_2)[E_1P] \tag{A6}$$

$$\frac{d[E_2P]}{dt} = k_2[E_1P] - (k_{-2} + k_3)[E_2P] \tag{A7}$$

These equations are the same as Eqns. 1 and 2.

Appendix B

We shall derive here a simplified expression for G_{rel} as a function of [ADP] (see Eqns. 26 and 27), using the information which we have obtained on k_{-2}, k_2 and k_3.

First we find an approximation for h (Eqn. 7):

$$h = \sqrt{[(k_{-1} + k_2) - (k_{-2} + k_3)]^2 + 4k_2k_{-2}}$$

$$= \sqrt{[(k_{-1} - k_3) + (k_2 - k_{-2})]^2 + 4k_2k_{-2}}$$

$$= \sqrt{(k_{-1} - k_3)^2 + (k_2 + k_{-2})^2 + 2(k_{-1} - k_3)(k_2 - k_{-2})}$$

Earlier we have shown that since $G_{rel} \geqslant 0$, $k_{-1} \geqslant k_3$ and we have also found that $k_{-2} << k_3 < k_2$. At this point we therefore conclude that:

$$h' = \sqrt{(k_{-1} - k_3)^2 + (k_2 + k_{-2})^2 + 2(k_{-1} - k_3)(k_2 + k_{-2})}$$

$$h' = (k_{-1} - k_3) + (k_2 + k_{-2}) \tag{B1}$$

is a very good approximation for h. We now define:

$$h' - h \equiv \epsilon \qquad (\text{note that } \epsilon = 0 \text{ for } k_{-1} = k_3) \tag{B2}$$

When we enter Eqns. B1 and B2 into Eqn. 27 and divide with $[E_1P]_{o,rel}$ we get:

$$\frac{G_{rel}}{[E_1P]_{o,rel}} = \frac{2(k_{-1} - k_3) - \epsilon(1 + [E_2P]_{o,rel}/[E_1P]_{o,rel})}{2(k_{-1} - k_3) - 2\epsilon + 2(k_2 + k_{-2})} \tag{B2}$$

Since $[E_2P]_{o,rel}/[E_1P]_{o,rel} \approx 1.5$ (see Eqn. 21), the last term in the numerator is close to 2ϵ. Rearranging Eqn. B3 and defining $[E_1P]_{o,rel} = A$ leads to:

$$\frac{1}{G_{rel}} \simeq \frac{1}{A}\left(1 + \frac{k_2 + k_{-2}}{(k_{-1} - k_3) - \epsilon}\right) \simeq \frac{1}{A}\left(1 + \frac{k_2 + k_{-2}}{k_{-1} - k_3}\right) \tag{B4}$$

Assuming spontaneous as well as ADP-dependent dephosphorylation of E_1P, as outlined under Results:

$$k_{-1} = k'_{-1} + k''_{-1} \cdot [ADP]$$

Eqn. B4 can be written:

$$\frac{1}{G_{rel}} = \frac{1}{A}\left(1 + \frac{(k_2 + k_{-2})/k''_{-1}}{(k'_{-1} - k_3)/k''_{-1} + [ADP]}\right) \tag{B5}$$

or, corresponding to the illustration by the Eadie-Scatchard plot:

$$\frac{G_{rel}}{(k'_{-1} - k_3)/k''_{-1} + [ADP]} = \frac{k''_{-1}}{k_2 + k_{-2}} \cdot (A - G_{rel}) \tag{B6}$$

Provided $(k'_{-1} - k_3) << k''_{-1} \cdot [ADP]$, i.e., if the rate constants for the spontaneous dephosphorylation of E_1P and E_2P are about the same, G_{rel}^{-1} will be a linear function of $[ADP]^{-1}$ and $G_{rel}/[ADP]$ a linear function of G_{rel}.

Acknowledgements

The excellent technical assistance of Mrs. Inge Raungaard is gratefully acknowledged. This work was supported by a grant from the Danish Medical Research Council, J. No. 512—10806.

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