ATP−ADP Exchange Reaction Catalyzed by Na^+,K^+-ATPase: Dephosphorylation by ADP of the E_1P Enzyme Form[†]

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ABSTRACT: We studied the effects of Mg^{2+} and of ADP and other nucleoside diphosphates on the dephosphorylation of the E_1P form of the partially purified pig kidney Na^+,K^+-ATPase at 20−22 °C. We report for the first time the rate of the reversal of ATP phosphorylation. The experiments were done on enzyme subjected to controlled chymotrypsin digestion consisting of a homogenous population of a truncated catalytic subunit. Under this condition the whole cycle is $E_1 \leftarrow (f_1 \cdot ATP, b_1) \rightarrow E_1ATP \leftarrow (f_2, b_2) \rightarrow E_1P \cdot ADP \leftarrow (f_d, b_d \cdot ADP) \rightarrow E_1P-(f_3) \rightarrow E_1$. The values of f_1, b_1 , f_2, and f_3 were independently estimated in the absence of ADP; those of f_d, b_d, and b_2 were obtained from the fit of ADP-dependent dephosphorylation data to the differential equation set. When $f_2 = 0$ or b_1 is very large, the model predicts that dephosphorylation by ADP gives a single exponential; in all other cases it predicts a biphasic dephosphorylation in a semilogarithmic plot.The fast phase is governed by $b_2 \cdot$ADP and the slow one by b_1.This was experimentally verified. Also, ADP stimulates E_1P breakdown without release of P_i, thus leading to ATP synthesis. The data indicate that the true substrate for ATP synthesis is free ADP, while Mg^{2+} inhibits mainly by a reduction in the free [ADP]; in addition, E_1P has a very low affinity for MgADP. The nucleotide structure is also very important; all ADP analogues tested were much less effective than ADP due to a reduced affinity for the E_1P and a poor capacity to reverse phosphorylation.

The Na^+,K^+-ATPase is a plasma membrane enzyme that belongs to the E_1-E_2 family of transport P-ATPases. It is in charge of exporting Na^+ ions from the cell in exchange for K^+ ions present in the extracellular space. During the overall cycle 3 Na^+ ions are expelled and 2 K^+ ions were taken up for each ATP molecule being hydrolyzed while a series of phospho and dephospho intermediates are formed. It can also work in reverse where 2 K^+ are expelled, 3 Na^+ are taken up, and one molecule of ATP is synthesized from ADP and inorganic phosphate (see ref *1* for references). Hence, this system can perform different total and partial reactions. However, the association of chemical and transport events is loose: in some cases there is a biochemical counterpart to the transport event, while in others one of them is missing. The whole cycle (either forward or reverse) can be arbitrarily and experimentally divided into two: a Na^+ and a K^+ half-cycle. The Na^+ part is related to the extrusion of Na^+ in the forward mode and to the uptake of that ion in the reverse mode. In detail, it includes phosphorylation of the enzyme from ATP in the presence of Na^+(i) and Mg^{2+}(i), the formation of the sodium "occluding" $MgE_1P(Na_3)$ intermediate with the release of ADP to the cell interior, and its transformation into the "deoccluding" MgE_2PNa_3 form and the release of Na^+ to the outside, leaving the MgE_2P state ready to accept extracellular K^+. Therefore, this fraction of the Na^+,K^+-ATPase cycle carries two related partial reactions: the phosphoryl group exchange between ATP and ADP (known as ATP−ADP exchange) and the (ATP + ADP)-dependent Na^+(i)−Na^+(o) exchange. The first reaction can take place without the second, but for the second the first is indispensable; the reason is that ATP synthesis requires binding of ADP to $MgE_1P(Na_3)$ while extracellular Na^+ binds to MgE_2P. That is why oligomycin and *N*-ethylmaleimide, which block the $MgE_1P(Na_3)$−MgE_2PNa_3 transition, actually enhance ATP−ADP exchange *(1)*.

There are still several unresolved questions about the ATP−ADP exchange. On the one hand, although there are values for the rate of MgE_1ATP−$MgE_1P(Na_3)$ transition, there are no available data for the transition in the opposite direction nor on the rate constants associated with ADP binding to E_1P. On the other hand is the question of the role(s) of Mg^{2+}. Magnesium is essential for this partial reaction (to allow enzyme phosphorylation from ATP), but concentrations higher than 25 μM become inhibitory *(2)*. The mechanism of that inhibition is not known, but it must be related to the reverse reaction (ATP synthesis) since the Na^+-ATPase activity (ATP hydrolysis in the absence of extracellular K^+) is almost completely insensitive to Mg^{2+}. One possibility is that the true substrate is free ADP *(2)*. This would be consistent with the fact that E_1P has Mg^{2+} bound to it and this cation is not released until dephosphorylation takes place *(3, 4)*. Finally, we also lack evidence on the nucleotide specificity for that backward reaction. These aspects of the ATP−ADP exchange catalyzed by the Na^+,K^+-ATPase are addressed in this work. We initially intended to use native partially purified pig kidney enzyme; but, and in agreement with others (see ref *5*), that preparation showed a negligible or nil ADP-stimulated EP breakdown at the Na^+ concentration employed, surely because practically all goes into the E_2P state *(6, 7)*. On the other hand, it is

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well-known that with high NaCl concentrations the phosphoenzyme, even formed with normal or low [NaCl], becomes sensitive to ADP. However, that preparation does not lose completely its sensitivity to potassium ions, retaining a sizable Na^+,K^+-ATPase activity (*5*; personal unpublished results). Under these conditions, the minimal reaction scheme would be more complicated that than proposed here (see below), including the ADP-sensitive and the K^+-sensitive phosphoenzymes and the unidirectional rate constants relating the transitions between them. That system would make it practically impossible to determine, with any acceptable level of accuracy, the ADP-associated rate constants we intend to investigate. Therefore, we decided to subject pig kidney Na^+,K^+-ATPase to selective chymotrypsin digestion (see Materials and Methods). The rationale for considering this proteolyzed enzyme as a reasonable model for E_1P is adressed in the Discussion. The results provided values for all rate constants associated with the reverse phosphorylation. In addition, they indicated that the true substrate for ATP synthesis is free ADP, while Mg^{2+} inhibition occurs mainly by a reduction in the free [ADP]. However, we cannot rule out a Mg^{2+} inhibitory site present in the native enzyme that was removed by chymotrypsin. Finally, we show that the nucleotide structure is crucial for dephosphorylation of E_1P. All ADP analogues tested were much less effective than ADP, in their ability both to bind to E_1P and to pick up the phosphoryl group from the enzyme.

MATERIALS AND METHODS

Theory

Reaction Cycle for the Chymotrypsin-Treated Enzyme. With this treatment the enzyme is phosphorylated by ATP in a (Mg^{2+} + Na^+)-dependent manner but it does not go into the E_2P state *(8)*. Under this condition the whole reaction cycle could be reduced to the following scheme:

model 1

$$E_1 \leftarrow (f_1\cdot ATP,\ b_1) \rightarrow E_1\cdot ATP \leftarrow (f_2,\ b_2) \rightarrow E_1P\cdot ADP \leftarrow (f_d,\ b_d\cdot ADP) \rightarrow E_1P - (f_3) \rightarrow E_1$$

The resulting simultaneous differential equations are

$$d[E_1]/dt = -f_1[E1][ATP] + b_1[E_1ATP] + f_3[E_1P] \quad (1)$$

$$d[E_1ATP]/dt = f_1[E_1][ATP] + b_2[E_1P\cdot ADP] - (f_2 + b_1)[E_1ATP] \quad (2)$$

$$d[E_1P\cdot ADP]/dt = f_2[E_1ATP] + b_d[E_1P][ADP] - (b_2 + f_3)[E_1P\cdot ADP] \quad (3)$$

$$d[E_1P]/dt = f_d[E_1P\cdot ADP] - f_3[E_1P] \quad (4)$$

These equations, solved by numerical integration, were used for simulation and data fitting for both E_1P formation and breakdown. The values of the unidirectional rate constants in chymotrypsin-treated enzyme were not known and had to be determined. By appropriate experimental designs *(9)* we determined f_1, b_1, f_2, and f_3 in the absence of ADP. The on and off rates for ADP binding to E_1P, b_d and f_d, respectively, and b_2 were obtained from the fit of ADP-dependent dephosphorylation data to the set of simultaneous differential equations 1−4 using the previously calculated rate constants.

Experimental Procedures

Source of Enzyme. Pig kidney Na^+,K^+-ATPase was partially purified in a discontinuous sucrose gradient according to Jorgensen *(10)*; the specific activity was 15-20 units/mg and remained stable for months when stored at −85 °C (3−5 mg of protein/mL) in 25 mM imidazole (pH 7.5 at 20 °C), 2 mM EDTA, and 10% sucrose. Immediately before use the enzyme was washed twice (1:10 v/v) and resuspended in a solution containing 15 mM Tris·HCl (pH 7.4 at 37 °C), 1 mM NaCl, and 0.1 mM EGTA (when chymotypsin treatment was needed) or 100 mM NaCl, 70 mM imidazole (pH 7.4 at 20 °C), and 0.1 mM EGTA for native enzyme; sucrose and EDTA were absent.

Chymotrypsin Digestion. Controlled chymotryptic digestion was carried out incubating the enzyme (final concentration 1 mg/mL) for 30 min at 37 °C in 15 mM Tris·HCl (pH 7.4 at 37 °C), 1 mM NaCl, 0.1 mM EGTA, and 100 μg/mL α-chymotrypsin. The reaction was stopped by dilution into an ice-cold solution of 150 mM NaCl, 30 mM imidazole (pH 7.4 at 20 °C), and 0.1 mM EGTA. The enzyme was then washed twice at 0 °C in 10 volumes of the stopping solution and resuspended in the working solution [100 mM NaCl, 70 mM imidazole (pH 7.4 at 20 °C) and 0.1 mM EGTA] at about 3 mg/mL total protein concentration.

Biochemical Assays. Na^+,K^+-ATPase activity was assayed as in ref *11* following the release of [^{32}P]P_i from [γ-^{32}P]-ATP labeled according to Glynn and Chappell *(12)* with modifications *(13)*. The incubation solution had 20 mM KCl, 130 mM NaCl, 30 mM imidazole hydrochloride (pH 7.4), 3 mM $MgCl_2$, 3 mM ATP, and 0.1 mM EGTA. ATP−ADP exchange was performed at 20 °C as in ref *14*. The solutions always contained 100 mM NaCl, 70 mM imidazole hydrochloride (pH 7.4), and 0.1 mM EGTA. Protein was determined by the method of Lowry et al. *(15)*. Lithium dodecyl sulfate−polyacrylamide gel electrophoresis (LDS−PAGE) was done as indicated in ref *16*.

Estimation of Unidirectional Rate Constants in Chymotrypsin-Treated Enzyme. Pre-steady-state phosphorylation and dephosphorylation were carried out at 20-22 °C with an Intermekron DSM 3-SF gas-driven rapid mixing apparatus (Uppsala, Sweden) *(17)* where Berger ball mixers *(18)* were substituted for the usual tangential mixers. Different arrangements had two syringes and one chamber or three syringes and two chambers. The volume delivery from each syringe was 1 mL, and syringe 1 always contained the enzyme at a total protein concentration of 50 μg/mL. In some cases the enzyme had been interacting with ligands prior to incorporation into the syringe. This was done to reduce the total tubing length that would result in loss of a major fraction of the protein; i.e., in conditions in which reaching steady-state phosphorylation would take too long (see below). The output of the final chamber went into the stopping solution consisting of 7 mL of ice-cold 12% perchloric acid, 1 mM ATP, and 10 mM inorganic phosphate. The length of the tubes connecting mixing chambers and the last mixing chamber with the stopping solution provided the time of the reactions. The denatured protein was collected on Whatman GF/F filters, washed with 5% trichloroacetic acid and 10 mM inorganic phosphate, and counted in a liquid scintillation counter. The protein retention of

theses filters, checked with the Ponceau staining method *(19)*, was around 90%. The number of phosphorylating sites in the enzyme was determined on the basis of the E-P formed after 10 s of incubation at 20 °C in the presence of 100 μM [γ-^{32}P]ATP. The stopping solution, collection of the denatured protein, and radioactivity counting were performed as indicated above. Heat- or acid-denatured enzyme prior to phosphorylation was used as blank. The phosphorylating sites in chymotrypsin-treated enzyme ranged from 0.6 to 0.9 nmol/mg of protein; this is much lower than the usual 1.6−1.9 nmol/mg of protein observed in native Na^+,K^+-ATPase. These estimations were performed in each experiment and the values were used in the kinetic calculations.

Release of [^{32}P]P_i from E_1P. In the same experiment the EP and the concentration of [^{32}P]P_i were measured in the acid quenching mixture that contained, in addition to protein, nonhydrolyzed [γ-^{32}P]ATP. A 1-mL aliquot of that mixture was used for [^{32}P]P_i determination and the rest was filtered and washed as usual for EP estimation. The [^{32}P]P_i determination is described in ref *20*. One milliliter of stopping mixture was added to 1 mL of a 2% charcoal suspension on ice and was vortexed intermittently for about 10 min. After centrifugation in the cold at 3000 rpm for 10 min, the supernatant was taken and the procedure was repeated. One milliliter of the second supernatant and 50 μL of a 10 mM K_2HPO_4 solution were added to 1.5 mL of 1:1 (v/v) 2-butanol/xylene solution saturated with bidistilled water, placed in a 60 mm × 125 mm screw-cap tube, and mixed briefly by vortexing. Then 0.8 mL of 5% ammonium molybdate (in 4 N HCl) was added, mixed, and let stand at room temperature. After 15 min, 4 mL of the 2-butanol/xylene solution saturated with bidistilled water was added and, with the cap well tightened mixed vigorously with a vortex mixer for 10 s three times with 10 s intervals. Once phase separation had occurred, 2 mL of the top layer was added to 5 mL of 1 N NaOH placed in a scintillation vial and mixed until colorless. The Cerenkov radiation was counted in a liquid scintillation counter.

Solutions. All solutions were made with deionized bidistilled water. NaCl and KCl were of spectrometric grade. Vanadium-free ATP and ADP were from Boehringer Mannheim; the other chemicals, of reagent grade, were obtained from Sigma Chemical Co. Inorganic [^{32}P]phosphate was purchased from the Comisión Nacional de Energía Atómica of Argentina. [^{14}C]ADP was from New England Nuclear. Using arsenazo (III) as Mg indicator *(21)* we obtained, under the conditions of our experiments, a K_d for MgATP of 86 ± 5 μM and a K_d for MgADP of 621 ± 9 μM; these values were used for calculations of free ADP concentrations.

Counting. Radioactivity assays were performed in a Beckman liquid scintillation counter using a toluene-based scintillation fluid; counting times were long enough to obtain standard errors of about 1%.

Curve fitting was performed with the Scop nonlinear regression computer program (National Biomedical Simulation Resource, Durham, NC).

RESULTS

Some Characteristics of the Chymotrypsin-Treated Enzyme. Both the theoretical and experimental approaches used here were based on the existence of an homogeneous enzyme population. This point was checked by performing LDS−PAGE on native and treated preparations (*16*) previously subjected to phosphorylation from [γ-^{32}P]ATP in the presence of Na^+ and Mg^{2+}. All protein bands were detected by Coomassie Blue staining and the catalytic subunits also by autoradiography. The results of one of these experiments are illustrated in Figure 1. As expected, the chymotrypsin-digested enzyme used in this work consisted of a homogeneous population with a catalytic subunit of a molecular mass lower than the native one [77−83 kDa (*8, 22*)].

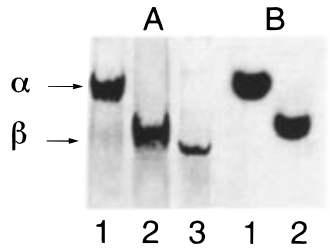

FIGURE 1: LDS−PAGE of native and chymotrypsin-treated Na^+,K^+-ATPase. Partially purified enzyme from pig kidney was subjected to controlled chymotrypsin treatment as described in Materials and Methods. Native and treated enzymes were phosphorylated by 1 s of incubation in 100 mM NaCl, 70 mM imidazole hydrochloride (pH 7.4), 0.1 mM EGTA, 10 μM Mg^{2+}, and 10 μM [γ-^{32}P]ATP. The reaction was stopped with 5% trichloroacetic acid, 10 mM P_i, and 1 mM ATP (final concentrations). After centrifugation, the pellet was washed twice in the same solution and then dissolved in the LDS-containing sample buffer. After 10 min at 37 °C, sucrose and pyronine Y were added and the electrophoresis was run at pH 2.4 and 4 °C as indicated in Materials and Methods. (A) Coomassie Blue staining where the α and β subunits are indicated; (B) autoradiography. Lanes 1 refer to native and lanes 2 to chymotrypsin-treated enzymes. Lane 3 in panel A corresponds to a bovine serum albumin standard. Note that (i) there is a difference in migration between native and treated enzyme α subunits and (ii) the gels do not indicate any heterogeneity in the α subunit populations.

In addition, this selectively proteolyzed enzyme had the following functional properties (not shown here): (i) There was no detectable Na^+,K^+-ATPase activity. (ii) The phosphoenzyme formed from ATP in the presence of Na^+ and Mg^{2+} was completely insensitive to K^+ ions but was dephosphorylated by ADP. (iii) When K^+ replaced Na^+ there was still EP formation from ATP; however, the EP levels were 30−40% of those seen with Na^+. (iv) An ATP−ADP exchange reaction was observed in Na^+-containing and also in Na^+-free Tris-buffered solutions. The exchange rate in the absence of Na^+ was about 25% of that seen in Na^+-containing medium. These results are in agreement with those reported by others (*8, 22, 23*).

Estimation of the Unidirectional Rate Constants of Model I in the Absence of ADP. We started by estimating all the unidirectional rate constants in model 1, except b_2, f_d, and b_d, in the absence of ADP at different Mg^{2+} concentrations. The lack of ADP made b_d meaningless but not f_d and b_2. We were aware that ignoring these last two constants could result in an underestimation of f_1 and/or f_2 in case b_2 was large and/or f_d was small. This could be of greater concern in chymotrypsin-treated enzyme, whose cycle does not have the almost irreversible E_1P-E_2P transition *(8)*. A way out of this problem would have been to use the f_1 and f_2 initially calculated to estimate b_2, f_d, and b_d and then take those values and reestimate the ATP-related constants until convergence was achieved. As shown below, the difference without and with consideration of the ADP-related constants was insignificant and that lengthy procedure was unnecessary. Before

Table 1: Unidirectional Rate Constants for ATP Phosphorylation and for Spontaneous Dephosphorylation of Chymotrypsin-Treated Na^+,K^+-ATPase in the Absence of ADP[a]

constant	concentration of ionized Mg^{2+}		
	none or 10 μM[b]	2 mM	5 mM
f_1 (ms^{-1} μM^{-1})	0.0248 ± 0.0010 (0.0261 ± 0.0009)	0.0264 ± 0.0012 (0.0275 ± 0.0009)	0.0151 ± 0.0012 (0.0163 ± 0.0008)
b_1 (ms^{-1})	0.0075 ± 0.0018	0.0069 ± 0.0019	0.0088 ± 0.0029
f_2 (ms^{-1})	0.0593 ± 0.0022 (0.0602 ± 0.0011)	0.0620 ± 0.0017 (0.0605 ± 0.0010)	0.0684 ± 0.0009 (0.0671 ± 0.0007)
f_3 (ms^{-1})	0.0006 ± 0.0003	0.0009 ± 0.0004	0.0007 ± 0.0001
K_{sATP} (b_1/f_1) (μM)	0.30	0.26	0.58

[a] The values of the rate constants were determined as described in Materials and Methods and ref *9*. The total number of phosphorylating sites was estimated separately on the basis of E-P formation from [γ-^{32}P]ATP. In parentheses are the values corrected by the introduction of the ADP-related constants in the absence of ADP in the solution, b_2 and f_d from Table 2. Note that there is no significant difference between the two procedures. Each entry is the mean ± SEM from the fits. [b] As some ionized magnesium was required for f_1 and f_2, we decided to use 10 μM.

attempting to measure f_1 and f_2, we needed the values of f_3 and b_1. These procedures and their rationale are described in detail in ref *9*.

(i) Rate Constant for Spontaneous E_1P Breakdown (f_3). Preliminary experiments showed that it required long times to attain steady-state phosphorylation with low concentrations of [γ-^{32}P]ATP. Actually, with the expected nucleoside concentration to be used in the ADP dephosphorylation experiments that would be about 100 ms. We therefore decided to start incubating the enzyme with ligands prior to its introduction into syringe 1. The total time elapsed between that operation and the actual mixing with the content of syringe 2 was about 10 s. Enzyme phosphorylation was initiated with 50 μg of total protein/mL in 100 mM NaCl, 70 mM imidazole hydrochloride (pH 7.4), 0.1 mM EGTA, 1 mM $MgCl_2$, and 3 μM [γ-^{32}P]ATP. Control experiments showed that after 10 s at 20 °C only 4−6% had been hydrolyzed while the amount of phosphoenzyme formed was indistinguishable from that obtained with 100 μM [γ-^{32}P]-ATP in 100 ms. The effluent from syringe 1 was mixed with 1 mL from syringe 2 containing 120 μM cold ATP (final concentration of 60 μM, which gives a 40/1 ratio of cold/radioactive), 2 mM *trans*-1,2-diaminocyclohexanetetraacetic acid (CDTA), and suitable [$MgCl_2$] to give none, 2 mM, or 5 mM final free [Mg^{2+}]. The dephosphorylation reaction was stopped at different times up to about 100 ms. The EP levels at $t = 0$ were obtained by delivering the content of syringe 1 into the acid stopping solution. The values of f_3 were obtained from the slope of the straight line given by the semilogarithmic plot of acid-stable EP as a function of the time passed between the mixing of both syringes and the stopping of the reaction.

(ii) Off Rate Constant for ATP Binding (b_1). One milliliter from syringe 1 containing 50 μg of total protein equilibrated with a (Na^+ + Mg^{2+})-free solution of 70 mM imidazole hydrochloride (pH 7.4), 0.1 mM EGTA and 0.1 mM CDTA was mixed with 100 μM [γ-^{32}P]ATP without or with 1 mM $MgCl_2$ coming from syringe 2. After 30 ms this suspension was mixed with 15 mM cold ATP and 1 mM free [Mg^{2+}] delivered by syringe 3; after variable times the effluent of that mixing chamber was mixed with 15 mM cold ATP, variable [$MgCl_2$] (to give variable final free [Mg^{2+}]), and 400 mM NaCl (final concentration 100 mM). Phosphorylation was stopped after 20 ms. Note that NaCl was present only during the phosphorylation reaction. The value of b_1 was calculated from the slope of the semilogarithmic plot of acid-stable phosphoenzyme as a function of the variable time between addition of cold ATP and NaCl.

(iii) Simultaneous Estimation of the On Rate of ATP Binding to E_1 (f_1) and the Rate for E_1ATP-E_1P Transphosphorylation (f_2). With the values of b_1 and f_3 already at hand, and neglecting b_2 and f_d in the absence of ADP, the fit of phosphorylation data to eq 1 contained three unknowns: f_1, f_2, and E_{tot}. The total number of phosphorylating sites was measured separately as indicated above. The estimation of f_1 and f_2 was done using two syringes following the time-dependent EP formation by varying the concentration of [γ-^{32}P]ATP at constant but different ionized [Mg^{2+}]. The elected [ATP] were 0.75, 1.5, 3, 5, 10, 25, 50, and 100 μM. The time-dependent EP formation rates with the eight ATP concentrations were simultaneously fitted to the equations from model 1; that gave two fitting parameters for around 80 data points.

The graphs from which the constants were calculated were identical to those already published for native enzyme *(9)*; for that reason we elected not to include them in this paper. All constant values determined in this section are in Table 1. The three conditions investigated were free (column 1), 2 mM (column 2), and 5 mM (column 3) [Mg^{2+}]. It must be pointed out that only b_1 and f_3 could be determined in true Mg^{2+}-free conditions (excess CDTA). Because there is no way to phosphorylate the enzyme in the absence of this cation, f_1 and f_2 were estimated in the presence of 10 μM ionized [Mg^{2+}]; although not zero, this concentration is much lower than the other two. With the rate constants from Table 1, model 1 predicts, in the absence of ADP, the time to reach steady state and the distribution of different enzyme forms at that point in time as a function of [ATP]. For instance, with 10 μM ATP, and at any of the three [Mg^{2+}] studied, steady state is attained in about 100 ms with the following distribution of enzyme forms: 0.3% E_1, 1.4% E_1-ATP, and 98.3% E_1P.

ADP Stimulated Dephosphorylation of E_1P in Na^+,K^+-ATPase Subjected to Controlled Chymotrypsin Digestion: Estimation of f_d, b_d, and b_2 and the Effects of Mg^{2+} Ions. All solutions employed contained three basic constituents: 100 mM NaCl, 70 mM imidazole hydrochloride (pH 7.4), and 0.1 mM EGTA. Aliquots of 50 μg of total protein/mL began phosphorylation outside the rapid mixing apparatus in the presence of 3 μM [γ-^{32}P]ATP and either 10 μM, 2 mM, or 5 mM $MgCl_2$. Once in syringe 1 this was mixed with 1 mL of solution coming from syringe 2, whose content varied. For Mg^{2+}-free conditions (phosphorylation with 10 μM $MgCl_2$) it had 1 mM CDTA. That [CDTA] would by itself stop any further phosphorylation. But, in addition, it also

had, as a control, the same cold [ATP] to be used to stop radioactive phosphorylation in the presence of Mg^{2+}. When the effects of [Mg^{2+}] were studied, cold ATP was included to give a final concentration of 60 μM after mixing, to halt radioactive phosphorylation. That concentration was 40 times the remaining radioactive nucleotide; in this case total $MgCl_2$ was also added to keep constant ionized Mg^{2+} at either 2 or 5 mM. For ADP-stimulated dephosphorylation, ADP was included together with the proper [$MgCl_2$]. The output of the mixing chamber went into the ice-cold acid stopping solution.

Preliminary experiments confirmed the expected insignificant or undetected effects of even millimolar ADP concentrations in ATP phosphorylated native Na^+,K^+-ATPase *(5)*; this was the case when dephosphorylation was followed without and with mM free Mg^{2+} concentrations (Not shown here). The results in chymotrypsin-treated enzyme are illustrated in Figure 2. They show the ADP-stimulated dephosphorylation, over a wide range of [ADP], in the absence (panel A) and in the presence of 2 mM (panel B) and 5 mM (panel C) ionized [Mg^{2+}]. In each graph the symbols correspond to experimental data obtained at the free ADP concentrations indicated in the caption. Two important findings are immediately noticeable. On the one hand, in all cases the ADP-stimulated dephosphorylation is biphasic; on the other, and as a difference from native RS vesiculated Ca^{2+}-ATPase *(24)* and oligomycin-treated Na^+,K^+-ATPase *(25)*, we were also able to measure the EP decline corresponding to the fast initial phase. The lines through the points in each figure correspond to the simultaneous best fits of all data points to the set of differential equations resulting from model 1 using the already estimated constants from Table 1. In general the fits were very good except for the initial points at the highest ADP concentrations. Possible implications will be dealt with in the Discussion. These simultaneous fits allowed the determination of the rate constants related to ADP-stimulated dephosphorylation and the way they are influenced by the ionized Mg^{2+} concentration. Their values are summarized in Table 2. The results show that Mg^{2+} is not at all required for ADP stimulation of E_1P breakdown: a huge rate is attained in its absence and with a singular high apparent affinity for the free nucleotide, which contrasts with the low affinity observed for total ADP in the presence of Mg^{2+}. In fact, for free ADP, going from Mg^{2+}-free to 5 mM [Mg^{2+}] produces a slight, 25% increase in b_2, no change in the off rate constant for ADP binding to E_1P (f_d), and about 50% reduction in the on rate for that ADP binding (b_d). Table 2 also shows that when total [ADP] is being considered, b_2 remains the same and there is a slight increase in f_d but a dramatic reduction in b_d (see Discussion). Two additional important points call for comments at this time: (i) With these rate constants we corrected those initially obtained for f_1 and f_2 (Table 1). The differences between the two set of values (the corrected values are shown in parentheses) was insignificant. (ii) In the simultaneous fitting, no matter what initial value for b_d we took [0.0001 μM^{-1} ms^{-1} or 1 μM^{-1} ms^{-1} as given by Hammes and Schimmel *(26)*], the system always converged to the same final values for the three. The same happened when different values were initially given to b_2 and f_d.

Lack of Extra [^{32}P]P_i Release during ADP Stimulation of EP Breakdown. So far the experiments have shown an increase in E_1P dephosphorylation due to ADP but they do not assure that this is due to a reverse reaction of the ATPase cycle. ADP stimulation of E_1P hydrolysis, although unlikely, cannot be ruled out *a priori*. One way to assess if we are in the presence of an actual synthesis of ATP is to follow the rate of [^{32}P]P_i release. If simultaneously with an increase in E_1P breakdown there is no extra [^{32}P]P_i release into the medium, the conclusion that it had been synthesis of ATP is inescapable. We checked that point in the experiments summarized in Table 3. It can be seen that, after 5.5 ms and expressed per milligram of total protein, the release of [^{32}P]P_i was the same, and indistinguishable from zero, in the absence and in the presence of 1 mM free ADP. That coincided with a marked stimulation of E_1P split, which went from almost nil in the absence of ADP to 0.379 ± 0.013 nmol/mg in its presence. Therefore, we can safely conclude that in our experiments all ADP stimulation of E_1P break-

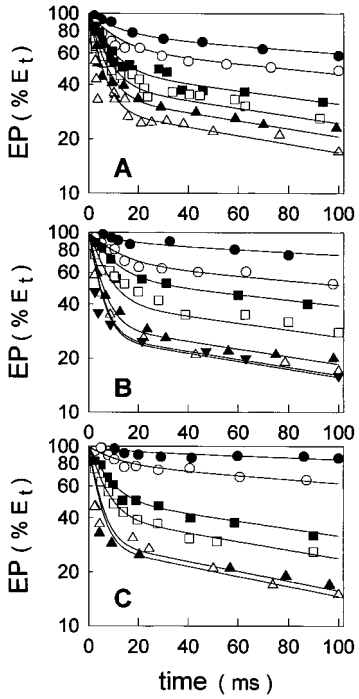

FIGURE 2: ADP stimulated dephosphorylation, in the absence and presence of variable [Mg^{2+}], of chymotrypsin-treated Na^+,K^+-ATPase after steady-state phosphorylation from ATP. The experiments were performed as indicated in the text at 20−22 °C with a Intermekron rapid mixing apparatus. All solutions employed contained three basic constituents: 100 mM NaCl, 70 mM imidazole hydrochloride (pH 7.4), and 0.1 mM EGTA. Aliquots of 50 μg of total protein/mL began phosphorylation outside the rapid mixing apparatus in the presence of 3 μM [γ-^{32}P]ATP and either 10 μM (A), 2 mM (B), or 5 mM (C) $MgCl_2$. Once in syringe 1 this was mixed with 1 mL of solution coming from syringe 2, containing 1 mM CDTA and cold ATP (60 μM final), and for ADP-stimulated dephosphorylation ADP was included together with the proper [$MgCl_2$] to keep constant ionized Mg^{2+} at either 2 or 5 mM. The output of the mixing chamber went into the ice-cold acid stopping solution. The data points are the values found experimentally. The lines are the best fits to model 1 using the rate constants from Table 1 on the basis of the following distribution of enzyme forms at steady state: 0.3% E_1, 1.4% E_1ATP, and 98.3% E_1P. The corresponding micromolar ADP concentrations, shown as free (total), were as follows. (A) Mg^{2+}-free: ●, 5 (5); ○, 10 (10); ■, 25 (25); □, 50 (50); ▲, 100 (100); △, 1000 (1000). (B) 2 mM Mg^{2+}: ●, 2 (10); ○, 8 (50); ■, 15 (100); □, 39 (250); ▲, 142 (1000); △, 520 (3000); ▼, 945 (5000). (C) 5 mM Mg^{2+}: ●, 1.5 (20); ○, 7.7 (100); ■, 38 (500); □, 75 (1000); ▲, 426 (5000); △, 992 (12000).

Table 2: Dephosphorylation by ADP of the E_1P Form of Chymotrypsin-Treated Na^+,K^+-ATPase: Effects of Mg^{2+} Ions[a]

	concentration of ionized Mg^{2+}		
constant	none	2 mM	5 mM
b_2 (ms^{-1})	0.161 ± 0.004	0.191 ± 0.004 (0.193 ± 0.005)	0.201 ± 0.008 (0.217 ± 0.006)
f_d (ms^{-1})	1.131 ± 0.012	1.067 ± 0.024 (1.655 ± 0.025)	1.194 ± 0.034 (1.816 ± 0.044)
b_d ($ms^{-1}\,\mu M^{-1}$)	0.0376 ± 0.0014	0.0287 ± 0.0018 (0.0064 ± 0.0002)	0.0185 ± 0.0021 (0.0019 ± 0.0001)
K_s (μM)	34	37 (259)	64 (958)

[a] The kinetic parameters correspond to the ADP-stimulated dephosphorylation of E_1P using model 1 from the text and the associated rate constants from Table 1. The values of the rate constants were estimated from the simultaneous fit of the data presented in Figure 2, panels A (no free Mg^{2+}), B (2 mM free Mg^{2+}), and C (5 mM free Mg^{2+}). Each entry is the mean ± SEM from the simultaneous best fits. Note that the values for total ADP are in parentheses.

Table 3: Absence of an Extra Release of Inorganic Phosphate into the Incubation Medium during the ADP Stimulation of E_1P Breakdown[a]

ADP during dephosphorylation (mM)	EP breakdown (nmol/mg of protein)	P_i released (nmol/mg of protein)
none	0.012 ± 0.020	0.012 ± 0.006
1	0.379 ± 0.013	0.010 ± 0.005

[a] The amount of EP and the concentration of $[^{32}P]P_i$ were measured, as described in Materials and Methods, in the acid quenching stopping solution after 5.5 ms of dephosphorylation in the absence and presence of 1 mM ADP. Each entry is the mean ± SEM from three different experiments. Note that the breakdown of E_1P induced by ADP takes place without any extra release of $[^{32}P]P_i$ into the medium.

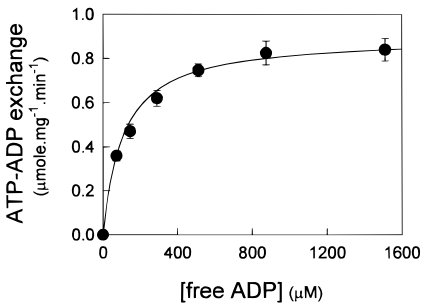

FIGURE 3: ADP stimulation of ATP−ADP exchange catalyzed by chymotrypsin-treated Na^+,K^+-ATPase in the presence of Na^+ ions. Phosphoryl group exchange between ATP and ADP was performed at 20 °C as indicated in ref *14* in solutions of the following composition: 100 mM NaCl, 70 mM imidazole hydrochloride (pH 7.4), 0.1 mM EGTA, 0.15 mM free Mg^{2+}, 3 mM ATP, and variable concentrations of $[^{14}C]$ADP. The symbols correspond to mean ± SEM of triplicate determinations. The line is the best fit to a Michaelian relationship between exchange and free [ADP]. Note that the K_m for free ADP is 108 ± 5 μM.

down represents the reverse reaction of the Na^+,K^+-ATPase cycle leading to ATP synthesis.

ADP Stimulation of ATP−ADP Exchange in Chymotrypsin-Treated Na^+,K^+-ATPase. The phosphoryl group exchange between ATP and ADP has forward and backward reactions; so far we have considered the backward reaction. Thus, if the ADP interactions with E_1P seen here are indeed related to the overall reaction we should observe an apparent affinity for ADP in the ATP−ADP exchange similar to, or at least not very different from, that seen above. The experiments to check this point are summarized in Figure 3. They were performed at the same temperature and in the same solution used for pre-steady-state kinetics. The only difference was the presence of 3 mM ATP at a constant Mg^{2+} of 0.15 mM. In the figure the rate of ATP−ADP exchange in the presence of Na^+ is plotted against the concentration of free ADP. The data points, fitted to a Michaelian equation, give a K_m for free ADP of 108 ± 5 μM. When a similar plot was made using the difference between the exchange in Na^+-containing and Na^+-free solutions, the rates were about 25% lower but the K_m was indistinguishable (112 ± 4 μM). Although on the high side, these K_m values are within the range of those observed for ADP dephosphorylation of E_1P (see Discussion).

Ability of Other Nucleoside Diphosphates To Stimulate E_1P Dephosphorylation. For triphosphate compounds it has already been shown that the nucleoside part of the molecule is crucial in determining its effectiveness on both the catalytic and regulatory roles in Na^+,K^+-ATPase *(27, 28)*. However, no information was available for diphosphate compounds in the reverse reaction cycle. Therefore, we decided to look into the structure−function relationship of nucleoside diphosphates in connection with their ability to dephosphorylate E_1P in the chymotrypsin-treated enzyme. Four ADP analogues (called here by the general name of NDP) were investigated in the absence of Mg^{2+}: CTP, GTP, IDP, and UDP. The experimental design was the same in all cases and was identical to that used before for ADP. On the other hand, the treatment of the data required a different approach. In these cases the E_1P formation before the start of dephosphorylation will be from ATP; after addition of NDP some rephosphorylation will come from the E_1NTP (N for analogue) complex. As the b_1 and f_2 values will correspond to these complexes, these experiments will provide us with the possibility to obtain additional up to now unknown information: the off rate constant for binding and the "transphosphorylation rate constant" for nucleoside triphosphates *other than ATP*. The calculation of unidirectional constants has more unknowns in these cases. Considering that E_1P spontaneous breakdown is independent of the source of the forward phosphorylation, f_3 is already known; but five constants, f_2, b_1, b_2, f_d, and b_d, had to be determined from the fits. Initially, we used the set of equations given by model 1, but the final values of the parameters, particularly those of b_2, f_d, and b_d, varied with the initial values given to them; however, the f_d/b_d ratio, an expression of the affinity of E_1P for NDP, remained constant. We tried to improve the fitting by an approximation that reduced the number of parameters. To that aim model 1 was modified by ignoring the $E_1P{\cdot}$ADP complex, and consequently f_d and b_d, replacing them by a K_m. Although less rigorous, this approach allowed

Table 4: Calculated Values of b_2 and K_m for the Dephosphorylation of E_1P in Chymotrypsin-Treated Na^+,K^+-ATPase by CDP, UDP, GDP, and IDP in the Absence of Mg^{2+} Ions[a]

constant	CDP	GDP	IDP	UDP
b_2 (ms^{-1})	0.0263 ± 0.0017	0.0375 ± 0.0017	0.0154 ± 0.0063	0.0415 ± 0.0011
K_m (μM)	625 ± 58	3931 ± 373	1357 ± 384	1222 ± 124

[a] The kinetic parameters correspond to the simultaneous fit of the nucleoside diphosphate-stimulated dephosphorylation of E_1P to model 2 from the text, where the E_1P·NDP complex is ignored and $b_{2app} = b_2[NDP]/(K_m + [NDP])$. The values of b_2 and K_m for each nucleotide were estimated from the data presented in Figure 4, considering also as parameters b_1 and f_2. Each entry is the mean ± SEM from the fits.

Table 5: Calculated Values of the Off Rate Constant for Binding to E_1 and E_1NTP-E_1P Transition for the Interaction of Different Nucleoside Triphosphates with Chymotrypsin-Treated Na^+,K^+-ATPase[a]

constant	CTP	GTP	ITP	UTP
b_1 (ms^{-1})	0.0696 ± 0.0006	0.0256 ± 0.0002	0.0183 ± 0.0067	0.0167 ± 0.0007
f_2 (ms^{-1})	0.1597 ± 0.0069	0.1456 ± 0.0093	0.1209 ± 0.0350	0.0363 ± 0.0021

[a] The rate constants were calculated from simultaneous fits of sets of curves relating the nucleoside diphosphate-stimulated dephosphorylation of E_1P to model 2 from the text, where the E_1P·NDP complex is ignored and $b_{2app} = b_2[NDP]/(K_m + [NDP])$. The values of b_1 and f_2 for each nucleotide were estimated from simultaneous fit of the data presented in Figure 4, considering also as parameters b_2 and K_m. Each entry is the mean ± SEM from the fits. See text for details. NTP refers to the nucleosides triphosphate CTP, GTP, ITP, and UTP.

us to obtain a quantification of the data. The resulting scheme is

model 2

$$E_1 \leftarrow (f_1 \cdot ATP,\ b_1) \rightarrow E_1ATP \leftarrow (f_2,\ b_{2app}) \rightarrow E_1P-(f_3) \rightarrow E_1$$

where

$$b_{2app} = b_2[NDP]/(K_m + [NDP])$$

and the fitting parameters were f_2, b_1, b_2, and K_m. With this modification the final values of these parameters were independent of the initial conditions. The data points and the simultaneous best fits are illustrated in Figure 4 while the values of the kinetic constants are in Table 4 for the NDPs and in Table 5 for the NTPs.

Table 4 shows that the NDP compounds are poor replacements for ADP in promoting E_1P dephosphorylation; this is seen both in the dephosphorylation rate and in the apparent affinity for the phosphoenzyme. The b_2 values are $^1/_4$ to $^1/_{10}$ of that with ADP, following the sequence UDP > GDP > CDP > IDP. The K_m differences are more dramatic, expressing a quite low affinity of the NDP compounds for E_1P. The K_m values are 20−120-fold higher than the K_S for ADP and the sequence in this case is GDP > UDP = IDP > CDP.

The off rate constant for binding (b_1) and the transphosphorylation rate constant (f_2) for the NTP compounds (triphosphate ATP analogues) are shown in Table 5. Comparing them with those for ATP in Table 2, it is obvious that there are differences, but they are not striking. For example, b_1 for CTP is 3-fold higher while those of the other nucleotides are within the range, or even slightly smaller, than that of ATP; i.e., the release from the catalytic site is not much affected by the nucleotide structure. The sequence for b_1 is CTP > GTP > ITP = UTP. For the transphosphorylation rate CTP, GTP, and ITP appear even better than ATP, while UTP is about the same. Their importance will be considered in the Discussion.

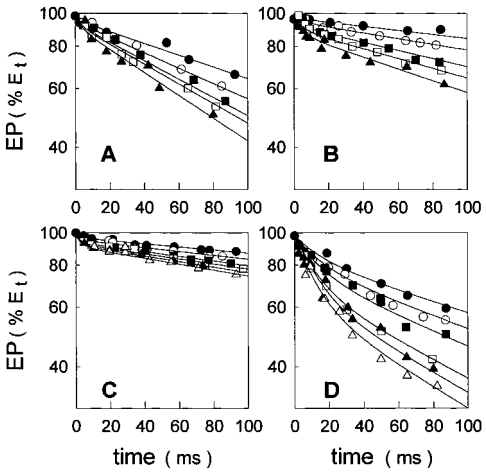

FIGURE 4: Effects of different nucleosides diphosphates (CDP, GDP, IDP, and UDP) in the absence of Mg^{2+} ions on the dephosphorylation of ATP-phosphorylated chymotrypsin-treated Na^+,K^+-ATPase. Chymotrypsin-treated Na^+,K^+-ATPase was phosphorylated for 10 s in the presence of 3 μM [γ-^{32}P]ATP, 10 μM $MgCl_2$, 0.1 mM EGTA, 100 NaCl, and 70 mM imidazole hydrochloride (pH 7.4). Time = 0 corresponds to the time phosphorylation was halted by adding excess CDTA. Dephosphorylation was carried out in the same solution without Mg^{2+}. The micromolar concentrations of the different nucleoside diphosphates were as follows. (A) CDP: ●, 500; ○, 1000; ■, 2000; □, 3000; ▲, 5000. (B) GDP: ●, 500; ○, 1000; ■, 2000; □, 3000; ▲, 5000. (C) IDP: ●, 500; ○, 1000; ■, 2000; □, 3000; ▲, 5000; △, 10 000. (D) UDP: ●, 500; ○, 700; ■, 1000; □, 2000; ▲, 3000; △, 5000. The lines through the points are the best simultaneous fit to model 2 in the text with the following parameters b_2, K_m, f_2, and b_1. Temperature was 20 °C.

DISCUSSION

The main objective of this work was to fill an important gap in the kinetic scheme of the Na^+,K^+-ATPase cycle: up to now there was no reported value for the rate of the reverse ATP phosphorylation reaction (E_1P·ADP−E_1·ATP). To optimize the response to ADP and to simplify the reaction scheme, so the ADP-related rate constants could be estimated from data fitting, we opted for the enzyme selectively digested with chymotrypsin. This preparation has been amply used in establishing important processes related to the Na^+ pump like the occlusion of Na^+ ions, the effects of vanadate, and the changes in conformations during the cycle *(1)*. The LDS−PAGE bands, detected with Coomassie Blue or previous phosphorylation from ATP, show that the chymotrypsin-treated Na^+,K^+-ATPase consists of a homogeneous population of catalytic subunits. This, which has

been also observed by others (*8, 22*), implies that reasons other that protein heterogeneity must account for the biphasic dephosphorylation kinetics. The reaction scheme used here considers that, when phosphorylated by ATP in the presence of Na^+ and Mg^{2+}, this preparation goes into E_1P while the transition into E_2P is blocked *(*see ref *8)*. Taking aside the fact that this is not a native enzyme (E_1P does not mean exactly the E_1P in the native state), the real issue is whether it can be used as a model for ADP interactions with E_1P. Let us consider some available data with the chymotrypsin-treated enzyme: (a) In the absence of phosphorylation from ATP it can occlude K^+ but not Na^+ ions, and ATP is ineffective as deoccluding agent (*29*). The authors proposed an ATP-insensitive E_2(K) form, but there may be other options, for instance, that chymotrypsin treatment is to K^+ what oligomycin is to Na^+ (*30*): both allow their occlusion in the unphosphorylated E_1 state. An observation in favor this idea is the fact that the chymotrypsin-treated Na^+,K^+-ATPase cannot be phosphorylated from P_i even in the presence of ouabain (*22*). As this P_i incorporation occurs only in the E_2 state (*31, 32*), it may very well be that this enzyme does not go into E_2. (b) In the presence of phosphorylation from ATP (*23, 33*), and the nonnucleotide fueling substrate acetyl phosphate (*33*), the enzyme occludes Na^+; in both instances Na^+ is released in the presence of ADP, a typical E_1P behavior. (c) Phosphorylation from ATP, which requires Mg^{2+}, can take place in the absence of Na^+; the EP levels, which in our hands are 30−40% of those seen in the presence of Na^+, can be detected also with K^+ as the main monovalent cation. The EPs formed in Na^+-containing or Na^+-free media are fully dephosphorylated by ADP with similar rates (*22*); this is also a key feature of the E_1P state. Therefore, the possibility that, in the presence of ATP, Mg^{2+}, and Na^+, all phosphoenzyme is in the MgE_1P(Na) state cannot be ruled out. (d) The enzyme phosphorylated from ATP in the presence of Na^+ and Mg^{2+} is not dephosphorylated by K^+ but it is wholly sensitive to ADP (*8, 22*; this work). From all the above it seems safe to conclude that the phosphorylated chymotrypsin-treated Na^+,K^+-ATPase remains in the E_1P form; this is even more obvious in the presence of Na^+. Therefore, this preparation can be used as a model for ADP−E_1P interactions; evidently, with the reservation that one cannot extrapolate all observations to the enzyme in the native state.

The results presented here are the first report of three main aspects related to this reverse phosphorylation: (i) the time course of the event, (ii) the rate of the E_1P−E_1ATP transition, and (iii) the values of the rate constants for the on and off ADP binding to E_1P. Additional relevant information has to do with the effects of [Mg^{2+}] and the ability of other nucleoside diphosphates to replace ADP.

The experiments were done in the absence and in the presence of 2 and 5 mM free Mg^{2+}. Early studies on the effects of Mg^{2+} on the rate constants related to ATP phosphorylation, without ADP, revealed similarities and differences between this preparation and native enzyme (see Table 2). For instance, the on rate constant for ATP binding (f_1) has values identical to those seen in native enzyme *(9)* and, likewise, it is relatively insensitive to [Mg^{2+}]: no effect up to 2 mM with a slight reduction at 5 mM Mg^{2+}. On the other hand, while in native enzyme the off rate constant for MgATP binding (b_1) is 4-fold lower than that for free ATP *(9, 34)*, both species are released at the same rate from

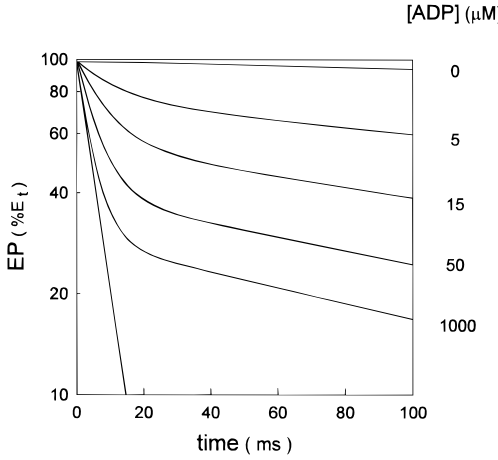

FIGURE 5: Computed simulation of ADP-stimulated dephosphorylation in the absence of Mg^{2+} of chymotrypsin-treated Na^+,K^+-ATPase after steady-state phosphorylation from ATP. Model 1 from the text and the unidirectional rate constants from Tables 1 and 2 were used. Time = 0 corresponds to the halting of phosphorylation after steady state has been reached. At this point the distribution of enzyme forms is 0.3% E_1, 1.4% E_1ATP, and 98.3% E_1P. The different curves are for [ADP] equal to 0, 5, 15, 50, and 1000 μM. Note that when [ADP] = 0 there is a slight *increase* in E_1P after stopping phosphorylation, which reaches its maximum at about 20 ms; from then on the E_1P has a linear decay whose rate constant is f_3. When [ADP] > 0 a reduction in E_1P starts at $t = 0$, but it is biphasic; an initial fast phase, which increases in size and rate with [ADP], is followed by a slow one. When [ADP] is large enough the initial part of the fast and the late part of the slow phases are linear; for $f_3 \ll b_2$ and $f_3 < b_1$, the initial slope is equal to b_2/2.303 and the late slope is approximately b_1/2.303. Note that the straight line coinciding with the initial slope at the highest [ADP] is obtained either when there is no rephosphorylation ($f_2 = 0$) or when there is a very fast rate of ATP release from E_1ATP (b_1 very large).

chymotrypsin-digested enzyme. However, the K_m for ATP (b_1/f_1) is around 0.3−0.6 μM; that is, about the same in both preparations. The transphosphorylation rate to form E_1P is not affected by Mg^{2+}; in native enzyme we followed the overall transphosphorylation from E_1ATP to E_2P, and that rate was stimulated by Mg^{2+} with a K_m of about 40 μM *(9)*. This may be another difference due to chymotrypsin; alternatively it might be due to a Mg^{2+}-stimulated E_1P−E_2P transition as suggested long ago (ref *35*; but see also ref *36*). Finally, in the chymotrypsin-digested preparation there is a slight, although not statistically significant, rate of spontaneous hydrolysis of E_1P that is also Mg^{2+}-independent.

In the nucleotide-stimulated dephosphorylation we followed the decay of the acid-stable phosphoenzyme. As such, there was no way to distinguish between free E_1P and the E_1P·ADP complex; therefore the E_1P estimates were always the sum of both species. The fact the ADP-stimulated E_1P decay was not associated with [^{32}P]P_i release into the medium (Table 3) indicates that it was related to ATP synthesis. An ADP-promoted E_1P hydrolysis had to be ruled out in view of other ADP-stimulated EP breakdowns not associated with the reverse reaction like those observed in Na^+,K^+- and SR Ca^{2+}-ATPase (*37, 38*). The theoretical model for the chymotrypsin-digested enzyme (model 1) predicts a biphasic response to ADP. This is very nicely seen in simulations. Figure 5 exemplifies a semilogarithmic plot of a computed simulation, using the rate constants from Tables 1 and 2 for free Mg^{2+}, of the ADP-stimulated dephosphorylation once phosphorylation from ATP has reached steady state. As it happened with the experimental data, E_1P refers to the sum

of the ADP-free and ADP-bound E_1P. Five values of [ADP] covered the whole range from zero to nonlimiting concentrations. When [ADP] = 0 there is a slight, and almost undetected, *increase* in EP after stopping phosphorylation; after about 20 ms E_1P has a linear decay whose rate constant is f_3. When [ADP] > 0 a reduction in E_1P starts at $t = 0$, but it is biphasic; an initial fast phase, which increases in size and rate with [ADP], is followed by a slow one. When [ADP] is large enough, the initial part of the fast phase and the late part of the slow phase are linear. For $f_3 \ll b_2$ and $f_3 < b_1$, the initial slope is equal to $b_2/2.303$ and the late slope is approximately $b_1/2.303$. The crucial step for the shape of the response to ADP in our system is the rephosphorylation of the enzyme from E_1ATP (the $E_1ATP-E_1P{\cdot}ADP$ transition). Notice from the computed simulation in Figure 5 that when rephosphorylation is prevented, either with a very slow rephosphorylation or a very fast release of ATP from E_1ATP, the ADP-dependent E_1P decay becomes linear with a rate equal to b_2. A biphasic response to ADP has also been seen in native sarcoplasmic reticulum Ca^{2+}-ATPase *(24, 37*−*39)*. In these experiments there are no data points in the fast phase and the slope of the slow phase has a *marked increase* together with [ADP]. A reason for the quantitative discrepancy with our data could be that in the Ca^{2+}-ATPase both the fast and the slow components are much faster than the equivalents in our system. In any event, that work and our own agree on a slow release of ATP from E_1ATP: 0.035 ms^{-1} for SR Ca^{2+}-ATPase at pH 7.0 and 25 °C and 0.008−0.034 ms^{-1} for pig kidney Na^+,K^+-ATPase at pH 7.4 and 20 °C. Relevant to this point are the data of Hobbs et al. in native and particularly in oligomycin-treated eel Na^+,K^+-ATPase *(25)*. When those enzymes phosphorylated from ATP were exposed to 5 mM ADP, a large fraction of the phosphoenzyme disappeared at a rate that could not be measured; this was particularly noticeable in the enzyme treated with oligomycin. It is possible that under those conditions, as may be the case with the SR Ca^{2+}-ATPase, the $E_1P{\cdot}ADP-E_1ATP$ transition is exceedingly fast. Alternatively, it has been proposed (Froehlich, personal communication) that this is indeed an artifact: upon reaching the acid medium of the quenching solution $E_1P{\cdot}ADP$ is quickly transformed into E_1ATP. If this suggestion is correct, one must assume that it does not happen in the chymotrypsin-treated Na^+,K^+-ATPase. Our interpretation of the results is in line with that of Pickart and Jencks *(24)*. These authors also consider the case of a single enzyme species where the rate constant governing the second phase is the slow release of ATP from E_1ATP. On the other hand, our model predicts, and our data substantiates, that the slow phase of E_1P decay will only slightly increase by [ADP] until reaching a slope controlled by b_1 (f_3 is very small). Actually, the validity of model 1 is further supported by the fact that similar values for b_1 can be obtained from the direct estimates reported in Table 1 and also from the slope of the slow component at saturating [ADP]. There is one detail that calls for comments. In the families of ADP-stimulated dephosphorylation curves (Figure 2), the initial points at the highest ADP concentrations lie to the left of the fitting curves. The data of these particular three curves could not be adequately fitted to the working model no matter what rate constants we took. There is no clear explanation for this behavior. One possibility is that it reflects a difficulty in assigning proper times at the initial short intervals for fast dephosphorylation rates; on the other hand, an additional ligand effect stimulating E_1P breakdown, although unlikely, cannot be completely ruled out. Whatever mechanism is involved, it does so by synthesis of ATP because the data in Table 3 clearly show that 1 mM free ADP leads to E_1P breakdown without release of free $[^{32}P]P_i$. At any rate, for the bulk of the data with ADP, and also with the other nucleoside diphosphates, the working model has proven to be appropriate.

We are giving here the first estimates for the rate constants associated with reverse ATP phosphorylation in Na^+,K^+-ATPase (Table 2). Although they were obtained in chymotrypsin-treated enzyme, considering the other unidirectional rate constants measured it is not unlikely that values of that order correspond also to the native preparation. The rate for the step of ATP synthesis is between 0.17 and 0.21 ms^{-1} (170−210 s^{-1}). Taking b_2 and the value of the transphosphorylation rate constant (0.059−0.068 ms^{-1}), this gives a $[E_1P{\cdot}ADP]/[E_1ATP]$ equilibrium of around 0.34. However, that equilibrium is offset by the large value of f_d. Actually, it is satisfying that the calculated values for the off rate for ADP binding to E_1P (f_d) of around 1.1 ms^{-1} (1000 s^{-1}) (Table 2) agree very well with the estimations of Froehlich and Heller *(37)* for the same constant in the SR Ca^{2+}-ATPase.

Magnesium ions are essential for ATP phosphorylation *(35)*. In addition, the $E_1ATP \rightarrow E_1P$ transition is required for the biphasic ADP-dependent E_1P decay observed both with and without free Mg^{2+}. There is convincing experimental evidence that Mg^{2+} is tightly bound to the enzyme while it stays phosphorylated *(3, 9, 34)*. Consequently, even in the absence of Mg^{2+} in the solution this cation must remain at the active site long enough to allow rephosphorylation of E_1ATP. Obviously, the same arguments apply to the biphasic E_1P decay produced by other nucleoside diphosphates. Closely linked to this point is the ADP species that acts as substrate for ATP synthesis. Our data shows that the K_S for total ADP increases 7.0-fold when going from Mg^{2+}-free to 2 mM Mg^{2+} and 15-fold when $[Mg^{2+}]$ was 5 mM. Under these conditions the K_S for free ADP did not change or increased just 90%, respectively. This latter increment is very small compared to those of total ADP. Thus, Mg^{2+} inhibiton of the ADP-dependent dephosphorylation of E_1P seems to occur mainly by a reduction in the free [ADP]. In other words, MgADP is not a substrate, and it is so simply because it does not fit into the ADP site. The exceedingly low affinity for MgADP is supported by the fact that high [MgADP] shows little effect on the dephosphorylation promoted by low [free ADP] (not shown). These observations also fit very well with the 112 μM K_m for free ADP stimulation of ATP−ADP exchange, particularly considering it is a kinetic K_m and not an equilibrium K_S. Actually, even if it were a K_S the slight discrepancy could be accounted for by ATP competing with ADP for the E_1P site with about 100 times lower affinity. Taken all together, these data show that free ADP is the true substrate for ATP synthesis in the reverse Na^+,K^+-ATPase cycle. A puzzling observation, for which we have no explanation, was the slight, although also statistically significant, rise in b_2 when $[Mg^{2+}]$ went from zero to 2 mM (15%) and 5 mM (31%).

This and previous works with several nucleoside di- and triphosphates indicate that the importance of the nucleotide structure depends on the partial reaction of the Na^+,K^+-ATPase being considered. It is crucial for dephosphorylation of E_1P by diphosphate nucleotides (this work) and for the

deocclusion of E_2 (K_2) *(28)*, but it does not seem so important for the release of NTP from E_1 or the E_1NTP−E_1P transphosphorylation (this work). Again, we are aware that there is no way to know if the data on chymotrypsin-treated enzyme can be fully extrapolated to native enzyme. If it were, the reduced capability of the analogues acting as substrates for the Na^+,K^+-ATPase should be found in their inability to act on specific steps of the reaction, mainly K^+ deocclusion *(28)*. Actually, the effectiveness correlation of these nucleotides in the deocclusion of Rb^+ and the Rb^+−Rb^+ exchange catalyzed by the Na^+,K^+-ATPase is very good: ATP high; CTP moderate; ITP and GTP slight; UTP none *(28)*. As far as the diphosphate compounds other than ADP, the data indicate that their poor ability to promote E_1P breakdown is a mixture of a low affinity for the E_1P binding site together with a difficulty to revert phosphorylation.

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