E_2P Phosphoforms of Na,K-ATPase. I. Comparison of Phosphointermediates Formed from ATP and P_i by Their Reactivity toward Hydroxylamine and Vanadate†

Natalya U. Fedosova,* Flemming Cornelius, and Irena Klodos

Department of Biophysics, University of Aarhus, Ole Worms Allé 185, DK-8000 Aarhus C, Denmark

Received March 26, 1998; Revised Manuscript Received June 29, 1998

ABSTRACT: The properties of Na,K-ATPase phosphoenzymes formed either from ATP in the presence of Mg^{2+} and Na^+ or from P_i in the absence of alkali cations were investigated by biochemical methods and spectrofluorometry employing the styryl dye RH421. We characterized the phosphoenzyme species by their reaction to *N*-methyl hydroxylamine, which attacks specifically the protein−phosphate bond. We studied reactions of the phospho- and dephospho-enzymes with vanadate, which is a transition-state analogue of phosphate in this enzyme. On the basis of substantial differences in the properties of the phosphoenzyme species formed either from ATP or P_i, especially in their reactivity to *N*-methyl hydroxylamine, it is suggested that the two phosphoenzyme species are two subconformations of the E_2P phosphoform. Analysis of the RH421 fluorescence responses under a variety of experimental conditions and comparing different enzyme sources suggested that the increase of RH421 fluorescence induced by inorganic phosphate in the absence of alkali cations is associated with the formation of the covalent acyl−phosphate bond.

The Na,K-ATPase or the sodium pump is an integral membrane protein found in all animal cells. It couples the active sodium transport out of the cell to the potassium transport into the cell at the expenditure of energy derived from the splitting of ATP. It is of vital importance for several cell functions including osmoregulation, energy coupling, water and salt balance, and generation of the membrane potential. The enzyme belongs to the P-type transport ATPases, i.e., it possesses phosphotransferase activity and performs hydrolysis in two steps: (i) transfer of the terminal phosphoryl group of ATP to a β-aspartyl residue; (ii) donation of the phosphoryl group to water. Both steps are reversible: when the cycle is forced to run backward, ATP can be synthesized from ADP and P_i (*1*, *2*).

In the presence of $MgCl_2$ and low [Na^+], phosphorylation from ATP (the so-called "physiological" route) leads to the formation of an acid-stable phosphoform, E_2P,[1] which can donate its phosphoryl group only to water. Inorganic phosphate in the presence of $MgCl_2$ can also phosphorylate Na,K-ATPase (the so-called "back-door" or "direct" route) forming an acid-stable phosphointermediate. Chemical characterization of the two phosphoforms, formed from ATP or P_i and isolated by acid denaturation indicated a close similarity. In both phosphoenzymes, the phosphate is covalently bound to the same aspartyl residue (*3*), which was later identified as Asp-369 (*4*). A similar conclusion was reached by Sontheimer et al. (*5*) who applied ^{31}P NMR method to peptic fragments of sarcoplasmic Ca-ATPase. Despite the chemical identity, the kinetic properties of the two phosphoforms are different. Although both phosphoenzymes are insensitive to ADP, their reactivity to K^+ differs: while the dephosphorylation of E_2P formed from ATP is activated by K^+, the dephosphorylation of the phosphointermediate formed from P_i in the presence of $MgCl_2$ and in the absence of alkali cations is almost insensitive to K^+ (*3*).

The purpose of the present investigation is to characterize in more detail and to compare the properties of E_2P phosphointermediates obtained either by the "physiological" or by the "direct" routes of phosphorylation. Vanadate is used to investigate the transition state of phosphate hydrolysis since its physical and chemical similarity to phosphate is generally accepted. A combination of biochemical and fluorescent methods has been applied to investigate phosphorylation and vanadate binding to the enzyme and to assess the nature of the acyl−phosphate bond in the enzyme derivative. The fluorescence measurements were performed with a styryl dye, RH421, which reports events in the reaction cycle of the Na,K-ATPase (*6*−*18*). Furthermore, enzymes from two different sources were compared: shark rectal gland and pig kidney Na,K-ATPase.

The results obtained indicate that the microenvironment of the phosphorylation site of the E_2P phosphointermediates formed from ATP or from P_i differs. A further characterization of the interactions of Na,K-ATPase and its phosphoenzymes with K^+ and other cations, described in the proceeding paper (*19*), lends additional support to the hypothesis that the two phosphointermediates are different subconformations of the E_2P-form of Na,K-ATPase. Preliminary results were reported in abstract form (*20*).

† This work was supported in part by The Danish Research Academy (fellowship to N.U.F.), The Danish Medical Research Council, and The Danish Biomembrane Research Centre, University of Aarhus.

* To whom correspondence should be addressed. Phone: +45 89422937. Fax: +45 86129599. E-mail: nf@biophys.au.dk.

[1] Abbreviations: E_1, Na,K-ATPase form with high affinity toward ATP and Na^+; E_2, Na,K-ATPase form with high affinity toward K^+ and low affinity to ATP; EP, phosphoenzyme; E_1P, ADP-sensitive phosphoenzyme; E_2P, K^+-sensitive phosphoenzyme; $E_2'P$, K^+-insensitive phosphoenzyme formed from P_i; EV, enzyme-vanadate complex; P_i, inorganic phosphate; V, vanadate, k_{obs}, observed rate constant, K_{ligand}, dissociation constant for a ligand.

S0006-2960(98)00703-X CCC: $15.00
Published on Web 09/05/1998

Table 1: Characterization of Na,K-ATPase Phosphoforms from Shark Rectal Glands and Pig Kidney Outer Medulla[a]

enzyme source	specific activity at 23 °C (U/mg)[b]	E_2P from ATP		E'_2P from P_i		EP from P_i + ouabain (nmol/mg)	EV, ΔF (%)
		(nmol/mg)	ΔF (%)	(nmol/mg)	ΔF (%)		
shark	10.5	2.5	177	1.25	80	3.1	60
pig	8.0	2.35	35	2.5	20	2.82	0

[a] The activity, phosphorylation levels, and fluorescence changes were measured as described in the Experimental Procedures. Fluorescence changes (ΔF) induced by a ligand are expressed as percentage of initial fluorescence level. Data are given as means of three to six independent experiments with standard error less than 10%. [b] U = μmol of ATP split/min.

EXPERIMENTAL PROCEDURES

Enzyme Preparation. Na,K-ATPase (EC 3.6.1.37)-enriched membrane fragments from shark rectal glands were purified as previously described (*21*). Na,K-ATPase from pig kidney was prepared according to Jørgensen (*22*) as modified by Jensen et al. (*23*). The specific hydrolytic activity measured at 37 °C and under standard conditions [according to Ottolenghi (*24*)] was 30−33 U/mg protein (U = μmol of ATP split/min) for shark enzyme and about 25 U/mg protein for pig kidney enzyme. The protein content was determined according to Lowry et al. (*25*), as described by Jensen and Ottolenghi (*26*), using bovine serum albumin as standard.

Reconstitution of Na,K-ATPase into Lipid Vesicles. Solubilization of shark Na,K-ATPase using $C_{12}E_8$ was performed as previously described (*27*). The specific activity of shark Na,K-ATPase at 23 °C after solubilization is 10−15 U/mg.

Reconstitution of Na,K-ATPase into lipid vesicles containing 260 mM sucrose was as previously described (*28*) and achieved by complete removal of detergent from a mixed-micelle suspension of protein:lipid:detergent by means of Bio-Beads. The final lipid:protein weight ratio was 20, and the proteoliposomes contained phosphatidylcholine:cholesterol in a molar ratio of 60:40. In each preparation, the protein orientation after reconstitution was determined by functional tests (*28*). About 10% of the total protein was oriented inside-out. The protein content of the proteoliposomes was determined by a modified Lowry method (*29*) including phospholipids as internal standards (*30*).

ATPase Activity. Na,K-ATPase activity of membrane preparations was followed spectrophotometrically at 20 °C by coupling the ADP production to NADH oxidation. The incubation medium contained 4 mM $MgCl_2$, 130 mM NaCl, 20 mM KCl, 3 mM ATP, 10 mM HEPES, 10 mM MES, pH 7.5, and the components of the coupled enzyme system (*31*). The Na,K-ATPase activity of reconstituted Na,K-ATPase and the Na-ATPase activity of membrane preparations were measured at 20 °C with [γ-^{32}P]ATP using the method of Lindberg and Ernster (*32*). The hydrolytic activity of reconstituted enzyme oriented exclusively inside-out was estimated by addition of ATP to the medium after preincubation with ouabain in the presence of MgP_i to inhibit nonoriented enzyme, as previously described (*33*).

Phosphorylation Reactions. Phosphorylation of the enzyme from $^{32}P_i$ was performed in 4 mM $MgCl_2$, 10 mM HEPES, and 10 mM MES, pH 7.5, and measured at either 0 or 20 °C in the presence or absence of ouabain (1 mM) according to Cornelius (*30*). Phosphorylation from [γ-^{32}P]-ATP (10 or 25 μM) was performed at 0 °C as previously described (*30*) in a medium containing 4 mM $MgCl_2$, 16 mM NaCl, and 30 mM imidazole, pH 7.4.

Spontaneous Dephosphorylation. Dephosphorylation after ATP phosphorylation was measured at 0 °C by chasing with unlabeled ATP (1 mM) in the presence of 10 mM $MgCl_2$ (*30*). Dephosphorylation after P_i phosphorylation was measured at 0 °C either by diluting the $^{32}P_i$ 50-fold or by chasing with 50 mM unlabeled P_i. After a chase period, dephosphorylation was stopped by an addition of 10% trichloroacetic acid and 2 mM sodium-pyrophosphate. Phosphoenzyme was measured as described previously (*30*). For fast reactions, a rapid mixing quenched-flow apparatus was employed.

Fluorescence Measurements. Equilibrium binding of P_i or vanadate was induced by addition of various concentrations of the ligand to 50 μg/mL Na,K-ATPase in the same medium as used for chemical measurements (4 mM $MgCl_2$ in 10 mM HEPES, 10 mM MES, adjusted with *N*-methyl-D-glucamine to pH 7.5). The change in fluorescence of RH421 (concentrations 200−600 nM) in response to those additions was recorded with a SPEX Fluorolog fluorometer at 20 °C in a cuvette (1 cm lightpath) with continuous stirring. In all experiments, the excitation wavelength was 580 nm (band-pass of 1 nm) and the fluorescence was measured with a 630 nm cutoff filter on the emission side.

Transient Fluorescence Measurements. Measurements of transient RH421 fluorescence responses were performed using a rapid-mixing stopped-flow spectroflurometer (Applied Photophysics). The flow volume was 200 μL. The excitation wavelength was either 550 nm (using a xenon lamp and band-pass of 18 nm) or 546 nm (using a mercury lamp and band-pass of 9 nm) and the fluorescence was measured with a 630 nm cutoff filter on the emission side.

Materials. ATP, purchased as sodium salt from Boehringer Mannheim, Germany, was converted to Tris salt by chromatography on a Dowex 1 column (from Sigma Chemicals Co., St. Louis, MO). RH421 was purchased from Molecular Probes, Inc., Eugene, OR, and dissolved in dimethyl sulfoxide. *N*-2-hydroxyethylpiperazine-*N*′-ethanesulfonic acid (HEPES), 2-*N*-morpholinoethanesulfonic acid (MES), *N*-methyl-D-glucamine, and *N*-methyl hydroxylamine hydrochloride were purchased from Sigma. All reagents used were reagent grade.

RESULTS

Phosphorylation of Na,K-ATPase by Inorganic Phosphate. Comparison of RH-Fluorescence Response and Measured EP Levels. Phosphorylation from P_i was originally measured by Albers et al. (*34*) and Lindenmayer et al. (*35*) in the presence of Mg^{2+}, K^+, and ouabain, but phosphoenzyme can be formed in the absence of ouabain and/or K^+ as well (*1*−*3*, *36*).

In our present experiments, maximum phosphorylation levels from P_i in the presence of ouabain obtained with both

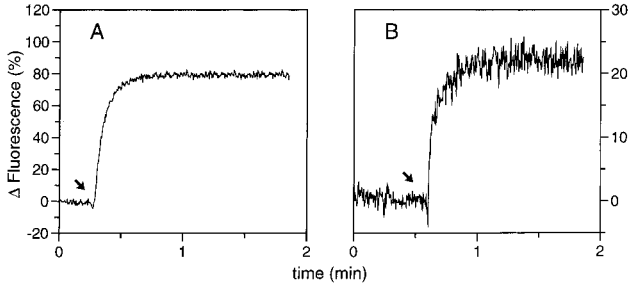

FIGURE 1: Change in RH421 fluorescence induced by addition of 3 mM inorganic phosphate (arrows) to Na,K-ATPase from shark rectal glands (panel A) or pig kidney (panel B) in 10 mM HEPES, 10 mM MES, and 4 mM $MgCl_2$, pH 7.5. The increase in fluorescence is expressed as percentage of the initial level.

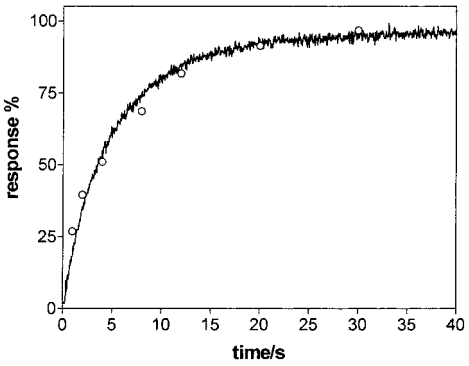

FIGURE 2: Comparison of time courses of the phosphorylation-induced RH421 fluorescence increase and the phosphorylation of Na,K-ATPase measured with $^{32}P_i$. Both experiments were performed with shark rectal gland enzyme at 20 °C in 10 mM HEPES, 10 mM MES, and 4 mM $MgCl_2$, pH 7.5. Concentration of P_i in both experiments was 4.45 mM. The fluorescence (line) was measured with rapid-mixing stopped-flow apparatus. The phosphorylation by $^{32}P_i$ (circles) was measured employing a rapid-mixing quenched-flow apparatus.

pig kidney and shark rectal gland Na,K- ATPases (Table 1) were about 3 nmol (mg of protein)$^{-1}$, a little higher than the phosphorylation level obtained from ATP [about 2.5 nmol (mg of protein)$^{-1}$]. In the absence of ouabain, phosphorylation of kidney enzyme by saturating [P_i] results in an EP level identical to that obtained with ATP. With shark enzyme, the EP level even at very high [P_i] did not exceed 50−60% of the number of active sites. Thus, there must be an intermediary phosphorylated acid labile E_2·P (cf. ref *37*), which transforms to the acid-stable E_2−P, as shown in

Scheme 1:

$$E + P_i \overset{K_P}{\rightleftharpoons} E_2{\cdot}P \overset{k_{+1}}{\underset{k_{-1}}{\rightleftharpoons}} E_2{-}P$$

The maximum level of E_2−P at [P_i] → ∞ is determined by the ratio of k_{+1}/k_{-1}, and this ratio must, therefore, be different for pig kidney and shark rectal gland enzymes.

For both shark and pig kidney Na,K-ATPase, addition of P_i in the presence of $MgCl_2$ in the absence of Na^+ and K^+ increased the fluorescence of RH421 (Figure 1). The time courses of the fluorescence increase and of the formation of acid-stable phosphointermediate are indistinguishable (Figure 2). The amplitude of the fluorescence response displayed a hyperbolic dependence on [P_i]. The estimated $K_{0.5P_i}$ for the fluorescence response to P_i addition, which according to Scheme 1 is equal to $K_P k_{-1}/(k_{+1} + k_{-1})$, was 330 and 29 μM for shark and pig kidney enzyme, respectively (Figure 3). Similar values were obtained from biochemical measurements of the acid stable phosphoenzyme level vs [P_i] performed under identical conditions (Figure 3, *insets*). The value of $K_{0.5}$ for pig kidney enzyme is identical to those previously obtained in other laboratories for Na,K-ATPase from pig kidney [32 μM (38)] and rabbit kidney [23 μM (*18*)].

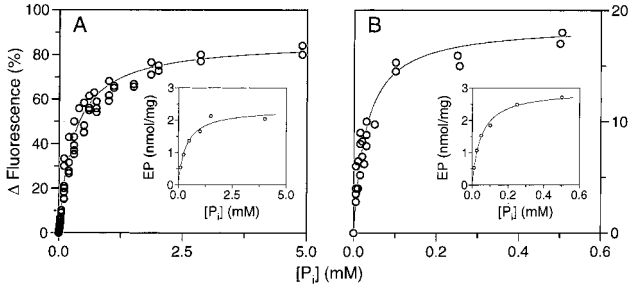

FIGURE 3: Equilibrium binding of inorganic phosphate to shark (panel A) and kidney (panel B) Na,K-ATPase estimated by RH421 fluorescence. Experiments were performed in 10 mM HEPES, 10 mM MES, and 4 mM $MgCl_2$, pH 7.5. The fluorescence change caused by phosphate addition is expressed as a percentage of the initial level. Note the difference in the scales between panel A and panel B. The levels of the acid stable phosphoenzyme formed under identical conditions are shown in the insets for comparison. All curves are hyperbolic fits to the data. For shark enzyme (panel A), $K_{0.5,fluor} = 330$ μM, $K_{0.5,chem} = 350$ μM. For kidney enzyme (panel B) $K_{0.5,fluor} = 29$ μM, $K_{0.5,chem} = 47$ μM.

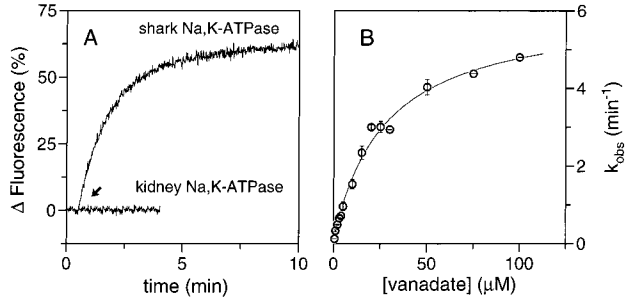

FIGURE 4: RH421 fluorescence following vanadate binding to Na,K-ATPase. Panel A shows a typical recording of RH421 fluorescence in broken Na,K-ATPase membrane preparation in response to addition of 3 μM vanadate (arrow) in the presence of 10 mM HEPES, 10 mM MES, and 4 mM $MgCl_2$, pH 7.5. In shark enzyme vanadate induced a slow monoexponential increase in fluorescence expressed as a percentage of the initial level. The observed rate constant of the fluorescence change, k_{obs}, vs vanadate concentration is shown in panel B. The values are the means of three experiments ± SE. The curve is a hyperbolic fit to the data.

The identity of the time courses for the fluorescence response and the phosphorylation measured with $^{32}P_i$, on one hand, and of $K_{0.5\ P_i}$ measured by both methods on the other indicate that the fluorescence response of RH421 is induced by formation of the acid stable E_2−P. It has to be mentioned, however, that the magnitude of the dye response with the two enzymes differs (Table 1, Figure 1), as previously discussed in Klodos et al. (*39*).

Binding of Vanadate to Na,K-ATPase Monitored by RH421 Fluorescence. To characterize the phosphointermediates in further details, we used the transition-state analogue vanadate as a substitute for phosphate. Vanadate binding to the shark enzyme induced a RH421 fluorescence response as depicted in Figure 4A (upper curve). The amplitude of the fluorescence change at saturating concentrations of

vanadate was smaller than that produced by phosphate and amounted to ~60% of the initial fluorescence level (Figure 4A). Vanadate addition to the pig kidney Na,K-ATPase was not accompanied by a fluorescence change of RH421 (Figure 4A, lower curve), although vanadate was bound to this enzyme as shown by its effect on P_i binding (see below) and by its inhibition of catalytic activity (not shown).

The rate of the fluorescence response to addition of vanadate to shark rectal enzyme was much lower than that to P_i. The observed rate coefficient of the monoexponential increase of the fluorescence (k_{obs}) showed a hyperbolic dependency on the vanadate concentration with a dissociation constant $K_V \approx 25\ \mu M$ (Figure 4B). The fact that k_{obs} is not a linear function of [V] excludes a one step interaction $E + V \rightleftarrows E{-}V$ and implies that the reaction with vanadate proceeds through formation of an intermediate complex ($E{\cdot}V$) comparable to the $E{\cdot}P$ complex formed with P_i (Scheme 1):

Scheme 2

$$E + V \overset{K_V}{\rightleftharpoons} E{\cdot}V \underset{k'_{-1}}{\overset{k'_{+1}}{\rightleftharpoons}} E{-}V$$

where K_V is the dissociation constant of the $E{\cdot}V$ complex, and k'_{-1} and k'_{+1} are the rate constants for the slow transition toward equilibrium. The observed rate coefficient for the formation of the high fluorescent $E{-}V$ form, k_{obs}, is equal to

$$k_{obs} = k'_{-1} + \frac{k'_{+1}[V]}{[V] + K_V}$$

From the data in the Figure 4B, both k'_{-1} (the intercept with the *y*-axis) and $k'_{+1} + k'_{-1}$ (equal to the value of k_{obs} at saturating [V]) can be evaluated. The ratio of k'_{-1}/k'_{+1} was at maximum 0.01, and therefore, the apparent dissociation constant of the $E{-}V$ complex, $K_{0.5\ V} = K_V k'_{-1}/(k'_{+1} + k'_{-1})$, was estimated to be in the nanomolar range.

Competition between Phosphate and Vanadate for Binding to the Na,K-ATPase. Addition of vanadate to phosphoenzyme formed at saturating [P_i] led to a monoexponential decrease in the fluorescence level for both pig renal and shark rectal enzyme reflecting the transition of the system toward a new equilibrium (*39*). As seen from Figure 4A, the final fluorescence level at saturating vanadate concentrations was about 60% above the initial level for shark enzyme and zero for the kidney enzyme. Thus, for both enzymes the final fluorescence level in the simultaneous presence of phosphate and vanadate was equal to that with vanadate alone, suggesting a complete replacement of phosphate with vanadate.

This fact together with the structural similarity of the two ligands could suggest a competitive type of interaction between P_i and vanadate:

Scheme 3

$$E{-}P \underset{k_{+1}}{\overset{k_{-1}}{\rightleftharpoons}} E{\cdot}P \underset{P_i}{\rightleftharpoons} E \overset{V}{\rightleftharpoons} E{\cdot}V \underset{k'_{-1}}{\overset{k'_{+1}}{\rightleftharpoons}} E{-}V$$

For such a scheme, the observed rate constant for the transition to the new equilibrium upon addition of vanadate

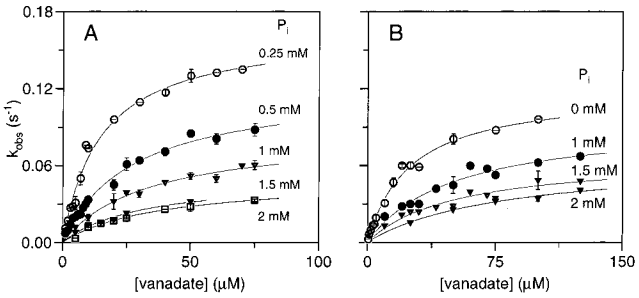

FIGURE 5: Effects of P_i and vanadate on the observed rate constant for the transition of the phosphoenzyme to the enzyme−vanadate complex measured by RH421 fluorescence. Data for pig kidney enzyme are shown in panel A, and data for shark enzyme in panel B. Na,K-ATPase was preequilibrated with different P_i concentrations in the presence of 10 mM HEPES, 10 mM MES, and 4 mM $MgCl_2$, pH 7.5. Addition of vanadate induced a transition to a new equilibrium accompanied by a decrease of RH421 fluorescence. In each case the observed rate constant (calculated from the monoexponential fit to the data) shows a hyperbolic dependence on vanadate concentration. [P_i] in the medium is indicated in the figure.

to the phosphoenzyme (k_{obs}) is described by the following equation:

$$k_{obs} = k'_{-1} + \frac{k'_{+1}[V]}{[V] + K_V\left(1 + \frac{[P_i]}{K_{0.5P_i}}\right)}$$

assuming that the transition $E{\cdot}V \rightleftarrows E{-}V$ is the rate-limiting step of the reaction and taking into account that $k'_{-1} \ll k'_{+1}$. From the equation, it is clear that for $[V] \rightarrow \infty$, k_{obs} approaches the same maximal value equal to $k'_{+1} + k'_{-1}$, independently of [P_i]. However, this is contrary to the experimental data shown in Figure 5, which demonstrate that at very high concentrations of vanadate, k_{obs} decreased with increasing [P_i] for both kidney (Figure 5A) and shark enzyme (Figure 5B). An attempt to fit the data with the same maximal value of k_{obs} failed, indicating that the simple competitive model of interaction (Scheme 3) does not describe the results adequately. Therefore, we suggest that at least one of the phosphoforms, either $E{-}P$ or $E{\cdot}P$ is able to bind vanadate and that this binding slows down the rate of transition toward the final $E{-}V$ complex.

Effect of Vanadate on Dephosphorylation of E_2P Formed from P_i. Investigation of the vanadate effect on the dephosphorylation of E_2P formed from P_i allows the detection of vanadate binding to $E{-}P$. In the experiments shown in Figure 6, phosphorylation of shark enzyme by 1 mM $^{32}P_i$ was interrupted by addition of an equal volume of chase solution containing various concentrations of vanadate, but no unlabeled P_i. Vanadate effectively prevented rephosphorylation by $^{32}P_i$, as shown by the fact that the final phosphoenzyme level (EP_∞) was close to zero (Figure 6), although the concentration of $^{32}P_i$ during the dephosphorylation was 0.5 mM. The time course of dephosphorylation in the presence of vanadate was the same as in control experiments (Figure 6), where the chase contained unlabeled P_i in the final concentration of 50 mM, showing that vanadate does not affect the dephosphorylation step (Figure 6, inset). These results imply that the rate-limiting step is the dephosphorylation itself and not the formation of the enzyme−

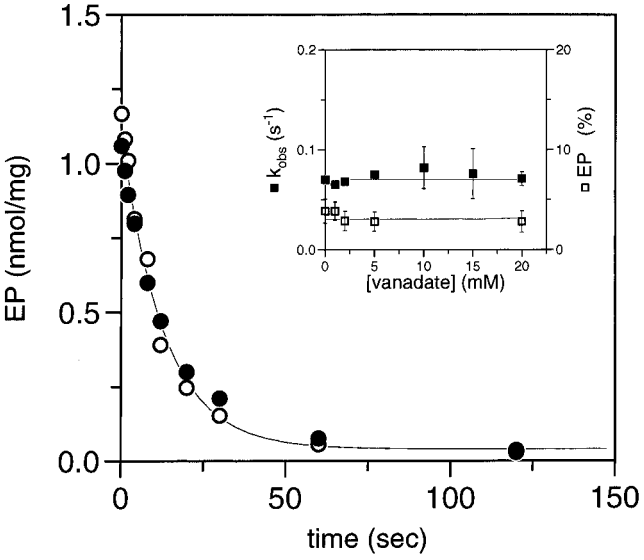

FIGURE 6: Time courses of shark enzyme dephosphorylation, when rephosphorylation from $^{32}P_i$ was prevented by addition of either 50 mM cold P_i (empty circles) or 1 mM vanadate (filled circles). Data are fitted to a monoexponential function $EP_t = EP_{max}(e^{-kt}) + EP_\infty$. The effect of an increasing vanadate concentration added to the chase solution on the derived values is shown in the inset.

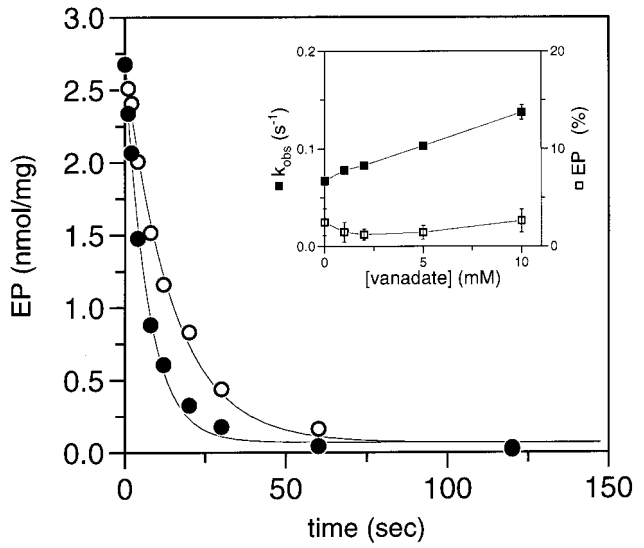

FIGURE 7: Effect of vanadate on the dephosphorylation of the E_2P formed from ATP. Time course of the dephosphorylation in the absence of vanadate (empty circles) and in the presence of 10 mM vanadate in the chase solution (filled circles). The experiments were performed with shark rectal gland Na,K-ATPase. Data were fitted to monoexponential functions, and derived values for the observed rate constants and residual EP_∞ levels obtained in the presence of different vanadate concentrations are shown in the inset.

vanadate complex. The noncompetitive interaction between vanadate and phosphate observed in the fluorescence experiments is due to vanadate binding to the E·P form. Whether it also binds to E−P and both this E−PV form and the E−P form dephosphorylate with identical rates cannot be resolved from the data.

Effect of Vanadate on Dephosphorylation of E_2P Formed from ATP. The time course of the dephosphorylation of shark Na,K-ATPase phosphoenzyme at 0 °C after phosphorylation from ATP is shown in Figure 7. The observed rate constants of the dephosphorylation of E_2P formed from ATP and EP formed from P_i were comparable and were approximately 0.07 s^{-1} at 0 °C, calculated from a monoexponential decay (Figure 6 and 7).

Vanadate (10 mM) increased the dephosphorylation rate of E_2P formed from ATP (Figure 7). It implies a low-affinity interaction between vanadate and the acid-stable phosphoenzyme E_2P formed from ATP at low Na^+ concentrations. This fact is in contrast to the experiments made with EP formed from P_i, where no effect of vanadate on the dephosphorylation rate constant was found (see Figure 6). It confirms that the E_2P phosphointermediate formed from ATP is different from the phosphorylated intermediate formed in the presence of P_i.

Sensitivity of the EP and EV Complexes to N-Methyl Hydroxylamine. Background. The protein−phosphate bond at the phosphorylation site has been identified as an acyl−phosphate bond. One of the characteristics of this bond is its reactivity toward hydroxylamine (or its analogue *N*-methyl hydroxylamine).

Hydroxylaminolysis of the acyl−phosphate bond should lead to a release of P_i and a formation of stable hydroxamic acid (*40*), which, in turn, should result in an irreversible inhibition of the enzyme activity. For Na,K-ATPase, it has been demonstrated that hydroxylaminolysis results in a discharge of $^{32}P_i$ from phosphointermediates, both after treatment with *N*-ethylmaleimide and after acid precipitation (*41*, *42*), but a complete inhibition of the overall activity was not observed (*43*, *44*). Post and Kume (*42*) suggested, therefore, that the sites for the nucleophilic attack of hydroxylamine differ for the native and the denatured phosphoenzymes: in native enzyme, hydroxylamine attacks the O−P bond and, thus, restores the active enzyme form. However, after protein denaturation, hydroxylamine attacks the C−O bond leading to the formation of a stable hydroxamate derivative. Although to the best of our knowledge the formation of such a derivative has not been demonstrated for the Na,K-ATPase, Post and Kume's suggestion emphasizes the significance of the microenvironment of the phosphorylation site in the reaction with hydroxylamine.

In the following, *N*-methyl hydroxylamine was used to characterize and to compare the properties of the enzyme−phosphate and the enzyme−vanadate bonds. We studied the effects of *N*-methyl hydroxylamine on (i) RH421 fluorescence of E−P and E−V forms, (ii) the dephosphorylation of phosphointermediates, (iii) the enzyme activity in the absence of K^+, and (iv) the reversibility of vanadate inhibition. We used *N*-methyl hydroxylamine instead of hydroxylamine since decomposition of hydroxylamine results in the release of NH_4^+, a very potent K^+-congener (*45*).

N-Methyl Hydroxylamine and RH421 Fluorescence. As shown in Figure 8, panels A and B, addition of *N*-methyl hydroxylamine to phosphoenzyme formed at saturating P_i resulted in the monoexponential decrease of RH421 fluorescence. The rate of the fluorescence decay increased with an increase in the concentration of *N*-methyl hydroxylamine (Figure 8, panels A and B). Similar effects were found for the vanadate-bound form (Figure 8, panels C and D).

The fact that *N*-methyl hydroxylamine in the presence of a supersaturating concentrations of P_i or vanadate causes the fluorescence to decrease with increasing observed rate constant when the [*N*-methyl hydroxylamine] is increased seems to exclude the possibility that the effect of *N*-methyl

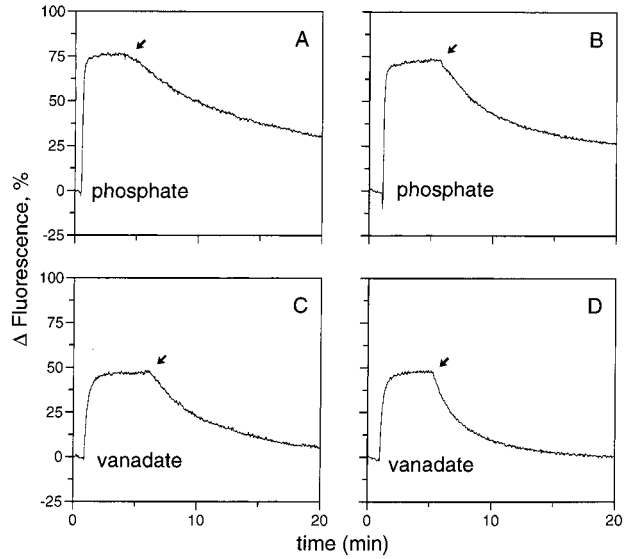

FIGURE 8: Effect of *N*-methyl hydroxylamine on the interaction of P_i or vanadate with shark Na,K-ATPase. The recordings of RH421 fluorescence were performed in the presence of 10 mM HEPES, 10 mM MES, and 4 mM $MgCl_2$, pH 7.5. The initial increase in the fluorescence was caused by equilibrium binding of 4 mM P_i (panels A and B) or 50 μM vanadate (panels C and D). The fluorescence drop was induced by 2 mM (panels A and C) or 6 mM (panels B and D) *N*-methyl hydroxylamine (the time of addition is indicated by the arrows).

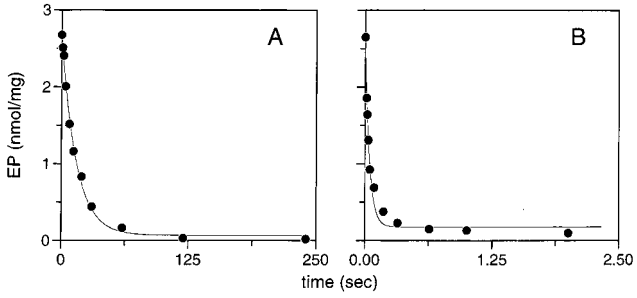

FIGURE 9: Sensitivity of phosphoenzyme formed from ATP to *N*-methyl hydroxylamine. Panel A, spontaneous dephosphorylation; panel B, dephosphorylation in the presence of 100 mM *N*-methyl hydroxylamine. Curves are monoexponential fits to the data (see Results). Note the difference in the scale of the time axis in panels A and B. The experiments were performed with shark rectal gland Na,K-ATPase.

hydroxylamine is an indirect one as a scavenger for free P_i or vanadate in the medium.

The results are consistent with a direct interaction of *N*-methyl hydroxylamine with the E−P or E−V complexes, respectively, and indicate that *N*-methyl hydroxylamine is able to shift the enzyme−P_i or enzyme−V equilibrium toward a non- or low-fluorescent form.

N-Methyl Hydroxylamine and Dephosphorylation. The direct effect of *N*-methyl hydroxylamine on phosphate release was investigated in dephosphorylation experiments by adding *N*-methyl hydroxylamine in the chase solution to the phosphoenzyme formed from either P_i or ATP. As seen from Figure 9, *N*-methyl hydroxylamine increased the observed rate constant for dephosphorylation of phosphoenzyme formed from ATP from 0.062 ± 0.003 s^{-1} to 36.4 ± 4.5 s^{-1}, i.e., more than 500-fold.

In contrast, dephosphorylation of the phosphoenzyme formed from P_i was inhibited 2-fold by *N*-methyl hydroxylamine (Figure 10A). This stabilizing effect of *N*-methyl hydroxylamine was also obvious in experiments where the dephosphorylation rate was decreased by ouabain (Figure 10B). The observed effects were not due to the *N*-methyl glucamine used to neutralize *N*-methyl hydroxylamine hydrochloride, or to an increase in the ionic strength, since dephosphorylation was unchanged with 100 mM choline chloride or with *N*-methyl glucamine chloride in the chase (not shown).

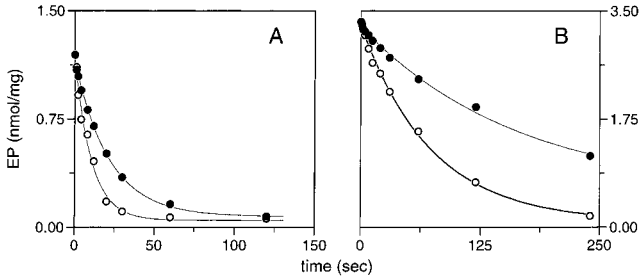

FIGURE 10: Effect of *N*-methyl hydroxylamine on the dephosphorylation of phosphoenzyme formed from either P_i alone (panel A), or P_i in the presence of ouabain (panel B). The time course of spontaneous dephosphorylation (empty circles) is compared with the dephosphorylation in the presence of 100 mM *N*-methyl hydroxylamine (filled circles). The experiments were performed with shark rectal gland Na,K-ATPase. Curves are drawn by eye.

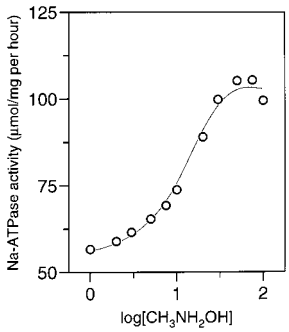

FIGURE 11: Effect of *N*-methyl hydroxylamine on the hydrolytic activity of Na-ATPase. Na-ATPase activity of shark rectal gland enzyme was measured with 25 μM [γ-^{32}P]ATP in the presence of 130 mM NaCl, 1 mM $MgCl_2$, and 30 mM histidine, pH 7.0 with shark rectal gland enzyme. The curve was drawn by eye.

Activation of the Na-ATPase by N-Methyl Hydroxylamine. To identify the site of the nucleophilic attack by hydroxylamine on the phosphoenzyme formed from ATP, we measured the effect of hydroxylamine on Na-ATPase activity (Figure 11) since it is generally assumed that the rate-determining step in this reaction is the hydrolysis of the phosphoenzyme. If the effect of *N*-methyl hydroxylamine is to accelerate the hydrolysis of the E−P bond without irreversible modification of the amino acid residue, one should observe a stimulation of the Na-ATPase activity. As seen from Figure 11, the Na-ATPase activity did increase with increasing concentrations of *N*-methyl hydroxylamine with a $K_{0.5} \approx 13$ mM (Figure 11).

It has been shown previously that various amines can interact with the K^+-binding sites (*46*). However, we could exclude a possible K^+-like effect of *N*-methyl hydroxylamine by employing a sided Na,K-ATPase preparation in which the enzyme was reconstituted into liposomes in the presence of *N*-methyl hydroxylamine and subsequently run through an anion-exchange column (*47*) to remove external *N*-methyl hydroxylamine. Preincubation with MgP_i and ouabain

Table 2: Sided Effects of *N*-Methyl Hydroxylamine on Na-ATPase Activity

N-methyl hydroxylamine inside/outside	−/−	+/−	+/+
hydrolytic activity (μmol/mg/hour)	165.3 ± 1.9	154.4 ± 2.3	211.7 ± 2.0

ensured that the only active enzyme was the one oriented inside-out (*33*). The proteoliposomes were then tested for Na-ATPase activity with or without *N*-methyl hydroxylamine in the test solution. With no *N*-methyl hydroxylamine in the test solution it was located only inside the vesicles and had access only to the extracellular (intravesicular) high-affinity K^+-sites. Even if some *N*-methyl hydroxylamine leaked from the proteoliposomes, its extravesicular concentration would be very low. In the other case where *N*-methyl hydroxylamine was present in the test solution, both intra- and extravesicular sites were exposed to high ligand concentrations. In Table 2 the results of a typical experiment are shown. As seen, *N*-methyl hydroxylamine activates Na-ATPase by a direct interaction with the cytoplasmic face of the pump and no effect was mediated by interaction with the extracellular K^+-binding sites.

Reversibility of the Vanadate Inhibition by N-Methyl Hydroxylamine. Figure 12 is a demonstration of a typical experiment where vanadate inhibition of Na,K-ATPase activity under optimal conditions was reversed by *N*-methyl hydroxylamine. As seen, addition of 30 μM vanadate led to a complete inhibition of hydrolysis, indicating that all enzyme molecules had tightly bound vanadate. In this case, addition of 100 mM *N*-methyl hydroxylamine was followed by a recovery of the Na,K-ATPase activity. It has to be mentioned that *N*-methyl hydroxylamine has some inhibitory effect on the Na,K-ATPase activity also in the absence of vanadate (not shown). The ability of *N*-methyl hydroxylamine to reverse the vanadate inhibition of Na,K-ATPase activity indicates a direct interaction with the enzyme−vanadate bond in a way comparable to that between *N*-methyl hydroxylamine and phosphoenzyme formed from ATP.

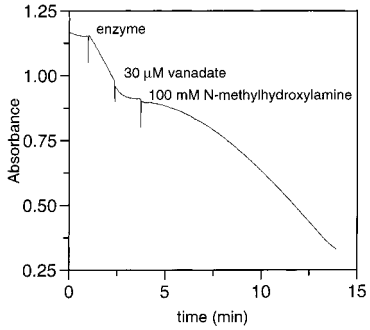

FIGURE 12: Reversibility of vanadate inhibition by *N*-methyl hydroxylamine. The shark rectal gland Na,K-ATPase reaction was followed spectrophotometrically at 340 nm by a coupled enzyme assay (see Experimental Procedures). The slope of the trace is proportional to the enzyme activity and vertical drops in the absorbance are due to addition of ligands. 30 μM vanadate caused inhibition of the Na,K-ATPase as shown by horizontal trace, while the subsequent addition of 100 mM *N*-methyl hydroxylamine partially restored the enzyme activity.

DISCUSSION

In the present study, a combination of biochemical and fluorescent methods was used to characterize the Na,K-ATPase reaction intermediates. This allows us to assign steady-state levels of fluorescence to biochemically well-characterized enzyme intermediates and to interpret the dynamic fluorescence responses in terms of transitions between intermediates in kinetic schemes. The fluorescence measurements were performed with the membrane-embedded styryl dye RH421 which responds to the formation of acid stable phosphoenzyme with a fluorescence increase, as shown by the following observations: (1) the time courses of the fluorescence increase and the formation of acid-stable phosphoenzyme are identical (Figure 2) and (2) the apparent P_i affinities for formation of acid stable phosphoenzyme on one hand and for RH421 fluorescence increase on the other are identical within the limits of experimental errors (Figure 3).

The study was performed with Na,K-ATPase from two sources—pig kidney and shark rectal glands. The two enzymes differ in several aspects, and although at present we do not know if the differences are due to different isoenzyme composition of the two preparations or to some other factors, the information obtained with the two enzymes adds to our knowledge about the reaction mechanism of the Na,K-ATPase in general. The enzymes from the two sources differ among others in (1) their apparent affinity to P_i (29 μM for the kidney enzyme and 330 μM for the shark enzyme), and (2) in the equilibrium level of acid stable phosphoenzyme obtained in P_i phosphorylation. For pig kidney enzyme, the EP level obtained with P_i is close to that obtained with ATP. With shark enzyme, it did not exceed 50−60% of that obtained with ATP even at very high [P_i] (Table 1). The latter observation indicates the presence of an appreciable amount of an intermediary phosphorylated acid labile and low fluorescence phosphoform, E_2•P, in shark enzyme. As mentioned in the Results, this difference in the reaction with P_i of the two enzymes lies mainly in the equilibrium between E_2•P $\rightleftarrows$ E_2−P. For pig enzyme, it is poised strongly toward the E_2−P, while in shark, the two forms are present in equal amounts. This difference also explains why the apparent affinity of kidney enzyme to P_i is found to be about 10 times higher than the apparent P_i affinity of the shark enzyme.

Although it is clear that the fluorescence response of the RH421 dye can be used to study the formation and the properties of acid-stable phosphoenzyme, there are still unanswered questions concerning the mechanism of the dye response (cf. ref *16*). One of the factors affecting the magnitude of the response appears to be lipid composition of the membrane (*39*), but we cannot exclude that the isoenzyme composition of the two enzyme preparations could also play a role of the response, since the kidney enzyme is an α_1 while the shark enzymes is an α_3 isoform.

Characterization of the Acyl−Phosphate Bond in Phosphoenzyme Formed from P_i. *N*-Methyl hydroxylamine addition to E−P formed from P_i resulted in a decrease of the rate of dephosphorylation (transient experiments) and in a decrease in the level of the high-fluorescence form (note that the level of the high fluorescence form was thought to reflect the level of the acid-stable phosphoform) in equilibrium experiments (Figure 8, panels A and B). Together these two observations suggest that *N*-methyl hydroxylamine does not interact with the enzyme−phosphoryl bond of this

phosphoform itself but modifies some amino acid residue(s) which slows down both dephosphorylation and phosphorylation processes. The effect on the latter process must be more pronounced as the equilibrium level of the phosphoenzyme is decreased.

Interaction of Na,K-ATPase with Vanadate. The mechanism of the interaction between enzyme and vanadate suggested by the kinetic analysis of the fluorescence responses, as mentioned in the Results (Scheme 2), is similar to that proposed for the interaction with P_i. It includes a rapid formation of an intermediate complex E·V followed by a slow transformation to an E−V state. As demonstrated by RH421, fluorescence the enzyme−vanadate complex and the phosphoenzyme formed from P_i apparently exhibited the same reactivity toward *N*-methyl hydroxylamine. The reasons for the fluorescence shifts, however, are quite different. As mentioned above, the acyl−phosphate bond of the phosphoenzyme formed from P_i was not directly affected by *N*-methyl hydroxylamine. In contrast, the fact that *N*-methyl hydroxylamine partially restored the Na,K-ATPase activity after complete vanadate inhibition suggests hydroxylaminolysis of the acyl−vanadate bond. An inhibitory effect of high concentrations of *N*-methyl hydroxylamine seen as a partial inhibition of the Na,K-ATPase activity (without or with vanadate, Figure 12) and decrease in the level of P_i phosphorylation is probably due to some modification of certain amino acids.

Restoration of the Na,K-ATPase activity after vanadate inhibition and the activation of Na-ATPase reaction by addition of *N*-methyl hydroxylamine (Figures 11 and 12) indicate a similarity between the bonding of phosphate in the phosphointermediate formed from ATP, E_2P, and of vanadate to the enzyme. This analogy implies a formation of a covalent bond between the enzyme and vanadate, an E−V form. The ability of vanadate to esterify hydroxyl groups and yield vanadate esters has previously been demonstrated (*48*, *49*).

Subconformations of E_2P. The analysis of the results obtained by fluorescent and biochemical methods revealed striking differences between the two phosphoforms previously characterized (see ref *18*) as E_2P forms. The present investigation demonstrates that depending on the route of formation—"physiological" from ATP or "direct" from P_i—the two phosphoenzymes have quite distinct properties. It has been previously shown by Post et al. (*3*) that the dephosphorylation of the two phosphointermediates differ in their sensitivity to K^+. In the present study, we suggest the existence of two distinct E_2P phosphoenzyme subconformations. The main evidence for this comes from experiments with *N*-methyl hydroxylamine indicating a difference in the structure of the phosphorylation site itself. In the following, the two phosphointermediates are denoted E_2P (formed from ATP) and $E_2'P$ (formed from P_i).

N-Methyl hydroxylamine slowed the rate of dephosphorylation of $E_2'P$ (see above) while it increased the rate of dephosphorylation of E_2P up to 500-fold. The latter effect of *N*-methyl hydroxylamine on E_2P is caused by a direct action of the ligand at the cytoplasmic face of the enzyme, as shown in experiments with Na,K-ATPase reconstituted into proteoliposomes and, thus, cannot be ascribed to a "K^+-like" effect. The β-aspartyl residue at the phosphorylation site, however, remains unmodified after the reaction with *N*-methyl hydroxylamine, as seen from its activation of the Na-ATPase reaction. If we assume that the acyl−phosphate bonds in E_2P and $E_2'P$ are covalently identical, it appears that the phosphorylation site of E_2P has a more "open conformation", i.e., it allows easier access of *N*-methyl hydroxylamine than the phosphorylation site of $E_2'P$. It has to be stressed that not all conformational changes of phosphointermediates coincide with changes in the accessibility of the phosphorylation site to hydroxylamine. It has been shown by Post and Kume (*42*) with *N*-ethyl maleimide modified Na,K-ATPase and recently by Ushimaru et al. (*50*) with the native enzyme that the dephosphorylation of E_1P is also accelerated by *N*-methyl hydroxylamine. The properties of the transition-state analogue complex E−V seem to be similar to those of E_2P, the phosphoenzyme formed from ATP, with respect to its reaction with *N*-methyl hydroxylamine. In other words, the transition state in the phosphoryl-transfer reaction appears to have an open conformation.

In conclusion, a difference in the structural organization of the phosphorylation site in the two E_2P forms produced by phosphorylation from either ATP or P_i is suggested on the basis of the differences in their reactivities toward *N*-methyl hydroxylamine. A proceeding manuscript (*19*) concerns the sensitivity of the two phosphoforms to various cations, i.e., it attempts to reveal the functional interactions between the substrate site and the cation-binding sites, interactions which are central for the coupling of hydrolytic and transport activities of Na,K-ATPase.

ACKNOWLEDGMENT

Hanne Zakarias, Henriette R. Petersen, Angielina Damgaard, and Jesper M. Kielsgaard are gratefully acknowledged for their excellent technical assistance. We would like to thank Dr. Bliss Forbush III with whom preliminary experiments on responses of RH-dyes to P_i binding were performed. Drs. R. L. Post and R. J. Clarke are gratefully acknowledged for critical reading of the manuscript and Dr. J. G. Nørby for stimulating discussions.

REFERENCES

1. Post, R. L., Taniguchi, K., and Toda, G. (1974) *Ann. NY Acad. Sci. 242*, 80−91.
2. Taniguchi, K., and Post, R. L. (1975) *J. Biol. Chem. 250*, 3010−3018.
3. Post, R. L., Toda, G., and Rogers, F. N. (1975) *J. Biol. Chem. 250*, 691−701.
4. Shull, G. E., Schwartz, A., and Lingrel, J. B. (1985) *Nature 316*, 691−695.
5. Sontheimer, G. M., Kalbitzer, H. R., and Hasselbach, W. (1987) *Biochemistry 26*, 2701−2706.
6. Bühler, R., Stürmer, W., Apell, H.,-J., and Läuger, P. (1991) *J. Membr. Biol. 121*, 141−161.
7. Forbush, B., III, and Klodos, I. (1991) in *The Sodium Pump: Structure, Mechanism and Regulation* (Kaplan, J. H., and De Weer, P., Eds.) pp 211−225, Rockefeller University Press, New York.
8. Läuger, P. (1991) in *The Sodium Pump: Structure, Mechanism and Regulation* (Kaplan, J. H., and De Weer, P., Eds.) pp 303−315, Rockefeller University Press, New York.
9. Stürmer, W., Bühler, R., Apell, H.-J., and Läuger, P. (1991) *J. Membr. Biol. 121*, 163−176.
10. Clarke, R. J., Schrimpf, P., and Schöneich, M. (1992) *Biochim. Biophys. Acta 1112*, 142−152.
11. Stürmer, W., and Apell, H.-J. (1992) *FEBS Lett. 300*, 1−4.

12. Pratap, P. R., and Robinson, J. D. (1993) *Biochim. Biophys. Acta 1151*, 89−98.
13. Heyse, S., Wuddel, I., Apell, H.-J., and Stürmer, W. (1994) *J. Gen. Physiol. 104*, 197−240.
14. Klodos, I. (1994) in *The sodium pump: structure mechanism, hormonal control and its role in disease* (Bamberg, E., and Schoner, W., Eds.) pp 517−528, Springer-Verlag, New York.
15. Schwappach, B., Stürmer, W., Apell, H.-J., and Karlish, S. J. D. (1994) *J. Biol. Chem. 269*, 21620−21626.
16. Fedosova, N. U., Cornelius, F., and Klodos, I. (1995) *Biochemistry 34*, 16806−16814.
17. Schulz, S., and Apell, H.-J. (1995) *Eur. Biophys. J. 23*, 413−421.
18. Apell, H.-J., Roudna, M., Corrie, J. E. T., and Trentham, D. R. (1996) *Biochemistry* 35, 10922−10930.
19. Cornelius, F., Fedosova, N. F., and Klodos, I. *Biochemistry* (submitted for publication).
20. Fedosova, N. U., Cornelius, F., Forbush, B., III, and Klodos, I. (1997) Na/K-ATPase & Related Transport ATPases. Structure, Mechanism and Regulation. in *Ann. NY Acad. Sci.* (Beauge, L. A., Gadsby, D. C., and Garrahan, P. J., Eds.) Vol. 834, pp 386−389.
21. Skou, J. C., and Esmann, M. (1979) *Biochim. Biophys. Acta 567*, 436−444.
22. Jørgensen, P. L. (1974) *Biochim. Biophys. Acta 356*, 36−52.
23. Jensen, J., Nørby, J. G., and Ottolenghi, P. (1984) *J. Physiol. (London) 346*, 219−241.
24. Ottolenghi, P. (1975) *Biochem. J. 151*, 61−66.
25. Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem. 193*, 265−275.
26. Jensen, J., and Ottolenghi, P. (1983) *Biochim. Biophys. Acta 731*, 282−289.
27. Esmann, M. (1988) *Methods Enzymol. 156*, 72−79.
28. Cornelius, F. (1988) *Methods Enzymol. 156*, 156−167.
29. Peterson, G. L. (1977) *Anal. Biochem. 83*, 346−356.
30. Cornelius, F. (1995) *Biochim. Biophys. Acta 1235*, 197−204.
31. Nørby, J. G. (1988) *Methods Enzymol. 156*, 116−119.
32. Lindberg, O., and Ernster, L. (1956) *Methods Biochem. Anal. 3*, 1−22.
33. Cornelius F., and Skou, J. C. (1988) *Biochim. Biophys. Acta 944*, 223−232.
34. Albers, R. W., Koval, G. J., and Siegel, G. J. (1968) *Mol. Pharmacol. 4*, 324−336.
35. Lindenmayer, G. E., Laughter, A. H., and Schwartz, A. (1968) *Arch. Biochem. Biophys. 127*, 187−192.
36. Kuriki, Y., Halsey, J., Biltonen, R., and Racker, E. (1976) *Biochemistry 15*, 4956−4961.
37. Kaplan, J. H., Kenney, L. J., and Webb, M. R. (1985) in *The Sodium Pump* (Glynn, I. M., and Ellory, J. C., Eds.) pp 415−421, The Company of Biologists, Cambridge, U.K.
38. Campos, M., and Beaugé, L. (1994) *J. Biol. Chem. 269*, 18028−18036.
39. Klodos, I., Fedosova, N. U., and Cornelius, F. (1997) in *Na/K-ATPase & Related Transport ATPases. Structure, Mechanism and Regulation. in Ann. NY Acad. Sci.* (Beauge, L. A., Gadsby, D. C., and Garrahan, P. J., Eds.) Vol. 834, pp 394−396.
40. Lipmann, F., and Tuttle, L. C. (1945) *J. Biol. Chem. 159*, 21−28.
41. Bader, H., Sen, A. K., and Post, R. L. (1966) *Biochim. Biophys. Acta 118*, 106−115.
42. Post, R. L., and Kume, S. (1973) *J. Biol. Chem. 248*, 6993−7000.
43. Hokin, L. E., and Dahl, J. L. (1972) in *Metabolic Pathways* (Hokin, L. E., Ed.) 3rd ed.; Vol. 6, pp 269−315, Academic Press, New York.
44. Whittam, R., and Wheeler, K. P. (1970) *Annu. Rev. Physiol. 32*, 21−60.
45. Skou, J. C. (1960) *Biochim. Biophys. Acta 42*, 6−23.
46. Forbush, B., III. (1988) *J. Biol. Chem. 263*, 7979−7988.
47. Penefsky, H. S. (1979) *Methods Enzymol. 56*, 527−530.
48. Gresser, M. J., and Tracey, A. S. (1985) *J. Am. Chem. Soc. 107*, 4215−4220.
49. Tracey, A. S., and Gresser, M. J. (1986) *Proc. Natl. Acad. Sci. 83*, 609−613.
50. Ushimaru, M., Shinohara, Y., and Fukushima, Y. (1997) *J. Biochem. 122*, 666−674.

BI980703H