Kinetics of the Phosphorylation of Na,K-ATPase by Inorganic Phosphate Detected by a Fluorescence Method[†]

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ABSTRACT: Phosphorylation by P_i of the Na,K-ATPase from rabbit kidney in the absence of Na^+ ions but in the presence of Mg^{2+} ions has been studied. In the absence of K^+ ions, unphosphorylated and phosphorylated states induce different fluorescence levels in the membrane-bound styryl dye RH421, and hence transitions between the two states were monitored. Transient kinetic studies of phosphorylation were initiated by manual addition of P_i or by photochemical release of P_i from 1-(2-nitrophenyl)ethyl phosphate (caged P_i) using laser flash photolysis at 308 nm. Equilibrium studies of phosphorylation showed that the apparent K_m for P_i was $23.0 \pm 0.3 \mu M$ (mean $\pm$ sem) at pH 7.1 and 21 °C. The dye fluorescence increased in a biphasic manner on addition of 500 μM P_i to the enzyme: a rapid phase ($t_{1/2} < 1$ s) and a slower exponential phase at $0.059 \pm 0.003 s^{-1}$. The rate of the rapid phase was studied by fast concentration-jump experiments and exhibited first-order kinetics in P_i up to 60 μM . Fluorescence records vs time were exponential, and a plot of the rate constant versus $[P_i]$ had a slope of $1.47 \times 10^5 M^{-1} s^{-1}$ and ordinate ($[P_i] = 0$) intercept of $3.1 s^{-1}$. Addition of 50 mM NaCl to the phosphorylated enzyme induced an exponential decay in the dye fluorescence from which a rate constant of $0.10 \pm 0.005 s^{-1}$ was determined. These data were interpreted in terms of transformations between conformational states E_1 and E_2 , and the phosphorylated state $P-E_2$ defined in the Post-Albers mechanism of the Na,K-ATPase [Läuger, P., (1991) *Electrogenic Ion Pumps*, Sinauer Associates Inc., Sunderland, MA] as follows: $E_1 \xrightleftharpoons[k_{-1}]{k_{+1}} E_2 + P_i \xrightleftharpoons[k_{-2}]{k_{+2}} P-E_2 + H_2O$, where $k_{+1} = 0.059 s^{-1}$, $k_{-1} = 0.023 s^{-1}$, $k_{+2} = 1.47 \times 10^5 M^{-1} s^{-1}$, and $k_{-2} = 3.1 s^{-1}$. The RH421 fluorescence of state $P-E_2$ was studied over the pH range 6–8.5. Fluorescence was greatest at pH 8.5 and lowest at pH 6.0 in a simple binding isotherm with pK 7.5. The apparent K_m for P_i rose cooperatively with increasing pH (pK_a 8.6 and a Hill coefficient of 2). Therefore in the absence of monovalent metal ions, occupation of the cation (K^+) binding sites by protons promotes phosphorylation by P_i .

The Na,K-ATPase is an ion-transport protein of the plasma membrane which is found in nearly all animal cells. Under physiological conditions it hydrolyzes one molecule of ATP to move three Na^+ ions out of the cell and to import two K^+ ions against the electrochemical potential gradients of each ion. The Na,K-ATPase is characterized by its requirement for ATP, Mg^{2+} , and Na^+ on the cytoplasmic side of the protein and K^+ on the extracellular side (Glynn, 1985; Jørgensen, 1992; Läuger, 1991). Scheme 1 shows that the transport cycle of the pump, usually termed the Post-Albers cycle, consists of a series of ordered reaction steps which comprise the three principal processes necessary to perform active pumping: (1) ion binding or release, (2) protein phosphorylation and dephosphorylation, and (3) conformational changes (Läuger, 1991). The transport functions can be modified by variation of the ionic composition at either membrane interface or by alteration of the phosphate source.

Besides normal pumping, several noncanonical flux modes have been identified and utilized to investigate properties of partial reactions and to gain understanding of the pumping process (Glynn, 1985; Läuger, 1991): pump reversal (ATP synthesis), 3 Na^+ /3 Na^+ exchange (without net ATP hydrolysis), 2 K^+ /2 K^+ exchange (characterized by ^{18}O exchange between P_i and water), uncoupled Na^+ efflux (3 Na^+ /zero exchange), and 3 Na^+ /2 Na^+ exchange (accompanied by ATP hydrolysis).

Here we extend these analyses of the Na,K-ATPase through investigation of the phosphorylation of the enzyme by P_i , the so-called "back-door" phosphorylation, that has previously been studied mainly by ^{32}P -phosphorylation methods. It has been shown that $P-E_2$ formation from P_i can occur provided Na^+ is absent (Campos & Beaugé, 1994; Cornelius, 1995; Post et al., 1975). Phosphorylation from P_i or ATP results in chemically identical phosphoenzymes (Bonting et al., 1979; Berberian & Beaugé, 1991). On this basis back-door phosphorylation represents the reversal of the hydrolysis step of the phosphoenzyme under physiological conditions. Phosphorylation from P_i or from ATP requires Mg^{2+} (Post et al., 1975). P_i and ATP compete with each other for the enzyme, and so mutually influence their respective K_m values (Campos & Beaugé, 1994). Back-door phosphorylation can in principle be split

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Scheme 1: Post-Albers Scheme of the Pump Cycle of Na,K-ATPase under Physiological Conditions, Adapted from Wuddel and Apell (1994)^a

^a E₁ and E₂ are conformations of the enzyme with binding sites facing the cytoplasm and the extracellular medium, respectively. In the occluded states (Na₃)E₁-P, E₂(K₂), and ATP·E₂(K₂), the bound ions are unable to exchange with the aqueous phase. The horizontal parentheses in state P-E₂Na₃ indicate that in this state the ion sites are accessible only through a narrow well. Dashes indicate covalent bonds, and dots indicate noncovalent bonds. When the enzyme is phosphorylated by ATP in state E₁, three Na⁺ ions become occluded (Na₃E₁·ATP → (Na₃)E₁-P). The spontaneous transition to conformation E₂ leads to release of the Na⁺ ions to the extracellular side. This is followed by subsequent binding of potassium, which initiates enzyme dephosphorylation and occlusion of K⁺ ions. Low-affinity binding of ATP shifts the conformation to state E₁, from which K⁺ is released to the cytoplasm.

into two reaction steps:

Reaction 1 is accelerated by ATP in the K⁺ pathway of the normal pump mode (i.e., E₂ → E₁) (Campos & Beaugé, 1994), but not in the 3 Na⁺/2 K⁺ exchange mode, at least in reconstituted systems (Apell et al., 1990).

We have investigated the kinetics and equilibria of enzyme phosphorylation by P_i and determined the net charge of the ion-binding domain in this pathway. The use of a photo-sensitive “caged” phosphate allowed synchronization of pump function by imposing a phosphate concentration jump. Back-door phosphorylation was monitored by changes in the fluorescence of the styryl dye RH421¹ that has previously been used to investigate the occupation of the extracellularly-presented binding sites in the Na,K-ATPase (Bühler & Apell, 1995; Heyse et al., 1994; Stürmer et al., 1991a). The dye is thought to insert into the lipid domains of Na,K-ATPase membrane fragments and to detect changes of electric field strength inside the membrane dielectric but not conformational transitions (Bühler et al., 1991). Such changes in electric field strength are caused by binding or release of ions and/or by movement of charge inside the membrane. The fluorescence levels allow discrimination between differently charged states of the enzyme and the fluorescence change between states can be assumed to be proportional to the change of the electric field in the membrane (Bühler et al., 1991; Grinvald et al., 1982). Back-door phosphorylation can occur whether K⁺ ions are present or not (Post et al.,

1975; Sen et al., 1969). In the absence of K⁺ the cation binding sites are (partly) empty, so the steady state of the Na,K-ATPase after phosphorylation can be detected by RH421 as the accompanying charge change is uncompensated.

For the most part results are interpreted in terms of reactions 1 and 2. Experimental protocols are described which enable these noncanonical flux modes of the ATPase to be monitored. These modes are related to the reverse of the normal mode of the pump from K₂E₁·ATP to P-E₂ (Scheme 1) when ATP and K⁺ ions are absent.

MATERIALS AND METHODS

Materials. Sodium dodecyl sulfate was obtained from Pierce Chemical, Rockford, IL. Phosphoenolpyruvate, pyruvate kinase, lactate dehydrogenase, NADH, and ATP (disodium salt, special quality) were from Boehringer Mannheim. RH421 was from Molecular Probes, Eugene, OR. Dye purity was checked by thin-layer chromatography. Purine nucleoside phosphorylase was the “bacterial” protein from Sigma, and MESG (2-amino-6-mercapto-7-methylpurine ribonucleoside) was a gift from Dr. Martin Webb. All other reagents were at least analytical grade.

Preparation of Membrane Fragments Containing Na,K-ATPase. Na,K-ATPase was prepared from the outer medulla of rabbit kidneys using procedure C of Jørgensen (1974). The specific ATPase activity was measured by the pyruvate kinase/lactate dehydrogenase assay (Schwartz et al., 1971), and the protein concentration was determined by the method of Lowry et al. (1951), using bovine serum albumin as a standard. For most preparations the specific activity was in the range 1900–2000 μmol of P_i formed per hour and mg of protein at 37 °C.

Synthesis of Caged Inorganic Phosphate. (±)-1-(2-Nitrophenyl)ethanol (1 mmol) was phosphorylated as previously described for the separate (+)- and (−)-enantiomers (Corrie et al., 1992), except that instead of precipitating the product as its Ba²⁺ salt, it was isolated by anion-exchange chromatography on a column of DEAE cellulose (2 × 40 cm) eluted with a linear gradient formed from 10 and 200 mM triethylammonium bicarbonate (each 1000 mL). Fractions containing the caged phosphate were evaporated under reduced pressure and then evaporated an additional three times with methanol to remove excess triethylamine. The recovered material (triethylammonium salt) was converted to its Li⁺ salt by exchange with Dowex 50 (Li⁺ form) and stored at −20 °C.

Fluorescence Measurements. An ethanolic stock solution of the fluorescent styryl dye RH421 was added to the aqueous suspension of membrane fragments to obtain a final concentration of 200–250 nM. Steady-state fluorescence measurements or those involving manual additions (e.g., Figure 1) were carried out with a Perkin Elmer LS50B luminescence spectrometer. In a typical experiment the cell contained a suspension of membrane fragments containing 5–15 μg of Na,K-ATPase per mL (i.e., 0.03–0.1 μM; Ovchinnikov et al., 1986) in a standard solution at 21 ± 1 °C containing 230 nM RH421, 10 mM MgCl₂, 0.1 mM EDTA, 25 mM histidine adjusted to pH 7.1 ± 0.1 with HCl. Other reagents were added as specified. The thermostated cell holder was equipped with a magnetic stirrer. The excitation wavelength was set to 580 nm (slit width 15 nm), and the emission wavelength was set to 650 nm (slit width 15 nm).

¹ Abbreviations: DEAE cellulose, (diethylamino)ethyl cellulose; HPLC, high-performance liquid chromatography; RH421, N-(4-sulfobutyl)-4-[4-(p-(dipentylamino)phenyl)butadienyl]pyridinium, inner salt; Tris, tris(hydroxymethyl)aminomethane.

FIGURE 1: Comparison of the effects of P_i and ATP on the Na,K-ATPase. The enzyme and RH421 in 10 mM $MgCl_2$, 0.1 mM EDTA, 25 mM histidine adjusted to pH 7.1 ± 0.1 were incubated at $21 \pm 1^\circ C$ as described in Materials and Methods. P_i , ATP (each as Tris salts), and NaCl were added as indicated during time records of RH421 fluorescence. Interpretation of the changes following the additions is in the text. F_0 is the fluorescence of RH421 bound to the membrane. RH421 fluorescence is recorded on successive additions of (a) P_i , ATP, and NaCl, (b) P_i and NaCl, and (c) ATP, NaCl, and P_i .

Transient fluorescence signals produced by photochemical release of P_i from its precursor, caged P_i , were measured as described previously for experiments with caged ATP, the P^3 -1-(2-nitrophenyl)ethyl ester of ATP (Stürmer et al., 1989). The optical cell was filled with 200–300 μL of the solution described above plus 5–600 μM caged P_i . After thermal equilibration at $21^\circ C$, the fluorescence signal was monitored. Fluorescence was excited by light from a 250 W tungsten-halogen lamp, and emitted light from the sample cell was collected by an ellipsoidal mirror and focused onto the cathode of the photomultiplier. Excitation (580 nm) and emission (650 nm) wavelengths were selected by interference filters (13 nm bandwidth). The current signal of the photomultiplier was converted to voltage, digitized by a 16-bit analog-to-digital converter (DSP 32C Loughborough Sound Images Ltd., Loughborough, Leics LE11 0QE, England), and stored on the hard disk of a personal computer. P_i was released in the cuvette from caged P_i by a light flash (wavelength, 308 nm; total energy, 150 mJ; duration, 10 ns) generated by an EMG 100 excimer laser (Lambda Physik, Göttingen). The rate constant for P_i release on photolysis of caged P_i at pH 7.1 and $21^\circ C$ is approximately $3 \times 10^4 s^{-1}$ (Dantzig et al., 1992).

Determination of Released P_i . Buffer solutions containing flash-photolyzed caged P_i were analyzed by HPLC by a method adapted from Corrie and Trentham (1992). Samples were injected in a Millipore-Waters equipment and chro-

matographed by a Whatman Partisphere SAX column (cat. no. 4621-0505) with mobile-phase flow rate at 1.5 mL/min and UV detection at 254 nm. The mobile-phase buffer contained 125 mM $(NH_4)H_2PO_4$ and 10% methanol (adjusted to pH 6.2 with NaOH). When samples containing protein were investigated, the suspension was centrifuged to remove the membrane fragments prior to injection onto the column. The peak areas of the caged compound before and after illumination were determined, and the yield of the release reaction was calculated from the initial concentration and the ratio of the areas. The conversion efficiency was $10.5 \pm 0.4\%$. That the released P_i was equal to the photolyzed caged P_i was established by measurement of released P_i in control experiments using a P_i colorimetric assay (Webb, 1992).

RESULTS

Generation of $P-E_2$ from P_i and from ATP and Its Hydrolysis. Figure 1 shows several protocols during which RH421 fluorescence is monitored. Thus in Figure 1a, 230 nM RH421 and 9 μg of Na,K-ATPase/mL were added to the standard buffer and equilibrated until the fluorescence reached a constant level, presumably that of a mixture of E_1 and E_2 . At this point 500 μM Tris phosphate was added. P_i caused a fluorescence increase of 55%, qualitatively in agreement with a change to $P-E_2$ (Schwappach et al., 1994) via reactions 1 and 2.

Addition of ATP causes apparent hydrolysis of $P-E_2$ through the reaction sequence $P-E_2 \rightarrow E_2 \rightarrow E_2 \cdot ATP \rightarrow E_1 \cdot ATP$. Thus in Figure 1a, 200 μM ATP induced a decrease of fluorescence to a low level characteristic of the state $E_1 \cdot ATP$. 200 μM ATP was shown to be saturating by repeating the protocol using lower ATP concentrations in titration experiments. The apparent K_m for ATP was $11.8 \pm 0.2 \mu M$ (mean $\pm$ sem) (data not shown). Addition of 50 mM NaCl to the ATP treated solution now caused an increase of fluorescence (Figure 1a) that was about 1.6-fold greater than the fluorescence increase induced by P_i . 50 mM NaCl is saturating on the cytoplasmic site of the enzyme ($K_m = 8$ mM). The second fluorescence increase is presumed to be caused by phosphorylation from ATP in agreement with the partial reaction $Na_3E_1 \cdot ATP \rightarrow P-E_2$ (Scheme 1; Stürmer et al., 1991a; Stürmer & Apell, 1992). The different fluorescence levels for the $P-E_2$ states, obtained after phosphorylation by P_i or ATP, will be investigated below.

Addition of saturating Na^+ to $P-E_2$ generates Na_3E_1 (Heyse et al., 1994). The rate of this process was measured by monitoring the fluorescence when 50 mM NaCl was added to $P-E_2$ generated by P_i phosphorylation (Figure 1b). The rate constant for this exponential process of Na_3E_1 formation was $0.023 \pm 0.001 s^{-1}$ in the presence of 500 μM P_i . Similar experiments were performed in the presence of 10–500 μM P_i . The observed rate constant depended on the P_i concentration in agreement with the reaction sequence $P-E_2 \rightleftharpoons E_2 + P_i \rightarrow E_1$ (data not shown). The rate constant determined as $[P_i] \rightarrow 0$ was $0.1 s^{-1}$. The much more rapid decay induced by ATP (Figure 1a) is consistent with a 200-fold rate activation for the $E_2 \rightarrow E_1$ conformational change found in the presence of K^+ (Heyse et al., 1994). Caged ATP which binds to the ATPase (Borlinghaus & Apell, 1988; Forbush, 1984) also induced a rapid fluorescence decay confirming that ATP hydrolysis is not necessary for the process (data not shown).

The different fluorescence levels induced by 500 μM P_i and by 200 μM ATP plus 50 mM NaCl are important in describing properties of the $\text{P}-\text{E}_2$ state (Figure 1a). In an altered protocol (Figure 1c), the solution with ATPase, RH421, and 200 μM ATP was treated with 50 mM NaCl followed by 500 μM P_i . The small drop in fluorescence upon addition of ATP is not specific since it can also be observed in the absence of Mg^{2+} . Most probably it is caused by adsorption of ATP to the lipid domains of the membrane fragments. No fluorescence change occurred (after dilution correction) on P_i addition. From these findings we propose that the phosphorylation in the presence of Na^+ ions produces a differently charged state of the protein. The relevance of this observation with respect to the amount of uncompensated charge within the protein dielectric is addressed in the section on pH dependence and in the Discussion. The data in Figure 1a and c also show that, in the absence of potassium ions, state $\text{P}-\text{E}_2$ in eq 2 is the steady-state intermediate of the ATPase.

Several control experiments were performed. Thus following the protocol of Figure 1a, no fluorescence change occurred on P_i addition either in the absence of Mg^{2+} or in the presence of 50 mM NaCl. Previously reported findings that the conformational change in the presence of K^+ , $\text{E}_1 + 2\text{K} \rightarrow \text{K}_2\text{E}_1 \rightarrow \text{E}_2(\text{K}_2)$, is not accompanied by a fluorescence change (Stürmer et al., 1991a; Klodos, 1994) were verified. In standard solution or solutions containing 25 mM imidazole instead of histidine the RH421 fluorescence was monitored while 20 mM KCl and 200 μM were added successively and no significant fluorescence change was detected on either addition. To check the effect of protons on the RH421 fluorescence the pH was changed between 7.1 and 8.0 in standard solution by addition of 4 mM Tris base and between pH 8.0 and 6.6 by addition of HCl to standard solutions and to solutions containing 20 mM NaCl (to confine the protein in one state, Na_3E_1) or 20 mM KCl [to maintain state $\text{E}_2(\text{K}_2)$]. Significant RH421 fluorescence changes could not be detected in any of these experiments. Therefore we conclude that binding of protons to ion binding sites or to amino acids, which are accessible to the aqueous phase, does not affect the RH421 fluorescence in unphosphorylated states of the protein as has been demonstrated previously (Klodos, 1994).

RH421 Signals Associated with the Dependence of Back-Door Phosphorylation on P_i Concentration. To explore further the nature of phosphorylation by P_i , equilibrium titration experiments were performed in standard buffer containing 230 nM RH421 and 9 μg of Na,K-ATPase/mL. Aliquots of a concentrated solution of Tris phosphate were added and the fluorescence was measured relative to the initial level, F_0 . Results are shown in Figure 2. The line represents a fit of a simple binding isotherm to the averaged data of three titrations with the same enzyme preparation. Such experiments were repeated with four different preparations. The average half-saturating concentration, K_m , of the phosphorylation reaction was $23.0 \pm 0.3 \mu\text{M}$ at pH 7.1 and 21 $^\circ\text{C}$. Titration experiments performed with 10 mM Tris initially present did not show a significant change in the measured K_m for P_i .

RH421 Signals Associated with the Kinetics of Phosphorylation of the Na,K-ATPase by P_i : Slow Phase. Inspection of Figure 1a shows that addition of saturating (500 μM) P_i resulted in a biphasic fluorescence response. The ratio of

FIGURE 2: Titration of RH421 fluorescence changes induced by P_i in standard buffer conditions. The relative fluorescence changes, $\Delta F/F_0$, were calculated with respect to the fluorescence intensity, F_0 , prior addition of P_i . The experimental data were fitted by a Michaelis–Menten function, $\Delta F/F_0 = \Delta F_{\text{max}}/F_0 ([\text{P}_i]/(K_m + [\text{P}_i]))$, with a maximum fluorescence increase $\Delta F_{\text{max}}/F_0 = 0.41$ and the half-saturating concentration of phosphate $K_m = 23.2 \mu\text{M}$.

FIGURE 3: Time course of RH421 fluorescence upon addition of 500 μM P_i at $t = 0$ to the enzyme in standard buffer observed with higher time resolution than in Figure 1a. The fluorescence signal can be fitted by the sum of two exponentials. The fast process represents the mixing time of P_i in the cuvette. The slower process with a rate constant of 0.058 s^{-1} is assigned to the conformational change $\text{E}_1 \rightarrow \text{E}_2$ coupled to the more rapid $\text{E}_2 + \text{P}_i \rightarrow \text{P}-\text{E}_2$ step. The amplitude ratio of the fast to slow processes is 1.98.

the amplitude of the fast and the slow phases and the rate of the slow phase were readily monitored following manual addition of P_i to the enzyme in the presence of RH421 (Figure 3). As already stated we interpret the overall fluorescence increase to be due to $\text{P}-\text{E}_2$ formation *via* reactions 1 and 2. It follows that the fast phase is due to $\text{P}-\text{E}_2$ formation from E_2 (reaction 2), and the slow phase is due to E_1 transformation to E_2 that is then rapidly converted to $\text{P}-\text{E}_2$. In terms of this model the ratio of the amplitudes of the fast and slow phase, measured to be 2.2 ± 0.7 , corresponds to the ratio of E_2 to E_1 concentrations prior to P_i addition (i.e., k_{+1}/k_{-1}).

The rate constant of the slow phase (i.e., k_{+1}) was $0.059 \pm 0.003 \text{ s}^{-1}$. It has already been inferred from data such as in Figure 1b that k_{-1} , the rate constant of $\text{E}_2 \rightarrow \text{E}_1$, is $0.023 \pm 0.001 \text{ s}^{-1}$ in the presence of 500 μM P_i . The ratio of the measured rate constants ($0.059/0.023$) is 2.6 ± 0.3 and, as required by the model, equals within experimental error the amplitude ratio of 2.2 ± 0.7 .

It is important to establish whether the E_1 state identified here by the slow phase of the fluorescence change induced

by P_i corresponds to the E_1 state identified in other experiments. Heyse et al. (1994) have estimated that the rate constant for the E_1 to E_2 isomerization in the presence of K^+ is 400 s^{-1} at pH 7.2 and 20°C . Schulz and Apell (1995) reported an apparent dissociation constant for K^+ of 0.15 mM from the E_1 conformation of the same preparation. The rate constant for the putative E_1 to E_2 isomerization we have observed is 0.059 s^{-1} . This is a rate constant that would be expected in the presence of 22 nM K^+ ($400 \text{ s}^{-1} \times 0.022 \mu\text{M}/150 \mu\text{M} = 0.059 \text{ s}^{-1}$). Neglecting the effect of small differences of experimental conditions similar estimates can be made from the data published by Smirnova et al. (1995) on the E_1 to E_2 isomerization in the presence of K^+ . From their results a rate constant of 0.059 s^{-1} would be expected in the presence of $1.9 \mu\text{M}$ K^+ . The dependence of the rate constant of the slow phase of the fluorescence change induced by P_i was analyzed over a K^+ concentration range from zero to 1 mM. The anticipated catalytic effect of K^+ did not occur (data not shown). The observed rate constant decreased in an approximately linear fashion with $[K^+]$ from 0.059 to 0.03 s^{-1} between zero and 1 mM K^+ . We conclude therefore that, while the slow fluorescence change induced by P_i may be associated with an E_1 to E_2 isomerization, there may well be subclasses of states within the well-characterized functionally and chemically distinguishable E_1 and E_2 conformations (Post et al., 1975).

RH421 Signals Associated with the Kinetics of Phosphorylation of the Na,K-ATPase by P_i : Fast Phase. Manual addition of P_i (Figures 1a and 3) was inadequate to determine the kinetics of the rapid phase of the RH421 fluorescence change. However, when P_i was released by flash photolysis of caged P_i , a complex time course of the fast phase was seen (Figure 4, trace 1). In a control experiment caged P_i was photolyzed as before, but with MgCl_2 omitted from the solution. In this condition phosphorylation of the ATPase by P_i will not occur. Trace 2 shows that a step decrease of fluorescence occurred, followed by a transient increase. Subtraction of trace 2 from trace 1 resulted in trace 3 and shows a small step increase, followed by the time course of the more rapid phase of the fluorescence change presumed to be due to phosphorylation of the ATPase by P_i . This phase of the trace could be described by a single exponential process with rate constant k .

In a further control experiment caged P_i was omitted from the solution (now containing 10 mM MgCl_2). The fluorescence record was identical to that in Figure 4 trace 2 except that the initial step decrease in fluorescence was much less. We conclude therefore that the transient shown in trace 2 is a property of the membrane bound dye when exposed to a UV-laser pulse. The mean time constant of this transient was $18.5 \pm 0.7 \text{ ms}$ and, when recorded as in Figure 4, was independent of caged P_i concentration. The initial step decrease in fluorescence was variable and could not be correlated with caged P_i concentration.

Experiments as presented in Figure 4 were repeated at caged P_i concentrations between 5.6 and $600 \mu\text{M}$. In Figure 5 the rate constants, k , as observed in Figure 4 trace 3, are plotted against the concentration of the released P_i . The data points can be fitted by a straight line according to pseudo-first-order reaction kinetics:

$$k = k_{+2}[P_i] + k_{-2}$$

FIGURE 4: RH421 fluorescence following photorelease of P_i from caged P_i . Trace 1 shows the fluorescence time course following flash photolysis of $129 \mu\text{M}$ caged P_i that released $17 \mu\text{M}$ P_i in the solution as described in Materials and Methods. Trace 2 is an identical trial except that 10 mM MgCl_2 was omitted. Trace 3 is a difference record of traces 1 and 2. The solid line through trace 3 is the best fit to a single exponential plus a linear component of fluorescence versus time. This linear component is associated with the initial part of the slower phase of RH421 fluorescence response to P_i (Figure 3).

FIGURE 5: Dependence of the rate of the rapid phase of fluorescence increase on photoreleased P_i concentration. The rate constants k were obtained from traces such as Figure 4, trace 3. The regression line fitted to the data resulted in a slope of $1.47 (\pm 0.05) \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and ordinate intercept of $3.07 (\pm 0.17) \text{ s}^{-1}$.

where k_{+2} and k_{-2} are rate constants and $[P_i]$ is the concentration of P_i after the light flash. Analysis of the data presented in Figure 5 gives $k_{+2} = 1.47 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and $k_{-2} = 3.1 \text{ s}^{-1}$. Repetition of such experiments in buffers of pH 6.0 or 8.1 did not change the slope of the line significantly (data not shown). k_{+2} and k_{-2} correspond with r_b and r_f , respectively, in the kinetic model of Wuddel and Apell (1995).

The amplitudes of the exponential phases (e.g., Figure 4, trace 3) increased with increasing photoreleased $[P_i]$, consistent with the expected relationship:

$$A_1/A_2 = (1 + k_{-2}/k_{+2}[P_i]_2)/(1 + k_{-2}/k_{+2}[P_i]_1)$$

FIGURE 6: Dependence on pH of equilibrium binding of P_i to the ATPase. The experiments were performed as in Figure 2 in the standard buffer in which pH values were adjusted by HCl or Tris. The data at pH 7.5 represent two experiments to show typical scatter between data sets. The experimental data were fitted by simple binding isotherms. The different symbols distinguish the experiments.

where A_1 and A_2 are amplitudes from photoreleased concentrations $[P_i]_1$ and $[P_i]_2$ respectively.

RH421 Signals Associated with the Dependence of P_i Phosphorylation on pH. The phosphorylated proteins formed by reaction of the ATPase with ATP and with P_i are chemically identical (Bonting et al., 1979), and the fluorescence increase that can be induced by P_i is close to maximum at 500 μ M P_i addition (Figure 2). An explanation is required for the differences in fluorescence levels induced by P_i and by ATP plus NaCl (Figure 1a) that may go beyond the presence of Na^+ in the latter case.

To check for a possible occupation of the ion-binding sites by protons, which would at least partly compensate the negative charges in the sites and in consequence reduce the level of fluorescence, the titration experiments were repeated at different pH values between 6 and 8.5. The buffer pH was adjusted by addition of HCl or Tris. A selection of the results is shown in Figure 6. At least four titration experiments were performed at each pH and averaged. Each series could be fitted well by a simple binding isotherm to determine both the corresponding maximum fluorescence changes ($\Delta F_{\max}/F_0$), when the protein can be assumed to be completely phosphorylated, and the K_m values. In Figure 7 the pH dependence of these parameters is plotted as a function of pH. Figure 7a shows that the fluorescence of the fully phosphorylated protein is pH dependent. As controls, the pH dependence of the RH421-labeled membrane fragments was determined in the absence of P_i and also in the presence of 50 mM NaCl plus 500 μ M ATP. These conditions maintain the protein in the E_1/E_2 states and the $P-E_2$ state, respectively and in both cases the fluorescence did not change over the pH range 6.0–8.5. Therefore it can be assumed that the fluorescence changes are caused by alterations of the amount of uncompensated charge within the protein dielectric. The data in Figure 7a were fitted by a binding isotherm with a pK_a of 7.5. This observation can be interpreted to mean that in the absence of Na^+ ions a proton may bind to the extracellularly-presented binding sites of the $P-E_2$ state of the Na,K-ATPase. At high pH the fluorescence ratio $\Delta F_{\max}/F_0$ approaches a value of 0.7, which is close to that obtained (0.8) by phosphorylation with ATP in the presence of NaCl. The dependence of the half-saturating P_i concentration on pH is shown in Figure 7b. For pH values above 7.5, a steep increase of the K_m was

FIGURE 7: Influence of pH on (a) the maximum fluorescence of RH421 obtained after ATPase phosphorylation by P_i and (b) on equilibrium binding of P_i to the ATPase. In (a) experiments as shown in Figure 2 were analyzed to obtain the maximum fluorescence increase, $\Delta F_{\max}/F_0$, at a given pH. The line drawn is a fit to a simple binding isotherm with pK of 7.5 and a $\Delta F_{\max}/F_0$ of 0.7 at high pH values. The fluorescence intensity ratio ($\Delta F_{\max}/F_0$) represents the condition when virtually all protein is phosphorylated. In (b) the half-saturating P_i concentration dependence on pH derived from experiments as in Figure 6 is shown. The lines drawn represent fits of Hill functions with Hill coefficients $n_H = 2$ with pK 8.6 (solid line) and $n_H = 1$ with pK 9.2 (dashed line).

observed which can be reproduced by a Hill curve with a Hill coefficient of 2 and pK_a of 8.6.

To check the possible pH dependence of the phosphorylation step, $E_2 + P_i \rightarrow P-E_2$ or $E_2(H_2) + P_i \rightarrow P-E_2(H_2)$, the fast phase of enzyme phosphorylation was determined by experiments as presented in Figure 4 in buffers of pH in the range 6.5–8.5. The concentration of P_i , photoreleased from caged P_i , was 37 μ M (data not shown). The relaxation rate $k = 8.6 \pm 0.16 \text{ s}^{-1}$ was constant over the whole pH range studied and agreed with the value from Figure 5 at 37 μ M released P_i . The amplitude of the fast component of fluorescence was pH dependent. It decreased with increasing pH according to a binding isotherm ($pK = 7.6$) with the fluorescence at pH 6.5 being 6.5-fold greater than at pH 8.5. The pH independence of the rate constants and the pH dependence of the amplitude of the fluorescence change of the relaxation toward a new steady state in the process $E_2 + P_i \rightleftharpoons P-E_2$ allowed the conclusion that the state of E_2 contributing to the observed fluorescence change is controlled by pH, e.g., as $E_2(H_2)$.

In addition we found that the overall fluorescence change, $\Delta F_{\max}/F_0$ (Figure 7a), a steady state parameter, is the sum of the contributions of the fast and slow phases of the enzyme phosphorylation kinetics. The different pH dependence of both phases is reflected in their contribution to $\Delta F_{\max}/F_0$. At pH 6–6.5 the fluorescence change is generated predomi-

nantly by the fast phase ($\Delta F_{\text{fast}}/F_0 \approx 0.7\Delta F_{\text{max}}/F_0$). At pH 8.5 the overall fluorescence change is carried mainly by the slow phase ($\Delta F_{\text{slow}}/F_0 \approx 0.95\Delta F_{\text{max}}/F_0$). These findings are in agreement with the pH dependence of the population of state $E_2(H_2)$ before phosphorylation.

DISCUSSION

In the present experiments the previously reported back-door phosphorylation of the Na,K-ATPase (Post et al., 1975; Campos & Beaugé, 1994) was detected by an optical method, which was previously applied successfully to other electrogenic partial reactions of the ion-transport cycle (Stürmer et al., 1991; Heyse et al. 1994). The reaction sequence $E_1 \rightarrow E_2 \rightarrow P-E_2$ is expected to be strongly electrogenic when the ion-binding sites are unoccupied during the process (Wuddel & Apell, 1995). The movement of two uncompensated negative charges in the ion-binding sites, which are close to the extracellular aqueous phase in state $P-E_2$ and on the dielectric surface of the cytoplasmic side in state E_1 , could contribute significantly to the energetics of this partial reaction. Therefore the experiments were designed to determine both the kinetics of the reaction pathway and a possible occupation of the binding sites.

We have characterized a two-step process on addition of P_i to the Na,K-ATPase that we have interpreted in terms of E_1 and E_2 states as in reactions 1 and 2. The interpretation that the more rapid phase is associated with P_i interacting with the E_2 state appears straightforward. However, as noted in the Results, the distinctly different character of the E_1 states described here and by Smirnova et al. (1995) means that much still has to be learnt about subpopulations among the various conformations of the ATPase. In addition, while reactions 1 and 2 are a plausible description of the mechanism of back-door phosphorylation, in the literature additional states are inferred from oxygen exchange data. Thus a state, presumably $E_2 \cdot P_i$, is implied because intermediate oxygen exchange is observed during Na,K-ATPase activity (Dahms et al., 1973; Skvortsevich et al., 1973). Furthermore the bound P_i in $E_2 \cdot P_i$ must undergo some form of isomerization, such as rotation within its binding site (Trentham et al., 1976).

Origin of the RH421 Fluorescence Changes. Recent publications dealt with the mechanism of the RH421 fluorescence changes observed on different proteins and membrane preparations and especially Na,K-ATPase containing membrane preparations similar to ours (Visser et al., 1995; Fedosova et al., 1995). Their findings demonstrate that conformational transitions of the protein cannot be excluded. Therefore we checked the effect of the conformational transition $E_1 \rightleftharpoons E_2$ and the effect of pH variation on the RH421 fluorescence. At pH 7.1 in the absence of Na^+ and K^+ ions and in the presence of Mg^{2+} ions in the millimolar range the enzyme is predominantly in its E_1 conformation (Grell et al., 1991). Addition of 20 mM K^+ ions transfers the protein quantitatively in to state $E_2(K_2)$ (Karlsh, 1980) and addition of saturating P_i also populates the state $P-E_2(K_2)$ (Karlsh & Stein, 1982). Due to the high affinity of the $P-E_2$ states for K^+ the binding sites will remain occupied under these conditions. Tracing this reaction sequence by RH421, no significant fluorescence changes are observed. When the RH421 is monitored with membrane fragments beginning at pH 8 and subsequently

adding H^+ (to reach pH 6.5) again no changes in fluorescence intensity are observed. When ATP is added to enzyme in state $E_2(K_2)$, the reaction $E_2(K_2) \rightarrow E_1ATP$ is triggered and again no change of RH421 fluorescence was observed (Stürmer et al., 1991b; Klodos, 1994). All these findings indicate that RH421 fluorescence does not report the conformational transition $E_1 \rightleftharpoons E_2$. Back-door phosphorylation in the presence of K^+ is also a reaction step which is not detected by RH421 fluorescence. Since this reaction occurs at parts of the protein exposed to the cytoplasm, we conclude by analogy that in the absence of K^+ and the presence of H^+ the same is valid.

The consequence of these observations is that the only remaining reaction step, the extracellular binding/release of H^+ , is detected by RH421. This is in agreement with the observation that the binding and release of Na^+ and K^+ are detected by RH421 (Stürmer et al., 1991a; Bühler & Apell, 1995) and with the pH titration of the steady state fluorescence of the back-door-phosphorylated enzyme (Figure 7a). The fluorescence changes observed in the back-door phosphorylation experiments as presented in Figures 1, 3, and 4 are therefore produced by subsequent (electrogenic) proton release from the extracellularly accessible binding sites. Due to the fact that ion-binding and release steps are fast processes compared to the precursor steps the RH421 fluorescence changes represent the undistorted kinetics of the electrically silent steps, $E_1 \rightleftharpoons E_2 + P_i \rightleftharpoons P-E_2$.

Detection of RH421 Fluorescence Changes by Addition of P_i . In earlier studies of the back-door phosphorylation the existence of state $P-E_2$ was detected by an acid-stable binding of ^{32}P to the enzyme (Post et al., 1975; Campos & Beaugé, 1994). Therefore experiments corresponding to previously published findings on phosphorylation from P_i had to be performed to ensure that the observed fluorescence changes are specific. Crucial control experiments were that the fluorescence increase occurred only when P_i was added in the presence of Mg^{2+} but in the absence of high Na^+ and ATP. Subsequent addition of ATP or Na^+ reversed the P_i -induced fluorescence increase (Figure 1) as it reversed enzyme phosphorylation (Campos & Beaugé, 1994). The P_i concentration dependence of the RH421 fluorescence levels could be fitted by a simple binding isotherm with a half-saturating concentration, K_m , of 23.2 μM (Figure 2) which is in reasonable agreement with a previous value of 32 μM for the half-saturating concentration for back-door phosphorylation of pig kidney enzyme (Campos & Beaugé, 1994).

Determination of Rate Constants. P_i concentration jumps produced by laser flash photolysis of caged P_i allowed synchronization of the phosphorylation reaction. The dependence of the protein-induced fluorescence signal on the concentration of released P_i was linear according to a one-step reaction. Together with the data collected by manual addition of P_i (Figure 3) the reaction sequence may be split into a conformational change and an enzyme phosphorylation/dephosphorylation, as described by reactions 1 and 2 with values of rate constants listed in Table 1. Using ^{32}P studies Campos and Beaugé (1992) measured k_{-2} to be 1.9 s^{-1} , a value comparable to ours. The overall equilibrium dissociation constant for the reactions 1 and 2 is given by $k_{-2}(1 + k_{-1}/k_{+1})/k_{+2} = 29 (\pm 3) \mu M$ where the error was calculated from the sum of sem's. The result is in satisfactory agreement with the value of $23.0 \pm 0.3 \mu M$ obtained

may be very low and the steady state shifted to the state E_1 since electrogenicity of this reaction step is very high. According to Wuddel and Apell (1995) it has a dielectric coefficient of 1.8: thus the state $E_2(\)$ is sparsely populated, and the reaction $E_2(\) + P_i \rightarrow P-E_2$ would not contribute significantly. In the H^+ dependent pathway the population of state $E_2(H_2)$ depends on the square of the H^+ concentration and consequently higher pH requires higher concentrations of P_i to reach half-saturation of the state $P-E_2$ in the reaction $E_2(H_2) + P_i \rightleftharpoons P-E_2H_2$. The K_m for P_i has been found to be cooperative in $[H^+]$ (Figure 7b), which may be accepted as evidence for this mechanism.

The independence of the phosphorylation kinetics on pH in the range 6.5–8.5 demonstrated that changes in nonspecific protein protonation or protonation of inorganic phosphate, P_i , did not interfere with the phosphorylation reaction. The effect of pH on the fluorescence amplitude of this step, however, supported the assumption by independent evidence that the binding sites are occupied by protons during back-door phosphorylation. As already noted the fluorescence amplitudes of the slow and the fast phases of the fluorescence changes such as in Figure 3 depend strongly on pH. At pH 8.5 the fluorescence amplitude of the fast process was less than 5% of the overall fluorescence change on P_i addition. The amplitude of the fast process depends primarily on the population of state $E_2(\)$ or $E_2(H_2)$. Only the latter is controlled by pH via $E_1 + 2H^+ \rightleftharpoons H_2E_1 \rightleftharpoons E_2(H_2)$ and will produce the observed pH dependence.

Summarizing all this evidence the pathway $E_1 \rightleftharpoons H_2E_1 \rightleftharpoons H_2E_2 \rightleftharpoons P-E_2H_2$ (lower track in Scheme 2) may be proposed as the most probable reaction sequence for the back-door phosphorylation/dephosphorylation in the absence of Na^+ and K^+ ions. Under these conditions the rate constants given in Table 1 reflect the parameters of the pathway $H_2E_1 \rightleftharpoons H_2E_2 \rightleftharpoons P-E_2H_2$.

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