Glutamic Acid 472 and Lysine 480 of the Sodium Pump α1 Subunit Are Essential for Activity. Their Conservation in Pyrophosphatases Suggests Their Involvement in Recognition of ATP Phosphates†

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ABSTRACT: P-type ATPases such as the Na^+,K^+-ATPase (sodium pump) hydrolyze ATP to pump ions through biological membranes against their electrochemical gradients. The mechanisms that couple ATP hydrolysis to the vectorial ion transport are not yet understood, but unveiling structures that participate in ATP binding and in the formation of the ionophore might help to gain insight into this process. Looking at the α- and *β*-phosphates of ATP as a pyrophosphate molecule, we found that peptides highly conserved among all soluble inorganic pyrophosphatases are also present in ion-transporting ATPases. Included therein are Glu48 and Lys56 of the *Saccharomyces cerevisiae* pyrophosphatase (SCE1-PPase) that are essential for the activity of this enzyme and have been shown in crystallographic analysis to interact with phosphate molecules. To test the hypothesis that equivalent amino acids are also essential for the activity of ion-transporting ATPases, Glu472 and Lys480 of the sodium pump α1 subunit corresponding to Glu48 and Lys56 of SCE1-PPase were mutated to various amino acids. Mutants of the sodium pump α1 subunit were expressed in yeast and analyzed for their ATPase activity and their ability to bind ouabain in the presence of either ATP, Mg^{2+}, and Na^+ or phosphate and Mg^{2+}. All four mutants investigated, Glu472Ala, Glu472Asp, Lys480Ala, and Lys480Arg, display only a fraction of the ATPase activity obtained with the wild-type enzyme. The same applies with respect to their ability to bind ouabain, where maximum ouabain binding to the mutants accounts for only about 10% of the binding obtained with the wild-type enzyme. On the basis of our results, we conclude that Glu472 and Lys480 are essential for the activity of the sodium pump. Their function is probably to arrest the α- and *β*-phosphate groups of ATP in a proper position prior to hydrolysis of the *γ*-phosphate group. The identification of these amino acids as essential components of the ATP-recognizing mechanism of the pump has resulted in a testable hypothesis for the initial interactions of the sodium pump, and possibly of other P-type ATPases, with ATP.

The sodium pump (Na^+,K^+-ATPase; EC 3.7.3.17) is an enzyme present in all animal cell plasma membranes. It consists of α and *β* subunits and belongs to the P-type ATPases, a family of ion-transporting ATPases that hydrolyze ATP[1] in order to form ion gradients across biological membranes (*1*−*5*). For each hydrolyzed ATP, the sodium pump transports three Na^+ ions out of the cell and two K^+ ions into the cell (*4*, *6*). This activity results in the formation of a Na^+ gradient, the driving force of all secondary active transport processes, and in the maintenance of the membrane potential, the prerequisite for the generation of action potentials and electric signal conduction in neurons. Cardiac steroids prevent the enzyme from hydrolyzing ATP and lead to the disruption of the Na^+ gradient or membrane potential.

The molecular basis of the mechanism that couples ATP hydrolysis to the transport of Na^+ and K^+ ions is not fully understood. It is known, however, that, in a first reaction step involving the binding of Na^+ ions to the enzyme and requiring Mg^{2+}, the sodium pump becomes phosphorylated by the terminal phosphate group of ATP at an aspartic acid (*7*, *8*). In the case of the α1 subunit this aspartic acid is localized at position 369 of the primary amino acid sequence (*9*, *10*). Next to this aspartic acid, other amino acids involved in ATP recognition were primarily identified by various compounds that covalently modify amino acids of the protein. Several of these protein-reactive compounds are derivatives of nucleotide triphosphates and can be thought of as ATP analogues (*11*−*13*). Others, like fluorescein isothiocyanate (FITC), seem to be recognized within the ATP binding site (*14*, *15*), although no apparent structural similarity exists to the sodium pump substrate ATP.

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[1] Abbreviations: YNB, yeast nitrogen base; AP_2PL, pyridoxal 5′-diphospho-5′-adenosine; ATP, adenosine 5′-triphosphate; 2-azido-ATP, 2-azidoadenosine 5′-triphosphate; 8-azido-ATP, 8-azidoadenosine 5′-triphosphate; BSA, bovine serum albumin; ClR-ATP, *γ*-[4-(*N*-(2-chloroethyl)-*N*-methylamino)benzyl]amide ATP; EDTA, ethylenediaminetetraacetic acid; FITC, fluorescein 5′-isothiocyanate; FSBA, 5′-(*p*-fluorosulfonylbenzoyl)adenosine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; H_2DIDS, dihydro-4,4′-diisothiocyanatostilbene-2,2′-disulfonate; PBS-T, phosphate-buffered saline plus Tween-20; PLP, pyridoxal 5′-phosphate; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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Compared to the large number of ATP analogues and ATP site-directed probes used for studying the mechanism and for the labeling of the sodium pump, the number of amino acids identified that might participate in the formation of the ATP binding site is very low. Identification of additional amino acids that participate in recognition of the ATP adenosine and ATP phosphates might help to understand various aspects of the catalytic cycle of the pump, gain some insight into the three-dimensional arrangement of the ATP binding site, and possibly lead to a model describing the coupling of ATP binding and hydrolysis to vectorial ion transport across the plasma membrane. Besides the use of protein-reactive ATP analogues, an additional way to identify amino acids involved in ATP recognition is via the use of protein databases, which allow one to search for and identify common primary structure motifs among enzymes that catalyze similar reactions. Such an approach helped recently to identify a peptide of the sodium pump α1 subunit involved in Mg^{2+} recognition (*16*) or to demonstrate that P-type ATPases, like the sodium pump, share common structural and catalytic features with hydrolases of the haloacid dehalogenase superfamily of enzymes (*17*). In the work presented here we use this method to identify amino acids involved in the recognition of ATP phosphates by comparing the primary structure of ion-transporting ATPases with that of soluble inorganic pyrophosphatases.

Soluble inorganic pyrophosphatases (PPases) are enzymes that regulate the concentration of pyrophosphate in the cytosol of every cell (*18*). Pyrophosphate is generated in cells by various reactions that utilize ATP and would be toxic if not hydrolyzed by this class of enzymes (*19*). On the surface, these soluble enzymes do not seem to have much in common with the sodium pump and other ion-transporting ATPases, which, besides being water insoluble as mentioned above, are mainly responsible for the generation of ion gradients across cell membranes. A closer look, however, reveals a common characteristic: both enzyme types hydrolyze phosphodiester bonds. Taking into consideration that phosphodiester bonds and pyrophosphate groups also exist within the triphosphate moiety of the ATP molecule, we searched for and found a sequence of the sodium pump that is highly homologous to peptides of PPases known to interact with the pyrophosphate molecule. This sequence is localized within the cytosolic loop of the sodium pump α1 subunit, a peptide stretch that includes all amino acids that have been labeled so far by ATP analogues and ATP site-directed probes and is therefore referred to as the ATP binding site. Site-directed mutagenesis of Glu472 and Lys480 that are contained within this sequence and analysis of the properties of the mutants expressed in *Saccharomyces cerevisiae* verify that these two amino acids are as essential for the interactions of the sodium pump with ATP as their equivalents, Glu48 and Lys56 of the *S. cerevisiae* PPase, are for the interactions of this enzyme with pyrophosphate.

EXPERIMENTAL PROCEDURES

Vectors and Strains. The shuttle vector pCGY1406α*β* (*20*) was used for expression of α1 and *β*1 subunits of Na^+,K^+-ATPase in the yeast *S. cerevisiae* strain 20B12 (*21*). Derivatives of the pBluescript II SK(+) plasmid (Stratagene, La Jolla, CA) were used for the introduction of mutations and the amplification of DNA in *Escherichia coli* strain DH5αF' (Gibco BRL, Berlin, Germany). Conditions for cell growth and media compositions are described elsewhere (*21*−*23*).

Introduction of Mutations. The pBluescript II SK(+) plasmid was modified to contain an *Eco*NI site in place of the original *Eco*RV restriction site. Afterward, the plasmid was digested with *Xho*I and *Eco*NI, and a *Xho*I/*Eco*NI fragment of the sheep sodium pump α1 cDNA was ligated into these sites. This plasmid, denoted EPSKH1-4, was used as a template for introducing mutations by inverse PCR (*24*). For mutations of Glu472 the forward primers were 5′ATCGTC**GAT**ATCCCG3′ (bold, Glu472Asp; underlined, diagnostic *Eco*RV site) and 5′ATCGTC**GCG**ATCCCG3′ (bold, Glu472Ala; underlined, diagnostic *Nru*I site), and the reverse primer was 5′CTTGGCATATCTCTC3′. For the mutations of Lys480 5′TCCACCAAC**AGG**TACCAGTTA3′ (bold, Lys480Arg; underlined, diagnostic *Kpn*I site) and 5′TCCACCAA**CGCG**TACCAGTTA3′ (bold, Lys480Ala; underlined, diagnostic *Mlu*I site) were used as forward primers, and 5′GTTGAACGGGATCTCGACGAT3′ was used as the reverse primer. The conditions for PCR are described elsewhere in detail (*22*). After 25 PCR cycles, the amplification product was isolated by agarose gel electrophoresis, treated with T4 DNA polymerase (Promega, Madison, WI) to remove the dA overhang produced by the *Tfl* polymerase (Biozym, Oldendorf, Germany), and circularized back to a plasmid by the use of T4 DNA ligase (MBI Fermentas, Vilnius, Lithuania). A 1394-bp *Xho*I/*Eco*NI fragment carrying the mutation was removed from the EPSKH1-4 plasmid and inserted into the corresponding sites of BS-N/Kα1, a derivative of pBluescript II SK(+) previously prepared containing the entire α1 subunit cDNA. Then a 492-bp *Ava*III/*Eco*91I fragment of this plasmid that had been completely sequenced (*25*) and verified to contain the targeted mutations was used to replace the corresponding fragment of BS-N/Kα1. This last step was done to eliminate possible accidentally introduced mutations by the PCR in DNA segments outside of the sequenced *Ava*III/*Eco*91I fragment. Afterward, a 1648-bp *Xho*I/*Eco*91I fragment of BS-N/Kα1 was used to replace the corresponding fragment of the wild-type α1 cDNA from the yeast expression vector pCGY1406α*β*. The pCGY1406α*β* vectors now carrying the desired mutations were used to transform yeast (*26*).

Isolation of Membranes Containing Native or Mutant Sodium Pumps. The methods involved in the isolation of membranes from yeast cells and for the preparation of microsomes enriched in sodium pump are described elsewhere in detail (*21*−*23*). Single yeast colonies transformed with the vectors carrying the wild-type or mutant α1 subunit cDNA were grown overnight with continual agitation at 30 °C in 5 mL of YNB medium [1.5 g of YNB without amino acids and 2.5 g of $(NH_4)_2SO_4$ per liter]. Thereafter, the cultures were transferred to flasks each containing 500 mL of YNB, and incubation was continued under the same conditions for an additional 20−24 h. Nontransformed yeast was grown under the same conditions except that tryptophan at 20 mg/L was supplemented in the YNB medium. At that point the OD_{600} of the cell cultures was 1−2.

Cells were then collected by centrifugation for 10 min at 3000*g* and were washed twice with ice-cold water. Then they were suspended at 1 g wet weight per 2 mL of a buffer made

of 200 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 2 mM dithiothreitol, 10% (w/w) glycerol, 1 mM Na_2EDTA, 0.5 mg/mL leupeptin, 0.7 mg/mL pepstatin, and 0.2 mM phenylmethanesulfonyl fluoride. Cells were broken in a Bead Beater (Bio-Spec, Bartlesville, OK) using glass beads of 0.4−0.5 mm in diameter. To avoid overheating of the solutions, braking was done by applying 15 short bursts of 15 s duration, each time interrupted by a cooling period of 50 s on ice. Then the homogenates were centrifuged at 7000*g* for 20 min at 4 °C to remove cell debris. Afterward, the supernatants of this last step were centrifuged at 100000*g* for 1 h at 4 °C. The pelleted microsomal fractions were suspended in 25 mM imidazole/1 mM EDTA, pH 7.4. Sodium was omitted in this buffer. The protein content of the microsomal preparations was determined by the method of Lowry (*27*) using bovine serum albumin as a standard.

Yeast membranes enriched in Na^+,K^+-ATPase were prepared following the method of Jorgensen for purification of the enzyme from renal microsomal membranes. A total of 12.3 mg of microsomal protein isolated from yeast cells was incubated with 4.8 mL of 100 mM imidazole-HCI, 4 mM EDTA, pH 7.4, and 0.29 mL of 100 mM ATP (Tris-form) at room temperature. While the solution was stirred, 2.53 mL of 1.9 mg of SDS/mL was added within 15 min. The final volume of the solution was 9.6 mL. At this point the concentrations of the various components in the solution were 1.28 mg of protein/mL, 0.5 mg of SDS/mL, 25 mM imidazole-HCl, 1 mM EDTA, pH 7.4, and 3 mM ATP. After an additional 5 min of stirring at room temperature, the microsomal solution was layered on a discontinuous gradient formed by 8 mL of 40% sucrose, 5.5 mL of 30% sucrose, and 3.5 mL of 25% sucrose in 25 mM imidazole/1 mM EDTA, pH 7.4. After centrifugation for 1.5 h at 140000g_{av} and 4 °C, a fraction enriched in Na^+,K^+-ATPase was recovered from the interface between 30% and 40% sucrose. This fraction was diluted 1:3 with 25 mM imidazole-HCl and 1 mM EDTA, pH 7.4, and was pelleted for 30 min at 140000*g* and 4 °C. The pellet was suspended in 0.2 mL of the imidazole/EDTA buffer and stored at −70 °C.

Assay of Sodium Pump Activity. The overall ATPase activity was determined at 37 °C by a coupled spectrophotometric assay (*21*). The assay medium contained 60 mM imidazole-HCl, pH 7.4, 3 mM $MgCl_2$, 60 mM NH_4Cl, l00 mM NaCI, 0.4 mM NADH + H^+, 0.4 mM phosphoenolpyruvate, 3.5 mM ATP, 3 units/mL lactate dehydrogenase, and 1.5 units/mL pyruvate kinase. In 1.5 mL of this assay various aliquots of the microsomal preparation were added, and the decrease of the absorption at 365 nm was measured in a spectrophotometer. To distinguish the sodium pump activity from other ATPases included in the microsomal fraction, 100 μM ouabain was included in control assays that were run in parallel. By applying the Lambert−Beer's law ($c = E/dA\epsilon$), c, the concentration of NADH + H^+, which directly corresponds to the concentration of ATP, can be determined at any time of the reaction. E is the absorbance at 365 nm, d is the light path length through the assay solution (1 cm), and ϵ = 3400 L/(mol·cm), is the molar absorbance for NADH + H^+ at 366 nm.

SDS Electrophoresis and Western Blotting. Microsomes containing a total of 60 μg of protein were suspended in 15 μL of a sample buffer made of 5% β-mercaptoethanol (v/v), 5% (w/v) SDS, 4 M urea, 125 mM Tris-HCl, pH 6.8, 12.5% (v/v) glycerol, and 1% bromophenol blue (saturated solution in 0.1% ethanol). After an incubation of 30 min at 70 °C, proteins were separated by SDS−polyacrylamide gel electrophoresis (*28*) on gels containing 10% polyacrylamide and 0.3% *N*,*N*′-methylenebis(acrylamide). Proteins were transferred onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany), blocked in PBS-T containing 5% (w/v) dried milk, and subsequently incubated with an anti-α1 subunit monoclonal antibody (Alexis Corp., Grünberg, Germany). Detection of α1 subunits was carried out by incubating the nitrocellulose membrane with an alkaline phosphatase-conjugated anti-mouse IgG (seroTec, Oxford, England) and the alkaline phosphatase substrates 5-bromo-4-chloro-3-indoyl phosphate (Molecular Probes, Eugene, OR) and nitro blue tetrazolium (Serva, Heidelberg, Germany).

Estimation of Wild-Type and Mutant α1 Subunits in Yeast Microsomal Preparations by an Antibody Capture Assay. To obtain an estimate of the amount of expressed wild-type or mutant α1 subunits in yeast microsomal preparations, an antibody capture assay was applied with minor changes from the original protocol (*29*). A total of 20 μg of yeast microsomal protein in 50 μL of phosphate-buffered saline containing 0.1% Tween-20 (PBS-T) was added to the wells of a microtiter plate (MaxiSorbJ, Nunc, Wiesbaden, Germany). After incubation for 2 h at room temperature, the microtiter wells were washed twice with PBS-T. Afterward, 150 μL of blocking buffer composed of 3% bovine serum albumin in PBS-T with 0.02% sodium azide was added overnight to saturate the remaining protein binding sites of the well. Then the wells were washed once again as described before. To each of the wells 50 μL of the primary anti-α1 subunit monoclonal antibody was added at a 1:200 dilution in PBS-T and incubation continued for 2 h at room temperature. Then unbound antibody was removed by four washes with PBS-T, and afterward 50 μL of the alkaline phosphatase-conjugated anti-mouse IgG (1:200 in PBS-T) was added to the wells. After four more washes with PBS-T, the wells were washed twice with 10 mM diethanolamine, pH 9.5, containing 5 mM $MgCl_2$. All further steps concerning the time course of the formation of *p*-nitrophenolate from *p*-nitrophenyl phosphate were carried out as described (*29*).

Binding of [^{3}H]Ouabain. A total of 125 μg of microsomal protein was incubated for 30 min at 30 °C in 10 mM Tris-HCl, pH 7.5, 20 nM [^{3}H]ouabain (3000 Ci/mmol; Amersham Buchler, Braunschweig, Germany), and 5 mM $MgCl_2$ in the presence of either 5 mM PO_4 (Tris form) or 50 mM NaCl plus100 μM ATP. The total volume of each sample was 250 μL. Thereafter, the protein was pelleted by centrifugation at 13000*g* for 2 min, washed twice with H_2O at 4 °C, and dissolved in 300 μL of 1 M NaOH by incubation at 80 °C for 15 min. After the addition of 300 μL of 1 M HCl, the samples were mixed with 3.5 mL of scintillation cocktail, and radioactivity was determined by scintillation counting.

Palytoxin-Induced K^+ Efflux and Its Inhibition by Ouabain. Yeast cells expressing either of the mutants or the wild-type sodium pumps were incubated at a concentration of 6×10^6 cells mL^{-1} with 50 nM palytoxin and various ouabain concentrations in a buffer made of 300 mM glucose, 200 mM NaCl, 0.5 mM boric acid, 1 mM $CaCl_2$, and 10 mM HEPES, pH 7.4. The final volume of each sample was 500

Table 1: Identification of Homologous Peptides in Inorganic Pyrophosphatases and Ion-Transporting ATPases[a]

Enzyme	Organism	Swiss-Prot Accession	Amino acid sequence																	Identity (%)	Homology (%)
Na^+,K^+-ATPase α1 subunit	Sheep	**p04074**	**470**	I	V	E	I	P	F	N	S	T	N	K	Y	Q	L	S	**484**	**100**	**100**
Na^+,K^+-ATPase α1 subunit	*Torpedo californica*	**p05025**	**477**	I	V	E	I	P	F	N	S	T	N	K	Y	Q	L	S	**491**	**100**	**100**
Na^+,K^+-ATPase α subunit	*Artemia salina*	**p17326**	**454**	V	S	E	I	P	F	N	S	A	N	K	Y	Q	V	S	**468**	**73.3**	**86.7**
Na^+,K^+-ATPase α2 subunit	Chicken	**p24797**	**472**	V	T	E	I	P	F	N	S	T	N	K	Y	Q	L	S	**486**	**86.7**	**93.3**
Na^+,K^+-ATPase α3 subunit	Chicken	**p24798**	**464**	V	A	E	I	P	F	N	S	T	N	K	Y	Q	L	S	**478**	**86.7**	**93.3**
H^+,K^+-ATPase α subunit	Rat	**p09626**	**486**	V	C	E	I	P	F	N	S	T	N	K	F	Q	L	S	**500**	**80**	**93.3**
Ca^{2+}-ATPase	*Saccharomyces* (bakers yeast)	**p13586**	**472**	V	Q	E	L	P	F	N	S	K	R	K	L	M	A	T	**486**	**46.7**	**66.7**
Cation-transporting ATPase	*Synechococcus* (blue algae)	**p37278**	**451**	Q	D	E	I	P	F	T	S	E	R	K	R	M	S	V	**465**	**40**	**40**
Na^+-ATPase	*Saccharomyces* (bakers yeast)	**p13587**	**532**	I	A	E	F	P	F	D	S	T	V	K	R	M	S	S	**546**	**53.3**	**53.3**
Pyrophosphatase (ECO-PPase)	*E. coli*	**p17288**	**18**	V	I	**E**	I	P	A	N	A	D	PI	**K**	Y	E	I	D	**33**	**33.3**	**66.7**
Pyrophosphatase (SCE1-PPase)	*Saccharomyces* (bakers yeast)	**p00817**	**46**	V	V	**E**	I	P	R	W	T	N	A	**K**	L	E	I	T	**60**	**33.3**	**66.7**
Pyrophosphatase (SCE2-PPase)	*Saccharomyces* (bakers yeast)	**p28239**	**49**	I	V	E	V	P	R	W	T	T	G	K	F	E	I	S	**63**	**46.7**	**80**
Pyrophosphatase	*Thermus thermophilus*	**P38576**	**19**	V	I	E	V	P	R	G	S	G	N	K	Y	E	Y	D	**33**	**40**	**66.7**
Pyrophosphatase	*Synechocystis* (blue algae)	**p80507**	**18**	L	I	E	I	P	A	G	S	K	N	K	Y	E	F	D	**32**	**46.7**	**66.7**
Pyrophosphatase	*Thermoplasma acidofilum*	**p37981**	**21**	I	V	E	I	P	R	G	S	R	V	K	Y	E	I	A	**35**	**53.3**	**73.3**
Pyrophosphatase	*Thermophilic bact. PS3*	**p19514**	**11**	F	I	E	I	P	T	G	S	Q	N	K	Y	E	F	E	**25**	**46.7**	**60**
Pyrophosphatase	Bovine	**p37980**	**47**	V	V	E	V	P	R	W	S	N	A	K	M	E	I	A	**61**	**33.3**	**66.7**

[a] All sequences are compared to the peptide sequence of the sheep sodium pump α1 subunit. Identical amino acids are shown in dark gray boxes and homologous amino acids in light gray. The amino acids Glu20 and Lys29 of ECO-PPase and Glu48 and Lys56 of SCE1-PPase, which have been shown by mutagenesis to be important for the activity of these enzymes, are shown in bold and are underlined. In the current investigation we assess the role of Glu472 and Lys480 in the activity of the sodium pump.

μL, and incubation proceeded for 2 h at 30 °C. Afterward, cells were spun down, and the total K^+ content in the supernatant was determined by flame photometry (*30*). To determine the total K^+ concentration (100% value), cells were lyzed by incubation for 20 min at 65 °C in 0.1% lithium dodecyl sulfate.

RESULTS

Identification of Homologous Peptides in Inorganic Pyrophosphatases and Ion-Transporting ATPases. Protein sequence comparison between soluble inorganic pyrophosphatases and ion-transporting ATPases shows that both classes of enzymes contain a highly homologous peptide (Table 1). This stretch of the PPase protein contains amino acids Glu20 and Lys29 that were shown to be essential for the catalytic activity of *E. coli* pyrophosphatase (ECO-PPase) (*18*) and Glu48 and Lys56 that are essential for the activity of yeast pyrophosphatase (SCE1-PPase) (*31*). These glutamic acids and lysines, highly conserved in PPases, are also conserved within a peptide of ion-transporting ATPases displaying high homology to the PPase sequence. This ion-transporting ATPase peptide is localized within the so-called ATP binding site of these enzymes.

For the sheep kidney Na^+,K^+-ATPase (sodium pump) α1 subunit, Glu472 and Lys480 correspond to Glu20 and Lys29 of ECO-PPase and to Glu48 and Lys56 of SCE1-PPase. These two amino acids are also present in all the other PPases and ATPases shown in Table 1. A third amino acid, equivalent to ECO-PPase Pro22, seems also to be highly conserved. The role of this proline for the activity of ECO-PPase or other PPases has never been investigated by mutagenesis, possibly because mutations of this amino acid would be expected to have a considerable impact on the structure of the proteins. Taking this possibility into account, mutations of the Na^+,K^+-ATPase α1 subunit at Pro474 were not made in the current investigation, and preference was given to investigating the role of Glu472 and Lys480 for ATP interactions of the enzyme.

Expression of Sodium Pump α1 Subunit Mutants in Yeast. To ensure that the mutants were adequately expressed, microsomal proteins isolated from transformed yeast were probed in a western blot with a monoclonal antibody raised against the sodium pump α1 subunit. Figure 1 shows that the wild-type sodium pump and each of the mutants, Glu472Asp, Glu472Ala, Lys480Arg, and Lys480Ala, are expressed in the yeast in comparable quantities. As shown in Figure 1, the antibody does not recognize any protein of approximately 100 kDa corresponding to the wild-type or mutant sodium pump α1 subunits in microsomes isolated from nontransformed yeast cells.

Nevertheless, quantification of the protein of wild-type or mutant sodium pump α1 subunits in yeast microsomes cannot

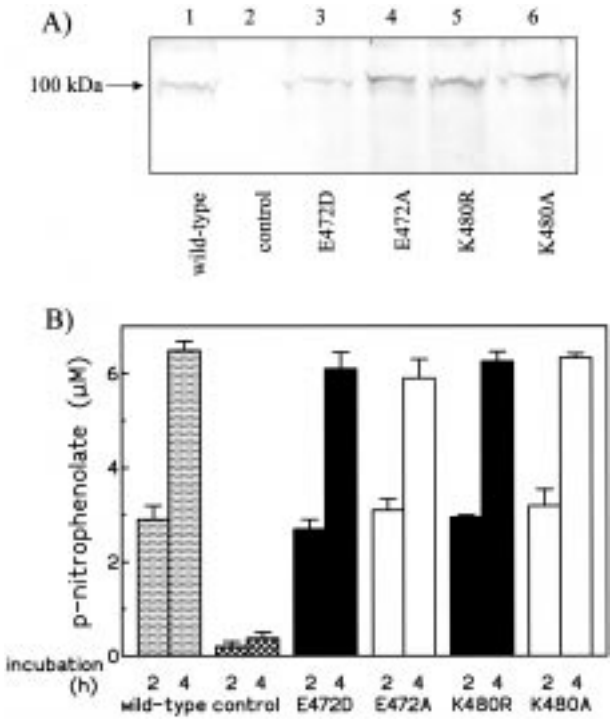

FIGURE 1: Expression and quantification of wild-type and mutant α1 subunits in yeast. (A) Detection of α1 subunits by western blotting. A total of 60 μg of microsomal protein was applied to each lane. Detection of wild-type (lane 1) and mutant (Glu472Asp, lane 3; Glu472Ala, lane 4; Lys480Arg, lane 5; Lys480Ala, lane 6) sodium pump α1 subunits in a western blot. A band at approximately 100 kDa, corresponding to the sodium pump α1 subunit, is not detected by the antibody in membrane preparations from nontransformed yeast cells (lane 2). (B) Quantification of wild-type and mutant α1 subunits in yeast membranes by an antigen capture assay. A total of 20 μg of microsomal protein per well was incubated for the times indicated in microtiter plates with an anti-α1 antibody and then with an alkaline phosphatase-conjugated secondary antibody (see Experimental Procedures). After the addition of *p*-nitrophenyl phosphate, the formation of the *p*-nitrophenolate anion was determined at 405 nm as a function of time. The similar time course of *p*-nitrophenolate formation for all membrane preparations indicates that wild-type and mutant sodium pump α1 subunits are expressed in similar quantities. Thus, after 4 h, 6.48 ± 0.2 μM *p*-nitrophenolate is formed when yeast membranes containing the wild-type α1 subunits are used in the assay. The use of the mutant α1 subunits delivers similar results that range between 5.9 ± 0.4 μM (Glu472Ala) and 6.33 ± 0.1 μM (Lys480Ala) *p*-nitrophenolate. The production of *p*-nitrophenolate is negligible when membranes from nontransformed yeast cells are used in the assay. The test was carried out with three different membrane preparations, and each time point was assayed twice. The values represent means ± SEM of these six measurements.

easily be addressed by the western blotting method. This was done by applying a variation of an antigen capture assay (*29*). The principle of this reaction is as follows: the antigen (here the wild-type or mutant α1 subunits) is affixed to a surface (microtiter plate wells). This affixed antigen is then recognized by a specific antibody (here an anti-α1 subunit monoclonal antibody). Subsequently, an alkaline phosphatase-conjugated secondary antibody (here an anti-mouse IgG) is added to the well. At this point antigen, primary antibody, secondary antibody, and alkaline phosphatase should be present at an equimolar stoichiometry. Thus, the amount of *p*-nitrophenyl phosphate hydrolyzed by the alkaline phosphatase directly depends on the number of α1 subunits that were originally present in each well, and therefore, the amount of *p*-nitrophenolate anion produced by the alkaline phosphatase can be used as a measure of the relative abundance of α1 subunits.

Figure 1B shows the results obtained by using microsomes from yeast expressing the wild-type or either of the mutants as an antigen. It is apparent that the time course of the formation of the *p*-nitrophenolate anion is in all cases very similar, ranging between 6.48 ± 0.2 μM (wild-type α1 subunit) and 5.9 ± 0.4 μM (Glu472Ala), indicating that similar quantities of wild-type or mutant α1 subunits are expressed in yeast. All other data are included in Table 2.

Effect of Palytoxin and Ouabain on Yeast Cells Expressing Wild-Type or Mutant Sodium Pumps. Palytoxin is produced by marine corals of the genus *Palythoa* and is the most potent animal toxin known thus far. Palytoxin causes K^+ efflux from all mammalian cells but is without any effect on *S. cerevisiae* cells (*30*, *32*). These cells become palytoxin-sensitive and lose intracellular K^+ when they express both α and β subunits of the mammalian sodium pump (*30*, *33*). Ouabain and other cardioactive steroids inhibit the palytoxin-induced K^+ efflux (*30*, *32*−*34*). Thus, determining the IC_{50} value for ouabain inhibition of the palytoxin-induced K^+ efflux allows one to obtain a measure for the relative affinity of the yeast-expressed enzymes for ouabain.

As shown in Figure 2, the maximum K^+ efflux induced by 50 nM palytoxin from yeast cells expressing either of the mutants is within the same range of the K^+ efflux obtained with cells expressing the wild-type enzyme. This further indicates that all expressed enzymes are present in the yeast plasma membranes in similar quantities.

Ouabain inhibits the palytoxin-induced K^+ efflux in a concentration-dependent manner (for simplicity, only the effect of 100 μM ouabain is shown in Figure 2). In the presence of 50 nM palytoxin, ouabain inhibits the palytoxin-induced K^+ efflux from cells expressing the wild-type sodium pump with an IC_{50} of 20.1 ± 1.8 μM (Table 2). The IC_{50} with cells expressing the Glu472Ala mutant is 14.0 ± 2.1 μM and with cells expressing the Glu472Asp mutant is 14.4 ± 0.9 μM. The IC_{50} values with the mutants Lys480Ala and Lys480Arg, 18.6 ± 2.6 and 20.3 ± 3 μM, respectively, were within the same range. Similar IC_{50} values indicate that the wild-type enzyme and all mutants recognize ouabain equally well, thus confirming the assumption that the mutations do not have a direct effect on the ouabain binding site.

ATPase Activity of Wild-Type and Mutant Sodium Pumps. The overall Na^+,K^+-ATPase activity of the wild-type and the mutant sodium pumps can be measured in yeast membrane preparations in the presence of Na^+, K^+, Mg^{2+}, and ATP as the fraction of the total ATPase activity that is sensitive to ouabain (*21*). As shown in Figure 3, the Glu472Asp mutant hydrolyzes 94.4 ± 20.4 nmol of ATP h^{-1} (mg of microsomal protein)$^{-1}$ (Table 2). Under the same conditions the Glu472Ala mutant is less active than the conservative mutant Glu472Asp and hydrolyzes only 46.3 ± 10.6 nmol of ATP h^{-1} mg^{-1}. The most active of all mutants is the Lys480Arg mutant, which hydrolyzes 99.2 ± 17.2 nmol of ATP h^{-1} mg^{-1}. The nonconservative mutant Lys480Ala, on the other hand, hydrolyzes only 56.7 ± 20 nmol of ATP h^{-1} mg^{-1}. Nevertheless, when compared to the ATPase activity of the wild-type sodium pump, which hydrolyzes 729 ± 75 nmol of ATP h^{-1} mg^{-1}, the ATPase activities of all mutants are significantly reduced and account

Table 2: Synopsis of the Effects of Mutations on Properties of Na^+,K^+-ATPase, ECO-PPase, and SCE1-PPase[a]

enzyme	mutation	ouabain-sensitive ATPase activity (%)	ouabain binding in the presence of ATP, Mg^{2+}, Na^+ (%)	ouabain binding in the presence of P_i and Mg^{2+} (%)	*p*-nitrophenolate formed in the antibody capture assay (μM/4 h)	IC_{50} for ouabain inhibition of the palytoxin-induced K^+ efflux (μM)
Na^+,K^+-ATPase	wild type	100 ± 10.3[b]	100 ± 14.4[c]	100 ± 12.8[d]	6.48 ± 0.2	20.1 ± 1.8
	Glu472Asp	12.9 ± 2.8	9.7 ± 3.4	1.7 ± 0.4	6.1 ± 0.35	14.4 ± 0.9
	Glu472Ala	6.4 ± 1.5	5.5 ± 1.7	6.3 ± 3.5	5.9 ± 0.4	14.0 ± 2.1
	Lys480Arg	13.6 ± 2.4	17.5 ± 2.8	5.9 ± 1.2	6.26 ± 0.2	20.3 ± 3
	Lys480Ala	7.8 ± 2.7	17.2 ± 3.8	7.8 ± 4.3	6.33 ± 0.1	18.6 ± 2.6
		PPase activity (%)				
ECO-PPase	wild type	100				
	Glu20Asp	16				
	Lys29Arg	2				
SCE1-PPase	wild type	100				
	Glu48Asp	14				
	Lys56Arg	2				

[a] All data concerning the properties of the Na^+,K^+-ATPase and its Glu472 or Lys480 mutants are from the current investigation. The data concerning the activity of ECO-PPase and its mutants are from Cooperman et al. (*18*). The data for the activity of SCE1-PPase and its mutants are taken from Heikinheimo et al. (*31*). [b] 729 ± 75 nmol of ATP h^{-1} mg^{-1}. [c] 1.18 ± 0.17 pmol mg^{-1}. [d] 0.78 ± 0.1 pmol mg^{-1}.

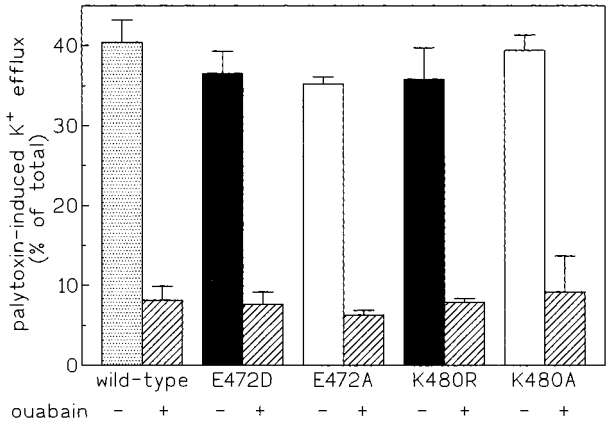

FIGURE 2: Inhibition of the palytoxin-induced K^+ efflux from yeast cells expressing wild-type or mutant sodium pumps by ouabain. Palytoxin causes K^+ efflux from all mammalian cells and from *S. cerevisiae* cells expressing mammalian sodium pumps (*30*, *32*). In the experiment shown in this figure, 50 nM palytoxin causes K^+ efflux that accounts for approximately 40% of the total K^+ content of yeast cells expressing either wild-type or mutant sodium pumps. Ouabain inhibits the palytoxin-induced K^+ efflux with similar IC_{50} values (see Table 2). Each value is the mean ± SEM of data from five independent experiments.

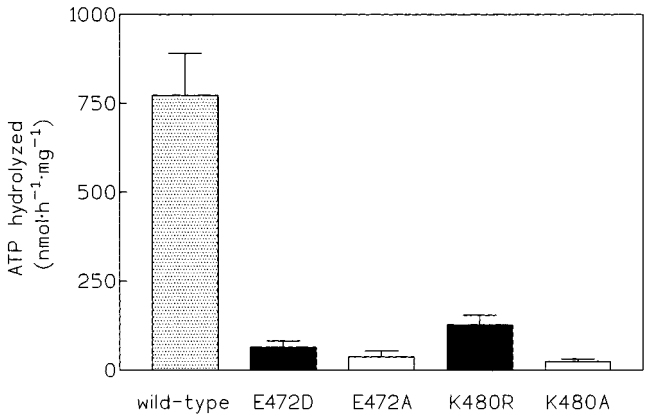

FIGURE 3: Ouabain-sensitive ATPase activity in membrane preparations from yeast cells expressing either wild-type or mutant sodium pumps. The overall sodium pump activity in membrane preparations from yeast expressing either wild-type or mutant sodium pumps was measured by a coupled spectrophotometric assay in the absence or presence of 1 mM ouabain (*21*). Each value is the mean ± SEM of data from 7 to 11 independent experiments.

for only a small fraction of the sodium pump activity of the wild-type enzyme. Depending on the mutation, the loss in ATPase activity parallels the loss in ouabain binding capacity (see next paragraph) and accounts for 86−94% of the ATPase activity obtained with the wild-type enzyme (Figure 3 and Table 2).

Binding of Ouabain to Wild-Type and Mutant Sodium Pumps in the Presence of ATP. In the presence of Na^+ and Mg^{2+} the sodium pump hydrolyzes ATP and becomes phosphorylated by the γ-phosphate group of ATP at Asp369 of the α1 subunit (*4*, *35*). This step leads to the occlusion of Na^+ within the protein and the subsequent release of the cations into the extracellular space (*4*). After release of Na^+, the phosphorylated enzyme is capable of binding ouabain with high affinity and forms a stable [phosphoenzyme·ouabain] complex that can easily be measured using [^{3}H]ouabain (*36*). Thus, in the presence of Na^+, Mg^{2+}, and ATP, ouabain binding can only take place when ATP is hydrolyzed and the enzyme is phosphorylated by the ATP terminal phosphate (*4*, *36*, *37*).

As expected, this property of the enzyme can clearly be demonstrated in experiments with microsomes from yeast cells expressing wild-type sodium pumps (Figure 4). Maximum binding of [^{3}H]ouabain to these membranes is 1.18 ± 0.17 pmol (mg of protein)$^{-1}$ (Table 2). Maximum binding of [^{3}H]ouabain to microsomes containing either the Lys480Arg or Lys480Ala mutants is considerably reduced. Only 0.206 ± 0.033 pmol of [^{3}H]ouabain is bound/mg of protein from microsomes containing the Lys480Arg mutant. Similarly, the equivalent value with membranes containing the Lys480Ala mutant is 0.203 ± 0.045 pmol mg^{-1}. An even more pronounced loss in ouabain binding capacity is obtained with membrane preparations containing either the Glu472Asp or the Glu472Ala mutants. Maximum ouabain binding to these mutants is 0.115 ± 0.04 or 0.065 ± 0.02 pmol mg^{-1}, respectively (Figure 4 and Table 2). Thus, each of the mutations has a very strong effect on ATP-promoted ouabain binding. Depending upon the nature of the mutation, the ouabain binding to the mutants accounts for only 5−13% of the total ouabain binding obtained with the wild-type sodium pumps (Table 2). Nevertheless, ouabain binding to the mutants is highly specific, since microsomes isolated

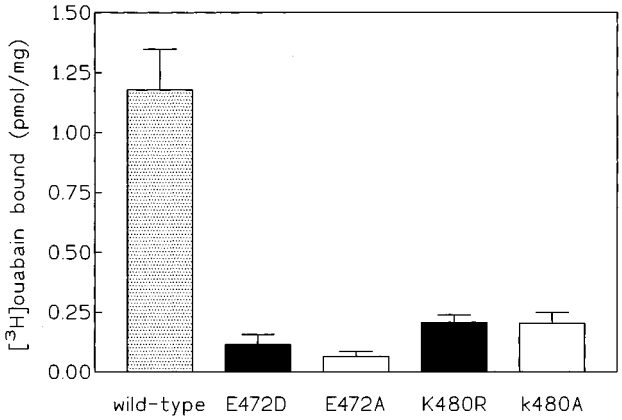

FIGURE 4: Binding of [^{3}H]ouabain to wild-type and mutant sodium pumps in the presence of ATP, Na^+, and Mg^{2+}. In the presence of ATP, Na^+, and Mg^{2+}, the sodium pump assumes the E1 conformation, which has a very low affinity for ouabain. Ouabain binding takes place when the enzyme has reached the E2-P conformational state after it has been phosphorylated by the terminal phosphate group of ATP (*4*, *36*, *37*). The results shown indicate that only the wild-type enzyme can assume the E2 state that subsequently leads to the formation of the [phosphoenzyme•ouabain] complex [E2-P•ouabain]. Each value is the mean ± SEM of data from 10 independent experiments.

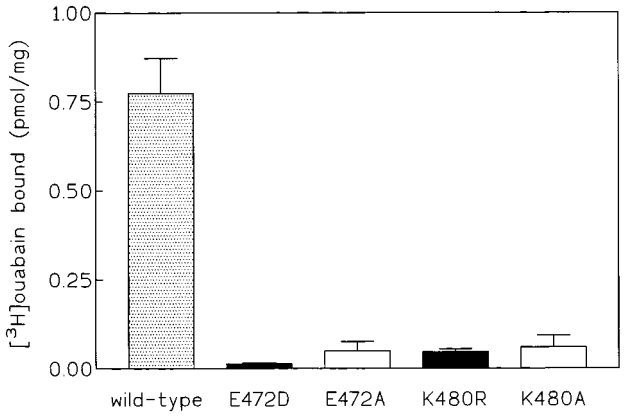

FIGURE 5: Binding of [^{3}H]ouabain to wild-type and mutant sodium pumps in the presence of phosphate and Mg^{2+}. The E2-P conformational state that binds ouabain with high affinity can be reached by incubating the enzyme with inorganic phosphate in the presence of Mg^{2+} (*4*, *37*). Only the wild-type sodium pump seems to be able to assume this conformational state. Each value is the average ± SEM of three independent experiments.

from nontransformed yeast do not bind ouabain under any conditions.

Binding of Ouabain to Wild-Type and Mutant Sodium Pumps in the Presence of Inorganic Phosphate. Ouabain binds with high affinity to the E2-P phosphoenzyme, formed in the presence of inorganic phosphate and Mg^{2+} (*4*, *37*). To evaluate whether the mutations have an effect on enzyme/phosphate interactions, microsomes containing mutants or wild-type sodium pumps were incubated with radioactive ouabain and phosphate, and the formation of the [phosphoenzyme•[^{3}H]ouabain] complex was determined by scintillation counting. As shown in Figure 5, ouabain binding to the mutants in comparison with binding to the wild-type enzyme is considerably reduced. The maximum ouabain bound to the wild-type enzyme is 0.78 ± 0.1 pmol (mg of protein)$^{-1}$. Under the same conditions, binding to the mutants is reduced by 91% (Lys480Ala) or 98% (Glu472Asp) (Table 2).

DISCUSSION

Active transport of ions by the sodium pump is initiated by the binding of Na^+, Mg^{2+}, and ATP to the enzyme followed by the hydrolysis and transfer of the γ-phosphate of ATP onto an aspartic acid (Asp369 of the sheep α1 subunit) and subsequent occlusion of Na^+ ions within the enzyme protein (*4*, *6*, *38*). The molecular basis for this reaction chain that couples the ATP hydrolysis to the opening of an ionophore for the translocation of ions against their electrochemical gradient, however, is not well understood. Identification of protein structures involved in ATP binding and hydrolysis, as well as in the formation of the ion translocation apparatus, might help to understand this process. The focus of the work presented here was placed on the identification of amino acids of the sodium pump that might be involved in ATP recognition.

A series of amino acids that participate in ATP recognition has been identified thus far by using various protein-reactive compounds. Among them, 2-azido-ATP and 8-azido-ATP are substrates of the sodium pump and label Gly502 and Lys480, respectively (*11*, *12*). Others, like 5′-(*p*-fluorosulfonylbenzoyl)adenosine (FSBA) or γ-[4-(*N*-(2-chloroethyl)-*N*-methylamino)benzyl]amide ATP (ClR-ATP), although they resemble nucleotide triphosphates and their interaction with the enzyme can be prevented by ATP, are not substrates of the sodium pump. As a result, some uncertainty exists with respect to Cys656 and Lys719, the FSBA labeling sites (*39*), and Asp710, the ClR-ATP labeling site (*40*), as to whether they are constituents of the ATP binding site. In contrast to these ATP-like substances, FITC, a protein-reactive probe, was shown to modify Lys501 of the sodium pump α1 subunit (*14*). Although no apparent similarity exists between FITC and ATP, the fact that ATP prevents modification of Lys501 by FITC led to the conclusion that Lys501 is localized within the adenosine-recognizing moiety of the α1 subunit of the sodium pump.

Thus, despite the large number of ATP analogues or ATP site-directed probes used for studying the properties of the sodium pump, only a limited number of amino acids have been identified thus far by use of presumably ATP site-directed, protein-reactive compounds. Possibly, this low yield is because not all ATP analogues can be synthesized in a suitable radioactive form for labeling studies. In addition, some of them display only a low affinity for the enzyme, and in some cases, the specificity of their interactions with the sodium pump is rather questionable.

In the work presented here, additional amino acids that may participate in ATP recognition were identified by a different approach, taking advantage of the accessibility of protein databases. Under the assumptions that homology of catalytic reactions might be reflected in homology of the protein primary structure and that ATP is a pyrophosphate analogue, a search was carried out to identify peptides that are conserved in soluble inorganic pyrophosphatases and ion-transporting ATPases. As a result, both enzyme groups were found to contain a peptide of high homology (Table 1). In the ion-transporting ATPases this peptide is a part of the cytosolic loop that is localized between membrane-spanning domains M4 and M5. This cytosolic loop contains Asp369,

the ATP phosphorylation site of the sodium pump α1 subunit, and all the other amino acids that have been identified thus far via their interaction with various ATP site-directed probes. In the PPases, this peptide contains a glutamic acid and a lysine, 2 out of a total of 17 polar amino acids that are identical in all soluble inorganic pyrophosphatases known to date (*18*). These are equivalent to Glu48 and Lys56 of the *S. cerevisiae* SCE1-PPase, which were shown by analysis of crystallographic data to be located within the active site of the enzyme (*41*). In *E. coli* ECO-PPase, the equivalent amino acids Glu20 and Lys29 were shown by mutational analysis to be important for the catalytic activity of the enzyme (*42*, *43*). They also seem to be localized in the catalytic site, a conclusion based on the analysis of protein crystals obtained in the absence or presence of sulfate, a phosphate analogue (*44*, *45*). The conserved glutamic acid of the pyrophosphatases corresponds to Glu472 of the sodium pump α1 subunit. Interestingly, the conserved PPase lysine is equivalent to the Lys480 of the α1 subunit, the same amino acid that has been modified by phosphate and ATP analogues (*11*, *13*) and whose importance for ATP recognition was questioned after the investigation of mutants of this amino acid (*46*). This Lys480 is highly conserved among ion-transporting ATPases, suggesting that it might be essential to function. It is also present in the phylogenetically very archaic blue algae (*Synechococcus*) cation-transporting ATPase (Lys461; Table 1) and appears to be equivalent to Lys492 of the sarcoplasmic reticulum Ca^{2+}-ATPase, which has been labeled by various ATP analogues (*47*−*50*) and was shown by mutational analysis to be essential for the function of this enzyme (*51*). This Lys492, however, is localized within a sequence that displays very low homology to the corresponding sequences of the other ion-transporting ATPases shown in Table 1 or to the Ca^{2+}-ATPase from plasma membranes of animal cells. Possibly, the phylogenetic distance between Ca^{2+}-ATPases and other ion-transporting ATPases, as proposed by others (*52*), accounts for the differences in the primary sequence.

Apart from the conserved amino acids that correspond to Glu48 and Lys56 of *S. cerevisiae*, a proline, corresponding to Pro50 of SCE1-PPase, is also present in all PPase and ATPase peptides shown in Table 1. The role of this proline for the activity of PPases has never been investigated by mutagenesis, possibly because mutations of this amino acid would be expected to have a considerable impact on the structure of the proteins. For the same reason, mutation of the sodium pump α1 subunit Pro474 was not carried out, and the focus of this investigation was on analyzing properties of Glu472 and Lys480 mutants.

To determine whether these two amino acids are as important for the catalytic activity of the sodium pump as they are for the activity of PPases, Glu472 and Lys480 were mutated by PCR to Asp472 or Ala472 and to Arg480 or Ala480, respectively, and the mutants were expressed together with sodium pump β subunits in the yeast *S. cerevisiae*. The simultaneous expression of α and β subunits is absolutely required in order to obtain functional sodium pumps, since the basic catalytic unit of the enzyme is formed by an α/β heterodimer (*21*, *30*).

Wild-type and mutant sodium pump α1 subunits are expressed in yeast, as shown in the western blot illustrated in Figure 1A. Although expression levels appear similar in this figure, application of a variation of an antigen capture assay (*29*) helped to further verify that wild-type and mutant α1 subunits are expressed in matching quantities in the transformed yeast cells (Figure 1B and Table 2). This was important in order to exclude that changes in kinetic parameters obtained with the mutant enzymes are not due to altered expression levels but rather to the mutations themselves. Therefore, the dramatic reduction of maximum [^{3}H]ouabain binding in the presence of ATP, Na^+, and Mg^{2+} (Figure 4) is not due to altered expression levels but rather due to the mutations.

The mutations do not have a direct effect on the ouabain binding site. This was verified in the experiments with palytoxin, where palytoxin causes K^+ efflux from whole yeast cells expressing the wild-type or the mutant enzymes with similar EC_{50} values (Table 2). In addition, maximum K^+ efflux is approximately the same with all cells, indicating that wild-type and mutant sodium pumps are present at similar quantities in the plasma membrane of the yeast cells. Ouabain inhibits the palytoxin-induced K^+ efflux from cells expressing the wild-type or mutant sodium pumps to the same extent and with similar IC_{50} values (Table 2). Since enzyme phosphorylation is not absolutely required for the palytoxin-induced K^+ efflux or its inhibition by ouabain, this experiment shows that the extracellularly localized ouabain binding site of the enzyme is not affected by the mutation. It is therefore quite unlikely that, in the experiments with microsomal preparations, the reduced [^{3}H]ouabain binding obtained with the mutants is due to a direct effect of the mutations on the ouabain binding site. Since, under these conditions, [^{3}H]ouabain preferentially binds to the phosphorylated enzyme formed by the transfer of the γ-phosphate group of ATP to Asp369, it can be concluded rather that the phosphorylation process is affected by the mutations. The same conclusion can be drawn from the experiments concerning the overall ATPase activity of the yeast-expressed enzymes (Figure 3). Depending on the mutation, the ATPase activity of the mutants accounts for only 6−14% of the ATPase activity obtained with the wild-type enzyme.

The results from both of these experiments, ouabain binding in the presence of ATP and overall Na^+,K^+-ATPase activity (Table 2), underline the importance of Glu472 and Lys480 for the enzyme/ATP interactions. Although in the present work we do not assess whether ATP binds to the mutants with reduced affinity or whether the rate of ATP hydrolysis is affected by the mutations, the data strongly suggest that Glu472 and Lys480 are as essential for the sodium pump as Glu48 and Lys56 and their counterparts are for the activity of the SCE1-PPase and other pyrophosphatases (*18*, *31*).

How might Glu472 and Lys480 be involved in the binding and hydrolysis of ATP? An answer to this question can be approached by taking into consideration known facts about the interaction of PPases with Mg^{2+} and pyrophosphate (*18*, *31*). Lys56 of SCE1-PPase is thought to directly interact with one of the phosphate oxygens, whereas Glu48 interacts primarily with Mg^{2+}, contributing to the destabilization of the phosphodiester bond formed between the two phosphates (*31*, *44*). This destabilization allows a nucleophilic attack of a water hydroxyl group on one of the phosphorus atoms and leads to the hydrolysis of the phosphodiester bond. Although Glu48 and Lys56 are not the only amino acids that interact

with the phosphates of the pyrophosphate molecule, their importance becomes more apparent when crystallographic data are taken into consideration. In all PPases, the peptide that extends between the glutamic acid and the lysine equivalent to Glu48 and Lys56 of SCE1-PPase forms a conserved structure. This structure is also present in the ECO-PPase, despite the additional Pro27, and resembles an inverted U with its tips formed by the glutamic acid and the lysine (Figure 6). Localized at this position, these amino acids appear to hold the phosphates of the pyrophosphate molecule in a forceps-like manner in the right position prior to hydrolysis. A similar interaction might take place within the catalytic site of the sodium pump or other ion-transporting ATPases. Thus, if this U-shaped structure is also formed by the peptide of the sodium pump α1 subunit, one might assume that Glu472 and Lys480 of the sodium pump α1 subunit possibly together with Mg^{2+} ions interact in a comparable way with the phosphodiester bond of the ATP molecule that is formed between α- and *β*-phosphate groups.

The fact that Lys480 is labeled by both AP_2PL and PLP indicates that this amino acid is involved in the recognition of phosphate groups, as correctly suggested by Hinz and Kirley (*13*). Thus, in this point of view, the labeling of the same Lys480 by 8-azido-ATP (*11*) does not necessarily indicate that Lys480 is directly interacting with the adenine moiety of the ATP molecule but is merely within reach of the highly reactive azido group of 8-azido-ATP. This proposition is supported by findings concerning the conformation of Mg^{2+}-complexed ATP analyzed by ^1H NMR and ultraviolet spectrophotometric methods. According to these reports, the α-phosphate group of the ATP molecule is in close proximity to the C8 atom of the adenine moiety (*53*). Therefore, if ATP is assumed to retain a similar conformation when bound within the ATP binding site, one can imagine that the C8-azido group of 8-azido-ATP labels Lys480, which originally interacts with the α-phosphate group of ATP. Taking into account the fact that the distance between Lys501 and Lys480, as determined by labeling experiments with H_2DIDS, is approximately 1.4 nm (*54*), one can imagine that the azido group of 8-azido-ATP labels Lys480, while the azido group of 2-azido-ATP labels Gly502.

The results obtained here and the labeling experiments with 8-azido-ATP and AP_2PL or PLP all suggest that Lys480 is an essential amino acid of the ATP binding site. Nevertheless, there is some controversy regarding the role of Lys480 in the activity of the sodium pump. An earlier investigation of mutants of Lys480 produced results leading to the conclusion that this amino acid is not an essential component for the interactions of the sodium pump with ATP (*46*). Since, in this former study, the Lys480 was also mutated to alanine and arginine, it is not apparent why, in contrast to the current study, no influence of the mutations on the enzyme interactions with ATP or phosphate was detected in this investigation. In the former study, however, a Lys480Glu mutant displayed considerably altered properties compared with the wild-type enzyme (*46*). This was interpreted to indicate electrostatic repulsion between the negatively charged glutamic acid and phosphate, consistent with the assumption of Hinz and Kirley that Lys480 is an amino acid that interacts with ATP phosphates.

In our experiments, this latter proposition seems to apply for both Lys480 and Glu472. As shown in Figures 4 and 5,

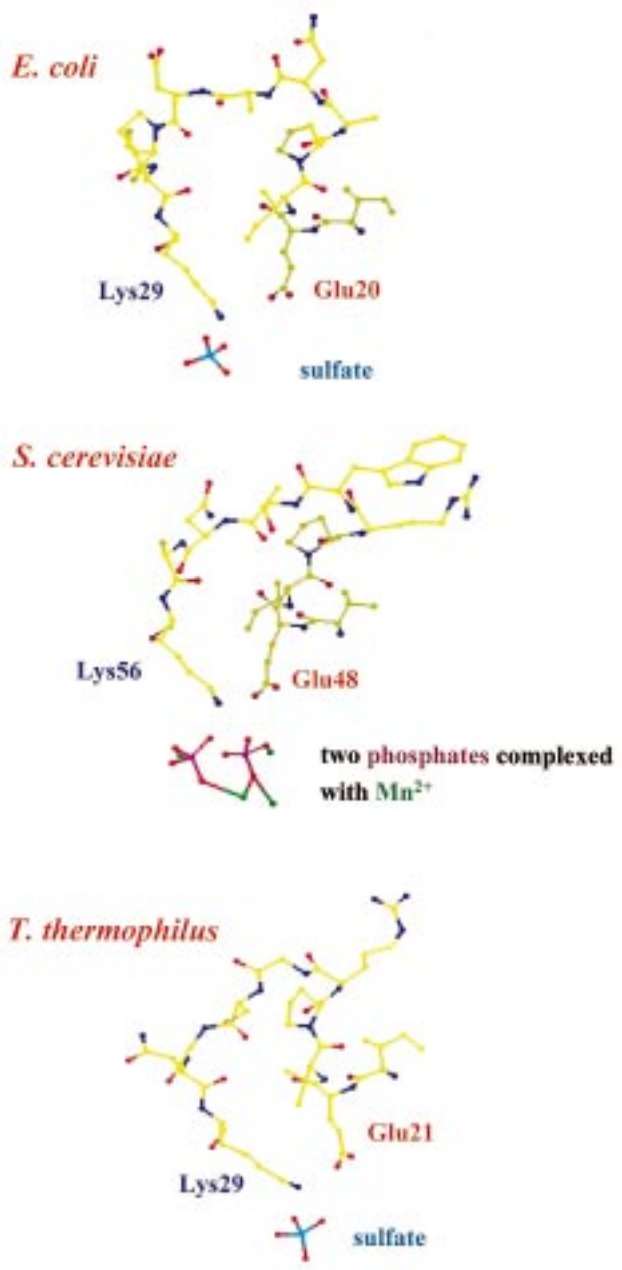

FIGURE 6: Interactions of conserved glutamic acids and lysines of PPases with sulfate and phosphate molecules. The structures of the peptides shown are extracted from published crystallographic data (*E. coli* from ref *45*; *S. cerevisiae* from ref *41*; *T. thermophilus* from ref *60*). In all cases, the glutamic acid and lysine residues are at the tips of the U-shaped peptide and interact either with phosphate or with the phosphate analogue sulfate. Interestingly, the presence of the additional Pro27 in *E. coli* does not influence the U-shape of the peptide or the positioning of the Lys29 and the Glu20 toward the substrate. Since the overall U-shape structure remains stable, one could assume that the corresponding Val471−Lys480 peptide of the α1 subunit of the sodium pump might be arranged in a similar way that allows Lys480 and Glu472 to interact with the α/*β*-phosphates of ATP.

ouabain binding in the presence of either ATP, Na^+, and Mg^{2+} or phosphate and Mg^{2+} to the mutants Glu472Ala, Glu472Asp, Lys480Ala, and Lys480Arg is considerably reduced. Ouabain binding under these conditions is promoted by the formation of a phosphoenzyme complex. The fact that with all mutants ouabain binding accounts for only a fraction of the value obtained with the wild-type enzyme is

probably due to a decreased formation of the phosphoenzyme complex as a result of the mutations. The phosphointermediate of the sodium pump, however, is formed at Asp369 of the α1 subunit (*35*, *55*−*57*). Thus, the question arises, how do mutations of Glu472 or Lys480 influence the formation of the carboxylic phosphoric acid anhydride at Asp369?

One possibility is to assume that replacing Lys480 or Glu472 results in an enzyme that in the presence of phosphate and Mg^{2+} is incapable of assuming the required conformational changes that lead to the formation of the phosphointermediate (E2-P), which subsequently binds and forms a stable complex with ouabain [E2-P·ouabain]. Taking into consideration, however, that lysines and glutamic acids of PPases equivalent to Lys480 and Glu472 of the α1 subunit have been shown to interact with sulfate or phosphate moieties (Figure 6), a different possibility might explain why the binding of ouabain to the mutants is reduced also in the presence of ATP, Na^+, and Mg^{2+}. On the basis of experiments showing that Lys480 is labeled by both AP_2PL and PLP, Hinz and Kirley suggested that the specificity of these two reagents is not determined by the presence of the adenosine moiety but rather by the presence of the phosphates of the ATP molecule and that the initial interaction of ATP with the ATP binding site might be initiated by the recognition of phosphates (*13*). Taking their idea a step further, we suggest that the initial step might be the recognition of the α/*β*-pyrophosphate of ATP at Glu472 and Lys480, followed by the binding of the adenosine moiety to the enzyme. The formation of the phosphorylation pocket, containing Asp369, is possibly accelerated as a result of conformational changes induced by these initial interactions. Although no evidence for this assumption can be provided at this time, such a mechanism involving an initial interaction of the α/*β*-pyrophosphate of ATP with Glu472 and Lys480 and the subsequent binding of the adenosine moiety would explain why ADP, which also binds to the sodium pump and to other ion-transporting ATPases, does not become hydrolyzed by the enzyme, while ATP formation from ADP and P_i is still possible under certain experimental conditions (*58*, *59*).

At present, it is not possible to propose a more detailed model for the interactions of Asp369, Glu472, Lys480, and Mg^{2+} with ATP phosphates and for the reaction step that leads to ATP hydrolysis. Some additional information, however, might be gleaned from a recent publication reporting structural similarities between ion-transporting ATPases and haloacid dehalogenases (*17*). These structural similarities are proposed to be reflected in similarities of the reaction mechanisms of the two classes of enzymes. Nevertheless, in contrast to the situation with haloacid dehalogenases or with PPases, there are no data from crystalline structure analysis available for any ion-transporting ATPase that can allow the direct assessment of single amino acids in the tertiary structure of the proteins. Since Glu48 and Lys56 of the SCE1-PPase are not the only charged amino acids contributing to the interactions of this enzyme with pyrophosphate (*31*, *44*), one might also expect that besides Glu472 and Lys480 additional amino acids might be contributing to the interactions of the sodium pump with ATP α- and *β*-phosphate groups. The fact that mutations of Glu472 or Lys480 do not result in a complete loss of Na^+,K^+-ATPase activity (Figure 3) or ATP-promoted ouabain binding (Figure 4) might be taken as an indication for the existence of such additional amino acids. Their identification will possibly help to understand not only how ATP binding and hydrolysis occur but also how these events are coupled to the vectorial ion transport catalyzed by ion-transporting ATPases.

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