Lys^{691} and Asp^{714} of the Na^+/K^+-ATPase α Subunit Are Essential for Phosphorylation, Dephosphorylation, and Enzyme Turnover†

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ABSTRACT: P-type ATPases such as the sodium pump appear to be members of a superfamily of hydrolases structurally typified by the L-2-haloacid dehalogenases. In the dehalogenase L-DEX-ps, Lys151 serves to stabilize the excess negative charge in the substrate/reaction intermediates and Asp180 coordinates a water molecule that is directly involved in ester intermediate hydrolysis. To investigate the importance of the corresponding Lys691 and Asp714 of the sodium pump α subunit, sodium pump mutants were expressed in yeast and analyzed for their properties. Lys691Ala, Lys691Asp, Asp714Ala, and Asp714Arg mutants were inactive, not only with respect to ATPase activity but also to interaction with the highly sodium pump-specific inhibitors ouabain or palytoxin (PTX). In contrast, conservative mutants Lys691Arg and Asp714Glu retained some of the partial activities of the wild-type enzyme, although they completely failed to display any ATPase activity. Yeast cells expressing Lys691Arg and Asp714Glu mutants are sensitive to the sodium pump-specific inhibitor PTX and lose intracellular K^+. Their sensitivity to PTX, with EC_{50} values of 118 ± 24 and 76.5 ± 3.6 nM, respectively, was clearly reduced by almost 7- or 4-fold below that of the native sodium pump (17.8 ± 2.7 nM). Ouabain was recognized under these conditions with low affinity by the mutants and inhibited the PTX-induced K^+ efflux from the yeast cells. The EC_{50} for the ouabain effect was 183 ± 20 μM for Lys691Arg and 2.3 ± 0.08 mM for the Asp714Glu mutant. The corresponding value obtained with cells expressing the native sodium pump was 69 ± 18 μM. In the presence of P_i and Mg^{2+}, none of the mutant sodium pumps were able to bind ouabain. When Mg^{2+} was omitted, however, both Lys691Asp and Asp714Glu mutants displayed ouabain binding that was reduced by Mg^{2+} with an EC_{50} of 0.76 ± 0.11 and 2.3 ± 0.2 mM, respectively. In the absence of Mg^{2+}, ouabain binding was also reduced by K^+. The EC_{50} values were 1.33 ± 0.23 mM for the wild-type enzyme, 0.93 ± 0.2 mM for the Lys691Arg mutant, and 1.02 ± 0.24 mM for the Asp714Glu enzyme. None of the neutral or nonconservative mutants displayed any ouabain-sensitive ATPase activity. Ouabain-sensitive phosphatase activity, however, was present in membranes containing either the wild-type (1105 ± 100 μmol of *p*-nitrophenol phosphate hydrolyzed min^{-1} mg of $protein^{-1}$) or the Asp714Glu mutant (575 ± 75 μmol min^{-1} mg^{-1}) sodium pump. Some phosphatase activity was also associated with the Lys691Arg mutant (195 ± 63 μmol min^{-1} mg^{-1}). The results are consistent with Lys691 and Asp714 being essential for the phosphorylation/dephosphorylation process that allows the sodium pump to accomplish the catalytic cycle.

The sodium pump (Na^+/K^+-ATPase, EC 3.6.3.8) is a member of the P_2-type ATPases (*1*). As an integral membrane protein of animal cells, it hydrolyzes ATP to transport Na^+ ions out of the cell and K^+ ions into the cell at a stoichiometry of 1:2:3 ($ATP/K^+/Na^+$) and produces both a chemical and an electrical gradient across cell membranes (*2*−*4*). The ion translocation is coupled to conformational transitions of the enzyme, which, like all P-type ATPases, in an intermediate step forms a phosphoric ester between a highly conserved aspartic acid and the terminal γ-phosphate group of the hydrolyzed ATP[1] (*5*−*7*).

New work suggests that the P-type ATPases also belong to a large superfamily of hydrolases structurally typified by the L-2-haloacid dehalogenase (HAD) (*8*). The L-2-haloacid dehalogenase from *Pseudomonas sp*. (L-DEX-ps; EC 3. 8. 1. 2), a member of the superfamily of HAD hydrolases that includes phosphatases, epoxide hydrolases, and L-2-haloacid dehalogenases (*9*), catalyzes the hydrolytic dehalogenation of L-2-haloalkanoates to produce the corresponding D-2-hydroxyalkanoates in a mechanism that is very reminiscent of the ATP hydrolysis catalyzed by ATPases (*10*, *11*).

The reaction of L-DEX-ps begins with a nucleophilic attack of Asp10 on the α-carbon atom of L-2-haloalkanoates, causing the release of a halide ion and the formation of an ester intermediate. This is subsequently hydrolyzed by an

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; HAD; haloacid dehalogenase; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)-aminomethane; PTX, palytoxin.

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activated water molecule, restoring the side-chain carboxyl group of the Asp10 (*8*, *12*, *13*).

In the reaction mechanism of the sodium pump, Asp369[2] assumes the same function as does the Asp10 of L-DEX-ps. Thus, its carboxyl oxygen can initiate a nucleophilic attack on the electron-rich γ-phosphate bond of ATP, resulting in phosphoester formation (phosphorylation). The phosphoenzyme is subsequently hydrolyzed (dephosphorylation), and the free energy of hydrolysis is utilized for ion transport.

Protein structure analysis at 2.5-Å resolution shows that the Asp10 of L-DEX-ps resides within a protein structure characterized by six parallel β-sheets (β-strand order 3-2-1-4-5-6) flanked by five α-helices (*14*). This so-called Rossmann fold is also found in the crystal structure of the sarcoplasmic reticulum Ca^{2+}-ATPase, and based on sequence homology analysis it most likely also exists in the sodium pump α subunit and in all other P_2-type ATPases as well (*15*).

In the crystal structure of L-DEX-ps, there is a water molecule located close to Asp10, Ser175, Asn177, and Asp180. The latter three residues supposedly enhance the nucleophilicity of the water molecule for hydrolysis of the ester intermediate. Lys151, on the other hand, is involved in stabilizing the excess negative charge in the substrate or reaction intermediates (*8*, *10*, *16*). Site-directed mutagenesis studies suggest that Lys151 and Asp180 are essential for the enzymatic activity of L-DEX-ps (*10*, *12*).

Protein sequence comparison reveals that amino acid residues Lys691 and Asp714, located in the phosphorylation domain of the sodium pump α1 subunit, correspond to the Lys151 and Asp180 in L-DEX-ps, which are highly conserved in the HAD superfamily and P-type ATPases. Asp714 is localized within a conserved sequence ^{709}GDGVND^{714} that contains the Mg^{2+} coordinating site Asp710 (*17*, *18*).

We therefore selected both Lys691 and Asp714 as targets, exchanging them via site-directed mutagenesis for other residues. All mutant α subunits were coexpressed with β subunits in *Saccharomyces cerevisiae*, which does not contain endogenous Na^+/K^+-ATPase (*19*−*21*), and were investigated with respect to their properties.

EXPERIMENTAL PROCEDURES

Cell Strains and Plasmids. Escherichia coli (*E. coli*) strain DH5αF′ (Gibco, Eggenstein/Germany) was used as a replication system for the plasmid pBluescript KS II + (pKS$^+$; Stratagene, La Jolla/CA) with native or mutated cDNA of the Na^+/K^+-ATPase α1 subunit.

The yeast expression vector pCGY1406αβ containing cDNA encoding both the sheep kidney α1 subunit and the dog kidney β1 subunit of Na^+/K^+-ATPase (*19*) was used to express the proteins in the *Saccharomyces cerevisiae* yeast cell line 30-4 (*22*).

Introducing Mutations. For the introduction of mutations, a new cloning vector was constructed by inserting an *Apa*I−*Bgl*II linker made by annealing the oligonucleotides CCTTAAGGGTGACCA and GATCTGGTCACCCTTAAGGGGCC (Roth, Karlsruhe/Germany) to generate a *BstE*II site between the original *Apa*I and *Bgl*II sites of the pKS$^+$. This new plasmid, referred to as pKS$^+$-*BstE*II, was then digested with *Bgl*II and *BstE*II to obtain a 2937-bp *BstE*II−*Bgl*II fragment as a vector that was purified from a 1% agarose gel after electrophoresis.

Table 1: Primers for Introducing Lys691 and Asp714 Mutations in the α1 Subunit by Inverse-PCR

mutation	oligonucleotide sequence
native Lys691	5′TCTCCGCAGCAG**GAA**CTCATCATTGTG3′
Lys691Ala	5′TCTCCGCAGCAG**GCG**CTCATCATTGTG3′
Lys691Arg	5′TCTCCGCAGCAG**AGG**CTCATCATTGTG3′
Lys691Asp	5′TCTCCGCAGCAG**GAT**CTCATCATTGTG3′
	5′GGTCCT*CGC*GAACACAATCTCCGTGTG3′ reverse primer with a new restriction site *Nru*I as the diagnostic signal

mutation	oligonucleotide sequence
native Asp714	5′GACGGTGTCAAT**GAC**TCCCCGGCTTTG3′
Asp714Ala	5′GACGGTGTCAAT**GCA**TCCCCGGCTTTG3′ with a new restriction site *Nsi*I
Asp714Arg	5′GACGGTGTCAAT**CGA**TCCCCGGCTTTG3′ with a new restriction site *Cla*I
Asp714Glu	5′GACGGTGTCAAT**GAA**TCCCCGGCTTTG3′ with an additional restriction site *Cfr*10I
	5′TCCAGTTACAGCCACAATGGCACCCTG3′ reverse primer

Thereafter, another *BstE*II−*Bgl*II fragment of 1010 bp from the sheep sodium pump α1 subunit cDNA was inserted into the same restriction site of the pKS$^+$−*BstE*II plasmid. This new construct of 3947 bp was denoted pKS$^+$-AS and served as the vector for site-directed mutagenesis by inverse PCR (*23*, *24*). The primers used for introducing the mutations are listed in Table 1.

The PCR products were then blunt-ended using T_4 DNA polymerase (*25*, *26*), phosphorylated by T_4 DNA polynucleotide kinase, separated by 1% agarose gel electrophoresis, and purified from the agarose gels by using the JETsorb kit (Genomed, Bad Oeynhausen, Germany). These PCR products carrying the mutations were then ligated into the pKS$^+$−*BstE*II that had been previously digested with *Bgl*II and *BstE*II and dephosphorylated by alkaline phosphatase.

The new constructs carrying the mutations were introduced into *E. coli* by electroporation (*27*, *28*). Isolated plasmids were sequenced entirely by an automated DNA sequencer (*29*).

Finally, *Bgl*II and *BstE*II fragments of the pKS$^+$-AS plasmids carrying the desired mutations were used to replace the corresponding part of the α1 subunit cDNA of the pCGY1406αβ yeast expression vector (*19*). The new constructs were used to transform (*30*) the yeast strain 30-4.

A single clone of yeast cells 30-4 expressing native or mutant Na^+/K^+-ATPase was incubated in 5 mL of YNBU (1.7 g/L yeast nitrogen base without amino acids, 5 g/L ammonium sulfate, 20 g/L glucose, 20 mg/L uracil) medium at 30 °C overnight. The culture was continuously shaken at 180 rpm on a orbital shaker. Then the cell culture was transferred to 200 mL of YNBU and incubated until the desired OD_{600} value of 1.5−2.0.

For large scale cultures, the 5-mL culture was transferred to 1.3 L of YNBU medium containing 13 mL of a 100-fold supplement mix (2 g/L each of adenine, L-histidine/HCl, L-arginine/HCl, and L-methionine; 3 g/L each of L-tyrosine, L-leucine, L-isoleucine, L-lysine/HCl; 5 g/L L-phenylalanine;

[2] Numbers correspond to the sheep Na^+/K^+-ATPase α1 subunit.

10 g/L each of L-glutamate acid and L-aspartate acid; 15 g/L L-valine; 20 g/l L-threonine; 40 g/L L-serine), and further incubated before use.

Palytoxin-Induced K^+ Efflux from Yeast Cells Expressing Native or Mutant Sodium Pumps. Single yeast clones expressing either wild-type or various mutant Na^+/K^+-ATPases were grown overnight at 30 °C in 5 mL of YNBU. The cell suspensions were transferred into 200 mL of YNBU medium and grown for an additional 24 h. Cells were then sedimented at 4 °C by centrifugation for 5 min at 3500*g* and washed twice using 50 mL of HBC (10 mM Hepes, 0.5 mM borate, 1 mM $CaCl_2$, pH 7.5) buffer. The cells were suspended in HBC buffer with 200 mM NaCl to reach a cell density of 5×10^6 cells/mL and were further incubated at 30 °C for 2 h with various concentrations of PTX dissolved in HBC buffer with 0.1% BSA. In some experiments, ouabain was included at various concentrations, while the PTX concentration was constant. The total reaction volume was in all cases 1 mL. Thereafter, cells were centrifuged for 2 min at 13000*g*, the supernatant was collected, and the concentration of K^+ ion was determined by flame photometry. The total K^+ concentration of the cells was measured after cell membranes were dissolved by incubation in 0.01% lithium dodecyl sulfate at 65 °C for 30 min.

Preparation of Yeast Microsomes and of SDS-Extracted Membranes. The preparation of microsomes from yeast cells and the preparation of yeast membranes enriched in sodium pump after SDS extraction and high-speed centrifugation through a sucrose gradient have been described in detail elsewhere (*19*).

SDS−Polyacrylamide Gel Electrophoresis (SDS−PAGE) and Immunodetection of Wild-Type and Mutant Sodium Pumps by Western Blotting. A total of 15 μg of SDS-extracted protein containing either native or mutant Na^+/K^+-ATPase was suspended in 10 μL of loading buffer was separated by SDS−PAGE following established protocols (*31*). Protein from untransformed yeast served as the negative control. Protein was then transferred onto nitrocellulose (NC) membranes following the instructions provided by the commercially available ECL Western blotting system PRN 2180 kit (Amersham Pharmacia Biotech, Freiburg, Germany). Following the same protocol, the α1 subunit or β1 subunit of the Na^+/K^+-ATPase were detected using specific antibodies (Alexis Corporation, Darmstadt, Germany) raised in mice, each used at a dilution of 1:2500. The secondary antibody was an HRP (horseradish peroxidase)-coupled anti-mouse IgG provided by the kit.

Na^+/K^+-ATPase Activity Assay. The overall activity of Na^+/K^+-ATPase was determined by a coupled spectrophotometric assay (*19*).

Phosphatase Activity of Wild-Type and Mutant Na^+/K^+-ATPase. A total of 30 μg of SDS-extracted protein containing either wild-type or mutant Na^+/K^+-ATPase was incubated in 1 mL of 10 mM Tris/HCl, pH 7.5 containing 3 mM $MgCl_2$ and 8 mM KCl. The reaction was started by adding *p*-nitrophenol phosphate at a final concentration of 3 mM. The effect of ATP on the phosphatase activity was investigated by adding the substance at 1 mM concentration to the above solution. In some experiments, Mg^{2+} was omitted. The Na^+/K^+-ATPase-specific phosphatase activity was determined by preincubating the protein with 100 μM ouabain. The formation of the *p*-nitrophenolate anion was measured using a spectrophotometer at 405 nm. The molar extinction coefficient is $\epsilon = 18\,500$ L mol^{-1} cm^{-1}.

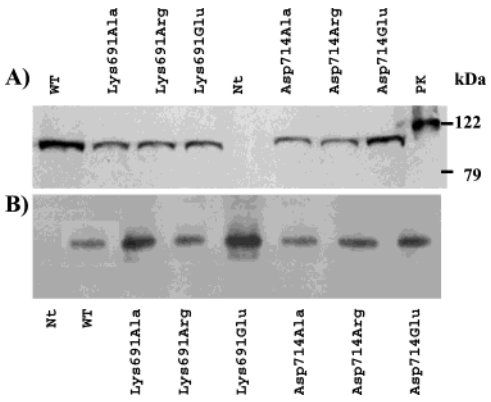

FIGURE 1: Expression of α1 and β1 subunits in yeast cells. A total of 15 μg of yeast cell membranes containing either wild-type (WT) or mutant Na^+/K^+-ATPase were separated by SDS−PAGE and then transferred to a nitrocellulose membrane (NC membrane). The blots were subsequently probed with monoclonal antibody against either the α1 or the β subunit of the Na^+/K^+-ATPase (see Experimental Procedures). (A) Immunodetection of wild-type and mutant α subunits. The Na^+/K^+-ATPase purified from pig kidney (PK) was used as the positive control. Yeast cell membranes from nontransformed calls (Nt) were used with the same procedure as the negative control. (B) Immunodetection of coexpressed β subunits.

Binding of [^{3}H]ouabain as a Function of [Mg^{2+}]. The effect of Mg^{2+} on [^{3}H]ouabain binding was investigated by incubating 250 μg of microsomal protein containing either wild-type or mutant Na^+/K^+-ATPase at 30 °C for 30 min with various concentrations of $MgCl_2$ in the presence of 10 mM Tris/HCl, pH 7.5, 50 nM [^{3}H]ouabain, and 5 mM Tris/PO_4, pH 7.5 in a final volume of 500 μL. Ionic strength was kept constant by adding choline chloride. Thereafter, the protein was pelleted by centrifugation at 13000*g* for 4 min in a benchtop centrifuge and washed twice with 500 μL of ice-cold H_2O. The protein was dissolved in 250 μL of 1 N NaOH at 75 °C for 15 min. After neutralization with 250 μL of 1 N HCl, 4.5 mL of scintillation fluid was added and the amount of bound [^{3}H]ouabain was determined by scintillation counting. The nonspecifically bound [^{3}H]ouabain was estimated in the presence of 1 mM nonradioactive ouabain.

Inhibition of [^{3}H]ouabain Binding by K^+. The conditions are the same as above with the only difference that various concentrations of K^+ was used instead of Mg^{2+}.

[^{3}H]Ouabain Binding to the Phosphoenzyme Formed from ATP. A total of 250 μg of microsomal protein containing either wild-type or mutant Na^+/K^+-ATPase was incubated at 30 °C for 30 min with 10 mM Tris/HCl, pH 7.5, 100 mM NaCl, and 100 μM Tris/ATP with or without 5 mM $MgCl_2$. The final volume of the sample was 500 μL. The amounts of bound [^{3}H]ouabain and the nonspecific binding were determined as described above.

RESULTS

Expression of Native or Mutant Na^+/K^+-ATPase in Yeast. SDS-extracted proteins from yeast membranes were probed in a Western blot with a monoclonal antibody raised either against the α1 or β1 subunit of Na^+/K^+-ATPase. As shown in Figure 1A, the wild-type and each of the mutated α1 subunits of Na^+/K^+-ATPase were detected at approximately

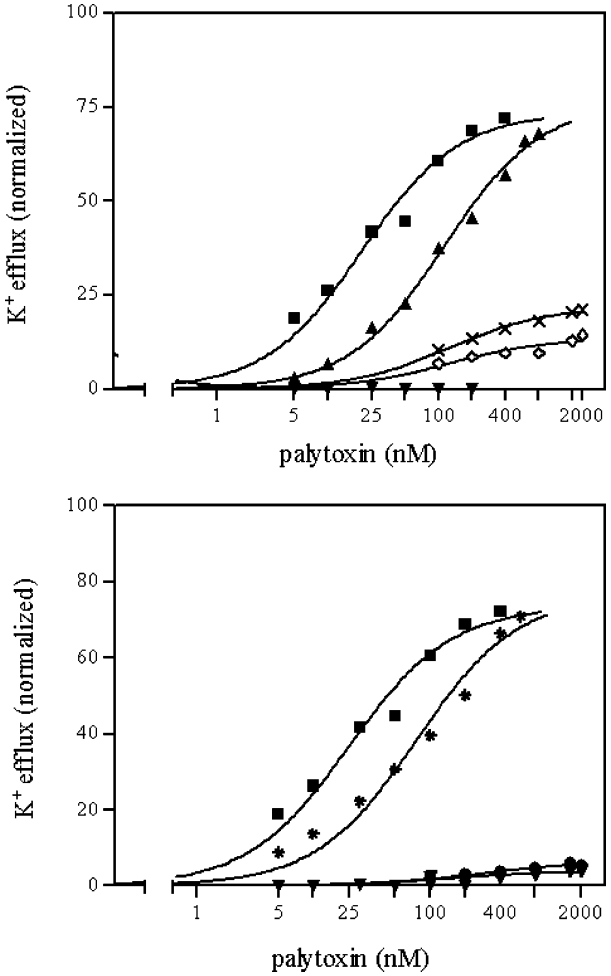

FIGURE 2: PTX-induced K^+ efflux from yeast cells expressing wild-type or mutant Na^+/K^+-ATPase. (A) Yeast cells (5×10^6 cells/mL) expressing either wild-type Na^+/K^+-ATPase (■) or its mutants Lys691Arg (▲), Lys691Ala (X), and Lys691Asp (◇) were incubated with various concentrations of PTX at 30 °C for 2 h as described in Experimental Procedures. Nontransformed yeast cells (▼) served as a control. (B) An analogous experiment was carried out with yeast expressing either wild-type Na^+/K^+-ATPase (■), or its mutants Asp714Glu (∗), Asp714Ala (▼), and Asp714Arg (●).

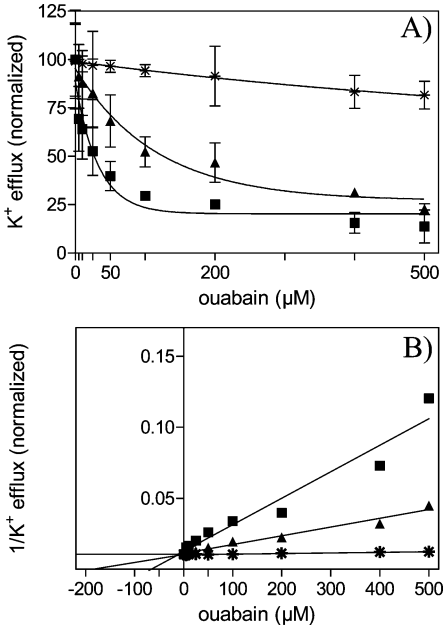

FIGURE 3: Ouabain inhibition of the PTX-induced K^+ efflux from yeast cells expressing either wild-type or Lys691Arg and Asp714Glu mutants of Na^+/K^+-ATPase. Yeast cells containing wild-type Na^+/K^+-ATPase (■) were incubated with 50 nM PTX and various concentrations of ouabain at 30 °C for 2 h, as described in Experi-mental Procedures. Cells expressing Lys691Arg (▲) or Asp714Glu (∗) mutants were incubated with 200 nM PTX under otherwise identical conditions. (A) Determination of K^+ concentration in the supernatant. (B) Determination of EC_{50} values for inhibition of the PTX-induced K^+ efflux by ouabain. The EC_{50} value is the negative of the intercept with the abscissa. For all experiments, $n = 4$; error bars represent ± SEM.

110 kDa as was the α1 subunit of the positive control of pig kidney Na^+/K^+-ATPase at 122 kDa. All mutants and the wild-type α1 subunit were present in similar quantities. The antibody did not recognize any protein of ∼110 kDa in SDS-extracted membranes from nontransformed yeast cells. Figure 1B shows the β1 subunit of the wild-type and all of the mutants of Na^+/K^+-ATPase expressed in yeast cells with a molecular mass of ∼39 kDa in SDS−polyacrylamide gels. Nontransformed yeast cells were used as the negative control.

PTX-Induced K^+ Efflux from Yeast Cells Expressing Either Wild-Type or Mutant Na^+/K^+-ATPase. PTX acts on Na^+/K^+-ATPase and H^+/K^+-ATPase, causing formation of a channel within the enzyme (*20*, *32*, *33*) that allows ions to flow down their electrochemical gradient. As expected, yeast cells expressing wild-type Na^+/K^+-ATPase displayed a significant PTX-induced K^+ efflux with an EC_{50} value of 17.8 ± 2.7 nM, whereas the nontransformed yeast cells did not show any PTX-induced K^+ efflux (Figure 2). The interactions of PTX with each of the Lys691 or Asp714 mutants of Na^+/K^+-ATPases, however, were considerably affected: the EC_{50} values obtained with cells expressing the conservative mutants Lys691Arg or Asp714Glu were about 7-fold (117.6 ± 23.8 nM) or 4-fold (76.5 ± 3.6 nM) higher than that of the wild-type, respectively. Maximal K^+ efflux from these cells was 68.1 ± 17.5% and 70.9 ± 28.7% and therefore within the same range obtained with cells expressing the wild-type with a corresponding efflux maximum of 72.2 ± 8.4%.

On the other hand, the other two neutral and two nonconservative mutants, Lys691Ala and Lys691Asp (Figure 2A) or Asp714Ala and Asp714Arg (Figure 2B), were very insensitive to PTX. At 2 μM PTX, the K^+ efflux from cells expressing Lys691Ala and Lys691Asp were 21.1 and 14.4% of the total K^+, respectively. Under similar conditions, the K^+ efflux from cells expressing either Asp714Ala or Asp714Arg mutants accounted for 3.8 and 5.4% of the total K^+ content of the cell, respectively (Figure 2B).

Ouabain Inhibition of the PTX-Induced K^+ Efflux from Yeast Cells Expressing Either Mutant or Wild-Type Na^+/K^+-ATPase. The PTX-induced K^+ efflux from yeast cells expressing the wild-type Na^+/K^+-ATPase was inhibited by ouabain to about 90% (here the maximum value is defined as the K^+ efflux obtained in the absence of ouabain and the minimum K^+ efflux the one obtained without PTX or ouabain; Figure 3A). The EC_{50} in the presence of 50 nM PTX was 69 ± 18 μM. The corresponding values obtained from cells expressing the Lys691Arg or Asp714Glu mutant of Na^+/K^+-ATPase were 183 ± 20 μM at 200 nM PTX and 2.35 ± 0.08 mM at 200 nM PTX, respectively (Figure 3B).

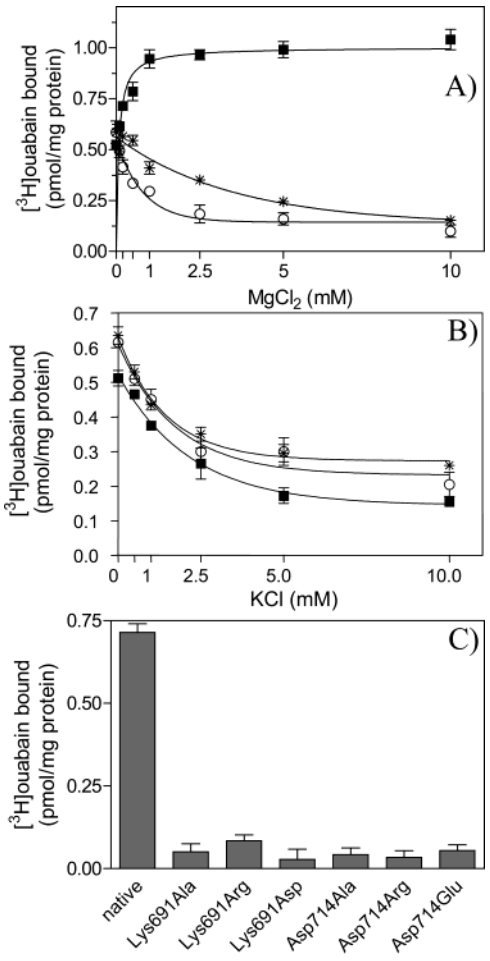

FIGURE 4: Binding of [^{3}H]ouabain under various conditions. (A) As a function of [Mg^{2+}]. Membrane protein (250 μg) from yeast expressing either wild-type (■), Lys691Arg (○), or Asp714Glu(∗) was incubated at 30 °C for 30 min with various concentration of Mg^{2+} in the presence of 5 mM phosphate and 50 nM [^{3}H]ouabain (see Experimental Procedures for details). (B) As a function of [K^+]. The conditions are the same as in panel A. K^+ reduces binding of ouabain to the wild-type and mutant enzymes with similar EC_{50} values; (■) wild-type, (○) Lys691Arg, (∗) Asp714Glu. (C) [^{3}H]ouabain binding in the presence of Na^+ and Mg^{2+}-ATP. Membrane protein (250 μg) was incubated with a reaction mixture containing 10 mM Tris/CL, pH 7.5, 100 mM NaCl, 100 μM ATP, and 5 mM $MgCl_2$ at 30 °C for 30 min. While ouabain binding to the mutants is highly reduced when compared to the binding obtained with the native enzyme, no ouabain binding was obtained with the Lys691Asp mutant under these conditions. Bound ^{3}[H]-ouabain was measured by scintillation counting, as described in Experimental Procedures. For all experiments $n = 6$; error bars represent ± SEM.

For the other four mutations, Lys691Ala, Lys691Asp, Asp714Ala, and Asp714Arg, this experiment was omitted because of their insensitivity toward PTX.

Measurement of [^{3}H]ouabain Binding to Yeast Membranes Containing Native or Mutant Na^+/K^+-ATPase. First attempts to investigate the binding of [^{3}H]ouabain to the mutants failed. Since the mutations were introduced within an area of protein that had previously been demonstrated to be involved in Mg^{2+} binding (*17*), the failure of ouabain binding to the mutants could have been due to some disturbance in Mg^{2+} recognition caused by the mutations. Thus, a Mg^{2+} titration experiment was carried out by keeping [^{3}H]ouabain constant at 50 nM. As expected (*34*), Mg^{2+} stimulated [^{3}H]-ouabain binding to the wild-type Na^+/K^+-ATPase with an EC_{50} of 0.30 ± 0.07 mM (Figure 4A). Increasing [Mg^{2+}], however, caused an inhibition of the [^{3}H]ouabain binding to the mutants (Figure 4A). An EC_{50} value of 0.76 ± 0.11 mM Mg^{2+} was calculated for the reduction of [^{3}H]ouabain binding to the Lys691Arg mutant and a corresponding value of 2.3 ± 0.2 mM Mg^{2+} was obtained with the Asp714Glu mutant.

Inhibition of [^{3}H]ouabain Binding by K^+. Ouabain binding is at least partially competitive with K^+ (*35*, *36*). Thus, when Mg^{2+} was omitted, K^+ reduced the binding of [^{3}H]ouabain to the Lys691Arg mutant with an EC_{50} of 0.93 ± 0.20 mM (Figure 4B). Binding of [^{3}H]ouabain to the Asp714Glu mutant was also reduced by K^+ with an EC_{50} of 1.02 ± 0.24 mM. The same effect was observed with membranes containing the wild-type sodium pump (EC_{50} = 1.33 ± 0.23 mM; Figure 4B).

[^{3}H]Ouabain Binding in the Presence of Na^+ and Mg^{2+}-ATP. In the presence of Na^+ and Mg^{2+}-ATP, the Na^+/K^+-ATPase hydrolyzes ATP and becomes phosphorylated by the γ-phosphate group of ATP at Asp369 of the α subunit (*5*, *21*). This phosphorylated enzyme can then be converted to E_2-P, which is capable of binding ouabain to form a stable [E_2*P·ouabain] complex. This can be measured when [^{3}H]-ouabain is used in the reaction.

As expected, yeast membranes containing wild-type Na^+/K^+-ATPase bind [^{3}H]ouabain under these conditions with a B_{max} of 0.72 ± 0.03 pmol/mg of protein. The maximal binding of [^{3}H]ouabain to each mutant of Na^+/K^+-ATPase was strongly reduced (Figure 4C).

Na^+/K^+-ATPase Activity Assay. The specific (ouabain-sensitive) ATPase activity of the wild-type Na^+/K^+-ATPase was 27.5 ± 9.1 mU/mg of microsomal protein, thus representing 32.2 ± 8.5% of the total ATPase activity of the preparation. With the mutants, Na^+/K^+-ATPase activity was undetectable under these conditions.

Phosphatase Activity of the Wild-Type and Mutant Enzymes. The K^+-stimulated phosphatase activity is a property of the Na^+/K^+-ATPase. The enzyme is also capable of hydrolyzing *p*-nitrophenol phosphate besides various phosphate esters.

Table 2 summarizes the results obtained with wild-type and mutant Na^+/K^+-ATPase. Preparations from yeast cells expressing wild-type Na^+/K^+-ATPase specifically hydrolyzed significant amounts of *p*-nitrophenol phosphate (1105 ± 100 μmol min^{-1} mg^{-1}). These were determined as the difference in *p*-nitrophenol phosphate hydrolysis in the absence or presence of 100 μM ouabain. Also, a significant phosphatase activity was determined with preparations containing the Asp714Glu mutant (575 ± 75 μmol min^{-1} mg^{-1}). With the Lys691Arg mutants, the specific phosphatase reaction was strongly diminished (195 ± 63 μmol min^{-1} mg^{-1}), but was nevertheless clearly measurable. Both of these mutants, as well as the wild-type enzyme, absolutely required Mg^{2+} for the phosphatase activity. Addition of ATP in the presence of Mg^{2+} completely inhibited the phosphatase activity (Table 2). The nonconservative mutants Lys691Asp and Asp714Arg did not display any ouabain-sensitive phosphatase activity. The two neutral mutants seemed to display some specific phosphatase activity, but this was too low to be investigated any further.

Table 2: Phosphatase Activity of the Wild-Type and Mutant Na^+/K^+-ATPase[a]

Na^+/K^+-ATPase	*p*-NPP hydrolyzed (μmol min^{-1} mg^{-1})	*p*-NPP hydrolyzed in the presence of 1 mM ATP (μmol min^{-1} mg^{-1})	*p*-NPP hydrolyzed in the absence of Mg^{2+} (μmol min^{-1} mg^{-1})
wild-type enzyme	1105 ± 100	none	none
Lys691Arg	195 ± 63	none	none
Lys691Ala	120 ± 88	ND	ND
Lys691Asp	none	ND	ND
Asp714Glu	575 ± 75	none	none
Asp714Ala	175 ± 100	ND	ND
Asp714Arg	none	ND	ND

[a] ND: not determined.

ENZYME	Motif I		Motif II				Motif III	
L-2-HALOACID DEHALOGENASE (*PSEUDOMONAS SP.*; EC 3.8.1.2)	10		118		151		180	
	9 FDLYGTL	(101)	LSNGS	(27)	VYKPD	(20)	VSSNAWDA	181
L-2-HALOACID DEHALOGENASE (*XANTHOBACTER AUTOTROPHICUS*; EC 3.8.1.2.)	9		115		147		176	
	7 FDAYGTL	(99)	LSNGA	(27)	VFKPH	(20)	VSSNGFDV	177
PHOSPHOSERINE PHOSPHATASE (*METHANOCOCCUS JANNASCHII*; EC 3.1.3.3.)	11		99		150		171	
	10 FDFDSTL	(81)	VSGGF	(45)	LEKIA	(12)	VGDGANDI	172
CALCIUM-TRANSPORTING ATPASE SARCOPLASMIC RETICULUM (*ORYCTOLAGUS CUNICULUS*; EC 3.6.3.9)	351		625		684		707	
	350 SDKTGTL	(267)	ITGDN	(53)	SHKSK	(14)	TGDGVNDA	708
SODIUM/POTASSIUM-TRANSPORTING ATPase ALPHA-1 CHAIN (*OVIS ARIES*; EC 3.6.3.8)	369		610		691		714	
	368 SDKTGTL	(234)	VTGDH	(75)	QQKLI	(14)	TGDGVNDA	715

FIGURE 5: Sequences surrounding the three motifs of the haloacid dehalogenase superfamily in sheep Na^+/K^+-ATPase. The absolutely conserved Asp residue in motif I and III, the highly conserved Lys residue in motif III, and the highly conserved residue Ser/Thr in motif II are underlined. The alignment is based on those of Koonin (*44*) and Aravind et al. (*8*), which were obtained with iterative approaches using haloacid dehalogenase as the starting sequence. The numbers in the parentheses correspond to the number of amino acids that are inserted between motifs.

DISCUSSION

Using iterated sequence comparison and position-specific iterated BLAST (PSI-BLAST) starting from haloacid dehalogenases, Aravind et al. have shown that these enzymes share three statistically significant motifs that are also present in P_2-type ATPases (*8*). In the first of these motifs, Asp10 of L-DEX-ps is homologous to Asp369 in the sheep Na^+/K^+-ATPase α1 subunit (Figure 5). The second motif contains a strictly conserved hydroxyl residue provided by either serine or threonine. The third motif contains Lys151 and Asp180 of L-DEX-ps that correspond to Lys691 and Asp 714 of the Na^+/K^+-ATPase α1 subunit, respectively. These three motifs comprise a typical haloacid dehalogenase Rossmann fold.

In the dehalogenase L-DEX-ps, the catalytic reaction begins with a nucleophilic attack of Asp10 on the α-carbon of the substrate L-2-haloalkanoate (Figure 6). In this initial step, Lys151 serves to stabilize the excess negative charge of the substrate. In the second step, the ester intermediate is hydrolyzed by an activated water attacking on the γ-carbon of the Asp10, and the Asp10 is regenerated while the product is formed. The water is proposed to be activated by the nucleophile Asp180. Both these amino acids are essential for the catalytic activity of L-DEX-ps (*10*), which provided the basis for our own investigations of the analogous amino acids Lys691 and Asp714 of the sodium pump.

Role of Lys691. On the basis of Aravind's proposal (*8*), Lys691, especially its positive charge, should be critical for the phosphorylation of the enzyme by ATP (Figure 6B). In this proposal, the positively charged ε-amino group of the lysine increases the electrophilicity of the γ-phosphate group of ATP, enabling it to be attacked by the carboxyl group of Asp369. This reaction leads to the formation of the phosphorylated intermediate of the sodium pump, denoted $E_1{\sim}P$. Thus, if the model is correct, the expectation was that neither the neutral nor the nonconservative mutations of Lys691 should lead to functional enzymes. The conservative mutant Lys691Arg, on the other hand, was expected to display some of the properties of the native enzyme.

Our results showed that Lys691Ala and Lys691Asp were rather inactive. Membrane preparations from cells expressing these mutants did not display any detectable ouabain-sensitive ATPase or phosphatase activity. Also in experiments with whole yeast cells, these enzymes more or less completely failed to interact with the highly specific sodium pump inhibitor PTX when it was applied at concentrations below 100 nM (Figure 2). Some K^+ efflux was observed at very high PTX concentrations, possibly indicating the presence of the mutants in the yeast plasma membrane.

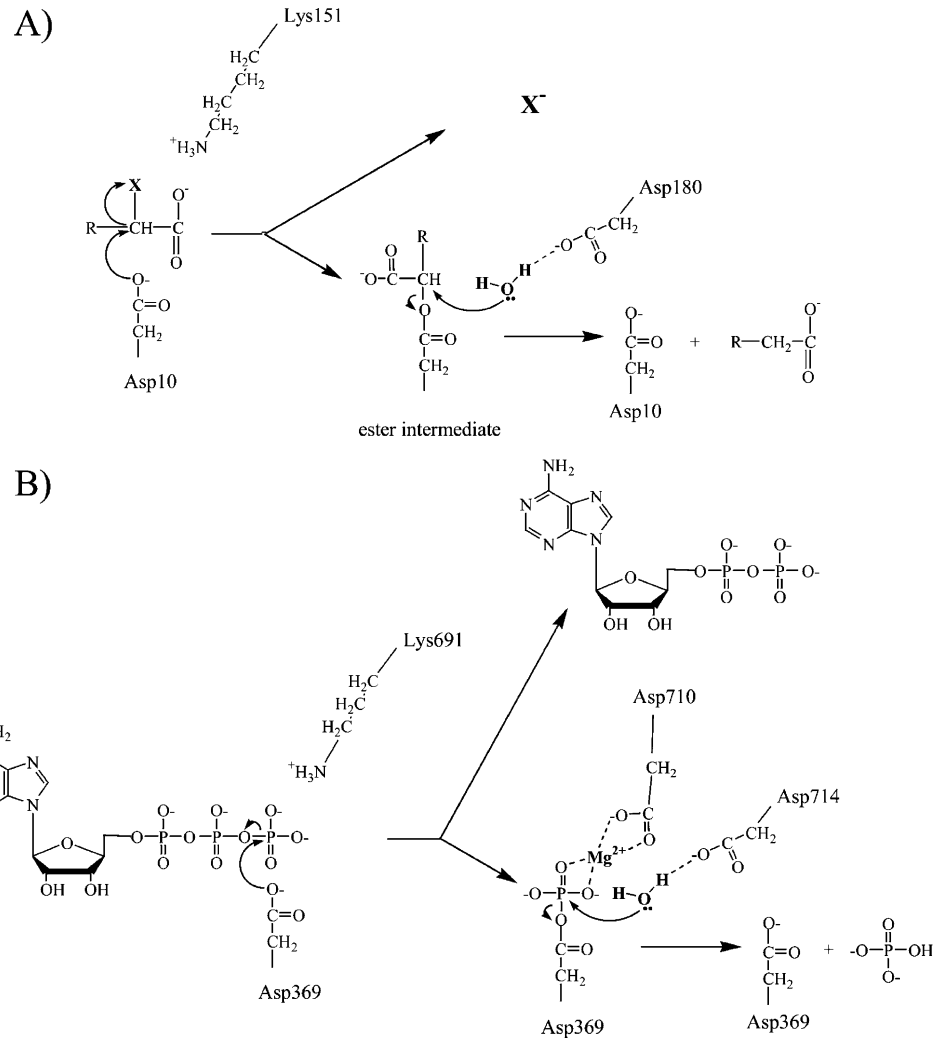

FIGURE 6: Proposed reaction mechanism of the haloacid dehalogenase L-DEX-ps and Na^+/K^+-ATPase. (A) Reaction of HAD L-DEX-ps (adapted from ref *8*). In the first step of the enzyme reaction, the relative nucleophile Asp10 attacks the α-carbon of the substrate to form an ester intermediate and a halide ion. In this step, Lys151 serves to stabilize the excess negative charge in the substrate/reaction intermediates. In the second step, the ester intermediate is hydrolyzed by an activated water attacking the γ-carbon of the Asp10, and the Asp10 is regenerated while the product is formed. The water is proposed to be activated by the nucleophile Asp180. (B) Proposed phosphorylations/dephosphorylation mechanism of Na^+/K^+-ATPase. The Asp369, Lys691, and Aps714 residues of the sodium pump correspond to Asp10, Lys151, and Asp180 in L-DEX-ps, respectively. As in the case of L-DEX-ps, the reaction begins with a nucleophilic attack of the aspartate (Asp369) carboxyl oxygen on the electron-rich γ-phosphate bond of ATP, resulting in phosphoester formation (phosphorylation). The phosphoenzyme is subsequently hydrolyzed (dephosphorylation) and the free energy of hydrolysis is utilized for ion transport. The Lys691 residue stabilizes the excess negative charge in the substrate/reaction intermediates and Asp 714 coordinates a water molecule that is directly involved in ester intermediate hydrolysis. The Mg^{2+} cation that is coordinated by Asp 710 (*17*) probably contributes to increasing the electrophilicity of the phosphorus atom.

Like the two other mutants, the Lys691Arg enzyme also completely failed to display any ATPase activity, indicating that this mutant enzyme is not capable of undergoing all conformational transitions required for the accomplishment of the catalytic cycle. Nevertheless, in contrast to the Lys691Ala and Lys691Asp mutants, the conservative mutant Lys691Arg retained several of the properties of the wild-type enzyme. Thus, yeast cells expressing this mutant become sensitive to PTX and lose cytosolic K^+. Their sensitivity to PTX was clearly reduced by almost 7-fold below the sensitivity of cells expressing the native sodium pump. Under these conditions, the plasma membrane-embedded Lys691Arg mutant recognized ouabain which inhibited the PTX-induced K^+ efflux from the yeast cells, albeit with about 3-fold lower sensitivity compared to the wild-type.

PTX is known to interact with the E_1 conformational state of the enzyme and its phosphorylated derivatives (*37*−*39*). Thus, in yeast cells where Na^+, Mg^{2+}, ATP, or ADP are present in the cytosol, PTX binding to the sodium pump is favored over ouabain binding, and therefore high ouabain concentrations are needed to inhibit the PTX-induced K^+ efflux. With the Lys691Arg mutant, the E_1 conformational state is probably stabilized by the inability of the enzyme to undergo the whole pump cycle, leading to a more pronounced insensitivity toward ouabain (Figure 3).

In the presence of 5 mM Mg^{2+}, membrane preparations containing the Lys691Arg mutant bind little [^{3}H]ouabain when used at 50 nM. When Mg^{2+} is omitted, the same membranes bind ouabain to almost the same extent as membrane preparations from yeast cells expressing the wild-type enzyme. Titration with Mg^{2+} revealed a reduction in ouabain binding. With the wild-type enzyme, Mg^{2+} has an opposite effect, thus promoting ouabain binding under these conditions (Figure 4).

The apparent discrepancy between the two opposing effects of Mg^{2+} can be explained by the fact that Mg^{2+} is known to stabilize the E_1 conformational state of the enzyme, as verified by trypsin-digestion patterns of the enzyme α subunit (*40*). This conformation does not display a high affinity for ouabain. On the other hand, in the membrane preparations where Mg^{2+} is omitted from the reaction mixture, an $E_1 \leftrightarrow E_2$ transition with the equilibrium toward the E_2 form (*41*) allows ouabain binding even at nanomolar ouabain concentrations. This is also supported by the fact that in the absence of Mg^{2+}, ouabain binding to the mutant is almost as K^+-sensitive as the wild-type enzyme.

In the E_1 conformational state, the sodium pump binds *p*-nitrophenol phosphate, which in the presence of K^+ is then hydrolyzed by the enzyme (*40*). This property is retained to some extent by the Lys691Arg mutant (Table 2). As with the wild-type enzyme, this partial activity is also fully dependent on Mg^{2+} and is completely inhibited when ATP is added (Table 2).

In summary, our results specify for the first time that Lys691 is a critical amino acid for the activity of the Na^+/K^+-ATPase. The ability of the mutant enzymes to hydrolyze ATP or phosphoric acid esters is considerably affected. Thus, by taking into consideration Aravind's proposal (*8*), we have to conclude that the Lys691 of the sodium pump α1 subunit is a functional homologue to the Lys151 of the dehalogenase L-DEX-ps (Figures 6 and 7).

Role of Asp714. In the dehalogenase L-DEX-ps, a water molecule that is directly involved in catalysis is coordinated by Asp180 (Figure 6A). According to Aravind's proposal (*8*), the negatively charged carboxyl group of the corresponding Asp714 of the sodium pump α subunit should interact with a water hydrogen, thus resulting in increased nucleophilicity of the water oxygen. This enables the oxygen to attack the phosphorus atom of the phosphoric ester formed between Asp369 and the ATP terminal phosphate and to cleave (hydrolyze) the latter (Figure 6B).

The prediction based on this model is that the activity of the Asp714Ala or Asp714Arg mutants should be considerably affected, which was found to be the case. No sodium pump-associated activities were detected with these mutants, despite sufficient expression of the mutant proteins. This finding is in good agreement with earlier investigations showing completely inactive Asp714Ala and Asp714Arg mutants (*17*).

In contrast, however, the Asp714Glu mutant retains several of the enzymatic properties, although no overall ATPase reaction was detected with SDS-extracted membrane preparations from yeast cells expressing this mutant. Thus, cells expressing the Asp714Glu mutant are sensitive to PTX and release intracellular K^+ upon exposure to the toxin. This PTX-induced K^+ efflux, however, is almost completely insensitive to ouabain. The EC_{50} for ouabain that was estimated from the slow inactivation of the K^+ efflux was greater than 2.3 mM, indicating either a very weak interaction of the mutant with the cardiac glycoside or a very stable formation of a mutant/PTX complex. The relatively low EC_{50} for PTX and the incapacity of membrane preparations from cells expressing this mutant to bind ouabain in the presence of Mg^{2+} and inorganic phosphate indicate that the Asp714Glu mutant has a rather reduced affinity for ouabain. This is probably due to the presence of ATP, ADP, and Mg^{2+} in the cytosol that arrest the enzyme in the E_1 conformational state, which does not favor ouabain binding to the enzyme. This assumption is also supported by the fact that membrane preparations containing the Asp714Glu mutant fail to bind ouabain unless Mg^{2+} is omitted. Addition of Mg^{2+} causes a reduction in ouabain binding (Figure 2A) possibly by stabilizing the E_1 conformational state (*40*). Omitting Mg^{2+} allows an $E_1 \leftrightarrow E_2$ transition with the equilibrium toward the E_2 form (*41*). This conformational state binds ouabain and is sensitive to K^+, which in the absence of Mg^{2+} reduces ouabain binding (Figure 4B).

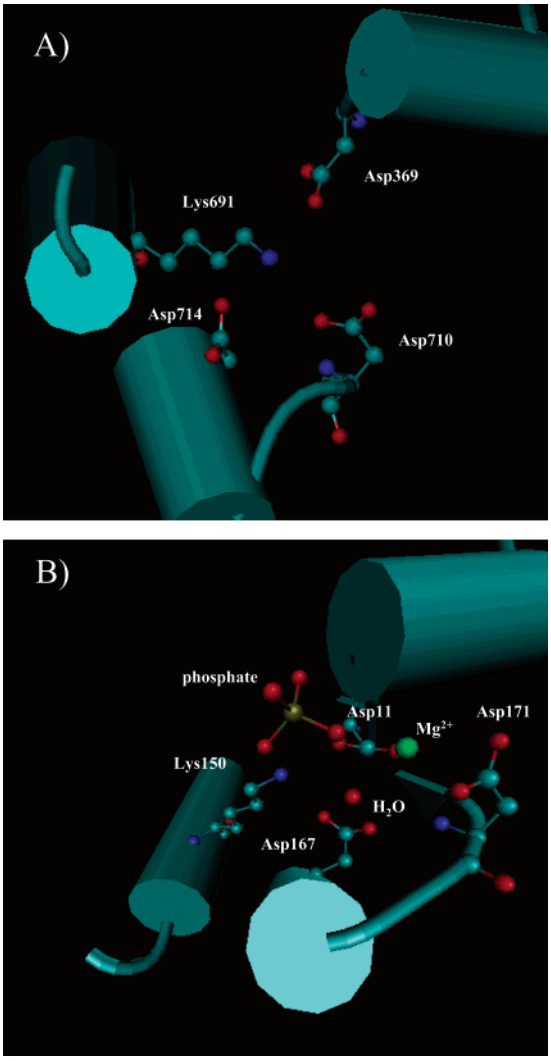

FIGURE 7: Catalytic sites of the sodium pump α subunit and of the phosphoserine phosphatase of *Methanococcus jannaschii*. (A) The catalytic site of the α subunit of the sodium pump is based on the model of Ettrich et al (*45*). (B) The catalytic site of the phosphoserine phosphatase is derived from enzyme crystal structure (*46*). Both figures were composed using the program VMD (Visual molecular Dynamics; http://www.ks.uiuc.edu/Research/vmd/).

From all mutants investigated, the Asp714Glu enzyme clearly displays the most pronounced phosphatase activity with *p*-nitrophenol phosphate as a substrate (Table 2). The binding of the *p*-nitrophenol phosphate in the presence of Mg^{2+} is thought to take place at the E_1 conformational state of the Na^+/K^+-ATPase, as determined by tryptic digestion patterns of the sodium pump protein (*40*). Addition of K^+ takes the enzyme into the E_2 conformational state, which might be the actual catalytic site for the hydrolysis of

p-nitrophenol phosphate (*40*). Apparently, the Asp714Glu mutant is capable of undergoing this $E_1 \leftrightarrow E_2$ transition that leads to the hydrolysis of *p*-nitrophenol phosphate. This phosphatase activity is, as expected, completely sensitive to ATP and requires Mg^{2+}. A similar result was obtained with membrane preparations containing the wild-type enzyme (Table 2). It is not clear yet, however, whether the hydrolysis of *p*-nitrophenol phosphate includes a phosphorylation/dephosphorylation step of the sodium pump. Nevertheless, the fact that all mutants investigated here except the Lys691Arg and Asp714Glu-mutants almost completely fail to hydrolyze *p*-nitrophenol phosphate indicates that the structure within which the *p*-nitrophenol phosphate hydrolysis takes place is the Rossmann fold formed around Asp369, the phosphorylation site of the sodium pump (Figure 7). This is also supported by the fact that the *p*-nitrophenol phosphatase activity of the Asp714Glu mutant is, although clearly defined and easy to measure, about half of that obtained with the wild-type enzyme. Taken together, the tryptic digestion experiments (*40*) and our results indicate that the E_1 or E_2 conformations of the pump are possibly only variations of the same catalytic site.

Role of Mg^{2+} and Water for the Catalytic Reaction. On the basis of our data, Aravind's proposal is most likely correct: Lys691 is essential for the phosphorylation of the sodium pump and Asp714 for the dephosphorylation process by coordinating a water molecule that cleaves the phosphoric ester bond.

While the involvement of Lys691 in the enzymatic reaction has not been previously investigated, Asp714 has been addressed by mutagenesis (*17*, *18*). In these latter studies, however, since the asparagine and alanine mutants of this amino acid produced enzymes without any ATPase activity, as in the current investigation, the studies were not carried any further. The focus was placed instead on Asp710, which was shown to be most likely involved in coordinating a Mg^{2+} that is essential for enzymatic activity. On the basis of this finding and on previously published theoretical studies attempting to model the active center of P_2-type ATPases onto the dehalogenase fold (*42*), the authors proposed that Asp714 might also be directly involved in Mg^{2+} binding and coordination (*17*, *18*).

Our current results, however, clearly show that the Asp714Glu mutant maintains several of the enzyme's partial reactions that either require or are affected by Mg^{2+}, thus allowing one to assume that Asp714 is not directly involved in Mg^{2+} coordination. This conclusion is indirectly also supported by the fact that Mg^{2+} is not important for the catalytic reaction of all haloacid dehalogenases and is absent in the crystal structure of the *Pseudomonas sp.* enzyme, which does not require this cation for its function. In contrast, the phosphoserine phosphatase of *Methanococcus jannaschii* requires Mg^{2+}, which coordinates with Asp167 in the crystal structure (Figures 5 and 7). This Asp167 is homologous to the Asp710 of the sodium pump α subunit (Figures 5 and 7), which indeed has been shown to coordinate Mg^{2+} (*17*, *18*). Asp714, however, is too far away from this Mg^{2+}, which is probably why it was suggested earlier that a water molecule might be placed between Asp714 and Mg^{2+} (*43*). Nevertheless, the fact that our mutants still recognize Mg^{2+} with only slightly altered apparent affinity compared to the wild-type, and the fact that mutation of Asp714 alters the hydrolytic activity of the enzyme without abolishing its ability to bind Mg^{2+}, as determined in the *p*-nitrophenol phosphatase experiment, suggests that it is not Mg^{2+} but rather H_2O that is coordinated by Asp714. A water molecule (HOH 222) is just 2.72 Å from the corresponding Asp171 of the phosphoserine phosphatase (corresponds to Asp714), which supports this conclusion (Figure 7). A similar situation is found with the L-DEX-ps, where a water molecule 2.73 Å from Asp180 is only found when substrate is bound to the enzyme (*16*). All of the above makes it very likely that Asp714 of the sodium pump α subunit is involved in the hydrolysis of the phosphoric ester bond formed at Asp369. The coordination of water results in increased nucleophilicity of the water oxygen, thus supporting the hydrolytic process. In addition, the Asp710-Mg^{2+}-phosphate coordination probably increases the electrophilicity of the phosphorus atom of the phosphate group (Figure 6B), thus supporting the same process that leads to the hydrolysis of the phosphoric acid and to the continuation of the catalytic cycle.

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