Site-Directed Mutagenesis of the Sodium Pump: Analysis of Mutations to Amino Acids in the Proposed Nucleotide Binding Site by Stable Oxygen Isotope Exchange†

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ABSTRACT: A model for the active site of P type ATPases has been tested by site-directed mutagenesis of amino acids in two conserved sequences of Mg^{2+}-dependent and Na^+- and K^+-stimulated ATPase. The mutants K501R, K501E, D586E, D586N, P587A, and P588A were expressed in yeast cells and compared with wild type. In addition to previously published assays of adenosine 5′-triphosphate binding and hydrolysis, measurements of ^{18}O exchange between P_i and water have been used to identify steps in the E_2 half of the reaction cycle affected by the mutations. The study supports the prediction that K501 in the KGAP sequence interacts with adenosine 5′-triphosphate. However, quantitative comparisons of the effect of mutation K501E on the activity with the effects of mutations to an enzyme of known structure that also catalyzes phosphoryl group transfer make a direct role for the positive charge on the side chain of K501 in catalysis by stabilizing the transition state unlikely. No evidence for the predicted interaction between D586 and the hydroxyl groups of ribose was found. However, the data do indicate that the spatial organization of the loop containing the DPPR sequence is critical for phosphorylation of the enzyme. A role for D586 in coordinating the Mg^{2+} that is required for activity is proposed.

Na,K-ATPase[1] is a member of a family of structurally related, integral-membrane proteins that function as primary transporters of ions across cell membranes against electrical and/or chemical potential gradients. Other physiologically important examples are the Ca-ATPases of sarcoplasmic reticulum and plasma membranes and the gastric H,K-ATPase. The active transporters or pumps in this class are called P type ion motive ATPases because ATP hydrolysis is catalyzed by formation of a covalent phosphoenzyme intermediate. A series of protein conformational changes have been postulated to explain coupling of the energy released by hydrolyzing ATP to ion movement. A conformational change that could account for the limited rate, ion specificity, and stoichiometry of ion transport by the sodium pump has been demonstrated experimentally (Lin & Faller, 1996), but many specific questions about the relationship between the structure of the molecule and how it functions remain unanswered.

One approach toward a better understanding of the mechanism of ATP-dependent ion transport by P type ATPases is identification of amino acids that are located in the active site where they might participate in catalysis or the conformational changes presumed to couple catalysis to transport. Serrano (1989) has identified six conserved sequences in the hydrophilic domains of P type ATPases by aligning primary structures and proposed that charged amino acids in the five conserved sequences located in the largest cytosolic loop interact with bound ATP. Taylor and Green (1989) predicted the secondary structure of a polypeptide containing three of those conserved sequences and compared allowed folds of the polypeptide with the known tertiary structures of ATP-binding proteins. The resulting three-dimensional model of the active site resembles the crystal structure of adenylate kinase (Müller & Schulz, 1992) and predicts interaction between lysine in the conserved sequence KGAP with the α and β phosphates of ATP and between aspartic acid in the conserved DPPR sequence with the ribose hydroxyl groups of the bound nucleotide.

MacLennan and co-workers (Maruyama & MacLennan, 1988; Maruyama *et al*., 1989; Clarke *et al*., 1990) have tested the theoretical model experimentally by changing amino

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[1] Abbreviations: Na,K-ATPase, Mg^{2+}-dependent and Na^+- and K^+-stimulated ATPase (EC 3.6.1.37); Ca-ATPase, Ca^{2+}- and Mg^{2+}-dependent ATPase (EC 3.6.1.38); H,K-ATPase, Mg^{2+}-dependent, H^+-transporting, and K^+-stimulated ATPase (EC 3.6.1.36); *x*-N_3-ATP, 2- or 8-azido-ATP; P_i, inorganic phosphate; PEP, phosphoenolpyruvate; FITC, fluorescein 5′-isothiocyanate; DTT, DL-dithiothreitol; TEA, triethylammonia; Tris, tris(hydroxymethyl)aminomethane; MES, 2-(*N*-morpholino)ethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; NADH, reduced nicotinamide adenine dinucleotide; GCMS, gas chromatograph−mass spectrometer; SIM, selected ion monitoring; WT, wild type; TN, turnover number; SD, standard deviation; SEM, standard error of the mean.

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acids in the predicted ATP binding site of sarcoplasmic reticulum Ca-ATPase and measuring the effects of the mutations on Ca^{2+} transport. Their results demonstrate that function is affected by amino acid substitutions in all five of the conserved sequences which were postulated to interact with ATP. The predicted interaction with the ribose hydroxyl groups could not be confirmed, but data consistent with interaction of the lysine in the KGAP sequence with the phosphate groups of ATP were obtained. In addition, progress was made toward defining the functional role of specific amino acids in ATP catalysis and Ca^{2+} transport by also studying the effects of the mutations on phosphorylation and dephosphorylation of the enzyme. Evidence that mutation of two amino acids in the DPPR sequence may eliminate transport either by preventing phosphorylation of the enzyme or by blocking the conformational change that follows phosphorylation of the enzyme by ATP was obtained.

One purpose of this study was to test the results of chemical modification experiments that seem to indicate that the lysine in the KGAP sequence of Na,K-ATPase interacts with the purine ring instead of the phosphate groups of bound ATP. We recently found that K480 is photochemically labeled by 8-N_3-ATP (Tran *et al*., 1994a), whereas G502 in the KGAP sequence is modified by 2-N_3-ATP (Tran *et al*., 1994b). Therefore, it is likely that both of these amino acids are located in the part of the ATP binding site that interacts with adenine because the photoactivatable azido groups are attached to the purine ring. G502 is adjacent to lysine 501, which is labeled by FITC (Farley *et al*., 1984; Kirley *et al*., 1984). K501 is probably located in the vicinity of adenine because ATP protects against fluorescein incorporation but may not be near the phosphate groups because the FITC-modified enzyme can still be phosphorylated by P_i (Karlish, 1980). On the other hand, both labeling of K480 by adenosine diphosphopyridoxal (Hinz & Kirley, 1990) and the effects of mutations of K480 on ATP and P_i binding (Wang & Farley, 1992) suggest a location near the phosphate groups of the bound nucleotide as well as near adenine. To explain the different reactivities of the azido group in the 2 and 8 positions at opposite ends of the purine ring, we have suggested an arrangement of amino acids around ATP in Na,K-ATPase that permits interaction of K480 with both adenine and phosphate group(s) but predicts interaction of K501 only with the purine ring (Tran *et al*., 1994a).

A second purpose of the study was to determine whether mutations in the DPPR sequence also affect phosphorylation or block a conformational change in Na,K-ATPase. Therefore, site-directed mutations of amino acids in the DPPR sequence of Na,K-ATPase, as well as mutations of K501, were studied. The effects of these mutations on catalytic activity and on the apparent affinity of the enzyme for ATP were determined. In addition, the interaction of the mutants with inorganic phosphate in a partial reaction catalyzed by the enzyme was examined by measuring the exchange of ^{18}O between P_i and water. The results of the study suggest that the lysine in the KGAP sequence of Na,K-ATPase does not participate directly in phosphoryl group transfer to the enzyme, but they confirm that the geometry of the region of the protein containing the DPPR sequence is critical for the phosphorylation reaction. We propose that D586 is involved in coordination of the Mg^{2+} required for activity. An abstract describing parts of this investigation has been published (Faller *et al*., 1995).

EXPERIMENTAL PROCEDURES

Materials

Plasmids and Mutations. Plasmid pCGY1406 $\alpha\beta$ (Horowitz *et al*., 1990) was used to express both the sheep kidney α subunit and the dog kidney β subunit of Na,K-ATPase in yeast cells lacking an endogenous sodium pump. For mutagenesis, a DNA fragment of about 2 kb from the α subunit cDNA was isolated from sheep kidney α subunit DNA by *Xba*I and *Eco*RI digestion and was inserted into the Bluescript plasmid pKS^+ (Stratagene) between the *Xba*I and *Eco*RI sites. Site-directed mutagenesis was performed using the Muta-Gene kit from Bio-Rad. Each mutation was confirmed by nucleotide sequencing. An 0.4 kb fragment containing the desired mutation was isolated from the Bluescript vector by *Nsi*I and *Bst*EII digestion and was ligated back into the *Nsi*I/*Bst*EII sites of the α subunit cDNA in another Bluescript pSK^+ plasmid. Finally, the mutations were introduced into the yeast expression plasmid pCGY1406$\alpha\beta$ by replacing the 1.6 kb *Xho*I/*Bst*EII fragment of the wild type α subunit cDNA with the corresponding fragment from the Bluescript plasmid containing the desired mutation.

Protein Expression and Membrane Preparation. The yeast expression plasmids were transformed into yeast strain 30-4 (MAT α trp1 ura3 Vn2 GAL^+), and cells were grown in selective minimal medium at 30 °C to an OD_{600} of 1−2. Cell lysis, membrane preparation, and detergent extraction of membranes were carried out as previously described (Wang & Farley, 1992; Horowitz *et al*., 1990; Eakle *et al*., 1994). Expression levels of wild type and mutant α subunits were estimated from Western blots of yeast membranes with monoclonal antibody #5 (D. Fambrough, Johns Hopkins) and alkaline phosphatase-conjugated goat anti-mouse second antibody. The proteins were stained with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium, and the expression level was quantified by densitometry. Serially diluted dog kidney Na,K-ATPase was used as the standard.

Labeled Inorganic Phosphate. $[^{18}O]P_i$ was synthesized (Stempel & Boyer, 1986) from ultrapure, dry PCl_5 (Alpha Products) and >99% ^{18}O-enriched water (Icon).

Methods

[^{3}H]Ouabain Binding to Phosphoenzyme Formed from Inorganic Phosphate. Membrane protein (250 μg) was incubated in 1 mL of 50 mM Tris-HCl (pH 7.4), 4 mM $MgCl_2$, 20 nM [^{3}H]ouabain (Du Pont-New England Nuclear, specific activity of 27−30 Ci/mmol), and 4 mM H_3PO_4. The reaction was carried out at 37 °C for 60 min. After cooling to 0−4 °C, the membranes were collected by centrifugation at 12000*g* for 20 min and were washed twice with 1 mL of ice-cold water. The amount of [^{3}H]ouabain associated with membrane pellets was quantified by scintillation counting. Nonspecific binding was determined by the addition of 1 mM nonradioactive ouabain to the assays. The affinity for ouabain was estimated from experiments under the same experimental conditions in which the concentration of P_i was fixed (4 mM), and the concentration of ouabain was increased from 1 nM to 1 μM.

[^{3}H]Ouabain Binding to Phosphoenzyme Formed from ATP. Membrane protein (250 μg) was incubated at 37 °C for 3 min in 1 mL of 50 mM imidazole hydrochloride (pH

7.5), 100 mM NaCl, 5 mM $MgCl_2$, 20 nM [^{3}H]ouabain, and ATP from 0 to 50 μM. After being incubated on ice for 5 min, the membranes were collected by centrifugation and washed with ice-cold water. The methods described in the preceeding paragraph were used to quantify the amount of [^{3}H]ouabain bound and correct for nonspecific binding. The apparent affinity for ATP was estimated as a K_m value by fitting the Michaelis−Menten equation to the data with the nonlinear, curve-fitting program (Levenberg−Marquardt algorithm) of Slidewrite Plus for Windows (Advanced Graphics Software, Inc., Carlsbad, CA).

Na,K-ATPase Enzymatic Assay. Na,K-ATPase activity was determined as previously described (Scheiner-Bobis & Farley, 1994) using SDS-extracted yeast membranes. Briefly, an aliquot of the membrane suspension isolated after SDS treatment containing 200 μg of protein was incubated for 30 min at 37 °C with 5 mM $MgCl_2$, 5 mM Tris-phosphate, and 5 mM sodium azide, with or without 1 μM ouabain, in 10 mM Tris-HCl at pH 7.4 (total volume of 100 μL). Ouabain-sensitive ATPase activity was determined spectrophotometrically by adding 80 μL of the mixture to 1 mL of a coupled-assay buffer preequilibrated to 37 °C containing 3.3 mM ATP, 3.3 mM $MgCl_2$, 100 mM NaCl, 20 mM KCl, 5 mM sodium azide, 1 mM EDTA, 1 mM DTT, 1.5 mM PEP, 0.35 mM NADH, 20 μg of lactate dehydrogenase, and 20 μg of pyruvate kinase in 20 mM HEPES−TES at pH 7.4 and recording changes in absorbance at 340 nm for 15 min.

Measurement of ^{18}O Exchange between P_i and Water. The method developed by Stempel and Boyer (1986) was used to measure the distribution of ^{18}O isotopomers [$P^{18}O_j{}^{16}O_{(4-j)}$, $0 \le j \le 4$] formed as a function of time when Na,K-ATPase catalyzes oxygen exchange between P_i and water. The reaction was initiated by adding enzyme to 0.5 mL of a solution containing 98−99% ^{18}O-enriched P_i. As a precaution, 0.1 mM DTT was added to the reaction mixture to prevent inhibition of the enzyme by oxidation, and 5 mM sodium azide was added to inhibit catalysis of ^{18}O exchange by F_oF_1 type ATPases that might be present in the yeast membranes. It was established in a separate experiment that ν_{ex} and P_c are unaffected by the 5 mM Na^+ when oxygen exchange is activated by 20 mM K^+ at the high ionic strength (0.2 M) used in our experiments. Other details of the assay are explained in the figure legends. The reaction was quenched by layering the assay mixture onto a 0.5 × 4 cm column packed with Dowex AGX4 (100−200 mesh) in the chloride form. After successive washes with 10 mL of water (18.2 MΩ), the column was acidified with 3.5 mL of 30 mM HCl, and the bound P_i was eluted with an additional 5 mL of 30 mM HCl. Volatile triethyl phosphate was formed by reacting the lyophilized phosphate with diazoethane, and the distribution of isotopomers in the product was analyzed with a Hewlett-Packard 5972 GCMS equipped with a polar capillary column and operated in the selected ion monitoring (SIM) mode. Masses 155−163 were measured.

Analysis of ^{18}O Exchange between P_i and Water. Equation 1 is the chemical equation for the reaction by which P type ion motive ATPases catalyze ^{18}O exchange between P_i and water (Boyer *et al*., 1977).

$$E + P_i \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}P \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E{-}P + H_2O \qquad (1)$$

The exchange rate (ν_{ex}) is the rate at which unlabeled oxygen enters P_i and is given by eq 2, in which k'_{-2} is a pseudo-first-order rate constant that includes the constant H_2O concentration.

$$\nu_{ex} = k'_{-2}[E{-}P] \qquad (2)$$

What makes ^{18}O exchange a uniquely powerful mechanistic probe is that the number of oxygen atoms exchanged per turnover depends on the number of times the second step in eq 1 recycles before P_i dissociates from the enzyme. The probability of oxygen exchange is given by the partition coefficient (P_c), which is the ratio of the rate constant for P_i entering the exchange reaction through loss of water and formation of the covalent phosphoenzyme bond (k_2) to the sum of this rate constant plus the rate constant for P_i escape from the exchange reaction by dissociation from the enzyme (k_{-1}).

$$P_c = \frac{k_2}{k_{-1} + k_2} \qquad (3)$$

Since the natural abundance of ^{18}O in water is negligible, at one extreme when P_c approaches 0 ($k_{-1} \gg k_2$), there is an obligatory exchange of one oxygen atom each time phosphoenzyme is formed. In this case, the distribution of ^{18}O in phosphate remains random (Bock & Cohn, 1978). At the other extreme when P_c approaches 1 ($k_2 \gg k_{-1}$), all four oxygen atoms are exchanged before P_i is released from the enzyme. At intermediate P_c values, the distribution of ^{18}O in phosphate is nonrandom. Assuming that all four oxygens of bound P_i are equivalent, calculation of the distribution of isotopomers as a function of time reduces to a problem in statistics (Hackney & Boyer, 1978). Hackney (1980) has derived equations for calculating nonrandom distributions of ^{18}O isotopomers from P_c for ^{18}O exchange between P_i and water. The exchange parameters ν_{ex} and P_c were estimated by simultaneously fitting the theoretical equations for the five ^{18}O isotopomers of P_i to measured isotopomer distributions with the SigmaPlot 5.01 nonlinear, least-squares program. SigmaStat 1.01 was used to compare the parameter estimates for mutants with wild type, and the probability (P) of obtaining the reported value when the mutation actually had no effect is quoted.

Estimation of the P_i Dissociation Constant from ^{18}O Exchange Measurements. The P_i dissociation constant was estimated as the Michaelis constant for the exchange reaction by fitting the Michaelis−Menten equation to estimates of ν_{ex} as a function of free P_i concentration. Since substrate as well as enzyme is regenerated in the exchange reaction (eq 1), the steady-state rate of ^{16}O incorporation into labeled P_i is measured. The first-order rate constant obtained by dividing the estimated maximum exchange rate (V_{ex}) by the total enzyme concentration is

$$k_{ex} = \frac{k_2 k'_{-2}}{k_2 + k'_{-2}} \qquad (4)$$

and the half-maximum free P_i concentration equals

$$K_m = \frac{K_p}{1 + \dfrac{k_2}{k'_{-2}}} \qquad (5)$$

where K_p (k_{-1}/k_1) is the substrate constant (Boyer *et al*., 1977).

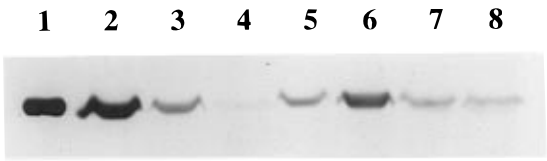

FIGURE 1: Expression of Na,K-ATPase mutants in yeast cells. Membranes were prepared from yeast cells containing the indicated Na,K-ATPase mutants or wild type, and 150 μg of membrane protein was loaded onto each lane of an SDS−polyacrylamide gel. After electrophoresis, the proteins were transferred to nitrocellulose and were incubated with a monoclonal antibody to chicken Na,K-ATPase (MAby #5; D. Fambrough). The nitrocellulose was then incubated with alkaline phosphatase-conjugated goat anti-mouse antibody, and proteins were stained with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium: lane 1, 0.3 μg of dog kidney Na^+,K^+-ATPase; lane 2, wild type; lane 3, K501E; lane 4, K501R; lane 5, D586E; lane 6, D586N; lane 7, P587A; and lane 8, P588A.

Mechanism of Na,K-ATPase-Catalyzed ^{18}O Exchange. Dahms and Boyer (1973) concluded from measurements of average isotope enrichment that ^{18}O exchange between P_i and H_2O is catalyzed by the E_2 conformation in the reaction cycle proposed by Albers (1967) and Post (Post *et al.*, 1969) for Na,K-ATPase. In an unpublished study,[2] we have shown that P_c for enzyme purified from pig kidneys is 0.24 ± 0.04 at pH 7.4, increasing dramatically to about 0.6 as the pH is lowered to 6.5. Mg^{2+} is required for catalysis and binds before P_i, so the Michaelis constant estimated from kinetic titrations with P_i is an apparent value given by eq 6

$$K_m(\text{app}) = K_m\left(1 + \frac{K_{Mg}}{[Mg^{2+}]}\right) \quad (6)$$

in which K_{Mg} is the equilibrium constant for Mg^{2+} dissociation from the E_2 conformation of the enzyme. All of the rate constants in eq 1 could be estimated, and the values for purified hog enzyme are recorded in the WT row of Table 4. In the Discussion, a value of K_{Mg} (2.8 mM) obtained from completely independent stopped-flow studies of the conformational change between E_1 and E_2 (Smirnova & Faller, 1993) and eqs 3−6 are used to compare the exchange parameters of wild type with those of purified enzyme and to predict the effects of changing individual rate constants on the exchange parameters.

RESULTS

The mutations K501R, K501E, D586E, D586N, P587A, and P588A were introduced into the α subunit of sheep Na^+,K^+-ATPase, and each mutant α subunit was expressed in yeast cells together with the β subunit of dog Na,K-ATPase using a single expression plasmid (Horowitz *et al.*, 1990). Wild type enzyme (WT) in the reported experiments is Na,K-ATPase, consisting of unmutated sheep α subunits and dog β subunits, expressed in yeast cells.

Expression Levels. Figure 1 is a Western blot showing that wild type and mutant α subunits were expressed at different levels. Mutants were expressed at 21−54% of the wild type level, except for K501R (lane 4) which was expressed at lower levels. The expression level of wild type and of each mutant also varied in different preparations. Therefore, in order to compare the effects of different mutations on enzymatic activities, turnover numbers (TNs) were calculated from the ratio of the activity measurement to the ouabain titer. The ouabain titer, defined as the maximum ouabain binding capacity of phosphoenzyme formed from P_i, was calculated from the measured amount of ouabain bound when the total concentration was 20 nM and the ouabain dissociation constant (K_d). The estimated K_d values and the ouabain titers for wild type and all of the mutants except D586E are reported in columns 4 and 5, respectively, of Table 1. Ouabain binding was dramatically affected by mutations to D586. Changing aspartic acid 586 to asparagine reduced the affinity for ouabain more than 50-fold. Not enough ouabain binding could be detected to estimate the K_d for D586E. The estimated ouabain K_d values for WT and the other mutants are all within two standard deviations of the mean, so the mean value (9.5 ± 2.0 nM) was used to calculate their ouabain titers. The ouabain titers (expressed as a percentage of WT) of mutants K501E (34%), D586N (57%), P587A (15%), and P588A (17%) agree satisfactorily with their expression levels (31, 54, 27, and 21% of WT, respectively) estimated by densitometry of the SDS−polyacrylamide gel in Figure 1.

Table 1: Effect of Mutations on Ouabain Binding and Enzymatic Activity

mutant	K_m(ATP)[a] (μM)	N [b]	K_d(ouabain)[c] (nM)	ouabain titer[d] (pmol mg^{-1})	TN[e] (s^{-1})	n[f]
WT	0.19 ± 0.06	2	6.4 ± 1.2	8.7 ± 2.7	44 ± 7	5
K501R			10.3 ± 2.8	0.46 ± 0.12	30 ± 5	5
K501E	1.8 ± 0.2	4	10.4 ± 2.1	3.0 ± 1.0	16 ± 5	2
D586E	0.23 ± 0.04	3				
D586N	0.23 ± 0.04	1	594 ± 63	4.9 ± 1.2	14 ± 3	4
P587A	0.17 ± 0.02	2	8.7 ± 1.0	1.3 ± 1.0	68 ± 21	5
P588A	0.10 ± 0.06	1	11.7 ± 1.8	1.5 ± 0.9	70 ± 14	5

[a] Apparent Michaelis constant for phosphorylation of enzyme by ATP estimated indirectly from ouabain binding to the steady-state concentration of phosphoenzyme formed from ATP. [b] Number of titrations. When $N > 1$, the mean $K_m \pm$ standard error of the mean (SEM) is reported. When $N = 1$, the estimate of $K_m \pm$ standard deviation (SD) is tabulated. The number of concentration points (n) per titration is 8 ($0 \leq [ATP]_o \leq 50$ μM). [c] Ouabain dissociation constant; $n = 16$ except for D586N ($n = 12$). [d] Maximum ouabain binding capacity of phosphoenzyme formed from P_i (Results). [e] Mean turnover number = ouabain-inhibitable ATPase activity (micromoles per milligram per second)/ouabain titer (micromoles per milligram) ± SEM. [f] Number of measurements.

Na,K-ATPase Activity. Although the measured enzymatic activity of some mutants was low, none of the mutations abolished the ability of the enzyme to hydrolyze ATP, indicating that the side chain functional groups of the amino acids studied are not essential for catalytic activity. The results of the Na,K-ATPase activity measurements for the mutants, except D586E whose ouabain titer could not be determined, and for wild type enzyme are summarized in column 6 of Table 1. The mean TN and standard error of the mean (SEM) are reported. The probability that none of the mutants affected catalytic activity is only 2%. However, only the turnover number of mutant D586N is different from that of wild type at the 95% ($P < 0.05$) confidence level.

ATP Binding Affinity. If the amino acids under investigation are located in the ATP binding site of the enzyme, the effects of mutations on Na,K-ATPase activity could result from changes in the structures of the side chains that affect the binding affinity of the enzyme for substrate. Na,K-ATPase is phosphorylated from ATP during the normal catalytic cycle, and ouabain binds with high affinity to the

[2] Unpublished results of M. Stengelin and V. N. Kasho.

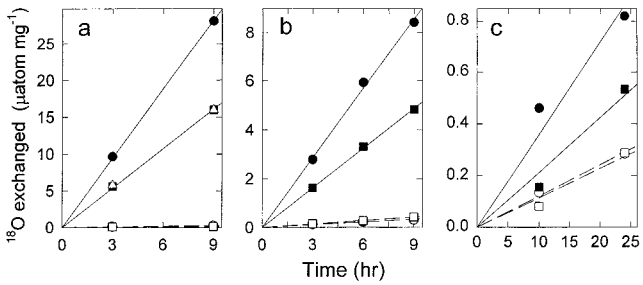

FIGURE 2: Ouabain-inhibitable ^{18}O exchange activity. The amount of ^{18}O exchanged in microatom per milligram of protein is plotted against the time in hours. Circles indicate measurements at pH 6.5, and squares or triangles indicate measurements at pH 7.5. With one exception, open symbols mean that ouabain was present, and closed symbols mean that ouabain was absent. The exception is the open triangle, which is used to denote overlapping data points collected in a separate experiment in the absence of ouabain. The reaction solution contained membrane protein with or without 0.1 mM ouabain, 2 mM [^{18}O]P_i, 20 mM KCl, 2 mM $MgCl_2$, 0.1 mM DTT, and 5 mM NaN_3 in 0.1 M Tris-HCl (pH 7.4) or MES-Tris (pH 6.5) at 30 °C, adjusted to a 200 mM ionic strength with choline chloride. (a) WT (0.42 mg mL^{-1}). From the slopes of the fitted lines, more than 99% of the exchange reaction is inhibited by ouabain at both pH values. (b) K501E (0.34 mg mL^{-1}). The percentage of the exchange inhibited by ouabain is 91% at pH 7.4 and 96% at pH 6.5. (c) D586N (0.85 mg mL^{-1}). This is one of three separate experiments with the mutant in which 66% of the activity was inhibited by ouabain at pH 6.5 and 46% at pH 7.4.

phosphorylated intermediate (Wallick & Schwartz, 1988). Therefore, in order to determine whether any of the mutations affected the apparent affinity of the enzyme for ATP, the dependence of ouabain binding on ATP concentration was measured.

The K_m values for ATP were estimated indirectly from ouabain binding to the steady-state level of phosphoenzyme formed from different ATP concentrations in a 3 min assay, and they are reported in column 2 of Table 1. The justification for considering the K_m obtained in this way as a measure of the enzyme's affinity for ATP is that the value estimated for wild type Na,K-ATPase was 0.19 ± 0.06 μM, which is equal within experimental error to the directly measured (Hegyvary & Post, 1971) equilibrium constant for dissociation of ATP from purified renal Na,K-ATPase (0.22 μM). There is no entry for mutant K501R because its low expression level precluded study. Only the K_m of mutant K501E is different from that of wild type at the 99% confidence level ($P < 0.01$), indicating that the affinity of the enzyme for the nucleotide was not affected by the D586E, D586N, P587A, or P588A mutations.

The apparent affinity of the K501E mutant for ATP is reduced nearly 10-fold compared to those of wild type and the other mutants. However, even in this case, enzymatic activity was measured under saturating conditions (3.3 mM ATP), so the turnover numbers are estimates of k_{cat} that can be compared directly. In Table 3, the k_{cat} values for the Na,K-ATPase activities of the mutants are expressed as a percentage of that of wild type and compared with published data (Maruyama & MacLennan, 1988; Clarke *et al*., 1990) for the effects of the corresponding mutations of Ca-ATPase on Ca^{2+} transport.

Ouabain-Inhibitable ν_{ex}. The amounts of ^{18}O exchanged between 2 mM P_i and water in the presence and absence of ouabain by yeast membranes containing wild type and mutant K501E are shown plotted as a function of time in panels a and b of Figure 2, respectively. The linear dependence demonstrates that ν_{ex} is constant for at least 9 h. In this time, the percentage of P_i in the $P^{18}O_4$ peak when ouabain was absent decreased to less than $^1/_{10}$ of its original value in the case of wild type and to less than $^1/_2$ of its original value in the case of the mutant. At pH 7.4, the ouabain-inhibitable activity calculated from the difference between the slopes of the least-square straight lines drawn through the points with and without ouabain present is 91% of the total measured ^{18}O exchange activity for the mutant (Figure 2b) and greater than 99% of the total measured ^{18}O exchange activity for wild type (Figure 2a). The ^{18}O exchange rates are nearly twice as fast at pH 6.5 in both cases.

Two mutants had to be incubated with P_i for a longer time in order to obtain measurable isotope exchange. This is shown for one experiment with mutant D586N in Figure 2c. Incubation of yeast membranes containing D586N with 2 mM P_i for 24 h decreased the percentage of P_i in the $P^{18}O_4$ peak to about 80% of its original value at pH 7.4 and to about 70% of its original value at pH 6.5. Averaging all of the D586N data, roughly 33% of the observed exchange rate was ouabain-inhibitable at pH 7.4, and the corresponding percentage at pH 6.5 was approximately 62%. The estimated ν_{ex} of mutant D586N was the same within experimental error at 10 and 24 h as indicated by the linear dependence of the amount of ^{18}O exchanged on time. The percentage of P_i in the $P^{18}O_4$ peak decreased only about 10% when P_i was incubated for 20 h with yeast membranes containing mutant D586E, and only about 7% of the observed exchange was ouabain-inhibitable. Therefore, ν_{ex} for mutant D586E could not be reliably estimated. The exchange rates for wild type and the other mutants at a total P_i concentration of 2 mM are reported as turnover numbers in columns 4 and 7 of Table 2. The mean values for wild type and mutant D586N include estimates from replicate measurements of the isotopomer distributions on different days with different aliquots of the membrane preparations.

Isotopomer Distribution. Figure 3 is a bar graph showing the isotopomer distribution after 9 h at 30 °C when ^{18}O exchange is catalyzed by yeast membranes in which mutant P588A was expressed. The open bars represent the isotopomer distribution of the starting [^{18}O]P_i incubated without yeast membranes. They show that inorganic phosphate does not exchange ^{18}O with water at a significant rate. The cross-hatched bars show the isotopomer distribution after incubation of yeast membranes with ouabain and 2 mM [^{18}O]P_i for the same length of time, and the filled bars indicate the isotopomer distribution after incubation of the membranes without ouabain. The difference between the filled and cross-hatched bars is a measure of the ^{18}O exchange activity of the mutant Na,K-ATPase.

The P_c and ν_{ex} of mutant P588A were estimated from the isotopomer distribution in the absence of ouabain at the 9 h time point using the isotopomer distribution in the presence of ouabain after this time (cross-hatched bars) as the approximate starting distribution. At pH 7.4, the sum of the absolute values of the residuals between the observed and calculated isotopomer distribution (not shown) for all five peaks was 0.13% of the total [^{18}O]P_i abundance. The corresponding aggregate discrepancy between the calculated and observed isotopomer distributions at pH 6.5 was 0.61%. The bar graph shows clearly that the isotopomer distributions at pH 7.4 and 6.5 are dramatically different after catalysis of ^{18}O exchange by P588A for the same length of time. The

Table 2: Effect of Mutations on P_i Binding and ^{18}O Exchange Parameters

	pH 7.4					pH 6.5		
mutant	K_m(app)[a] (mM)	n[b]	TN[c] (s^{-1})	P_c[d]	N[e]	TN (s^{-1})	P_c	N
WT	1.5 ± 0.2	6	86 ± 1	0.20 ± 0.02	4	149 ± 2	0.53 ± 0.03	2
K501R	1.8 ± 0.1	7	43 ± 3	0.17 ± 0.01	3	59 ± 14	0.44 ± 0.01	3
K501E	3.1 ± 0.3	7	66 ± 1	0.17 ± 0.01	3	117 ± 2	0.44 ± 0.02	3
D586N			0.4 ± 0.1	0.02 ± 0.02	5	1.3 ± 0.1	0.08 ± 0.01	4
P587A	4.1 ± 1.1	7	20 ± 1	0.25 ± 0.01	3	53 ± 3	0.55 ± 0.02	3
P588A	3.0 ± 0.1	11	86 ± 1	0.16 ± 0.01	3	195 ± 1	0.45 ± 0.01	3

[a] Apparent Michaelis constant for ^{18}O exchange ± SD. [b] Number of concentration points in titration. [c] Mean turnover number = ouabain-inhibitable ^{18}O exchange activity (micromoles per milligram per second)/ouabain titer (micromoles per milligram) ± SEM. [d] Mean partition coefficient (eq 3) ± SEM. [e] Number of estimates.

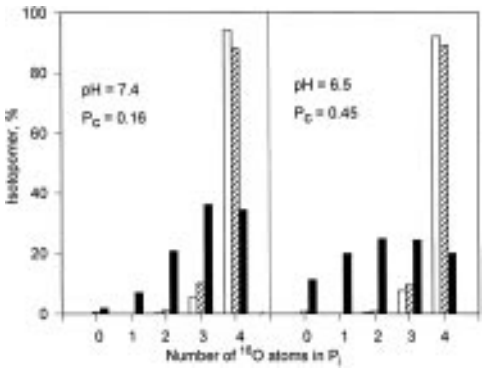

FIGURE 3: Phosphate isotopomer distribution. The percentage of P_i present as each isotopomer after 9 h for mutant P588A is plotted against the number of ^{18}O atoms in the isotopomer. The reaction conditions were identical to those described in the legend of Figure 2, except that the protein concentration was 1.00 mg mL^{-1}. No yeast membranes were present in the experiment indicated by open bars. The filled bars denote the experiment in which yeast membranes were present, and the cross-hatched bars denote the experiment in which yeast membranes were incubated with ouabain. The pH at which the experiment was conducted and the P_c values estimated from the isotopomer distributions are indicated on the bar graphs.

estimated P_c value increases from 0.17 at pH 7.4 to 0.45 at pH 6.5. From the analysis of reaction aliquots stopped at two shorter times (3 and 6 h), P_c as well as ν_{ex} were found to be independent of time for mutant P588A at both pH values.

A different procedure was used to estimate the exchange parameters for D586N because one-third or more of the observed exchange was not inhibited by ouabain. In this case, apparent ν_{ex} and P_c values for catalysis of ^{18}O exchange by endogenous phosphatases in the yeast membranes were estimated from the measured isotopomer distribution in the presence of ouabain using the measured isotopomer distribution in the absence of yeast membranes as the starting isotopomer distribution. The apparent exchange parameters were then used to calculate the isotopomer distribution resulting from catalysis of ^{18}O exchange by endogenous phosphatases. This distribution at the midpoint of the ouabain-inhibitable exchange reaction was used as the approximate starting distribution for estimation of ν_{ex} and P_c for catalysis of ^{18}O exchange by D586N. In other words, a linear change in the background isotopomer distribution with time was assumed. The estimates of P_c for wild type and for the different mutants of Na,K-ATPase that were studied are recorded in columns 5 and 8 of Table 2. Only the P_c of mutant D586N is different from that of wild type at the 99% confidence level at both pH values.

P_i Binding Affinity. The total concentration of P_i (2 mM) used for the comparative experiments summarized in columns 4−9 of Table 2 was selected to optimize the ^{18}O exchange measurements and is not saturating. Therefore, ν_{ex} was measured as a function of P_i concentration in order to estimate the K_m for P_i of wild type and each of the mutants at pH 7.4. The resulting titration curves were hyperbolic (not shown), and K_m was estimated from the functional dependence of ν_{ex} on the concentration of free P_i. Since ν_{ex} for catalysis of ^{18}O exchange by Na,K-ATPase depends on the free Mg^{2+} concentration as well as the free P_i concentration,[2] the total concentration of Mg^{2+} was adjusted using a value of 8.5 mM for the dissociation constant of Mg^{2+} from P_i (Smirnova *et al*., 1989) to keep the concentration of free Mg^{2+} constant and approximately equal to the free Mg^{2+} concentration in the experiments reported in columns 4−9 of Table 2. Purified Na,K-ATPase catalyzes ^{18}O exchange between P_i and H_2O by an ordered binding mechanism in which Mg^{2+} binds first.[2] Therefore, the Michaelis constant estimated from the empirical half-maximum free P_i concentration or $K_{0.5}$ is an apparent value [K_m(app)] given by eq 6.

Although different membrane preparations were used for the titrations, all of the exchange rates estimated from measurements at a single P_i concentration that were used to calculate the turnover numbers in column 4 of Table 2 fell on the titration curves except one, and the exception is the mutant (D586N) with a low affinity for ouabain (Table 1) that could result from reduced affinity for P_i. The estimates of P_c at pH 7.4 from the titrations were independent of P_i concentration and numerically indistinguishable from the values in column 5 of Table 2. Consistent with reduced affinity of mutant D586N for P_i, only a lower limit on the value of K_m(app) (>25 mM) could be estimated because ν_{ex} increased linearly with free P_i concentration in the concentration range 0−10 mM (n = 6). However, ν_{ex} values within $^1/_3$ of the estimated saturating or maximum value (V_{ex}) were measured for wild type and each of the other mutants, and the estimates of K_m(app) for P_i are recorded in column 2 of Table 2. The estimates of K_m(app) for mutants K501E, P587A, and P588A are different from that of wild type at the 99% confidence level.

The estimates of K_m(app) from the titration curves were used to calculate the maximum turnover number of wild type and each mutant at pH 7.4 from the TN estimates at 2 mM P_i in Table 2. The maximum turnover number is equivalent to k_{ex} (eq 4) because V_{ex} is independent of Mg^{2+} concentration,[2] and the values of k_{ex} are reported as percentages of the wild type value in column 3 of Table 3. The k_{ex} estimates for mutants K501R, P587A, and P588A are different from that of wild type at the 95% confidence level. Since tighter

Table 3: Comparison of Na^+,K^+-ATPase and Ca-ATPase Mutants

	Na,K-ATPase			Ca-ATPase[a]			
	ATPase	^{18}O exchange					
mutant	k_{cat}[b] (%)	k_{ex}[c] (%)	P_c	transport (%)	E−P	$E_1P \rightarrow E_2P$	mutant
WT	100 ± 16	100 ± 7	0.20	100			WT
K501R	68 ± 22	55 ± 10	0.17	60			K515R
K501E	37 ± 18	115 ± 18	0.17	5			K515E
D586E				<5	−		D601E
D586N	32 ± 12		0.02	<5	+	×	D601N
P587A	155 ± 72	42 ± 14	0.25	83			P602L
P588A	159 ± 57	147 ± 16	0.16	<5	+	×	P603L
				<5	−		P603G

[a] Maruyama and MacLennan (1988) and Clarke *et al.* (1990). [b] Percentage of wild type k_{cat} (44 ± 7 s^{-1}). [c] Percentage of wild type k_{ex} (163 ± 12 s^{-1}).

Table 4: Theoretical Prediction of Exchange Parameters

	assumed rate constants				predicted exchange parameters		
mutant	k_1 (M^{-1} s^{-1})	k_{-1} (s^{-1})	k_2 (s^{-1})	k'_{-2} (s^{-1})	k_{ex} (s^{-1})	K_m(app)[a] (mM)	P_c
WT[b]	1.3×10^6	1.5×10^3	500	500	250	1.5	0.25
K501R	1.3×10^6	1.5×10^3	500	108	89	0.5	0.25
K501E	6.5×10^5	1.5×10^3	500	500	250	3.1	0.25
D586N	1.3×10^6	2.5×10^4	500	500	250	26	0.019
P587A	1.3×10^6	1.5×10^3	500	131	69	0.4	0.25
P588A	1.3×10^6	1.5×10^3	500	1.2×10^3	355	2.2	0.25

[a] Equation 6 with a K_{Mg} of 2.8 mM (Smirnova & Faller, 1993b). [b] The assumed rate constants for WT are the estimates from the unpublished work cited in footnote 2.

binding at lower pH could explain part or all of the inverse dependence of the turnover number on pH (Table 2), membranes containing wild type or mutant K501E were also titrated with P_i at pH 6.5. The K_m(app) of wild type was not significantly different at pH 6.5 (1.3 ± 0.1 mM) from the value at pH 7.4, but the K_m(app) of K501E was approximately halved (1.4 ± 0.1 mM) by lowering the pH. The k_{ex} value of wild type at pH 6.5 (254 ± 12 s^{-1}) is roughly 50% larger than the value at pH 7.4 (163 ± 12 s^{-1}), but the k_{ex} value of mutant K501E at pH 6.5 (206 ± 12 s^{-1}) is not significantly different from the value at pH 7.4 (188 ± 15 s^{-1}).

DISCUSSION

A three-dimensional model proposed by Taylor and Green (1989) for the active site of P type ion motive ATPases has been tested by site-directed mutagenesis of Na,K-ATPase. The amino acids investigated are in two conserved sequences KGAP and DPPR, and the model predicts interaction of their charged side chains (K and D) with the phosphate and ribose hydroxyl groups of bound ATP, respectively. Effects of the mutations on enzymatic activity, ATP binding, and phosphorylation of the enzyme by inorganic phosphate were measured. The corresponding amino acids in sarcoplasmic reticulum Ca-ATPase were investigated earlier by MacLennan and co-workers (Maruyama & MacLennan, 1988; Clarke *et al.*, 1990), so to facilitate the discussion, the results obtained by mutating homologous amino acids in the two P type enzymes are compared in Table 3.

In addition to a transport assay that assesses overall function, MacLennan and co-workers studied a partial reaction in the Ca-ATPase cycle with ^{32}P-labeled substrates to learn the specific reaction step affected by a mutation. The plus and minus signs in Table 3 indicate whether the authors concluded from autoradiography of gels that mutations in the DPPR sequence of Ca-ATPase prevented phosphorylation by ATP and P_i (−) or permitted phosphorylation by both substrates (+). A cross (×) is used to indicate that they concluded the mutation slowed or blocked the conformational change which normally occurs after phosphorylation by ATP. We have dissected the reaction cycle of Na,K-ATPase by a different method. Since catalysis of ^{18}O exchange between inorganic phosphate and water has not been used previously to study mutants of any P type ATPase, we begin by discussing experiments designed to demonstrate the feasibility of applying this technique to mutants of Na,K-ATPase expressed in yeast cells.

Feasibility of ^{18}O Exchange Measurements. Figure 2 shows that the exchange rates (eq 2) of wild type and mutants expressed in yeast cells can be measured with high precision. The value of k_{ex} calculated from the experimentally estimated TN for WT in Table 2 at pH 7.4 (163 ± 12 s^{-1}) differs from the value in Table 4 (250 s^{-1}) predicted from the estimated rate constants for purified enzyme[2] by only 35%. The experimentally determined values of P_c and K_m(app) in Table 2 can be compared directly with the predicted values in Table 4 and are also in reasonable agreement. More than 90% of the exchange observed in Figure 2b is catalyzed by mutant K501E (Results), even though only about 0.03% of the protein in the yeast membranes was mutant Na,K-ATPase, on the basis of the ouabain titer (Table 1). By comparison, as little as 18% of the observed ATPase activity of yeast membranes in which mutants of Na,K-ATPase are expressed may be ouabain-inhibitable (Scheiner-Bobis & Farley, 1994). The most probable explanation of the higher signal to noise ratio for exchange of ^{18}O between P_i and water than for ATPase activity is that the K_m for catalysis of the exchange reaction by the yeast plasma membrane, proton ATPase (Amory *et al.*, 1982) is 40−100 times larger (177 mM) than the K_m(app) for catalysis of the exchange reaction by Na,K-

ATPase expressed in yeast cells (Table 2), whereas both enzymes bind ATP with comparable affinity.

Figure 2 also demonstrates that mutants of Na,K-ATPase expressed in yeast cells are stable for a long enough time to significantly populate all five of the isotopomer peaks (Figure 3). Therefore, the partition coefficient (eq 3) for the exchange reaction can be estimated with high precision from data at a single time point because five equations, one for each of the five isotopomers, must be satisfied simultaneously. The sum of the absolute values of the residuals between the observed and fitted distributions (Results) was less than 1% for all of the parameter estimates reported in Table 2. Since both exchange parameters were found to be independent of time, three quasi-independent estimates of P_c and ν_{ex} could be obtained from measurements at three time points. The ouabain titer for the P588A preparation assayed was 0.6 pmol mg^{-1}, so less than 2 pmol (six 0.5 mL aliquots ± ouabain) of the mutant was required to obtain the estimates of the exchange parameters recorded for this mutant at pH 7.4 in Table 2.

Figure 3 also shows the dramatic dependence of P_c on pH that is characteristic of P type ATPases (Faller & Diaz, 1989). Measurements of P_c at two pH values were made to obtain an additional criterion for judging whether a mutant differs from wild type. The P_c estimate for WT at pH 6.5 (Table 2) is in satisfactory agreement with the value quoted earlier in Methods for purified hog enzyme (0.6).[2]

Mutations of K501. Changing K515 in the KGAP sequence of Ca-ATPase to arginine or glutamate reduced Ca^{2+} transport relative to that of wild type (Table 3). Neutralization of the charge on lysine by substituting alanine or glutamine (Murayama & MacLennan, 1988; Maruyama *et al*., 1989) gave intermediate reductions of transport (25−30%). Therefore, the decrease in transport activity paralleled the progressive change in charge on the side chain from positive to negative, which is consistent with the predicted interaction of the positively charged lysine side chain with the negatively charged α and β phosphoryl groups of bound ATP. However, changing K515 to alanine did not affect phosphorylation of the enzyme by either ATP or P_i (Murayama *et al*., 1989), suggesting a more complex role for this amino acid in catalysis and transport than simply functioning as a ligand for the phosphate groups of ATP (Clarke *et al*., 1990). The results of mutating the homologous lysine (K501) in Na,K-ATPase also implicate the side chain in both ATP binding and subsequent steps in the catalytic cycle.

Our results indicate that changing K501 affects P_i as well as ATP binding. The order-of-magnitude increase in the K_m inferred from ouabain binding for phosphorylation of Na,K-ATPase by ATP when lysine is changed to glutamic acid (Table 1) is indirect evidence for K501 participation in ATP binding. The apparent affinity of the enzyme for P_i also decreases when a negative charge is substituted for the positive charge on K501 (Table 2). The expression for K_m (eq 5) contains the rate constants for covalent bond formation and cleavage as well as the substrate constant. However, since neither the k_{ex} nor the P_c of K501E is different from that of wild type (Table 3), decreased affinity of the mutant for P_i is the most probable cause of the observed increase in K_m(app). Alternatively, the mutation could affect P_i binding indirectly by affecting Mg^{2+} binding. The affinity of the enzyme for Mg^{2+} would have to decrease (eq 6) in order to explain the observed increase in K_m(app). This seems unlikely because introducing a negative charge into the pocket where both Mg^{2+} and P_i bind (Stewart *et al*., 1989) would be expected to increase the enzyme's affinity for Mg^{2+} and indirectly decrease the apparent affinity of the enzyme for P_i. On the other hand, a decrease in the second-order rate constant for formation of the Michaelis complex (k_1) is reasonable because the rate constant for a diffusion-limited reaction is approximately proportional to the product of the charges on the reacting species (Eigen, 1960), and the mutation decreases the mean net charge on the protein from Z to $Z - 2$. The predictions for K501E in Table 4 show that numerically halving k_1 can explain the observed exchange parameters of the mutant.

The effects of the mutations on k_{cat} indicate that more than the affinity of the enzyme for ATP is affected (Table 3). The apparent decrease in Na,K-ATPase activity of mutant K501E ($P = 0.053$) cannot be explained by the decreased affinity for ATP because the ATP concentration used in the assay (3.3 mM) was still sufficient to saturate the catalytic sites. It can also be concluded that reaction steps not involved in catalyzing ^{18}O exchange are affected by the mutations because the exchange reaction is not rate-limiting. Even in the case of mutant K501R which catalyzes ^{18}O exchange only about half as fast as wild type, k_{ex} for the exchange reaction (89 ± 9 s^{-1}) is greater than the estimate of k_{cat} for the ATPase activity of the mutant (30 s^{-1}). Although we could not measure the apparent affinity of K501R for ATP, it is unlikely that the conservative replacement of lysine with arginine could cause the nearly 10^4-fold increase in K_m that would be needed to explain the apparent reduction in ATPase activity of the mutant ($P = 0.14$). Therefore, steps between ATP binding and the reactions in the E_2 half of the cycle that catalyze oxygen exchange (eq 1) are probably affected by mutating K501.

The ^{18}O exchange data for K501R also indicate that reactions other than substrate binding and phosphorylation of E_2 are affected by the mutation. The predictions for K501R in Table 4 were made by assuming that the lower rate of ^{18}O exchange by K501R results entirely from a smaller rate constant for the hydrolysis step in eq 1. The calculation shows that K_m(app) should decrease unless Mg^{2+} binding (K_{Mg}) is also affected by the mutation. Alternatively, the reduced ν_{ex} could result from overestimation of the steady-state E−P concentration. The derivation of eqs 4 and 5 assumes that increasing P_i converts all of the enzyme into the forms with P_i bound (E·P and E−P). For catalysis of ^{18}O exchange by Na,K-ATPase, there is the additional requirement that phosphate be bound to the E_2 conformation, and the equilibrium between enzyme conformations depends upon the concentrations of Na^+, K^+, and Mg^{2+}. All of wild type is in the reactive E_2K form under the assay conditions (20 mM K^+, 5 mM Na^+, and 2 mM Mg^{2+}) used in this study (Smirnova & Faller, 1993). However, a mutation could affect the conformational equilibrium either indirectly by altering the enzyme's affinity for one or more of the metal ions or directly by changing the rate constants for the conformational change so that dividing the estimated maximum exchange rate by the ouabain titer gives a value of k_{ex} that is too low. Regardless of whether the correct explanation of the reduced exchange rate of K501R is a smaller k'_{-2} or lower steady-state E−P concentration, the ^{18}O exchange

data indicate that replacing lysine by arginine affects more than one reaction step.

We do not have enough structural information to understand how the mutations affect the rate and equilibrium constants. Nevertheless, some insight into the role K501 might play in catalysis can be gained by comparing our activity data with the results of mutations to an ATPase of known tertiary structure that probably catalyzes phosphoryl group transfer by a similar mechanism. Both Na,K-ATPase and adenylate kinase catalyze transfer of the γ-phosphoryl group of ATP to another molecule. In the case of Na,K-ATPase, P_i is transferred to a side chain carboxyl group of the enzyme (D369). It is known from ^{18}O exchange experiments that Na,K-ATPase catalyzes hydrolysis by direct attack of a carboxylate oxygen on phosphorus (Dahms *et al*., 1973), and it is thought from the tight binding of orthovanadate to the enzyme that the transition state involves a pentacoordinate intermediate (Cantley *et al*., 1978). Therefore, it is likely that the mechanism of phosphoenzyme formation resembles the mechanism of γ-phosphoryl group transfer from ATP to bound AMP catalyzed by adenylate kinase. The high-resolution structure of this enzyme with P^1,P^5-bis(5′-adenosyl) pentaphosphate bound (Müller & Schulz, 1992) confirmed the linear arrangement of the nucleoside and phosphate groups expected for an in-line, associative displacement reaction (Knowles, 1980) that would explain the experimentally observed inversion of the stereochemistry of the groups attached to phosphorus (Richard & Frey, 1978). Bound divalent cations and/or positive charges on an enzyme can accelerate this type of reaction by stabilizing the increased negative charge relative to the ground state on the oxygen atoms in the pentacoordinate transition state (Knowles, 1980). However, mutations to positively charged amino acids that are close enough in the crystal structure of adenylate kinase to hydrogen bond to the γ phosphate of ATP reduced the k_{cat} of that enzyme 3−4 orders of magnitude more than changing K501 reduced either the k_{cat} for ATP hydrolysis or the k_{ex} for ^{18}O exchange by Na,K-ATPase. Therefore, it is unlikely that K501 of Na,K-ATPase is close enough to phosphate bound at the γ-phosphoryl site to function as a proton donor in hydrogen bond formation and participate directly in the catalytic mechanism.

The order-of-magnitude decrease in K_m observed for mutation K501E could mean that lysine 501 is hydrogen bonded to the α or β phosphate of bound ATP as predicted by Taylor and Green (1989) because mutations of arginines in the adenylate kinase structure that are hydrogen bonded to the α phosphate of bound AMP have comparable effects on the affinity of the enzyme for AMP (Müller & Schulz, 1992). Locating K501 near the α and/or β phosphate groups, even though the adjacent amino acid (G502) is modified by 2-N_3-ATP (Tran *et al*., 1994b), is not necessarily inconsistent with evidence that K480, which is modified by the 8-N_3-ATP (Tran *et al*., 1994a), also interacts with the phosphate groups of bound ATP (Hinz & Kirley, 1990; Wang & Farley, 1992) because a 13-amino acid sequence between K480 and K501 is predicted to form random coil (Taylor & Green, 1989). One way, suggested by molecular models, that K480 and K501 could both interact with one or more phosphate groups and be labeled by the azido group in the 2 and 8 positions at opposite ends of the conjugated ring system of adenine is formation of a loop which places either K480 or K501 above and the other amino acid below the plane of the purine ring.

Mutations of the DPPR Sequence. Even the conservative substitution of glutamic for aspartic acid in the DPPR sequence of Ca-ATPase had a profound effect on the properties of the sarcoplasmic reticulum enzyme, essentially eliminating transport and phosphorylation of the enzyme by either ATP or P_i (Table 3). Adamo *et al*. (1995) have recently reported that the corresponding mutant of plasma membrane Ca-ATPase (D672E) retains 15% of wild type Ca^{2+} transport activity and can be phosphorylated by ATP but is dephosphorylated only about one-third as fast as wild type. The mutant of sarcoplasmic reticulum Ca^{2+}-ATPase in which aspartic acid was replaced by asparagine (D601N) could still be phosphorylated by either ATP or P_i, but transport was effectively eliminated, dephosphorylation slowed, and the phosphoenzyme formed from ATP was ADP sensitive, suggesting that neutralization of the negative charge on the side chain blocked the conformational change between E_1P and E_2P. The mutation P602L reduced transport only slightly. However, changing P603 to either leucine or glycine had dramatic effects on function analogous to the effects of mutations D601N and D601E, respectively. Both substitutions markedly inhibited transport, P603G by preventing phosphorylation and P603L by slowing the conformational change that follows phosphorylation by ATP. Since parallel reductions in transport occurred when acetyl phosphate was substituted for ATP as the energy source and no phosphorylation of mutants D601E and P603G by either P_i or ATP could be detected, MacLennan and co-workers (Clarke *et al*., 1990) concluded that the DPPR sequence is critical for the integrity of the phosphorylation site.

Mutations of D586. Mutations of the amino acid in Na,K-ATPase homologous to D601 of Ca-ATPase support the conclusion that this residue is more important for phosphate binding than for nucleotide binding. The affinity of the enzyme for ATP is not affected by substituting either glutamic acid or asparagine for aspartic acid (Table 1). Mutations that disrupted hydrogen bonds between the 2′- and 3′-hydroxyl groups of ribose and the phenolic oxygen of a tyrosine residue reduced the affinity of adenylate kinase for ATP 2−4-fold (Müller & Schulz, 1992). Therefore, this study does not provide any support for the proposed interaction of the glutamic acid in the DPPR sequence of Na,K-ATPase with the hydroxyl groups of ribose (Taylor & Green, 1989).

However, replacing aspartic acid in the DPPR sequence with asparagine reduces the measured ^{18}O exchange rate nearly 2 orders of magnitude (compare amount of exchange and time scales in Figure 2a,c). The K_d for ouabain is dramatically affected by this mutation (Table 1), but good agreement between the ouabain titer (Table 1) expressed as a percentage of WT (57%) and the relative expression level (54%) estimated from densitometry of the gel in Figure 1 indicates that the explanation of the reduced exchange activity is not lower expression of enzyme that can be phosphorylated.

The lower affinity of D586N for ouabain in the coupled binding assay could result from reduced affinity for P_i, and the ^{18}O exchange data provide direct evidence for an effect of mutations to D586 on P_i binding. Not enough ^{18}O exchange was catalyzed by D586E in 20 h to reliably estimate P_c and ν_{ex}, consistent with failure to detect phosphorylation of the corresponding mutant of Ca-ATPase from

sarcoplasmic reticulum (Clarke *et al*., 1990). However, the exchange parameters could be estimated for D586N, in agreement with the report that the analogous mutant of Ca-ATPase can be phosphorylated by P_i (Clarke *et al*., 1990). Substituting asparagine for aspartic acid at position 586 reduces the affinity of Na,K-ATPase for P_i by at least 1 order of magnitude (Results), and the P_c for exchange of ^{18}O is also lowered 10-fold (Table 2). The simplest explanation of these results is that the mutation increases the rate constant for P_i dissociation (k_{-1}) and does not affect the other rate constants in eq 1. The increase in k_{-1} that would be required to obtain the order-of-magnitude lower P_c for ^{18}O exchange estimated for mutant D586N compared to the wild type value (Table 3) was calculated with eq 3. The predictions made for D586N in Table 4 assuming that only the value of k_{-1} is affected by the mutation show that the theoretical K_m(app) exceeds the lower limit (25 mM) inferred from the titration curve for the mutant (Results). Of course, we cannot exclude the possibility that the mutation also causes compensating changes in other rate constants in eq 1 that do not affect the composite parameters we measure.

Mutations of the Proline Residues. Substituting alanine for proline did not affect the estimate of either K_m (Table 1) or k_{cat} (Table 3) for ATP hydrolysis within experimental error. For example, there is a 13 and 30% chance, respectively, that the ostensibly higher k_{cat} values calculated for mutants P587A and P588A are not really different from the wild type value. On the other hand, the affinity of both mutants for P_i is significantly lower than that of wild type (Table 2). The values of k_{ex} for ^{18}O exchange (104 ± 23 s^{-1}) and k_{cat} for ATP hydrolysis (101 ± 31 s^{-1}) are the same within experimental error for P587A, suggesting that the E_2 half of the catalytic cycle may become rate-limiting for this mutant. The prediction for P587A in Table 4 shows that the observed reduction in the ^{18}O exchange rate caused by the mutation cannot be explained by a decrease in k'_{-2} because K_m(app) should decrease and experimentally the apparent affinity for P_i increases (Table 2). Decreasing just k'_{-2} to explain the lower ^{18}O exchange rate of mutant K501R also predicted a decrease in K_m(app) that was not observed, and possible alternative explanations of the data were discussed. Applying analogous reasoning to the data for P587A, either the mutation affects Mg^{2+} binding in addition to k'_{-2} or the fraction of the enzyme in the E_2 conformation under the assay conditions is affected by substituting alanine for proline. The increase in k_{ex} for ^{18}O exchange by mutant P588A over that of wild type is evidence that mutations to the proline residues in the DPPR sequence can affect the efficiency of phosphoenzyme hydrolysis. It is likely that this mutation increases k'_{-2} because the affinity of the mutant for P_i is lower and P_c is not significantly different from the wild type value. The prediction for P588A in Table 4 shows that increasing k'_{-2} by 47% (240 s^{-1} for P588A compared to 136 s^{-1} for WT) could explain the observed increases in both k_{ex} and K_m(app).

The ATPase data for mutation P588A in the DPPR sequence of Na,K-ATPase seem to conflict with the observed inhibition of transport caused by analogous changes to the homologous amino acid of Ca-ATPase. However, the decreased affinity of mutant D586N for ouabain in a P_i-dependent assay and the ^{18}O exchange data support the major conclusion of MacLennan and co-workers (Clarke *et al*., 1990), that this region of the protein is critical for formation of the phosphorylation site. Proline disrupts α-helices, and the DPPR sequence is predicted to lie in a loop between a β-sheet and an α-helix (Taylor & Green, 1989). Therefore, mutations to the prolines could affect the geometry of the active site and transitions between the E_1 and E_2 conformations. A possible, alternative explanation of the profound effect of mutations to the aspartic acid residue in the DPPR sequence is that the side chain carboxyl group is involved in chelating Mg^{2+}, which in turn could interact electrostatically with P_i and stabilize the transition state in the phosphoryl group transfer reaction. A Mg^{2+} that is chelated by the enzyme in the absence of substrate has been shown to interact with the γ phosphate of bound ATP by ^{31}P NMR (Klevickis, 1982), and Mg^{2+} coordinates with oxygens of the β and γ phosphates of AMP−PNP in the crystal structure of F_1-ATPase (Abrahams *et al*., 1994), which is also thought to catalyze ATP hydrolysis via a pentacoordinate intermediate. The dramatic effect of the additional methylene group in the side chain of mutant K601E on Ca^{2+} transport by Ca-ATPase and the evidence that mutations of the proline residues in the DPPR sequence of Na,K-ATPase affect the ^{18}O exchange parameters are both consistent with the conclusion that small perturbations of the geometry in this region of the molecule interfere directly with catalysis.

Conclusions. In this paper, we have introduced a new method for studying site-directed mutants of Na,K-ATPase that has a number of advantages over previously described assays. First, ^{18}O exchange between P_i and H_2O can be precisely measured even when the level of expression in yeast cells is low. This is illustrated by the relatively low percentage errors in the estimated exchange parameters (Table 2). Second, exchange is catalyzed by a partial reaction of the enzyme (eq 1), whose mechanism is relatively well understood. Third, the isotopomer distribution, which depends upon the ratio of rate constants for elementary reaction steps (eq 3), as well as the rate of isotope exchange can be measured. Therefore, the effects of mutations in the active site of Na,K-ATPase could be narrowed to specific reaction steps and quantitatively compared with mutations to an enzyme of known structure that is folded similarly and also catalyzes phosphoryl group transfer.

Two predictions of a proposed model (Taylor & Green, 1989) for the active site of P type ion motive ATPases were tested by site-directed mutagenesis of Na,K-ATPase. The results confirm that mutations of lysine in the conserved KGAP sequence affect ATP binding. However, the data argue strongly against direct coordination of the γ phosphate by K501 or a role for the positive charge on the side chain in catalysis by stabilizing the transition state formed during phosphoryl group transfer. No evidence to support the prediction that the aspartic acid residue in the DPPR sequence interacts with the hydroxyl groups of ribose was obtained, but the data do support the conclusion of MacLennan and co-workers (Clarke *et al*., 1990) from studies of homologous mutations to Ca-ATPase that the DPPR sequence is critical for formation of the phosphorylation site. A role for D586 in coordinating Mg^{2+}, which may be involved in accelerating phosphorylation of the enzyme, is proposed that is supported by ordered binding of Mg^{2+} and then P_i in the ^{18}O exchange reaction.[2]

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