Mutations were introduced in the motif \(884^\text{D}DRW887\) from an extracellular peptide of the sodium pump \(\alpha\) subunit localized between M7 and M8 membrane spans to investigate a possible role of this structure in ion recognition. A homologous sequence \(399^\text{QDCW}^{402}\) that occurs in the P-loops of Na\(^+\) channels was shown earlier to be important for ion gating. Mutant sodium pumps were expressed in yeast and subsequently investigated for their behavior toward ouabain, Na\(^+\), K\(^+\), and ATP. Native enzyme and D884A, D884R, D885A, D885E, or D885R mutants all bind ouabain in the presence of phosphate and Mg\(^2+\). The \(K_o\) values determined from Scatchard analysis are in the range 5–8 nm for the native enzyme and the D884A, D885E, or D885A mutants, and 15.7 ± 2.04 and 30.1 ± 4.32 nm for mutants D884R and D885R, respectively. This ouabain binding is reduced in the presence of K\(^+\) in a similar way for both native or mutant sodium pumps with relative affinities (\(K_{o,2}\)) for K\(^+\) ranging from 1.4 to 3.7 mm. Ouabain binding in the presence of 100 \(\mu\)M ATP is promoted by Na\(^+\) with \(K_{o,2} = 1.64 ± 0.01\) mm for the native enzyme and \(K_{o,2} = 8.6 ± 1.35\) mm for the D884R mutant. The \(K_{o,2}\) values of the two enzymes for ATP are 0.66 ± 0.16 mm and 1.1 ± 0.12 mm, respectively. Ouabain binding as a function of Na\(^+\) concentration, on the other hand, is very low for the D885R mutant, even at an ATP concentration of 2 mm. Phosphate or eosin, however, are recognized by this mutant enzyme, so that a major conformational change within the ATP-binding site appears unlikely.

The inability of the D885R mutant to bind ouabain in the presence of Na\(^+\) and ATP could be explained by assuming that the M7/M8 connecting extracellular loop, which also contains the mutated amino acids, is invaginated within the plane of the plasma membrane and possibly involved in acceptance and/or release of Na\(^+\) ions coming from cytosolic areas of the protein. In this case, the placement of an additional positive charge might repel Na\(^+\) ions and interrupt their flow, thus not allowing the enzyme to assume the proper conformational state for ouabain binding. Such invaginated hydrophilic protein structures, such as the P-loops of Na\(^+\) and K\(^+\) channels, are already known and have been shown to participate in ion conduction.

The sodium pump from plasma membranes of animal cells is an oligomeric enzyme that consists of an \(\alpha\) and a \(\beta\) subunit. The enzyme hydrolyzes ATP to actively transport Na\(^+\) ions out of the cell and K\(^+\) ions into the cell. Ouabain and other cardioactive steroids inhibit ATP hydrolysis and the transport of ions. The amino acids or the membrane spans involved in the formation of the ionophoric part of the sodium pump are not yet known although in several investigations mutations of single amino acids from the membrane spans of the \(\alpha\) subunit were shown to affect interactions of the enzyme with ions. Additional experiments, however, demonstrate that the \(\beta\) subunit also affects the behavior of the enzyme toward the transported ions. Based on today’s knowledge, interaction of \(\alpha\) and \(\beta\) subunits does not seem to occur between their transmembrane spans but rather between extracellular sequences of the two subunits. A hydrophilic sequence of 26 amino acids of the sodium pump \(\alpha\) subunit, localized between its M7 and M8 membrane spans (1), was shown earlier to interact with an extracellular part of the \(\beta\) subunit (2). Since ionophore formation is generally accepted to occur by interactions of membrane spans, the finding that an extracellular interaction of hydrophilic areas of the two proteins affects behavior of the enzyme toward the transported ions might appear rather unexpected.

Nevertheless, participation of extracellularly localized sequences in ion conduction has been demonstrated with voltage-dependent Na\(^+\) or K\(^+\) channels (3, 4). In these channels, a so-called “P-loop” structure (pore-forming loop) that is formed by a hydrophilic extracellular part of the channel protein is proposed to invaginate into the membrane bilayer, being surrounded by the hydrophobic membrane spans of the protein (5). This amphiphilic behavior of the P-loop, interactions with membrane spans and the water surface, is possibly the basis for the control of Na\(^+\) or K\(^+\) fluxes through the membrane-spanning protein of Na\(^+\) or K\(^+\) channels. Similar mechanisms have been proposed for the “hour-glass” structure of aquaporin (6).

In a recent investigation from this laboratory dealing with the transmembrane topology of \(\alpha\) and \(\beta\) subunits of the sodium pump, a structure similar to the P-loop of Na\(^+\) or K\(^+\) channels was proposed to be formed between the M7 and M8 membrane spans of the \(\alpha\) subunit (7). In the proposed model, this structure also contains the 26 amino acids known to interact with the \(\beta\) subunit. A sequence comparison of the hydrophilic loop localized between M7 and M8 spans of the \(\alpha\) subunit (7) in the proposed model, this structure also contains the 26 amino acids known to interact with the \(\beta\) subunit. A sequence comparison of the hydrophilic loop localized between M7 and M8 spans of the \(\alpha\) subunit (7) in the proposed model, this structure also contains the 26 amino acids known to interact with the \(\beta\) subunit. A sequence comparison of the hydrophilic loop localized between M7 and M8 spans of the \(\alpha\) subunit (7) in the proposed model, this structure also contains the 26 amino acids known to interact with the \(\beta\) subunit. A sequence comparison of the hydrophilic loop localized between M7 and M8 spans of the \(\alpha\) subunit (7) in the proposed model, this structure also contains the 26 amino acids known to interact with the \(\beta\) subunit.
The 5th membrane domain and the terminal sequence of the predicted total volume of the reaction was 100 m
The sodium pump mutants were expressed in the yeast Saccharomyces cerevisiae and their properties toward Na⁺ ions were investigated. The results obtained indicate a possible involvement of Asp884 and Asp885 in the release of Na⁺ to the extracellular space and allow us to conclude that single amino acids within the structure between the M7 and M8 membrane spans of the α subunit are important in ion recognition and conduction by the sodium pump.

MATERIALS AND METHODS
Vectors and Strains—For transformation of yeast strain 20B12 (8) the shuttle vector pCGY1406αβ was used (9). For DNA manipulation in Escherichia coli strains DH5α F' (Life Technologies Inc., Gaithersburg, MD) the plasmid pBlueScript II SK+ (Stratagene, La Jolla, CA) was used. Conditions for cell growth and media are described elsewhere (9).

Mutations by PCR—For the production of mutants from the postulated P-loop-like structure between the M7 and M8 membrane spans of the α subunit the plasmid LPSKH 5–7 was constructed using pBlueScript® II SK+ (Stratagene, La Jolla, CA) as a template and used for bacteria or yeast transformations. For DNA manipulation in Escherichia coli strains DH5α F' (Life Technologies Inc., Gaithersburg, MD) the plasmid pBlueScript II SK+ (Stratagene, La Jolla, CA) was used. Conditions for cell growth and media are described elsewhere (9).

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Table I

| Primers used in PCR generation of mutations | 
|--------------------------------------------|
| Native sequence | 5′-AGGCTTGAGGATCGCCTAGTGAAC-3′ |
| D884A | 5′-AGGCTTGAGGATCGCCTAGTGAAC-3′ |
| D884R | 5′-AGGCTTGAGGATCGCCTAGTGAAC-3′ |
| D885A | 5′-AGGCTTGAGGATCGCCTAGTGAAC-3′ |
| D885R | 5′-AGGCTTGAGGATCGCCTAGTGAAC-3′ |
| Reverse primer | 5′-ACCGTGAAGACGACGTGAC-3′ |

The abbreviations used are: PCR, polymerase chain reaction; Na⁺,K⁺-ATPase, sodium- and potassium-activated adenosine 5'-triphosphatase (EC 3.6.1.37).

Isolation of Microsomes—Single yeast cell colonies transformed with the vector pCGY1406αβ (9) carrying, besides the β subunit cDNA, also native or mutant α subunit cDNA were grown overnight in 5 ml of YNB medium. Thereafter the cell suspension was transferred into 1 liter of YNB supplemented with all amino acids except tryptophan (13) and was allowed to grow overnight to an A660 of 2–3. Cells were centrifuged and washed twice with H₂O. Breaking of the cells occurred under the conditions described previously through the use of a bead-beater (14).

Microsomes were isolated as described (9, 14) and suspended in 25 mM imidazole, 1 mM EDTA (Tris form), pH 7.4. Protein was determined by the method of Lowry et al. (15). Before use in experiments, microsomes were centrifuged in a bench-top centrifuge at 13,000 × g for 5 min and washed twice with 10 mM Tris-HCl, pH 7.4. This procedure was repeated twice and the pellet was suspended in Tris-HCl, pH 7.4. Protein concentration was determined again and adjusted to 1 mg/ml. Due to this last washing procedure, the sodium or potassium content in the protein suspension was less than 0.05 mM, as assessed by flame photometry.

Scatchard Analysis of [³H]Ouabain Binding—A total of 250 μg of microsomal protein was incubated in equilibrium (60 min) at 30 °C with various concentrations of [³H]ouabain (Amersham, Little Chalfont, United Kingdom; 48 mCi/mmol) in 5 mM PO₄ (Tris form), 5 mM MgCl₂, and 10 mM Tris-HCl, pH 7.4. The final volume of each incubation mixture was 250 μl. Thereafter the protein was pelleted at room temperature by centrifugation for 2 min at 13,000 × g in a bench-top centrifuge. The pellet was washed twice with 1 ml of ice-cold water and centrifuged again under the same conditions. The protein pellet was then dissolved in 250 μl of 1 M NaOH at 70 °C for 15 min, and after neutralization with 250 μl of 1 M HCl, the amount of bound [³H]ouabain in the pellet was determined by scintillation counting. Nonspecifically bound [³H]ouabain was determined in the presence of excess nonradioactive ouabain (3 nm). Maximum ouabain binding (Bmax) and dissociation constants (Kd) were calculated from a Scatchard plot (16).

Promotion of [³H]Ouabain Binding by ATP—Microsomes from yeast cells expressing either the native enzyme or mutants D884A, D884R, D885A, D885R, or D885E were incubated at a concentration of 1 mg/ml for 5 min at 30 °C with 50 mM NaCl, 5 mM MgCl₂, and 50 mM [³H]ouabain and various concentrations of ATP (Tris form) in 10 mM Tris-HCl, pH 7.4. The final volume of each sample was 250 μl. Afterward microsomes were centrifuged at 13,000 × g for 2 min and the pellets were washed twice with 1 ml of H₂O at 4 °C. Bound [³H]ouabain was determined as described for the Scatchard analysis protocol.

Promotion of [³H]Ouabain Binding by Na⁺—A total of 125 μg of the microsomal protein isolated from yeast cells expressing either the native enzyme or mutants D884A, D884R, D885A, D885R, or D885E was incubated at a concentration of 1 mg/ml for 5 min at 30 °C with 50 mM NaCl, 5 mM MgCl₂, and 50 mM [³H]ouabain and various concentrations of NaCl. The final volume of each sample was 250 μl. Bound [³H]ouabain was determined as described above. Nonspecifically bound [³H]ouabain was determined in the absence of ATP or in the presence of 3 mM nonradioactive ouabain.

Promotion of [³H]Ouabain Binding by Inorganic Phosphate—A total of 125 μg of the microsomal protein was incubated in 250 μl of 10 mM Tris-HCl, pH 7.4, containing, 50 mM [³H]ouabain, 5 mM MgCl₂, and various concentrations of PO₄ (Tris form), pH 7.4. Incubation was carried out for 60 min at room temperature. Bound [³H]ouabain was determined as described above.

Reduction of [³H]Ouabain Binding by K⁺—The reduction of the binding of [³H]ouabain by K⁺ was measured in incubations of the microsomal protein in 250 μl of 10 mM Tris-HCl, pH 7.4, containing 5 mM PO₄ (Tris form), pH 7.4, 50 mM [³H]ouabain, 5 mM MgCl₂, and various concentrations of KCl. Incubation was carried out for 60 min at room temperature. Bound [³H]ouabain was determined as described above.

Interactions with Eosin Y—Microsomal protein from yeast cells expressing either native or the D884R mutant sodium pumps was incubated at a concentration of 50 μg/ml in 10 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 10 mM NaCl, and 5 mM eosin Y (Sigma). Membranes from nontransformed yeast cells were used in a control experiment. Fluorescence was measured in a Hitachi 3000P fluorometer using an excitation wavelength of 530 nm and monitoring the emission at 545 nm. The band passes for the excitation or the emission light were each 5 nm. After the addition of 12.5 μM ouabain changes in fluorescence were monitored every 5 min. During this process samples were continuously stirred at room temperature.
RESULTS

Scatchard Analysis of [3H]Ouabain Binding to Native and Mutant Na+,K+-ATPase—In its E2 state, Na+,K+-ATPase becomes phosphorylated from inorganic phosphate in the presence of Mg2+ (E2-P; Scheme 1). Ouabain binds with high affinity to this E2-P form of the enzyme, producing a stable (E*2-P, Ouabain) complex (17) that can be easily detected when radioactive ouabain is used. By keeping all other parameters constant, ouabain binding can be measured as a function of ouabain concentration. From a Scatchard plot it is then possible to determine the affinity and the maximal binding capacity of the enzyme for ouabain.

Membranes from yeast cells expressing native Na+,K+-ATPase or any of the mutants bind [3H]ouabain with high affinity under these conditions (Table II). Fig. 1 shows a representative experiment. From Scatchard analyses performed in triplicate a Kd of 5.67 ± 1.51 nM was determined for the E*2-P, Ouabain complex with native enzyme microsomes. Similar Kd values were obtained with microsomes containing the D884A, D885A, or D885E mutants (Table II). In comparison with these values, 3- and 5-fold higher Kd values of 15.7 ± 2.04 and 30.1 ± 4.32 nM were determined for the formation of the same complex with microsomes containing either D884R or D885R mutants of the sodium pump, respectively (Fig. 1 and Table II). Maximum ouabain binding was similar with all membrane preparations. In the representative experiment depicted in Fig. 1, maximum ouabain binding was 1.6 nM with microsomes containing native sodium pumps and 1.0 or 1.3 nM with membranes containing D884R or D885R mutants, respectively.

Effect of K+ on the Binding of [3H]Ouabain to Native or Mutant Sodium Pumps—The amount of the E*2-P, Ouabain complex decreases as a function of the K+ concentration (Scheme 1; Refs. 17 and 18). Therefore, using radioactive ouabain, by determining the amount of the enzyme for K+ it is possible to elucidate the relative affinity of the sodium pump for K+. Ouabain as a function of the Na+ or ATP concentration can be measured using radioactive ouabain.

Microsomes isolated from yeast cells expressing the native Na+,K+-ATPase incorporated [3H]ouabain as a function of the Na+ concentration in the presence of Mg2+ and ATP. Maximal binding of [3H]ouabain was 0.78 pmol/mg protein (Fig. 3). Na+ ions promoted binding of [3H]ouabain with Kd = 1.64 ± 0.01 mM. Similar results were obtained with membranes containing the D884A or the D885A mutants (Table II). Like these enzymes, the D884R mutant of the sodium pump also bound ouabain as a function of the Na+ concentration (Fig. 3), but the relative affinity for Na+ was considerably reduced. The Kd for Na+ promotion of ouabain binding was 8.6 ± 1.35 mM and therefore significantly higher than the Kd determined with the native enzyme. Maximal binding of ouabain to the D884R mutant was 0.65 pmol/mg protein (Fig. 3). Under the same conditions, however, ouabain binding to microsomes containing the D885R mutant of the pump was not sensitive to Na+ (Fig. 3), so that a calculation of Kd was not meaningful. An increase in [3H]ouabain binding was not detected even at rather high (50–500 mM) Na+ ion concentrations (not shown).

To investigate whether this lack of ouabain binding to the D885R mutant was due to a decreased affinity of the mutant for Na+ or to a decreased apparent affinity of this enzyme for ATP, measurement of ouabain incorporation was repeated with various ATP concentrations in the presence of 50 mM NaCl. ATP promotion of ouabain binding to microsomes from yeast expressing the D885R mutant of the enzyme was very low (Fig. 4) and could not be used to calculate the Kd value of the reaction. Use of ATP concentrations as high as 2 mM (not shown) did not result in a considerable increase in ouabain binding for this mutant. Fig. 4, however, also demonstrates

\[ K_{d, \text{Na}^+} \text{ (mM)} \]  

TABLE II

Dissociation constants and relative affinities determined for native and mutant sodium pumps

|            | Kd, ouabain (nM) | Kd, ATP (µM) | Kd, P (µM) | Kd, Na+ (mM) | Kd, K+ (mM) |
|------------|------------------|--------------|------------|--------------|-------------|
| Native     | 5.67 ± 1.51      | 0.66 ± 0.16  | 45.1 ± 0.98| 1.64 ± 0.01  | 3.70 ± 0.75 |
| D884A      | 6.64 ± 1.03      | 0.89 ± 0.13  | ND*        | 2.15 ± 1.36  | ND          |
| D884R      | 15.7 ± 2.04      | 1.01 ± 0.12  | ND         | 8.60 ± 1.35  | 1.90 ± 0.44 |
| D885E      | 8.39 ± 3.10      | 0.98 ± 0.10  | ND         | ND           | ND          |
| D885A      | 5.04 ± 0.36      | 1.09 ± 0.05  | ND*        | 5.0 ± 0.65   | 1.60 ± 0.67 |
| D885R      | 30.1 ± 4.32      | 1.63 ± 0.05  | 153 ± 11.8 | 1.30 ± 0.34  | 1.39 ± 0.34 |

* ND, not determined.  
* k, very low affinity.
that ouabain binding to microsomes containing either native or D884R mutant Na\(^{+}\)-K\(^{+}\)-ATPase was promoted by ATP with \(K_{0.5}\) values of 0.66 ± 0.16 or 1.01 ± 0.12 \(\mu\)M, respectively. Table II summarizes the results obtained from three experiments with microsomes isolated from various clones. ATP promoted in all cases the binding of ouabain with a \(K_{0.5}\) of roughly 1 \(\mu\)M.

**Promotion of [\(^{3}H\)Ouabain Binding by Inorganic Phosphate**—Based on the reaction shown in Scheme 1, ouabain binding in the presence of Mg\(^{2+}\) can be analyzed as a function of inorganic phosphate concentration. As shown in Table II, inorganic phosphate (Tris form) promoted \([^{3}H]\)ouabain binding to membrane preparations containing either native or mutant sodium pumps. Based on three similar experiments involving membranes isolated from various yeast clones, phosphate was found to promote the binding of ouabain to membranes containing native sodium pumps with a \(K_{0.5}\) of 45.1 ± 0.98 \(\mu\)M (Table II). A similar \(K_{0.5}\) value of 50.5 ± 7.65 \(\mu\)M was obtained with membranes containing the D885A mutant. The \(K_{0.5}\) value of 153 ± 11.8 \(\mu\)M obtained with membranes containing the mutant D885R is approximately 3-fold higher than the \(K_{0.5}\) values obtained with the native enzyme or the D885A mutants.

**Binding of Eosin to Native or Mutant Sodium Pumps**—Eosin binding experiments were performed to investigate whether the D885R mutant retains an ATP-binding site capable of recognizing ATP. Eosin, like its covalently binding analog fluorescein isothiocyanate (19, 20), has been shown in a series of experiments to bind within the ATP-binding site of the Na\(^{+}\),K\(^{+}\)-ATPase with high affinity and to display all characteristics of an ATP analog (21–23). Binding to the ATP-binding site causes an increase in fluorescence, which is inhibited or reversed by ouabain (22). A similar behavior was observed upon incubation of yeast membranes that contain native Na\(^{+}\),K\(^{+}\)-ATPase. The initial fluorescence decreased by addition of ouabain in a time-dependent manner with \(\tau_{1/2} = 3.14\ min^{-1}\) (Fig. 5). Similarly, a decrease in fluorescence was also observed with membranes containing the D885R mutant with a \(\tau_{1/2} = 1.98\ min^{-1}\) (Fig. 5). When membranes from nontransformed yeast cells were used in an analogous experiment, no decrease in fluorescence was observed (Fig. 5).

**DISCUSSION**

A direct comparison of the P-loops of voltage-sensitive Na\(^{+}\) channels from skeletal or heart muscle with the hydrophilic part of the sodium pump \(\alpha\) subunit that is located between its M7 and M8 membrane spans reveals a homology in primary structure of about 50% (Fig. 6). Since within these peptides an area of 17 amino acids is 60% homologous (Fig. 6), coincidence appears very unlikely. Within these homologous peptides, Trp\(^{405}\) from the motif Ser\(^{399}\)QDCW\(^{402}\) was shown earlier to be important for the gating of voltage-gated Na\(^{+}\) channels from human skeletal muscle (4). In a recent investigation based on mutagenesis experiments, Asp\(^{406}\) is thought to be localized within the mouth of the pore and to participate in the formation of the selectivity filter of Na\(^{+}\) channels (24). A homologous motif is present in the P-loop of the equivalent channel from heart muscle (\(^{399}\)QDYW\(^{402}\)) and within the hydrophilic loop of the sodium pump \(\alpha\) subunit (\(^{884}\)DDR\(^{887}\)) that connects M7 and M8 membrane spans. This obvious homology leads to the question of whether amino acids within this motif might also be essential for Na\(^{+}\) recognition/conduction by the sodium pump. Comparison of these three sequences reveals that the second (aspartate) and the fourth amino acids (tryptophan) are identical, the first amino acids are homologous and the third amino acids variable. This suggests that the second and fourth, and possibly also the first amino acids of the motif are important for Na\(^{+}\) recognition/conduction. Carboxyl groups were shown in various kinetic experiments to be involved in ion recognition by

**FIG. 2. [\(^{3}H\)Ouabain binding as a function of K\(^{+}\) concentration.** K\(^{+}\) ions cause a decrease in phosphate-- and Mg\(^{2+}\)-dependent ouabain binding. In the experiment shown performed as described under “Materials and Methods” K\(^{+}\) causes a reduction of ouabain binding to membranes containing the native sodium pumps (\(\square\)) or the D884R (\(\Delta\)) or D885R (\(\bullet\)) mutants. Data are from one experiment that is representative of results from three similar experiments. Each point represents the mean of duplicate measurements ± range. From a Dixon-like plot (inset), \(K_{0.5}\) values for K\(^{+}\) inhibition of ouabain binding can be calculated from the intercept of the straight lines with the abscissa.

**SCHEME 2. Binding of ouabain to the sodium pump in the presence of Na\(^{+}\) and ATP.**

![Scheme 2](image-url)
the sodium pump (25–27). For this reason and because mutations of the comparatively bulky tryptophan might result in enzymes with altered conformational states, preference was given in the current study to mutating the two neighboring aspartic acid residues within the884DDRW887 motif of the sodium pump

To investigate their properties, mutants were expressed in the yeast Saccharomyces cerevisiae, a cell that does not contain endogenous sodium pumps or any other proteins that could bind [3H]ouabain. Therefore, ouabain binding is not only a good tool to demonstrate the expression of functional sodium pumps in yeast (9) but also to investigate the properties of mutant enzymes (14). As expected, microsomes derived from yeast cells expressing the native sodium pumps bind [3H]ouabain in the presence of phosphate and Mg2+ with a $K_D$ value of 1.6 mM and 8.9 mM, respectively (see “Materials and Methods” for experimental protocol). Under these conditions ouabain binding to membranes containing the D885R mutant (●) is very low and cannot be used for the calculation of $K_D$ value for Na+. Data are from one experiment that is representative of results from three similar experiments. Each point is the mean (± range) of duplicate measurements.

that mutations did not affect the interactions of the enzymes with the steroid. Microsomes from cells expressing either the D884R or D885R mutants, however, bind ouabain with 3–5-fold higher $K_D$ values of 15.7 ± 2.04 and 30.1 ± 4.32 nM, respectively (Fig. 1; Table II). It is apparent that these mutations somehow affect the affinity of the enzyme for ouabain. Nevertheless, both mutants still bind ouabain with $K_D$ values that clearly indicate high affinity ouabain-binding forms of the enzyme. The concentration of expressed native or mutant sodium pumps is approximately the same, as indicated from the intercepts of the straight lines with the abscissa, ranging from 1.3 to 1.6 nM (Fig. 1). It was demonstrated earlier that a mutation of Arg880 from the M7/M8 connecting loop to Pro880 also causes a reduction in ouabain affinity, thus indicating a possible involvement of Arg880 in recognition of the cardioactive steroid (30). Although this might be the case, conformational effects were not excluded (30). Nevertheless, as detailed further below, it appears that the M7/M8 loop and the amino acids localized therein might be directly involved in the binding of ouabain. The fact, however, that in our experiments the
mutations D884A, D885A, or D885E do not affect ouabain binding, whereas the mutations D884R or D885R cause a reduction of ouabain binding affinity, could be interpreted to indicate some slight alteration of the conformational arrangement of the protein due to the introduction of the positively charged arginines. These conformational changes could result in reduced affinities for ouabain.

Binding of ouabain to native or mutant sodium pumps is inhibited by K⁺ (Scheme 1) (28). Thus, the amount of ouabain bound to the enzyme as a function of K⁺ concentration can serve as a measure of relative affinity, K₀.5, of the enzyme for K⁺ ions (31). As shown in Fig. 2, K⁺ reduces the binding of ouabain to native or mutant phosphoenzymes formed in the presence of phosphate and Mg²⁺ (Scheme 1). The K₀.5 values for the K⁺ effect determined with membranes from cells expressing native enzyme, D884R, or D885R mutants are 3.7 ± 0.75, 1.9 ± 0.44, and 1.39 ± 0.34 mM, respectively (Table II). These values are all within the same order of magnitude, underlining the fact that mutations do not appreciably alter K⁺ recognition by the enzyme. The K₀.5 value for K⁺ determined with the D885R mutant is slightly lower than the K₀.5 value determined with the native enzyme. This apparently higher sensitivity, however, might be due to the decreased affinity of this enzyme for ouabain and is not necessarily the result of an alteration in the affinity of the enzyme for K⁺ ions. The K₀.5 values obtained indicate the action of K⁺ ions on extracellular K⁺-binding sites of the enzymes. They also demonstrate that the mutations did not affect the interaction of α and β subunits with each other, a possibility that had to be taken into consideration since the area where the mutations were introduced is known to be essential for α and β subunit contact (1, 2).

When ouabain incorporation in the presence of ATP and Mg²⁺ was determined as a function of the Na⁺ concentration (Scheme 2), it became apparent that ouabain binding to membranes containing the native enzyme or either of the D884A or D885A mutants is similarly promoted by Na⁺ in the presence of 0.1 mM ATP with K₀.5 values of 1.64 ± 0.01, 2.15 ± 1.36, and 1.60 ± 0.67 mM, respectively. These values are in good agreement with previously published results and indicate the action of Na⁺ ions at cytosolic sites. Na⁺ is known to promote ouabain binding in the presence of ATP and Mg²⁺ with an apparent affinity of 0.6 mM (32) and to stimulate phosphorylation of the enzyme by ATP by acting on cytosolic sites with a K₀.5 value of 1.6 mM (33). The corresponding value of 8.60 ± 1.35 mM obtained with membranes containing the D884R mutant was significantly higher than the values obtained with the native enzyme or the D884A and D885A mutants (Fig. 3; Table II). Apparently the placement of a positive charge in place of Asp⁸⁸⁴ affects the ability of the enzyme to respond to Na⁺. A much more pronounced effect was observed by the substitution D885R. Binding of the steroid to membranes containing the D885R mutant was very low (Fig. 3); thus, a calculation of a K₀.5 value for Na⁺ was not possible. This experiment clearly demonstrates a fundamental change in some of the properties of the enzyme due to the D885R mutation. It does not verify, however, whether the reduction in ouabain binding is associated with an inability of the mutant enzyme to recognize ATP, Na⁺, or both.

To clarify whether the reduced binding observed for these Asp → Arg mutants is due to a reduction in the affinity of the mutants for Na⁺ or ATP, the effects of ATP on ouabain binding were examined under the conditions described in Scheme 2. Fig. 4 shows that ATP promotes ouabain binding to membranes containing either native or D884R mutant Na⁺,K⁺-ATPase with K₀.5 values of 0.66 ± 0.16 and 1.01 ± 0.12 mM, respectively (Table II). Similar values were obtained with the D884A, D885A, and D885E mutants. These values are in good agreement with K₀.5 values determined for ATP binding to sodium pumps from mammalian tissues (34) and demonstrates that the recognition of ATP by the D884R mutant is unchanged. In the same experiment, however, the stimulation of ouabain binding to membranes containing the D885R mutant by ATP was very low (Fig. 4). Ouabain binding under these conditions depends on the conversion of the enzyme from the E₂P to the E₂P conformational state that can only occur after the enzyme is phosphorylated from ATP and Na⁺ is released to the extracellular space (Scheme 2). Therefore, the inability of this mutant enzyme to bind ouabain might be due either to its inability to recognize ATP or to become phosphorylated.

To evaluate whether the enzyme can become phosphorylated, ouabain binding was measured as a function of the concentration of inorganic phosphate. Phosphate promotes ouabain binding to the native enzyme or to the D885A mutant with K₀.5 values of 45.15 ± 0.98 and 50.5 ± 7.65 μM, respectively (Table II). Promotion of ouabain binding by phosphate was also observed for the D885R mutant. Although the K₀.5 value determined, 153 ± 11.8 μM, is 3-fold higher than the other values, the experiment clearly demonstrates that the D885R mutant can still recognize phosphate. Since the D885A mutant recognizes inorganic phosphate with the same relative affinity as the native enzyme, the reduction in affinity observed with the D885R mutant possibly reflects conformational alterations of this enzyme due to the insertion of the positively charged Arg⁸⁸⁴ in place of the negatively charged Asp⁸⁸⁴ and does not indicate a direct involvement of Asp⁸⁸⁴ in the binding of phosphate. In the latter case, one would also expect to detect alterations in phosphate affinity with the D885A mutant.

The results thus far point to a defect in either Na⁺ or ATP binding by the D885R mutant. It was difficult to address the question of whether the D885R mutant can recognize ATP by

| SEQUENCE | SOURCE | REF. | NCBI-ENTREZ |
|----------|--------|------|-------------|
| 334YDYTSNRALAMAWQALRRNFTQFVTETM591 | human | (43) | 184039 |
| 334YDYTSNRALAMAWQALRRNFTQFVTETM591 | human skeletal muscle Na⁺ channel | (44) | 1331037 |
| 867AENGFLPTFLVLYQDAKYMIEEPVYD508 | shop sodium pump α₁ subunit | (45) | 114377 |
| 867AENGFLPTFLVLYQDAKYMIEEPVYD508 | human sodium pump α₁ subunit | (46) | 114374 |
measuring phosphoenzyme formation from ATP, however, because yeast membrane preparations contain H+ -ATPase, an enzyme that utilizes ATP and forms a phosphorylated intermediate with the phosphate derived from the γ-phosphate group of the ATP molecule. Therefore, a different approach was chosen to evaluate the interactions of the mutant enzyme with ATP. Eosin, like its structural analog fluorescein isothiocyanate (19, 20), was shown in several investigations to be an ATP analog that binds within the ATP-binding site of the Na+ -K+ -ATPase (21–23). This binding, which can be detected as an increase in fluorescence, is inhibited or can be reversed by ouabain (22). Thus, the question of whether the D885R mutant can recognize ATP was addressed by investigating the interactions of this enzyme with eosin in the presence of ouabain. As shown in Fig. 5, ouabain causes a time-dependent decrease of the initial fluorescence obtained by the incubation of microsomes containing either the native enzyme or the D885R mutant of the sodium pump with eosin. A similar effect was not observed with microsomes from nontransformed yeast cells, underlining the specificity of the eosin binding. Thus, the D885R mutant is most likely capable of binding ATP in a normal manner.

Taken together, the experimental data indicate that the inability of the D885R mutant to respond to ATP or Na+ is not likely to be due to a major conformational change of the enzyme protein caused by the mutation. First, ouabain binding and its sensitivity to K+ is not essentially affected by either of the mutations, indicating that α and β subunits still assemble to form a functional complex that recognizes K+ or ouabain. Second, if the observed altered properties of the D885R mutant are specific, some alteration of the Kα,β for Na+ should also be observed with the D884R mutant. This indeed is the case. The increased Kα,β value of this mutant for Na+, as compared with the value obtained for native enzyme, clearly shows that the region of the protein where the mutations were introduced is associated with enzyme-Na+ interactions. Finally, the fact that the D885R mutant recognizes phosphate and eosin within the ATP-binding site clearly shows that no major conformational changes occur as a result of the mutation.

Two important questions remain. Why is ouabain binding to the D885R mutant in the presence of Na+ and ATP considerably reduced, although the enzyme is capable of recognizing phosphate and binding the ATP analog eosin? Also, why does a mutation that is, based on today’s knowledge, localized in an extracellular area of the α subunit (1, 2) influence Na+ and ATP recognition at cytosolic sites (32, 33, 35)?

A possible interpretation of the data can be made by taking into consideration the reaction shown in Scheme 2. In this scheme, the binding of ATP and Na+ to the enzyme (E1) leads to enzyme phosphorylation and Na+ occlusion (E1-P(3Na+)) followed by Na+ release from extracellularly accessible Na+ sites. Thus, it may be that Asp884 and Asp885 are localized within a region responsible for occlusion and/or release of Na+ to the extracellular side. In such a case, mutations would result in inhibition of the flow of Na+ ions through the ionophore part of the sodium pump. With the D884R mutant this inhibition can be overcome by increasing either ATP or Na+ concentrations and is experimentally manifested as reduced relative affinities for these substances. The mutation of Asp885 to Arg885, however, seems to be more crucial for Na+ conduction, since an increase in neither ATP nor Na+ concentrations can overcome the inability of the mutant enzyme to bind ouabain.

It should not be concluded, however, that Asp885 as a solitary amino acid constitutes in itself the binding site for Na+ ions coming from the cytosol. If this would have been the case, some significant changes in the interaction of the other mutants with Na+ should have been measured, underlining an absolute requirement for Asp885. Therefore, we have to conclude that it is not the single amino acids Asp884 or Asp885 but a larger and more complex structure that is involved in Na+ conduction. In a recent investigation Asp884 and Asp886 were shown to be involved in the coordination of Na+ and K+ ions (36). It might be that the three-dimensional arrangement of the protein allows Asp884 and Asp885 to participate in the formation of this structure. Consistent with the conclusion that a larger structure is involved in ion conduction and not a single amino acid, neutral or conservative mutations of Asp884 and Asp885 do not affect the interactions of the enzymes with Na+ ions. Rather, the positively charged groups of the D884R and D885R mutations within the structure involved in Na+ conduction possibly have a repellent effect on Na+ ions coming from cytosolic sites, and result in decreased or discontinued conduction and release of Na+ ions to the extracellular space.

Both Asp884 and Asp885 are part of the hydrophilic sequence of the α subunit that is localized between M7 and M8 membrane spans. Although detection of this sequence with antibodies does not always bring clear results regarding its topology (37–39), the fact that this peptide (1) interacts with an extracellular sequence of the β subunit (2) provides sufficient proof for its extracellular localization.

At first glance it might appear unlikely that extracellularly localized amino acids or peptides might be involved in conduc-
tion of ions, but analogous structures known as P-loops (pore-forming loops) have been documented for ion channels and their role in ion conduction is widely accepted, if not undisputed. In a recent investigation from this laboratory (7), it was proposed for the first time that the loop between M7 and M8 membrane spans of the α subunit might form a structure analogous to the P-loops of K⁺ or Na⁺ channels (3–5) or to the hour-class structure of aquaporin (6). In this case, the loop would be invaginated within the plane of the plasma membrane (Fig. 7), possibly interacting with membrane spans. In analogy to the ion channels, this structure could also participate in pore formation and ion conduction. The results obtained in the investigation presented here support this idea. If the M7/M8 loop is accessible from the intracellular side, one could imagine that cytosolic Na⁺ is transferred to this peptide and subsequently released to the extracellular side by a yet unknown mechanism (Fig. 7). This would require a rather flexible protein structure. The fact that the insertion of the sequence QDC(Y)W from the P-loop of Na⁺ is transferred to this peptide and subsequently released to the extracellular side by a yet unknown mechanism (Fig. 7). This would require a rather flexible protein structure. The fact that the insertion of the sequence QDC(Y)W from the P-loop of Na⁺ is transferred to this peptide and subsequently released to the extracellular side by a yet unknown mechanism (Fig. 7). This would require a rather flexible protein structure. The fact that the insertion of the sequence QDC(Y)W from the P-loop of Na⁺ is transferred to this peptide and subsequently released to the extracellular side by a yet unknown mechanism (Fig. 7).}

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