Random Mutagenesis of the Sheep Na,K-ATPase α1 Subunit Generating the Ouabain-resistant Mutant L793P*

(Received for publication, October 20, 1995, and in revised form, April 12, 1996)

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The polymerase chain reaction was used to randomly mutagenize a cDNA cassette encoding amino acids 691-946 of the sheep Na,K-ATPase α subunit. The mutagenized cassettes were used to replace the wild-type region in the full-length cDNA, and pools of mutants were transfected into HeLa cells. After the generation of resistant cells via selection in 0.5 μM ouabain, polymerase chain reaction was used to amplify the mutagenized cassette from the genomic DNA of the stable transfectants. Sequence analysis of the polymerase chain reaction product revealed three amino acid substitutions: I729V, L793P, and K836R. Subsequent site-directed mutagenesis experiments showed that only L793P was important for resistance. To elucidate the role of L793 in ouabain inhibition, additional mutations at this position were prepared. L793A and L793I mutants were constructed and expressed in HeLa cells. Only L793A survived selection using ouabain, which suggested that resistance is not due to the specific substitution of leucine with proline. To explore the mechanism of resistance, apparent affinities of the L793P mutant for sodium and potassium were compared to the wild-type HeLa pump. Although the apparent affinities were comparable for sodium, the mutant had a 2-fold higher apparent affinity for potassium. This suggests that the mechanism of ouabain insensitivity of L793P is due to a perturbation in the region of the enzyme that may include the K⁺ binding site.

Na,K-ATPase is located on the cell membrane of all eukaryotic cells and maintains the electrochemical gradient of the cell. It consists of a larger α subunit and a smaller, glycosylated β subunit. The former is the catalytic subunit; it pumps three Na⁺ ions out in exchange for two K⁺ ions pumped into the cell at the expense of ATP. It is also the site of interaction with cardiac glycosides, compounds that are commonly used to treat congestive heart failure. There is considerable interest in the characterization of the ouabain binding site of Na,K-ATPase to better understand the ouabain binding site with the aim of promoting rational drug design.

The site of cardiac glycoside binding is not fully understood, in part because the three-dimensional structure of the enzyme has not been elucidated. The amino-terminal half of the protein was found to be important after a chimera comprising the amino-terminal half of the ouabain-resistant rat α1 enzyme and the carboxyl-terminal half of the ouabain-sensitive sheep α1 enzyme proved to be ouabain resistant (1). Subsequently, several amino acids in the amino-terminal half of the protein were identified that affected inhibition of the enzyme by ouabain. Using the numbering of Shull et al. (2), these include the cysteine and tyrosine in the first transmembrane domain: Cys-104 (3, 4) and Tyr-108 (4); Gln-111 (1, 2), Pro-118 (4), Asp-121 (5), and Asn-122 (1, 6) in the first extracellular domain; and Tyr-308, located in the extracellular domain between the putative H3-H4 transmembrane domains (7).

The possibility that the carboxy half of the protein may interact with ouabain was initially suggested by the finding that a monoclonal antibody localized to the H1-H2 extracellular domain increased ouabain binding (8). Furthermore, a chimera that was made using the amino-terminal half of the H,K-ATPase and the carboxyl-terminal half of the rat α1 Na,K-ATPase retained sensitivity to ouabain (9). Soon thereafter, Thr-797 (10-12) and Arg-880 (4) were identified as being important in conferring sensitivity to ouabain.

Based on the hypothesis that the carboxy-terminal half of this protein, like the amino-terminal half, potentially has several residues that affect the interaction with ouabain, we have reexamined a region of the sheep α1 subunit that includes amino acids 691-946 using PCR based random mutagenesis. This region encompasses a cytoplasmic region, the putative transmembrane domains H5 and H6, and two large predicted extracellular domains.

**EXPERIMENTAL PROCEDURES**

Materials—Molecular biology reagents were from Amersham Corp., Boehringer Mannheim, Pharmacia Biotech Inc., Promega, QIAGEN Inc., Stratagene, and U. S. Biochemical Corp. Reagents used in enzyme assays were obtained from Aldrich, and National Diagnostics (Atlanta, GA). Tris-ATP was from Sigma, γ[32P]ATP from DuPont NEN, and ouabain from Boehringer Mannheim. Bio-Rad was the source for electrophoresis equipment. Tissue culture supplies were obtained from Life Technologies, Inc., and the Lineberger Cancer Center Tissue Culture Facility at the University of North Carolina. All other reagents were of the highest quality available. The eukaryotic expression vector constructs were described previously (1). The vector, pKC4, has the complete cDNA inserts of either sheep α1 (S1p-1pCK4) or rat α1 (rα1-pCK4) Na,K-ATPase subunits. The sheep α1 cDNA is divided by endonuclease restriction sites into discrete cassettes that are compatible with the M13 multiple cloning site.

Random Mutagenesis—PCR-based random mutagenesis, based on a protocol by Leung et al. (13), is outlined in Fig. 1. The most salient features are that the reactions were conducting using a 10 mM concentration of each of the four deoxynucleotide triphosphates except for dATP, which was at a concentration of 0.2 mM. Furthermore, 0.5 mM MnCl₂ was added so that, with MgCl₂, the total ion concentration was 0.4%. Under these conditions, the fidelity of the Taq DNA polymerase is compromised. It was estimated that the error rate increased from 0.4% under normal conditions to approximately 2% in the altered con-
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Cell Culture and Transfection—HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum and antibiotics. Cells were prepared for transfection as described previously by Burns and Price (10). Each electroporation combined 20 μg of linearized plasmid with 1 × 10⁶ HeLa cells. Electroporation of the randomly mutagenized plasmid used a total of 1 mg of pooled mutant plasmid that was transfected into a total of 5 × 10⁵ cells in 50 separate electroporations. Each electroporation was plated to a 100-mm tissue culture plate. Cells that were resistant to ouabain were isolated using a cloning cylinder. These cell lines were expanded in medium containing 0.5 μM ouabain.

RNA Analysis—Analysis of all ouabain-resistant cell lines used the protocol described by Price and Lingrel (1). Recovery of Mutagenized Cassettes from Stably Transfected Ouabain-resistant Cell Lines and Sequencing—Genomic DNA was prepared for amplification as described previously (10) and was essentially as described by Higuchi (15). The two oligonucleotide primers were the same as those used to amplify the cassette for random mutagenesis. The PCR reactions were essentially the same as above, except that the dNTPs were used in equimolar concentrations and no MnCl₂ was added to the reaction. PCR products were gel purified with the Qiaex gel extraction kit (QIAGEN) and sequenced directly by adapting the methods of Kretz et al. (16) and Kusukawa et al. (17) to more readily accommodate the annealing reaction as outlined in the Sequenase 2.0 kit from U. S. Biochemical Corp. The primer-template ratio was set at approximately 100:1. After the addition of annealing buffer and deionized distilled water, the total volume was 10 μl. This was incubated at 95 °C for 2 min, then put on ice immediately. Sequencing followed, according to the instructions in the Sequenase 2.0 kit, which is based on the method of Sanger et al. (18). To control for the possibility of errors introduced by Taq DNA polymerase, PCR products from eight separate reactions based on the original resistant cell line were pooled. Sequence of site-directed mutants was based on a pool of five separate PCR reactions.

Preparation of Crude Plasma Membranes from HeLa Cells—Membranes were prepared from wild-type HeLa cells and cells transfected with Lys-mutants or rat α1. This was done essentially as described by Price and Lingrel (1), except that cells from five confluent T150 flasks were harvested and cells were homogenized using 30–40 strokes of a Dounce-type homogenizer, and the final preparation was suspended in...
1 mM Tris and 1 mM EDTA, pH 7.4. Protein yields were determined (19) using bovine serum albumin as the standard. Microsome preparations were snap frozen in liquid nitrogen, stored at −70 °C, and used within 3 months.

Na,K-ATPase Activity Assay and Data Analysis—Enzyme activity was determined by the release of γ-32P from γ-[32P]ATP in an assay that was essentially as described by J ewell and Lingrel (20) in a method modified from Brown (21). In brief, assays were prepared in 1.7-mI tubes, split into triplicates, and incubated at 37 °C for 1 h. Tubes were placed on ice, and 250 μl of acid molybdate (5% ammonium molybdate in 2 n H2SO4 containing 9.17% trichloroacetic acid and 2 mM phosphate) was added. Tubes were shaken for 15 s three times at intervals of 5 min and centrifuged briefly; then 500 μl of scintillation fluid. Counts were taken to give an error of <±1%. Less than 20% of the ATP was hydrolyzed over the course of 1 h in any tube. Release of phosphate over the hour was linear in all assays.

Na,K-ATPase activity was determined against increasing concentrations of ouabain, NaCl, and KCl. In ouabain sensitivity experiments, specific enzyme activity was taken as the difference between the activity at 10−4 M ouabain and the test concentrations of ouabain. Activity in ouabain-free medium was taken as 100% activity. The rate of phosphate release was linear at each concentration of ouabain. The specific conditions for the ouabain experiments were as detailed by J ewell and Lingrel (20). All experimental data were analyzed for goodness of fit to either a one-site or two-site Michaelis-Menten equation for microsome preparations expressing mutant enzyme, as well as those from the host cell line (22). Cell lines expressing mutants fit best to the two-site Michaelis-Menten equation, and the test concentrations of ouabain. The higher affinity site was within the standard error for the IC50 of wild-type HeLa cells using the one-site model.

In the ion dependence experiments, specific Na,K-ATPase activity was taken as the difference between activity in ion-free medium and the test concentrations of ion. ATPases that were non-Na,K-ATPases were not dependent on ion concentration. When preparations containing mutant enzyme were assayed, 1 μM ouabain was in the assay medium to inhibit the native ouabain-sensitive pump of HeLa cells. The specific conditions for the ion dependence experiments were as detailed by J ewell and Lingrel (20). In these experiments, 100% activity was activity at Vmax according to the equation,

\[ V_{\text{max}} = \frac{1 + EC_{50}^{B/(B+|B|)}}{1 + EC_{50}^B/(B+|B|)} \quad (\text{Eq. 1}) \]

where \( n = 3 \) for Na+ and \( n = 2 \) for K++; this equation assumes that each ion binding site is independent. Data were also fit to the equation,

\[ V = V_{\text{max}} |B|^{(B+|B|)} \]

(\text{Eq. 2})

where \( n = 3 \) for Na+ and \( n = 2 \) for K++; this equation does not assume independence between ion binding sites. Both equations are from Garay and Garrahan (23). These models were fit using nonlinear regression with SigmaPlot (Jandel Scientific), which fits by minimizing the sum of squares of differences between data and model.

RESULTS

The pool of mutated cDNAs resulted in one resistant clone from a total of 50 tissue culture dishes when transfected cells were selected in 0.5 μM ouabain. This clone, designated RMPCR1, was expanded into a stable cell line and maintained in 0.5 μM ouabain. Genomic DNA was isolated from these cells and PCR was used to amplify the integrated, randomly mutated cassette. The amplion was the expected size based on the cDNA (~780 base pairs; data not shown). Product from endogenous Na,K-ATPase gene of the HeLa genome would be expected to be significantly larger because introns would be included in the PCR product. Northern analysis using a probe targeted to a region coding for the small t intron of the SV40 vector that is inserted between the cDNA and the polyadenylation sequence indicated that the original resistant clone expressed this vector-derived RNA (data not shown). The PCR product recovered from RMPCR1 genomic DNA is therefore likely the result of stable integration of the randomly mutagenized vector into the HeLa genome. The resistant phenotype is likely due to the presence and expression of the mutant sheep α1 cDNA.

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| Construct* | Trial 1 | Trial 2 | Trial 3 | Trial 1 | Trial 2 | Trial 3 |
|------------|--------|--------|--------|--------|--------|--------|
| None       | 0      | 0      | 0      | 0      | —      | —      |
| Sheep α1   | 0      | 0      | 0      | 0      | —      | —      |
| Rat α1     | 11     | 24     | 20     | 16     | 28     | 25     |
| L729V + L793P | 12     | 14     | 16     | 12     | 14     | 16     |
| L793P      | 31     | 16     | 27     | 0      | 0      | 0      |
| L793I      | —      | —      | —      | 0      | 0      | 0      |
| L793A      | —      | —      | —      | 3      | 5      | 6      |

*Construction of sheep α1 Na-K-ATPase mutants is described under "Experimental Procedures." Each transfection used 20 μg of linearized plasmid and 1 × 106 HeLa cells.

b —, not performed.
Fig. 2. **Na,K-ATPase activity inhibited by ouabain.** The inset table shows the IC$_{50}$ mean ± S.E. from the respective cell lines. Each mean was derived from three experiments, each done in triplicate. Membranes were prepared from wild-type HeLa cells and clones were transfected with site-directed mutants, as described under “Experimental Procedures.” All values were corrected for background activity measured in 10$^{-2}$ M ouabain. Percent activity was determined by setting the activity in the ouabain-free assay to 100%. , wild type (wt) HeLa; , rat a1; , L793P-1; , L793P-2. The specific Na,K-ATPase activity (μmol of ATP hydrolyzed per mg of protein/h) of each microsome preparation without ouabain was: wild type HeLa = 33.9 ± 0.54; rat a1 = 29.35 ± 0.73; L793P1 = 11.63 ± 0.13; and L793P2 = 27.55 ± 0.60. Plots for mutants are representative of three assays from at least two separate microsome preparations. Solid lines indicate data fit to the one-site Michaelis-Menten equation; dashed lines indicate data fit to the two-site equation.

**Quence described above (data not shown).** Furthermore, sequencing of PCR product derived from genomic DNA verified that the cDNA that was integrated into the genomic DNA of the host cell contained the site-directed L793P change.

Crude membranes from the two L793P cell lines, as well as from wild-type HeLa cells, and a stable cell line expressing rat a1 were prepared and assayed for the ability of ouabain to inhibit Na,K-ATPase activity (Fig. 2). The L793P1 and L793P2 cell lines are comparable in their resistance to ouabain, which approaches that of the cell line expressing the rat a1 subunit (Fig. 2, inset).

These mutant Na,K-ATPases were also characterized for their dependence on Na$^+$ and K$^+$ ions for activity (Table I) and were compared to wild-type HeLa Na,K-ATPase. These data show that little difference in the dependence of Na$^+$ exists between the L793P mutants and the wild-type enzyme, whereas significant differences are seen in the dependence on K$^+$ on activity. The measured dependence on K$^+$ suggests that the mutant ouabain-resistant enzymes have a higher affinity for K$^+$ than does the wild-type enzyme. Data were fit to both independent and cooperative models, but there was no difference in conclusions derived from these models.

To test the hypothesis that the mechanism of resistance introduced by this substitution is due to a localized change in conformation that may be specific to substitution with proline, two other amino acids with nonpolar side chains were substituted: isoleucine to maintain the size and polarity of leucine, and alanine, to vary the size. As with all site-directed mutants, the mutagenized cassette was sequenced in its entirety before being transfected into the host cells. Table I indicates that resistance of the Leu-793 substitution is not specific to proline substitution, because L793A generated ouabain-resistant colonies. The identities of the L793A clones were verified, as described previously, by collecting genomic DNA from three clonal cell lines, using PCR to amplify the pertinent cassette, and sequencing the PCR product. All three cell lines produced the correct size of amplicon, and sequence analysis revealed a sheep-specific sequence that contained the engineered base changes.

**Discussion**

There were three amino acid changes produced using this PCR-based random mutagenesis method. Interestingly, each amino acid is conserved in all Na,K-ATPases reported to date (24). However, L793P accounted for most if not all of the resistance of the original triple mutant. This was supported by the fact that the IC$_{50}$ values for the L793P stable cell lines were comparable to the IC$_{50}$ for rat a1 construct, a sodium pump that is highly resistant to ouabain (20) (Fig. 2). Although neither the L729V nor the K836R mutations could confer resistance to ouabain, this does not exclude these residues from contributing to ouabain sensitivity, as might have been seen if less conservative substitutions had occurred at these sites.

One possible mechanism of resistance could have been an increase in affinity for both ions, resulting in a more efficient pump. Because enzyme activities in response to increasing concentrations of sodium were not significantly different between the mutant and the host cell, any increase in the efficiency of this enzyme with regard to Na$^+$ is probably negligible. Alternatively, the conformational change that accompanies the catalytic cycle may occur more readily, even in the presence of ouabain. Activity in response to an increasing concentration of potassium showed that the mutant pump could achieve comparable rates of activity at half the concentration of potassium when compared to the host cell response, suggesting that the affinity for potassium is increased in the L793P mutant. Johnson et al. (25) have shown that an increase in K$^+$ concentrations correlates with decreased ouabain binding in the sheep Na,K-ATPase. It is possible that increased affinity for K$^+$ causes increased competition with ouabain at the ouabain binding site, and this may be the mechanism of resistance in the L793P mutant. Although K$^+$ and ouabain binding sites are not thought to be identical (25), leucine 793 may be a residue that these two sites share.

The L793P inserts a proline between two other prolines in a region that is predicted to be a transmembrane domain (2). Because a polyproline sequence forms a left-handed helix with three prolines per turn, it is expected that the local confor-
tion of the protein will be disrupted regardless of the original conformation. However, because the host HeLa cells cannot survive in 0.5 \( \mu \)M ouabain, the L793P mutant must be a functioning protein. To determine whether resistance was specific to the L793P substitution, two conservative substitutions, L793I and L793A, were constructed, expressed in HeLa cells, and selected for their ability to resist inhibition by ouabain. L793I is the most conservative change, so it was not expected that this construct would survive. Because L793A did survive (Table I), resistance is not isolated to any conformational changes inherent to substitution with proline alone. It is difficult to predict what the effects of the alanine substitution may have on this area. It is possible that resistance is due to conformational shifts in the protein that disrupt the relative position of at least part of the fifth transmembrane domain so that interactions between residues of juxtaposed transmembrane domains may redefine either or both of the ouabain and K\(^+\) binding site.

In summary, leucine 793 has been identified as an amino acid in the carboxyl-terminal half of the protein, which plays a significant role in the interaction between ouabain and the sodium pump. This residue resides in the putative fifth transmembrane domain and lies very close to threonine 797, an additional residue recently shown to be involved in ouabain inhibition (10–12). Therefore, this region is clearly important for the interaction between ouabain and the Na,K-ATPase. These findings also support the utility of PCR-based random mutagenesis in the study of proteins, the tertiary structure of which is not available.

Acknowledgments—We thank Dr. Jerry B Lingrel for both the sheep \( \alpha_1 \) and the rat \( \alpha_1 \) subunit cDNA constructs and Dr. Elizabeth Jewell-Motz for invaluable guidance with the Na,K-ATPase assays.