Expression of an Active Na,K-ATPase with an α-Subunit Lacking All Twenty-three Native Cysteine Residues*

Received for publication, May 3, 2000, and in revised form, July 13, 2000
Published, JBC Papers in Press, July 20, 2000, DOI 10.1074/jbc.M003737200

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We have constructed a mutant Na,K-ATPase α-subunit with all native cysteine residues replaced. Using the baculovirus system, this cysteine-less α-subunit and wild-type β-subunit were expressed in High Five cells. After 3 days of infection, cells were fractionated, and endoplasmic reticulum, Golgi apparatus, and plasma membranes were isolated. The molecular activity of the cysteine-less mutant in the plasma membranes was close to the wild-type protein (8223 min⁻¹ versus 6655 min⁻¹). Cation and ATP activation of Na,K-ATPase activities revealed that replacing all 23 cysteines resulted in only a 50% reduction of Kₘ for Na⁺, a 2-fold increase in Kₘ for K⁺, and no changes in Kₘ for ATP. The distribution of α-subunits among the membranes showed a high percentage of cysteine-less protein in the endoplasmic reticulum and Golgi apparatus compared with the wild-type protein. Furthermore, the cellular stability of the αβ assembly appeared reduced in the cysteine-less mutant. Cells harvested after more than 3 days of infection showed extensive degradation of the cysteine-less α-subunit, which is not observed with the wild-type enzyme. Thus the Na,K-ATPase contains no cysteine residues that are critical for function, but the folding and/or assembly pathway of this enzyme is affected by total cysteine substitution.

Na,K-ATPase (EC 3.6.1.3) is a heterodimeric membrane protein that utilizes the energy of hydrolysis of one ATP molecule to transport three sodium ions and two potassium ions against their electrochemical potential gradients. This enzyme is present in the plasma membrane of most eukaryotic cells and is a member of the P-type ATPase family (1) which forms a phosphorylated enzyme intermediate during the reaction cycle. The Na,K-ATPase is composed of a catalytic α-subunit (110 kDa) and a glycosylated β-subunit (~55 kDa); both subunits are required for function. Various isoforms of each of the subunits have been cloned from different species and tissues, and their enzymatic activities have been characterized (2–5).

The most commonly accepted model for the reaction mechanism of the Na,K-ATPase involves the sequential formation of two acyl-phosphate intermediates. The first intermediate, E₆P₃, forms on the transfer of the terminal phosphate of ATP to Asp⁶⁵⁶ in a sodium-dependent fashion. This form of phosphoenzyme intermediate is ADP-sensitive and K⁺-insensitive. When the sodium ions leave the protein, E₆P₃ is converted to E₆P₂, which is a potassium-sensitive and ADP-insensitive intermediate. This is the basis of the Albers-Post model (6) for the reaction mechanism of the Na,K-ATPase, as well as for most P₂-type ATPases (1). Early important observations using N-ethylmaleimide delineated these phosphoenzyme forms and showed that a population of sulphydryl residues, when modified, blocked the E₆P to E₆P transition and inhibited ATPase activity (7). These observations were among the first to demonstrate that cysteine residues may play a key role in the Na,K-ATPase enzymatic cycle. Later works have identified cysteine residues that may be involved in ouabain binding (8, 9), ATP hydrolysis (10, 11), and αβ association (12). In addition, several sulphydryl-reactive reagents have been shown to inactivate the Na,K-ATPase (13–16). However, the modified cysteine residues were not identified, and the functional significance of these residues are unknown. We have shown recently that a Cys-specific maleimide, MIANS,¹ inactivates the Na,K-ATPase activity by selectively modifying Cys⁵⁷⁷ and that inactivation is prevented by preincubation with ATP (11). Although data from our labeling studies demonstrated that Cys⁵⁷⁷ is located in the ATP-binding domain of the Na,K-ATPase α-subunit, it is not clear whether the sulphydryl side chain of this residue is directly involved in ATP binding.

In a very recent work, Shi et al. (17) constructed a panel of ouabain-resistant sheep α-subunit mutants, in each of which one or several of the 23 native cysteine residues were substituted. The α-subunits that contained the single mutations were expressed in HeLa cells, and all but two mutants containing the C242A or C242S substitution were able to support cell growth under ouabain-selective pressure. They concluded in that study that the expression of a functional cysteine-less α-subunit would not be possible because Cys⁴⁴² may play an important role in enzyme function. However, they did not rule out the possibility that Cys⁴⁴² is not essential for function but rather plays a role in protein folding or trafficking and that the expression system used in their study does not result in plasma membrane localization of this particular mutant protein. Of course, the ability to individually substitute single cysteines and produce functional Na,K-ATPase mutants, each with small changes in enzymatic properties, does not mean that a Na,K-ATPase mutant with all 23 cysteines simultaneously substituted would retain activity.

In the present work we have constructed a form of the sheep renal Na,K-ATPase α-subunit in which all 23 of its native cysteine residues (Fig. 1) are removed. This cysteine-less α-sub-

* This work was supported by National Institutes of Health Grant GM39500 (to J. H. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: MIANS, 2-(4’-maleimidylanilino)naphthalene-6-sulfonic acid; ER, endoplasmic reticulum; CAPS, 3-(cyclohexylamino)propanesulfonic acid.
Functional Expression of a Cysteine-less Na,K-ATPase α-Subunit

unit and the wild-type β1-subunit were heterologously expressed in insect cells using a baculovirus expression system. This expression system, first employed in studies of Na,K-ATPase by Mercer and co-workers (18) and recently by us to define the membrane topology of the Na,K-ATPase α-subunit (19), provides the opportunity to study expressed Na,K-ATPases in an environment containing little or no endogenous Na,K-ATPase activity (18, 19). We have examined the properties of this novel Na,K-ATPase mutant lacking all the α-subunit cysteines and find that the Na+, K+, and ATP concentration dependence and turnover number of the phosphoenzyme are only slightly different from the native enzyme. The availability of a cysteine-less α-subunit provides the groundwork for future structure-function studies that utilize introduced cysteines and sulphydryl chemistry.

EXPERIMENTAL PROCEDURES

Plasmids and Construction of Mutants—A sheep α1-subunit cDNA was cloned into the pOCUS-2 vector (Novagen) as a NcoI and Sse8387I fragment. The 23 native cysteine residues in the α-subunit (Fig. 1) were then substituted with alanines or serines via polymerase chain reaction overlap extension mutagenesis (20, 21) using primers listed in Table I. Recombinant baculovirus containing the sheep β1-subunit and the wild-type or cysteine-less α1-subunit cDNA was produced by following the protocols described previously (19). The genomic DNA of the recombinant baculoviruses was isolated by using the Easy-DNA Kit (Invitrogen) and was sequenced to ensure the appropriate cysteine mutations in the α-subunit.

Protein Expression and Purification—Log-phase high viability High Five cells were infected with the recombinant baculoviruses for 3 days and were harvested for protein purification (19). The harvested High Five cell membranes were fractionated on a five-step sucrose gradient, and the ER (endoplasmic reticulum), Golgi apparatus, and plasma membrane fractions of the infected cells were isolated by sucrose gradient fractionation. The plasma membrane fractions of the 3-day infected High Five cells contained the highest Na,K-ATPase activities (reported in Table II) and were used to measure the ligand-binding activities as well as the cation and ATP activation of the enzymes.

The ATP phosphorylation levels of the wild-type and cysteine-less versions of the Na,K-ATPases were measured, and the molecular activities for the two enzymes were calculated. As can be seen in Table II, although the specific activity of the cysteine-less mutant is less than the wild-type protein, both forms have equivalent molecular activity. In other words, despite the lower copy number of the cysteine-less mutant in the plasma membrane, each α(cysteine-less)/β complex is as active as the wild-type protein. The [3H]ouabain-binding activities of the expressed proteins were also determined, which were 18.50 ± 1.00 pmol·mg⁻¹ protein for the wild-type enzyme and 4.36 ± 0.35 pmol·mg⁻¹ protein for the cysteine-less mutant enzyme. This is a further indication of the lower expression level of the cysteine-less protein, and these estimates agree well with those of the phosphorylation levels.

The ouabain-sensitive Na,K-ATPase activities of the expressed proteins were measured in media containing various concentrations of Na⁺ and K⁺, and the corresponding curves for representative experiments are shown in Figs. 2 and 3, respectively. The kinetic parameters of the cation activation are described in Table III, which shows that the cysteine-less mutant displayed a higher apparent affinity for Na⁺ ions and a lower apparent affinity for K⁺ ions when compared with the expressed wild-type enzyme. However, these differences were only about 2-fold in each case and occurred without any changes in the Hill coefficient for ion activation. Similarly, we compared the ATP concentration dependence of the Na,K-ATPase activities of the expressed wild-type and cysteine-less enzymes. Typical results are summarized in Table III. The half-maximal concentrations for ATP were 0.23 mM and 0.17 mM for the wild-type and cysteine-less Na,K-ATPases, respectively.

Sensitivity to Sulphydryl Modification—The wild-type and cysteine-less versions of the Na,K-ATPases were incubated
with MIANS, a cysteine-modifying reagent previously shown to inactivate the wild-type enzyme isolated from renal tissue (11). Typical results are represented in Fig. 4, which shows that, although MIANS inactivated the ATPase activity of the wild-type enzyme, the ATPase activity of the cysteine-less enzyme was not affected.

**Cellular Stability of the Expressed Na,K-ATPases—** After 3 days of infection, the ER and Golgi apparatus membrane fractions contained most of the expressed cysteine-less a-subunits (Fig. 5A, compare lanes 4 and 5 with lane 6), whereas with the expressed wild-type protein a greater proportion is found in the plasma membrane (Fig. 5A, lanes 1–3). The ATPase activity for the wild-type a-subunits remained constant from 3 to 5 days postinfection (10.7 μmol of P_i mg⁻¹ protein h⁻¹ in the plasma membrane), and Western analyses showed that these proteins are not degraded (Fig. 5B, lanes 1–3). The cysteine-less a-subunit, on the other hand, showed lower ATPase activity 5 days postinfection (2.18 μmol of P_i mg⁻¹ protein h⁻¹ in the plasma membrane compared with 3.34 μmol of P_i mg⁻¹ protein h⁻¹ for 3 days postinfection), and Western blots of the membrane fractions showed that the cysteine-less proteins are highly degraded (Fig. 5B, lanes 4–6). Comparable amounts of the β-sub-

### Table I

Oligonucleotide primers used for site-directed mutagenesis

| Mutations | PCR primers (5’–3’) |
|-----------|---------------------|
| C86S      | GGGTCAAGTTCGCGAGCGC |
| C104S     | GATTTGGAACATTTGGCCAG |
| C138S     | GCCTGACATGAGGACGCGC |
| C204S     | GAGGCTGACATGAGGACGCGC |
| C242S     | GAGGCTGACATGAGGACGCGC |
| C336A     | GGGCCTGACATGAGGACGCGC |
| C349S     | GAGGCTGACATGAGGACGCGC |
| C367S     | GGGCCTGACATGAGGACGCGC |
| C421S     | GGGCCTGACATGAGGACGCGC |
| C452S     | GGGCCTGACATGAGGACGCGC |
| C456S,C457S | GGGCCTGACATGAGGACGCGC |
| C511S     | GGGCCTGACATGAGGACGCGC |
| C577S     | GGGCCTGACATGAGGACGCGC |
| C599S     | GGGCCTGACATGAGGACGCGC |
| C656S     | GGGCCTGACATGAGGACGCGC |
| C698S     | GGGCCTGACATGAGGACGCGC |
| C802A     | GGGCCTGACATGAGGACGCGC |
| C911S     | GGGCCTGACATGAGGACGCGC |
| C930A     | GGGCCTGACATGAGGACGCGC |
| C964A     | GGGCCTGACATGAGGACGCGC |
| C983S     | GGGCCTGACATGAGGACGCGC |

### Table II

Characteristics of Na,K-ATPases isolated from plasma membrane preparations of baculovirus-infected High Five cells

| Na,K-ATPase activity | ATP phosphorylation | Molecular activity |
|----------------------|---------------------|--------------------|
|                      | μmol P_i mg⁻¹ protein h⁻¹ | pmol mg⁻¹ protein min⁻¹ |
| Wild type            | 11.02 ± 0.35          | 27.6 ± 2.16         | 6655 ± 562         |
| Cys-less             | 3.34 ± 0.53           | 6.77 ± 0.50         | 8223 ± 1439        |

**Fig. 2.** Na⁺ activation of Na,K-ATPases expressed in High Five insect cells. Na,K-ATPase activity of High Five plasma membranes expressing the wild-type (wt) sheep a1β1 (closed circle) or a1(cysteine-less)β1 (open square) was determined in assay medium containing 0–125 mM NaCl as described under “Experimental Procedures.” Each value below represents the means of duplicate determinations from at least three different membrane preparations.
units were detected by Western analysis in all membrane fractions and did not appear to be degraded (data not shown).

**DISCUSSION**

In the present work we have constructed a version of the sheep renal Na,K-ATPase in which all 23 of the native cysteine residues in the a1-subunit have been replaced by alanines or serines. This cysteine-less a1-subunit and the sheep b1-subunit were heterologously expressed in baculovirus-infected insect cells, and the resulting cell plasma membrane fractions were isolated for functional characterization. We obtained evidence demonstrating that the removal of all 23 cysteines from the native a-subunit does not compromise enzymatic activity.

**Enzymatic Activities of the Expressed Proteins**—Our data show that the expression level of the wild-type Na,K-ATPase is higher than that of the cysteine-less protein (Fig. 5), and this difference is reflected in their ouabain-sensitive ATPase activities (Table II). However, the molecular activities (represented by the phosphoenzyme turnover numbers) of these two Na,K-ATPases, which are independent of the expression levels, are essentially the same within the experimental errors (Table II). The expressed Na,K-ATPases were further characterized by activation with cations and ATP, and the results are summarized in Table III. The cysteine-less version of the Na,K-ATPase, when compared with the expressed wild-type protein, displays a higher apparent affinity for Na$^+$ ions and a lower apparent affinity for K$^+$ ions. This may come about from a small change in a rate constant that results in a shift in $E_1/E_2$ equilibria where $E_1$ forms are favored over $E_2$ forms in the cysteine-less mutant compared with the wild type. In any case, the cation affinities for the two proteins do not differ greatly (Na$^+$ ions: 16.38 mM for wild type, 8.57 mM for cysteine-less; K$^+$ ions: 3.40 mM for wild type, 7.24 mM for cysteine-less) and agree
regarding the functions of the 23 native cysteines in the Na,K-ATPase α-subunit. First, it is clear that none of the cysteines is essential for the structural or functional integrity of the Na,K-ATPase. Our total cysteine-less α-subunit was expressed and targeted to the plasma membranes of baculovirus-infected insect cells (Fig. 5A, lane 6) and showed molecular activity that is similar to the wild type (Table II).

Second, it seems likely that all previous studies have shown that inactivation or modification of the properties of the Na,K-ATPase following selective modification of sulfhydryls is due to the introduction of bulky moieties into the cysteine side-chains and not due to the modification of an essential or important cysteine side chain per se. We have previously shown using chemical modification that Cys 577 is located in the ATP-binding domain (11), i.e., modification of this cysteine inhibits activity and inhibition is prevented by ATP. Data presented here establish that the sulfhydryl side chain of Cys 577 is not directly involved in substrate binding. Several other Cys-directed reagents have also been used to inhibit and study the structure-function relationships of the Na,K-ATPase (13–16). Although the locations of these modified cysteines have not been identified, our data show that none of these residues would be critical for function. Similar observations have been made previously with the Neurospora crassa H⁺-ATPase, another P₂-ATPase. Enzyme inactivation with N-ethylmaleimide led to the suggestions of important cysteine residues in the ATP-binding or catalytic domain of this protein. However, site-directed mutagenesis and analyses of the expressed mutants revealed that cysteines play no essential role in the enzymatic mechanism of the H⁺-ATPase (26, 27).

The third conclusion that can be drawn from our experiments is that Cys 242 plays no functional role in the Na,K-ATPase α-subunit. As described earlier (see Introduction), Shi and co-workers have constructed a panel of ouabain-resistant sheep α₁-subunit mutants in which one to three of the 23 native cysteine residues were substituted (17). The mutants were expressed in HeLa cells, and all but the Cys 242-substituted mutants were able to support cell growth under ouabain-selective pressure. Although Cys 242 does not appear to be located within the catalytic domains, it was concluded in their study that Cys 242 is important for function, and that construction of a cysteine-less α-subunit would not be possible. Their conclusions are at odds with our findings, which clearly show that an entire cysteine-less α-subunit is expressed and fully functional. One explanation to account for this would be that Cys 242 plays a role in protein folding or trafficking, and that the expression system used in that study does not result in plasma membrane localization of this particular mutant protein. Our cysteine-less α-subunit, though fully functional, does appear to have altered properties in protein folding and/or assembly (Fig. 5, lanes 4–6). Using our expression system, it would be interesting to study the trafficking pattern and degradation of a protein in which Cys 242 is introduced back into the cysteine-less background.

Site-directed mutagenesis is a powerful tool that has been used in structure-function studies of membrane proteins by identifying individual residues important for structure and activity. The construction of cysteine-less membrane proteins, such as H⁺-ATPase (26), lactose permease (28), erythrocyte plasma membrane anion exchange protein (29), and rhodopsin (30) has been reported and utilized to study the structure-function relationships of these important proteins. The total replacement of cysteine residues in the Na,K-ATPase α-subunit has enabled us to eliminate any important role for cysteine residues in the α-subunit of the Na,K-ATPase. We have shown here for the first time that the 23 native cysteine residues in
the Na,K-ATPase α-subunit have no essential role in the protein and that these residues can all be simultaneously replaced without any deleterious effect on enzyme function. The successful expression of a cysteine-less Na,K-ATPase α-subunit should be useful for future structure-function studies where individual cysteine residues will be incorporated into different parts of the protein so that spectroscopic methods can be employed to obtain dynamic structure data.

Acknowledgments—We thank Dr. Craig Gatto (Illinois State University) for helpful discussions and advice and Jeremy Johnston and Keiki Hinami for technical assistance.

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