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Functional role of cysteine residues in the (Na,K)-ATPase α subunit

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Abstract

The structural-functional roles of 23 cysteines present in the sheep (Na,K)-ATPase α1 subunit were studied using site directed mutagenesis, expression, and kinetics analysis. Twenty of these cysteines were individually substituted by alanine or serine. Cys452, Cys455 and Cys456 were simultaneously replaced by serine. These substitutions were introduced into an ouabain resistant α1 sheep isoform and expressed in HeLa cells under ouabain selective pressure. HeLa cells transfected with a cDNA encoding for replacements of Cys242 did not survive ouabain selective pressure. Single substitutions of the remaining cysteines yielded functional enzymes, although some had reduced turnover rates. Only minor variations were observed in the enzyme Na^+ and K^+ dependence as a result of these replacements. Some substitutions apparently affect the E1 ↔ E2 equilibrium as suggested by changes in the K_m of ATP acting at its low affinity binding site. These results indicate that individual cysteines, with the exception of Cys242, are not essential for enzyme function. Furthermore, this suggests that the presence of putative disulfide bridges is not required for α1 subunit folding and subsequent activity. A (Na,K)-ATPase lacking cysteine residues in the transmembrane region was constructed (Cys104, 138, 336, 802, 911, 930, 964, 983Xxx). No alteration in the $K_{1/2}$ of Na^+ or K^+ for (Na,K)-ATPase activation was observed in the resulting enzyme, although it showed a 50% reduction in turnover rate. ATP binding at the high affinity site was not affected. However, a displacement in the E1 ↔ E2 equilibrium toward the E1 form was indicated by a small decrease in the K_m of ATP at the low affinity site accompanied by an increase in IC_{50} for vanadate inhibition. Thus, the transmembrane cysteine-deficient (Na,K)-ATPase appears functional with no critical alteration in its interactions with physiological ligands.

Keywords: (Na,K)-ATPase; Cysteine; Structure-function; Mutagenesis; Transmembrane

1. Introduction

The (Na,K)-ATPase transports sodium and potassium ions against their electrochemical gradients across the plasma membrane of eukaryotic cells. The enzyme consists of two major subunits, α (M_r = 112 000) and β (M_r = 35 000 for the protein component). Although various topological models have been proposed for the catalytic α subunit, in recent years a model comprised of ten transmembrane segments has emerged from hydrophobic analysis, protein chemistry studies, and comparison with other members of the P2-type ion transport ATPase family [1–3] (Fig. 1). The large cytoplasmic loop between H4 and H5 transmembrane fragments of the

Abbreviations: Sheep RD α, (Na,K)-ATPase α1 subunit modified by substitutions Gln111Arg and Asn122Asp; TM-Cys-All, α subunit carrying the following substitutions: Cys104, 138, 336, 802, 911, 930, 964, 983Xxx; P_i, inorganic phosphate

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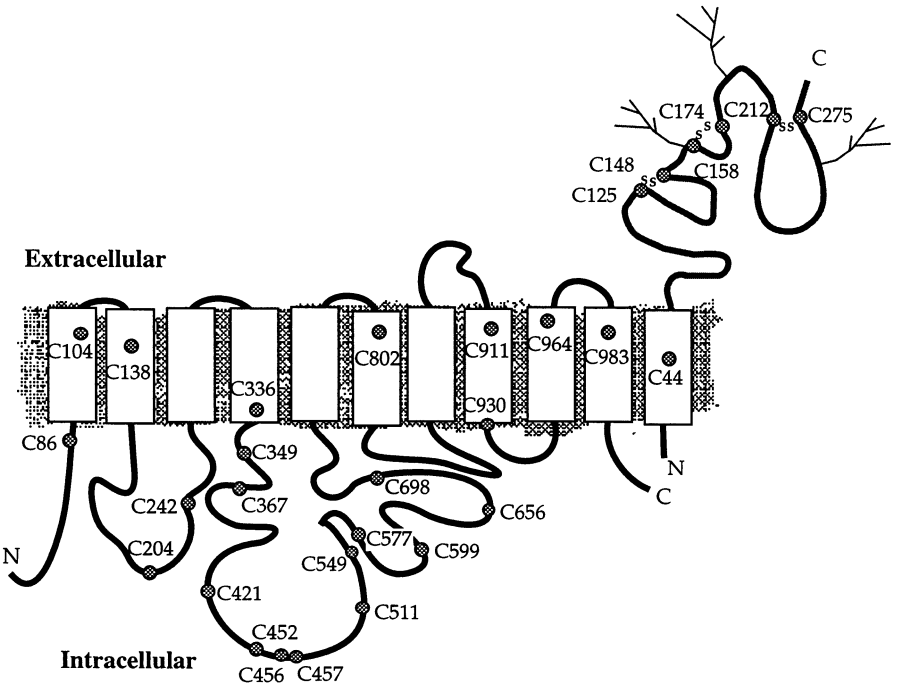

Fig. 1. Membrane topology of the (Na,K)-ATPase. The scheme shows the primary structure positions of cysteines in the α and β subunits and the location of disulfide bridges in the β subunit.

α subunit constitutes the ATP binding and hydrolysis domain [2,4,5]. Within this region, Asp369[1] was identified as the carboxyl residue phosphorylated by ATP during the catalytic cycle [6,7], and Asp710 and Asp714 have been described as being required for ATP binding [8,9]. On the other hand, the cation binding and transport domain includes several transmembrane segments [10,11]. In particular, the hairpin formed by transmembrane segments H5 and H6 plays a key role in ion transport [11,12]. Ser775, Glu779, Asp804, and Asp808, all located in these transmembrane segments, seem involved in cation binding and transport [13–19].

Cysteine amino acids in the (Na,K)-ATPase have been the target of chemical modification and mutagenesis studies directed toward establishing their accessibility, participation in ligand binding, and oxidation state [9,20–37]. Fig. 1 shows the location of these cysteine residues in the primary structure of the sheep α1 and β1 subunits and in relation to the membrane topology. The roles of cysteines in the β subunit have been established [29,34]. This subunit has seven cysteine amino acids [38]. Six of them located in the extracellular portion of the protein, form disulfide bridges [29] (Fig. 1). Site directed mutagenesis of these amino acids indicated that the replacement Cys44Ser does not affect α-β subunit assembly or enzyme activity. Mutagenesis of cysteines involved in Cys158-Cys174 or Cys212-Cys275 disulfide bridges led to a β subunit that did not assemble with the α subunit. Disruption of the Cys125-Cys148 bond produced a tightly assembled but inactive enzyme [34].

In the case of the α subunit, the functional roles of cysteines and the presence of disulfide bonds is less clear. A number of cysteine-specific reagents (some of them ATP analogues) react with the enzyme in an ATP protectable fashion, suggesting the participation of sulfhydryl groups in ATP binding [20–25,36]. Most cysteines modified in these studies have not been identified, although it has been described that Cys656 is a target of 5′-*p*-fluorosulfonylbenzoyladenosine [25] while Cys549 is modified by erythrosin 5′-isothiocyanate [36]. Nevertheless, site directed mutagenesis studies have indicated that these two residues would not be required for enzyme function [9,33].

[1] Throughout this report the amino acid positions corresponding to the sheep α1 and β1 sequences will be used to simplify reading and comparison among systems.

Quantification of free sulfhydryl groups in the α subunit of the enzyme has suggested the presence of disulfide bridges in this protein [23,27,28,30]. Gevondyan et al. [27] indicated the presence of a disulfide bond in the α subunit, suggesting that it might be functionally important. These authors later proposed that peptides containing Cys452, Cys456, Cys457, Cys511 and Cys549 would be cross-linked via a disulfide bond [28]. However, Lane [33] showed that replacement of these cysteines did not inactivate the enzyme nor did they affect the interaction of the enzyme with ATP at the low affinity site. Kirley [30] proposed the presence of three disulfide bridges in the α subunit. Although the involved cysteines were not located, it was suggested that they would be buried within hydrophobic regions of the enzyme since the disulfide bonds were not accessible for reduction by β-mercaptoethanol. Thus, the presence of disulfide bonds in the α subunit of the enzyme has not been established.

Site directed mutagenesis studies have targeted some of the cysteine residues in the α subunit. Those directed to locate amino acids involved in ouabain binding have replaced Cys104 and Cys802 [31,32]. These substitutions did not lead to enzyme inactivation. Wang and Farley [37] proposed the participation of Cys911, together with other amino acids, in α-β assembly. However, the single Cys911Gly substitution did not affect subunit assembly, overall enzyme activity or ouabain binding. Lane et al. [9,33] substituted several cysteines, in order to study their participation in ATP binding (Cys367 and Cys656) or essential disulfide bonds (Cys452, Cys456, Cys457, Cys511 and Cys549). They observed no decrease in activity or alterations in the ATP binding to the low affinity site. However, because of their particular aims, all these studies involved only a partial characterization of the cysteine-substituted enzymes.

Considering the probable participation of cysteines in ATP binding and the putative presence of structurally relevant disulfide bridges, we systematically examined the role of each wild-type cysteine residue in enzyme function. Our objective was also to generate a collection of functional cysteine-deficient enzymes that would facilitate future structural studies. Complementing this goal we generated a functional α subunit devoid of cysteines in the transmembrane region. As with other membrane proteins, a (Na,K)-ATPase with a reduced or minimal number of cysteines could be probed with various sulfhydryl reagents (radioactive, fluorescent, spin-labeled, etc.) to obtain structural information [39–42]. Moreover, cysteine-deficient proteins may be the key for locating those cysteines involved in disulfide cross-links observed after chemical reduction of the (Na,K)-ATPase [43,44].

2. Materials and methods

2.1. Mutagenesis and expression

The eukaryotic expression vector pKC4 was used in these studies. This vector contains the sheep (Na,K)-ATPase α1 subunit cDNA modified by substitutions Gln111Arg and Asn122Asp to encode a form of the enzyme with low affinity for ouabain (RD α) [45]. Site directed mutagenesis was performed by the 'mega-primer' method [46]. In addition to wild-type unique restriction sites, silent restriction sites were engineered in order to divide the RD α1 cDNA into cassettes encoding no more than one transmembrane segment: *Xho*I (aa 59), *Bst*Z17I (aa 110), *Xba*I (aa 229); *Pml*I (aa 309), *Pst*I (aa 511), *Hin*dIII (aa 691), *Kpn*I (aa 797), *Sac*II (aa 830), *Mlu*I (aa 879), *Bgl*II (aa 945), and *Bsu*36I (aa 970). Nucleotide substitutions were made to produce the following amino acid replacements: Cys86Ala, Cys104Ser, Cys138Ser, Cys204Ser, Cys242Ala, Cys242Ser, Cys336Ala, Cys349Ser, Cys367Ser, Cys421Ser, Cys452,456,457Ser, Cys511Ser, Cys549Ser, Cys577-Ser, Cys599Ser, Cys656Ser, Cys698Ala, Cys802Ala, Cys802Ser, Cys911Ala, Cys911Ser, Cys930Ala, Cys964Ser, and Cys983Ala. Each mutant RD α1 cDNA was verified by dideoxynucleotide sequencing. Subcloning of cassettes carrying Cys104Ser, Cys138-Ser, Cys336Ala, Cys802Ala, Cys911Ala, Cys930Ala Cys964Ser, and Cys983Ala mutations allowed the construction of a cDNA encoding the multiple cysteine replacement Cys104, 138, 336, 802, 911, 930, 964, 983Xxx. The resulting protein will be referred to as TM-Cys-All in this report. HeLa cells were transfected with these vectors using liposomes (Lipofectase, Gibco BRL) and selected by inclusion of 1 μM ouabain in the culture medium. Ouabain resistant colonies were isolated from different transfec-

tions and expanded into stable cell lines. HeLa cells were maintained in Dulbecco's modified Eagle's media supplemented with 10% calf serum at 37°C in humidified air at 5% CO_2.

2.2. Membrane preparation

Crude membranes from HeLa cells were prepared using a NaI treatment [13]. Protein determinations were performed in accordance to Bradford [47] using bovine serum albumin as standard.

2.3. Enzyme activity assay

The (Na,K)-ATPase was measured in a medium (mM): NaCl 130; KCl, 20; EGTA, 0.5; $MgCl_2$, 3; ATP, 3; and histidine, 50; pH (20°C) 7.4; 0.3 mg/ml BSA; approx. 1 μg/ml of membrane protein, and either 10 or 0.01 mM ouabain [13]. Choline-Cl was added to the assay media to maintain ionic strength when the cation concentrations were changed (total [monovalent cation] = 200 mM). The assay was performed at 37°C for 30 min and the released inorganic phosphate (P_i) measured by the method of Lanzetta et al. [48]. The (Na,K)-ATPase corresponding to expressed protein was calculated by subtracting the activity observed in the presence of 10 mM ouabain (inhibits both the endogenous human and the heterologously expressed sheep enzymes) from that detected with 0.01 mM ouabain (inhibits the human enzyme). The Na-ATPase was measured in a similar medium, with 200 mM NaCl and 0 mM KCl. The apparent cation affinities were estimated as the $K_{1/2}$ for ATPase activation, fitting activity versus [cation] curves to the equation $v = V_{max}\ L^n/L^n + K_{1/2}$, where L is the cation concentration. The K_m of ATP at the low affinity site was calculated from activity versus [ATP] curves fitted to simple Michaelis kinetics. Vanadate IC_{50} values were obtained by fitting activity versus [inhibitor] curves to the equation: $v = V_{max}/(1+([I]/IC_{50}))$ [16].

2.4. Phosphorylation assays

Na^+ activated phosphorylation was carried out as previously described [14] in medium containing 50 mM NaCl, 1 mM $MgCl_2$, 5 μM [γ-^{32}P]ATP, 0.04 mM EGTA, 100 μg/ml oligomycin, 0.01 mM ouabain, 75 mM HEPES/imidazole, pH (20°C) 7.2, and 0.15 mg/ml membrane protein. In blank tubes NaCl was substituted by 50 mM KCl and oligomycin was omitted. Reactions were initiated by the addition of [γ-^{32}P]ATP and stopped after 30 s at 0°C with 5 vols. of 5% trichloroacetic acid, 1 mM P_i. Radioactivity was measured in a scintillation counter.

3. Results

3.1. Individual cysteine substitution

The sheep (Na,K)-ATPase α1 subunit has 23 cysteines in its sequence [7]. In order to assess their structural-functional role we systematically substituted each single cysteine in the α subunit for Ala or Ser (Table 1), except Cys452, Cys456, and Cys457 which were simultaneously replaced by serine due to their proximity. All cysteine residues were initially replaced by serine except Cys86, Cys242, Cys698, Cys930, and Cys983 where the substitution by alanine facilitated the initial screening of mutated DNA by restriction analysis. In this study we used an expression-ouabain selection system that requires functional heterologous enzymes to support cell growth [45]. We observed that most cysteine-substituted enzymes were able to generate the Na^+ and K^+ gradients required to maintain cell viability. However, cells transfected with cDNA encoding for replacements Cys242Ser or Cys242Ala were unable to survive in the presence of 1 μM ouabain. Increased extracellular KCl concentration had no effect on growth of cells transfected with cDNAs encoding Cys242 substitutions [13]. Cells transfected with cDNA encoding substitutions Cys802Ser and Cys911Ser were also unable to yield colonies in the presence of ouabain; however, the alternative replacements Cys802Ala and Cys911Ala appeared functional and stable expression was possible. Thus, Cys242 appears to be the only cysteine that cannot be removed in the sheep α1 subunit without affecting its expression in this system. The particular structural-functional alterations that the replacements of Cys242 produce were not investigated, but will be the subject of future studies.

Table 1 shows the effect of the introduced substitutions on enzyme activities and phosphoenzyme lev-

Table 1
(Na,K)-ATPase activity, Na-ATPase activity, phosphoenzyme level and turnover number of RD control, and cysteine substituted enzymes

Replacement	(Na,K)-ATPase activity (μmol/mg/h)[a]	Na-ATPase activity (% of total (Na,K)-ATPase)	Phosphorylation (pmol/mg)[b]	Turnover number (1/min)[c]
RD α control	20.9 ± 5.0[d]	12.5 ± 1.9	52.1 ± 13.0	6702 ± 344
Cys86Ala	17.7 ± 5.6	10.3 ± 5.8	64.4 ± 22.2	5072 ± 1102
Cys104Ser	9.4 ± 1.4	< 5[e]	45.8 ± 12.9	3674 ± 476
Cys138Ser	18.2 ± 2.1	9.2 ± 6.5	61.3 ± 4.5	5045 ± 827
Cys204Ser	10.3 ± 5.1	19.1 ± 1.6	45.2 ± 5.8	1844 ± 493
Cys336Ala	6.2 ± 1.2	6.1 ± 3.2	45.9 ± 12.6	2694 ± 383
Cys349Ser	3.0 ± 0.5	< 5	35.5 ± 0.3	1218 ± 214
Cys367Ser	4.9 ± 0.8	22.8 ± 4.3	37.79 ± 9.8	2334 ± 316
Cys421Ser	5.6 ± 1.5	20.0 ± 1.4	80.51 ± 18.0	1509 ± 242
Cys452,456,457Ser	5.3 ± 1.5	20.0 ± 2.5	52.7 ± 6.74	1726 ± 523
Cys511Ser	12.4 ± 4.5	< 5	134.3 ± 32.4	2238 ± 919
Cys549Ser	15.6 ± 3.7	17.4 ± 4.8	70.2 ± 11.8	3724 ± 1006
Cys577Ser	12.4 ± 4.1	22.3 ± 7.0	91.9 ± 35.7	2509 ± 355
Cys599Ser	9.1 ± 2.3	< 5	82.2 ± 28.9	2077 ± 329
Cys656Ser	8.8 ± 3.8	< 5	69.7 ± 20.7	2187 ± 599
Cys698Ala	4.8 ± 1.8	21.3 ± 3.3	58.5 ± 18.8	1989 ± 760
Cys802Ala	15.0 ± 6.4	10.1 ± 7.7	52.8 ± 7.0	4538 ± 1416
Cys911Ala	16.4 ± 3.2	15.0 ± 1.5	95.5 ± 35.1	3497 ± 1255
Cys930Ala	4.6 ± 1.0	13.9 ± 2.9	66.0 ± 28.6	1352 ± 233
Cys964Ser	7.3 ± 1.7	17.5 ± 8.9	59.2 ± 6.5	2044 ± 335
Cys983Ala	7.0 ± 1.1	< 5	32.6 ± 1.9	3642 ± 699

[a]Maximum activity as μmol of hydrolyzed ATP per mg of membrane protein per h.
[b]pmol of phosphoenzyme per mg of membrane protein.
[c]The turnover number was calculated independently for each preparation as the ratio of ATPase activity to phosphoenzyme level.
[d]Values are the mean ± S.E. of $n = 3$ independent clones, each clone was assayed at least in duplicate.
[e]Na-ATPase activity was low and could not be determined accurately.

els. It was observed that most replacements lead to some reduction in enzyme turnover. This effect was larger (more than 50% reduction) in enzymes carrying substitutions Cys204Ser, Cys336Ala, Cys367Ser, Cys349Ser, Cys421Ser, Cys452,456,457Ser, Cys511-Ser, Cys577Ser, Cys599Ser, Cys656Ser, Cys698Ala, Cys930Ala and Cys964Ser. Noticeably most of these are replacements of cysteines in the large cytoplasmic loop (Fig. 1). To determine maximum phosphoenzyme levels, phosphorylation by ATP was measured in the presence of oligomycin [49]. Substituted enzyme phosphorylation levels were similar to those of the RD α control enzyme and the observed variations are likely associated with differences in expression levels. The Na-ATPase activity (measured in the absence of K^+) was also examined. An increase of this activity has been associated with alterations in dephosphorylation steps of substituted enzymes [14,15,50]. None of the studied cysteine-deficient enzymes showed a major effect on this parameter.

Table 2 presents the effects of cysteine residues substitution on the Na^+ and K^+ dependence of the enzyme. The Na^+ dependence of the enzyme was apparently not affected by these replacements, except for small variations in the cooperativity of the activation (Cys367Ser, Cys983Ala). Some substitutions showed a higher apparent affinity for K^+ (Cys86Ala, Cys204Ser, Cys421Ser, Cys549Ser, Cys577Ser, Cys599Ala, Cys656Ser, Cys698Ala, and Cys964Ser) and modest changes in the Hill coefficients of activation curves (Cys204Ser, Cys549Ser, Cys983Ala). Table 2 also shows the ATP dependence of the activity. ATP in the submillimolar range acts at its low affinity site accelerating K^+ deocclusion and the transition to the E1 conformation [49]. Several substitutions (Cys86Ala, Cys204Ser, Cys421Ser, Cys511Ser, Cys656Ser and Cys911Ala) led to significant in-

Table 2
Na^+, K^+ and ATP dependence of the (Na,K)-ATPase activity of RD α control, and cysteine substituted enzymes

Replacement	Na^+ activation		K^+ activation		ATP
	$K_{1/2}$ (mM)	n_{Hill}	$K_{1/2}$ (mM)	n_{Hill}	$K_{1/2}$ (mM)
RD α control	4.34 ± 0.51[a]	2.00	1.17 ± 0.22	1.15	0.225 ± 0.034
Cys86Ala	5.32 ± 0.66	1.82	0.62 ± 0.02	1.17	0.546 ± 0.092
Cys104Ser	6.05 ± 1.26	1.87	1.31 ± 0.26	1.38	0.193 ± 0.049
Cys138Ser	6.33 ± 0.27	2.04	1.57 ± 0.26	1.17	0.118 ± 0.032
Cys204Ser	5.94 ± 1.27	2.07	0.66 ± 0.06	2.07	0.527 ± 0.190
Cys336Ala	6.07 ± 1.35	2.15	1.07 ± 0.11	1.76	0.292 ± 0.058
Cys349Ser	2.02 ± 0.47	2.61	1.85 ± 0.68	0.95	0.332 ± 0.110
Cys367Ser	5.15 ± 1.13	1.51	0.78 ± 0.04	1.11	0.216 ± 0.042
Cys421Ser	5.40 ± 1.33	2.61	0.57 ± 0.17	1.05	1.031 ± 0.164
Cys452,456,457Ser	4.89 ± 1.22	1.88	0.75 ± 0.20	1.05	0.378 ± 0.127
Cys511Ser	4.55 ± 0.34	2.88	0.78 ± 0.17	1.27	0.868 ± 0.237
Cys549Ser	4.49 ± 0.49	2.38	0.55 ± 0.10	1.70	0.315 ± 0.056
Cys577Ser	6.61 ± 0.64	1.80	0.55 ± 0.08	1.33	0.427 ± 0.169
Cys599Ser	5.56 ± 0.48	1.81	0.53 ± 0.26	1.37	0.349 ± 0.164
Cys656Ser	4.91 ± 1.04	2.27	0.56 ± 0.23	1.22	0.539 ± 0.067
Cys698Ala	4.75 ± 0.74	1.48	0.54 ± 0.25	1.82	0.393 ± 0.124
Cys802Ala	3.34 ± 0.03	2.63	1.55 ± 0.04	1.16	0.154 ± 0.080
Cys911Ala	5.31 ± 0.62	2.39	0.81 ± 0.16	1.47	0.723 ± 0.092
Cys930Ala	5.02 ± 0.41	2.03	1.60 ± 0.15	1.06	0.177 ± 0.016
Cys964Ser	6.07 ± 0.44	2.81	0.33 ± 0.11	1.26	0.194 ± 0.052
Cys983Ala	5.97 ± 0.24	1.64	1.09 ± 0.24	2.03	0.212 ± 0.046

[a]Values are the mean ± S.E. of $n = 3$ independent clones, each clone was assayed at least in duplicate.

creases (at least twofold) in the K_m of ATP, which suggest a tendency of these enzymes to remain in E2 conformation. On the other hand, Cys138Ala- and Cys698Ser-substituted enzymes showed a reduced K_m of ATP, indicating an inclination of these enzymes toward E1 conformations.

3.2. *Functional characteristics of the α1 subunit devoid of cysteines in the transmembrane region*

A 'cysteine-less' enzyme would be a formidable tool in studies where cysteine amino acids are engineered at particularly relevant positions and probed with reporter reagents. It seemed unlikely that a 'cysteine-less' (Na,K)-ATPase α1 subunit could be produced, since Cys242 seems essential for enzyme function and replacement of several other cysteines leads to significant reduction in turnover rates (Tables 1 and 2). However, the construction of a molecule for structural studies where cysteines in the transmembrane region have been removed appeared as an achievable goal. To this end, a RD α1 cDNA was constructed encoding for the Cys104Ser, Cys138Ser, Cys336Ala, Cys802Ala, Cys911Ala, Cys930Ala

Table 3
(Na,K)-ATPase activity, Na-ATPase activity, phosphoenzyme level and turnover number of RD α control, and TM-Cys-All enzymes

Replacement	(Na,K)-ATPase activity (μmol/mg/h)[a]	Na-ATPase activity (% of total (Na,K)-ATPase)	Phosphorylation (pmol/mg)[b]	Turnover number (1/min)[c]
RD α control	20.9 ± 5.0[d]	12.5 ± 1.9	52.1 ± 13.0	6702 ± 344
TM-Cys-All	7.6 ± 3.7	9.6 ± 4.7	59.8 ± 10.7	3238 ± 875

[a]Maximum activity as μmol of hydrolyzed ATP per mg of membrane protein per h.
[b]pmol of phosphoenzyme per mg of membrane protein.
[c]The turnover number was calculated independently for each preparation as the ratio of ATPase activity to phosphoenzyme level.
[d]Values are the mean ± S.E. of n = 3 independent clones, each clone was assayed at least in duplicate.

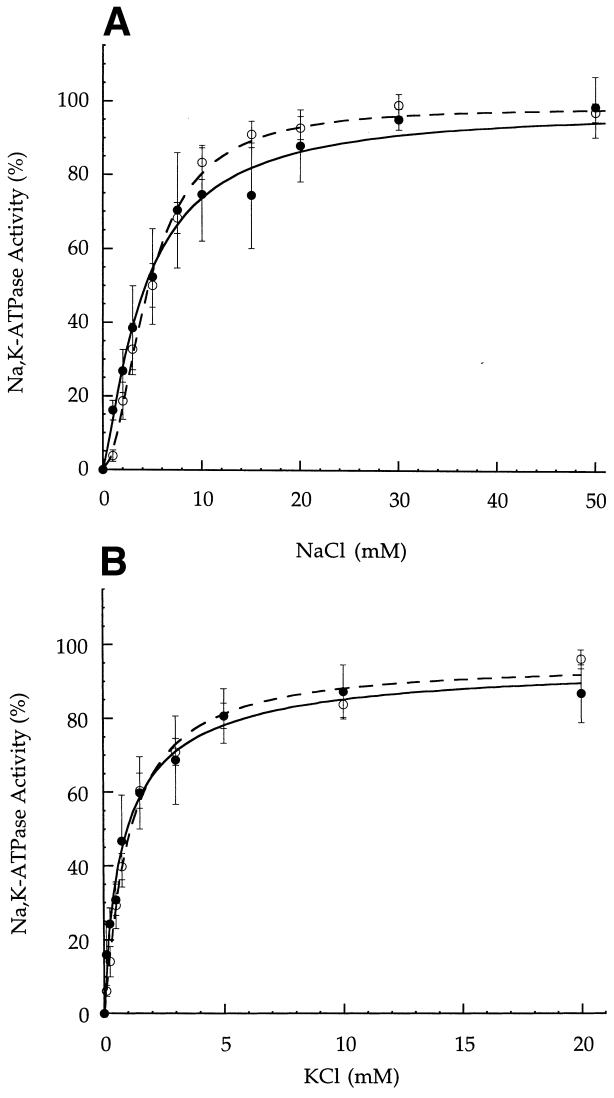

Fig. 2. Na^+ (A) and K^+ (B) dependence of (Na,K)-ATPase activity of RD α control and TM-Cys-All substituted enzymes. The (Na,K)-ATPase activity was determined as indicated in Section 2. The Na^+ dependence was measured in the presence of 20 mM KCl while the Na^+ concentration was varied. The K^+ dependence was measured in the presence of 30 mM NaCl while the K^+ concentration was varied. The values are the mean of results obtained with membrane preparations from three independent clones, each one measured in duplicate. The (Na,K)-ATPase activities corresponding to 100% were similar to the values presented in Table 3. The $K_{1/2}$ values (mM) and Hill coefficients were as follows: (A) Na^+: RD α control enzyme 4.58 ± 0.18, n = 1.89 (○); TM-Cys-All enzyme 4.20 ± 0.43, n = 1.25 (●). (B) K^+: RD α control enzyme 1.02 ± 0.10, n = 1.12 (○); TM-Cys-All enzyme 0.87 ± 0.10, n = 0.87 (●).

Cys964Ser, and Cys983Ala multiple replacements. This cDNA yielded a protein, TM-Cys-All, capable of supporting cell growth in our ouabain selection-expression system. Although TM-Cys-All showed a lower activity compared with that of RD control enzyme (52% reduced turnover number), alterations were not observed in the enzyme steady-state phosphorylation level and the Na-ATPase activity (Table 3).

The TM-Cys-All enzyme was characterized in terms of its dependence on Na^+, K^+ and ATP for activity. Fig. 2 shows that both cations stimulate TM-Cys-All in a manner similar to the RD α control enzyme. No significant changes were detected in Na^+ $K_{1/2}$, K^+ $K_{1/2}$, although a reduction in the cooperativity for Na^+ stimulation of (Na,K)-ATPase activity was observed. The K_m of ATP for (Na,K)-ATPase stimulation was reduced in the TM-Cys-All enzyme (Fig. 3), suggesting a tendency of the enzyme to undergo the conformational change E2(K) → E1+K. Vanadate inhibits the (Na,K)-ATPase by interacting with the E2 form of the enzyme [49]. Thus, the vanadate IC_{50} has been used to test the E1 ↔ E2 equilib-

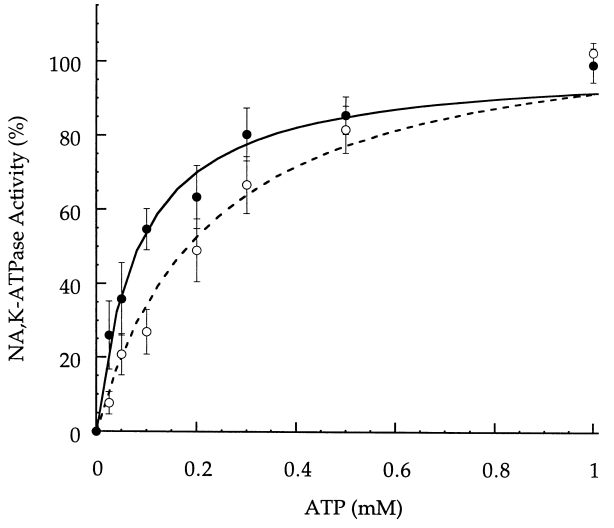

Fig. 3. ATP dependence of (Na,K)-ATPase activity of RD α control and TM-Cys-All substituted enzymes. The (Na,K)-ATPase activity was determined as indicated in Section 2. The ATP concentration was varied as indicated. The values are the mean ± S.E. of results obtained with membrane preparations from three independent clones, each one measured in duplicate. The (Na,K)-ATPase activities corresponding to 100% were similar to the values presented in Table 3. The K_m values of ATP (mM) were as follows: RD α control enzyme 0.225 ± 0.034 (○); TM-Cys-All enzyme 0.083 ± 0.013 (●).

rium of mutated proteins [17,50,51]. In agreement with the reduced K_m of ATP, an increase in the IC_{50} of vanadate was observed for the TM-Cys-All enzyme (Fig. 4). To verify that these multiple substitutions in the transmembrane region have no long-range structural effect on the large cytoplasmic loop, the nucleotide concentration dependence of phosphorylation by ATP was determined (Fig. 5). As expected, the TM-Cys-All enzyme behaved as the control enzyme in this partial reaction. The high affinity interaction of ATP with the enzyme was not affected by the substitutions introduced in the TM-Cys-All protein, suggesting the structural integrity of the nucleotide binding site.

4. Discussion

The results presented in this paper indicate that cysteines in the (Na,K)-ATPase α1 subunit, with the exception of Cys242, are not required for enzyme function. Furthermore, a (Na,K)-ATPase devoid of transmembrane cysteines was constructed and characterized. This enzyme appeared functional and showed no apparent alterations in its interaction with Na^+, K^+, or ATP.

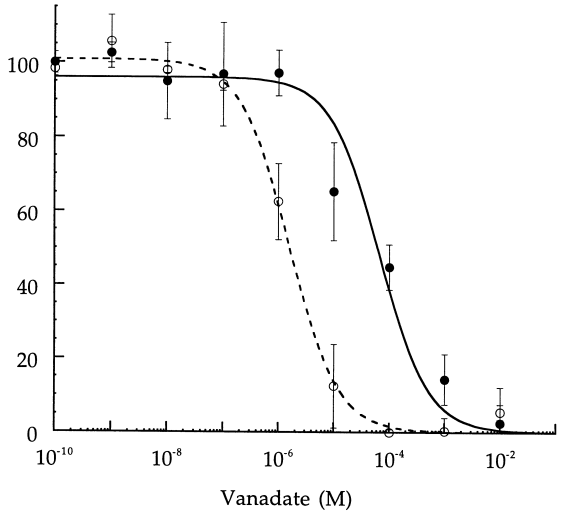

Fig. 4. Vanadate inhibition of (Na,K)-ATPase activity of RD α control and TM-Cys-All substituted enzymes. The (Na,K)-ATPase activity was determined as indicated in Section 2. The vanadate concentration was varied as indicated. The values are the mean ± S.E. of results obtained with membrane preparations from three independent clones, each one measured in duplicate. The (Na,K)-ATPase activities corresponding to 100% were similar to the V_{max} values presented in Table 3. IC_{50} values (μM) were: RD α control enzyme 1.57 ± 0.20 (○); TM-Cys-All enzyme 66.74 ± 2.31 (●).

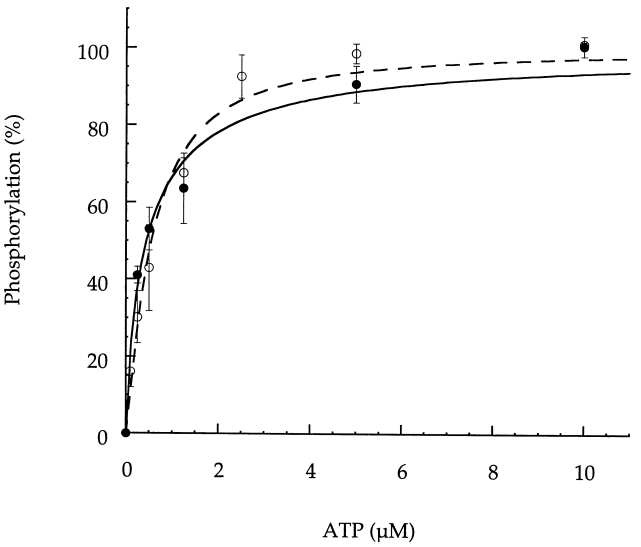

Fig. 5. Phosphorylation by ATP of RD α control and TM-Cys-All substituted enzymes. The phosphorylation levels were determined as indicated in Section 2. The ATP concentration was varied as indicated. The values are the mean ± S.E. of results obtained with membrane preparations from three independent clones, each one measured in duplicate. Phosphoenzyme levels corresponding to 100% were similar to the values presented in Table 3. The K_m values of ATP (μM) were as follows: RD α control enzyme 0.55 ± 0.06, $n = 1.22$ (○); TM-Cys-All enzyme 0.44 ± 0.07, $n = 0.84$ (●).

4.1. Functional roles of cysteine residues in the (Na,K)-ATPase

Most of the 23 cysteines in the (Na,K)-ATPase α subunit are conserved in isoforms from different species. However, a number of them (those at positions 204, 242, 456, 457, 577, 656, 930, 964 and 983) are not conserved in *Drosophila melanogaster*, *Artemia* sp., *Ctenocephalides felis*, or *Hydra vulgaris* isoforms. Moreover, Cys457 is not conserved in α3 and α4 isoforms while Cys456 is absent in α2, α3 and α4, the isoforms found in higher organisms. This suggested that these cysteines would not be required for enzyme function. Surprisingly Cys242 substituted enzymes could not be expressed in our system. One possibility is that replacement of Cys242 leads to functional alterations in the substituted enzymes and consequently these are unable to replace the en-

dogenous isoform function in our expression system. Although it is known that the H2-H3 loop would not be necessary for ATP binding or hydrolysis [4,5], the proximity of this loop to the ATP binding domain and its importance in conformational changes [51] might be related to the effects of Cys242 replacement. Alternatively, replacement of Cys242 might affect the expression and targeting of the heterologous subunit to the plasma membrane. It has been observed that some residue substitutions in different regions of the enzyme affect this process [34,50,52].

Characterization of functional single-cysteine-deficient enzymes indicates that the replacements do not have a major effect on cation-enzyme interactions, or on the ATP-enzyme interaction at the low affinity nucleotide binding site. Comparing these variations with changes produced when relevant residues in the enzyme are replaced [9,13–19], it seems likely that the modest changes observed by substituting cysteines result from small conformational changes rather than from disruption of any specific function of the involved cysteine. These structural changes might be responsible for the reduced turnover numbers observed in some mutants. In this way, the observed results suggest that, although individual cysteines would no participate in the binding of a particular ligand, their replacement introduces structural changes that affect rate limiting steps within the catalytic cycle. Further studies of key cysteines would be necessary to identify the affected steps. Functional substitutions of cysteines at positions 367, 452, 456, 457, 511, 549, 656, and 911 have been described [9,33,37]. Our characterization of these enzymes complements and is in agreement with results previously reported. Considering in particular the replacements of Cys367 and Cys656, Lane et al. [9] described large turnover numbers for the heterologous enzyme and no change in this parameter when comparing substituted and control proteins. The differences in the reported turnover values, as well as the lower turnover described here for Cys367- and Cys656-substituted enzymes, might be due to methodological differences. Nevertheless, a similar overall conclusion can be extracted from these reports, that these cysteines are not essential for enzyme function.

Various studies have postulated the presence of disulfide bridges in the α subunit of the (Na,K)-ATPase [23,27,28,30]. Mutagenesis studies of cysteines participating in disulfide bonds in the β subunit suggest that their replacement, i.e., removal of the disulfide bond, leads to significant functional alterations. Similarly in the sarcoplasmic reticulum Ca-ATPase replacement of either Cys877 or Cys890 (proposed to form a disulfide bridge) produces enzyme inactivation [53,54]. In the case of the (Na,K)-ATPase α subunit, all but one individual cysteine can be replaced without significantly impairing enzyme function. The effects of replacing Cys242 are unlikely due to its participation in a disulfide bond, since similar alterations would have been observed upon replacing its counterpart in the bond. Thus, these results would indicate that disulfide bridges in the (Na,K)-ATPase α subunit, if present, are not required for enzyme function.

4.2. *The α1 subunit lacking cysteines in the transmembrane region*

Diverse studies have used chemical modification of engineered cysteines to obtain structural information on membrane proteins [39–42]. However, in most cases this approach requires the removal of wild-type cysteines that may react with the probes. A partially cysteine-deficient (Na,K)-ATPase would be useful, for instance in scanning cysteine accessibility analysis [41]. The simultaneous replacement of several cysteines without major effects on enzyme function has been achieved in two other P-type ATPases, the sarcoplasmic reticulum Ca-ATPase [54] and the yeast H-ATPase [55]. Considering this, we constructed an α1 subunit without cysteines in the transmembrane region. This cysteine-deficient enzyme proved to be functional, although its activity was reduced. The reduced turnover number of this enzyme might be associated with its probable tendency to remain in E1 conformation as suggested by a reduced low affinity K_m of ATP and the increased vanadate IC_{50}. However, the functional characteristics of the TM-Cys-All enzyme indicate that the introduced substitutions would not produce major structural alterations in the protein. For instance, considering the ATP dependence of phosphorylation, the high affinity nucleotide binding site appears intact in this enzyme. Furthermore, taking into account the relative proximity of the replacements to the putative cation binding sites, it is relevant to point out that the re-

placement of transmembrane cysteines does not lead to any apparent change in the interaction of the enzyme with Na^+ or K^+. Consequently, relevant information might be obtained using the TM-Cys-All enzyme in studies directed to understanding the structure of the enzyme.

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