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Reactivity of Cysteines in the Transmembrane Region of the Na,K-ATPase α Subunit Probed with Hg^{2+}

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Abstract. To gain insight into the structure and conformational coupling in the Na,K-ATPase, this study characterized the reaction of the α1 subunit transmembrane cysteines with a small probe. Intact HeLa cells expressing heterologous Na,K-ATPase were treated with (μM) $HgCl_2$ after placing the enzyme predominantly in either of two conformations, phosphorylated E2P.Na/E2P or dephosphorylated ATP.E1.K/ATP.E1. Under both conditions the treatment led to enzyme inactivation following a double exponential kinetic as determined by ouabain-sensitive K^+ uptake measurements. However, the rate constant of the slow reacting component was ten times larger when the protein was probed in a medium that would favor enzyme phosphorylation. Enzymes carrying mutations of cysteines located in the α1 subunit transmembrane region were used to identify the reacting –SH groups. Replacement Cys104Ser reduced enzyme inactivation by removing the slow reacting component under both treatment conditions. Replacement of Cys964 reduced the inactivation rate constant of the fast reacting component (79%) and removed the slow reacting component when the dephosphorylated enzyme was treated with Hg^{2+}. Moreover, Cys964Ser substituted enzyme was insensitive to Hg^{2+} when treated under phosphorylation conditions. These results indicate that Cys964 is involved in the fast inactivation by Hg^{2+}. Although the double mutant Cys964, 104Ser was still partially inactivated by treatment under nonphosphorylating conditions, an enzyme devoid of transmembrane cysteines was insensitive to Hg^{2+} under all treatment conditions. Thus, this enzyme provides a background where accessibility of engineered transmembrane cysteines can be tested.

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Key words: Na,K-ATPase — Na-pump — Cysteine — Transmembrane — Hg^{2+} — K-uptake

Introduction

The Na,K-ATPase is responsible for maintaining the Na^+ and K^+ gradients across eukaryotic plasma membranes. This protein belongs to the P2-type ion transport ATPase family, which is characterized by the phosphoryl-enzyme intermediate formed during the catalytic cycle (Lutsenko & Kaplan, 1995). In recent years efforts have been directed toward understanding the structure of these enzymes (Horisberger, 1994; Lingrel & Kuntzweiler, 1994; MacLennan et al., 1997). Crystallographic electron microscopy studies of the sarcoplasmic reticulum Ca-ATPase (Zhang et al., 1998) and *Neurospora crassa* H-ATPase (Auer et al., 1998) have produced 8Å resolution structures. These studies have shown important structural characteristics of the P2-type ATPases, particularly in the transmembrane region. However, they do not provide information on the nature of cation binding sites or the identity of transmembrane segments forming the cation path.

The membrane topology of the Na,K-ATPase has been established for both enzyme subunits, α and β. The catalytic α subunit has ten transmembrane fragments and a large cytoplasmic loop between the fourth and fifth transmembrane segments (H4 and H5), while β has a single transmembrane segment and most of its mass toward the extracellular side of the protein (Horisberger, 1994; Lingrel & Kuntzweiler, 1994; Lutsenko & Kaplan, 1995). Although little is known about the conformational changes that couple ATP hydrolysis with cation transport, it has been established that ATP binding and hydrolysis occurs in the large cytoplasmic loop (Horisberger, 1994; Lingrel & Kuntzweiler, 1994), while ion

binding and transport takes place in the transmembrane region of the protein (Karlish et al., 1990; Argüello & Kaplan, 1994; Lutsenko et al., 1995). In particular, the importance of the α subunit fifth and sixth transmembrane segments (H5 and H6) for cation binding and energy transduction has been established (Argüello & Kaplan, 1994; Argüello & Lingrel, 1995; Lutsenko et al., 1995; Kuntzweiler, Argüello & Lingrel, 1996). For instance, residues Ser775, Glu779, Asp804, and Asp808 in these two transmembrane segments appear critical for Na^+ and K^+ interaction with the enzyme (Argüello & Lingrel, 1995; Argüello et al., 1996; Kuntzweiler et al., 1996; Blostein et al., 1997; Pedersen et al., 1997; Nielsen et al., 1998). On the other hand, extensive mutagenesis targeting oxygen-containing residues located in various transmembrane segments has failed to reveal additional amino acids essential for Na^+ and K^+ binding (Argüello et al., 1999; Vilsen, 1993; Kuntzweiler et al., 1995; Vilsen, 1995; Yamamoto et al., 1996; Vasilets et al., 1998). Therefore, although it is likely that additional transmembrane segments may be lining the Na,K-ATPase cation path, only H5 and H6 have been identified thus far.

Scanning cysteine accessibility mutagenesis has been used to explore the ion path of various transporters (Akabas et al., 1992; Lü & Miller, 1995; Kuner et al., 1996; Sun et al., 1996; Bainbridge et al., 1998; Kimura et al., 1998). This approach involves the replacement of individual transmembrane amino acids with cysteines and the subsequent probing of the resulting proteins with membrane-impermeable, cysteine-specific reagents. In the case of the Na,K-ATPase, this methodology may provide unique information on the structure of the ion path. However, a required initial step for these studies is to analyze the reactivity of wild-type transmembrane cysteines with this type of probes, producing a cysteine-deficient enzyme insensitive to the probe. This enzyme could in turn be used as an appropriate background for scanning cysteine accessibility mutagenesis.

Membrane-impermeable reagents modify cysteines in the transmembrane region of the Na,K-ATPase α subunit. N-hydroxysuccinimidyl digoxigenin-3-methylcarbonyl-E-aminocaproate modifies Cys104 in the first transmembrane segment (Antolovic et al., 1995). Lutsenko et al. (1997) have indicated that Cys911 and Cys964 are also exposed to the extracellular media, since the large membrane-impermeable reagent 4-acetamido-4′-maleimidylstilbene-2-2′-disulfonic acid, disodium salt (SDSM) labeled these residues. However, prolonged treatment led to only a partial enzyme inactivation. On the other hand, treatment with membrane-impermeable thiosulfonate derivatives like [2-(trimethylammonium)ethyl] methanethiosulfonate (MTSET) does not inhibit the wild-type Na,K-ATPase by reacting with transmembrane cysteines (L. Mikhaylova and J.M. Argüello, *unpublished results*). The ligand-dependent reversible inhibition of Na,K-ATPase by treatment of membrane preparations with Hg^{2+} has been described (Anner, Moosmayer & Imesch, 1990, 1992; Vassallo et al., 1999). In particular, the extracellular treatment with Hg^{2+} also leads to inactivation of the Na,K-ATPase (Wang & Horisberger, 1996). Replacement of Cys104 partially reduces this inactivation (Wang & Horisberger, 1996). The involvement of other transmembrane cysteines in inactivation by Hg^{2+} has not been explored.

Toward establishing conditions for scanning cysteine accessibility mutagenesis, this work characterized the reaction of wild-type transmembrane cysteines in the Na,K-ATPase α subunit with a small membrane-impermeable cysteine-specific probe such as Hg^{2+} and identified those cysteines whose modification leads to enzyme inactivation. Enzymes carrying single and multiple substitutions of wild-type cysteine residues located in the transmembrane region of the enzyme were used in these studies. The simultaneous or individual replacement of these cysteines with alanine or serine yielded functional proteins (Shi et al., 2000). Although these substitutions in certain cases led to some reduction in enzyme turnover, they had little or no apparent effect on the enzyme interaction with Na^+, K^+ or ATP (Shi et al., 2000). Thus, these enzymes were suitable tools for testing the reactivity of transmembrane cysteines with an externally applied probe.

Materials and Methods

Mutagenesis, Expression, and Tissue Culture

The eukaryotic expression vector pKC4 was used in these studies. This vector contained 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 α1) (Price & Lingrel, 1988). Site directed mutagenesis were performed by the "megaprimer" method (Sarkar & Sommer, 1990). Nucleotide substitutions were made to produce the following amino acid replacements: Cys104Ser, Cys138Ser, Cys336Ala, Cys802Ala, Cys911Ala, Cys930Ala, Cys964Ser, and Cys983Ala. The multiple cysteine replacement (Cys104, 138, 964Ser, Cys336, 802, 911, 930, 983Ala) (All-TM-Cys) was constructed by subcloning cassettes encoding single amino acid substitutions as previously described (Shi et al., 2000). These proteins were stably expressed by HeLa cell lines maintained under 1 μM ouabain selective pressure in Dulbecco's Modified Eagle's media supplemented with 10% calf serum, at 37°C in humidified air with 5% CO_2 (Price & Lingrel, 1988).

Hg^{2+} Treatment of Intact Cells Expressing Cysteine-Deficient Enzymes

HeLa cells expressing the heterologous proteins were grown in 24 well plates. Cells were rinsed with (mM) 60, NaCl; 100, CholineCl; 5, glucose; 0.5, $MgCl_2$; 4, Pi-Tris, pH 7.4 (Solution A) and then treated in two different systems that differ in the Na^+ and K^+ concentrations.

All procedures were performed at 37°C. "*Na-medium*": The cells were preincubated for 5 min in a solution containing (mM) 5, NaCl; 135, CholineCl; 5, glucose; 0.5, $MgCl_2$; 4, Pi-Tris; pH 7.4; 0.01, bumetanide; 0.012, monensin; and either 0.001 or 2, ouabain. After preincubation, the cells were treated in this medium plus the indicated $HgCl_2$ concentration for various periods of time. "*K-medium*": The cells were preincubated during two 5 min periods in a solution containing (mM) 5, KCl; 135, CholineCl; 5, glucose; 0.5, $MgCl_2$; 4, Pi-Tris; pH 7.4; 0.01, bumetanide; 0.012, monensin; and either 0.001 or 2, ouabain. The solution was changed after each 5 min preincubation period. Following preincubation, the cells were treated in this medium plus the indicated $HgCl_2$ concentration for various periods of time.

K^+ Transport Assays

K^+ uptake into HeLa cells, using $^{86}Rb^+$ as congener, was measured as described by Munzer et al. (1994). After Hg^{2+} treatment under different conditions (either *Na-medium* or *K-medium*) cells were rinsed and incubated 5 min at 37°C in Solution A plus (mM) 5, KCl; 0.01, bumetanide; 0.012, monensin; and either 0.001 or 2, ouabain. Uptake was initiated by replacing the media with Solution A plus (mM) 1.5, KCl; $^{86}Rb^+$ (0.5 μCi); 0.01, bumetanide; 0.012, monensin; and either 0.001 or 2, ouabain. The assay was performed at 37°C for 10 min and ended by removing the media and washing the cells with ice-cold Solution A. The cells were solubilized with 0.2 M NaOH and aliquots were taken for protein and radioactivity quantification. Protein determinations were performed in accordance to Bradford (1976) and radioactivity was measured in a scintillation counter. K^+ uptake corresponding to expressed protein was calculated by subtracting the uptake observed in the presence of 2 mM ouabain (inhibits both the endogenous human and the heterologously expressed sheep RDα1 enzymes) from that measured in the presence of 0.001 mM ouabain (inhibits the human enzyme). Values in the plots are the mean ± SE of three independent clones measured at least in duplicate.

Curve Fitting

Activities *vs.* treatment time curves were fit assuming a two-step inactivation process (Ray & Koshland Jr., 1961; Argüello & Kaplan, 1990). Thus the rate of inactivation is described by:

$$-\delta[E]/\delta t = \delta[E_i']/\delta t + \delta[E_i'']\delta t \quad (1)$$

Upon integration:

$$[E]/[E_o] = \alpha e^{-k1't} + (1 - \alpha)e^{-k2't} \quad (2)$$

Where:

$$k' = k[I]^n \quad (3)$$

In these equations E represents the active enzyme form, E_i' and E_i'' are partially inhibited forms of the enzyme, I is Hg^{2+} concentration in the treatment medium, k_i are the rate constants and n the average order of the reaction for each site.

Activities *vs.* inhibitor curves were measured after 1 min treatment when the contribution to the inactivation by the slow reacting component is minimal. Thus, these curves were fit to a single inhibitor site model using $v = V_{max}/(1 + ([I]/IC_{50}))$ (Eq. 4). Curve fitting was carried out using commercially available software (KaleidaGraph, Synergy Software, Reading, PA).

Abbreviations

The abbreviations used are: SDSM, 4-acetamido-4′-maleimidylstilbene-2-2′-disulfonic acid, disodium salt; MTSET, [2-(trimethylammonium)ethyl] methanethiosulfonate; sheep RD α1, Na,K-ATPase α1 subunit modified by substitutions Gln111Arg and Asn122Asp; TM-Cys-All, α subunit carrying the multiple substitutions Cys104, 138, 964Ser, Cys336, 802, 911, 930, 983Ala.

Results

RD α1 Control Enzyme Inactivation by Extracellular Hg^{2+} Treatment

The goal of this work was to characterize the inactivation of the Na,K-ATPase by modification of transmembrane cysteines with a probe applied at the extracellular side of the enzyme. HeLa cells expressing the RDα1 control enzyme were treated with Hg^{2+} and the resulting inactivation was followed by measuring ouabain-sensitive K^+-uptake. Considering that proposed movements of transmembrane segments during the catalytic cycle might affect transmembrane cysteine modification (Nagai et al., 1986; Argüello & Kaplan, 1991, 1994; Lutsenko et al., 1995; Sarvazyan, Modyanov & Askari, 1995), the enzyme was probed under nonturnover conditions and while it was driven into two particular conformations. This was achieved by modifying the intracellular Na^+ and extracellular Na^+ and K^+ concentrations during treatment. Cells expressing heterologous enzymes were treated with the probe under two conditions: (i) "*Na-medium*", cells were allowed to equilibrate in a 5 mM extracelllular Na^+ (0 mM K^+) medium in the presence of monensin (Munzer et al., 1994). Under these conditions the enzyme turnover would be negligible (Garrahan & Glynn, 1967*a,b;* Glynn, 1985) and >80% of the protein would remain predominantly in the E2P.Na/E2P conformations (Beaugé & Glynn, 1979, *see also* Table I in Heyse et al., 1994). (ii) "*K-medium*", cells were preincubated in 5 mM extracellular K^+ (0 mM Na^+) medium in the presence of monensin. Under this condition, 0 mM free intracellular [Na^+] has been previously assumed (Harootunian et al., 1989; Zahler et al., 1997) and experimental data has showed no net Na^+ efflux (Zahler et al., 1997). Furthermore, we have assumed that at 0 mM (or extremely low) Na^+ and in the presence of millimolar K^+ the enzyme is driven into an ATP.E1.K/ATP.E1 conformation (Glynn, 1985; Heyse et al., 1994), even when a small K^+:K^+ exchange might be present (Karlish & Stein, 1982; Glynn, 1985). Thus, the enzyme was treated under conditions favoring either its dephosphorylated (*K-medium*) or phosphorylated (*Na-medium*) forms, with ions likely occupying their binding sites.

Figure 1 shows the reduction of ouabain sensitive K^+-uptake by the RD α1 control enzyme after 1 min

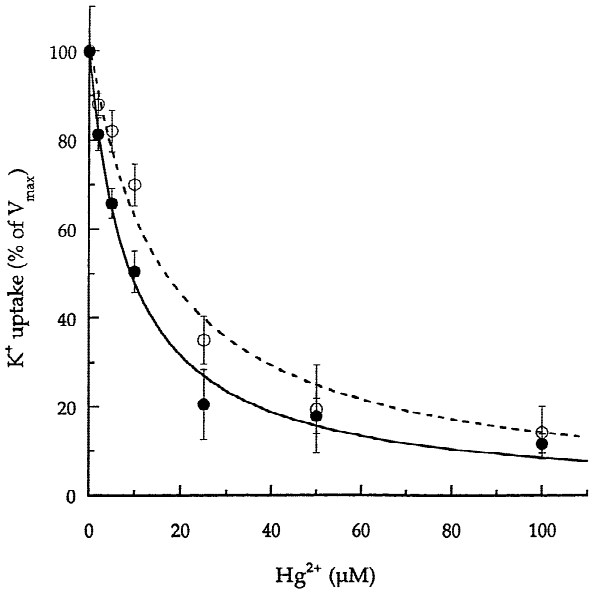

Fig. 1. Inactivation of RDα1 Enzyme by Extracellular Treatment with Hg^{2+}. HeLa cells expressing RDα1 enzyme were treated for 1 min with the indicated [Hg^{2+}] in *K-medium* (●) or *Na-medium* (○) as indicated in Materials and Methods. The K^+ uptake corresponding to 100% was 120 nmol/mg of cell protein. No differences were observed in the maximum K^+ uptake values after preincubation in *K-medium* or *Na-medium*. Background uptake measured in the presence of 2 mM ouabain was not modified by Hg^{2+} treatment. The data were fit to a single site inhibitor model (Eq. 4). IC_{50} (μM) were 10 ± 1 μM, *K-medium;* 22 ± 3 μM, *Na-medium*.

treatment of whole cells with $HgCl_2$. Ouabain-insensitive (background) K^+-uptake was not affected by the probe under any conditions used in this study. At this short treatment time only a fast reacting site was evident (*see below*) and the inactivation was analyzed by a single inhibitor site model. Under both treatment conditions Hg^{2+} fully inactivated the Na,K-ATPase with an IC_{50} slightly influenced by the enzyme conformation (10 ± 1 μM in the *K-medium* and 22 ± 3 μM in the *Na-medium*).

Figure 2 shows the time course of inactivation of the RD α1 enzyme by extracellular treatment with various Hg^{2+} concentrations under the two described treatment conditions. Thiols form strong complexes with Hg^{2+} ($K_D \approx 10^{-40}$–10^{-44}) (Jocelyn, 1972). Taking into account the absence of competing ligands in the treatment media, we assumed a primarily irreversible reaction of Hg^{2+} with the enzyme. Then, inactivation kinetic curves were best described by a two-step model where a fast and a slow reacting component are observed (Ray & Koshland Jr., 1961). Due to methodological constraints (manual exchange of solutions and endpoint Rb^+ uptake determinations), the shortest treatment time studied was 1 min. Consequently, the reaction rates of the fast reacting components were probably underestimatd by the curve-fitting analysis. Nevertheless, the fitting param-

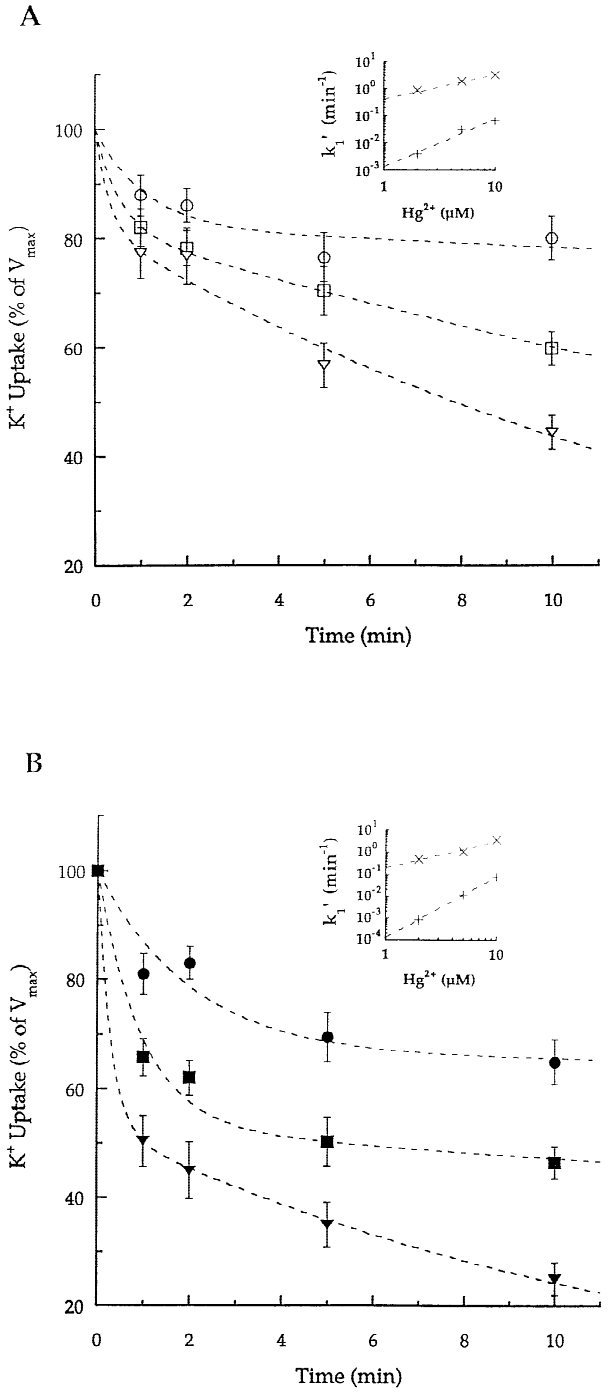

Fig. 2. Time Course of RDα1 Enzyme Inactivation by Extracellular Treatment with Hg^{2+}. HeLa cells expressing RDα1 enzyme were treated during different times with 2 μM (○, ●), 5 mM (□, ■), or 10 μm (▽, ▼); [Hg^{2+}] in *Na-medium* (*A*) (○, □, ▽) or *K-medium* (*B*) (●, ■, ▼) as indicated in Materials and Methods. The data were fit to a two-step inactivation model (Eq. 2). To determine the inactivation rate constants k_1 and k_2 (Eq. 3); k_1' and k_2' were plotted against [Hg^{2+}] (inserts). Fitting parameters were as follows: (*A*) $\alpha = 0.18$; $k_1 = 0.41 \pm 0.01$ min^{-1} μM^{-n}, $n = 0.9$; $k_2 = 0.0014 \pm 0.0001$ min^{-1} μM^{-n}, $n = 1.7$; (*B*) $\alpha = 0.47$; $k_1 = 0.21 \pm 0.02$ min^{-1} μM^{-n}, $n = 1.2$; $k_2 = 0.00012 \pm 0.00005$ min^{-1} μM^{-n}, $n = 2.8$.

eters (α, k_1', k_2', and from these k_1, k_2, n) provide an initial description of the Na,K-ATPase inactivation by Hg^{2+}. The effects of the enzyme conformation on the inactivation characteristics was evident in the rates of inactivation and in the fraction of enzyme (α) undergoing inactivation through the fast reacting site. For instance, this latter parameter was larger when the enzyme was treated in *K-medium* ($\alpha = 18$, *Na-medium;* $\alpha = 47$, *K-medium*). The inserts in Fig. 2 show the linear relationships between the log k′ and log [Hg^{2+}] (Eq. 3). The inactivation rate constants of each component (k_1 and k_2, respectively) were obtained from the intercepts of these lines. The analysis of these parameters indicated that while the inactivation rate constant of the fast reacting component was slightly affected by the enzyme conformation ($k_1 = 0.2 - 0.4$ min^{-1} μM^{-n}), the slow component appeared to react ten times faster when the enzyme was treated in *Na-medium* ($k_2 = 0.0014$ min^{-1} μM^{-n}, *Na-medium;* $k_2 = 0.00012$ min^{-1} μM^{-n}, *K-medium*). The presence of more than one reaction site was also suggested by the exponential dependence of the slow component apparent rate constant on Hg^{2+} concentration ($n = 2.8$, *K-medium;* $n = 1.73$, *Na-medium*) (inserts of Fig. 2). Conversely, the order of the reaction for the fast component ($n \approx 1$) is consistent with only one modification site involved in this particular reaction.

An additional aspect of the reaction of the enzyme with Hg^{2+} is that, as previously observed (Anner et al., 1992; Vassallo et al., 1999), the inactivation was largely removed by including 2 mM Cysteine in the washing and incubation media used after Hg^{2+} treatment. For instance, 95–98% activity was recovered by this method after 10 min treatment with 10 μM Hg^{2+}.

Cysteine-Deficient Enzymes Inactivation by Extracellular Hg^{2+} Treatment

The characteristics of the Na,K-ATPase inactivation by Hg^{2+} suggest that several cysteines were reacting with the probe. To identify these cysteines, HeLa cells expressing enzymes carrying substitutions of single transmembrane cysteines were tested. It was expected that replacement of reacting cysteines would prevent inactivation or significantly reduce its rate. The Na,K-ATPase has eight cysteines in the transmembrnae region of its α subunit (Cys104, Cys138, Cys336, Cys802, Cys911, Cys930, Cys964, and Cys983) (Shull et al., 1985), plus one in the transmembrane region of the β subunit (Cys46) (Shull et al., 1986). Replacement of the α subunit cysteines, individually or simultaneously, produces functional enzymes (Shi et al., 2000).

Figure 3 shows the effects of treatment of cysteine-deficient enzymes with Hg^{2+} in *Na-medium.* When HeLa cells expressing cysteine-deficient enzymes were treated with 10 μM Hg^{2+} in the *Na-medium* for 10 min, it was apparent that only replacements Cys104Ser and Cys964Ser could prevent, at least partially, enzyme inactivation (Fig. 3*A*). A more detailed analysis showed that replacement of Cys802, Cys911, Cys930, or Cys983, had not appreciable effect on enzyme inactivation kinetics (*not shown*), while replacements Cys138Ser and Cys336 led to greater inactivation by Hg^{2+} due to a larger fraction of enzyme being inactivated by modification of the fast reacting component (*not shown*). Figure 3*B* shows the inactivation kinetics of Cys104Ser substituted enzyme. This latter replacement partially removed the slow reacting component. The curve fitting yielded k_2' values close to (or lower than) zero preventing estimation of k_2. On the other hand, the fast component rate constant was affected to a less extend ($k_1 = 0.41 \pm 0.01$ min^{-1} μM^{-n}, RDα1 enzyme; $k_1 = 0.13 \pm 0.02$ min^{-1} μM^{-n} Cys104Ser substituted enzyme). As a result of the reduction of the slow reacting component a larger fraction of the enzyme was modified at the fast reacting site ($\alpha = 30$) (Fig. 3*B*). Figure 3*C* shows that the replacement of Cys964 completely prevented inactivation of the enzyme treated in *Na-medium* (the small decrease in K^+ uptake was within the experimental error). Although these results are consistent with the participation of Cys104 and Cys964 in the inactivation by Hg^{2+}, it is intriguing that the substitution of a single cysteine (Cys964) removed the apparent multicomponent inactivation. However, it is possible that as a result of the Cys964 replacement, the reaction of other sulfhydryl groups in the molecule was prevented.

Treatment of the enzyme in a *K-medium* resulted in different observations (Fig. 4*A*). In this case, as in the *Na-medium* treatment condition, a larger fraction of the Cys138Ser substituted enzyme was inactivated through the fast reacting component (*not shown*), while replacement of Cys336, Cys802, Cys911, Cys930, or Cys983 had no effect on the inactivation kinetics (*not shown*). The most interesting differences were again detected in Cys104Ser and Cys964Ser enzymes. Mutation Cys104Ser greatly reduced the apparent inactivation rates of the slow reacting component (Fig. 4*B*). However, this replacement had no significant effect on the kinetics of the fast reacting component ($k_1 = 0.21 \pm 0.02$ min^{-1} μM^{-n}, RDα1 enzyme; $k_1 = 0.16 \pm 0.04$ min^{-1} μM^{-n} Cys104Ser substituted enzyme). These results support the hypothesis that this cysteine might be involved in the slow inactivation of the enzyme. On the other hand, the Cys964Ser substituted enzyme showed a reduction in the inactivation rates of both components when the enzyme was treated in *K-medium* (Fig. 4*C*). In this way, the slow reacting component was not evident in this enzyme except at high (10 μM) Hg^{2+}; while the fast reacting component inactivation rate was reduced 79% with respect to control enzyme (0.21 ± 0.02 min^{-1} μM^{-n}, RDα1 enzyme; 0.046 ± 0.003 min^{-1} μM^{-n}

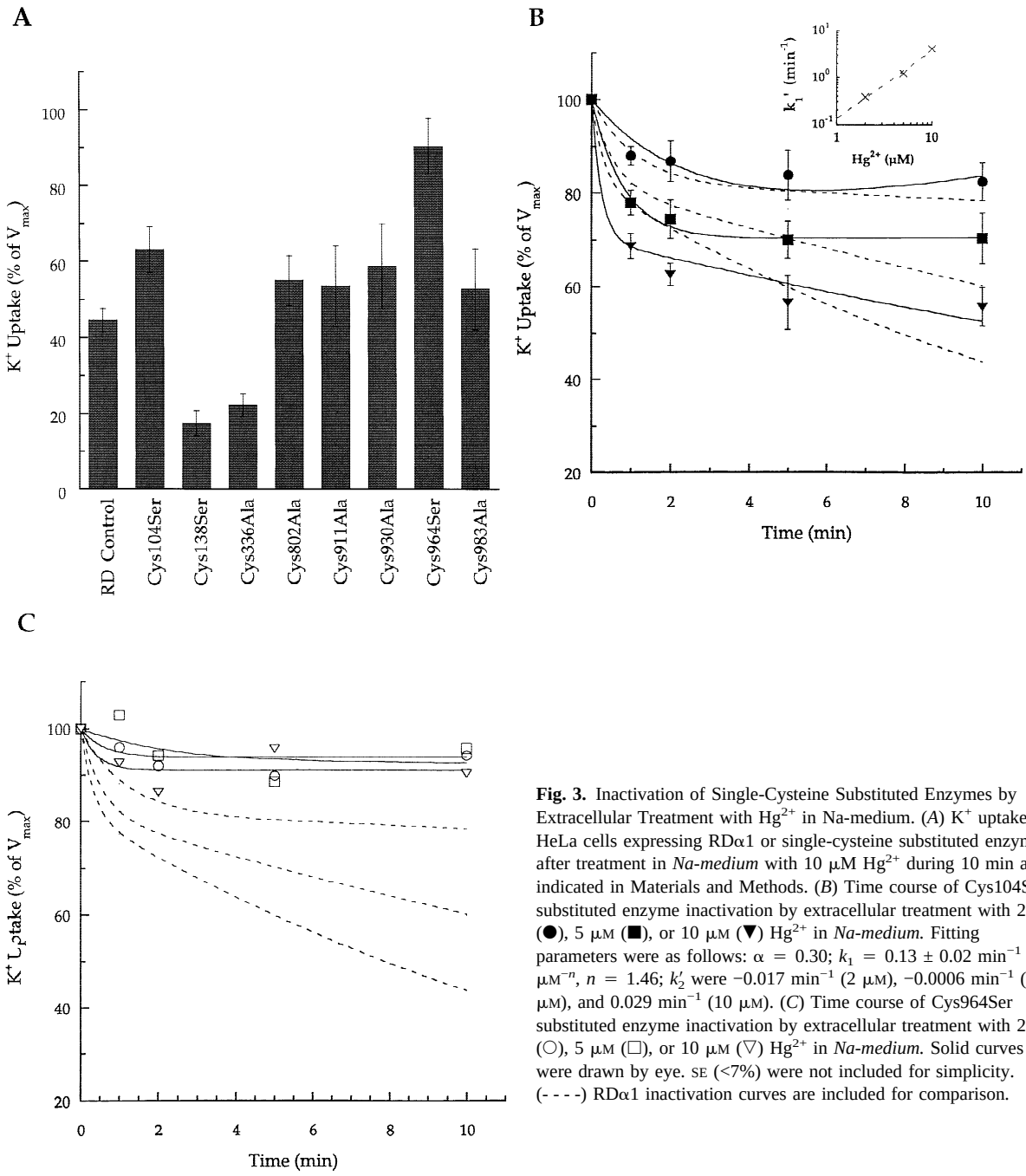

Fig. 3. Inactivation of Single-Cysteine Substituted Enzymes by Extracellular Treatment with Hg^{2+} in Na-medium. (*A*) K^+ uptake by HeLa cells expressing RDα1 or single-cysteine substituted enzymes after treatment in *Na-medium* with 10 μM Hg^{2+} during 10 min as indicated in Materials and Methods. (*B*) Time course of Cys104Ser substituted enzyme inactivation by extracellular treatment with 2 μM (●), 5 μM (■), or 10 μM (▼) Hg^{2+} in *Na-medium.* Fitting parameters were as follows: $\alpha = 0.30$; $k_1 = 0.13 \pm 0.02$ min^{-1} μM^{-n}, $n = 1.46$; k_2' were -0.017 min^{-1} (2 μM), -0.0006 min^{-1} (5 μM), and 0.029 min^{-1} (10 μM). (*C*) Time course of Cys964Ser substituted enzyme inactivation by extracellular treatment with 2 μM (○), 5 μM (□), or 10 μM (▽) Hg^{2+} in *Na-medium.* Solid curves were drawn by eye. SE (<7%) were not included for simplicity. (- - - -) RDα1 inactivation curves are included for comparison.

Cys9644Ser substituted enzyme) (insert Fig. 4*C*). This reduction in the reaction of the fast component would suggest that Cys964 might be the site modified at a faster rate.

Considering these findings, i.e., the apparent association of Cys104 with the slow reacting site and Cys964 with the fast reacting one, an enzyme carrying the double substitution Cys104, 964Ser was tested (Fig. 5). However, this enzyme presented inactivation kinetics similar to the single Cys964Ser mutant (Compare Fig. 5*B* with Fig. 4*C*); i.e., no additional protection was observed. Thus, it was apparent that upon replacement of Cys104 and Cys964, additional cysteines are probably modified when of the enzymes is treated with Hg^{2+} in *K-medium.*

All-TM-Cys Enzyme is not Inactivated by Extracellular Hg^{2+} Treatment

Under dephosphorylation conditions (*K-medium*), all of the single-cysteine substituted enzymes and the enzyme

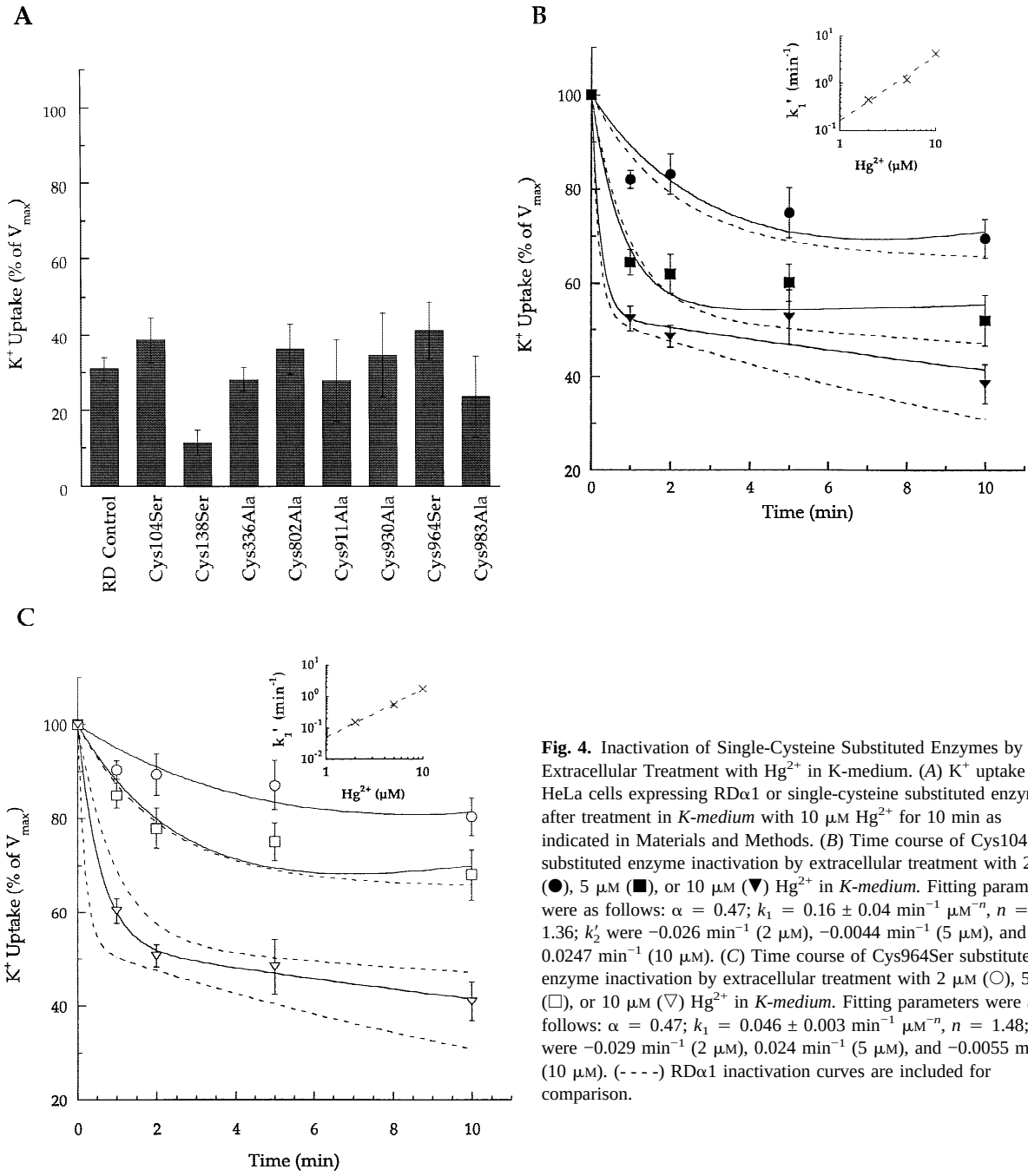

Fig. 4. Inactivation of Single-Cysteine Substituted Enzymes by Extracellular Treatment with Hg^{2+} in K-medium. (*A*) K^+ uptake by HeLa cells expressing RDα1 or single-cysteine substituted enzymes after treatment in *K-medium* with 10 μM Hg^{2+} for 10 min as indicated in Materials and Methods. (*B*) Time course of Cys104Ser substituted enzyme inactivation by extracellular treatment with 2 μM (●), 5 μM (■), or 10 μM (▼) Hg^{2+} in *K-medium.* Fitting parameters were as follows: $\alpha = 0.47$; $k_1 = 0.16 \pm 0.04$ min^{-1} μM^{-n}, $n = 1.36$; k_2' were -0.026 min^{-1} (2 μM), -0.0044 min^{-1} (5 μM), and 0.0247 min^{-1} (10 μM). (*C*) Time course of Cys964Ser substituted enzyme inactivation by extracellular treatment with 2 μM (○), 5 μM (□), or 10 μM (▽) Hg^{2+} in *K-medium.* Fitting parameters were as follows: $\alpha = 0.47$; $k_1 = 0.046 \pm 0.003$ min^{-1} μM^{-n}, $n = 1.48$; k_2' were -0.029 min^{-1} (2 μM), 0.024 min^{-1} (5 μM), and -0.0055 min^{-1} (10 μM). (- - - -) RDα1 inactivation curves are included for comparison.

encoding the double Cys104,964Ser substitution, were at least partially inactivated by the probe (Figs. 4 and 5). Therefore, it was important to verify that the probe was in fact reacting only with transmembrane cysteines. To this end, we studied the effect of treatment with extracellular Hg^{2+} on a protein carrying the multiple substitutions Cys104, 138, 964Ser, Cys336, 802, 911, 930, 983Ala, i.e., with no cysteines in the transmembrane region. Figure 6 shows that, as expected, the probe did not inactivate the All-TM-Cys. Only a small decrease of approximately 10% (within our experimental error) in ouabain-sensitive K^+ uptake was observed under both treatment conditions. These data indicate that the enzyme inactivation is due to modification of transmembrane cysteines, and that removal of the reacting groups does in fact prevent the enzyme inactivation. These results also provide information concerning the only reduced cysteine in the β subunit of the enzyme, Cys46. The heterologous sheep α1 and endogenous human β subunits constitute the All-TM-Cys enzyme. Consequently, al-

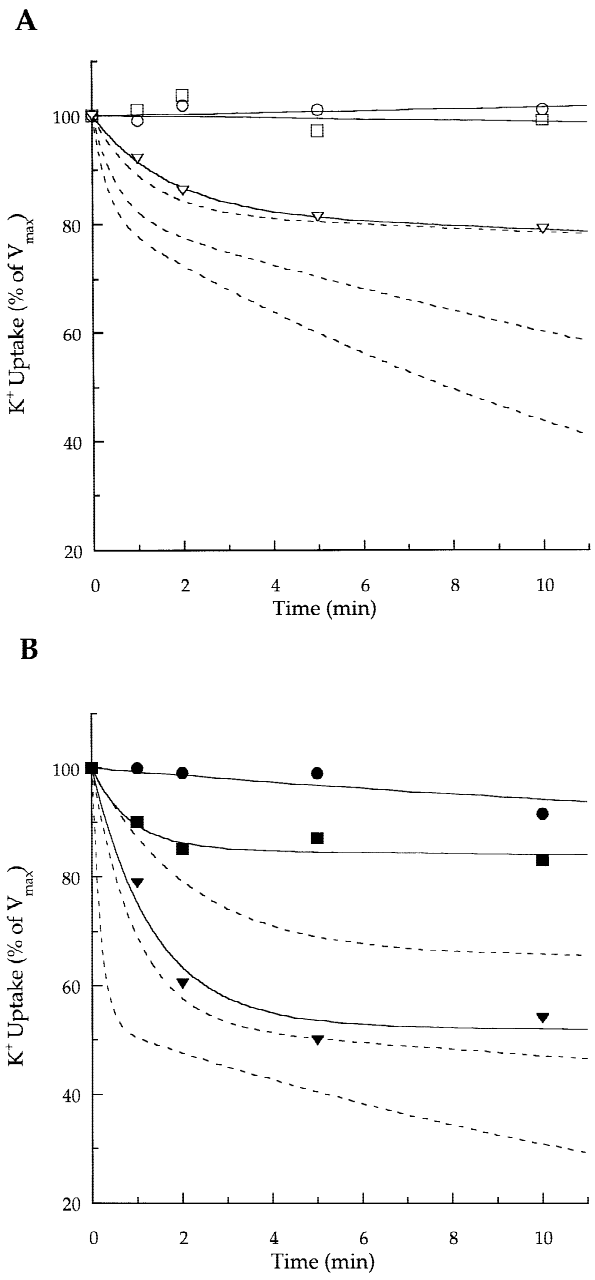

Fig. 5. Inactivation of Cys104,964Ser Substituted Enzyme by Extracellular Treatment with Hg^{2+}. (*A*) Time course of Cys104,964Ser substituted enzyme inactivation by extracellular treatment with 2 μM (○, ●), 5 μM (□, ■), or 10 μM (▽, ▼); [Hg^{2+}] in *Na-medium* (*A*) (○, □, ▽) or *K-medium* (*B*) (●, ■, ▼). Solid curves were drawn by eye. SE (<7%) were not included for simplicity. (- - - -) RDα1 inactivation curves are included for comparison.

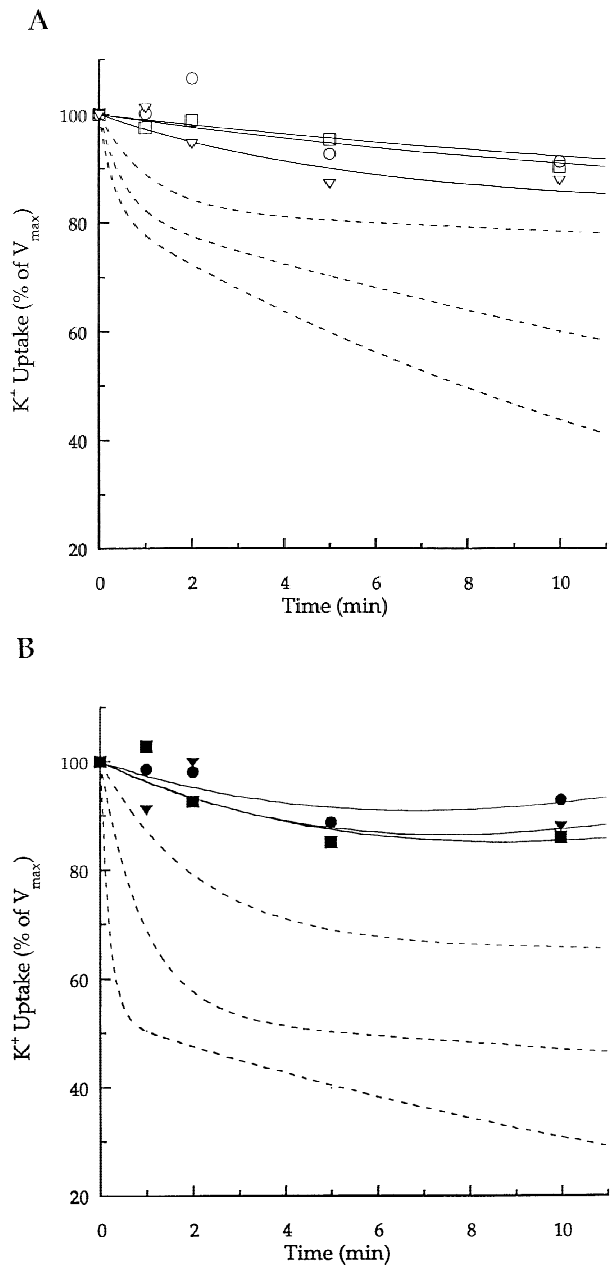

Fig. 6. Inactivation of All-TM-Cys Enzyme by Extracellular Treatment with Hg^{2+}. (*A*) Time course of All-TM-Cys enzyme inactivation by extracellular treatment with 2 μM (●, ○), 5 μM (□, ■), or 10 μM (▽, ▼); [Hg^{2+}] in *Na-medium* (*A*) (○, □, ▽) or *K-medium* (*B*) (●, ■, ▼). Solid curves were drawn by eye. SE (<7%) were not included for simplicity. (- - - -) RDα1 inactivation curves are included for comparison.

though all α subunit transmembrane cysteines have been removed, Cys46 is still present in the transmembrane region of the tested enzyme. However, it is apparent that Cys46 in the β subujnit is not accessible to react with Hg^{2+} or, alternatively, its modification does not lead to inactivation.

Discussion

In this study the reactivity of solvent-accessible transmembrane cysteines in the Na,K-ATPase sheep α1 subunit with Hg^{2+} was investigated. Treatment of RDα1 enzyme with Hg^{2+} under the conditions of our system led

to enzyme inactivation. This was associated with the modification of solvent accessible transmembrane cysteines since washing the probe with millimolar cysteine restored the enzyme activity. The kinetics of inactivation suggested that the probe modified several cysteines. Previous studies by Wang and Horrisberg (1996) showed that external treatment with Hg^{2+} of *Xenopus laevis* Na,K-ATPase under turnover conditions led to a two-component inactivation. Although a large percentage (40–60%) of the inactivation occurred within 20 sec upon initiation of treatment, a slower reacting component following thereafter was described (Fig. 1*B* in Wang & Horrisberg, 1996). The slow reacting component was characterized by rate constants approximately 60 times faster than those reported here. These differences could be due to the different enzyme sources or treatment protocols (turnover *vs.* nonturnover conditions).

Chemical modification of Glu779 (Argüello & Kaplan, 1991, 1994) and various transmembrane cysteines (Cys802, Cys911, Cys964) (Nagai et al., 1986; Lutsenko et al., 1995; Lutsenko et al., 1997), as well as the formation of disulfide bridges generated by cysteine oxidation with Cu-Phenanthroline (Sarvazyan et al., 1995), are influenced by the presence of enzyme ligands in the treatment media. Investigators have proposed that the corresponding transmembrane segments undergo ligand-dependent conformational transitions that are likely to be relevant during the catalytic cycle. Considering this, the reactivity of cysteines was tested after driving the enzyme into different conformations. To achieve this goal without disrupting the cell integrity, the extracellular and intracellular cation concentrations were manipulated such as nonturnover and particular enzyme conformations could be expected to be preponderant (*K-medium,* dephosphorylated form; *Na-medium,* phosphorylated form). The clear differences in the inactivation kinetic of control and cysteine-deficient enzymes treated under these two conditions suggest that distinct enzyme conformations were attained and that these forms have noticeable effects on transmembrane cysteine accessibility.

The location of cysteines reacting with Hg^{2+} was attempted using cysteine-deficient enzymes in which the α subunit eight transmembrane cysteines have been individually substituted. It was expected that the replacement of involved cysteines would at least partially prevent inactivation, decreasing the rate of inactivation of a particular component. Our results suggest that Cys802, Cys911, Cys930 or Cys983 do not react with Hg^{2+} or that their modification does not affect K^+ uptake by the enzyme. Cys138Ser and Cys336Ala substituted enzymes were inactivated by the probe to a larger extent (compared to the control enzyme). The simplest explanation for this observation is that replacements of Cys138 and Cys336 indirectly affect the reactivity of other transmembrane cysteines.

The replacement Cys104Ser significantly reduced the slow reacting component of the inactivation curves. It has been proposed that Hg^{2+} modifies Cys104, since replacement of this cysteine partially reduced enzyme inactivation after treatment under turnover conditions (Wang & Horisberger, 1996). Similar results (i.e., replacement of Cys104 removes the slow reacting component) were obtained in this study when the enzyme was treated under phosphorylating conditions (Fig. 4*A*). Considering that E1P/E2P apparently are the preponderant forms of the enzyme under turnover conditions (Glynn, 1985; Heyse et al., 1994), the concordance of these results was expected. On the other hand, the significant influence of the enzyme conformation on the reaction rate constants of this site (evident in the inactivation of the RDα1 control enzyme) suggests that the accessibility of Cys104 is affected by the enzyme conformation. Thus, when the enzyme is phosphorylated, Cys104 would be more exposed reacting ten times faster with the probe. This observation is relevant considering the participation of the H1–H2 extracellular loop in ouabain binding (Price & Lingrel, 1988; Lingrel & Kuntzweiler, 1994; Antolovic et al., 1995; Palasis et al., 1996). It is known that the phosphorylated form of the Na,K-ATPase binds ouabain with higher affinity (1×10^{-9} M in the presence of Mg^{2+} and P_i) compared with the unphosphorylated form (2×10^{-8} M in the presence of Mg^{2+} alone) (Yoda & Yoda, 1982; Wallick & Schwartz, 1988). Then, the larger exposure of this domain in the phosphorylated form, as suggested by a faster reaction rate for Cys104, might be directly related to and explain the higher affinity for ouabain observed in the phosphorylated enzyme.

The replacement of Cys964 greatly reduced the inactivation rate constants under both treatment conditions. In fact, Cys964Ser appeared to be the only replacement that significantly reduced the rate constants of the fast reacting component. These results strongly suggest the modification and the exposure of Cys964 to the aqueous media, supporting previous observations with SDSM (Lutsenko et al., 1997). Furthermore, the complete protection under *Na-medium* conditions (phosphorylated enzyme) indicates that the replacement of Cys964 somewhat changes the membrane disposition of other transmembrane cysteines and this effect appears to be dependent on the enzyme conformation.

Although several constructs encoding for multiple cysteine substitutions were tested, only the All-TM-Cys enzyme was resistant to Hg^{2+} treatment. This suggests a complex reaction of Hg^{2+} with several transmembrane cysteines or that upon replacement of Cys104 and Cys964 other sulfhydryl groups become accessible. In addition, it also points out that, at the tested concentra-

tions, the probe would react only with cysteines in the transmembrane region of enzyme. Consequently, the described experimental system appears promising for testing the accessibility of cysteines engineered in the background of the All-TM-Cys protein.

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