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Similarities and Differences between the Properties of Native and Recombinant Na^+/K^+-ATPases[1]

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Progress of mutagenesis studies on the relation of the structure of Na^+/K^+-ATPase to its reaction mechanism has been impeded by the paucity of information on the properties of small amounts of impure recombinant enzyme obtained in the currently available expression systems, and the uncertainty of whether expression in a new environment alters the various catalytic activities of this membrane enzyme. Hence, our aim was to make a detailed comparison of the properties of the extensively studied canine kidney Na^+/K^+-ATPase with those of its α_1,β_1 subunits expressed in the baculovirus-infected Sf-9 cells. The active fraction of the recombinant enzyme, containing 10–20% of the expressed α subunits, was found to have normal molar activity, all the partial reactions, and the ability to catalyze ATP-dependent Na^+/K^+ exchange after reconstitution into proteoliposomes. Comparison of steady-state kinetics of the hydrolytic activities of recombinant and native enzymes showed that (a) ATP and Na^+ plots of Na^+-ATPase were the same in the two preparations; (b) apparent K^+ affinity of K^+-phosphatase of recombinant enzyme was lower than that of kidney enzyme; and (c) for Na^+/K^+-ATPase activity, apparent K^+ affinity of recombinant enzyme was lower, and its apparent Na^+ and ATP affinities were higher than those of kidney enzyme. The two enzymes had similar ADP- and K^+-sensitive phosphointermediates, identical affinities for ouabain, and similar ligand sensitivities of dissociation rates of ouabain–enzyme complexes. Evidently, the recombinant enzyme has reduced affinity at cytoplasmic K^+ sites, but no changes at multiple Na^+, ATP, and ouabain binding sites. Likely causes of this selective change include altered glycosylation state of β and interactions among active and inactive recombinant enzymes. The present results provide the necessary database for the appropriate use of an expression system in structure–function studies on canine α_1,β_1 isoform of Na^+/K^+-ATPase, and indicate the need for similar studies on recombinant Na^+/K^+-ATPases obtained in other expression systems.

Key Words: recombinant Na^+/K^+-ATPase; Na^+-ATPase; K^+-phosphatase; Sf-9 insect cells; canine kidney; ouabain.

Na^+/K^+-ATPase[3] is the intrinsic enzyme of the plasma membrane that catalyzes the active transports of Na^+ and K^+ in most eukaryotic cells. The enzyme consists of α and β subunits, both of which are required for function. In recent years, important information on structure–function relationship of the enzyme has been obtained through mutagenesis studies, in spite of the absence of a system for significant overproduction of the enzyme and its mutants (1). Small quantities of the active enzyme have been expressed in *Xenopus* oocytes, several mammalian cell lines, yeast, and insect cells (1). Each of these expression systems has specific advantages and limitations. For the purpose we had in mind, i.e., structure–function studies related to the enzyme's reaction mechanism; the baculovirus-infected insect cell was the most promising for two reasons: First, it seemed to be the most convenient way of obtaining relatively larger amounts of active recombinant enzyme suitable for extensive biochemical studies; and, second, the Sf-9 cell contained little or no endogenous Na^+/K^+-ATPase relative to the level of the expressed enzyme (2). In order to use this expression system for

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[3] Abbreviations used: K^+-MFPase, K^+-dependent 3-*O*-methylfluoresceinphosphatase; Chaps, 3-[3-cholamidopropyl)dimethylaminoiol]-1-propanesulfonic acid; Mops, 4-morpholinepropanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

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such structure–function studies, however, it was necessary to determine (a) if the various reactions and partial reactions of the enzyme could be characterized in sufficient detail with the available quantities of the expressed enzyme, and (b) whether the properties of the enzyme were significantly altered upon expression in a new environment. For this purpose, we chose the Na^+/K^+-ATPase of canine kidney outer medulla consisting of α_1 and β_1 isoforms because its purified preparations have been used extensively in our laboratory and others for studies on structure and reaction mechanism of Na^+/K^+-ATPase (e.g., 3–7). Here we report the results of studies in which the cDNAs of the canine α_1 and β_1 subunits were used to express active Na^+/K^+-ATPase in Sf-9 insect cells; procedures were developed for the study of numerous biochemical properties of the small quantities of the expressed enzyme; and comparisons were made between the appropriate properties of the recombinant and the canine kidney enzymes. Results pertinent to the status of the related endogenous activities of the Sf-9 cells are also reported.

MATERIALS AND METHODS

Cells and viral infections. Construction of recombinant baculovirus and infections of Sf-9 cells were carried out as described previously (2), except that cDNAs encoding canine α_1 subunit (8) and β_1 subunit (9) were cloned into the dual recombinant expression vector p2Bac (Invitrogen). Cells, at a density of 10^7 per 150-mm dish, were infected, incubated at 27°C for 3 days, suspended in the culture medium by gentle scraping, and collected by centrifugation at 1000*g* for 5 min.

Sf-9 membrane preparations. For a typical preparation to be used in biochemical assays, cells collected from four dishes were suspended in 80 ml of a solution containing 0.25 M sucrose, 2 mM imidazole, and 1 mM EDTA (pH 7.0), centrifuged, and washed twice more in the same solution. Cells were then disrupted, and crude membranes treated with NaI were prepared exactly as described by Lane *et al.* (10) for the preparation of membranes from HeLa cells. The final membrane pellet was thoroughly washed and suspended in the same sucrose–imidazole–EDTA solution described above. Using this procedure, the yield of membranes from four dishes of cells infected with the recombinant virus was about 1.2–1.6 mg of protein. The ouabain-sensitive Na^+/K^+-ATPase activities of such preparations (assayed as indicated below) constituted 50–60% of their total ATPase activities, and were in the range of 1.5–2.5 μmol ATP hydrolyzed/mg protein/h. When membranes were prepared by a more laborious procedure described previously (2), specific activities were 4–8 times higher, but the yields were lower. Unless stated otherwise, the NaI-treated membranes were used in the reported experiments.

Canine kidney preparations. Frozen canine kidney medulla (Pel-Freeze Biologicals) was homogenized in 0.25 M sucrose, 30 mM histidine, 1 mM EDTA (pH 6.8) and centrifuged for 30 min at 1000*g*. The supernatant was centrifuged at 100,000*g* for 1 h to obtain a crude microsomal preparation with specific Na^+/K^+-ATPase activity of 40–150 μmol hydrolyzed ATP/mg protein/h. These microsomes were either treated by the "rapid" version of the procedure of Jorgensen (11) to obtain the purified enzyme (sp act 1000–1500 μmol/mg/h) or treated with NaI, as indicated for Sf-9 membranes, to obtain preparations with specific activities in the range of 150–450 μmol/mg/h. Unless stated otherwise, when direct comparisons of the biochemical properties of the Sf-9 and canine kidney enzymes were desired, the NaI-treated microsomes were used. It was established that these microsomes did not contain sealed vesicles.

Assays. Na^+/K^+-ATPase activity was assayed at 37°C by measuring the initial rate of release of $^{32}P_i$ from [γ-^{32}P]ATP (12) in a solution containing 100 mM NaCl, 25 mM KCl, 3 mM $MgCl_2$, 1 mM EGTA, 2 mM ATP, 5 mM NaN_3, and 50 mM Tris–HCl (pH 7.4). The solution for the assay of Na^+-ATPase was the same except that it contained 50 mM NaCl, 1 μM ATP, and no KCl. Each ATPase assay was performed in the presence and absence of 1 mM ouabain to assess the ouabain-sensitive component of the activity. Assay of K^+-MFPase activity was performed at 37°C as described before (13), using a recording fluorimeter, in a solution containing 20 μM 3-*O*-methylfluorescein phosphate, 4 mM $MgCl_2$, 1 mM EGTA, 10 mM KCl, and 80 mM Tris–HCl (pH 7.4). When the K_m of a substrate or an activator ion was to be determined in any of the above assays, reaction mixtures had the same compositions as indicated above, except for the varying concentrations of the indicated ligand. When Na^+ was to be varied in ATPase assays, NaN_3 was omitted from the reaction mixtures. K_m values were determined through computer fitting of the data to Michaelis–Menten equation by nonlinear regression analysis.

Active site concentration was determined by the assay of either the maximal level of the enzyme's phosphointermediate or the maximal level of bound ouabain. The two have been shown to be equal for the canine kidney enzyme (12, 14). Phosphorylation by [γ-^{32}P]ATP was carried out at 0°C by incubating the enzyme or the membrane preparation with 2 mM $MgCl_2$, 100 mM NaCl, 10 μM ATP, and 50 mM Tris–HCl (pH 7.4) for 15 s. This was sufficient to achieve the maximal level of phosphointermediate formation (7, 12). Labeling obtained in the presence of KCl instead of NaCl was used to determine the Na^+-dependent component of phosphorylation. After denaturation with perchloric acid, the samples were filtered and counted (12).

To study decomposition of the phosphointermediate, EDTA was added to a final concentration of 20 mM to stop phosphorylation. After indicated periods, perchloric acid was added, and remaining phosphoenzyme was assayed as above. ADP (2 mM) or 25 mM KCl was added along with EDTA to test for ADP and K^+ sensitivities of the phosphoenzyme. To detect the ^{32}P-labeled phosphoenzyme on gels, acid-denatured samples were dissolved in SDS, subjected to electrophoresis on acid gels, and autoradiographed (7).

Maximal level of bound ouabain was assayed as described previously (12, 14), using a saturating concentration (0.4 μM) of [^{3}H]-ouabain either in the presence of 100 mM NaCl, 2 mM ATP, 3 mM $MgCl_2$, 25 mM Mes, 25 mM Tris (pH 7.0) or in the presence of 3 mM $MgCl_2$, 3 mM P_i (added as Tris phosphate), and 25 mM Mes, 25 mM Tris (pH 7.0). After incubation at 37°C for 10 min, the samples were cooled on ice, sedimented and washed at 4°C, and counted (12). Corrections were made for nonspecific binding determined in the presence of 3 mM unlabeled ouabain. For determination of ouabain dissociation rates (14), the ouabain–enzyme complexes formed as above were suspended in solutions containing 5 mM EDTA, 25 mM Mes, 25 mM Tris (pH 7.0), and the indicated ligands. After appropriate intervals, samples were cooled, washed, and counted as above.

Ouabain-stimulated phosphorylation of the enzyme by P_i (12) was carried out at 37°C for 10 min in the presence of 20 μM $^{32}P_i$, 2 mM $MgCl_2$, 50 mM Tris–HCl (pH 7.2), and the indicated ouabain concentrations. The acid-denatured phosphoenzyme was then subjected to SDS–gel electrophoresis at pH 2.4, autoradiographed, and quantified by densitometry (7).

Western blots were performed and quantified as described previously (15). The antibodies used were a polyclonal against canine β subunit prepared in our laboratory (15), and an α-reactive monoclonal (2F) provided by Dr. D. Fambrough (Johns Hopkins University). Method of Lowry *et al.* (16) was used for protein determination in all samples except those in proteoliposomes, where the method of Kaplan and Pedersen (17) was used.

Reconstitution and assay of transport activity. Crude membranes obtained from homogenates of Sf-9 cells (4×10^7 cells) prior to NaI treatment were suspended in 1 ml of a solution containing 160 mM KCl, 20 mM Mops (pH 7.2), 20% glycerol, and 1% Chaps, using a hand homogenizer. After standing on ice for 40 min with intermittent homogenization, the suspension was centrifuged at 130,000*g* for 30 min. The soluble supernatant was mixed with phosphatidylcholine (30 mg/ml; type IV-S from soybean, Sigma) and vortexed. The resulting suspension was placed on a 5-ml Sephadex G-25-300 column which had been equilibrated with 160 mM KCl, and 20 mM Mops (pH 7.2), allowed to stand for 1 h at 4°C, and centrifuged at 2000*g* for 5 min. The collected suspension of proteoliposomes was centrifuged at 150,000*g* for 2 h, and the sedimented proteoliposomes were resuspended in a solution containing 160 mM choline chloride, 2 mM KCl, and 20 mM Mops (pH 7.2) at a density of 2–4 mg of protein/ml. The same procedure was used to prepare proteoliposomes from canine kidney microsomes (4–5 mg of protein).

To assay ATP-dependent Na^+/K^+-exchange, 20 μl of the above proteoliposome suspension was added to 80 μl of a reaction mixture containing 150 mM choline chloride, 5 mM ^{22}NaCl, 0.2 mM EGTA, 3 mM $MgCl_2$, 2 mM ATP, 20 mM Mops (pH 7.2), and other additions as indicated. ATP was omitted from the control to measure ATP-independent uptake. After indicated periods of incubation at 24°C, 3 ml of an ice-cold stop solution containing 160 mM KCl and 20 mM Mops (pH 7.2) was added, and the mixture was passed through a 0.45-μm Millipore filter. The filter was washed three times with the same stop solution and counted. To measure total Na^+/K^+-ATPase of the proteoliposomes, the sealed vesicles were opened by treatment with 0.1 mg alamethicin/mg protein (18) and then assayed for initial rate of Na^+/K^+-ATPase activity as described above.

RESULTS

Active and inactive components of the enzyme expressed in insect cells. In experiments of Fig. 1 membrane preparations from uninfected Sf-9 cells, and from those infected with the recombinant virus, were subjected to SDS–gel electrophoresis, and probed with α- and β-specific antibodies. A purified canine kidney Na^+/K^+-ATPase preparation was also included for comparison. The membranes of control cells exhibited no immunoreactivity, while both α- and β-reactive materials were noted in the infected cells and the purified enzyme (Figs. 1A and 1B). Based on the densitometric comparisons of the α-reactive bands in different amounts of the preparations applied to gels (Fig. 1A), it was readily estimated that the α-content of the purified enzyme was about 100 times that of the Sf-9 membrane preparation. The mobilities of several β-reactive bands in the Sf-9 membranes were higher than those of the single glycosylated band of the purified kidney enzyme (Fig. 1B). This is in agreement with previous observations on the expression of rodent β in Sf-9 cells (2), indicating that the Sf-9 membrane preparation contained a mixture of unglycosylated β polypeptide and those whose glycosylation states are different from those of the kidney enzyme.

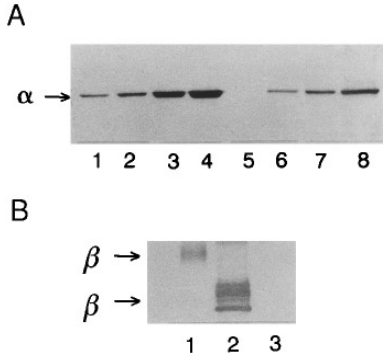

FIG. 1. Comparison of immunoblots of canine α_1 and β_1 subunits of Na^+/K^+-ATPase expressed in Sf-9 cells with those of the purified canine kidney enzyme. (A) Probed with an α-specific monoclonal antibody. Lanes 1–4, different quantities of purified kidney enzyme: 0.05, 0.1, 0.5, and 1 μg protein. Lane 5, membrane preparation of uninfected Sf-9 cells, 25 μg protein. Lanes 6–8, different quantities of membranes of α,β-infected Sf-9 cells: 5, 10, and 25 μg protein. (B) Probed with β-specific polyclonal antibody. Lane 1, purified kidney enzyme; lane 2, α,β-infected Sf-9 membranes; lane 3, uninfected Sf-9 membranes.

To assess the functional state of the expressed enzyme, membranes from eight preparations of α,β-infected cells similar to those of Fig. 1, and eight control preparations made from cells that were infected with the wild-type virus, were assayed for ouabain-sensitive Na^+/K^+-ATPase. The detected activities (μmol/mg/h $\pm$ SE) were 1.71 $\pm$ 0.09 for the α,β-infected cells and 0.29 $\pm$ 0.01 for the control cells. It is not clear that the latter activity is indeed due to an endogenous Na^+/K^+-ATPase (see below, and Discussion). Even if it is, however, these data indicate that a minimum of 80–90% of the activity of the α,β-infected cells is due to the expressed canine subunits.

The specific activity of the purified kidney enzyme used in experiments of Fig. 1A was 1200 μmol/mg/h. Since the ratio of this activity to that of the α,β-infected cells was far greater than the ratio of α protein in the two preparations (Fig. 1A), it was necessary to evaluate the quality of the enzyme expressed in Sf-9 cells by determining its molar activity. Using the procedures described under Materials and Methods, active site concentrations were determined in three α,β-infected preparations by the assay of maximal level of Na^+-dependent phosphoenzyme formed from ATP, and in three other preparations by the assay of maximal level of bound ouabain. (Using the same procedures, accurate assay of active site concentration in cells infected with the wild-type virus was not possible.) The combined data for the six α,β-infected preparations showed that active site concentration (pmol/mg $\pm$ SE) was 3.02 $\pm$ 0.4; and that Na^+/K^+-ATPase activity (μmol/mg/h $\pm$ SE) was 1.61 $\pm$ 0.13. The calculated molar activity of about 8900 min^{-1} is comparable to the molar activities of purified and undamaged preparations of the enzyme from a variety of sources (19). Taken together, the above data indicate that only 10–20% of the expressed α subunits are involved in enzyme units with normal

TABLE I

Comparison of Na^+/K^+-ATPase, Na^+-ATPase, and K^+-MFPase Activities of the Membranes of α,β-Infected Sf-9 Cells and Kidney Microsomes

Source	Na^+/K^+-ATPase (μmol/mg/h)	Na^+-ATPase (nmol/mg/h)	K^+-MFPase (nmol/mg/h)
Sf-9 membranes	1.64 ± 0.07	7.12 ± 0.06	67.5 ± 6.6
Kidney microsomes	134 ± 0.4	919 ± 90	5841 ± 221

Note. Assays were performed as described under Materials and Methods. Each value is the mean ± SE of assays in five different preparations of Sf-9 membranes, and triplicate assays of the same kidney microsomal preparation.

molar activity, and that the remainder exhibit no Na^+/K^+-ATPase activity.

It is well established that Na^+/K^+-ATPase activity of the enzyme may be inhibited without inactivation of some of its partial reactions. For example, fluorescein isothiocyanate inhibits Na^+/K^+-ATPase but not the K^+-phosphatase activity of the enzyme (20); and thimerosal inhibits Na^+/K^+-ATPase but not Na^+-ATPase and K^+-phosphatase activities (21). To determine if the expressed subunits that did not have Na^+/K^+-ATPase activity exhibited some partial reactions, several recombinant preparations and a canine kidney preparation were assayed for Na^+/K^+-ATPase, Na^+-ATPase, and K^+-MFPase activities. The results (Table I) showed that the ratio of a partial reaction to Na^+/K^+-ATPase activity in the α,β-infected cells did not exceed the same ratio in the kidney enzyme, indicating that the larger fraction of the expressed subunits that do not have Na^+/K^+-ATPase activity also do not catalyze the partial reactions. Therefore, the remaining data presented below characterize the various activities of the fraction of the expressed enzyme that is fully active.

K^+ and ADP sensitivities of the phosphoenzyme. In the Albers–Post reaction cycle of the enzyme, ADP-sensitive and K^+-sensitive phosphointermediates (E_1P and E_2P) are formed consecutively (7). To examine the properties of the phosphointermediate of the expressed enzyme of the α,β-infected cells, the membrane preparation was phosphorylated with ^{32}P-labeled ATP in the presence of 100 mM Na^+, and the effects of ADP or K^+ on dephosphorylation were examined. The results (Fig. 2C) showed that the phosphoenzyme was sensitive to both K^+ and ADP, but more so to ADP. This was surprising, since a large number of previous studies on the phosphoenzymes of the purified canine and pig kidney enzymes had shown that with NaCl concentrations of 100 mM or lower, the phosphoenzyme is predominantly K^+-sensitive, and that much higher NaCl concentrations are required to reveal the ADP-sensitive phosphoenzyme (22). It was confirmed that the phosphoenzyme of purified canine kidney enzyme formed in the presence of 100 mM Na^+ was indeed insensitive to ADP and sensitive to K^+ (Fig. 2A). To explore the cause of this apparent difference between the properties of the purified kidney enzyme and the recombinant enzyme, we examined the K^+ and the ADP sensitivities of the canine kidney enzyme in crude microsomes and in NaI-treated microsomes of canine kidney. The results were identical (shown only for crude microsomes in Fig. 2B), and similar to the results obtained with the enzyme expressed in Sf-9 cells. Evidently, it is the purification process involving SDS that alters the relative ADP and K^+ sensitivities of the kidney enzyme.

Kinetics of Na^+-ATPase, K^+-MFPase, and Na^+/K^+-ATPase activities. We used Na^+-ATPase activity to examine the high-affinity ATP catalytic site and the Na^+ sites of the expressed enzyme. The apparent K_m values of ATP (about 0.4 μM) were the same in the canine kidney enzyme and the expressed enzyme (Fig. 3), and similar to the values determined before either in Na^+-ATPase experiments (23) or those on Na^+-dependent phosphoenzyme formation (7). The Na^+ activation curve in the expressed and the kidney enzymes (Fig. 4) were also identical, showing the characteristic intermediary plateau that has been observed in numerous preparations of the enzyme (24).

Assay of K^+-MFPase activity as a function of K^+ con-

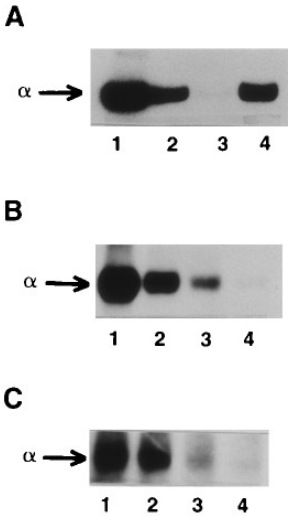

FIG. 2. ADP and K^+ sensitivities of the phosphointermediates of the canine kidney enzyme and the enzyme expressed in Sf-9 cells. Purified kidney enzyme (A), crude kidney microsomes (B), and membranes of α,β-infected Sf-9 cells (C) were phosphorylated with [γ-^{32}P]ATP in the presence of 2 mM Mg^{2+} and 100 mM Na^+. The phosphoenzymes were resolved on acid gels as described under Materials and Methods after 15 s of phosphorylation (lane 1), or after 15 s of phosphorylation followed by 5 s of dephosphorylation initiated by the addition of EDTA (lane 2), EDTA + K^+ (lane 3), and EDTA + ADP (lane 4).

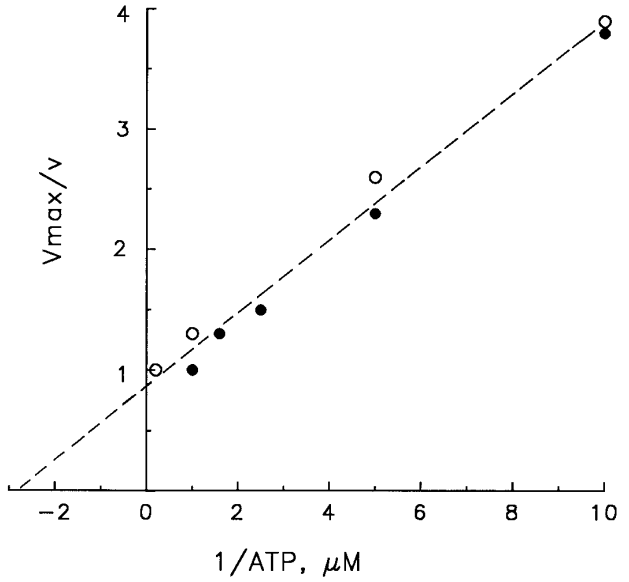

FIG. 3. Na^+-ATPase activity as a function of ATP concentration. Membranes of α,β-infected Sf-9 cells (○). Kidney microsomes (●). Mean of three determinations. Standard errors are smaller than symbol sizes.

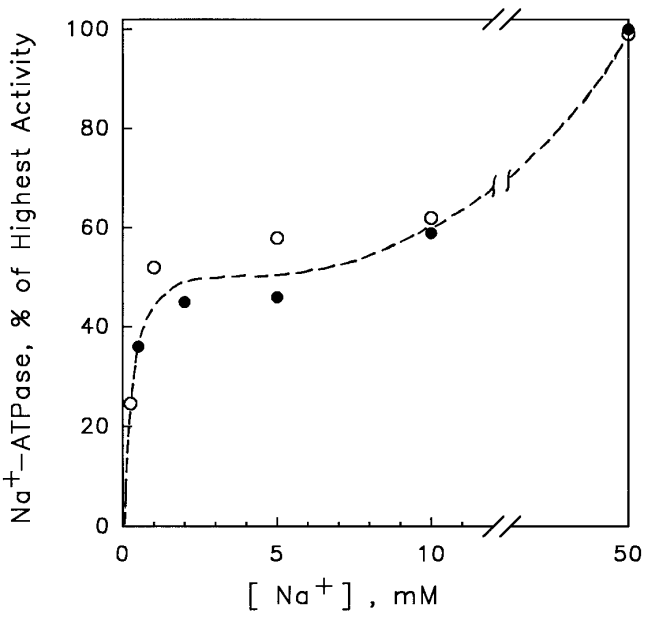

FIG. 4. Na^+-ATPase activity as a function of Na^+ concentration. Membranes of α,β-infected Sf-9 cells (○). Kidney microsomes (●). Mean of three determinations. Standard errors are smaller than symbol sizes.

centration (Fig. 5) showed that the apparent K_m of K^+ for the activity of the recombinant enzyme (1.6 ± 0.5 mM) was about sevenfold higher than that of the canine kidney enzyme (0.25 ± 0.08 mM).

Experiments of Fig. 6 showed that the significant change in apparent K^+ affinity of the phosphatase was also reflected in the K^+ activation curve of Na^+/K^+-ATPase activity: The apparent K_m of K^+ in the expressed enzyme (2.86 ± 0.43 mM) was significantly higher than that of the canine kidney enzyme (0.55 ± 0.15 mM). Consistent with this difference, there were changes in the opposite direction in the apparent affinities of ATP and Na^+. The results of Fig. 7 showed that the apparent K_m of ATP in Na^+/K^+-ATPase activity of the expressed enzyme (0.11 ± 0.01 mM) was significantly lower than that of the kidney enzyme (0.74 ± 0.05 mM); and similar experiments on the Na^+ activation curve (not shown) indicated that the apparent K_m of Na^+ in the recombinant enzyme (4.7 ± 0.3 mM) was significantly lower than that of the kidney enzyme (10.3 ± 0.4 mM).

K^+ also has an inhibitory effect on Na^+-ATPase when ATP concentration is low enough such that the low-affinity ATP site remains largely unoccupied (25). Experiments of Fig. 8 showed that this effect of K^+ was also less pronounced in the enzyme expressed in Sf-9 cells than in the canine kidney enzyme.

Ouabain interaction with the enzyme. Ouabain inhibits Na^+/K^+-ATPase, Na^+-ATPase, and K^+-phosphatase hydrolytic activities of the enzyme, but it stimulates the phosphatase activity in the absence of K^+ (26), and also stimulates the enzyme's phosphorylation by P_i when P_i is suboptimal (12). To compare the ouabain sensitivities of the expressed and the kidney enzymes without interference of possible differences in K^+ and

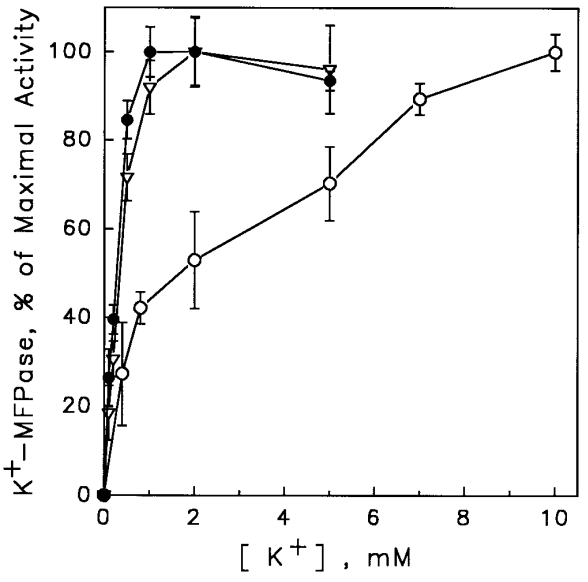

FIG. 5. K^+-MFPase activity as a function of K^+ concentration. Membranes of α,β-infected Sf-9 cells (○). Kidney microsomes (●). Purified kidney enzyme (▽). Mean ± SE of assays in seven different preparations.

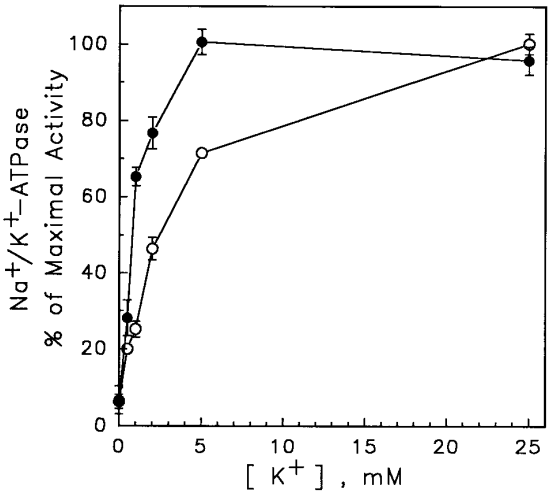

FIG. 6. Na^+/K^+-ATPase activity as a function of K^+ concentration. Membranes of α,β-infected Sf-9 cells (○). Kidney microsomes (●). Mean ± SE of assays in six different preparations.

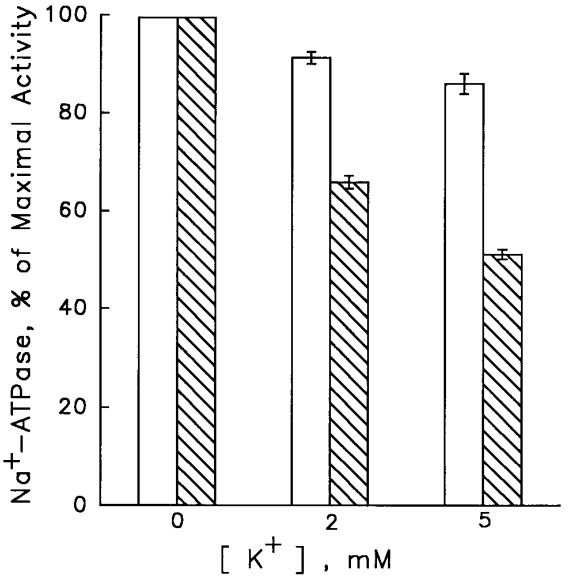

FIG. 8. Inhibitory effect of K^+ on Na^+-ATPase activity. Open bars, membranes of α,β-infected Sf-9 cells; shaded bars, kidney enzyme. Mean ± SE of assays in four different preparations.

Na^+ affinities, the effects of varying ouabain concentrations on phosphorylations of α subunits of both preparations by 20 μM $^{32}P_i$ were studied. The results (Fig. 9) showed the near identical sensitivities of the preparations to ouabain. When ouabain sensitivities of Na^+/K^+-ATPase activities of the two preparations were compared under the standard assay conditions described under Materials and Methods (data not shown), the resulting K_i values (about 0.1 μM) were not significantly different.

Ouabain is known to bind to canine kidney enzyme forming two different enzyme–ouabain complexes (I and II) with characteristic dissociation rates and distinct ligand sensitivities (14). To see if similar complexes were formed with the recombinant enzyme, experiments shown in Fig. 10 were performed. The results were identical to those obtained with the canine kidney enzyme (14), showing that (a) complex II formed in the presence of P_i and Mg^{2+} is more stable than complex I formed in the presence of $Na^+ + Mg^{2+} +$ ATP; (b) K^+ binds to complex I to stabilize it; and (c) Na^+ and ATP bind to complex II to destabilize it.

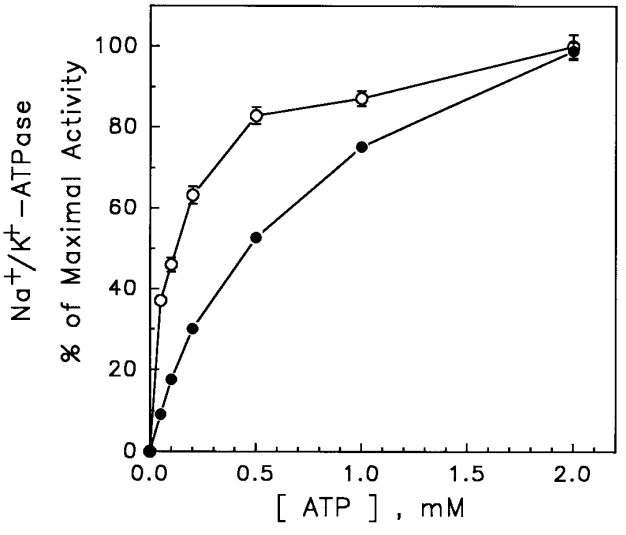

FIG. 7. Na^+/K^+-ATPase activity as a function of ATP concentration. Membranes of α,β-infected Sf-9 cells (○). Kidney microsomes (●). Mean ± SE of assays in seven different preparations.

Ion transport by the reconstituted enzyme. When membrane preparations isolated from α,β-infected cells were solubilized and reconstituted into proteoliposomes, they exhibited an ATP-dependent ^{22}Na-uptake into K^+-loaded vesicles (Fig. 11). The sensitivities of this uptake to vanadate and digitoxigenin (Fig. 11), in addition to its ATP-dependence, characterized it as being due to Na^+/K^+ exchange through the reconstituted enzyme of the inside-out vesicles. To explore the possibility that reconstitution may have activated the inactive fraction of the expressed subunits in Sf-9 cells, and to see if transport activity could be reconstituted from the control Sf-9 cells, rates of ATP-dependent $^{22}Na^+$-uptake were compared in proteoliposomes prepared from uninfected Sf-9 cells, α,β-infected cells, and canine kidney microsomes. Ouabain-sensitive Na^+/K^+-ATPase activity of each preparation was also assayed after the liposomes were made leaky with alamethicin. The results (Table II) did not indicate the reactivation of a significant fraction of the inactive subunits of the α,β-infected cells. The specific activity of Na^+/K^+-ATPase in reconstituted membranes of α,β-infected

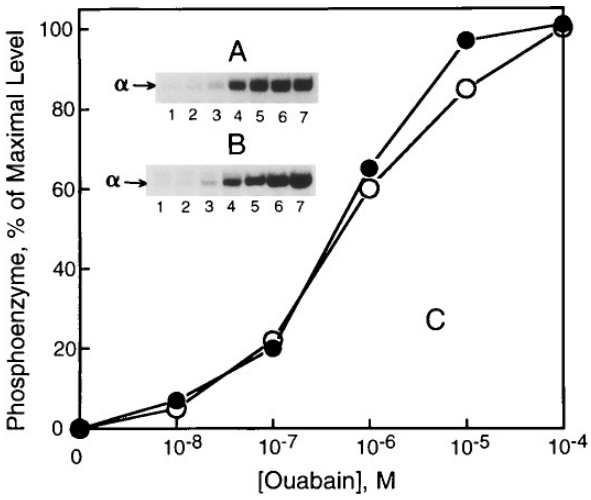

FIG. 9. Activating effects of varying ouabain concentrations on phosphorylation of the α subunit by 20 μM $^{32}P_i$. (A) Kidney microsomes. (B) Membranes of α,β-infected Sf-9 cells. Lanes 1–7, samples containing equal amounts of protein from reaction mixtures containing the following ouabain concentrations (M): 0, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}. (C) Plot of the gels in A and B after densitometric quantitation.

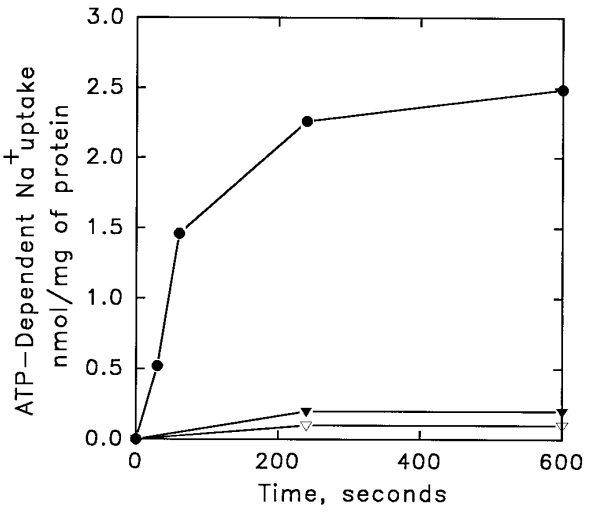

FIG. 11. Inhibitory effects of digitoxigenin and vanadate on the time course of ATP-dependent $^{22}Na^+$ uptake by proteoliposomes prepared from membranes of α,β-infected Sf-9 cells. Proteoliposome preparations and assays were carried out as described under Materials and Methods. ●, control liposomes; ▼, liposomes pretreated for 10 min with 0.1 mM digitoxigenin; ▽, liposomes pretreated for 10 min with 0.1 mM vanadate. Values are means from four different experiments. Standard errors are smaller than symbol sizes.

cells (Table II) was about the same as those of the membranes without reconstitution (Table I); and the ratio of transport activity to Na^+/K^+-ATPase activity in proteoliposomes of α,β-infected cells was double the same ratio in canine kidney proteoliposomes. While the different ratios may suggest some reconstitution-induced activation of inactive subunits of α,β-infected cells, they are more likely due to different fractions of inside-out vesicles in the two preparations.

ATP-dependent transport activity was not detected in vesicles prepared from uninfected cells, in spite of the presence of some ouabain-sensitive ATPase activity in these preparations (Table II).

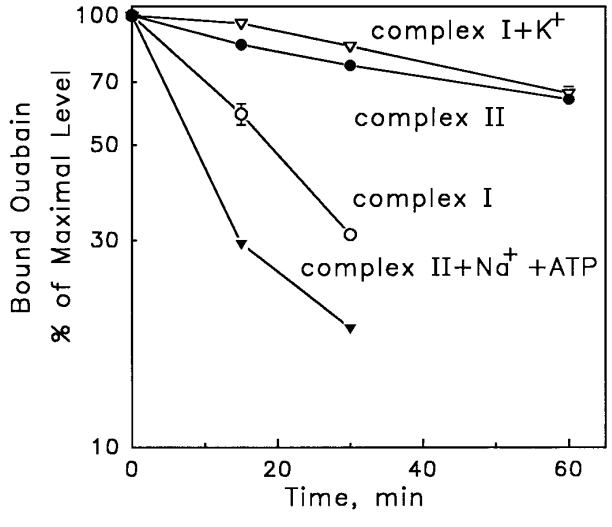

FIG. 10. Time course of release of ouabain from two types (I and II) of ouabain–enzyme complexes prepared from the recombinant enzyme. The two complexes were formed as indicated under Materials and Methods. They were incubated in 5 mM EDTA to determine spontaneous dissociation rates, or in 5 mM EDTA containing the indicated ligands: 10 mM K^+, 0.8 M Na^+, 2 mM ATP. Each value is the mean ± SE of four assays in each of two preparations.

DISCUSSION

The purpose of this study was to express α_1 and β_1 subunits of the canine Na^+/K^+-ATPase in Sf-9 insect cells, and to compare the properties of the resulting enzyme with those of the extensively studied canine kidney Na^+/K^+-ATPase, in order to assess the suitability of this expression system for structure–function studies related to the enzyme's reaction mechanism.

Before we consider the similarities and differences between the properties of the kidney enzyme and the enzyme expressed in Sf-9 cells, it is important to address the issue of the endogenous activity of these insect cells. In previous studies on the expressions of rodent isoforms of Na^+/K^+-ATPase in Sf-9 cells (2, 27), it was noted that membranes of uninfected cells exhibited small amounts of ouabain-sensitive Na^+/K^+-ATPase activity and high-affinity ouabain binding capacity, but that the phosphointermediate was barely detectable, and ouabain-sensitive $^{86}Rb^+$ uptake by intact uninfected cells was extremely low. These observations are confirmed and extended by our results: The

TABLE II

ATP-Dependent $^{22}Na^+$ Uptake and Total Na^+/K^+-ATPase Activity in Proteoliposomes

Source of proteoliposome	Na^+-uptake (nmol/mg/min)	Na^+/K^+-ATPase (μmol/mg/h)
Kidney microsomes	78.6 ± 3.6	142.6 ± 2
α,β-infected Sf-9 cells	1.45 ± 0.14	1.32 ± 0.05
Uninfected Sf-9 cells	Not detectable	0.24 ± 0.01

Note. Proteoliposomes were prepared and assayed as indicated under Materials and Methods. Na^+/K^+-ATPase is the total ouabain-sensitive activity assayed in leaky proteoliposomes. Each value is the mean ± SE of assays in four preparations.

membranes of both uninfected cells and cells infected with the wild-type virus clearly contain some ouabain-sensitive ATPase activity (Results and Table II); however, no ATP-dependent Na^+ transport is detectable in the reconstituted membranes (Table II). Also, while we noted (results not shown) high-affinity ouabain binding in some membrane preparations of uninfected cells equal to 5–10% of that observed in α,β-infected cells, neither this binding nor Na^+-dependent phosphorylation was observed consistently to permit the definite characterization of an endogenous Na^+/K^+-ATPase. In addition, using the highly sensitive fluorimetric assay of K^+-MFPase, we failed to detect any K^+-phosphatase activity in the uninfected cells. Taken together, these experiments create serious doubts about the existence of an endogenous enzyme of Sf-9 cells with properties similar to any of the known Na^+/K^+-ATPase isoforms. Xu (28) has presented evidence to suggest that several P-ATPases other than Na^+/K^+-ATPase may have binding sites with low affinities for cardiac glycosides; and Modyanov *et al.* (29) have shown that a human gene encodes a protein that seems to be a H^+/K^+-ATPase with relatively high ouabain sensitivity. Perhaps the apparent endogenous ouabain-sensitive activity of Sf-9 cells is not a Na^+/K^+-ATPase, but a distantly related P-ATPase.

Only a fraction of the α subunits (10–20%) expressed in Sf-9 cells is assembled as active Na^+/K^+-ATPase with a normal molar activity (Fig. 1 and Results). What causes the formation of the inactive enzyme is not known. However, our data establish that the inactive fraction is unable to catalyze any of the partial reactions of the enzyme, and that the detected partial activities, i.e., Na^+-ATPase and K^+-MFPase, are entirely due to the same fraction that exhibits Na^+/K^+-ATPase activity (Table I). This permits the use of all three hydrolytic activities for the comparison of the active recombinant enzyme with those of the kidney enzyme. An obvious advantage of the two partial hydrolytic activities is that they allow the examination of Na^+ kinetics in the absence of K^+, and the K^+ kinetics in the absence of Na^+. Also, while both high- and low-affinity ATP sites are involved in Na^+/K^+-ATPase activity, the assay of Na^+-ATPase allows the characterization of high-affinity catalytic site alone.

Comparison of Na^+-ATPase kinetics of the kidney enzyme and the enzyme expressed in Sf-9 cells shows the identical affinities of the catalytic sites of the two preparations for ATP (Fig. 3), and their similar Na^+ activation curves (Fig. 4). This complex Na^+ curve has been noted repeatedly previously and represents Na^+ binding at several transport and allosteric sites on cytoplasmic and extracellular sides (24; and references therein). The data clearly indicate, therefore, that the affinities and interactions of these multiple Na^+ sites are the same in the two preparations.

In contrast to the remarkable similarities of the Na^+-ATPase kinetics, there are clear differences between the K^+ activation curves of the K^+-MFPase and Na^+/K^+-ATPase of the two preparations (Figs. 5 and 6). As in the case of the Na^+ sites, there are also multiple transport and allosteric K^+ sites of the enzyme (30, 31), and it is necessary to see if changes in K_m values obtained from steady-state kinetics can be assigned to one or more of these sites. There is some evidence to indicate that activation of phosphatase is due to K^+ binding at a cytoplasmic site (32). As pointed out by others (33), however, there is also evidence to indicate that phosphatase K^+ sites may be a composite of cytoplasmic and extracellular sites. That the apparent K^+ affinity detected in Na^+/K^+-ATPase activity is also lower in the recombinant enzyme than it is in the kidney enzyme (Fig. 6) may seem to place the altered K^+ site at the extracellular side, since it is extracellular K^+ that accelerates dephosphorylation of E_2P and activates the overall ATPase activity. There is also evidence, however, to indicate an additional activating effect of cytoplasmic K^+ sites on Na^+/K^+-ATPase activity (34). Therefore, since in the unsided enzyme preparations used for the assay of Na^+/K^+-ATPase both the cytoplasmic and the extracellular sites are exposed to ions, it is not possible to identify the K^+ sites whose affinities are different in experiments such as those of Fig. 6. On the other hand, inhibition of Na^+-ATPase by K^+, when ATP concentration is low, is certainly due to K^+ effect at cytoplasmic sites (25, 34). Therefore, the data of Fig. 8 point to the different K^+ affinities of the two preparations at the cytoplasmic sites. The lower K_m of ATP in Na^+/K^+-ATPase activity of the enzyme expressed in Sf-9 cells (Fig. 7) is also consistent with the antagonistic effects of ATP at the low-affinity site, and K^+ at cytoplasmic sites (25). The well-established antagonism between Na^+ and K^+ at the cytoplasmic site accounts for the lower K_m of Na^+ in Na^+/K^+-ATPase activity expressed in Sf-9 cells (Results). On the whole, these data clearly show differences of the two enzymes

at the cytoplasmic K^+ sites, but do not rule out differences at extracellular K^+ transport sites. These considerations point to the well-established, but often neglected, difficulty of the interpretation of the complex kinetics of the hydrolytic activities of this enzyme in the unsided isolated preparations. The ability to reconstitute the recombinant enzyme in proteoliposomes (Fig. 11 and Table II) will permit the control of internal and external ligands in these sided preparations, and the future resolution of the questions raised by the technically simpler studies on the hydrolytic activities.

Regardless of the nature of the K^+ sites whose affinities are altered, it is important to consider why this change occurs when the canine enzyme is expressed in Sf-9 cells. One possibility is suggested by the different glycosylation states of the β subunit in the two enzyme preparations (Fig. 1). The role of β subunit in the regulation of K^+ interaction with the enzyme is now well established (35–37). Although glycosylation of β is not essential for activity (38, 39), we know of no data on the possible role of different glycosylation states on K^+ affinity. Another potential explanation is the different lipid environments of the subunits within the kidney and Sf-9 plasma membranes as suggested previously (40). This alternative may be difficult to test, however, since the tightly bound annular lipids may not be easily exchanged by solubilization–reconstitution. A possibility that should be considered seriously is that the lower K^+ affinity of the active enzyme in Sf-9 membranes may be due to the large excess of the inactive units of the enzyme. In the purified kidney enzyme there are α,β and α,α interactions (3, 15); and it is well established that interactions among functional α,β protomers affect the properties of various ligand binding sites (41), and that E_1–E_2 transition within the reaction cycle also affects the nature of protomer–protomer interactions (42). More recent studies (43, 44) have also shown the existence of specific α,α interactions among the subunits that are not turning over. It is reasonable, therefore, to assume that active and inactive subunits may also interact, and to consider that the excess of the latter in the Sf-9 membranes may affect some ligand interactions with the active enzyme. In this regard, it is of interest that two mutants of the sheep Na^+/K^+-ATPase that exhibited significantly lower apparent K^+ affinities than the control were also expressed along with large quantities of inactive enzyme (45). We should also note that in expression systems where there are significant amounts of endogenous Na^+/K^+-ATPase possible interactions of endogenous and recombinant enzymes must be considered.

The remaining data of this paper point to similarities rather than differences of the properties of the kidney and the recombinant enzymes. Our initial experiments showed that the phosphointermediate of the expressed enzyme had properties different from those previously reported of the phosphointermediate of the highly purified canine kidney enzyme. However, the less purified preparations of the kidney enzyme behaved the same as the recombinant enzyme (Fig. 2). The issue of why purification of the kidney enzyme, involving SDS treatment, alters the ADP and K^+ sensitivities of the phosphointermediate is an important one, but not relevant to the main point of the present communication. Suffice it to say that there is evidence for detergent effects, at both solubilizing and nonsolubilizing concentrations, on the subunit interactions of the enzyme (23, 42), and for alterations in E_1P/E_2P ratio due to these detergent effects (46).

The similarities of ouabain interactions with the kidney and the recombinant enzymes (Figs. 9 and 10) have twofold significance: First, the identical ouabain affinities of the two preparations, along with similar properties of the Na^+ and high-affinity ATP sites in the two preparations (Figs. 3 and 4), emphasize the specificity of noted changes in the apparent K^+ affinities. Second, the demonstration of the existence of the two classical ouabain complexes in the recombinant enzyme (Fig. 10) indicates that in future studies it will be possible to use the complexes of the expressed enzyme and its mutants, just as it was done in the purified enzyme (14), to characterize a variety of ligand binding sites through changes in ouabain dissociation rates. Such studies should complement the characterization of ligand binding sites through steady-state kinetic studies on the hydrolytic activities of the enzyme.

The studies presented here clearly show that even with the modest levels of expression of active Na^+/K^+-ATPase in baculovirus-infected insect cells crude membrane preparations of these cells are suitable for studies on (a) kinetics of Na^+/K^+-ATPase and partial hydrolytic activities; (b) the characteristics of the different conformational states of the phosphointermediate and their dephosphorylation rates; (c) ouabain binding to the enzyme, and ligand effects on ouabain release kinetics; and (d) the transport functions of the enzyme in reconstituted proteoliposomes. Having used such studies to compare the recombinant enzyme with the prototype kidney enzyme, our findings show similarities between the majority of the properties of the two preparations, and a highly selective difference in their K^+ affinities. The present data, combined with the large body of available information on structure and function of the purified canine kidney enzyme, constitute an appropriate database for mutagenesis studies on the relation of the enzyme's structure to its reaction mechanism.

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