Functional consequences of mutation Asn^{326}→Leu in the 4th transmembrane segment of the α-subunit of the rat kidney Na^+,K^+-ATPase

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Abstract **Site-specific mutagenesis was used to replace Asn^{326} in transmembrane segment M4 of the ouabain-insensitive α_1-isoform of rat kidney Na^+,K^+-ATPase. Mutant Asn^{326}→Leu was functional as demonstrated by the ability of COS cells expressing the mutant enzyme to grow in the presence of ouabain. In three independent assays encompassing Na^+ titrations of Na^+,K^+-ATPase activity, Na^+-ATPase activity, and phosphorylation from ATP, the Asn^{326}→Leu mutant displayed a reduced apparent affinity for Na^+. By contrast, this mutant exhibited a slightly increased apparent affinity for K^+ relative to the wild-type enzyme. In the presence of Na^+ without K^+, the Asn^{326}→Leu mutant hydrolyzed ATP at a high rate corresponding to 32% of the maximal Na^+,K^+-ATPase activity, and the rate of dephosphorylation of the phosphoenzyme intermediate was enhanced in the mutant relative to that of the wild-type enzyme. Oligomycin, known to stabilize the Na^+-occluded phosphoenzyme intermediate, reduced the dephosphorylation rate of the mutant and increased the steady-state phosphoenzyme level formed by the mutant at least 3-fold, whereas an increase in the steady-state phosphoenzyme level of only 10–15% was determined for the wild-type enzyme. The molecular turnover number for the Na^+,K^+-ATPase reaction, calculated when the steady-state phosphoenzyme level obtained in the presence of oligomycin was taken as a measure of the concentration of active sites, was slightly reduced relative to that of the wild-type enzyme. The data are discussed in terms of a role for Asn^{326} in binding of cytoplasmic Na^+ and in mediation of inhibition of dephosphorylation.**

Key words: Asparagine; Na^+/K^+-pump; Na^+ site; Mutation; Oligomycin

1. Introduction

The Na^+,K^+-ATPase (EC 3.6.1.37) is an integral membrane protein belonging to the P-type family of ion-transporting ATPases that catalyze ATP hydrolysis through the formation and breakdown of an aspartyl phosphorylated intermediate [1]. To understand the molecular mechanism of cation transport by the Na^+,K^+-ATPase and related ion pumps, it is essential to determine the amino acid residues involved in cation binding and in the discrimination between the different cations. While the residues taking part in ATP binding and catalysis are located in the cytoplasmic domain, those involved in the binding and transport of the cations are likely to reside in the transmembrane region of the pump protein [2–6]. In recent mutagenesis studies of the Na^+,K^+-ATPase, the residues with oxygen-containing side chains Glu^{329}, Glu^{781}, and Thr^{809} in putative transmembrane segments M4, M5, and M6, respectively, were shown to be important to cation binding at the high affinity Na^+- and/or K^+-transport sites [7–9]. This is similar to the demonstration that the homologous counterparts Glu^{309}, Glu^{771}, and Thr^{799} in another family member, the sarcoplasmic reticulum Ca^{2+}-ATPase, are critical to Ca^{2+}-occlusion [5,10,11]. The number of residues needed to form the selective Na^+- and/or K^+-liganding structure(s) in Na^+,K^+-ATPase is unknown. While the above-mentioned residues in M4, M5, and M6 are highly conserved among the P-type ATPases, another M4 residue with cation binding potential, Asn^{326}, is conserved only among the four isoforms of the Na^+,K^+-ATPase and the closely related X,K^+-ATPases of colon and kidney [12,13]. In the present study, the role of Asn^{326} of the α_1-isoform of rat kidney Na^+,K^+-ATPase has been analysed by mutagenesis. Asn^{326} was replaced by the hydrophobic residue leucine to remove the oxygen containing side chain and at the same time retaining the approximate size of the side chain. The Asn^{326}→Leu mutant resembles the previously described mutant Glu^{781}→Ala [8] in the sense that it was found to hydrolyze ATP at a high rate in the absence of K^+ and display strongly reduced affinity for Na^+ relative to the wild- type. On the other hand, unlike the Glu^{781}→Ala mutant, which displayed low apparent affinity for K^+ [8], the apparent affinity of the Asn^{326}→Leu mutant for K^+ was slightly increased relative to the wild-type. The data suggest a role for Asn^{326} in cytoplasmic Na^+ binding and in mediation of inhibition of dephosphorylation by external Na^+, whereas Asn^{326} does not seem to be important for K^+ binding at the external activation sites.

2. Materials and methods

2.1. Construction of mutant cDNAs and expression in COS-1 cells

Most of the methodology used here has previously been described in detail [7,14]. In brief, oligonucleotide-directed site-specific mutagenesis was carried out on cDNA encoding the ouabain-resistant rat α_1-isoform of the Na^+,K^+-ATPase α-subunit, and mutations were verified by nucleotide sequencing. The full-length cDNAs encoding wild-type or the Asn^{326}→Leu mutant contained in the expression vector pMT2 [15] were transfected into COS-1 cells, and several ouabain-resistant cell lines containing the cDNA stably integrated in the chromosomes were selected in the presence of 5 μM ouabain [7,14].

2.2. Enzyme preparation and Na^+,K^+-ATPase activity assays

A crude plasma membrane fraction was prepared and the vesicles opened with deoxycholate as previously described [7,14]. Protein concentration was determined by the dye binding method of Bradford [16] using bovine serum albumin as standard. The ATPase activity of the wild-type or mutant at various concentrations of Na^+, K^+, and ATP was measured on leaky membrane vesicles at 37°C by determination of the production of inorganic phosphate [7,14] and by a NADH-

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Abbreviations: E1, conformation with high Na^+ and ATP affinities; E2, K^+-occluded conformation with low affinity for ATP; $K_{0.5}$, concentration giving half-maximal activation; M1–M10, the putative transmembrane helices numbered from the NH_2-terminal end of the peptide.

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coupled assay [17]. The ouabain-resistant ATPase activity associated with the expressed exogenous enzyme was calculated by subtraction of the background ATPase activity measured at 10 mM ouabain from the ATPase activity measured at 10 μM ouabain. At least three separate membrane preparations corresponding to different clonal isolates of cells transfected with the same cDNA were assayed in duplicate [7,14]. There were no significant differences between different clonal isolates with respect to the Na^+, K^+, and ATP concentrations ($K_{0.5}$) giving half-maximal activation.

2.3. Phosphoenzyme formation and decay

Phosphorylation of the leaky membrane suspension (10 μg total membrane protein) was carried out for 10 s at 0°C in a reaction mixture containing 2 μM [γ-^{32}P]ATP, 20 mM Tris (pH 7.4), 3 mM $MgCl_2$, 1 mM EGTA, 10 μM ouabain, and various concentrations of NaCl or 50 mM KCl, the latter condition representing the background level of phosphoenzyme [7]. In experiments in which the effect of oligomycin was studied, the leaky membrane suspension was preincubated with 20 μg oligomycin/ml (a mixture of oligomycin A, B, and C components, from Sigma, dissolved in ethanol on the day of the experiment) in the presence of the indicated concentration of NaCl and 3 mM $MgCl_2$ for 10 min at 20°C, and the phosphorylation was carried out at the same oligomycin concentration.

Dephosphorylation was studied following addition of a chase solution containing unlabeled ATP to produce a final concentration of 1 mM. The reaction mixture (270 μl) was stirred continually in a thin-walled glass cylinder immersed in ice-water, and the chase solution (30 μl) was added manually from syringes.

Following acid quenching, the phosphorylated protein was subjected to SDS-polyacrylamide gel electrophoresis at pH 6 to separate the phosphorylated Na^+,K^+-ATPase from other phosphorylated proteins present in the crude plasma membrane preparation [7]. Autoradiograms were produced from the dried gels, and quantitation was obtained by liquid scintillation counting of gel slices.

3. Results

3.1. Expression

The mutant $Asn^{326} \rightarrow Leu$ of the ouabain-resistant Na^+,K^+-ATPase was found to be active as indicated by its ability to allow transformed COS cells to grow under selective conditions in the presence of ouabain to inhibit the endogenous COS cell Na^+,K^+-ATPase. To allow a detailed characterization of the functional properties of the exogenous expressed $Asn^{326} \rightarrow Leu$ Na^+,K^+-ATPase mutant, it was important to optimize the expression level of the mutant protein. Hence, the COS cells were grown in the presence of a low K^+ concentration (900 μM) which resulted in a 3- to 4-fold upregulation of the expressed exogenous mutant protein as previously described for the wild-type and other mutants [8].

3.2. ATPase activity

All the Na^+,K^+-ATPase activity measurements described below were carried out on leaky plasma membranes opened up by treatment with sodium deoxycholate, and the concentrations of Na^+ and K^+ on the two sides of the membrane were therefore identical. The internal and external cation binding sites in the wild-type Na^+,K^+-ATPase enzyme can be distinguished with this experimental system, due to the large difference in their preference for Na^+ and K^+. Thus, the internal sites display high affinity for Na^+ while the external sites display low affinity for Na^+ and high affinity for K^+.

The ATPase activity of the expressed mutant enzyme was measured at a variety of conditions and compared with the ATPase activity of a similar preparation of the wild-type rat kidney Na^+,K^+-ATPase expressed in COS cells. First, the ouabain dependence of Na^+,K^+-ATPase activity was titrated at

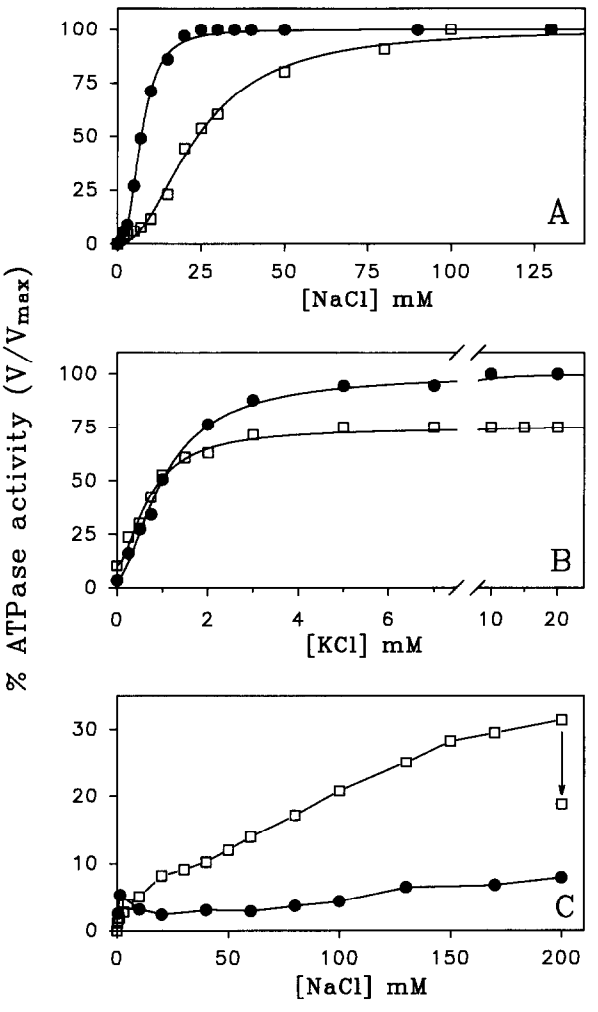

Fig. 1. Cation dependencies of ATPase activities of plasma membranes isolated from COS-1 cells transfected with cDNA encoding either wild-type or the mutant $Asn^{326} \rightarrow Leu$. The ATPase measurements were carried out at 37°C, pH 7.4, 3 mM ATP, 3 mM $MgCl_2$, 1 mM EGTA, 10 μM ouabain, and (A) various concentrations of NaCl in presence of 20 mM KCl, or (B) various concentrations of KCl in presence of 40 mM NaCl, or (C) various concentrations of NaCl in the absence of KCl. In all the experiments the ionic strength was kept constant by addition of choline chloride. The arrow in (C) indicates the effect of preincubation with oligomycin. The average values resulting from duplicate measurements are presented as percentage of the maximum Na^+,K^+-ATPase activity, as calculated after subtraction of background. The differences between the duplicate measurements were smaller than the size of the symbols. The respective values for the specific Na^+,K^+-ATPase activities corresponding to 100%, the $K_{0.5}(Na^+)$ values (A), and the $K_{0.5}(K^+)$ values (B) were the following: (A) wild-type (●), 7.9 μmol/h/mg, 7.1 mM; mutant $Asn^{326} \rightarrow Leu$ (□), 18.9 μmol/h/mg, 24.0 mM; (B) wild-type (●), 18.3 μmol/h/mg, 1.0 mM; mutant $Asn^{326} \rightarrow Leu$ (□), 18.9 μmol/h/mg, 0.8 mM; (C) wild-type (●), 24.3 μmol/h/mg; mutant $Asn^{326} \rightarrow Leu$ (□), 18.9 μmol/h/mg.

saturating Na^+, K^+, and ATP concentrations. Like the wild-type enzyme the mutant enzyme displayed a 500–1000-fold lower apparent affinity for ouabain compared to the endogenous COS cell Na^+,K^+-ATPase (not shown).

Fig. 1A and B show the Na^+- and K^+-dependencies of Na^+,K^+-ATPase activity measured in the mutant and the wild-type in the presence of 10 μM ouabain, i.e. at conditions in which the contribution of endogenous COS cell Na^+,K^+-ATP-

ase constitutes less than 5% of the wild-type activity (cf. [14]). The apparent affinity for Na^+ was reduced as much as 3–4-fold in the $Asn^{326} \rightarrow Leu$ mutant relative to the wild-type (Fig. 1A), whereas the apparent affinity for K^+ was slightly increased in the mutant (Fig. 1B). In the presence of 40 mM Na^+ and saturating concentrations of K^+, the Na^+,K^+-ATPase activity of the mutant reached only 75% that of V_{max} (Fig. 1B), reflecting the reduction in apparent Na^+ affinity described above. When the Na^+ concentration was increased to 100 mM to saturate the Na^+ sites, the $K_{0.5}(K^+)$ values of the mutant and the wild-type were both displaced towards higher K^+ concentrations, but the mutant still exhibited a slightly higher apparent affinity for K^+ relative to the wild-type ($K_{0.5}(K^+)$ values of 1.2 mM and 1.6 mM for the mutant and the wild-type, respectively, not shown).

Previously, a mutant was described ($Glu^{781} \rightarrow Ala$) which surprisingly was able to sustain high Na^+-ATPase activity in the absence of K^+ [8]. As seen from the point of intersection between the K^+ titration curve and the ordinate in Fig. 1B, and from the data in Fig. 1C, this was also the case for the mutant $Asn^{326} \rightarrow Leu$, which thus represents the second mutant of this phenotype described to date. The $Asn^{326} \rightarrow Leu$ mutant displayed a V_{max} for Na^+-ATPase activity of as much as 32% of the maximal Na^+,K^+-ATPase activity, corresponding to a 4–5-fold increase of V_{max} for the Na^+-ATPase activity relative to that of the wild-type. As seen in Fig. 1C, the low Na^+-ATPase activity exhibited by the wild-type follows a rather complex multiphasic activation pattern, which has previously been discussed [8]. From the Na^+-ATPase profile displayed by the mutant $Asn^{326} \rightarrow Leu$, it appears that the high-affinity stimulatory phase corresponding to Na^+ binding at cytoplasmic sites was displaced towards higher Na^+ concentrations relative to the wild-type. The phase corresponding to inhibitory Na^+ binding is hard to distinguish, while low affinity activation corresponding to Na^+ binding at external sites occurred in the same concentration range as for the wild-type with V_{max} being reached at a Na^+ concentration close to 200 mM.

The ATP concentration dependence of Na^+,K^+-ATPase activity was determined for the $Asn^{326} \rightarrow Leu$ mutant and the wild-type Na^+,K^+-ATPase in the presence of 130 mM Na^+ and 20 mM K^+ (Fig. 2). The mutant displayed a $K_{0.5}$ value for ATP (108 μM) 2–3-fold lower than that of the wild-type.

3.3. Characterization of the phosphoenzyme intermediate

In the normal Na^+,K^+-ATPase and Na^+-ATPase reaction cycles of the wild-type enzyme, ATP hydrolysis proceeds through an aspartyl phosphoenzyme intermediate. The phosphoenzyme intermediate of the mutant $Asn^{326} \rightarrow Leu$ was characterized in the experiments described in Figs. 3 and 4. Fig. 3A presents the results of experiments in which the effect of oligomycin on the steady-state phosphorylation level formed from ATP was studied in the absence of K^+. Oligomycin is known to promote occlusion of Na^+ and to decrease the rate of dephosphorylation so that all enzyme accumulates in the phosphorylated form in the wild-type enzyme. As seen in Fig. 3A, oligomycin was able to increase the steady-state phosphoenzyme level in the mutant at least 3-fold, whereas an increase of only 10–15% was observed in the wild-type.

The low steady-state phosphorylation level observed for the mutant in the absence of oligomycin can be explained either by a reduced rate of phosphorylation or by an increased dephosphorylation rate. Microscale methodology was therefore adapted to examine the dephosphorylation kinetics of expressed mutant and wild-type Na^+,K^+-ATPase. The dephosphorylation was measured in the presence and absence of oligomycin, following addition of a chase solution containing non-radioactive ATP diluting the radioactive ATP 500-fold. As seen in Fig. 3B, the dephosphorylation rate of the $Asn^{326} \rightarrow Leu$ mutant was found to be significantly higher than that of the wild-type in the absence of oligomycin, but following incubation with oligomycin the phosphoenzyme of the mutant was stabilized to the same extent as that of the wild-type, consistent with the inhibitory effect exerted by oligomycin on Na^+-ATPase activity (arrow, Fig. 1C).

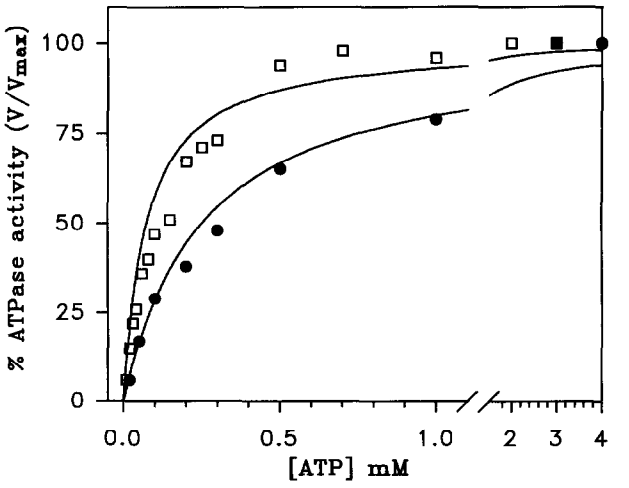

Fig. 2. ATP dependence of Na^+,K^+-ATPase activity of plasma membranes isolated from COS-1 cells transfected with cDNA encoding either wild-type or the mutant $Asn^{326} \rightarrow Leu$. Na^+,K^+-ATPase measurements were carried out at 37°C, pH 7.4, in the presence of 130 mM NaCl, 20 mM KCl, $MgCl_2$ (0.1–4.0 mM, keeping $[Mg^{2+}]$ constant), 1 mM EGTA, 10 μM ouabain, and various concentrations of ATP. The average values resulting from duplicate measurements are presented as percentage of the maximum Na^+,K^+-ATPase activity as calculated after subtraction of background ATPase activity measured at the same ATP concentration. The differences between the duplicate measurements were smaller than the size of the symbols. The respective values for the specific ATPase activities corresponding to 100% and the $K_{0.5}$(ATP) values were the following: wild-type (●), 7.6 μmol/h/mg, 0.28 mM; mutant $Asn^{326} \rightarrow Leu$ (□), 18.9 μmol/h/mg, 0.11 mM.

Because the apparent affinity for Na^+ determined in the ATPase assay is dependent on the competition from K^+ and the rate of dephosphorylation, it is important to minimalize these factors in order to be able to evaluate the true affinity for Na^+ at the intracellular transport sites. To this end, phosphorylation from ATP at various concentrations of Na^+ was measured in the absence of K^+ and in the presence of oligomycin (Fig. 4). Under these conditions the mutant displayed a 6–7-fold reduced affinity for Na^+ relative to the wild-type. When oligomycin was omitted from the reaction mixture, a $K_{0.5}$ value for Na^+ almost identical to that determined in presence of oligomycin was obtained for the wild-type (not shown). This is consistent with the low rate of dephosphorylation observed with the wild-type even in the absence of oligomycin. Due to the low level of phosphoenzyme formed by the mutant in the absence of oligomycin, it was not possible to obtain a reliable $K_{0.5}$ value for Na^+ activation of phosphorylation of the mutant under these conditions.

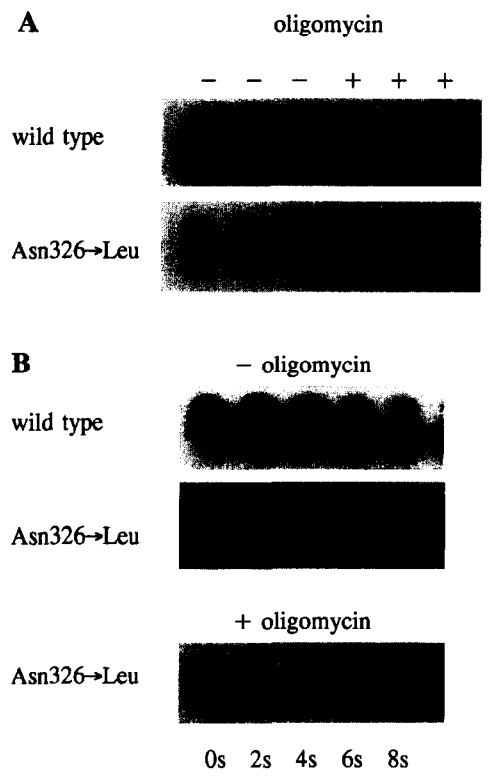

Fig. 3. Effect of oligomycin on steady-state phosphorylation levels and dephosphorylation rates of the wild-type and the mutant $Asn^{326} \rightarrow Leu$. In (A), phosphorylation was carried out as described under section 2 in the presence of 150 mM NaCl with (+) or without (–) oligomycin present, and acid quenching was performed at steady-state. Autoradiographs of the dried SDS-polyacrylamide gels corresponding to triplicate determinations are shown. In (B), phosphorylation was carried out in the presence of 40 mM NaCl with or without oligomycin, followed by addition of unlabeled ATP to a final concentration of 1 mM, and acid quenching at serial time intervals as indicated. The autoradiographs of the dried SDS-polyacrylamide gels are shown. Each lane was loaded with 7 μg of plasma membrane protein. The maximum specific Na^+,K^+-ATPase activities of the samples used for phosphorylation were the following: wild-type, 18.3 μmol/h/mg; mutant $Asn^{326} \rightarrow Leu$, 18.9 μmol/h/mg.

4. Discussion

The present study has demonstrated that the mutant $Asn^{326} \rightarrow Leu$ displays low Na^+ affinity in the Na^+,K^+- and Na^+-ATPase assays, as well as in the phosphorylation assay carried out in the absence of K^+ under conditions where dephosphorylation was blocked. These data suggest a direct or indirect role for Asn^{326} in high-affinity Na^+ binding. The oxygen-containing side chain of Asn^{326} may possibly participate as a liganding group in one or more of the three intracellular Na^+ sites in the conformation with high Na^+ and ATP affinities (E1 form). Since the mutant exhibited a 2–3-fold increased apparent affinity for ATP in the Na^+,K^+-ATPase assay relative to the wild-type, an indirect effect on Na^+ affinity exerted by a poise of the E1–E2 equilibrium in favor of the E2 form, which exhibits lower Na^+ and ATP affinities than E1, seems to be excluded [7].

On the basis of a low apparent affinity for Na^+ detected in the phosphorylation assay with mutants, three additional residues Glu^{329}, Glu^{781}, and Thr^{809}, have been classified as potential Na^+ ligands at the high affinity cytoplasmic sites in the E1 form of the Na^+,K^+-ATPase [5,8]. The residues Asn^{326}, Glu^{329}, Glu^{781}, and Thr^{809} are located in putative transmembrane segments M4, M5, and M6, which are likely to form a cavity for cation occlusion both in Na^+,K^+-ATPase and Ca^{2+}-ATPase [5]. Asn^{326} is located only 3 residues N-terminal to Glu^{329} and, hence, one turn below Glu^{329} in the putative α-helical structure of transmembrane segment M4. Unlike the other 3 residues mentioned, Asn^{326} is neither conserved in the Ca^{2+}-ATPase nor in the gastric H^+,K^+-ATPase, whereas it is conserved in all four isoforms of the Na^+,K^+-ATPase and in the X,K^+-ATPases from colon and kidney, which in the putative transmembrane sector show higher homology with the Na^+,K^+-ATPase than the gastric H^+,K^+-ATPase [13,18]. Asn^{326} may, therefore, contribute to the specific selectivity towards Na^+ in the E1 form of the Na^+,K^+-ATPase. The lack of conservation of Asn^{326} at the homologous position in the gastric H^+,K^+-ATPase is consistent with the finding that the K^+ affinity was unchanged or even slightly increased in the $Asn^{326} \rightarrow Leu$ mutant. On the basis of this result Asn^{326} is not likely to participate in K^+ binding in the 'K^+ forms', E2 and E2P, of the Na^+,K^+-ATPase. This represents a significant difference from the residues Glu^{329} and Glu^{781}, which were shown to be important not only for Na^+ binding but also for K^+ binding in the Na^+,K^+-ATPase [5,7,8].

The mutant $Asn^{326} \rightarrow Leu$ was found to be able to sustain ATP hydrolysis at a high rate even in the absence of K^+, the maximum Na^+-ATPase activity of the mutant amounting to as much as 32% that of the maximum Na^+,K^+-ATPase activity (Fig. 1C). Only one of the above-mentioned mutants with reduced Na^+ affinity, namely $Glu^{781} \rightarrow Ala$, has previously been shown to display similar characteristics [8]. Both in the $Asn^{326} \rightarrow Leu$ mutant (Fig. 3A) and in the $Glu^{781} \rightarrow Ala$ mutant [8], the steady-state phosphoenzyme level was low compared with the wild-type phosphoenzyme level. The dephosphorylation data obtained in the present study showed that in the $Asn^{326} \rightarrow Leu$ mutant the phosphoenzyme decayed at a rate that was significantly increased relative to that of the wild-type (Fig. 3B). Addition of oligomycin led to a reduction of the Na^+-ATPase activity and of the dephosphorylation rate, and the steady-state phosphoenzyme level increased at least 3-fold in the presence of oligomycin. Therefore, it appears likely that the high rate of dephosphorylation of the $Asn^{326} \rightarrow Leu$ mutant is a major factor determining the high Na^+-ATPase activity and the low steady-state phosphorylation level measured in the absence of oligomycin. On the other hand, from the measurements of the Na^+,K^+-ATPase activity and the enzyme concentration, given by the maximum steady-state phosphoenzyme level obtained in the presence of oligomycin, a molecular turnover number for the Na^+,K^+-ATPase reaction in the $Asn^{326} \rightarrow Leu$ mutant could be calculated, which was slightly reduced (80–90%) relative to that of the wild-type. Hence, it is not excluded that the rate of the phosphorylation reaction was reduced to some extent in the mutant, thereby contributing to the low steady-state phosphorylation level. In the present study, it was not possible to detect any reduction of the phosphorylation rate in the mutant (data not shown), but since a manual mixing technique was used, for which the lower reaction time limit is 1–2 s, the phosphorylation rate would have to be more than 10-fold reduced relative to that of the wild-type to be measurable.

To understand the reason why in the mutant Na^+ was able

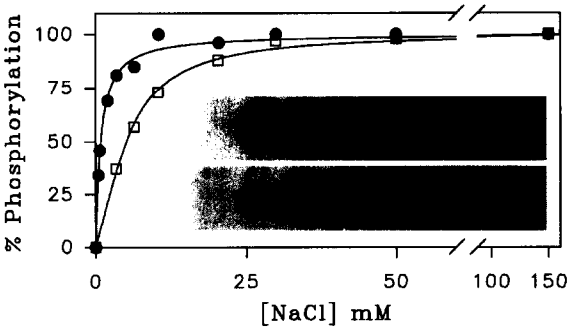

Fig. 4. Na^+ dependence of phosphorylation from ATP of the wild-type and mutant $Asn^{326} \rightarrow$ Leu. Phosphorylation was carried out as described in section 2, in the presence of oligomycin and various concentrations of Na^+ as indicated on the abscissa. The ionic strength was kept constant by addition of choline chloride. The data are averages of duplicate measurements and are presented as percentage of the maximum steady-state phosphorylation level. The insets show examples of autoradiographs of the dried SDS-polyacrylamide gels corresponding to the following Na^+ concentrations: 0 mM, 3.4 mM, 6.4 mM, 10.4 mM, 20.4 mM, 30 mM, 50 mM, and 150 mM (from left to right) (●) and upper panel, wild-type; (□) and lower panel, mutant $Asn^{326} \rightarrow$ Leu. Each lane was loaded with 7 μg of plasma membrane protein. The $K_{0.5}(Na^+)$ values were the following: wild-type, 0.8 mM; mutant $Asn^{326} \rightarrow$ Leu, 5.1 mM.

to substitute for K^+ in the activation of dephosphorylation it should be noted that the complex multiphasic Na^+ activation pattern of the Na^+-ATPase activity in the wild-type enzyme is the result of several effects [19]: (i) stimulation of phosphorylation by Na^+ binding to internal high-affinity sites in the E1 form; (ii) inhibition by Na^+ binding at external site(s) possessing intermediate affinity; (iii) reversal of that inhibition by Na^+ binding at external activation site(s) of lower affinity in the E2P form. Although accurate $K_{0.5}$ values for the various Na^+ effects exerted by binding to the external sites cannot be determined from the measurement of overall Na^+-ATPase activity, it is clear that the Na^+-ATPase activity of the $Asn^{326} \rightarrow$ Leu mutant, like that of the wild-type enzyme, reached V_{max} at a Na^+ concentration close to 200 mM, and the affinity for Na^+ at the external low-affinity site(s) on the E2P form responsible for activation of Na^+-ATPase activity thus seems to be little changed following mutation of Asn^{326} to Leu. Hence, the K^+-like effect exerted by Na^+, which enhances the dephosphorylation rate and Na^+-ATPase activity in the $Asn^{326} \rightarrow$ Leu mutant, may arise primarily from an increased efficiency of Na^+ as activator of dephosphorylation (V_{max} effect on Na^+-ATPase) rather than from an increased affinity for Na^+ at the external activation site(s). The increased efficiency of Na^+ activation in the mutant might be due to disruption of an inhibitory external Na^+ site [19], or the replacement of Asn^{326} with Leu might in some other way enhance the signal transduction to the catalytic domain leading to dephosphorylation.

In conclusion, it is likely that Asn^{326} and Glu^{781} both participate in binding of intracellular Na^+ at the high-affinity activation sites and, later in the cycle, in addition in the signal transduction leading to inhibition of dephosphorylation by external Na^+. Because the apparent affinity for K^+ was normal or slightly increased in the $Asn^{326} \rightarrow$ Leu mutant, whereas the K^+ affinity was reduced in the previously characterized $Glu^{781} \rightarrow$ Ala mutant, only Glu^{781} contributes directly to K^+ binding at external activation site(s) involved in K^+ occlusion and transport. If Asn^{326} is part of an inhibitory external Na^+ site which is disrupted by the mutation to Leu, this site is most likely not identical to one of the external K^+-activation sites.

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