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Site-directed mutagenesis of Asp-376, the catalytic phosphorylation site, and Lys-507, the putative ATP-binding site, of the α-subunit of *Torpedo californica* Na^+/K^+-ATPase

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Point mutations of Asp-376 of the α-subunit of *Torpedo californica* Na^+/K^+-ATPase (the site of phosphorylation during the catalytic cycle) to Asn, Glu or Thr led to virtual abolishment of Na^+/K^+-ATPase activity and ouabain-binding capacity. Replacement of Lys-507 of the same subunit (the putative ATP-binding site) by Met resulted in decreases in Na^+/K^+-ATPase activity and ouabain-binding capacity. These results are in agreement with those reported for rabbit sarcoplasmic reticulum Ca^{2+}-ATPase (Maruyama, K. and MacLennan, D.H. (1988) Proc. Natl. Acad. Sci. USA 85, 3314–3318).

Introduction

Cation pumps such as H^+-, Ca^{2+}- and Na^+/K^+-ATPase are transiently phosphorylated during the catalytic cycle and are called P-type ATPase [1,2]. In these enzymes, the carboxyl group of an aspartyl residue, the sequence around which is highly conserved [-Cys-Ser-Asp(P)-Lys-], has been shown to be phosphorylated [3]. Replacement of the phosphorylatable aspartic acid residue by other amino acids, e.g., asparagine, in yeast H^+-ATPase by site-directed mutagenesis has been reported to result in a functional enzyme with a normal phosphorylated intermediate [4], whereas the same point mutation in rabbit sarcoplasmic reticulum Ca^{2+}-ATPase has been shown to result in its complete inactivation [5].

In this report, as a third example for P-type ATPase, we examined the effects of mutations of Asp-376, the phosphorylation site, of the α-subunit of *Torpedo californica* Na^+/K^+-ATPase on the catalytic function of the enzyme. The effect of mutation of Lys-507 of the same subunit was also studied. This lysine residue is specifically modified by fluorescein isothiocyanate (FITC) and thought to be involved in ATP binding [6,7].

Materials and Methods

Site-directed mutagenesis. A 606-bp fragment [KpnI (497)–BamHI(1103)] and a 502-bp fragment [BamHI (1103)–HindIII(1605)] were excised from pSPT(α) containing the entire coding region of *T. californica* Na^+/K^+-ATPase α subunit [8], and used for Asp-376 and Lys-507 mutation, respectively. Both fragments were purified and subcloned into M13 mp19. The oligonucleotide primers used for mutagenesis (Table I) were prepared in an Applied Biosystems Model 380A DNA synthesizer. Site-directed mutagenesis of the α subunit gene fragment was performed according to the kit sup-

TABLE I

Sequences of mutagenic oligonucleotide

Mutation	Oligonucleotide (changes underlined)
Asp-376 to Asn	5′-TTTGCTCAAACAAAACT-3′
Asp-376 to Glu	5′-TGCTCAGAGAAAACTGG-3′
Asp-376 to Thr	5′-TTTGCTCAACCAAAACTG-3′
Lys-507 to Met	5′-GGTGATGATGGGAGCAC-3′

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plier's instructions (Amersham (code RPN 1523)) [9]. The mutated fragments were sequenced to verify that the desired mutants had actually been obtained. The gene fragments carrying the mutations were cut off from the replicative form of M13 mp19 and used to replace the corresponding segments of the α subunit gene that had been inserted into pSP65.

Expression in Xenopus oocytes. The linearized plasmids were transcribed in vitro with the aid of SP6 RNA polymerase and the mRNAs synthesized were injected into *Xenopus laevis* oocytes as described [10] except that the oocytes were isolated with collagenase (2 mg/ml at 19°C for 6 h) instead of scissors and a forceps. The concentration of mRNA was 0.5 μg/μl for each mRNA and about 20 nl, on an average, was injected per oocyte. The injected oocytes were incubated at 19°C for 3 days in modified Barth's medium containing 100 μg/ml each of ampicillin, streptomycin and cefmenoxime and 15 μg/ml nystatin. Labeling of translation products was carried out as described [10] except that L-[4,5-^{3}H]leucine (142 Ci/mmol; final concentration, 0.3 mCi/ml) was used instead of L-[U-^{14}C]leucine. Preparation of oocyte microsomes and determination of ATPase activity and [21,22-^{3}H]ouabain-binding capacity were performed as described [10,11].

Materials. Enzymes used for site-directed mutagenesis and in vitro transcription were purchased from Takara Shuzo Co., Nippon Gene and Toyobo. Antisera raised against the α- and β-subunits of *T. californica* Na^+/K^+-ATPase have been described [10]. Other chemicals and biochemicals were obtained from Nacalai Tesque Inc. and were of reagent or higher grade.

Results and Discussion

Site-directed mutagenesis was used to analyze the functions of two important residues of the α subunit of *T. californica* Na^+/K^+-ATPase, one is Asp-376, which has been shown to be the site of catalytic phosphorylation [3], and the other is Lys-507, which is thought to be the ATP-binding site on the basis of the observation that ATP protects Lys-507 against its modification with FITC[6,7]. For this purpose, point mutations were performed to replace Asp-376 by Asn, Glu or Thr and Lys-507 by Met. The mutant genes were transcribed in vitro and the mRNAs thus synthesized were expressed in *Xenopus* oocytes.

The oocytes, to which a mutated mRNA for the α-subunit of the ATPase had been injected together with the wild-type mRNA for the β-subunit, were incubated for 3 days in the presence of L-[^{3}H]leucine. The oocytes were then treated with Triton X-100 and subjected to immunoprecipitation with a mixture of anti-α and anti-β subunit anti-sera [10]. The immunoprecipitates were examined by SDS-polyacrylamide gel electrophoresis and subsequent fluorography. As shown in Fig. 1, the α subunit (about 100 kDa) was detected as a heavily exposed band irrespective of the mutation introduced. For the β-subunit, the fully glycosylated (βm about 60 kDa) as well as the core-glycosylated forms (βc, about 40 kDa) were present, though the latter was very faint. The levels of the α- and β-subunits expressed were similar to each other and seemed to be high enough to assay their activities for all samples [10,11]. We then examined ouabain-inhibitable ATPase activity of microsomes prepared from oocytes injected with mRNAs. Microsomes from oocytes that had not received mRNAs were used as a control to assess the contribution of Na^+/K^+-ATPase inherent to the oocytes. As shown in Fig. 2 and Table II, the increments of ATPase activity from the control level in mutants in which Asp-376 had been mutated to Asn, Glu or Thr were very slight. On the other hand, the ATPase activity of microsomes from oocytes injected with mRNA for the Lys-507 to Met mutant was definitely higher than that of control, although it was lower than that of microsomes from oocytes injected with mRNA for the wild-type α-subunit.

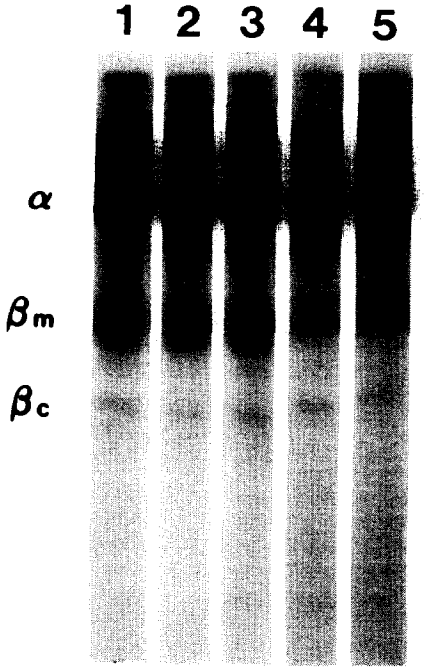

Fig. 1. Fluorogram of translation products from Na^+/K^+-ATPase subunit-specific mRNAs in *Xenopus* oocytes. Cell extracts from oocytes injected with wild-type (lane 5) or Asp-376 to Asn (lane 1), Asp-376 to Glu (lane 2), Asp-376 to Thr (lane 3) or Lys-507 to Met (lane 4) mutated α-subunit were subjected to immunoprecipitation with a mixture of anti-α and anti-β subunit sera. In every case, mRNA for the wild-type β-subunit of *T. californica* was injected together with mRNA for the α-subunit.

In order to elucidate the causes for the difference in enzymatic activity among the mutants, the Na^+/K^+-ATPase expressed on the cell surface was estimated by measuring the ouabain-binding capacity of oocytes [10]. As ouabain binds specifically and stoichiometrically to

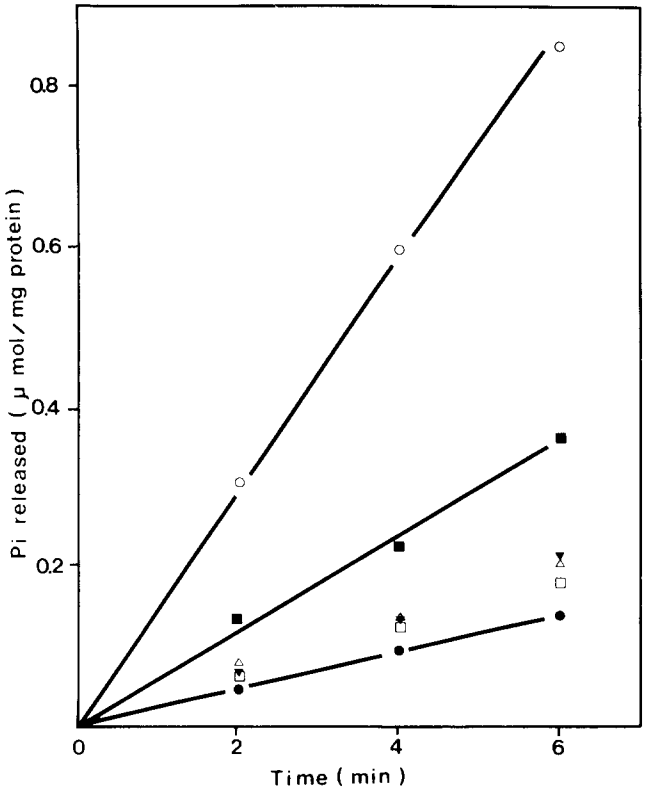

Fig. 2. Na^+/K^+-ATPase activity in microsomes from non-injected *Xenopus* oocytes (●) and from oocytes injected with mRNA for the wild-type α-subunit (○); or Asp-376 to Asn (▼), Glu (△) or Thr (□) mutant; or Lys-507 to Met (■) mutant. mRNA for the wild-type β-subunit was simultaneously injected.

the catalytically active form of the enzyme [12], the ouabain-binding capacity of an oocyte should reflect the amount of functionally active Na^+/K^+-ATPase on the cell surface. As shown in Table II, the ouabain-binding capacity of the oocyte injected with mRNA for the Lys-507 mutant was obviously higher than that of control, but lower than that of oocytes injected with the wild-type mRNA. As is evident from Table II, the ratio of ouabain-binding capacity to ATPase activity was not significantly different between the wild-type and Lys-507 mutant, suggesting that molar activities of these two enzymes were nearly the same. Hence, it is likely that the reduced activity of Lys-507 mutant was due to the reduced amount of the functionally active enzyme expressed on the cell surface and not caused by partial reduction of molar activity of the enzyme.

For the Asp-376 mutants, a little ouabain-binding was observed in agreement with the results for ATPase activity (Fig. 2 and Table II). When mRNA for the β-subunit was injected alone, the oocytes exhibited a slight increase in Na^+/K^+-ATPase activity probably due to the induction of the α-subunit of the host cell (Noguchi, unpublished results). Hence, it is plausible to consider that the hybrid Na^+/K^+-ATPase between *Xenopus* α- and *Torpedo* β-subunit exhibited small ATPase activity and ouabain-binding capacity in oocytes injected with mRNA for Asp-376 mutated α-subunit and mRNA for the wild-type β-subunit. It was, therefore, concluded that all the Asp-376 mutants were inactive, though they were expressed in sufficient quantities. We could not, however, say whether this lack of activities in the Asp-376 mutants resulted from a structural defect of the enzyme due to the mutations or from the prevention of catalytic phosphoenzyme formation even if the over-all structure of the mutated enzymes were essentially normal. In any case, replacement of Asp-376 by any of the three amino acids (Asn, Glu, Thr) resulted in the loss of functional enzyme formation, supporting the results for Ca^{2+}-ATPase reported by Maruyama and MacLennan [5].

TABLE II

Ouabain-binding and Na^+/K^+-ATPase activity of mutants expressed in Xenopus oocytes

Xenopus oocytes were injected with mRNAs for the mutant subunit shown in the table together with the mRNA for the wild-type β-subunit and incubated for 3 days at 19° C. The oocytes were deprived of follicular cells and assayed for [^{3}H]ouabain-binding capacity. For Na^+/K^+-ATPase activity, microsome preparations which had been treated with 1 M NaSCN, were assayed in the presence and absence of 1 mM ouabain as described in Ref. 10, and the ouabain-sensitive activity was taken as Na^+/K^+-ATPase.

	[^{3}H]Ouabain-binding (fmol/oocyte)	Na^+/K^+-ATPase activity (μmol P_i/mg per h)
Control *	16.5± 2.5(n = 5)	1.3±0.4(n = 4)
Wild type	56.3±18.3(n = 5)	5.7±1.7(n = 4)
Asp-376 to Asn	23.6± 5.2(n = 5)	1.7±0.6(n = 3)
Asp-376 to Glu	25.2± 3.5(n = 5)	1.7±0.4(n = 4)
Asp-376 to Thr	24.5± 3.5(n = 5)	1.6±0.5(n = 4)
Lys-507 to Met	32.7± 5.3(n = 4)	3.0±0.8(n = 4)

* No mRNAs were injected.

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