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Autoregulation of the phosphointermediate of Na^+/K^+-ATPase by the amino-terminal domain of the α-subunit

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Chymotryptic cleavage of the α-subunit of the canine kidney Na^+/K^+-ATPase in the presence of Na^+ abolishes ATPase activity and yields an 83 kDa peptide from Ala 267 to the COOH-terminus. To test the proposal that E_1 to E_2 conformational transition is blocked in this modified enzyme, we have made a detailed comparison of its phosphorylation with that of the native enzyme by ATP. While phosphorylation of α is dependent on Na^+ and prevented by K^+, that of the 83 kDa peptide is modestly stimulated by Na^+; and only this stimulation, but not the Na^+-independent phosphorylation is inhibited by K^+. Ouabain, which inhibits α-phosphorylation by ATP, activates Na^+-independent phosphorylation of the 83 kDa peptide by ATP, and inhibits the Na^+-stimulation of this process. While there is a ouabain-stimulated phosphorylation of α by P_i, the 83 kDa peptide is not phosphorylated by P_i with or without ouabain. In its sensitivity to ADP, and insensitivity to K^+, the phosphopeptide is similar to the E_1P of the native enzyme; however, the spontaneous decomposition rate of the phosphopeptide is orders of magnitude lower than that of the native EP. Na^+ has no effect on the spontaneous decomposition of the phosphopeptide; but at high Na^+ concentrations ($K_{0.5}$ = 350 mM) the ADP sensitivity of the phosphopeptide is reduced. The phosphopeptide, like the native EP, is acid-stable, alkaline-labile, and sensitive to hydroxylamine and molybdate. The chymotrypsin-treated enzyme catalyzes an ADP-ATP exchange activity that is stimulated by Na^+. The Na^+-independent part of this exchange, unlike that of the native enzyme, is activated by ouabain. Our findings establish that (a) the phosphorylation process and its control by Na^+, K^+ and ouabain are autoregulated by the NH_2-terminal domain of the α-subunit; and (b) the often repeated assumption that the primary role of this domain is in the regulation of E_1-E_2 transitions is not valid.

Introduction

Studies on the controlled proteolysis of the α-subunit of Na^+/K^+-ATPase have provided invaluable information about the organization of this transport enzyme within the plasma membrane, and the relation of the enzyme's structure to its function [1]. Some of the conclusions based on such studies, however, are less solid than others. For example, while it is well established that the reaction of chymotrypsin with the Na^+ form of the enzyme (E_1) leads to the cleavage of a specific bond on the NH_2-terminal side of the aspartyl group that is phosphorylated in the course of ATP hydrolysis, the often repeated conclusion that the cleavage of this bond stabilizes the enzyme in the E_1 state [2–6] is supported by some observations [2,4] but not by others [7]. To clarify these uncertainties we have subjected the enzyme to controlled proteolysis, and we have examined the characteristics of the phosphorylated products of the proteolytic fragments in more detail than it has been done before. The results contradict some previous assumptions, and provide new insights into the role of the NH_2-terminal segment of the α-subunit in the autoregulation of the enzyme function.

Materials and Methods

The enzyme from canine kidney medulla was prepared and assayed as described before [8]. Reaction of the enzyme with chymotrypsin was done as described by Jørgensen and Petersen [4]. Briefly, the enzyme (0.5 mg/ml) was incubated at 37°C for varying periods in a solution containing 10 mM NaCl, 15 mM Tris-HCl (pH 7.4), and 15 μg/ml of α-chymotrypsin. The reaction was stopped by the addition of trypsin-chymotrypsin inhibitor (100 μg/15 μg of chymotrypsin). The enzyme

Abbreviations: SDS, sodium dodecyl sulfate; CDTA, 1,2-cyclohexylenedinitrilotetraacetic acid.

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was collected by centrifugation in cold, and washed prior to use. Sequential treatment with chymotrypsin and trypsin was done as described before [4].

Unless indicated otherwise, the various enzyme preparations were phosphorylated with [γ-^{32}P]ATP at 0°C by incubation in a medium containing 20 mM Tris-HCl (pH 7.2), and the indicated concentrations of Mg^{2+}, ATP, Na^+, K^+ and ouabain. All such incubations were done for 90 s. Preliminary experiments had shown that this was sufficient to assure the attainment of the maximal level of phosphorylation. Reactions were stopped by the addition of equal volumes of an ice-cold solution of 10% trichloroacetic acid containing 200 mM unlabeled P_i and 50 mM unlabeled ATP. The sediments were either filtered and counted [8], or subjected to SDS-gel electrophoresis on 7.5% polyacrylamide gels at pH 2.4 [9]. The gels were stained and autoradiographed by conventional procedures. Phosphorylation by [^{32}P]P_i was done as described before [8].

To measure dephosphorylation, the chymotrypsin-treated enzyme was phosphorylated with labeled ATP as indicated above, an ice-cold solution of Tris-CDTA was added to obtain a final concentration of 20 mM CDTA; and aliquots were removed at indicated times, diluted with the trichloroacetic acid solution as indicated above, filtered, and counted. It was established experimentally that 20 mM CDTA was sufficient to stop phosphorylation of the enzyme under all conditions used here.

For the assay of ADP-ATP exchange, the enzyme was incubated at 37°C in a solution containing 0.1 M Tris-HCl (pH 7.2), and the indicated concentrations of ATP and [^{14}C]ADP, and other ligands as specified. The rate of incorporation of labeled ADP into ATP was measured as described [10].

For the determination of the NH_2-terminal sequence of the 83 kDa peptide, the chymotrypsin-treated enzyme was subjected to SDS-gel electrophoresis as indicated above, electroblotted onto polyvinylidene difluoride membranes [11], and sequenced on an Applied Biosystems model 477A sequencer.

α-chymotrypsin (type II, bovine pancrease), trypsin (type III-s, bovine pancrease), trypsin-chymotrypsin inhibitor (soybean), 'Vanadium-free' ATP, ADP, and ouabain were obtained from Sigma. [γ-^{32}P]ATP, [^{14}C]ADP, and [^{32}P]P_i were obtained from DuPont-New England Nuclear.

Results

In agreement with previous observations [4,5], when the canine kidney enzyme was exposed to chymotrypsin in a medium of low ionic strength containing Na^+, the major product of the cleavage of the α-subunit was a peptide with the molecular mass of about 83 kDa (Fig. 1a). The NH_2-terminal sequence of this fragment was

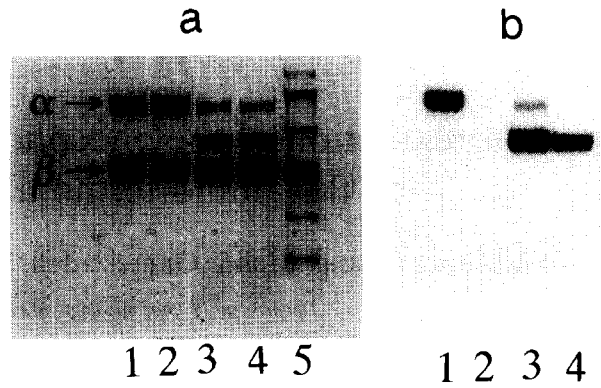


Fig. 1. Effects of Na^+ and K^+ on the phosphorylation of the native and the chymotrypsin-treated enzyme. The enzyme was exposed to chymotrypsin for 15 min as described in Materials and Methods. The control enzyme (lanes 1 and 2) and the treated enzyme (lanes 3 and 4) were reacted with 0.3 μM [γ-^{32}P]ATP in the presence of 2 mM Mg^{2+} and either 100 mM Na^+ (lanes 1 and 3) or 20 mM K^+ (lanes 2 and 4). Acid-denatured samples and molecular mass markers (lane 5) were subjected to SDS-gel electrophoresis at pH 2.4. The gels were stained (a) and autoradiographed (b). The marker proteins, from top to bottom, had the following molecular mass (kDa): 180, 116, 84, 58, 48.5, 36.5 and 26.6. See Materials and Methods for other details.

ASGLEGGQTXIAAXI which is in agreement with that of the 83 kDa fragment of the pig kidney enzyme [5]; indicating that chymotrypsin cleaves between Leu-266 and Ala-267 *. Also in agreement with other data [4], exposure of the native and the cleaved enzymes to ATP in the presence of Na^+ and Mg^{2+}, resulted in the phosphorylations of the α-subunit and the 83 kDa fragment (Fig. 1b). However, when the Na^+ of the phosphorylation medium was replaced with K^+, while the α-subunit was not phosphorylated as expected, the 83 kDa fragment was phosphorylated (Fig. 1b); indicating that chymotryptic cleavage alters the cation requirements of phosphorylation. To explore the nature of these alterations in more detail, the reaction of the enzyme with chymotrypsin was extended to obtain a preparation whose ATPase activity was less than 2% of the control, and contained little or no α-subunit as judged by the Coomassie blue staining of the SDS-gels. This preparation was then exposed to [γ-^{32}P]ATP in the presence of various ligands, and placed on SDS-gels. The results (Fig. 2) showed that (a) the 83 kDa fragment was the only peptide that was phosphorylated; and [b] its phosphorylation did not require either Na^+ or K^+, and was not inhibited by ouabain.

Preparations similar to those of Fig. 2 (i.e., devoid of α-subunit) were used in the following experiments to characterize phosphorylation and dephosphorylation of the 83 kDa peptide through quantitation of the

* Because the complete sequence of the canine kidney α-subunit is not known, for convenience the numbering of the pig kidney α-subunit [5] is used.

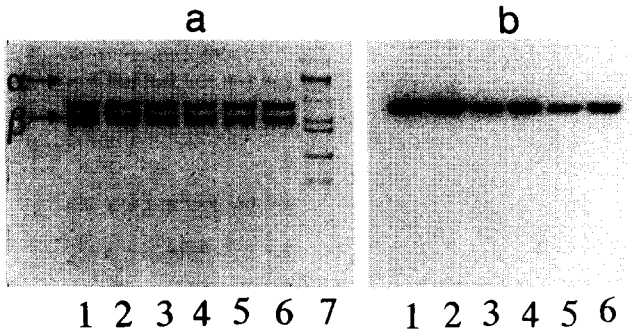


Fig. 2. Effects of Na^+, K^+, and ouabain on the phosphorylation of the 83 kDa peptide of the chymotrypsin-treated enzyme. The enzyme was exposed to chymotrypsin as in Fig. 1, but the reaction period was extended to 60 min. The treated enzyme was reacted with 0.3 μM [γ-^{32}P]ATP as indicated in Materials and Methods and with the various samples containing the following additional ligands: lane 1, $Mg^{2+}+Na^+$; lane 2, $Mg^{2+}+Na^++$ouabain; lane 3, $Mg^{2+}+K^+$; lane 4, $Mg^{2+}+K^++$ouabain; lane 5, Mg^{2+}; lane 6, $Mg^{2+}+$ouabain. The ligand concentrations were: 2 mM Mg^{2+}, 100 mM Na^+, 20 mM K^+ and 1 mM ouabain. To allow ouabain binding, all samples were preincubated for 10 min before the addition of Na^+, K^+ and ATP. After electrophoresis, gels were stained (a) and autoradiographed (b) as in Fig. 1. Lane 7 contained the markers.

phosphopeptide on filters rather than on gels; and to study the ADP-ATP exchange activity of the peptide.

Phosphorylation of the 83 kDa Fragment by ATP

Experiments of Fig. 3 showed that in the presence of 2 mM Mg^{2+}, and in the absence of Na^+ and K^+, the half-maximal phosphorylation of the 83 kDa peptide was obtained at 50 nM ATP. Without Mg^{2+}, ATP did not phosphorylate. In the presence of 0.3 μM ATP, half-maximal phosphorylation occurred with 0.1 mM Mg^{2+} (data not shown). Mg^{2+} could be replaced with several other divalent cations. With 0.3 μM ATP and 2 mM divalent cation, the relative phosphopeptide levels

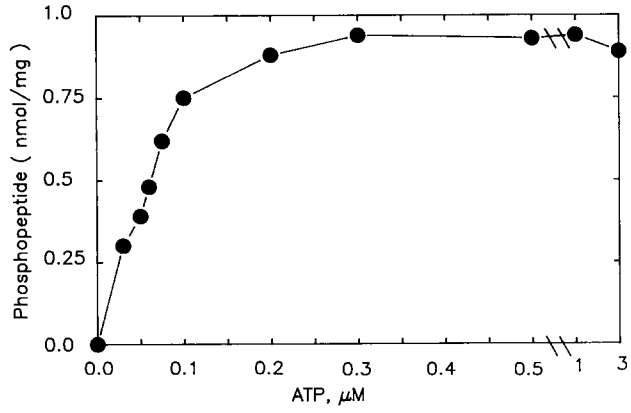


Fig. 3. Effects of varying ATP concentrations on the phosphorylation of the 83 kDa peptide of the chymotrypsin-treated enzyme. The enzyme was treated with chymotrypsin as in Fig. 2 and phosphorylated in the presence of 2 mM Mg^{2+} and the indicated ATP concentrations. The acid-stable phosphopeptide was collected on filters and counted as indicated in Materials and Methods.

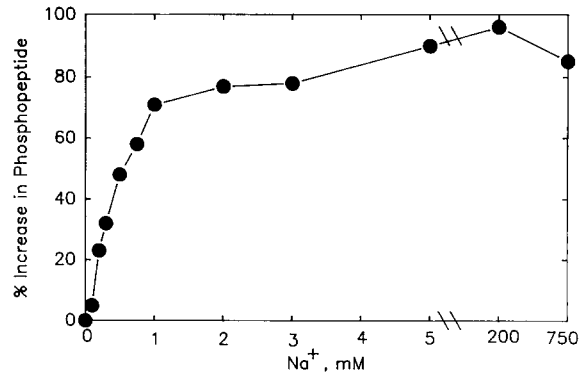


Fig. 4. Stimulatory effects of varying Na^+ concentrations on the phosphorylation of the 83 kDa peptide of the chymotrypsin-treated enzyme by ATP. Experiments were done as in Fig. 3 in the presence of 2 mM Mg^{2+}, 0.3 μM ATP and the indicated Na^+ concentrations.

were: Mg^{2+}, 1; Co^{2+}, 1; Mn^{2+}, 1.3; Ca^{2+}, 0.75; and Zn^{2+}, 0.4.

The maximal level of the phosphopeptide formed in the presence of ATP and Mg^{2+} was increased when Na^+ was present. The $K_{0.5}$ value of Na^+ for this stimulation was about 0.5 mM (Fig. 4). In the presence of 100 mM Na^+, the $K_{0.5}$ value of ATP for phosphorylation was about the same as that in the absence of Na^+ (Fig. 3). In eight different chymotrypsin-treated preparations with gel patterns similar to those of Fig. 2, the maximal level of the phosphopeptide (nmol/mg protein) obtained with 0.3 μM ATP and 2 mM Mg^{2+} was 0.62 ± 0.06 (S.E.) in the absence of Na^+, and 1.16 ± 0.10 (S.E.) in the presence of 100 mM Na^+.

K^+ had no effect on phosphorylation induced by ATP + Mg^{2+}, but it antagonized the stimulatory effect of Na^+ (Fig. 5). This effect of K^+ which seems to be exerted with high affinity (Fig. 5) is also inconsistent with the notion that the 83 kDa peptide remains in the E_1 state.

Because of the reported effects of imidazole and Tris on phosphorylation of the native enzyme by ATP [12], the effects of these compounds on the phosphorylation

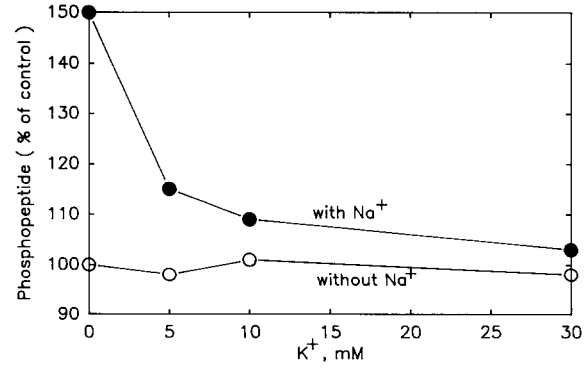


Fig. 5. Effects of varying K^+ concentrations on the phosphorylation of the 83 kDa peptide of the chymotrypsin-treated enzyme by ATP. Experiments were done as in Fig. 4 in the absence of Na^+ and in the presence of 100 mM Na^+.

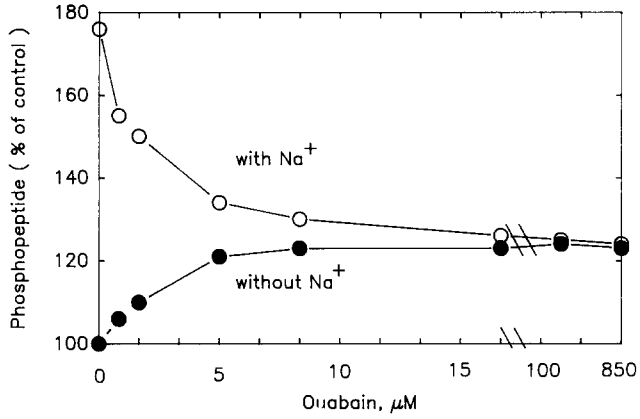


Fig. 6. Effects of varying ouabain concentrations on the phosphorylation of the 83 kDa peptide of the chymotrypsin-treated enzyme by ATP. The treated enzyme, prepared as in Fig. 2, was preincubated with 2 mM Mg^{2+} and the indicated ouabain concentrations before reaction with 0.3 μM ATP in the absence of Na^+ and in the presence of 100 mM Na^+.

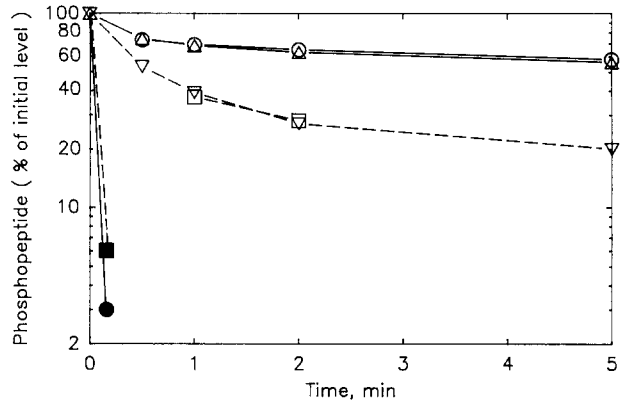


Fig. 7. Effects of K^+ and ADP on the time-course of dephosphorylation of the 83 kDa phosphopeptide of the chymotrypsin-treated enzyme. The chymotrypsin-treated enzyme that was devoid of α-subunit was prepared as in Fig. 2. In one series of experiments, the treated enzyme was exposed to 0.3 μM ATP+2 mM Mg^{2+}. After 90 sec (zero time), phosphorylation was stopped by the addition of either 20 mM CDTA (△), or 20 mM CDTA+20 mM K^+ (○), or 20 mM CDTA+10 μM ADP (●). The phosphopeptide levels were then determined at indicated times. In the other similar series, the treated enzyme was exposed to 0.3 μM ATP+2 mM Mg^{2+} +100 mM Na^+; and phosphorylation was terminated by the addition of either 20 mM CDTA (▽), or 20 mM CDTA+20 mM K^+ (□), or 20 mM CDTA+10 μM ADP (■).

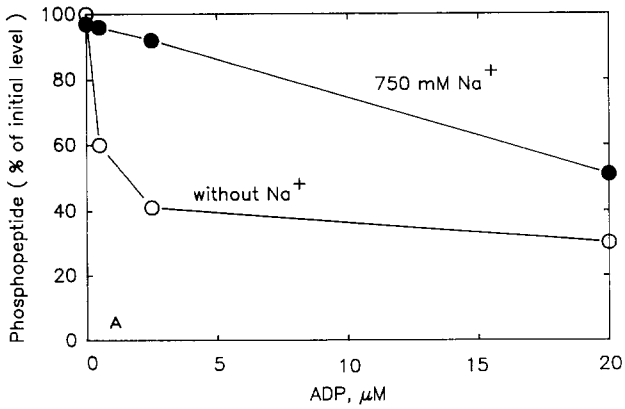
of the chymotrypsin-treated enzyme were studied. With 2 mM $Mg^{2+}$and 0.3 μM ATP, variation of Tris-HCl (pH 7.2) concentration in the range of 20–160 mM had no effect on the maximal level of phosphopeptide; and stimulation by 100 mM Na^+ was the same at all Tris concentrations. Similarly, variation of imidazole concentration in the range of 20–160 mM had no significant effect on the phosphopeptide either in the absence or the presence of Na^+. Change of pH in the range of 6.1–8.0 also had no significant effect on the level of the phosphopeptide and on its stimulation by Na^+.

Ouabain stimulated phosphorylation induced by ATP + Mg^{2+}, but it antagonized activation of phosphorylation by Na^+ (Fig. 6).

Dephosphorylation of the 83 kDa peptide

Phosphorylation of the peptide in the presence of ATP and Mg^{2+} was terminated by the addition of excess CDTA in order to examine the characteristics of dephosphorylation, and the effects of various ligands added along with CDTA. The spontaneous dephosphorylation was not mono-exponential (Fig. 7). The predominant component decomposed slowly with a rate constant (0.085 min^{-1}) that was orders of magnitude lower than that of the spontaneous dephosphorylation of the native phosphoenzyme (e.g., Ref. 13).

K^+ had no effect on the dephosphorylation of the phosphopeptide that was formed in the presence of ATP + Mg^{2+}, but ADP caused its rapid dephosphorylation (Fig. 7). Na^+, at concentrations up to 750 mM, also



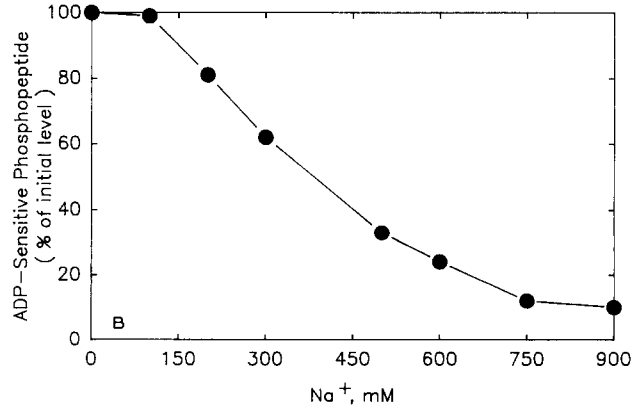


Fig. 8. Effect of Na^+ on the ADP sensitivity of the 83 kDa phosphopeptide of the chymotrypsin-treated enzyme. The chymotrypsin-treated enzyme was prepared as in Fig. 2 and phosphorylated in the presence of 0.3 μM ATP+2 mM Mg^{2+}. In experiments of Fig. 8a phosphorylation was terminated by the addition of 20 mM CDTA containing the indicated concentrations of ADP. The CDTA solutions contained either 750 mM Na^+ (●) or no Na^+ (○). The phosphopeptide levels were determined at zero time and after 10 sec of dephosphorylation. Experiments of Fig. 8b were similar to the above, except that the CDTA solutions contained 2 μM ADP and the indicated concentrations of Na^+.

had no effect on dephosphorylation rate (data not shown), but Na^+ reduced the sensitivity of the phosphopeptide to ADP (Fig. 8a). Experiments of Fig. 8b showed that the $K_{0.5}$ for this effect of Na^+ (350 mM) was quite different from the $K_{0.5}$ value of 0.5 mM for Na^+ activation of phosphorylation (Fig. 4). Experiments similar to those of Fig. 8 showed that K^+ did not affect the ADP sensitivity of the phosphopeptide, and it did not antagonize the effect of Na^+ on ADP sensitivity (data not shown).

When the phosphopeptide was formed in the presence of 100 mM Na^+, in addition to ATP + Mg^{2+}, the dephosphorylation rate was again nonlinear, but the ratio of the rapidly decomposing component to the slowly decomposing component was higher than the same ratio obtained in the absence of Na^+ (Fig. 7). In its insensitivity to K^+, and sensitivity to ADP, the phosphopeptide obtained in the presence of 100 mM Na^+ was similar to that obtained in the absence of Na^+ (Fig. 7).

Ouabain had no effect on the dephosphorylation of the phosphopeptide that was formed in the presence of ATP + Mg^{2+} (data not shown). When the phosphopeptide was formed in the presence of ouabain, its dephosphorylation characteristics were the same as those shown in Fig. 7 for the phosphopeptide that was formed in the presence of ATP + Mg^{2+}.

In experiments the results of which are not shown, the stability of the phosphorylated 83 kDa peptide was compared with that of the phosphoenzyme in solutions with pH values in the range of 3–10. The results for both were similar to those reported before for the phosphoenzyme [14], showing that the phosphate bond was acid-stable and alkaline-labile. Also in agreement with observations on the phosphointermediate of the native enzyme [14,15], the phosphopeptide was sensitive to hydroxylamine and molybdate, consistent with its being an acylphosphate.

Nonreactivity of the 83 kDa peptide with P_i

In the native enzyme ouabain is known to prevent the Na^+-dependent phosphorylation by ATP [13] and stimulate phosphorylation by P_i [8]. Since in the chymotrypsin-treated enzyme ouabain stimulated phosphorylation by ATP + Mg^{2+} (Figs. 2 and 6), it was important to examine the possibility of the phosphorylation of 83 kDa peptide by P_i. Experiments of Fig. 9 confirmed the ouabain stimulation of the phosphorylation of the native α-subunit by P_i, but showed that over a wide range of P_i concentrations the 83 kDa peptide was not phosphorylated either in the presence or the absence of ouabain.

Phosphorylation of the 83 kDa peptide in the presence of 3 mM Mg^{2+} and 0.3 μM ATP was not affected by P_i concentrations as high as 1 mM (data not shown).

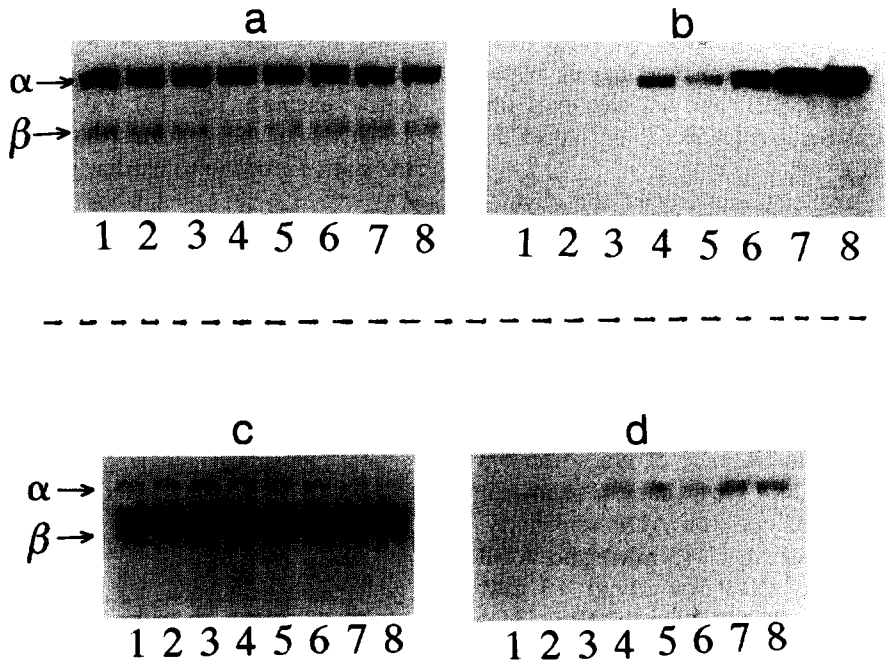


Fig. 9. Comparison of the effects of P_i and ouabain on the native and the chymotrypsin-treated enzymes. The enzyme was exposed to chymotrypsin for 30 min as indicated in Materials and Methods. The native (a and b) and the treated enzyme (c and d) were exposed for 15 min at 24°C to the indicated concentrations of [^{32}P]P_i, with and without 2 mM ouabain. All solutions contained 4 mM Mg^{2+}. The reaction mixtures of lanes 1, 2, 3 and 4 contained 2 μM P_i, 4 μM P_i, 20 μM P_i, and 40 μM P_i respectively. Those of lanes 5, 6, 7 and 8 contained 1 mM ouabain in addition to the same P_i concentrations as lanes 1, 2, 3 and 4. The acid-denatured samples were placed on SDS-gels at pH 2.4. The gels were stained (a and c) and autoradiographed (b and d).

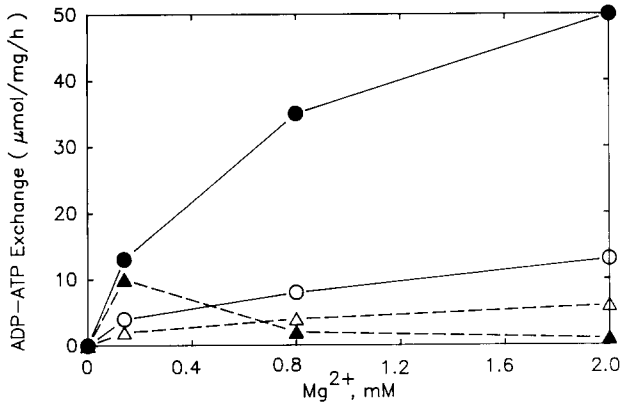


Fig. 10. Comparison of the effects of varying Ma^{2+} concentrations on the ADP-ATP exchange activities of the native (△,▲) and the chymotrypsin-treated (○,●) enzymes. The chymotrypsin-treated enzyme was prepared as in Fig. 2. Exchange activity was assayed as indicated in Materials and Methods in the presence of 4 mM ATP, 1 mM ADP and the indicated $MgCl_2$ concentrations. The solutions contained either 100 mM Na^+ (▲,●) or no Na^+ (△,○).

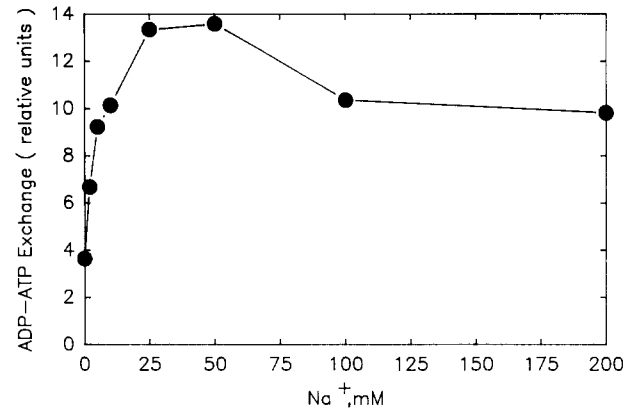


Fig. 11. Effects of varying Na^+ concentrations on the ADP-ATP exchange activity of the chymotrypsin-treated enzyme. The treated enzyme was prepared as in Fig. 2 and assayed as in Fig. 10 in the presence of 3 mM Mg^{2+} and the indicated Na^+ concentrations.

ADP-ATP exchange activity of the 83 kDa peptide

Experiments whose data are not shown indicated that the ADP-induced decomposition of the phosphopeptide (Fig. 7) was not accompanied by P_i release. This, and the previous limited observations on the ADP-ATP exchange activities of the chymotrypsin- and trypsin-treated enzymes [4,16,17], prompted the following experiments. Our results (Fig. 10) confirmed the well established fact (e.g., Ref. 18) that the Na^+-activated exchange of the native enzyme is evident only when Mg^{2+} concentration is low relative to those of the nucleotides. The chymotrypsin-treated enzyme, however, exhibited Na^+-activated exchange at all tested Mg^{2+} concentrations (Fig. 10), and its maximal observed activity was considerably greater than that of the native enzyme (Fig. 10).

Stimulation of the exchange activity of the chymotrypsin-treated enzyme reached a maximal level at 50 mM Na^+ ($K_{0.5}$ = 5 mM Na^+), but declined with further increase in Na^+ concentration (Fig. 11). This decline is consistent with the ability of high concentrations of Na^+ to reduce the sensitivity of the phosphopeptide to ADP (Fig. 8b). With fixed concentrations of Mg^{2+} (3 mM) and ATP (4 mM), K_m of ADP for exchange was 0.3 mM in the absence of Na^+, and 0.8 mM in the presence of 100 mM Na^+ (data not shown); also confirming an effect of Na^+ on ADP sensitivity. With fixed concentrations of Mg^{2+} (3 mM) and ADP (0.6 mM), the K_m value of ATP was about 0.3 mM either in the presence or absence of 100 mM Na^+ (data not shown).

K^+, at concentrations up to 50 mM, had no effect on the exchange activity of the chymotrypsin-treated enzyme (data not shown); but 2 mM K^+ was sufficient to block the stimulation of the exchange by 25 mM Na^+ (Fig. 12).

Ouabain stimulated the exchange activity of the chymotrypsin-treated enzyme, but maximal activation by ouabain was lower than that obtained with Na^+ (Fig. 12). When both ouabain and Na^+ were present, activation was about the same as that obtained with ouabain alone (Fig. 12).

Ouabain is known to inhibit the ADP-ATP exchange activity of the native enzyme [18]. In experiments of Fig. 13 this inhibitory effect of ouabain was compared with its activating effect on the exchange activity of the chymotrypsin-treated enzyme. The ouabain-sensitivity of the latter activity was about an order of magnitude lower than that of the native enzyme activity (Fig. 13). The ouabain sensitivity of the exchange activity of the native enzyme (Fig. 13) was in accord with the lower sensitivity of the E_1 forms than those of the E_2 forms of the native enzyme to cardiac glycosides [19].

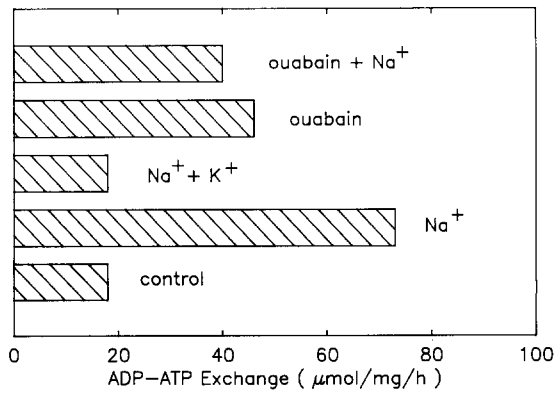


Fig. 12. Effects of Na^+, K^+, and ouabain on the ADP-ATP exchange activities of the chymotrypsin-treated enzyme. The treated enzyme was prepared as in Fig. 2; and assayed for exchange activity in the presence of 4 mM ATP + 1 mM ADP + 3 mM Mg^{2+} (control) and the following ligands: 25 mM Na^+, 2 mM K^+, and 1 mM ouabain, as indicated.

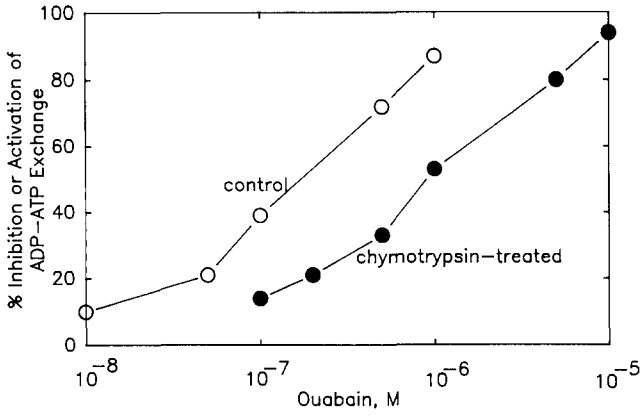


Fig. 13. Comparison of the inhibitory effects of ouabain on the Na^+-dependent ADP-ATP exchange of the native enzyme with the activating effects of ouabain on the Na^+-independent ADP-ATP exchange of the chymotrypsin-treated enzyme. The treated enzyme was prepared as in Fig. 2. Exchange activity of the control enzyme was assayed in the presence of 4 mM ATP, 1 mM ADP, 100 mM Na^+ and 0.1 mM Mg^{2+}. The exchange activity of the treated enzyme was assayed in the presence of the same nucleotide concentrations and 3 mM Mg^{2+}. Both enzymes were preincubated for 5 min with Mg^{2+} and the indicated ouabain concentrations prior to the start of the exchange assay.

Phosphorylation of the chymotrypsin, trypsin-treated enzyme

Exposure of the enzyme to trypsin in the presence of K^+, cleaves the α-subunit between Arg-438 and Ala-439 resulting in a 48 kDa fragment that contains the phosphorylation site (Asp-369). Exposure to chymotrypsin in the presence of Na^+, followed by exposure to trypsin in the presence of K^+, yields an 18.7 kDa peptide

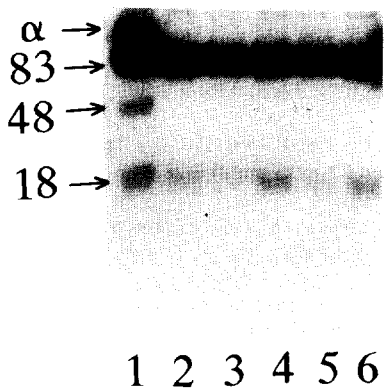


Fig. 14. Effects of Na^+, K^+, and ouabain on phosphorylation by ATP of the α-subunit, the 83 kDa, the 48 kDa, and the 18 kDa peptides of the chymotrypsin, trypsin-treated enzyme. The enzyme was treated sequentially with chymotrypsin and trypsin as indicated in Materials and Methods. Identical quantities of the treated enzyme were phosphorylated by ATP in the presence of indicated ligands under conditions described in legend to Fig. 2. The acid-denatured samples were subjected to SDS-gel electrophoresis at pH 2.4 and autoradiographed. In addition to ATP, the reaction mixtures contained the following: lane 1, $Na^+ + Mg^{2+}$; lane 2, $Na^+ + Mg^{2+}$ + ouabain; lane 3, $K^+ + Mg^{2+}$; lane 4, $K^+ + Mg^{2+}$ + ouabain; lane 5, Mg^{2+}; lane 6, Mg^{2+} + ouabain.

containing the phosphorylation site [4,5]. Such sequential cleavage with chymotrypsin and trypsin was conducted to obtain a preparation that included all three fragments (83 kDa, 48 kDa, and 18.7 kDa) that contain the phosphorylation site. The preparation was then reacted with ATP under various conditions. The results (Fig. 14) showed that phosphorylation of the 48 kDa fragment, like that of the α-subunit, was dependent on Na^+, and inhibited by ouabain; whereas the 18.7 kDa fragment, like the 83 kDa peptide, was phosphorylated in the absence of Na^+, and its phosphorylation was stimulated by either Na^+ or ouabain.

Experiments with the pig kidney enzyme

To see whether or not the above observations were limited to the canine kidney enzyme, some of the studies were repeated using the purified pig kidney enzyme. In experiments similar to those of Figs. 1 and 2, it was established that the phosphorylation characteristics of the 83 kDa peptide of the pig enzyme were the same as those of the canine enzyme.

Discussion

The present data show that similarities between the properties of the phosphopeptide of the chymotrypsin-cleaved enzyme and E_1P of the native enzyme are superficial. While both are sensitive to ADP and insensitive to K^+, the phosphopeptide is far more stable than the native phosphoenzyme (Fig. 7); and the effects of Na^+, K^+, and ouabain on the formation and the decomposition of the two differ considerably. The most striking of these differences are the following: (1) While the phosphorylation of the native enzyme by ATP is dependent on Na^+ (at a high-affinity intracellular site) and prevented by K^+, that of the 83 kDa fragment is modestly stimulated by Na^+ at this site; and only this Na^+ stimulation, but not the Na^+-independent phosphorylation, is prevented by K^+ (Figs. 2 and 5). (2) Ouabain, which is an inhibitor of the phosphorylation of the native enzyme by ATP (Ref. 13 and Fig. 1), is an activator of the Na^+-independent phosphorylation and the Na^+-independent ADP-ATP exchange activities that are catalyzed by the 83 kDa peptide (Figs. 6, 12, and 13). (3) While the occupation of a low-affinity Na^+ site in the native enzyme increases the ratio of ADP-sensitive to K^+-sensitive phosphoenzyme [20], in the 83 kDa peptide the occupation of this site simply decreases the sensitivity of the K^+-insensitive phosphopeptide to ADP (Fig. 8). Taken together, these results clearly indicate that the previous assumptions that the cleavage of the NH_2-terminal of the α-subunit by chymotrypsin stabilizes the enzyme in the E_1 form, and that the primary role of this domain is in the control of E_1-E_2 transitions, are no longer justified. Needless to say, the conclusions

of those studies that have been based on the above assumptions (e.g., Refs. 2–6) should be reexamined.

Our findings, however, have implications beyond the above. In studies and discussions of the structure-function relationships of this and related membrane proteins (e.g., Refs. 21, 22), there has been a tendency to imply or assume the existence of distinct domains with separate functions and well-defined interactions (e.g., ion binding domain, energy transduction domain, etc.). To some extent the assumptions of such semiautonomous domains are justified and supported by observations. Consider, for example, our data showing that the multitude of changes that are induced in the properties of the phosphorylation site after chymotryptic cleavage, are not altered by the further tryptic cleavage (Fig. 14). This clearly suggests the irrelevance of a large segment of the COOH-terminal end to the control of the phosphorylation site by the NH_2-terminal domain. On the other hand, our findings should also serve as warning against the temptation of assigning a distinct and limited regulatory role to a structural segment based on the finding that an amino acid replacement or modification in that segment alters a tested function. In this regard our observations on ouabain effects on phosphorylation and the exchange activity of the 83 kDa peptide (Figs. 6, 12 and 13) are of particular interest. Based on previous work [23] which had established that the first extracellular domain (H_1-H_2) of the α-subunit regulates the ouabain sensitivity of the enzyme, we had expected different ouabain sensitivities of the native enzyme and the 83 kDa peptide from which the H_1-H_2 domain had been detached. While this expectation was indeed fulfilled (Fig. 13), the accompanied reversal of the nature of the ouabain effect was totally unexpected. Evidently, a seemingly limited structural alteration produces a multitude of both quantitative and qualitative functional changes. Adding to the complexity of the situation is the absence of evidence for or against the possibility that the fragments resulting from controlled proteolysis, and retained in the membrane, may continue to interact; albeit in a different way than they do in the native enzyme. It is clear, therefore, that the determination of the mechanism of the autoregulation of the enzyme by its NH_2-terminal domain requires further studies.

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