Restoration of phosphorylation capacity to the dormant half of the α-subunits of Na^+,K^+-ATPase

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Abstract Purified kidney Na^+,K^+-ATPase whose α-subunit is cleaved by chymotrypsin at Leu^{266}–Ala^{267}, loses ATPase activity but forms the phosphoenzyme intermediate (EP) from ATP. When EP formation was correlated with extent of α-cleavage in the course of proteolysis, total EP increased with time before it declined. The magnitude of this rise indicated doubling of the number of phosphorylation sites after cleavage. Together with previous findings, these data establish that half of the α-subunits of oligomeric membrane-bound enzyme are dormant and that interaction of the N-terminal domain of α-subunit with its phosphorylation domain causes this half-site reactivity. Evidently, disruption of this interaction by proteolysis abolishes overall activity while it opens access to phosphorylation sites of all α-subunits.

Key words: Na^+,K^+-ATPase; Phosphoenzyme; Half-site reactivity; Oligomeric structure; Chymotrypsin

1. Introduction

Na^+,K^+-ATPase couples hydrolysis of ATP to the active transports of Na^+ and K^+ across the plasma membranes of most animal cells [1]. In the course of ATP hydrolysis, Asp^{369} of the α-subunit of the enzyme is phosphorylated and dephosphorylated [1]. Although the properties of this acid-stable phosphoenzyme have been thoroughly studied in relation to the reaction mechanism of the enzyme [2], there is a long-standing uncertainty as to whether all or half of the α-subunits of the functional unit of the enzyme are phosphorylated under optimal conditions. This controversy which has been reviewed repeatedly [3–5] is caused by disagreements on the extent of contaminants in the commonly used purified preparations of this membrane-bound enzyme and on the relative merits of different methods of protein assay used for the quantitation of enzyme subunits in these preparations. In the course of our recent structure–function studies on Na^+,K^+-ATPase preparations that are subjected to controlled proteolysis [6–9], it became evident that determination of relative phosphorylation capacities of the native enzyme and the enzyme whose α-subunit is cleaved at Leu^{266}–Ala^{267} may resolve the above controversy. Here, we present the results of such determinations, showing that, while this cleavage inhibits enzyme activity, it restores phosphorylation capacity to half of the phosphorylation sites that are dormant in the native enzyme.

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2. Materials and methods

Purified membrane-bound Na^+,K^+-ATPase of canine kidney medulla, with specific activity in the range of 1000–1600 μmol of ATP hydrolyzed/mg/h, was prepared and assayed as indicated [9,10]. The Na^+-dependent treatment with α-chymotrypsin [6,9], phosphorylation with [γ-^{32}P]ATP in the presence of Na^+ and Mg^{2+} at 0°C [6,7,10], assay of total phosphoenzyme on filters [10], resolution and assays of ^{32}P-labeled peptides on acid gels either by autoradiography [6,7] or by counting of gel slices [11,12], and analysis of N-terminal sequences [6,9] were done by procedures the details of which we have described before. The relative quantities of stained peptide bands on gels were determined by densitometry [7,9]. The total amount of enzyme protein applied to each gel was in the range of 1–10 μg. It was established experimentally that under these conditions there were linear relationships between the amount of native or cleaved enzyme applied to the gel and the densities of stained α-subunit, β-subunit, and 83-kDa peptide bands.

3. Results

Na^+,K^+-ATPase was exposed to chymotrypsin in the presence of Na^+ for various times and assayed for ATPase activity and for the relative contents of α- and β-subunits. The stained gels (Fig. 1) confirmed previous observations [6,13], showing that the major product of the cleavage of α-subunit was a 83-kDa peptide whose N-terminal residue has been determined to be Ala^{267} [6,14]. The data also showed (Fig. 2) that the rate of decline of Na^+,K^+-ATPase activity was the same as the rate of disappearance of α-subunit and that there was little or no change in the amount of β-subunit during exposure to chymotrypsin.

When partially cleaved samples of Na^+,K^+-ATPase were exposed to [γ-^{32}P]ATP in the presence of Mg^{2+} and Na^+, resolved on acid gels, and autoradiographed (Fig. 3), it was evident that the 83-kDa peptide retains the ability to form the acid-stable phosphointermediate. Although previous studies of our laboratory and others [6,13,15] had shown this property of the 83-kDa fragment, no quantitative data relating the extent of cleavage of α-subunit to the level of phosphoenzyme were reported in these studies. Therefore, in experiments in Fig. 4, we exposed Na^+,K^+-ATPase to chymotrypsin and, at various time intervals, we removed aliquots to assay for the relative amounts of remaining α-subunit, the resulting 83-kDa peptide, and total acid-stable phosphoenzyme formed in the presence of ATP, Mg^{2+}, and Na^+. Phosphorylation conditions were chosen to assure the formation of maximal levels of phosphoenzyme from α-subunit and 83-kDa peptide [6,7]. The results clearly showed that as α-subunit decreased and 83-kDa peptide increased with time, there was a time-dependent increase in the level of total phosphoenzyme, followed by a subsequent decline (Fig. 4). Further analysis of these data revealed the following.

(1) In Fig. 4, the measured levels of stained α-subunit and

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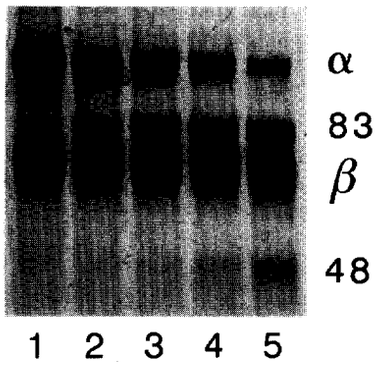

Fig. 1. Time-dependent cleavage of the α-subunit of Na^+,K^+-ATPase by chymotrypsin. The enzyme (0.5 mg/ml) was exposed to α-chymotrypsin (25 μg/ml) in the presence of 10 mM Na^+, resolved on SDS gels, and stained with Coomassie blue. Exposure times (min) for lanes 1–5: 0, 10, 20, 40, 60.

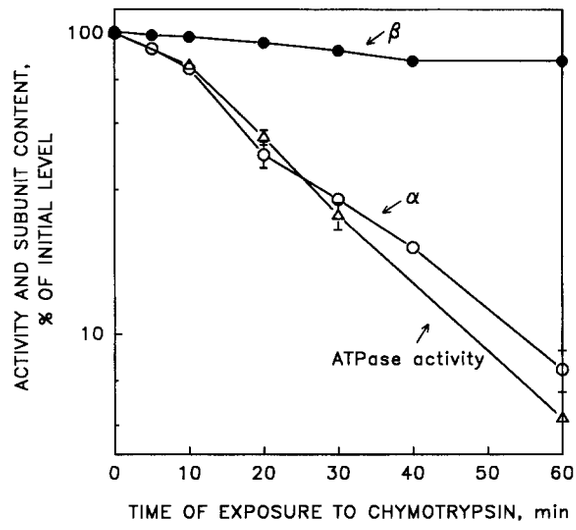

Fig. 2. Time-dependent effects of chymotrypsin on Na^+,K^+-ATPase activity and α- and β-subunit contents. Proteolytic cleavage and assays were done as in Fig. 1 and Section 2.

83-kDa peptide bands at various times were expressed relative to control level of α-subunit at zero time. We then made the reasonable assumption that unit weights of the two peptides were stained with equal intensities, and, based on the actual amounts of lost α-subunit at each time point, we calculated the theoretical yield of 83-kDa peptide relative to control level of α-subunit (Fig. 4). The equalities of actual and theoretical values of 83-kDa peptide at early time points (10 min and 20 min) indicated the validity of the above assumption on the identical staining characteristics of α-subunit and 83-kDa bands. Disagreements between the found and calculated values of 83-kDa bands at later time points (Fig. 4) suggested the secondary digestion of the 83-kDa peptide by chymotrypsin. The occurrence of such secondary digestion was also indicated by the appearance of peptides of about 48-kDa after prolonged exposure to chymotrypsin (Fig. 1, lane 5), and by the identification of these as cleavage products of 83-kDa peptide.*

(2) An obvious explanation for increase in total phosphoenzyme after exposure to chymotrypsin (Fig. 4) is that proteolysis unmasks dormant phosphorylation sites. We assumed, as suggested before [5,16], that half of the original α-subunits were dormant and that the unmasked sites were the result of the cleavage of this dormant half. Based on the actual amounts of α-subunit and 83-kDa peptide found at each time point, we then calculated the expected amount of total phosphoenzyme. At 10 and 20 min after exposure to chymotrypsin, the calculated and found values of total phosphoenzyme were nearly identical (Fig. 4), supporting the validity of the above assumption on the dormancy of half of the sites. At later time points, calculated total phosphoenzyme values were higher than the found values (Fig. 4), suggesting that prolonged proteolysis causes not only secondary digestion but also some non-specific inactivation of phosphorylation capacity in 83-kDa peptide, or α-subunit, or both.

Although experiments in Fig. 4 clearly showed an increase in total phosphorylation capacity of the enzyme after proteolysis, it was necessary to explore the possibility that the increase may have been due to unmasking of phosphorylation sites in impurities of low molecular mass that have been said to be present in some purified enzyme preparations [18]. Therefore, control enzyme and partially cleaved samples were phosphorylated with labeled ATP, resolved on acid gels, and assayed for ^{32}P-contents of the separated α-subunit and 83-kDa bands. The results showed that the sum of the ^{32}P-contents of the two bands in the partially cleaved preparations clearly exceeded the ^{32}P-content of the control α-subunit (Fig. 5), indicating that the unmasked sites were entirely within the α-chains and not due to impurities.

4. Discussion

The data of Figs. 4 and 5 show that in the early phases of proteolysis the number of phosphorylation sites of the α-subunits are doubled. The significance of this finding must be considered in relation to previous data and controversies regarding half-site reactivity of this enzyme.

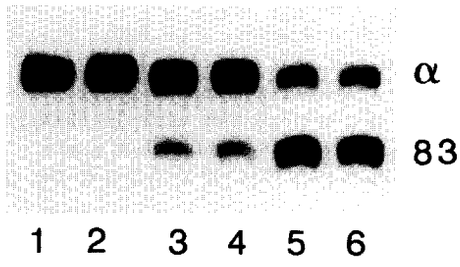

Fig. 3. Phosphorylations of α-subunit and 83-kDa peptide by ATP. Control enzyme (lanes 1 and 2) and samples cleaved for 10 min (lanes 3 and 4) or 20 min (lanes 5 and 6) by chymotrypsin were phosphorylated with [γ-^{32}P]ATP, resolved an acid (pH 2.4) gels in duplicate, and autoradiographed as indicated in Section 2.

*N-terminal sequence analysis of this band showed that it was a mixture of peptides with N-termini at positions 582–585 of the α-subunit (A. Ivanov and N.N. Modyanov, unpublished observations). The late appearance of the band during exposure to chymotrypsin, and the fact that at earlier time points for every α-subunit lost an 83-kDa peptide is produced (Fig. 4), indicates that the peptides of 48-kDa band are products of the secondary digestion of the 83-kDa peptide. It is also of interest to note, as we have indicated before [9], that to date it has not been possible to identify on these gels the other expected product of the primary chymotryptic cleavage, i.e., the 30-kDa N-terminal fragment of the α-subunit. Studies on the characterization of this product are in progress.

It is now well-established that in highly purified preparations of Na^+,K^+-ATPase used in most laboratories the concentration of active sites (high-affinity nucleotide binding sites, or ouabain binding sites, or phosphorylation sites) is about half of the α-subunit concentration, when Lowry protein assays are used for these determinations [5,16,17]. The conclusion of these studies, i.e., that half of the α-subunits of an oligomer of α,β-protomers are dormant, has been criticized on the grounds that the protein assay used in these studies underestimates protein content [3,18] and that purified preparations exhibiting apparent half-site reactivity contain large quantities of impurities [18] and/or denatured Na^+,K^+-ATPase [18,19]. The importance of our data presented here is that they also indicate the dormancy of half of the active sites of native α-subunits but that this conclusion is independent of the presence of any impurities and the shortcomings of methods used to determine the subunit protein content of these preparations. To explain our findings on the basis of the presence of denatured enzyme, one must make the unreasonable assumptions that half of the enzyme is coincidentally denatured in an unspecific manner, and that this half is renatured in respect to its phosphorylation capacity by the same highly specific Na^+-dependent proteolysis that cleaves the native and the denatured α-subunits at the same site. Clearly, the Na^+-specific production of the 83-kDa peptide from all α-subunits would be more logical if active and dormant halves were interacting within a functional oligomer of α,β-protomers.

Although our findings in conjunction with those reviewed before [3–5,16,17], indicate the dormancy of half of the α-subunits, our data taken alone have an alternative explanation: that all native α-subunits are phosphorylated but that an additional dormant site on each α-subunit is unmasked upon proteolysis. This possibility, however, is unlikely for two reasons. First, although the phosphoenzymes formed from intact α-subunit and the 83-kDa peptide differ in their ligand sensitivities [6,13], their identical pH and hydroxylamine sensitivities [6] suggest that their phosphorylation sites are identical. Second, while there is some evidence suggesting the possibility of multiple ATP binding sites on an α-subunit [20,21], there are no previous experimental data to suggest the existence of multiple phosphorylation sites on a single α-chain. Nevertheless, future studies should be done to see if an acylphosphate other than Asp^{369} can be identified in the phosphorylated 83-kDa peptide.

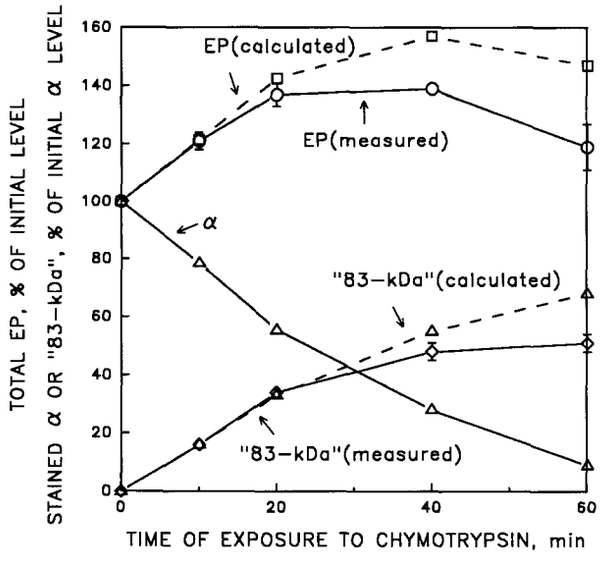

Fig. 4. Relation between the extent of chymotryptic cleavage and total capacity for phosphoenzyme (EP) formation. After exposure to chymotrypsin as in Fig. 1, aliquots were phosphorylated at 0°C for 90 s in the presence of 3 μM ATP, 2 mM Mg^{2+}, and 100 mM Na^+; and total acid-stable EP was assayed on filters (Section 2). Samples were also resolved on gels as in Fig. 1, and stained α and 83-kDa bands were assayed by densitometry relative to control level of α. Equal loading of gels was assessed by normalizing against β contents. The indicated calculated values were obtained as described in the text, based on relative molecular weights of 112 and 83 for α and 83-kDa peptide. The measured value at each time point is mean ± S.E. of 4–8 determinations. Standard errors are not shown when smaller than symbol size.

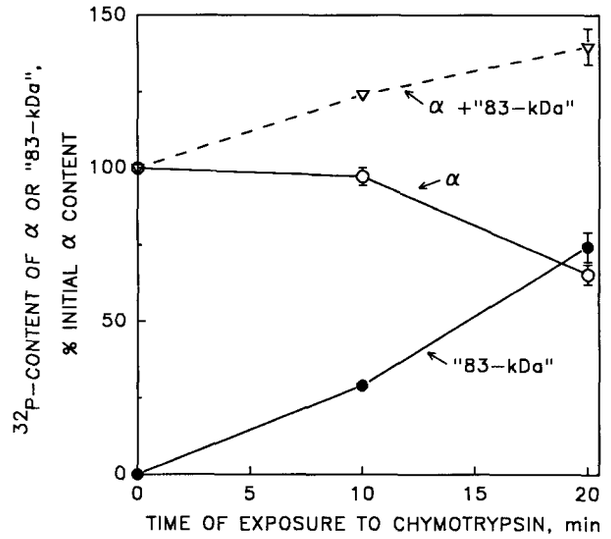

Fig. 5. ^{32}P-Contents of α-subunit and 83-kDa fragment in control and partially cleaved enzymes after phosphorylation. Chymotryptic cleavage and phosphorylation were done as in Fig. 4, and the samples were resolved on acid gels. Replicate gels were assayed for radioactivity of the separated bands after slicing of the gels and counting of the appropriate slices. Values are mean ± S.E. of six determinations. Standard errors not shown when smaller than symbol size.

That membrane-bound Na^+,K^+-ATPase is an oligomer with interacting α,β-protomers has been established [5,20–28] independent of the questions regarding its reaction mechanism and its half-site reactivity. Our data presented here, along with previous studies of our laboratory and others [5,20–22,29,30], provide unambiguous evidence for its half-site reactivity. We suggest that debates on whether the membrane-bound enzyme is a half-site reactive oligomer should now be replaced by efforts focused on the clarification of the functional significance of the enzyme's dormant sites.

Restoration of the phosphorylation capacity of the dormant sites by cleavage at Leu^{266}-Ala^{267} shows that N-terminal domains of α-subunit regulate the phosphorylation domain across the cleavage site. In previous studies we have also shown the regulation of the phosphorylation site by C-terminal domains of α [7,8], interactions between the N-terminal and C-terminal intramembrane helices of the α-chain [9], contact between the same helices of the α-subunit and the single intramembrane helix of β-subunit [9], and interactions between phosphorylation and dimerizing domains of α-subunit

[8]. Together, these findings clearly indicate that a multitude of intrasubunit and intersubunit domain–domain interactions regulate the functions of the oligomeric Na^+,K^+-ATPase.

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