DISSOCIATION CONSTANTS OF THE PRONASE FRAGMENT OF THE PHOSPHORYLATED $(Na^+ + K^+)$-DEPENDENT ATPase

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It has been shown previously that the phosphorylated intermediate of the $(Na^+ + K^+)$-dependent ATPase is an acyl phosphate (Nagano et al., 1965, Hokin et al., 1965 and Bader, Sen and Post, 1966) and that it can be solubilized by proteolytic enzymes (Albers, Fahn and Koval, 1963, Hokin et al., 1965 and Bader, Sen and Post, 1966). Pronase releases an acyl phosphate containing fragment with an average molecular weight of about 380 determined by gel filtration (Bader, Post and Jean, 1967). This report shows that the pronase fragment has five dissociation constants determined by electrophoresis at different pH. It consists at least of one dibasic and one dicarboxylic amino acid. The phosphate is probably bound to the ω-carboxyl group. The dibasic amino acid seems to be arginine or lysine.

METHODS

$(Na^+ + K^+)$-dependent ATPase from rabbit kidney cortex was phosphorylated from $[\gamma\ ^{32}P]$ ATP in the presence of Na^+ and Mg^{++}, according to Bader, Sen and Post, 1966. The phosphorylated and

washed enzyme was incubated with pronase for 20 minutes at 23° C and pH 7.0 (Bader, Post and Jean, 1967). Under these conditions about 80% of the enzyme bound ^{32}P could be solubilized. The solubilized material was subjected to electrophoresis either without further treatment or after incubation with 0.5 M $NaNO_2$ in 1.5 M acetic acid for 20 minutes at 23° C to convert amino groups to hydroxyl groups (Van Slyke, 1911). Electrophoresis was carried out on Whatman 3MM paper with 2500V and 50 mA for 60 minutes. The buffer used was the twice diluted phosphate-citrate-borate buffer described by Teorell and Stenhagen, 1938. Caffeine was used to determine any movement of the origin during electrophoresis.

RESULTS

The figure shows the electrophoretic mobility of the different compounds tested at different pH. According to its first and second dissociation constant, the curve of the inorganic phosphate shows one inflexion point at pH 2 and another one around pH 7, with a plateau in between. A second plateau appears above pH 8. This agreement of inflexion points with dissociation constants is a measure of the accuracy of the method. The untreated pronase fragment was neutral at pH 4, as negative and positive charges cancelled each other at this pH. The pronase fragment increased its speed toward the cathode proportional to decreasing pH. With increasing pH, it became more and more negatively charged. There were two plateaus, one between pH 5.5 and 7.5, and another one between pH 9.5 and 10.5. The increase in speed from pH 7.5 to 9.5 seemed to be due to a removal of a positive charge from an amino group, since the step from 6 cm to 11 cm disappeared after nitrous

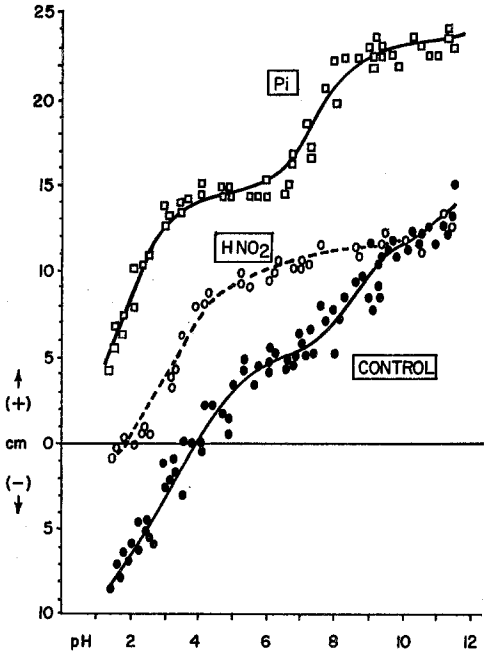

Figure. pH electrophoresis of pronase fragment.
● , pronase fragment untreated,
○ , pronase fragment treated with nitrous acid,
□ , inorganic phosphate

acid treatment and the whole curve between pH 1.3 and 8 was moved 5 to 6 cm toward the anode. The pronase fragment was then neutral at pH 2 and had a large plateau from pH 5 to pH 10.5. The removal or addition of one charge seemed to displace the pronase fragment for about 5 to 6 cm. If this distance was used to estimate the different dissociation constants, the following pK values were obtained: 1.3, 3, 2, 4, 5, 8.5 and one above 11. The first three dissociation constants were too close together to show any plateau between them in the pH electrophoresis curve. The observed pK values of the pronase fragment were compared with the dissociation constants of different groups taken from the literature (Table). The first pK coincided with the first dissociation

constant of acyl phosphates. The second pK coincided with the dissociation constant of an α-carboxyl group in peptides. An ω-carboxyl group would have agreed with the third pK, but the third pK was more likely due to the second pK of the acyl phosphate group. The fourth pK agreed with an α-amino group, and the fifth pK was in the neighborhood of a guanidinium group, although an ω-amino group could not be excluded.

TABLE

APPROXIMATE pK_a VALUES OF THE PRONASE FRAGMENT

	pK_a Values	
	Observed	From Literature
pK_1	1.3	acyl - PO_3^- 1.2 (a)
pK_2	3.2	α - COO^- 3.3 (b)
pK_3	4.5	ω - COO^- 4.5 (b), acyl - PO_3^{--} 4.8 (a)
pK_4	8.5	α - NH_3^+ 8.0 (b)
pK_5	11.5 - 12	ω - NH_3^+ 10.5, Guanidinium 12 (b)

(a) Lipmann and Tuttle, 1947; (b) Greenstein and Winitz, 1961.

DISCUSSION

The results indicate that the pronase fragment contains a dibasic and a dicarboxylic amino acid. The molecular weight of the pronase fragment of about 380 (Bader, Post and Jean, 1967) would agree with a dibasic-dicarboxylic-dipeptidyl phosphate; such compounds have a range of molecular weights from 361 to 403. The phosphate seems to be bound to the ω-carboxyl group according to the pK_2 which arises, most likely, from an α-carboxyl group. The data do not show clearly the identity of the dibasic amino acid. A histidine was ruled out earlier

by photo-oxidation with rose bengal (Bader, Post and Jean, 1967). The nitrous acid treatment apparently removed only one amino group. This seems to speak against a lysine. Under the conditions used, the α-amino group of a control lysine was completely removed and most of the ε-amino group. But it may be possible that due to special circumstances in the pronase fragment the harder removable ω-amino group was not yet attacked by the nitrous acid and, therefore, the presence of a lysine residue was obscured. In favor of an arginine residue is the high pK_5 value and the fact that the pH dependent hydrolysis of the pronase fragment is identical with that of N-CBZ-arginyl phosphate (Bader and Jean, 1967). Both show the typical shoulder between pH 5 and 8 as already described with the whole phosphorylated ATPase and the peptic peptides (Bader, Sen and Post, 1966). The only difference between the pronase fragment and N-CBZ-arginyl phosphate was that the pronase fragment was exactly twice as stable over the whole pH range tested (pH 0.5 to 12.0).

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