BBA 45561

FURTHER CHARACTERIZATION OF A PHOSPHORYLATED INTERMEDIATE IN ($Na^+ + K^+$)-DEPENDENT ATPase

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(Received January 13th, 1967)

SUMMARY

1. A radioactive acyl phosphate intermediate in the ($Na^+ + K^+$)-dependent ATPase (ATP phosphohydrolase, EC 3.6.1.3) of guinea-pig kidney was treated with proteolytic enzymes. The soluble products were separated by paper electrophoresis at pH 2.1.

2. Differential digestion with pepsin (EC 3.4.4.1) yielded a sequence of six fragments. Their molecular weights ranged from about 10000 to about 2200 as estimated by gel filtration.

3. All fragments were positively charged and contained at least one sulfhydryl group and no disulfide bond.

4. Hydrolysis of the intermediate or the peptic fragments with pronase gave a positively charged fragment with a molecular weight of 380. This fragment contained no sulfhydryl group. At least part of the positive charge was not that of histidine or lysine. The pronase fragment was not arginyl phosphate.

5. The results provide further evidence that the acyl phosphate group is attached to a protein containing one or more sulfhydryl and basic groups.

INTRODUCTION

Proteolytic enzymes solubilize the phosphorylated part of an intermediate[1] in the ($Na^+ + K^+$)-dependent ATPase transport system of cell membranes[2–4]. The soluble fragments are probably peptides[3,4] connected to the phosphate group with an acyl phosphate bond[3–5]. Hydrolysis kinetics have indicated only one type of bond[5]. This report presents further characterization of radioactive fragments released by proteolytic enzymes.

METHODS

Labelling of the ($Na^+ + K^+$)-dependent ATPase system with ^{32}P from [γ-^{32}P]-ATP, enzymatic hydrolysis of the labelled denatured enzyme with pepsin (EC 3.4.4.1), trypsin (EC 3.4.4.4), and papain (EC 3.4.4.10) (all from Worthington Biochemical

Abbreviation: Z-, benzyloxycarbonyl group.

Corp., Freehold, N.J.), electrophoresis, performic oxidation of the digests and molecular weight estimation by gel filtration with Bio-Gels P2, P10 and P20 (Bio-Rad Laboratories, Richmond, Calif.) were carried out as described earlier[4].

Pronase digestion of the peptic digest

0.1 ml of the supernatant of the peptic digest[4], containing about 500 $\mu\mu$moles and 100000 counts/min of ^{32}P was incubated with 50 μl of 0.1 M sodium phosphate (pH 7.3) and 5 mg of pronase (from Calbiochem, Los Angeles, Calif.) at 23°. The final pH was 6.5. The reaction was stopped after 15 min by adding 10 μl of 3 M trichloroacetic acid and the denatured protein was sedimented by centrifugation at 30000 $\times$ g for 20 min. All the ^{32}P stayed in the supernatant.

Treatment with N-ethylmaleimide

20 μl of the peptic digest in 10 mM HCl, containing about 100 $\mu\mu$moles and 20000 counts/min of ^{32}P, were incubated with 10 μl of 50 mM histidine-free base and 20 mM *N*-ethylmaleimide for 15 min at 23°. The final pH was 6.3. After cooling to 0°, 20 μl were applied to Whatman 3 MM filter paper for electrophoresis at pH 2.1. 2 μl of performic acid were added to the remaining 20 μl, which were applied after incubation for 2 min at 0°. The reactions were carried out in glass-stoppered weighing bottles holding 0.2 ml (Kimble, Cat. No. 51050C, Owens-Illinois, Toledo, Ohio).

Treatment with sulfite

20 μl of the peptic digest in 10 mM HCl, containing about 100 $\mu\mu$moles ^{32}P with 20000 counts/min were incubated with 2 μl of 1 M sodium sulfite (pH 6.0) and 20 μl of 10 mM $CuSO_4$ for 30 min at 23°. After cooling at 0°, paper electrophoresis and performic oxidation were carried out as described following *N*-ethylmaleimide treatment.

Synthesis of L-*arginyl phosphate*

L-Arginyl phosphate (acylphosphoarginine) was synthesized by a modification of the method of NISHIMURA, DODD AND MEISTER[6]. Acyl phosphate was determined as the hydroxamate[7]. 1.5 mmoles of *N*-α-Z-L-arginine (from Pierce Chemical Co., Rockford, Ill.) were incubated at 23° in 3 ml of 90 % aqueous pyridine containing 1.5 mmoles of *N*,*N*'-dicyclohexylcarbodiimide (from Pierce Chemical Co., Rockford, Ill.) and 1.5 mmoles of H_3PO_4. After 10 min the mixture was filtered through a Millipore filter into 5 ml ice-cold ether and centrifuged at 5000 rev./min for 10 min at 0°. The oily precipitate was washed three times with 5 ml of ice-cold ether by centrifugation at 2000 rev./min for 10 min and resuspension. The yield of *N*-α-Z-L-arginyl phosphate was between 20 % and 30 %. The final residue was dried *in vacuo* and then subjected for 20 min at 23° to a stream of dry HBr to split off the carbobenzoxy group. The residue was dispersed in 1.5 ml 2 M Tris (final pH about 4.5). The mixture was shaken with 1 ml CCl_4 and 3 μl of the clear aqueous phase were taken for electrophoresis. About 30 % of the *N*-α-Z-L-arginyl phosphate were recovered as L-arginyl phosphate. Complete hydrogenation or further purification was not attempted because of the lability of the acyl phosphate bond. The electrophoretogram was sprayed with molybdate for phosphate[8], with ninhydrin for amino groups and with Sakaguchi reagent for guanidine groups[9].

RESULTS

Differential peptic digestion

In an earlier study[4] pepsin released one fragment rapidly, which was converted after some time into two fragments with a molecular weight about 2200. To bring out details, graded digestion was performed with different amounts of pepsin. The lability of the acyl phosphate bond made it impractical to stop digestion by raising the pH and thus observe the release of fragments in a true time sequence. Com-

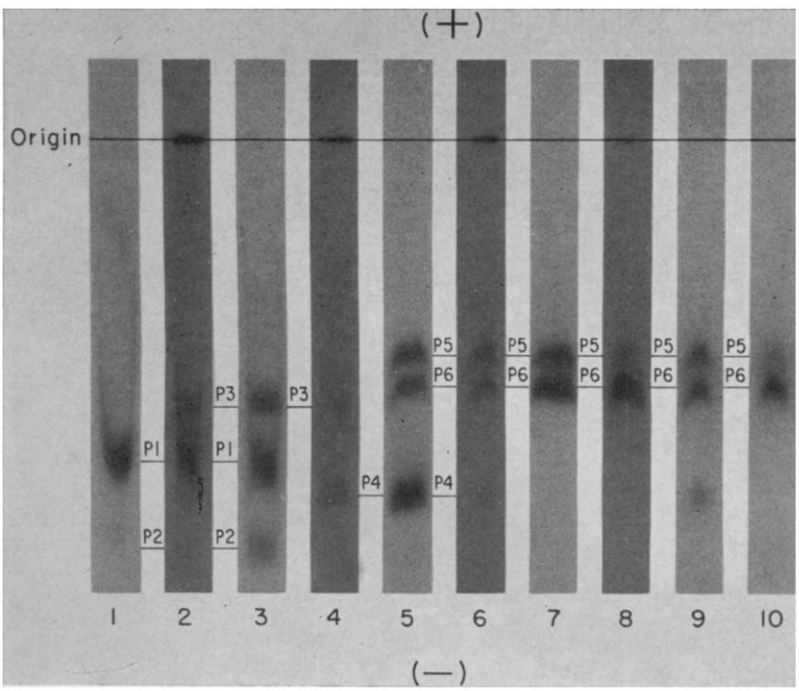
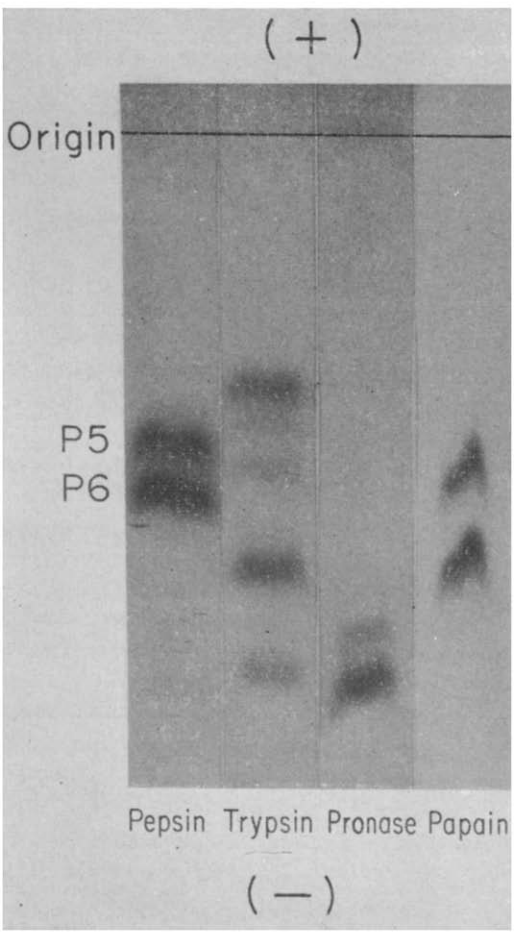


Fig. 1. Differential peptic digestion of the ^{32}P-intermediate. The ^{32}P-labelled, acid-washed ATPase preparation was incubated with various pepsin concentrations for various times at various temperatures. About 3 mg of protein of the labelled ATPase preparation were added to 0.3 ml of 10 mM HCl. The preparation contained about 15000 counts/min per mg protein. Samples 1 through 8 are from one ATPase preparation; Samples 9 and 10 are from another. The pepsin to ATPase protein ratios (w/w) were: Samples 1 and 2, 0.0006; Samples 3 and 4, 0.006; Samples 5 and 6, 0.06; Samples 7 and 8, 0.6; and Sample 9, 0.4. Sample 10 contained only material solubilized by digestion of Sample 9. Its pepsin concentration was 2 mg pepsin in 0.1 ml. Samples 1, 3, 5, 7 and 9 were incubated with pepsin for 30 min at 23°. After cooling to 0°, the samples were centrifuged at 30000 × *g* for 20 min. Three-quarters of the supernatant were taken out and 5000–10000 counts/min, representing about 50–100 $\mu\mu$moles of ^{32}P, were applied to paper for electrophoresis. The precipitates of Samples 1, 3, 5, and 7, containing the undigested material and most of the pepsin, were rehomogenized with the residual supernatant and brought to 0.1 ml with 10 mM HCl. These homogenates were kept for 24 h at 2°. After this time a sample of each whole homogenate was taken for electrophoresis (Samples 2, 4, 6 and 8). 0.1 ml of the supernatant of Sample 9 was reincubated with 2 mg pepsin for 30 min at 37°. After centrifugation at 30000 × *g* for 20 min at 0° a sample of the supernatant was taken for electrophoresis (Sample 10). Electrophoresis was carried out in 1% formic acid, at 2100 V and 46 mA for 105 min at 0°. The labels P1–P6 designate the various radioactive spots obtained by peptic digestion. Spot P6 moved 7.5 cm from the origin. The anodal side of the electrophoretogram is not shown; it contained only the $^{32}P_i$ spots (compare Fig. 6).

parison of various samples suggested a sequence of six fragments. All fragments were positively charged at pH 2.1. Fig. 1 shows the results. After brief digestion with the smallest amount of pepsin an intense spot, P1, appeared with a very faint spot, P2, moving well ahead of it (Sample 1). After longer digestion with this amount of pepsin (Sample 2) or after brief digestion with 10-fold more pepsin (Sample 3), a third spot, P3, appeared moving more slowly than P1. After longer digestion with this amount of pepsin Spot P3 was still present, but Spots P1 and P2 had disappeared. Instead



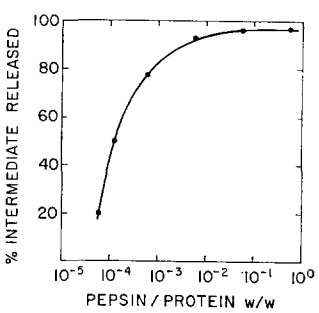


Fig. 2. The dependence of the release of the ^{32}P-intermediate on the ratio of pepsin to ATPase protein. The data are from Samples 1, 3, 5 and 7 in Fig. 1 and Sample 1 in Fig. 5. Incubation was for 30 min at 23°.

Fig. 3. Digestion of the ^{32}P-labelled ATPase with various proteolytic enzymes. 4 mg of ATPase protein, containing about 100000 counts/min were placed in 0.2 ml. The buffer was 10 mM HCl for pepsin, 60 mM Tris phosphate at pH 7.3 for trypsin and pronase, and 50 mM sodium acetate, 5 mM Na_2EDTA and 5 mM cysteine (pH 5.0) for papain. 4 mg of the proteolytic enzyme were added. Incubation was for 30 min at 23°. After cooling and the addition of 10 μl of 3 M trichloroacetic acid the samples were centrifuged at 30000 $\times$ g for 20 min at 0°. A sample of the supernatant was taken for paper electrophoresis. Electrophoresis was as in Fig. 1. The labels P5 and P6 at the left side refer to the peptic fragments.

a new spot, P4, appeared moving slightly faster than P1 (Sample 4). After brief digestion with a further 10-fold increase in pepsin Spot P3 was absent, Spot P4 was prominent and two slow spots, P5 and P6, appeared. P5 was slightly more intense than P6 (Sample 5). After longer digestion Spot P4 became fainter (Sample 6). Brief digestion with 10-fold more pepsin failed to yield Spot P4 (Sample 7) and after longer digestion Spot P6 became slightly more intense than P5 (Sample 8). The same shift in intensity from P5 to P6 appeared after repeated peptic digestion (Samples 9 and 10). Spot P6 appeared to be the smallest peptic fragment. Spot P4 is faint in Sample 9 and disappeared in Sample 10. The radioactive spots remaining at the origin in Samples 2, 4, 6 and 8 represent intermediate not yet released by peptic digestion. The release of ^{32}P was almost complete at the highest pepsin concentration (Sample 8). The release is shown in Fig. 2. For half-maximal release after 30 min at 23° the pepsin concentration was 0.0036 % (w/v) or 1 μM.

Digestion with other enzymes

Other endopeptidases also solubilized the phosphorylated intermediate[4]. Fig. 3 shows paper electrophoretograms of digests obtained with pepsin, trypsin, papain and pronase. Pronase, papain and pepsin, solubilized almost all of the intermediate but trypsin released only 45 %. Pronase and papain each produced two spots; trypsin released five spots.

Since pronase hydrolyzes nearly all peptide bonds[10], it was desirable to test

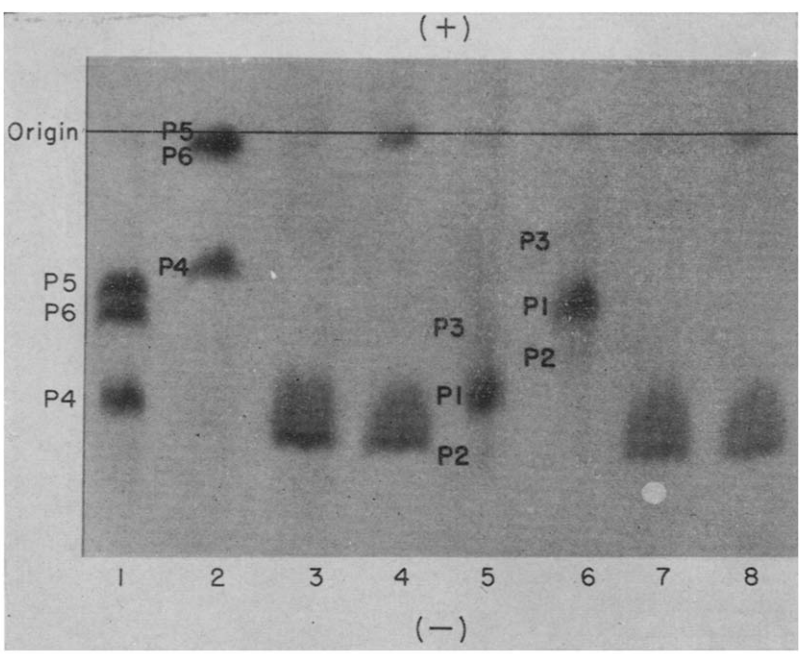


Fig. 4. Hydrolysis of peptic fragments P1–P6 by pronase. Conditions for peptic digestion were as in Fig. 1. The ratio of pepsin to ATPase protein in Samples 1–4 was 0.4 and in Samples 5–8 was 0.00013. Incubation was for 30 min at 23°. Samples 1, 2, 5 and 6 had peptic digestion only. Samples 3 and 4 derive from Sample 1 and Samples 7 and 8 from Sample 5 after further incubation with pronase. Samples 2, 4, 6 and 8 were treated with performic acid after proteolytic digestion. Electrophoresis was as in Fig. 1.

its action on the peptic fragments. Although the optimal pH for pronase is 8.5 (ref. 11), pH 6.5 was used because of the lability of the acyl phosphate at alkaline pH (refs. 3–5). The results appear in Fig. 4. After incubation of Fragments P1–P3 and Fragments P4–P6 with pronase, the more prominent electrophoretic spot (Samples 3 and 7) had a mobility similar to that of the more prominent spot from the whole ATPase (compare Fig. 3). The trailing edge of the slower pronase fragment in Fig. 4, in contrast to the sharp spot in Fig. 3, may be due to the shorter time of digestion in Fig. 4. Performic oxidation decreases the mobility of the peptic fragments[4]. Oxidation of the pronase fragments (Samples 4 and 8) did not change the mobility and gave only a small additional spot near the origin. In contrast, the untreated peptic fragments responded normally (Samples 2 and 6). Pronase appeared to remove an oxidizable group from the peptic fragments.

A sulfhydryl group

The sensitivity of the peptic fragments to performic oxidation suggests the presence of a sulfhydryl group[4]. For a confirmatory test the peptic fragments were treated with *N*-ethylmaleimide, $CuSO_4$, and sulfite. In each case the sensitivity of the product to performic oxidation was also tested. Under the conditions used, *N*-ethylmaleimide alkylates sulfhydryl groups specifically[12], Cu^{2+} catalyzes the oxidation of sulfhydryl groups by O_2 to disulfides[13], and sulfite splits a disulfide into a sulfhydryl group and an *S*-sulfonate group[14]. *N*-Ethylmaleimide prevented the

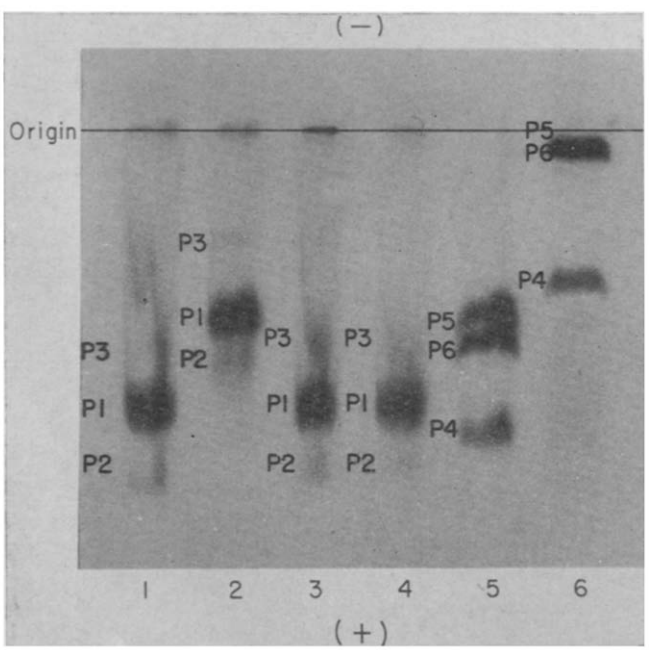


Fig. 5. *N*-Ethylmaleimide treatment of peptic fragments P1–P3. Conditions for peptic digestion were as in Fig. 1. The ratio of pepsin to ATPase protein was 0.00013. Incubation was for 30 min at 23°. Sample 1: peptic fragments P1–P3, control; Sample 2: performic oxidation of Sample 1; Sample 3: *N*-ethylmaleimide treatment of Sample 1; Sample 4: performic oxidation of Sample 3; Sample 5: peptic fragments P4–P6, control (this is the same sample as Sample 1 in Fig. 6); Sample 6: performic oxidation of Sample 5. Electrophoresis was as in Fig. 1.

reduction in mobility produced by performic oxidation of all the peptic fragments, P1–P6 (Sample 4 in Figs. 5 and 6), although it did not change the electrophoretic mobility by itself (Samples 3 in Figs. 5 and 6). In the experiment of Fig. 6 $CuSO_4$ changed Fragments P4–P6 to a row of blurred spots which moved faster (Sample 5). Performic oxidation of this material had much the same effect (Sample 6) as in the absence of $CuSO_4$ treatment (Sample 2). After $CuSO_4$ treatment *N*-ethylmaleimide had no effect on the mobility of Fragments P4–P6 (Sample 7) nor did it prevent the reduction in mobility produced by oxidation (Sample 8). Sulfite by itself affected neither mobility nor sensitivity to oxidation (Samples 11 and 12). In combination with $CuSO_4$ it had the same effect as performic oxidation (Sample 9). Performic oxidation of this material had no further effect (Sample 10).

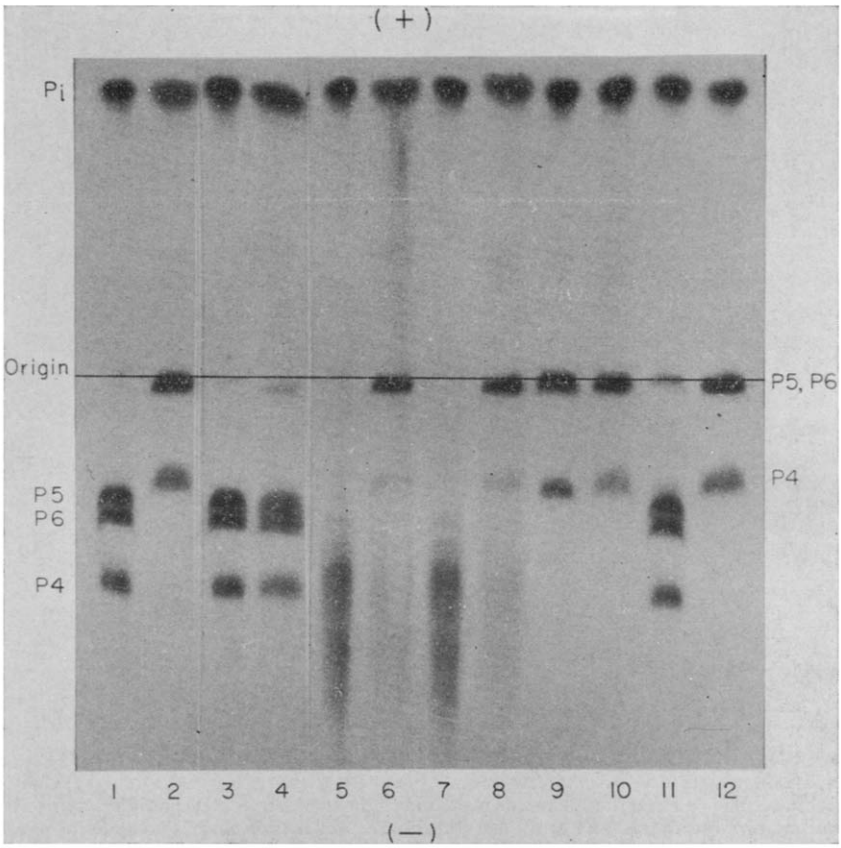


Fig. 6. Treatment of peptic fragments P4–P6 with sulfhydryl and disulfide reagents. Conditions for peptic digestion were as in Fig. 1. The ratio of pepsin to ATPase protein was 0.4 and incubation was for 30 min at 23°. Samples with odd numbers show the peptic fragments P4–P6 before treatment with performic acid. Samples with even numbers show the same fragments after treatment with performic acid. The labels at the left side designate the peptic fragments before and at the right side the same fragments after performic acid treatment. Samples 1 and 2, controls; Samples 3 and 4, treatment with *N*-ethylmaleimide; Samples 5 and 6, treatment with $CuSO_4$ for 30 min at 23° in 50 mM histidine–HCl (pH 6.3); Samples 7 and 8, Sample 5 treated with *N*-ethylmaleimide; Samples 9 and 10, treatment with sulfite and with $CuSO_4$; Samples 11 and 12, treatment with sulfite without $CuSO_4$. Electrophoresis was as in Fig. 1.

Molecular weights

Gel filtration of peptic fragment P1 on Bio-Gel P10 and P20 indicated an apparent molecular weight of about 10000. Gel filtration of the pronase fragments on Bio-Gel P2 indicated an apparent molecular weight of about 380.

The pronase fragment

Since the pronase fragment was the smallest, a further effort was made to

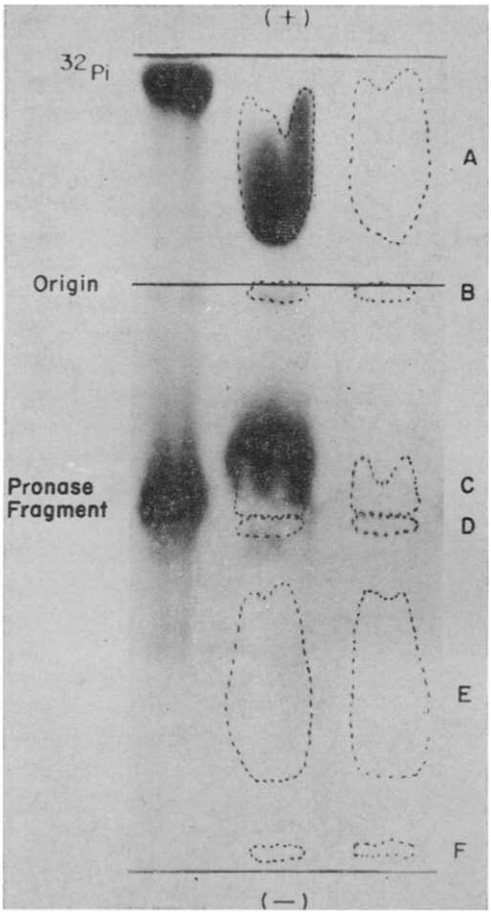


Fig. 7. Comparison of the electrophoretic mobility of the pronase fragment and synthetic L-arginyl phosphate. The pronase fragment was prepared as described in Fig. 3. Sample 1: pronase digest; Sample 2: pronase digest and L-arginyl phosphate preparation simultaneously applied to the paper; Sample 3: L-arginyl phosphate preparation. Electrophoresis was carried out in 1% formic acid at 2500 V and 50 mA for 60 min at 0°. The letters on the right side designate the various spots obtained from the L-arginyl phosphate preparation after spraying the electrophoretogram with molybdate, ninhydrin or Sakaguchi reagent (dotted outlines in Samples 2 and 3). Spots A, B and D stained for phosphate; Spots D, E and F stained for amino groups; Spots B, C, D, E and F stained for a guanidine group. Spot A: inorganic phosphate (overloaded); Spot B: *N*-α-Z-arginyl phosphate; Spot C: *N*-α-Z-L-arginine (overloaded); Spot D: L-arginyl phosphate; Spot E: L-arginine (overloaded); Spot F: probably arginylarginine formed from L-arginyl phosphate and L-arginine[18]. In this electrophoretogram the mobility of P_1 was reduced by its approach to the edge of the paper. The data in Figs. 4 and 6 show that the mobility of the pronase fragment is about 85% of that of P_1.

characterize it. At pH 2.1 it moved toward the cathode and therefore carried a positive charge. By analogy with monoacetyl phosphate its first dissociation constant may be about 1.17 (ref. 15) and most of its phosphate group may be in the monoionic form. This form would neutralize only one cationic group, such as that of an N-terminal amino acid. To account for the observed mobility at least one more cationic group is required. It might be provided by a dibasic amino acid such as histidine, lysine or arginine. To test for histidine the pronase fragment was subjected to photooxidation in the presence of rose bengal[16]. A control sample of histidine was destroyed by this procedure but the mobility of the pronase fragment was unchanged. To test for lysine the pronase fragment was treated with nitrous acid[17]. A control sample of lysine was destroyed by this procedure and the mobility of the pronase spot was reduced to zero. By the reasoning above every cationic group except one was destroyed by nitrous acid. The stable group could not have been an amino group of lysine.

To test for arginine directly was not possible because of the alkali lability of the phosphate group. Instead, arginyl phosphate was synthesized. The electrophoretic mobility of the synthetic L-arginyl phosphate was compared with that of the pronase fragment (Fig. 7). The pronase fragment (Sample 1) moved 9.6 cm and only slightly more slowly than L-arginyl phosphate (Spot D in Samples 2 and 3). However, when the pronase fragment was applied to the paper together with the L-arginyl phosphate preparation (Sample 2), it moved much more slowly than L-arginyl phosphate and the spot was also distorted. The distortion was due to a large *N*-α-Z-L-arginine spot (Spot C) which separated the pronase fragment from L-arginyl phosphate. The pronase fragment was therefore not simply arginyl phosphate.

DISCUSSION

Proteolysis and electrophoresis of a phosphorylated intermediate in the $(Na^+ + K^+)$-dependent ATPase of cell membranes yielded 15 fragments in all (Figs. 1 and 3). In particular, graded digestion with pepsin yielded a sequence of six fragments (Fig. 1) with molecular weights from 10000 to 2200 (ref. 4). Pronase converted all the peptic fragments to one or two fragments with a molecular weight of about 380. These results indicate that the fragments are probably peptides. The ease with which the peptides were released suggests that the phosphorylated intermediate is superficial on the membrane. The combination of an acyl phosphate[3–5] with a peptide indicates that the phosphorylated intermediate of the $(Na^+ + K^+)$-dependent ATPase system may be an acyl phosphoprotein. Such a phosphoprotein is different from the *O*-phosphoprotein envisioned by HEALD[19] and AHMED AND JUDAH[20] as a participant in active cation transport.

Prolonged hydrolysis of the intermediate with pepsin yielded a single fragment, P6 (Fig. 1). This single fragment is electrophoretic evidence for the homogeneity of the intermediate and extends the evidence of NAGANO *et al.*[5], which was based on first-order hydrolysis kinetics.

Treatment of the peptic fragments with performic acid, *N*-ethylmaleimide, $CuSO_4$ and sulfite gave clear evidence for a sulfhydryl group (Figs. 5 and 6). There was no evidence of a disulfide bond. All six fragments were protected from oxidation by *N*-ethylmaleimide, which reacts only with sulfhydryl groups and not with disul-

fides. Mild oxidation with $CuSO_4$ produced a smudge of spots This was probably due to disulfide bond formation between the labelled fragments and unlabelled peptides released by the pepsin. Subsequent stronger oxidation with sulfite abolished the electrophoretic mobility of most of the fragments; the changes are similar to those produced by performic oxidation. In both cases a sulfonic acid would be expected. The only difference is that treatment with sulfite leaves an $R–S–SO_3^-$ group[13] while treatment with performic acid leaves an $R–SO_3^-$ group. The free sulfhydryl group may be that of a cysteine.

Hydrolysis with pronase of the ^{32}P-labelled ATPase or the peptic peptides produced a small fragment insensitive to oxidation. The insensitivity indicates that the sulfhydryl group had been split off. The pronase fragment carried a net positive charge at least part of which was not that of histidine or lysine. It might contain arginine although it was not simply arginyl phosphate. A dipeptidyl phosphate is a possibility. Determination of the structure by direct analysis will require preparation and purification of larger amounts of the pronase fragment than are now available. The difficulties are that the enzyme preparation is crude and insoluble and that the intermediate is unstable. By present methods one person with two centrifuges could make 0.01 μmole every 6 weeks, not counting losses due to hydrolysis. The fragment would still require purification and it has not yet been recovered from the electrophoresis paper.

ACKNOWLEDGEMENTS

We are indebted to Dr. G. H. BOND and to Dr. A. K. SEN for helpful advice. We wish to thank Mrs. M. KOPECKY, Mrs. B. ORCUTT and Mrs. FRANCES NOAH for excellent technical assistance.

This work was supported by grants 5RO1-HE-01974-12 from the National Heart Institute, National Institutes of Health, U.S. Public Health Service and Ba 97/3 from the Deutsche Forschungsgemeinschaft.

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