Evidence for a β-Aspartyl Phosphate Residue in the Phosphorylated Intermediate of the Red Beet Plasma Membrane ATPase

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ABSTRACT
A borohydride reduction method was used to identify the phosphorylated amino acid in the phospho-enzyme of the red beet (Beta vulgaris L.) plasma membrane ATPase. Plasma membrane fractions were phosphorylated with unlabeled ATP in the presence of MgSO4 at pH 6.5 and then treated with sodium [3H]borohydride. The borohydride-treated samples were subjected to hydrolysis in 6 normal HCl at 110°C for 22 hours and then analyzed by high voltage paper electrophoresis and thin layer chromatography. This analysis demonstrated the formation of labeled homoserine as the major reduction product when phosphorylated membrane samples were treated with sodium [3H]borohydride. This suggests that the phospho-group in the plasma membrane ATPase of red beet storage tissue is attached to the β-carboxyl side chain of an aspartic acid residue in the active site of the enzyme.

There is strong evidence for the presence of an ATP-dependent, electrogenic proton efflux pump at the plasma membrane of higher plant cells (17 and references therein) and it has been postulated that a plasma membrane-associated ATP phosphenoloprotein hydrolyase (ATPase) may represent the enzymatic machinery responsible for this process (13, 14). The plasma membrane ATPase isolated from corn roots (2–4), oat roots (18), and red beet storage tissue (6) has been shown to form a rapidly turning over phosphorylated intermediate on a 100,000 D polypeptide, presumably the catalytic subunit of the enzyme. This property is similar to what has been observed for the animal cell Na+, K+-ATPase (12), sarcoplasmic reticulum Ca2+-ATPase (12), and the fungal cell plasma membrane H+-ATPase (11).

The phosphorylated intermediate of the plasma membrane ATPase from corn roots (2), oat roots (18), and red beet storage tissue (6) is acid stable, alkali labile, and hydrolyzed by hydroxylamine. These properties suggest that the protein-phosphate bond is an acyl phosphate linkage formed by the side chain of a glutamic or aspartic acid residue in the active site of the enzyme (20). The acyl phosphate bond is too labile to allow a direct determination of the phospho-amino acid in these intermediates (20); however, Degani and Boyer (9) have presented a borohydride reduction method which can be used for characterizing the acyl phosphate linkage in proteins. This method involves reductive cleavage of the acyl phosphate bond with sodium [3H]borohydride followed by protein digestion in acid and analysis of the labeled hydroxylamine in acid. In this report, the borohydride reduction method was used to identify the catalytic phosphorylated amino acid residue in the plasma membrane ATPase from red beet storage tissue.

MATERIALS AND METHODS
Plant Material. Red beet (Beta vulgaris L.) storage roots were purchased commercially. The tops of the plants were removed and the storage roots were stored for up to 3 months in moist vermiculite at 5°C.

Isolation of Plasma Membrane Fractions. Plasma membrane-enriched fractions were isolated from red beet storage tissue as previously described (5) with minor modifications. The homogenizing medium was modified from that previously described (5) by the addition of 15 mM β-mercaptoethanol. The presence of β-mercaptoethanol tended to reduce some of the variability in the levels of ATPase activity observed previously (5). Plasma membrane fractions isolated from sucrose gradients were diluted with 250 mM sucrose, 1 mM Tris Mes (pH 7.2), 1 mM dithioerythritol (pH 7.2), 1 mM dithioerythritol (pH 7.2), 1 mM dithioerythritol (pH 7.2), and then centrifuged at 227,000g (47,000 rpm) for 30 min in a Beckman Ti70 rotor. The resultant pellets were suspended in suspension buffer at a protein concentration of about 10 mg/ml and then frozen under liquid N2 for up to 4 d without significant loss in ATPase activity.

Phosphorylation. Plasma membranes were phosphorylated in a 1.0-ml reaction volume containing 40 µM unlabeled Tris ATP (pH 6.5), 40 µM MgSO4, 30 mM Tris Mes (pH 6.5), and 500 µg membrane protein. The reaction was carried out for 5 s at ice temperature and stopped by the rapid addition of 25 ml ice cold 10% TCA containing 40 mM NaH2PO4, 5 mM Na2P2O7, and 1 mM Na2 ATP (quenching solution). Rapid mixing was provided by a magnetic stir bar. The quenched samples were centrifuged at 27,000g (15,000 rpm) for 15 min in a Sorvall SS-34 rotor. The resultant pellets were suspended in 0.5 ml of quenching solution using a chilled glass rod, and suspended pellets from six tubes (about 3 mg protein) were pooled and diluted to 26 ml with quenching solution in a single centrifuge tube. The pooled sample was centrifuged at 27,000g (15,000 rpm) for 15 min in a Sorvall SS-34 rotor, and the resultant pellet was then subjected to reduction with sodium [3H]borohydride. Control samples containing unphosphorylated membranes were prepared by adding the quenching solution to the membrane samples prior to the addition of ATP. These samples were then treated in an identical manner as the phosphorylated membrane samples.

Sodium [3H]Borohydride Reduction. Phosphorylated and unphosphorylated plasma membrane samples containing about 3 mg

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FIG. 1. High voltage paper electrophoresis of hydrolysates of sodium [3H]borohydride-treated phosphorylated and unphosphorylated plasma membrane fractions. Homoserine (H) and homoserine lactone (HL) (produced by treatment of homoserine with 0.25 N HCl for 72 h, room temperature) standards were located by ninhydrin staining. Fifteen μl of solution containing 0.05 mg of the standards and 15 μl of protein hydrolysate were applied to the paper. Electrophoresis and the determination of radioactivity were carried out as described in "Materials and Methods."

FIG. 2. High voltage paper electrophoresis of alkali-treated sodium [3H]borohydride-reduced plasma membrane fractions. Hydrolysates of sodium [3H]borohydride-treated phosphorylated and unphosphorylated plasma membrane fractions were incubated in 0.25 N NaOH for 72 h at room temperature. Electrophoresis and the determination of radioactivity were carried out as described in "Materials and Methods." The standards for electrophoresis were the same as for Figure 1.

Protein were suspended in 0.5 ml dimethyl sulfoxide, and 40 μl of 40 mm sodium [3H]borohydride (625 mCi/mmol) was added. After 15 min of incubation at room temperature, the reaction was stopped by the addition of 5 ml 0.44 M HClO4. The precipitate was collected by centrifugation at 13,000 g (10,500 rpm) for 20 min in a Sorvall SS-34 rotor and washed three times by suspension in 5 ml 0.44 M HClO4 and recentrifugation.

The [3H]borohydride-treated samples were hydrolyzed in evacuated sealed tubes in 6 N HCl at 110°C for 22 h. The protein hydrolysates were evaporated to dryness at 35°C. The residue was suspended in 1.0 ml H2O and dried twice in succession to remove exchangeable tritium. The final residue was suspended in 50 μl H2O and titrated to pH 2.5 with pyridine, or 5 μl of 3.0 N NaOH was added to give a final concentration of 0.25 N NaOH. Samples titrated to pH 2.5 were used immediately for electrophoresis while samples treated with 0.25 N NaOH were incubated for 72 h at room temperature prior to electrophoresis.

High Voltage Paper Electrophoresis and TLC. High voltage paper electrophoresis was conducted on Whatman No. 3 MM paper at pH 2.5. Generally, about 15 μl of sample was applied per sample lane and the electrophoresis buffer contained 2% (v/v) HCOOH (pH 2.5). The electrophoresis was first run at 500 v for 30 min (to remove salts) and then at 1500 v for 1 h. The paper was dried, and then 2-cm-wide strips were cut down each sample lane. These strips were then cut into 1 cm segments, and the radioactivity in each segment was determined by liquid scintillation spectrometry in 5 ml of a dioxane cellosolve scintillation cocktail (3).

TLC was carried out on aluminum backed silica gel plates (silica gel-60, Merck). Samples (10 μl total volume) were spotted to the plates and chromatography was carried out with a butanol-acetic acid:NaOH (4:1:1, v/v/v/v) solvent system. The TLC plate was developed twice in succession to enhance resolution. Following chromatography, the silica gel plate was dried and 2-cm-wide strips were cut down the sample lanes. These strips were cut into 0.5-cm segments, and the radioactivity in these segments was determined in 5 ml dioxane cellosolve scintillation cocktail.

For both high voltage paper electrophoresis and TLC the location of authentic standards was determined by staining with ninhydrin reagent (0.5% w/v ninhydrin in absolute ethanol). Homoserine was obtained from Sigma while α-amino-δ-hydroxy-
valeric acid was synthesized by the method of Gauntry (10). The data shown are for representative experiments which were repeated at least two times.

RESULTS AND DISCUSSION

A phosphorylated membrane sample and a corresponding unphosphorylated membrane sample from red beet plasma membranes were treated with sodium [3H]borohydride essentially as described by Degani and Boyer (9). The reaction was carried out in dimethyl sulfoxide in order to decrease the competitive hydrolysis reaction of the acyl phosphate bond (9). Following digestion in 6 N HCl, aliquots of the samples were analyzed by high voltage paper electrophoresis (Fig. 1). Treatment of the membrane samples with sodium [3H]borohydride resulted in a substantial amount of non-specific tritium incorporation as indicated by the amount of radioactivity present in the unphosphorylated sample. The peptide bond can undergo a reductive cleavage reaction with sodium borohydride to produce β-amino alcohol derivatives (7, 16) and this may represent a major contribution to the non-specific incorporation of tritium in these samples (9). Despite substantial non-specific labeling, it is clear that the phosphorylated membrane sample contains a major peak of radioactivity that co-migrates with homoserine lactone (Fig. 1).

When both the phosphorylated and unphosphorylated samples were treated with 0.25 N NaOH for 72 h prior to electrophoresis, the distribution of radioactivity shown in Figure 2 was observed. As before a substantial amount of non-specific labeling was observed, however, the major radioactive peak associated with the phosphorylated sample shifted to co-migrate with homoserine.

The co-migration of the radioactive peak with homoserine lactone and its shift to co-migrate with homoserine after treatment with base suggest that homoserine is the major reduction product when phosphorylated samples are treated with sodium [3H]borohydride. The homoserine can form the lactone ring during acid treatment in protein digestion which can be opened by treatment with base. Formation of homoserine would be consistent with the acyl phosphate group in the ATPase phospho-enzyme being attached to the β-carboxyl side chain of an aspartic acid residue in the active site of the enzyme.

To further analyze the borohydride-treated samples, a two-dimensional separation was carried out using both high voltage paper electrophoresis and TLC. Phosphorylated membrane samples were treated with sodium [3H]borohydride and subjected to high voltage paper electrophoresis. The region of the paper corresponding to the location of homoserine lactone was excised from the electropherogram and extracted in 5 ml of H2O for 72 h. The extract was evaporated to dryness and the residue was treated with either 0.25 N NaOH or 0.25 N HCl for 72 h at room temperature. The samples were then analyzed by TLC with a butanol:acetic acid:H2O solvent system (Fig. 3). When the residue was treated with 0.25 N HCl, radioactivity co-migrated with homoserine lactone. However, when the residue was treated with 0.25 N NaOH, the position of the radioactive peak shifted to co-migrate with homoserine. The position of the radioactive peaks in the phosphorylated samples did not correspond to the position of α-amino-β-hydroxyvaleric acid treated with either 0.25 N NaOH or 0.25 N HCl (its migration did not change, i.e. the lactone was not readily formed) and chromatographed with this system. These results with a two-dimensional separation suggest, as above, that the reduction product of the acyl-phosphate group is homoserine; the hydroxyamino acid derivative of a phospho-aspartate group. This is further supported by the lack of correspondence with α-amino-β-hydroxyvaleric acid; the reduction product of a phospho-glutamate group.

The results of this study suggest that the plasma membrane ATPase of red beet storage tissue catalyzes the hydrolysis of ATP via the phosphorylation of an aspartic acid residue. This result is significant since this is identical to the catalytic intermediates observed for the animal cell Na+,K+-ATPase (15), sarcoplasmic reticulum Ca2+-ATPase (9), and plasma membrane H+-ATPase of fungi (1, 8). This result is also consistent with the preliminary findings of Walderhaug et al. (19) which indirectly suggest the presence of an aspartyl phosphate group in the phosphorylated intermediate of the corn root plasma membrane ATPase. These workers found that pronase digests of phosphorylated corn root plasma membrane ATPase contained a labeled polypeptide with similar electrophoretic mobility and pH stability properties as the active site polypeptide of Na+,K+-ATPase produced by a similar digestion. Thus, the plasma membrane ATPase of higher plants may share a common reaction mechanism with other well characterized cation transporting ATPases.

LITERATURE CITED