Short Communication

Role of Magnesium in the Plasma Membrane ATPase of Red Beet

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ABSTRACT

The phosphorylation technique was used to assess the role of Mg in the red beet (Beta vulgaris L.) plasma membrane ATPase. When an excess of ethylenediaminetetraacetate (Tris salt, pH 6.5) was added to phosphorylation reactions at steady-state, the phosphorylation level declined exponentially and the rate constant for dephosphorylation was similar to that observed when phosphorylation reactions were chased with unlabeled ATP. When KCl was included with the EDTA chase, a 2.4-fold increase in the turnover of the phosphoenzyme was observed. Thus, the formation of the phosphorylated intermediate but not its breakdown requires free Mg to be present. When an excess of unlabeled ATP containing MgSO4 was added to plasma membranes incubated for 20 seconds with [γ-32P]ATP in the absence of MgSO4, a burst of phosphorylation was observed that declined exponentially. The rate constant for this decline was similar to that observed for phosphoenzyme turnover after initial labeling in the presence of MgSO4. Extrapolation of this kinetic plot to zero time indicated that ATP binding can occur when MgSO4 is absent. It is proposed that Mg has a specific role in the transphosphorylation reaction of the terminal phosphate group of ATP to the enzyme.

It is widely believed that an ATPase associated with the plasma membrane of higher plant cells may be involved in driving energy-dependent proton efflux (9, 10, 12). While the plant plasma membrane ATPase has not been fully purified, its properties have been well characterized in partially purified membrane fractions from a limited number of species (4, 9, 10). Similar to the cation transport ATPases of animal cells (7) and the plasma membrane H+-ATPase of fungal cells (6), the plant ATPase forms a phosphorylated intermediate on a 100,000 D polypeptide when incubated with [γ-32P]ATP (2, 3, 5). This property is in contrast to the proton pumping ATPases of prokaryotes and organelles which are not phosphorylated when ATP is hydrolyzed (11).

The plasma membrane ATPase from higher plants is Mg activated and further stimulated by monovalent cations (9, 10). Whether or not this stimulation of the activity by monovalent cations is a manifestation of direct transport of these species by the enzyme is as yet unresolved (10). The requirement for Mg is believed to occur because the true substrate for the enzyme is the Mg-ATP complex (1). This role of Mg has been proposed for a number of the transport ATPases from animal cells (7) while an additional role of Mg in increasing the turnover of the phosphoenzyme has been proposed for the Ca²⁺-ATPase of the sarcoplasmic reticulum (7) and red cell membrane (14). In this report, the phosphorylation technique was used to clarify the specific role of Mg in activating the ATPase of red beet plasma membranes.

MATERIALS AND METHODS

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. Plasma membrane-enriched fractions were isolated as previously described (4) and immediately frozen under liquid N₂ for up to 4 d without significant loss in ATPase activity. ATPase phosphorylation was measured at pH 6.5 and ice temperature by the incorporation of radioactive phosphate from [γ-32P]ATP into TCA-insoluble protein as previously described (2, 3, 5). The details of the phosphorylation conditions are given for each experiment. The data shown represent the mean values for experiments which were repeated three times.

RESULTS AND DISCUSSION

In previous work, it was demonstrated that the plasma membrane ATPase from red beet required Mg ion and maximal activity occurred when Mg and ATP were present at the same concentration (4). Although these results from steady-state ATPase assays imply a substrate-related role for Mg (1), it is impossible to state the precise function of this cation in the mechanism of the enzyme. Initial evidence from phosphorylation studies suggested that Mg acted in the formation of the phosphorylated intermediate and that the subsequent breakdown of the phosphoenzyme did not require free Mg to be present (5).

Further evidence of this role for Mg is shown in Figure 1. When phosphorylation reactions were allowed to reach steady-state (20 s phosphorylation) and an excess of EDTA (Tris salt, pH 6.5) was applied, the phosphorylation level quickly declined. The decrease in phosphorylation was exponential with time and a first-order rate constant of 0.0084 s⁻¹ was calculated from the slope of the plot. Although the addition of EDTA to the reaction caused the [γ-32P]ATP concentration to decrease from 40 to 36.4 μM, simple dilution of the substrate cannot account for the observed decrease in the steady-state phosphorylation level. The rate constant for dephosphorylation is similar to that found when an excess of unlabeled ATP sufficient to prevent measurable labeling was applied to phosphorylation reactions at steady-state (5). Removal of Mg thus has the effect of stopping the further incorporation of label while allowing the breakdown of the previously labeled phosphoenzyme to proceed at its usual rate. This could mean that the dephosphorylation reaction is truly independent of Mg or, alternatively, that bound Mg is not released until the end of the catalytic cycle. It should be pointed out that, in order to obtain

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Fig. 1. Decline of steady-state phosphorylation level in response to an addition of EDTA. Plasma membranes were phosphorylated at ice temperature in a 1.0-ml reaction volume containing 40 μM [γ-32P]ATP, 40 μM MgSO4, 2 mg carrier BSA, and 30 mM Tris-Mes (pH 6.5) for 20 s. Following the 20-s phosphorylation, 20 μmol EDTA (Tris salt, pH 6.5) containing 55.5 μmol KCl (when present) in 100 μl were added, and the reactions were quenched at the indicated times by the rapid addition of 25 ml of ice-cold 10% TCA containing 40 mM Na2HPO4, 5 mM Na2EDTA, 1.0 mM Na2ATP. Protein-bound radioactivity was determined as previously described (2, 3, 5). The values were expressed relative to the 20-s phosphorylation level. Best fit lines were determined by linear regression and first-order rate constants were calculated as $K_{obs} = -2.303$ (slope). The rate constants were 0.0084 s⁻¹ in the absence of KCl and 0.020 s⁻¹ in the presence of KCl.

De-phosphorylation rate constants similar to those observed with an unlabeled ATP chase (5), the EDTA-concentration had to be increased to twice the previously used concentration (5). This may indicate that Mg is tightly bound to the enzyme so that a 500-fold molar excess of EDTA is required to prevent completely the incorporation of radioactive label. When 50 mM KCl (final assay concentration) was included with the EDTA chase, the turnover of the phosphoenzyme increased 2.4-fold. Therefore, the effect of KCl on the turnover of the phosphoenzyme, which was also observed with an unlabeled ATP chase (3, 5) does not require free Mg to be present. The absence of a requirement for free Mg in the dephosphorylation reaction is similar to that observed for the animal cell Na⁺,K⁺-ATPase (7, 13) but differs from that observed for the Ca²⁺-ATPase of the sarcoplasmic reticulum (7) and red cell membrane (14) where Mg greatly accelerates phosphoenzyme breakdown.

Unlike the dephosphorylation kinetic plots observed with an unlabeled ATP chase (5), extrapolation back to zero time reveals values of phosphoenzyme much less than the steady-state phosphorylation level (Fig. 1). This may imply that an additional rapid phase of phosphoenzyme breakdown is present in addition to the phase observed after 5 s in the presence of EDTA. Inasmuch as the rate constant of the main phase of phosphoenzyme breakdown would be sufficient to account for the observed ATPase activity of these preparations (5), this rapid phase may represent a side reaction unrelated to the productive catalytic cycle.

In order to determine the specific role of Mg in phosphoenzyme formation, the experiment shown in Figure 2 was performed. Plasma membranes were incubated with [γ-32P]ATP for 20 s in the absence of MgSO4 and EDTA was included to chelate any free Mg present in the reaction solution. Following the 20-s incubation, unlabeled ATP (Tris salt, pH 6.5) or unlabeled ATP containing MgSO4 was added and the reactions were subsequently quenched. The results were then compared to a control reaction where the membranes were phosphorylated for 20 s in the presence of MgSO4. In all cases MgSO4 was added so that at least a 40 μM concentration was achieved. In the absence of MgSO4, the phosphorylation level was about 6.7% of the control and this level remained constant when the reaction was chased for 6 or 15 s with unlabeled ATP in the absence of MgSO4. When MgSO4 was included with the ATP chase, a burst of phosphorylation was observed that declined exponentially with a rate constant similar to that observed for reactions performed initially with MgSO4 and chased with unlabeled ATP (5). Extrapolation of the plot to zero time reveals a value close to the control phosphorylation level.

These results suggest that binding of ATP to the enzyme can occur which is independent of MgSO4 but subsequent transphosphorylation of the terminal phosphate from ATP to a group on the enzyme requires Mg to be present. During the 20-s phosphorylation in the absence of MgSO4, the enzyme can bind ATP. When unlabeled ATP is added and the radioactive label is diluted out, if MgSO4 is present, any bound [γ-32P]ATP can then phosphatoty the enzyme. The similarity between the extrapolated phosphorylation level and that of the control implies that ATP binding occurs equally well whether or not MgSO4 is present and that transphosphorylation is much faster than the exchange of bound ATP. These results concerning the role of Mg in phosphoenzyme formation are qualitatively similar to that observed for the animal cell Na⁺,K⁺-ATPase (13).

Therefore, while steady-state assays of ATP hydrolytic activity allow the general statement that the productive substrate for hydrolysis with the plasma membrane ATPase is the MgATP complex, the phosphorylation technique has allowed an assessment of the specific role of Mg in the reaction mechanism of the enzyme. The requirement of Mg for transphosphorylation of the terminal phosphate from ATP to the enzyme may imply a specific
role of this cation in stabilizing an enzyme-ATP intermediate or in improving the effectiveness of ADP as a leaving group by binding the \( \alpha \) and \( \beta \) phosphoryl groups of ATP (8).

LITERATURE CITED
