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Characterization of the Solubilized Plasma Membrane ATPase of Red Beet[1]

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ABSTRACT

The plasma membrane ATP-phosphohydrolase (ATPase) from red beet (*Beta vulgaris* L.) storage tissue was solubilized with the zwitterionic detergent Zwittergent 3-14 from a plasma membrane-enriched fraction which was extracted with the anionic detergent, sodium deoxycholate. For both the extraction of extraneous proteins by deoxycholate and the solubilization of active plasma membrane ATPase by Zwittergent 3-14, the optimal concentration of detergent was 0.1% (weight per volume) with a detergent to protein ratio of 1.0 (milligram per milligram). The properties of the solubilized ATPase were found to be similar to the membrane-bound enzyme with respect to pH optimum, substrate specificity, inhibitor sensitivity, and kinetics of K^+ stimulation. The solubilized ATPase preparation formed a rapidly turning over phosphoenzyme, the breakdown velocity of which was increased in the presence of 50 millimolar KCl. Solubilization with 0.1% Zwittergent 3-14 following extraction with 0.1% deoxycholate resulted in an increase in both ATPase activity and steady state phosphoenzyme level; however, a direct correspondence between the increase in ATPase activity and phosphorylation level did not exist. It is proposed that this discrepancy may be the result of a detergent-mediated modification of kinetic rate constants in the mechanism of the enzyme.

The electrogenic primary transport of protons from the cytoplasm to the cell exterior appears to be a common property of higher plant cells (24 and references therein). It is widely believed that a plasma membrane bound ATP phosphohydrolase (ATPase) may be responsible for driving this process (16, 17, 24), and recent studies with partially purified preparations of this enzyme reconstituted into sealed membrane vesicles would support this proposal (22, 28).

Current work on the plasma membrane ATPase of higher plant cells has revealed striking similarities to transport ATPases present in the membranes of other eukaryotic organisms. These enzymes include the H^+-transport ATPase present at the plasma membrane of fungal cells (14) and transport ATPases present in animal cell membranes such as the Na^+, K^+-ATPase (15), the sarcoplasmic reticulum Ca^{2+}-ATPase (15), and the gastric mucosal H^+, K^+-ATPase (26). Like these other transport enzymes, the plasma membrane ATPase of higher plant cells forms a covalent phosphorylated intermediate on a 100,000 mol wt catalytic subunit during the course of ATP hydrolysis (3–5, 7, 8, 27, 29). The protein phosphate bond formed by the plant ATPase is an acyl-phosphate (3, 7, 27, 29) on the β-carboxyl side chain of an aspartic acid residue in the active site of the enzyme (9, 30). This also appears to be a common property shared by those transport enzymes which form phosphorylated intermediates (15, 26, 30).

A major goal of our work is to understand the molecular basis of energy coupling to membrane transport mediated by the plant plasma membrane ATPase. To achieve this goal, it will be necessary to have a detailed understanding of the reaction mechanism of this enzyme. In this paper, we describe the solubilization of the plasma membrane ATPase isolated from red beet storage tissue. The solubilized enzyme preparation may be useful for further studies on the reaction mechanism of the enzyme since both enzyme activity and phosphorylation levels are substantially increased above the levels present in previous preparations (6–8). In addition, preliminary evidence is presented which suggests that detergent treatment may modify kinetic rate constants of the plasma membrane ATPase. This observation is an important consideration in studies on the reaction mechanism of the enzyme and may partially explain the observed activation of the enzyme by detergents (10, 12).

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 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 (6, 8) with minor modifications. This isolation procedure includes a KI extraction step to decrease membrane associated nonspecific phosphatase (6). Since it became apparent that the buoyant density of the plasma membrane depends upon the metabolic status of the storage tissue (25), plasma membranes were isolated from tissue maintained for at least three weeks in storage to be consistent with earlier studies (6, 7). To increase the yield on a protein basis, sucrose gradients were modified to contain a 25%/35% (w/w) sucrose interface. Plasma membrane-enriched fractions isolated from sucrose gradients were diluted with 250 mM sucrose, 1 mM Tris Mes (pH 7.2), 1 mM DTE (suspension buffer) to 28 ml and then centrifuged at 227,000*g* (47,000 rpm) for 30 min in a Beckman Ti 70 rotor. The resultant pellets were suspended in suspension buffer at a protein concentration of about 10 mg/ml and then frozen under liquid N_2 for up to 4 d without significant loss in ATPase activity.

Detergent Treatment of Plasma Membrane Fractions. The frozen plasma membrane fraction was thawed, transferred to a 13.5 ml polycarbonate centrifuge tube, and diluted with suspension buffer to a protein concentration of 2.0 mg/ml. For treatment with sodium deoxycholate the membranes were treated as described by Briskin and Leonard (4) with minor modifications. The detergent solutions contained DTE instead of DTT, and

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following incubation with deoxycholate, the samples were centrifuged for 30 min at 200,000*g* (47,000 rpm) in a Beckman Ti 70.1 rotor. The supernatant was decanted, and the 200,000*g* pellet (deoxycholate pellet) suspended in suspension buffer.

ATPase activity was solubilized with Zwittergent 3-14 (Calbiochem-Behring) by suspending the 200,000*g* deoxycholate pellet in suspension buffer at a protein concentration of 2.0 mg/ml and adding dropwise an equal volume of suspension buffer with 40% (v/v) glycerol containing twice the desired concentration of Zwittergent 3-14. The detergent-treated membranes were incubated on ice for 20 min and then centrifuged at 200,000*g* (47,000 rpm) for 30 min in a Beckman Ti 70.1 rotor. The supernatant was decanted, and the 200,000*g* pellet resuspended in suspension buffer.

Enzyme Assay. Phosphohydrolase activity was analyzed as previously described (6). The standard assay was carried out in a 1.0-ml reaction volume with 3 mM substrate, 3 mM $MgSO_4$, 30 mM Tris Mes (titrated from 0.3 M stocks of Tris and Mes to desired pH), and 50 mM KCl (when present). Following incubation for 30 min at 38°C, the released Pi was determined by the method of Ames (1). Potassium-stimulated activity represented the difference between activity measured in the presence and absence of 50 mM KCl. ATP was purchased from Boehringer and converted to the Tris salt by cation exchange on Dowex 50 resin. All other substrates were purchased from Sigma and were used as sodium salts. Any variations on the assay conditions are indicated in "Results."

Phosphorylation. ATPase phosphorylation was measured at pH 6.5 and ice temperature by the incorporation of radioactive phosphate from [γ^{32}P]ATP into TCA-insoluble protein as previously described (4, 5, 7, 8). Specific details are given for each experiment.

Protein Assay. Protein was determined by two methods in this study. For the determination of enzyme specific activity and protein distribution in detergent treatments, protein was assayed by the method of Peterson (23) following a TCA precipitation to remove interference by DTE. When the protein concentration of membrane samples was being adjusted prior to detergent treatment, protein was determined by the method of Bradford (2) since this technique was quite rapid.

RESULTS

Detergent Treatment of the Plasma Membrane Fraction. Plasma membrane fractions isolated from red beet storage tissue were first treated with the detergent deoxycholate in order to selectively extract proteins other than the plasma membrane ATPase. The goal was to produce a pelletable membrane fraction enriched in plasma membrane ATPase which would be useful for subsequent treatment with other detergents. This 'negative' purification step provided substantial increases in ATPase activity when carried out with corn root plasma membrane fractions (4) and would have the effect of increasing the selectivity of a subsequent detergent solubilization step.

When the red beet plasma membrane fraction was treated with a range of deoxycholate concentrations (Fig. 1), maximal enrichment of pelletable ATPase specific activity occurred when the detergent was present at a concentration of 0.1% (w/v). This represented a detergent to protein ratio of 1.0 (mg/mg). Higher concentrations of the detergent resulted in an inactivation of the pelletable ATPase activity. Substantial amounts of protein could be solubilized by deoxycholate; however, the solubilization of substantial amounts of active ATPase was not observed. Glycerol present at either 10% or 20% (v/v) was not found to increase the solubilization of ATPase by this detergent (data not shown). These results concerning the effects of deoxycholate extraction upon a plasma membrane fraction from red beet were similar to those previously observed for a plasma membrane fraction isolated from corn roots (4). Therefore, treatment of the red beet plasma membrane fraction with 0.1% deoxycholate was carried out prior to treatment with other detergents.

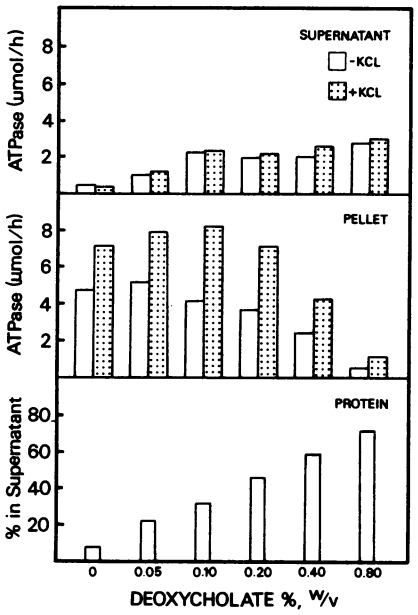

FIG. 1. Apparent solubilization of ATPase activity and protein from a red beet plasma membrane fraction by sodium deoxycholate. The plasma membrane fraction was treated with the indicated concentrations of sodium deoxycholate (see "Materials and Methods") and then centrifuged at 200,000*g* for 30 min. ATPase activity represents the total activity present in 0.5 ml of supernatant and suspended pellet.

Solubilization of the plasma membrane ATPase from deoxycholate extracted membranes was attempted with several detergents (Triton X-100, deoxycholate, Zwittergent 3-16); however, Zwittergent 3-14 proved to be the most effective. The Zwitterionic detergent was able to solubilize substantial amounts of protein and ATPase activity (Fig. 2). Maximal solubilization of ATPase (measured in the presence of 50 mM KCl) and K^+-stimulated ATPase occurred when the Zwittergent 3-14 concentration was 0.1% (w/v) with a detergent to protein ratio of 1.0 (mg/mg). Higher concentrations of the detergent reduced the solubilized ATPase activity. It was found that the presence of 20% glycerol (v/v) was required in order to obtain active solubilized ATPase and the specific activity remained fairly constant for 1 to 2 h if the preparation was maintained at ice temperature. Zwittergent 3-14 solubilized preparations could be stored for several weeks at −80°C without significant loss of ATPase activity if the samples were initially frozen under liquid N_2.

Extraction of the plasma membrane fraction with 0.1% deoxycholate and subsequent solubilization of the ATPase with 0.1% Zwittergent 3-14 yielded considerable increases in both ATPase specific activity and phosphoenzyme level above those found for the plasma membrane fraction (Table I). The final 0.1% Zwittergent 3-14 supernatant fraction contained about 29% of the protein of the plasma membrane fraction and had a nearly 3.4-fold increase in ATPase activity (measured in the presence of 50 mM KCl) but only 1.7- fold increase in the steady state level of phosphoenzyme. This discrepancy between the fold enrichment of ATPase and the fold enrichment of phosphoenzyme will be discussed in more detail later in this paper.

Characterization of the Solubilized Plasma Membrane ATPase. The enzymic properties of the solubilized ATPase were examined so that they could be compared with the observed properties of the membrane-bound enzyme (6). The pH optimum for ATPase measured in the presence of 3 mM $MgSO_4$ was

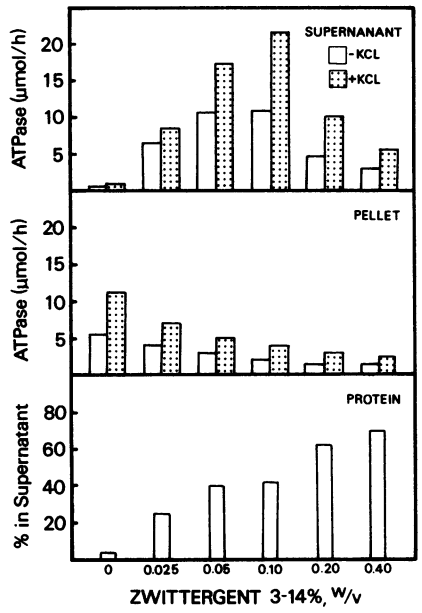

FIG. 2. Apparent solubilization of ATPase activity and protein by Zwittergent 3-14 from deoxycholate-extracted plasma membrane fraction from red beet. The 0.1% (w/v) deoxycholate-extracted red beet plasma membrane fraction was treated with the indicated concentration of Zwittergent 3-14 (see "Materials and Methods") and then centrifuged at 200,000*g* for 30 min. ATPase activity represents the total activity present in 0.5 ml of supernatant and suspended pellet.

7.0, and this declined to 6.5 when 50 mM KCl was included in the assay (Fig. 3). Stimulation of enzyme activity by KCl was also maximal at pH 6.5 but declined to near zero as the assay pH was increased to 8.5. The stimulatory effect of KCl appeared to represent a direct effect upon the enzyme and not the dissipation of a membrane potential since this enzyme preparation was unaffected by ionophores and thus did not represent 'sealed' membrane vesicles (data not shown).

The enzyme showed a clear preference for ATP as the substrate for hydrolysis and all aspects of the activity were substrate specific for ATP (Table II). As has often been observed (16, 17), the highest degree of substrate discrimination occurred for the K^+-stimulated component of the activity.

The effects of various phosphohydrolase inhibitors on the activity of the solubilized ATPase was examined (Table III). The activity was insensitive to the mitochondrial ATPase inhibitors oligomycin and azide and also insensitive to ouabain, a specific inhibitor of the animal cell Na^+, K^+-ATPase (15). Molybdate, an inhibitor of nonspecific phosphatase in plants cells (13) slightly affected the activity while DCCD,[3] DES, and Na_3VO_4, inhibitors of plasma membrane ATPase (13, 16, 17) strongly inhibited the enzyme.

When the kinetics of the K^+-stimulation of ATPase were examined for the solubilized enzyme, the kinetic profile did not fit the Michaelis-Menten relationship as shown by the lack of a linear relationship when the data were analyzed with an Eadie-Hofstee plot (Fig. 4). The data were instead complex and suggestive of either negative cooperativity (16, 17, 20) or multiple sites of K^+ action.

Phosphoenzyme Formation and Breakdown by the Solubilized Enzyme. The time course of phosphoenzyme formation and breakdown was characterized in Figure 5. A steady state phosphorylation level was rapidly established and maintained throughout the duration of the time course. When excess unlabeled ATP was added to reactions at steady state, the phosphorylation level rapidly declined indicating that the phosphorylation was rapidly turning over. If KCl was included with the unlabeled ATP chase, the rate of phosphoenzyme turnover was increased. When the time course of phosphoenzyme breakdown was replotted in a semi-logarithmic manner (Fig. 5, inset) the first order rate constants were determined to be 0.016 and 0.030 s^{-1} for phosphoenzyme breakdown in the absence and presence of 50 mM KCl.

Based on these results, it is apparent that the phosphorylation observed for the solubilized preparation is rapidly turning over and representative of an enzyme reaction intermediate (4, 5, 7, 8). The detergent treatments do not appear to unmask endogenous protein kinase activities so that the short term phosphorylation values shown in Table I most likely represent estimates of steady state ATPase phosphoenzyme. As previously observed (5, 7, 8), KCl increased the turnover of the phosphorylated intermediate, consistent with its effect upon the ATPase activity of this preparation.

DISCUSSION

The plasma membrane ATPase of red beet storage tissue was solubilized from deoxycholate-extracted membranes with the zwitterionic detergent, Zwittergent 3-14. This solubilized ATPase preparation may be useful for further studies on the reaction mechanism of the enzyme since the preparation shows enrichment in both ATPase specific activity and phosphorylation level, the preparation method is fairly rapid and, if necessary, enzyme samples can be stored for several weeks at −80°C when frozen initially in liquid N_2.

Although deoxycholate, an anionic bile salt detergent, was useful in extracting extraneous proteins from the plasma membrane fraction, the solubilization of substantial amounts of active plasma membrane ATPase by this agent was not observed. While others have used this detergent in the solubilization of the plant plasma membrane ATPase (22), the results presented in this study may reflect our inability to stabilize the soluble enzyme in the presence of this detergent. On the other hand, Zwittergent 3-14 allowed solubilization of substantial amounts of ATPase activity from the deoxycholate-extracted membranes, and the activity was stable enough to allow routine enzymic assays and storage. This zwitterionic detergent has also been used in the

[3] Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; DES, diethylstilbesterol; PNP, *p*-nitrophenylphosphate.

Table I. *Distribution of ATPase Activity, Phosphoenzyme Formation, and Protein during Deoxycholate Extraction and Zwittergent 3–14 Solubilization of a Red Beet Plasma Membrane Fraction*

Fraction	ATPase Activity			Phosphoenzyme Formation[a]	Total Protein
	−KCl	+KCl	KCl stimulation		
	μmol Pi/mg·h			*pmol/mg*	*mg*
Plasma membrane	21.8	34.9	13.1	221.0	3.25
0.1% DOC pellet	31.5	52.4	20.9	306.4	2.37
0.1% 3–14 supernatant	56.6	117.1	60.5	384.6	0.94

[a] Measured for 20 s at ice temperature.

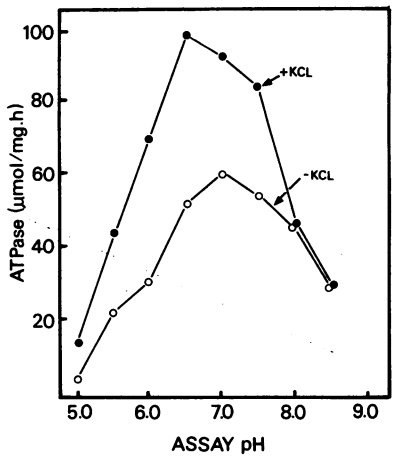

FIG. 3. Effect of assay pH on the ATP hydrolytic activity of the solubilized plasma membrane ATPase.

Table II. *Activity of the Solubilized Red Beet Plasma Membrane ATPase with Various Substrates*

Substrate	Phosphate Hydrolyzing Activity		
	−KCl	+KCl	KCl stimulation
	μmol Pi/mg·h		
ATP	56.6	117.2	60.6
ADP	13.5	19.7	6.2
AMP	1.7	0.72	0
GTP	23.4	24.7	1.3
UTP	24.7	27.2	2.5
PNP	18.7	17.8	0

Table III. *Effect of Inhibitors on the Solubilized Red Beet Plasma Membrane ATPase*

Treatment	ATPase Activity		
	−KCl	+KCl	KCl stimulation
	μmol Pi/mg·h		
Control	50.3	93.8	43.5
Ethanol, 1%	48.9	93.4	44.5
Oligomycin, 10 μg/ml[a]	53.8	90.6	36.8
NaN_3, 1 mM	51.4	95.2	43.8
Ouabain, 10 μM[a]	62.9	101.4	38.5
DCCD, 10 μM[a]	23.0	24.3	1.3
DES, 100 μM[a]	9.9	11.4	1.5
Na_3VO_4, 50 μM	22.6	15.2	0
Na_2MoO_7, 100 μM	51.4	80.7	29.3

[a] Assayed in the presence of 1% ethanol.

purification of the yeast plasma membrane ATPase (21) and in the partial purification of the plasma membrane ATPase from oat roots (28). In the latter case, the enzyme was solubilized with the Zwittergent from oat root plasma membranes which were previously extracted with Triton X-100 and KCl, thus representing a procedure similar to the one employed in this study. One interesting difference, however, was that these authors (28) required four times the Zwittergent concentration (detergent/protein ratio 4:1) used in this study to obtain optimal solubilization of the ATPase. This may imply that, unlike prior extraction with Triton X-100 and KCl, prior extraction with deoxycholate may facilitate the subsequent solubilization with Zwittergent 3-14. This interaction between the detergent treatments may also

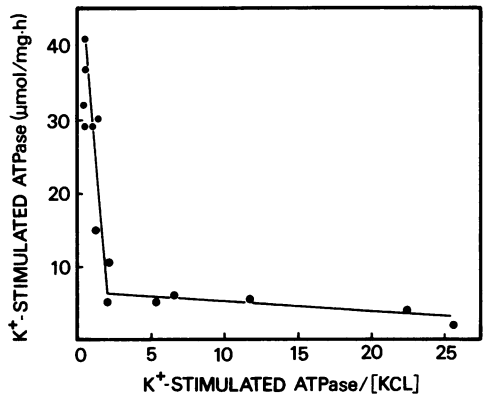

FIG. 4. Effect of increasing concentrations of KCl on the ATP hydrolytic activity of the solubilized ATPase. ATPase activity was assayed in the presence of increasing concentration of KCl (0.05–70 mM). The data are presented as an Eadie-Hofstee plot.

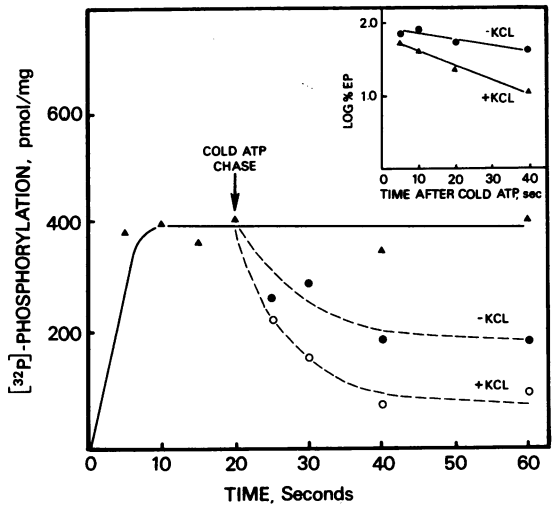

FIG. 5. Time course of phosphoenzyme formation and breakdown for the solubilized plasma membrane ATPase from red beet. Phosphorylation was carried out as described in "Materials and Methods." After 20 s, 1.5 μmol of unlabeled ATP (Tris salt, pH 6.5) and 50 μmol KCl (if indicated) were added, and the reactions were quenched at the indicated times. When replotted as a semi-log plot (inset), first order rate constants for dephosphorylation were calculated from the time course of phosphoenzyme breakdown. 'Best fit' lines were determined by linear regression, and first order rate constants were calculated as $K_d = -2.303$ (slope). The rate constants for dephosphorylation were 0.016 and 0.030 s^{-1} in the absence and presence of KCl, respectively.

explain why the solubilized red beet ATPase is inactivated substantially at the 4 to 1 detergent to protein ratio used by Vara and Serrano (28) to obtain active solubilized oat root plasma membrane ATPase (Fig. 2).

Characterization of the solubilized enzyme with respect to pH optimum (Fig. 3), substrate specificity (Table II), and inhibitor sensitivity (Table III) demonstrated properties similar to the membrane-bound enzyme (6). When the kinetics of K^+ stimulation of ATPase were examined for the solubilized plasma membrane ATPase, a complex kinetic profile was observed (Fig. 4) similar to that observed for the membrane-bound enzyme (6). This result differed, however, from that observed for the plasma membrane ATPase of corn roots where the complex kinetic relationship of K^+ stimulation of ATPase observed for the membrane-bound enzyme (19) changed to fit the Michaelis-Menten relationship following solubilization with octyl-glucoside and

fractionation with $(NH_4)_2SO_4$ (11). If the complex kinetic profile is the result of negative cooperativity (20), as originally proposed by Leonard and Hodges (18), then these different results could be explained by the different degrees to which these detergent treatments disrupt the protein-protein interactions required for cooperative effects (20).

When the solubilized plasma membrane ATPase was incubated in the presence of [γ-^{32}P]ATP, a rapidly turning over phosphorylated intermediate was observed (Fig. 5). The turnover rate of the phosphorylated intermediate was increased with the presence of KCl; consistent with KCl effects upon stimulating ATPase and similar to KCl effects upon the phosphoenzyme of the membrane-bound enzyme (6). As with ATPase activity, the steady state phosphorylation level has increased in the solubilized enzyme (Table I). However, it appeared that ATPase activity was enriched to a greater degree than the level of steady state phosphorylation. If it is assumed that the kinetic rate constants within the ATPase reaction mechanism remain unchanged throughout the detergent treatments and kinetic competence for phosphoenzyme turnover is established for the membrane bound enzyme (7), then this result could suggest enrichment of an ATP hydrolytic activity that does not form a phosphorylated reaction intermediate (*i.e.* one would expect a correspondence between the enrichment of sites of ATP hydrolysis [measured by phosphorylation] and ATPase activity). However, this would not be consistent with the observed properties of the solubilized ATPase preparation (Fig. 3; Tables II and III) which indicate enrichment in plasma membrane ATPase. This observed discrepancy then suggests the possible modification of kinetic rate constants by the detergent treatments used in this study. This is supported by the observation that the first order rate constants for phosphoenzyme breakdown in the solubilized ATPase (Fig. 5) are 1.3- to 1.7-fold greater than the rate constant observed for the membrane-bound enzyme (7, 8). Modification of kinetic rate constants by detergent treatments could imply difficulties in establishing a true quantitative description of the purification (*i.e.* fold purification, % yield) of integral membrane proteins where detergents are required for the separation of membrane components. Modification of kinetic rate constants by detergent treatments could also explain the observed detergent activation of the corn root plasma membrane ATPase (12) which could not be accounted for by a detergent-mediated increase in the accessibility of substrate (10) or dissipation of ion gradients. These detergent effects may reflect a modification of the membrane environment which in turn affects the mechanistic properties of the ATPase.

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