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Asparagine-Linked Carbohydrate Does Not Determine the Cellular Location of Yeast Vacuolar Nonspecific Alkaline Phosphatase

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The nonspecific alkaline phosphatase of *Saccharomyces* sp. strain 1710 has been shown by phosphatase cytochemistry to be exclusively located in the vacuole. *para*-Nitrophenyl phosphate-specific alkaline phosphatase is not detected by this procedure because the activity of this enzyme is sensitive to the fixative agent, glutaraldehyde. To determine whether the oligosaccharide of nonspecific alkaline phosphatase is necessary to transport the enzyme into the vacuole, protoplasts were derepressed in the absence or in the presence of tunicamycin, an antibiotic which interferes with the glycosylation of asparagine residues in proteins. The location of the enzyme in the tunicamycin-treated protoplasts, as determined by electron microscopy and subcellular fractionation, was identical to its location in control protoplasts. In addition, carbohydrate-free alkaline phosphatase was found in vacuoles from tunicamycin-treated protoplasts. Our findings indicate that the asparagine-linked carbohydrate moiety does not determine the cellular location of the enzyme.

Saccharomyces yeasts produce a variety of asparagine-*N*-acetylglucosamine glycoproteins that are to be found both inside and outside of the cell membrane. External invertase and acid phosphatase are highly glycosylated molecules (approximately 50% mannose) (6, 30), whereas the intracellular glycoproteins, alkaline phosphatase and carboxypeptidase Y, are noncytoplasmic and have a smaller percentage of carbohydrate (8 and 15% respectively) (17, 31). Phosphorylated mannose residues have been found in the carbohydrate chains of both external (7) and internal glycoproteins (14). The exact role of the carbohydrate moiety of these molecules is not yet clear.

Several enzymes that hydrolyze phosphomonoesters at alklaine pH are found in *Saccharomyces* cells. Two of the yeast alkaline phosphatases have very specific substrate requirements. The *para*-nitrophenyl phosphate (pNPP)-specific alkaline phosphatase (also called constitutive alkaline phosphatase [26]) is unusual in that its only known substrate is an artificial phosphomonoester, pNPP (2, 3). A second substrate-specific enzyme is L-histidinol phosphate phosphatase, one of the enzymes in the biosynthetic pathway for L-histidine (13, 25). A third phosphatase, nonspecific alkaline phosphatase (also called repressible alkaline phosphatase [26]), can hydrolyze practically any phosphomonoester and is the best characterized yeast alkaline phosphatase (2, 13, 26, 31).

Several studies have shown that alkaline phosphatase in intact cells or in protoplasts of *S. cerevisiae* (24, 37, 40) is an internal enzyme, but there is some disagreement on the exact cellular location. Bauer and Sigarlakie (4, 5) have located alkaline phosphatase activity in *S. cerevisiae* by using cytochemical staining procedures on frozen thin sections with pNPP as substrate. They showed that alkaline phosphatase is present in the vacuole, especially along the innerside of the vacuolar membrane and in cytoplasmic vesicles. More recently, Wiemken et al. (41) have examined the cellular location of a variety of yeast enzymes including alkaline phosphatase. Only 42% of the alkaline phosphatase activity measured in a protoplast lysate was derived from the vacuole. Since both of these groups used pNPP as substrate for alkaline phosphatase assay, they could not discriminate between the activity of pNPP-specific and nonspecific alkaline phosphatases.

The purpose of this study was to define the cellular location of yeast nonspecific alklaine phosphatase and to ascertain whether this loca-

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tion is determined by the asparagine-linked carbohydrate. To accomplish this, we treated protoplasts with tunicamycin (TM), an antibiotic that prevents the addition of oligosaccharides to asparagine residues in proteins (22, 36, 38), and then investigated the location and nature of the nonspecific alkaline phosphatase. Yeasts treated with TM do not synthesize active acid phosphatase or external invertase (21), and the rate of active carboxypeptidase Y synthesis is decreased (15). In contrast, the rate of synthesis of alkaline phosphatase is not affected by the antibiotic (31).

In the remainder of this article, the term alkaline phosphatase will refer to an enzyme which can hydrolyze the phosphomonoester bond of pNPP at alkaline pH. When it is necessary to distinguish between the alkaline phosphatase enzymes, the terms "pNPP-specific" or "nonspecific" will be used.

MATERIALS AND METHODS

Abbreviations used in this report are: pNPP, *para*-nitrophenyl phosphate; TM, tunicamycin; and fast red TR, 4-chloro-*o*-toluidine-1,5-diazonium naphthalene disulfonate.

Yeast strains and growth conditions. Diploid strain 1710 was selected for catabolite-resistant synthesis of invertase from the mutagenesis of *Saccharomyces* strain 303-67 (27). *S. cerevisiae* AL-20-3c (**a**, *pho3*, *pho8*) is defective in the synthesis of nonspecific alkaline phosphatase and constitutive acid phosphatase (19, 39). This organism was obtained from Y. Oshima (Osaka, Japan). Cells were grown in Vogel medium N (9) supplemented with vitamins (20) and 2% glucose. Mid-log phase cells were converted to protoplasts with Glusulase (Endo Laboratories Inc., Garden City, N.Y.) or Zymolyase 60,000 (gift of Y. Horie, Kirin Brewery, Takasaki, Japan) by the method of Kuo and Lampen (20). Protoplasts were derepressed for alkaline phosphatase by the method of Onishi et al. (31). The magnitude of derepression (two- to threefold) was similar to that reported at that time (31).

Enzyme assays. Assays for alkaline and acid phosphatase were performed as described by Onishi et al. (31). A unit of activity was defined as the amount of enzyme that hydrolyzed 1 μmol of substrate in 1 min at 30°C.

Nondenaturing polyacrylamide gel electrophoresis and detection of activity in gels. Polyacrylamide gel electrophoresis in nondenaturing conditions was based on the procedure of Davis (8). Alkaline phosphatase activity was detected in the gels either by the simultaneous capture method (11) or by using the chromogenic substrate, pNPP. In the first method, the slab gel was incubated in 0.033 M Tris-hydrochloride (pH 8.5) containing 0.001 M $MgCl_2$, 0.01 M α-naphthylphosphate (Sigma Chemical Co., St. Louis, Mo.) and fast red TR salt (Sigma) at a concentration of 1 mg/ml. The gels were incubated for approximately 15 min at 30°C. In the second method, staining for activity with pNPP, the gels were incubated in 0.033 M Tris-hydrochloride buffer (pH 8.5) containing 0.001 M $MgCl_2$ and 0.01 M pNPP. The gels were incubated for 30 to 90 min at 30°C. Gels were photographed or scanned at 410 nm with a Gilford model 250 spectrophotometer equipped with a model 2520 gel scanner.

Column chromatography. Bio-Gel A-0.5m (Bio-Rad Laboratories, Richmond, Calif.) column chromatography was performed under the conditions described by Onishi et al. (31). The column (2 by 95 cm) was eluted at 10 ml/h, and 3-ml fractions were collected. Concanavalin A affinity chromatography was performed in Pasteur pipettes under the conditions described by Abrams et al. (1).

Effects of glutaraldehyde. Fractions from Bio-Gel A-0.5m column chromatography containing enzyme activities of peaks I, II, or III (see Fig. 2) were mixed with an equal volume of the same prefixation buffer used for alkaline phosphatase cytochemistry but with the strength increased twofold and without mannitol. The level of glutaraldehyde was varied to determine its effect over a range of concentrations. Three 0.05-ml samples were taken immediately and again after 30 min of incubation on ice. Each sample was immediately placed in 0.8 ml 1 M Tris-hydrochloride (pH 8.9) containing 0.001 M $MgCl_2$ and assayed for alkaline phosphatase activity.

For the in vivo glutaraldehyde tests, washed protoplasts were divided into two portions. One was suspended in 5 ml of prefixation buffer; the other was in 5 ml of prefixation buffer that lacked glutaraldehyde but contained all other components and served as a control. The suspensions were gently mixed, and 2 ml was immediately removed and diluted into 20 ml of 0.8 M sorbitol, centrifuged at 600 × *g* for 3 min and washed with 0.8 M sorbitol. The sample was then suspended in 2 ml of 1 M Tris-hydrochloride (pH 8.9) containing 0.001 M $MgCl_2$, to which 0.02 ml of toluene-ethanol (1:1, vol/vol) was added. The protoplasts were shaken vigorously for 5 min and were assayed for alkaline phosphatase activity. After 30 min of incubation in the prefixation buffer, 2.0-ml samples were taken and treated in the same way as the initial time samples.

Beryllium sensitivity. The sensitivity of alkaline phosphatase to beryllium was tested by assaying the alkaline phosphatase enzymes that were partially purified by Bio-Gel A-0.5m column chromatography. The alkaline phosphatase in these fractions was assayed in the presence of 0.0005 M beryllium sulfate in the usual method for alkaline phosphatase assay, with the exception that the Tris concentration was lowered to 0.05 M.

Vacuole isolation. Vacuoles were isolated from protoplasts by the procedure of Oshumi and Anraku (32). The preparations appeared homogeneous by phase contrast microscopy and contained only low levels of patent alkaline phosphatase or α-glucosidase (cytosolic).

Electron microscopy cytochemistry. Protoplasts were harvested by centrifugation at 600 × *g* for 5 min and washed twice with 0.8 M sorbitol. The protoplasts (approximately 150 mg wet weight) were suspended in 5 ml of 0.05 M Tris-maleate (pH 7.4) containing 0.001 M $MgCl_2$, 3% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, Pa.), 2.5% dimethyl sulfoxide, and 12% (wt/vol) mannitol (prefixation buffer) for 30 min on ice. After prefixation, the protoplasts were washed twice in 0.05 M Tris-maleate (pH 8.5)

containing 0.001 M $MgCl_2$, 2.5% dimethyl sulfoxide, and 12% mannitol (wash buffer).

Staining was carried out by a modification of the Gomori procedure (12). The protoplasts were incubated in 5 ml of 0.05 M Tris-maleate (pH 8.5) containing 0.001 M $MgCl_2$, 0.01 M pNPP, and 0.025 M $Pb(NO_3)_2$. The $Pb(NO_3)_2$ was added just before use, and the staining solution was stirred continuously to prevent precipitation. Controls included omission of pNPP, omission of $Pb(NO_3)_2$, and the addition of 1 mM $BeSO_4$ to inhibit the nonspecific alkaline phosphatase.

The stained protoplasts were washed twice with wash buffer adjusted to pH 7.2 and were fixed overnight on ice in 0.05 M Tris-maleate (pH 7.2) containing 3% glutaraldehyde, 2% formaldehyde, and 2.5% dimethyl sulfoxide. The fixed protoplasts were washed twice with 0.112 M Veronal-acetate buffer (pH 6.9) and then postfixed in 2% OsO_4 (wt/vol, in Veronal-acetate) for 24 h in the dark. After a wash in distilled water, the protoplasts were stained for 1 h in aqueous 2% uranyl acetate. The protoplasts were then dehydrated in a standard ethanol series and embedded in Spurr resin (35).

Sections (80 to 120 nm) were cut on an OmU3 (C. Reichert, Austria) ultramicrotome. The sections were picked up on 2% Formvar carbon-coated grids, treated with Reynolds stain (33), and examined with a JEM-100CX electron microscope (JEOL, Ltd., Tokyo, Japan).

RESULTS

Alkaline phosphatases synthesized by *Saccharomyces* sp. strain 1710 protoplasts. To characterize the alkaline phosphatases produced by *Saccharomyces* sp. strain 1710, we subjected protoplast lysates to Bio-Gel A-0.5m column chromatography. In this chromatographic profile there were three peaks of alkaline phosphatase activity (Fig. 1). The enzyme activity in peak II was identified as the nonspecific alkaline phosphatase, and the enzyme in peak I was identified as an aggregate form of the nonspecific alkaline phosphatase that eluted in the void volume (31). The enzyme in peak III was identified as the pNPP-specific alkaline phosphatase by its electrophoretic mobility and by substrate specificity tests. The enzyme hydrolyzed pNPP, but did not hydrolyze α-naphthylphosphate, β-glycerophosphate, glucose-6-phosphate, or fructose-6-phosphate. The separation in this chromatography is not entirely based on gel filtration principles since elution of all three enzymes required NaCl and Triton X-100 (H. R. Onishi, Ph.D. thesis, Rutgers, The State University of New Jersey, New Brunswick, 1979); recovery of activity was in the 70 to 80% range.

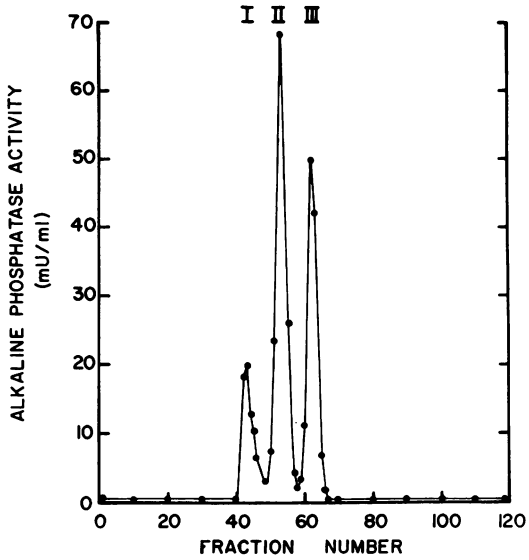

FIG. 1. Alkaline phosphatase synthesis by *Saccharomyces* sp. strain 1710 protoplasts. Protoplast cultures were centrifuged and lysed in 0.05 M Tris-hydrochloride buffer (pH 8.5) containing 0.001 M $MgCl_2$, 0.1 M NaCl, and 0.1% Triton X-100. The final pH was 7.5. To separate and partially purify the enzymes, we subjected protoplast lysates to gel filtration on a Bio-Gel A-0.5m column (2 by 95 cm). The column was eluted at 10 ml/h with lysis buffer at pH 7.5, and 3-ml fractions were collected.

Effect of glutaraldehyde on alkaline phosphatase activity. Enzyme cytochemistry procedures include a fixation step with glutaraldehyde before cytochemical staining to make the cell permeable to the staining reagents and to preserve the ultrastructure of the cell. It was therefore necessary to determine the effect of glutaraldehyde on the activities of the various alkaline phosphatases. Samples of peaks I, II, and III from Bio-Gel A-0.5m column chromatography of repressed and derepressed protoplasts were mixed with an equal volume of the prefixation buffer (twice the usual concentration) used in the cytochemical procedure. Since the concentration of glutaraldehyde in the cell may be less than the 3% in the prefixation buffer, the enzymes were also exposed to a variety of lesser glutaraldehyde concentrations. Samples were taken initially and after 30 min and then diluted in alkaline phosphatase assay buffer and assayed for hydrolytic activity on pNPP.

The pNPP-specific enzyme was quite sensitive to glutaraldehyde, even at a concentration of 1.5% (Fig. 2). However, the nonspecific alkaline phosphatase and the aggregated form of the nonspecific enzyme (peaks II and I, respectively, in Fig. 1) were relatively insensitive to even 3.0% glutaraldehyde. It was observed that the loss of activity of the pNPP-specific enzyme was extremely rapid. Within the 3 to 5 s from the addition of 3% glutaraldehyde to initial sampling, greater than 50% of the activity was lost.

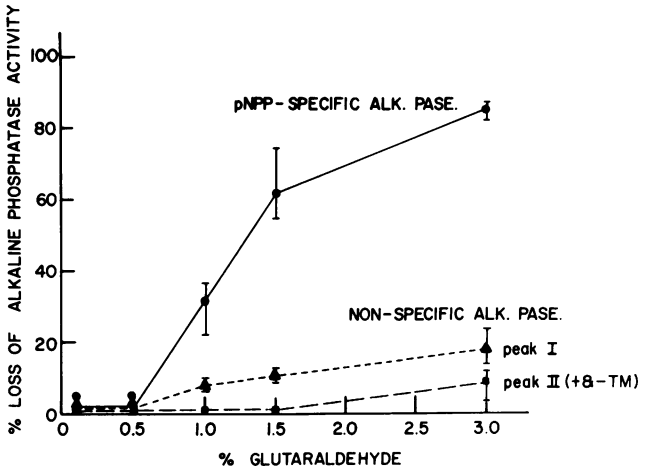

FIG. 2. The effect of glutaraldehyde on partially purified alkaline phosphatases (ALK. PASE.). Fractions from Bio-Gel A-0.5m column chromatography containing enzyme activities of peaks I, II, or III (Fig. 1) were mixed with an equal volume of the same prefixation buffer used for alkaline phosphatase cytochemistry but with the strength increased twofold and without mannitol. The level of glutaraldehyde was varied to determine its effect over a range of concentrations. Three 0.05-ml samples were taken after 30 min of incubation on ice. Each sample was immediately placed in 0.8 ml of 1 M Tris-hydrochloride (pH 8.9) containing 0.001 M $MgCl_2$ and assayed for alkaline phosphatase activity. Symbols: ●, peak III; ▲, peak I; ■, peak II.

This rapid loss of activity was not characteristic of the inactivation of the nonspecific enzyme.

To determine the in vivo effect of glutaraldehyde fixation, we suspended strain 1710 protoplasts in prefixation buffer and incubated for 30 min on ice. Samples were taken at the beginning and end of the incubation period. These samples were diluted with 0.8 M sorbitol, centrifuged, washed with 0.8 M sorbitol, made permeable with toluene-ethanol (1:1, vol/vol) (10), and assayed for hydrolytic activity on pNPP. The results of these experiments showed a 67% loss of pNPP hydrolytic activity.

Beryllium sulfate, at 0.5 mM, inhibited 100% of the nonspecific alkaline phosphatase activity, but inhibited the pNPP-specific alkaline phosphatase by only 50% (D. W. Clark, Ph.D. thesis, Rutgers—The State University of New Jersey, New Brunswick, 1982). Assaying glutaraldehyde-treated protoplasts, in the presence and absence of 0.5 mM beryllium sulfate, showed that all of the pNPP hydrolytic activity that remained after glutaraldehyde treatment was sensitive to beryllium. This suggested that all of the hydrolytic activity on pNPP that remains after glutaraldehyde treatment is caused by the nonspecific enzyme.

To show conclusively that the activity of pNPP-specific alkaline phosphatase is completely destroyed by the glutaraldehyde fixation, protoplasts of strain AL-20-3c, which cannot synthesize the nonspecific alkaline phosphatase, were treated with 1.5 and 3.0% glutaraldehyde. Samples were taken immediately and after 30 min. The samples treated with 1.5% glutaraldehyde lost 97% of their original alkaline phosphatase activity in 30 min. In the 3.0% glutaraldehyde-treated samples, less than 1% of the enzyme activity remained after 30 min of incubation. Once again there was a rapid loss of the pNPP-specific alkaline phosphatase activity. In the 3 to 5 s between the addition of 3% glutaraldehyde and the initial sampling, greater than 60% of the activity was lost, and in the sample with 1.5% glutaraldehyde, greater than 15% was lost.

These results demonstrate that the pNPP-specific alkaline phosphatase is completely destroyed by 3.0% glutaraldehyde treatment under the conditions used for the cytochemical detection of alkaline phosphatase. The in vitro glutaraldehyde tests demonstrated that partially purified pNPP-specific alkaline phosphatase was very sensitive to glutaraldehyde. Secondly, beryllium sulfate inhibited all of the pNPP hydrolytic activity remaining after glutaraldehyde treatment of strain 1710 protoplasts. Finally, all of the pNPP hydrolytic activity of strain AL-20-3c, which does not synthesize the nonspecific enzyme, was lost after 3.0% glutaraldehyde treatment. Consequently, protoplasts treated by 3.0% glutaraldehyde before alkaline phosphatase cytochemistry should be devoid of any stain that can be attributed to pNPP-specific alkaline phosphatase.

Nonspecific alkaline phosphatase is located in

the vacuole. The observation that glutaraldehyde treatment completely destroys the activity of the pNPP-specific alkaline phosphatase made it possible to determine the cellular location of the nonspecific alkaline phosphatase by electron microscopy cytochemistry. The method developed for alkaline phosphatase localization is a modification of the Gomori method (12) and uses pNPP as substrate. With this technique, it is possible to localize the nonspecific enzyme and to identify, in protoplasts treated with TM, any changes in the cellular location of the nonspecific enzyme that could be attributed to the lack of glycosylation.

Protoplasts repressed for alkaline phosphatase and stained for pNPP hydrolytic activity after glutaraldehyde fixation showed most of the stain deposits in the vacuoles (Fig. 3). Some deposits are also seen on the outside of the plasma membrane. We are convinced, based on the following observations, that these external deposits are artifacts. (i) The stained deposits cannot be due to acid phosphatase activity (a yeast extracellular glycoprotein), as the pH of the staining buffer is alkaline, and staining in the presence of NaF, an inhibitor of acid phosphatase, did not decrease the amount of external deposits (results not shown). (ii) Intact cells of strain 1710 lack external alkaline phosphatase activity, and several other studies have failed to demonstrate any alkaline phosphatase activity with intact cells or protoplasts of *S. cerevisiae* (24, 37, 40). (iii) The stained deposits cannot be from Glusulase preparations (which contain a trace of alkaline phosphatase activity) because protoplasts produced with Zymolyase 60,000 (which lacks any detectable alkaline phosphatase activity) also produced these external stain deposits (results not shown). (iv) Cells which were treated with Glusulase but still had some cell wall remaining, had stain deposits on the outside of the cell wall but no deposits opposed to the plasma membrane (results not shown). The clusters of stain on the outide of the plasma membrane have the same amorphous appearance as stained material sometimes seen inside vacuoles when the fixation of the vacuolar interior is more complete. The most likely explanation, based on the available evidence, is that these clusters are vacuolar enzyme released by protoplast lysis during handling.

The vacuoles of repressed protoplasts tended to be smaller and greater in number than those of derepressed protoplasts. Frequently, vacuoles in repressed protoplasts are seen clustered together, and those that are adjacent have membranes with poor definition. This suggests that the vacuole membrane is breaking down in the process of vacuole fusion. In repressed protoplasts with several small vacuoles, one or two will usually contain most of the stain.

Effect of TM on alkaline phosphatase synthesis. Extracts of TM-treated and control protoplasts of strain 1710 were subjected to nondenaturing polyacrylamide gel electrophoresis followed by detection of the enzyme with α-naphthylphos-

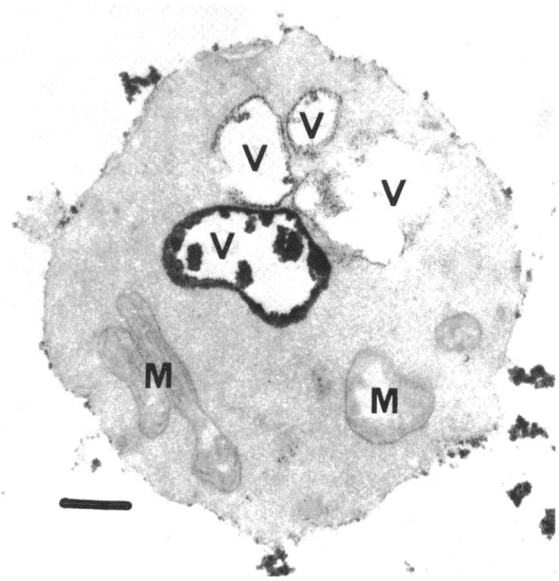

FIG. 3. The cellular location of nonspecific alkaline phosphatase. Protoplasts of strain 1710 repressed for alkaline phosphatase were prepared for alkaline phosphatase cytochemistry, sectioned, and examined under an electron microscope. V, Vacuole; M, mitochondria. Bar, 1.0 μm.

phate-fast red TR as substrate (Fig. 4). The TM-treated protoplasts showed an additional major and a faint minor band (Fig. 4, lane C) with electrophoretic mobilities greater than those in the control samples. These bands are the nonspecific alkaline phosphatase without its carbohydrate component (see Fig. 6). There was, however, some glycosylated nonspecific alkaline phosphatase present that was synthesized in the repressed protoplasts before the addition of TM.

The rate of synthesis of active alkaline phosphatase in strain 1710 is not affected by TM (31); this is in contrast to active external invertase and acid phosphatase, the syntheses of which are completely inhibited (21). To determine whether the level of active pNPP-specific alkaline phosphatase during derepression is affected by TM, protoplasts of strain AL-20-3c were incubated in derepressive medium in the presence and absence of TM. The levels of pNPP-specific alkaline phosphatase did not change in the 3-h derepression period (results not shown). Therefore, the two- to threefold increase of alkaline phosphatase activity in strain 1710 protoplasts (31) in the presence of TM during derepression is due to an increase of the activity of only the nonspecific alkaline phosphatase. Furthermore, the pNPP-specific alkaline phosphatase in extracts of AL-20-3c TM-treated protoplasts showed no change in the electrophoretic mobility from that in extracts of control protoplasts (results not shown).

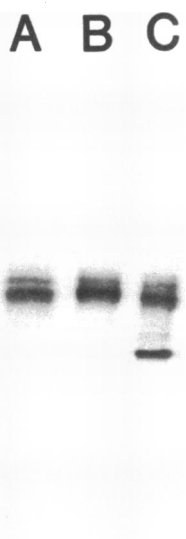

FIG. 4. Polyacrylamide gel electrophoresis of control and TM-treated protoplasts (strain 1710) derepressed for alkaline phosphatase. Protoplast cultures were incubated in the presence and absence of TM (10 μg/ml) for 3 h at 30°C. After the incubation, the protoplasts were harvested, washed, and lysed in 0.05 M Tris-hydrochloride buffer (pH 8.5) containing 0.001 M $MgCl_2$, 0.1 M NaCl, and 0.1% Triton X-100. The lysates were centrifuged at 30,000 × *g* for 30 min, and the supernatants (75 to 100 mU of alkaline phosphatase activity) were subjected to electrophoresis under denaturing conditions as described in the text. The gel was stained for activity by the simultaneous capture method with α-naphthylphosphate-fast red TR. Lanes: A, purified nonspecific alkaline phosphatase prepared by the method of Onishi et al. (31); B, control protoplast lysate; C, lysate of TM-treated protoplasts.

Effect of TM on the cellular location of alkaline phosphatase. Tests of glutaraldehyde sensitivity showed that the nonspecific alkaline phosphatase synthesized in the presence of TM was as resistant to 3.0% glutaraldehyde as the glycosylated nonspecific alkaline phosphatase (Fig. 2), consequently both forms should be detected by the cytochemical procedure. To determine whether the lack of carbohydrate affects the location of nonspecific alkaline phosphatase, repressed protoplasts were derepressed in the presence or absence of TM, and alkaline phosphatase cytochemistry was performed (Fig. 5). We did not notice any ultrastructural changes in protoplasts brought about by exposure to the antibiotic. The stain deposits in TM-treated protoplasts were seen, as in the control protoplasts, in the vacuole. This indicates that the nonspecific alkaline phosphatase devoid of carbohydrate is also found in the vacuole, and the absence of carbohydrate did not cause any change in the location of the enzyme.

To verify that nonspecific alkaline phosphatase is present in this organelle even after TM treatment, intact vacuoles were isolated from TM-treated protoplasts and control protoplasts by the method of Oshumi and Anraku (32), a procedure which gives microscopically homogeneous preparations with very little patent alkaline phosphatase. The vacuoles were lysed by osmotic shock and the entire lysates were subjected to nondenaturing polyacrylamide gel electrophoresis. The gel lanes were cut out and the alkaline phosphatase activity was detected with pNPP. The gel tracings (Fig. 6) showed that nonspecific alkaline phosphatase is present in vacuoles from both TM-treated and control protoplasts. With the vacuoles from TM-treated protoplasts, a new band was obtained that had an electrophoretic mobility greater than the nonspecific alkaline phosphatase. This band corresponds to the lowest band of activity from an extract of TM-treated protoplasts (Fig. 4, lane C). When the lysates of vacuoles from TM-treated protoplasts were subjected to concanavalin A affinity chromatography before nondenaturing polyacrylamide gel electrophoresis, the fastest moving band was still present, but the band corresponding to the glycosylated nonspe-

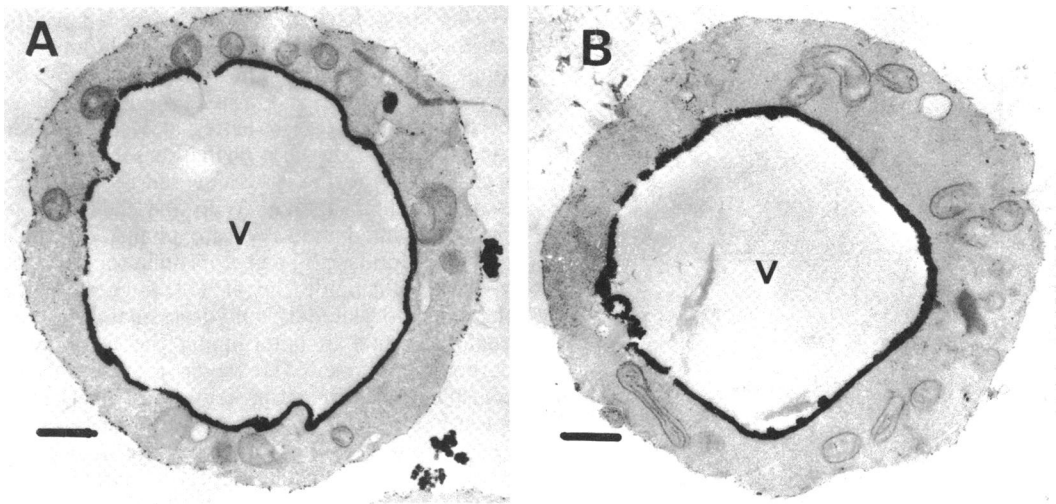

FIG. 5. Electron micrographs of control derepressed (A) and TM-treated derepressed (B) protoplasts of strain 1710 stained for alkaline phosphatase activity. Protoplast cultures were incubated in the presence and absence of TM (10 μg/ml) for 3 h at 30°C. Protoplasts were then collected by centrifugation, washed, and stained for alkaline phosphatase activity. V, Vacuole. Bar, 1.0 μm.

cific alkaline phosphatase was retained by the column (Fig. 6). This identifies the fastest moving band as the nonglycosylated nonspecific alkaline phosphatase and demonstrates that vacuoles from TM-treated cells contain both glycosylated and nonglycosylated nonspecific alkaline phosphatase. There was no band that corresponded to the pNPP-specific alkaline phosphatase, even after prolonged staining.

DISCUSSION

Saccharomyces sp. strain 1710 produces the pNPP-specific alkaline phosphatase, nonspecific alkaline phosphatase, and L-histidinol phosphate phosphatase. Onishi et al. (31) fractionated protoplasts and obtained a phosphatase fraction (peak III) that had activity on pNPP and L-histidinol phosphate, but was different from the nonspecific alkaline phosphatase in other biochemical criteria (e.g., ion requirements, pH optimum, and pH stability). Our detailed examination of the phosphatase enzymes produced by *Saccharomyces* sp. strain 1710 showed that the phosphatase activities of peak III (Fig. 1) were actually those of at least two separate enzymes, pNPP-specific alkaline phosphatase and L-histidinol phosphate phosphatase, which have similar chromatographic properties on a Bio-Gel A-0.5m column. We have also detected in strain 1710 the particulate nonspecific alkaline phosphatase characterized by Mitchell et al. (26), but we have no evidence, other than its particulate nature, that suggests that it is a different enzyme than the soluble nonspecific alkaline phosphatase purified by Onishi et al. (31). Kaneko et al. (19) have recently shown that the *PHO8* locus is the structural gene of the nonspecific alkaline phosphatase. Mitchell et al. (26) reported that the specific activity of the particulate nonspecific alkaline phosphatase is considerably lower in a *pho8* mutant than in wild type. Therefore, the particulate nonspecific alkaline phosphatase is probably a modified form of the soluble nonspecific alkaline phosphatase, rather than a different enzyme.

We have demonstrated that the nonspecific alkaline phosphatase is a vacuolar protein, confirming the observations of Bauer and Sigarlakie (5) that alkaline phosphatase is located in the vacuole of cells derepressed for alkaline phosphatase synthesis. Bauer and Sigarlakie (4), however, were unable to detect alkaline phosphatase when they stained ultrathin frozen sections of cells repressed for alkaline phosphatase. By using the cytochemical procedure that we developed, we were able to localize the nonspecific alkaline phosphatase in protoplasts repressed for alkaline phosphatase, but we were unable to obtain protoplasts that had vacuoles in which the interior was completely preserved. Usually, the inner side of the vacuolar membrane was fixed and the interior of the vacuole was devoid of biological material. Therefore we cannot conclude that the nonspecific alkaline phosphatase is restricted to or concentrated in the inner side of the vacuolar membrane. Although stain deposits were observed in vesicle structures that resemble endoplasmic reticulum, it is unlikely that they result from alkaline phos-

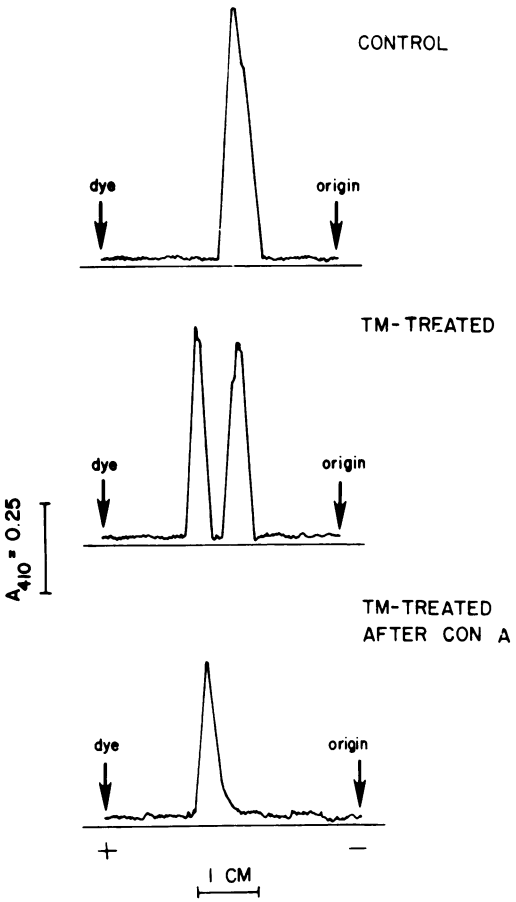

FIG. 6. Alkaline phosphatases of vacuoles from control and TM-treated derepressed strain 1710 protoplasts. Vacuoles were isolated as described in the text, lysed in 0.05 M Tris-hydrochloride buffer (pH 8.5) containing 0.001 M $MgCl_2$, and the lysates were subjected to centrifugation at 100,000 × *g* for 1 h at 4°C. The supernatants were either subjected to concanavalin A affinity chromatography and then polyacrylamide gel electrophoresis or were immediately subjected to polyacrylamide gel electrophoresis. After electrophoresis, the gels were stained for activity with pNPP and were scanned at 410 nm.

phatase activity since control protoplasts (lacking substrate during staining) also had deposits in vesicle structures. Furthermore, we were unable to find any evidence that the pNPP-specific alkaline phosphatase is located in the vacuole. This would explain the results obtained by Wiemken et al. (41), who demonstrated that only a part of the alkaline phosphatase is vacuolar. The alkaline phosphatase activity that they found in isolated vacuoles was presumably from the nonspecific alkaline phosphatase. The nonvacuolar alkaline phosphatase activity was probably the pNPP-specific alkaline phosphatase.

The yeast vacuole can be regarded as the functional equivalent to the mammalian lysosome (23). Several studies (29) have provided evidence that phosphorylated mannose residues of the carbohydrate moieties of lysosomal enzymes serve as recognition markers that mediate translocation to the lysosome and receptor-mediated cellular uptake. With the detection of phosphorylated carbohydrate chains on yeast carboxypeptidase Y, a glycoprotein found in the vacuole, Hashimoto, et al. (14) suggested that the phosphorylated carbohydrate chains have an analogous role in determining the location of carboxypeptidase Y. However, the presence of phosphate in a mannoprotein from yeasts does not necessitate a vacuolar location, since extracellular glycoproteins of yeasts are also phosphorylated (7). Furthermore, the presence of nonglycosylated, nonspecific alkaline phosphatase in vacuoles isolated from TM-treated protoplasts indicates that the asparagine-linked carbohydrate moiety on this enzyme does not determine the location of the nonspecific enzyme. Recently a similar conclusion was reached for carboxypeptidase Y by Schwaiger et al. (34). For most secretory proteins, the determinants of cellular location are not yet known. For nonspecific alkaline phosphatase, and probably for other vacuolar proteins of yeasts, the carbohydrate moiety does not contain the cellular "address." There is evidence (16, 18, 28) that carboxypeptidase Y is synthesized as a larger precursor molecule. The peptide portion that is cleaved from the precursor during formation of mature protein of yeasts may contain the information that directs the molecule to the vacuole.

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