Glycoprotein Nature of Yeast Alkaline Phosphatase

FORMATION OF ACTIVE ENZYME IN THE PRESENCE OF TUNICAMYCIN*

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The nonspecific alkaline phosphatase of yeast (Saccharomyces strain 1710) has been purified by ion exchange, hydrophobic, and affinity chromatography. This vacuolar enzyme has a molecular weight of 130,000 and is composed of subunits (probably of 66,000 molecular weight). It also has a small quantity of covalently associated carbohydrate; hydrolysis yielded mannose and glucosamine. The endo-β-N-acetylglucosaminidase of Streptomyces plicatus released carbohydrate indicating that the latter was attached to protein through an N-acetylgalcosaminylasparaginyl bond.

Synthesis of active alkaline phosphatase by yeast protoplasts is not depressed by tunicamycin, an inhibitor of dolichol-mediated protein glycosylation. Unlike the enzyme normally produced, the alkaline phosphatase which is formed in the presence of the antibiotic does not interact with concanavalin A and, therefore, is deficient in or lacking carbohydrate. We infer that there is no regulatory link in yeast between the glycosylation of a protein and its synthesis. The fact that other Asn-GlcNAc-type glycoprotein enzymes of yeast such as acid phosphatase are not produced in their active forms by tunicamycin-treated protoplasts may mean that, as unglycosylated proteins, they cannot be correctly folded or processed.

Protoxplants derepressed for phosphatase production contained substantial amounts of a second alkaline phosphatase which differed from the purified enzyme in substrate specificity, sensitivity to calcium, and reactivity with concanavalin A.

Yeast of the genus Saccharomyces produce a nonspecific alkaline phosphatase (EC 3.1.3.1) that has catalytic properties resembling those of alkaline phosphatases from a variety of other sources (1). Although the enzyme is produced when the cells are grown in the presence of inorganic phosphate, its synthesis increases severalfold when the phosphate becomes depleted (2). Most of the alkaline phosphatase activity in a yeast cell is located along the inner face of the vacuolar membrane (3–5).

During the course of investigations in our laboratory on glycoprotein biosynthesis in yeast, it was noted that alkaline phosphatase binds to concanavalin A-agarose.† Prompted by this suggestion that the protein is glycosylated, we have purified the enzyme and have found that there is carbohydrate linked to it through an N-acetylgalcosaminylasparaginyl bond.

Other yeast enzymes such as invertase (EC 3.2.1.26), acid phosphatase (EC 3.1.3.2), and carboxypeptidase Y (EC 3.4.12.8) are Asn-GlcNAc-type glycoproteins (6–8). Tunicamycin, an inhibitor of dolichol-mediated protein glycosylation (9, 10), depresses the production of active forms of these enzymes in protoplasts (11) and cells (12). However, the antibiotic does not alter the formation rate of active alkaline phosphatase. To gain insight into the cause of this differential effect of tunicamycin, we have characterized the alkaline phosphatase produced in the presence of tunicamycin

RESULTS

The nonspecific alkaline phosphatase of Saccharomyces strain 1710 was purified 6400-fold from derepressed cells by the procedure summarized in Table I. The final product appeared to be homogeneous by gel electrophoresis under non-denaturing conditions (Fig. 2); bands staining for protein, carbohydrate, and phosphatase activity had identical mobility. When the preparation was analyzed by SDS-polyacrylamide gel electrophoresis, three protein bands were evident (Fig. 3). The major component, Band B (Mr = 66,000), represented approximately 90% of the staining by Coomassie blue; Band A (Mr = 92,000) and Band C (Mr = 46,000) represented less than 1% and 10% of the staining, respectively.

In view of the seemingly nonstoichiometric relationship of the three peptides, we conclude that impurities in the alkaline phosphatase preparation are responsible for Bands A and C and that the native enzyme, which has a molecular weight of 130,000 (Fig. 4), is a dimer composed of subunits each having a molecular weight of 66,000 (Band B).

The pH stability, pH optimum, and substrate specificity of the purified phosphatase are documented in Fig. 5 and Tables II and III.

Carbohydrate Content—Two observations suggested that carbohydrate was associated with the alkaline phosphatase: (a) enzymatic activity could be removed from solution with conc A-agarose,1 and (b) periodate-reactive material migrated

† Portions of this paper (including “Experimental Procedures,” Figs. 1, 4, and 5, Tables I, II, and III, and some references) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full-size photocopies are available from the Journal of Biological Chemistry, 9560 Rockville Pike, Bethesda, Md. 20014. Request Document 79M-1465, cite author(s), and include a check or money order for $2.25 per set of photocopies.

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FIG. 2. Polyacrylamide gel electrophoresis of purified alkaline phosphatase. A sample of alkaline phosphatase (specific activity 2360 units/mg of protein) was subjected to electrophoresis under nondenaturing conditions as described under “Experimental Procedures,” in the miniprint. Gels to be stained (A) for protein with Coomassie Blue G250 or (B) for carbohydrate by the periodate-Schiff procedure were loaded with 25 μg of protein. The gel to be stained (C) for activity by the simultaneous capture method with α-naphthylphosphate/fast red TR was loaded with 100 milliunits of activity. Migration is from top to bottom.

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The sugars in the preparation were identified by gas-liquid chromatography (Table IV); mannose, glucose, and glucosamine were detected. Glucosamine and mannose are characteristically found in Asn-GlcNAc-type glycoproteins, but glucose is usually not a component of such glycoprotein molecules once their biosynthetic processing has been completed. Danishefsky et al. (44) found that the glucose present in purified antithrombin III was part of a tightly adhering contaminant, β-glucosyl ceramide, which could be removed by extraction with the phosphatase during electrophoresis under nondenaturing conditions (Fig. 2). Fig. 6 shows that two of the three proteins in the purified enzyme preparation could be adsorbed by con A. The preparation was incubated with con A-agarose, and the proteins remaining in solution following removal of the gel were identified by SDS-polyacrylamide gel electrophoresis. Only Band A was found (Fig. 6, lower). The adsorption of Bands B and C was largely prevented if 0.5% α-methylmannnoside was present during the incubation (Fig. 6, middle) indicating that their interaction with the lectin was not the result of a nonspecific hydrophobic adhesion. No adsorption was noted when unsubstituted agarose was employed in place of con A-agarose (see Fig. 3 and Fig. 6, upper).

The adsorption of protein species in the alkaline phosphatase preparation to con A-agarose. Adsorption was tested as described under “Experimental Procedures,” in the miniprint. The test mixtures were centrifuged and the supernatant fluids were analyzed by SDS-polyacrylamide gel electrophoresis. The gel scans represent the following: upper, preparation incubated with agarose; middle, preparation incubated with con A-agarose in the presence of 0.5% α-methylmannnoside; lower, preparation incubated with con A-agarose.
Carbohydrate composition of alkaline phosphatase and the effect of extraction with chloroform/methanol

Table IV

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GlcN</th>
<th>Man</th>
<th>Glc</th>
<th>Man/Glc</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>98</td>
<td>232</td>
<td>206</td>
<td>1:1</td>
</tr>
<tr>
<td>CHCl/CH₂OH</td>
<td>Protein residue</td>
<td>N.D.*</td>
<td>268</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>Organic extract</td>
<td>N.D.</td>
<td>0</td>
<td>49</td>
</tr>
</tbody>
</table>

* N.D., not determined.

with chloroform/methanol (2:1, v/v). We applied their extraction procedure to the alkaline phosphatase preparation with the result that the glucose content of the protein was greatly diminished while the level of mannose remained unchanged (Table IV). Unfortunately, the recovery of glucose in the organic solvent was lower than expected, and the reason for this is not known. Nevertheless, this finding together with the fact that a second unextracted preparation of alkaline phosphatase with a similar proportion of Band B and Band C contained a different ratio of glucose to mannose (data not presented) suggests that glucose is not covalently bound to protein in the purified preparation. We did not attempt to identify the glucose-containing contaminant.

Linkage between Carbohydrate and Protein—In many glycoproteins that contain mannose and N-acetylglucosamine, 2 residues of the latter sugar (linked to form a di-N-acetylchitobiosyl moiety) serve as a bridge between an asparaginyl residue in the protein and an oligosaccharide composed of mannosyl residues. Certain endo-β-N-acetylglucosaminidases release oligosaccharides from such glycoproteins by hydrolyzing the (1 → 4) linkage within the di-N-acetylchitobiosyl portion of the structure. We have employed endo-β-N-acetylglucosaminidase H of Streptomyces plicatus to determine whether this carbohydrate to protein linkage is present in yeast alkaline phosphatase.

The purified alkaline phosphatase was denatured with SDS in the presence of 2-mercaptoethanol to insure that the action of the endoglycosidase would not be sterically impeded (34). Following incubation with endo H at pH 5.0, the proteins in the mixture were examined by SDS-polyacrylamide gel electrophoresis. Each band's migration (expressed as apparent molecular weight) was compared to that of the corresponding band in a control mixture that had been incubated without the endoglycosidase (Table V). No change was observed in the case of Band A; this result was expected in view of the protein's lack of reactivity with con A-agarose (Fig. 6). The molecular weight of Band B decreased by approximately 8% and Band C showed a loss of about 15%. These decreases indicate that both of the proteins which were adsorbed by con A-agarose (Fig. 6) are Asn-GlcNAc-type glycoproteins.

To confirm that carbohydrate was being removed from alkaline phosphatase, a second experiment was performed with endo H under conditions which would preserve the enzymatic activity of the phosphatase. In this way, the affinity of the phosphatase for con A could be assessed after treatment with the endoglycosidase. The enzymes were incubated together at pH 7.0 and 37°C for 18 h in the absence of SDS and 2-mercaptoethanol. The recovery of phosphatase activity was 87% in this sample and 89% in a sample incubated under the same conditions without endo H. To precipitate the glycosylated species, con A was added along with various amounts of yeast mannan as carrier. Assay of the mixtures before removal of the resulting precipitates revealed that con A did not inhibit phosphatase activity. The per cent of phosphatase remaining soluble after sedimentation of the precipitates is shown as a function of mannan concentration in Fig. 7. The results, interpreted as antigen-antibody precipitation reactions, indicate that the population of endo H-treated enzyme molecules contained less carbohydrate than the phosphatase molecules in the untreated control. Removal of all susceptible carbohydrate units was not anticipated in this experiment because the conditions employed were not optimal for endo H activity.

In summary, the results in Fig. 7, taken together with those of Fig. 6 and Tables IV and V, support the conclusion that the alkaline phosphatase is an Asn-GlcNAc-type glycoprotein.

Nature of the Alkaline Phosphatase Formed in the Presence of Tunicamycin—The effect of tunicamycin on the formation of active alkaline and acid phosphatases was examined with protoplasts prepared from Saccharomyces strain 1710 (Fig. 8). The appearance of alkaline phosphatase activity was not disturbed by the antibiotic whereas the formation of acid phosphatase activity was completely suppressed. Similar results obtained with a related yeast, Saccharomyces strain 1016, have been reported previously (11).

To determine whether tunicamycin interfered with the glycosylation of alkaline phosphatase in this experiment, we

Table V

<table>
<thead>
<tr>
<th>Protein band</th>
<th>10⁻³ X molecular weight</th>
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<tr>
<td>Control</td>
<td>Endo H</td>
</tr>
<tr>
<td>A</td>
<td>92</td>
</tr>
<tr>
<td>B</td>
<td>66</td>
</tr>
<tr>
<td>C</td>
<td>46</td>
</tr>
</tbody>
</table>

Fig. 7. Affinity of endo H-digested alkaline phosphatase for con A. Alkaline phosphatase (6.1 µg) was incubated with or without 3.8 µg of endo H under nondenaturing conditions as described under "Experimental Procedures," in the miniprint. Samples of the incubated preparations were mixed with 50 µg of con A and various amounts of yeast mannan as carrier. After 2 h, the samples were assayed for total alkaline phosphatase activity, then centrifuged and the supernatant fluid assayed for soluble activity. ○, control, not treated with endo H; ●, endo H-digested enzymes.
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Fig. 8. The effect of tunicamycin on the appearance of acid phosphatase and alkaline phosphatase activities in cultures of protoplasts from *Saccharomyces* strain 1710. Protoplasts suspended in osmotically stabilized minimal medium with phosphate omitted were incubated with gentle agitation at 30°C either (A) without tunicamycin or (B) in the presence of the antibiotic at 10 μg/ml. Samples of the culture were periodically withdrawn, diluted to lyse the protoplasts, and assayed for total alkaline phosphatase activity (○) or acid phosphatase activity (●).

Fig. 9. Forms of alkaline phosphatase in control and tunicamycin-treated protoplasts. The protoplast cultures from the experiment shown in Fig. 8 were centrifuged after 3 h at 30°C, and the cells were lysed in 0.05 M Tris-HCl buffer, pH 8.5, containing 0.001 M MgCl₂, 0.1 M NaCl, and 0.1% Triton X-100. The final pH was 7.5. To separate and partially purify the enzyme forms, samples from the control lysate (1.4 units of alkaline phosphatase) or from the tunicamycin (TM)-treated preparation (1.3 units) were subjected to gel filtration on a column of Bio-Gel A-0.5m (2 x 95 cm). The column was eluted at 10 ml/h with the lysis buffer at pH 7.5, and 2.1-ml fractions were collected.

Protoplasts. Peak II had the same relative elution volume (V₆/V₀) as the purified alkaline phosphatase. It contained 49% of the alkaline phosphatase activity in the control protoplasts and 37% in the protoplasts incubated with the antibiotic. Peak I accounted for 9% of the total activity in each preparation. For reasons which will be mentioned in the next section, we feel that the enzymes in Peaks I and II are related, Peak II possibly arising by aggregation as described by Tonino and Steyn-Parve (4). Peak III represented 42% of the activity in the control protoplasts and 55% in the treated sample. As shown below, the enzymatic properties of this alkaline phosphatase species are markedly different from those of the Peak II enzyme. When the ability of con A to precipitate the active material in each of the peaks was determined, the results shown in Table VI were obtained. At least 95% of the activity in Peaks I and II from control protoplasts was precipitated; in contrast, only 50 to 60% of the activity in the corresponding peaks from the tunicamycin-treated culture was precipitable. In neither case was the activity in Peak III removed from solution by con A.

The amount of alkaline phosphatase produced by the protoplasts during incubation with tunicamycin equaled the amount present at the outset of the experiment (Fig. 8). Hence, 90% of the total activity at the time of harvest was synthesized before tunicamycin was present. This estimate should also apply to the enzyme in each peak because the relative proportion of phosphatase activities in the peaks did not change drastically in response to the antibiotic. Therefore, from the change in precipitability by con A, seen in the case of Peak I and Peak II (Table VI), we conclude that almost all of the Peak I and II enzyme which was synthesized in the presence of tunicamycin was devoid of carbohydrate.

Characteristics of the Peak III Enzyme—The alkaline phosphatase which we have purified extensively was extracted from cells grown to late-log phase in a complex medium. With these cells there was no evidence for the presence of additional alkaline phosphatase species (see Miniprint Supplement). On the other hand, protoplasts which were prepared from early to mid-log phase cells grown on chemically defined medium contained three alkaline phosphatase species (Fig. 9). Preliminary experiments which will not be presented in detail have yielded the following information concerning the relationship of these species with one another and with the purified enzyme.

When protoplast lysates were subjected to gel permeation chromatography on Bio-Gel A-0.5m, satisfactory recovery of alkaline phosphatase activity could be attained only if 0.1% Triton X-100 and 0.1 M NaCl were added to the elution buffer. Without these supplements only Peak II could be eluted from the column. Inclusion of the detergent resulted in the appearance of Peak III enzyme. When the ability of con A to precipitate the active material in each of the peaks was determined, the results shown in Table VI were obtained. At least 95% of the activity in Peaks I and II from control protoplasts was precipitated; in contrast, only 50 to 60% of the activity in the corresponding peaks from the tunicamycin-treated culture was precipitable. In neither case was the activity in Peak III removed from solution by con A.

The alkaline phosphatase activity released upon lysis of the protoplasts was fractionated on Bio-Gel A-0.5m into three peaks (see Fig. 9). The contribution of nonglycosylated species of alkaline phosphatase to the total activity in each peak was determined by testing the precipitability of active enzyme by con A at various concentrations of yeast mannan (added as carrier). The values represent the minimum percentage of each enzyme fraction remaining soluble after exposure to the lectin.

TABLE VI

<table>
<thead>
<tr>
<th>Protoplasts</th>
<th>Activity remaining soluble</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Peak I</td>
</tr>
<tr>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>Tunicamycin-treated</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>46</td>
</tr>
</tbody>
</table>
ance of Peak I. Peak III was observed only when both detergent and salt were present. In view of these requirements, it is likely that the separation achieved was not based solely upon size. As noted in the preceding section, the purified alkaline phosphatase eluted in the position of Peak II.

Unlike the purified alkaline phosphatase (Fig 7) and the enzymes in Peaks I and II, the alkaline phosphatase in Peak III (Table VI) does not bind to con A. Inasmuch as all glycosylated proteins from Saccharomyces species thus far examined are mannoproteins, this finding implies that this species of alkaline phosphatase is not a glycoprotein.

On the basis of three enzymatic parameters, the species in Peaks I and II differ markedly from the enzyme in Peak III but are indistinguishable from one another. First, the rate of p-nitrophenyl phosphate hydrolysis by the Peak I or II enzyme increases steadily above pH 7.5 to a maximum between 8.5 and 9.0, whereas the rate of hydrolysis by the Peak III enzyme is maximal between pH 8 and 9, but falls sharply and II are identical and contrast sharply with the substrate profile for Peak III. For example, the enzyme in Peak I or II hydrolyzes l-histidiol phosphate at 10% of the rate for p-nitrophenyl phosphate; the Peak III enzyme has a relative rate of 190% on l-histidiol phosphate. Third, the activity in Peak III is severely inhibited by CaCl\textsubscript{2}; the hydrolysis of p-nitrophenyl phosphate is decreased by 50% in the presence of 50 \( \mu \text{M} \) CaCl\textsubscript{2}. In 10 times that concentration of CaCl\textsubscript{2}, the enzymatic activity in Peak I or II is still 90% of the control level. Purified alkaline phosphatase had enzymatic properties which most closely resembled those of Peaks I and II, especially with respect to the lack of inhibition by Ca\textsuperscript{2+}.

**DISCUSSION**

Yeast of the genus Saccharomyces synthesize a number of glycosylated enzymes. Some of the enzymes are components of the vacuole (e.g. carboxypeptidase Y); others, such as invertase and acid phosphatase, are exported across the plasma membrane and are retained at the cell surface by the cell wall. The oligosaccharides associated with these enzymes are composed of mannose and N-acetylglucosamine, with residues of the latter forming di-N-acetylchitobiosyl units that serve to attach the oligomannosides to asparaginyl residues in the proteins. Generally, the oligosaccharides of the vacuole enzymes are less numerous and somewhat smaller than those of the wall glycoenzymes. For example, carboxypeptidase Y has four carbohydrate moieties per peptide chain, \( M \approx 51,000 \) (8), whereas invertase has 9 per peptide chain, \( M \approx 60,000 \) (6, 45, 46). The average mannose content of oligosaccharides from carboxypeptidase Y is about 13 residues (8); in invertase, there are two populations of oligosaccharides, one with an average mannoseyl content of 26 residues and the other averaging 54 residues (45). Structurally, the carbohydrate moieties of the vacuolar enzymes from yeast are analogous to those of mammalian Asn-GlcNAc glycoproteins of the "high-mannose" type. Those of the wall enzymes, on the other hand, may be viewed as "complex" units with high mannose cores (47) and peripheral chains which differ from the peripheral units of mammalian glycoproteins by being larger, branched, and composed of mannose. This view is supported by genetic data which indicate that the mannoseyltransferases responsible for the synthesis of the outer regions of the oligosaccharides in wall glycoproteins are distinct from those involved in the assembly of the core (48). Also, there are indications from cell fractionation experiments that the two sets of transferases are located in different subcellular compartments (49).

In 1975, Bauer and Sigalakie reported that the vacuole is the primary location of alkaline phosphatase activity within a yeast cell (5). This conclusion was based upon cytochemical results obtained through electron microscopy. Although reports on the nonspecific alkaline phosphatase of yeast describing its enzymatic properties as well as its genetic regulation had appeared (2-4, 38, 42, 50-52), little was known about the physical properties of the enzyme. Using chromatographic procedures developed for the isolation of mammalian alkaline phosphatases, we have purified the yeast enzyme from Saccharomyces strain 1710. The initial preparation was nearly homogeneous and provided evidence that the phosphatase, like other vacuolar enzymes in yeast, is a glycoprotein. The purified phosphatase could be stained by the periodate-Schiff procedure after polyacrylamide gel electrophoresis (Fig. 2) and was bound by concanavalin A (Fig. 1). Analysis of one preparation by gas-liquid chromatography showed the presence of mannose and glucosamine; some glucose was also found, but most of it was removed by extraction with chloroform/methanol. Analysis of the sugars in a second batch of the phosphatase gave the same qualitative results but a lower ratio of glucosamine to protein. Because of this, reliable estimates of the oligosaccharide units per peptide chain and the average oligosaccharide size could not be made. Incubation with endo-\( \beta \)-N-acetylgalactosaminidase \( H \) decreased the apparent molecular weight of the phosphatase and reduced its reactivity with con A. Sensitivity to the endoglucosaminidase suggests that protein to carbohydrate linkages of the Asn-GlcNAc type are present in the phosphatase. Moreover, the magnitude of the molecular weight change serves as an indication of the approximate content of asparagine-linked carbohydrate (54). Band B (Fig. 3), which probably represents alkaline phosphatase, was reduced by 8% in molecular weight.

The glycosylation of asparaginyl residues in vivo can be prevented with tunicamycin. This antibiotic specifically blocks the synthesis of dolichyl N-acetylglucosaminyl pyrophosphate, a key intermediate in the formation of core oligosaccharides for Asn-GlcNAc-type glycoproteins (9, 10, 53). In animal cells, inhibition of glycosylation with the antibiotic seems to have no effect upon the synthesis of the protein portion of a glycoprotein. This was first demonstrated with virus-infected cells (54, 55) and has since been documented in several other systems (56-61). Consequently, it is reasonable to anticipate that yeast, upon exposure to tunicamycin, will synthesize the carbohydrate-free forms of their glycoenzymes. We have found this to be the case for alkaline phosphatase. The rate at which the phosphatase activity appears in protoplasts is not altered by the antibiotic, and the enzyme molecules that are produced in the presence of the drug do not interact with concanavalin A indicating that they are not glycosylated. However, the formation of active acid phosphatase or of active invertase (11) is completely suppressed by tunicamycin. In the case of invertase, if other aspects of its biosynthesis had occurred normally, one would have expected the carbohydrate-free molecule to be active because activity is retained when carbohydrate is removed with endo H (49). Yet another pattern of response is shown by carboxypeptidase Y: tunicamycin depresses the appearance of the active enzyme severely but not completely (12). The active molecule that is made in antibiotic-treated cells is thought to be unglycosylated because its molecular weight equals that of the protein portion of the enzyme normally produced (8). Conceivably, immunologically cross-reacting but enzymatically inactive invertase and carboxypeptidase Y may accumulate as the result of tunicamycin action. Alternatively, these enzymes, as unglycosylated proteins, may be more susceptible to proteolytic destruction within the cell. These possibilities have been explored both in our laboratory and by others (8), but no

4 T. Mizunaga and J. S. Tkacz, unpublished data.
blocked provide indirect evidence in support of this view. The on modifications in conformation resulting from the absence found that the migration of viral envelope proteins from the peptide will be influenced substantially by the presence of the peptide is still a nascent chain extending from the ribosome has been made. However, the types of dysfunctions which are glycosyl groups. No direct experimental test of this hypothesis has been made. However, the types of dysfunctions which are associated with protein moieties whose glycosylation had been blocked provide indirect evidence in support of this view. The gross consequences vary but all conceivably could be based on modifications in conformation resulting from the absence of the usual carbohydrate chains. Thus, Leavitt et al. (63) found that the migration of viral envelope proteins from the site of synthesis in the rough endoplasmic reticulum of the host cell to the plasma membrane is impaired when the proteins are not glycosylated. Improper folding is thought to be the basis for this because the unglycosylated proteins are much less soluble and have a higher tendency to aggregate than their normal counterparts (63, 64). A similar explanation was offered as one of the possible reasons for the apparent accumulation of immunoglobulin molecules in the endoplasmic reticulum of tunicamycin-treated plasma cells (65). The unglycosylated fibronectin synthesized by tunicamycin-treated chick embryo fibroblast cells and the hemagglutinin of fowl plague virus produced by tunicamycin-treated host cells (64, 61) were shown to be unusually susceptible to proteolytic digestion. In contrast, the nonglycosylated polyprotein precursor of the envelope proteins in Rauscher murine leukemia virus formed in the presence of tunicamycin did not undergo processing (66). Such alterations in sensitivity to protease activity are also possible manifestations of modifications in protein conformation. The importance of oligosaccharides in guiding protein folding is probably modulated by other structural features of the glycoprotein and by environmental factors (64). However, the number of glycosylated asparaginyl residues in the structure of the glycoprotein may be an appropriate parameter by which to predict the importance of carbohydrate in the acquisition of proper conformation. Hickman and Kornfeld (59) have examined the effect of tunicamycin on the secretion of various immunoglobulins by mouse plasmacytoma cells and have noted that the extent of inhibition is correlated with the usual carbohydrate content of the immunoglobulin.

The effects of inhibiting glycosylation on the formation of the several yeast glycoprotein enzymes can be readily interpreted in terms of the proposed relationship between glycosylation and protein folding. For invertase and acid phosphatase, which are heavily glycosylated proteins, the consequences of tunicamycin action are severe. Carboxypeptidase Y has less carbohydrate than invertase but slightly more than alkaline phosphatase. The peptidase apparently represents an intermediate situation. Possibly, blocking glycosylation reduces the thermodynamic advantage which normally favors folding into an active conformation. Without this usual advantage, folding into a functional conformation may occur less frequently. Alkaline phosphatase, with a content of asparagine-linked carbohydrate probably amounting to less than 10% of the weight of the molecule, attains full activity when glycosylation is prevented. In this case, the conformation of the carbohydrate-free enzyme is probably also different from that of the glycosylated version but these differences could be reflected in effects more subtle than those which would have been detected by the methods we have employed. The moderate nature of the effects which might be expected is illustrated by the finding that carboxypeptidase Y from Anheuser-Busch bakers’ yeast, glycosylated to an extent of 15 to 20%, reacts with a larger apparent bimolecular rate constant ($k_{cat}$/$K_m$) does than the same enzyme containing 9% carbohydrate from Pleishmann bakers’ yeast (67). This line of reasoning based on the effect of carbohydrate on protein folding suggests some new areas for exploration. For example, if inability of the unglycosylated invertase protein to attain or maintain an active conformation is the explanation for the influence of tunicamycin upon the appearance of invertase activity in yeast protoplasts, then the protein must accumulate in a form so unlike the native enzyme that it has escaped solubilization and detection with antibodies. We are investigating this possibility. We are also investigating the influence of glycosylation on the subcellular location of alkaline phosphatase.

With respect to enzymatic properties such as substrate specificity, pH optimum, pH stability, and ion requirements, the alkaline phosphatase which we have purified from Saccharomyces strain 1710 does not differ from yeast alkaline phosphatases studied previously (2, 4, 38, 50). Our analytical data regarding the glycoprotein nature of the enzyme do not rule out the possibility that serine- or threonine-linked oligomannosides like those found in yeast cell wall polymers are present in addition to the asparagine-linked carbohydrate moieties. However, this possibility seems unlikely because tunicamycin which interferes only with the glycosylation of asparaginyl residues elicits the production of alkaline phosphatase molecules that do not interact with concanavalin A. Also, glycosylated seryl or threonyl residues have never been convincingly demonstrated in any of the highly purified yeast glycoproteins, and to date, there is no evidence that asparagine-linked sugar is present in the same wall polymers that bear serine-linked or threonine-linked oligosaccharides (48).

The alkaline phosphatase activity extracted from protoplasts included a previously undescribed enzyme which has high activity on histidinol phosphate but is still less specific than the histidinol phosphatase of yeast (38, 68). It also differs from our purified alkaline phosphatase in that it is readily inactivated by Ca$^{2+}$ salts and does not appear to contain carbohydrate. It can account for nearly half of the alkaline phosphatase activity in protoplasts, but was not detected in extracts prepared from derepressed cells (late exponential phase, phosphate limited). Furthermore, the yields during purification of the glycoprotein enzyme are sufficiently good to make it unlikely that a substantial amount of another phosphatase was lost at any single stage in the procedure. A study of the properties of this novel phosphatase and the conditions governing its formation is under way.

Acknowledgment—We thank James Flor of Merck and Co. for supplying tunicamycin.

REFERENCES

Tunicamycin and Yeast Alkaline Phosphatase Biosynthesis

37. Millay, R. H., Jr., and Houston, L. L. (1973) Biochemistry 12, 2591-2592

Additional references, 13 to 43, appear on p. 11952.
Glycoprotein Nature of Yeast Alkaline Phosphatase: Formation of Active Enzyme in the Presence of Tunicamycin

H. Russell Coster, Ian S. Thace, and J. Oliver Lennam

SUPPLEMENTAL MATERIAL

Tunicamycin and Yeast Alkaline Phosphatase Biosynthesis

Alkaline Phosphatase -- Enzyme activity was assayed with p-nitrophenylphosphate (Sigma Chemical Co., St. Louis, MO). The assay mixture was composed of 0.8 ml of 1 M Tris-HCl buffer, pH 8.8, containing 0.001 M MgCl2, 0.1 M of 0.04 M pNPP in water, and 0.1 ml of enzyme sample. The reaction was initiated by the addition of substrate and terminated by the addition of 0.5 ml of 1 M NaOH. The extent of hydrolysis was determined from the absorbance of the liberated p-nitrophenol at 410 nm using an extinction coefficient of 1.77 x 104 M-1 cm-1.

Purification of Alkaline Phosphatase. All steps in the purification were carried out at 4°C. Fractions collected in the several 280 run or 230 nm. Protein concentrations of pooled fractions were determined by the Lowry assay with bovine serum albumin (Sigma Chemical Co., St. Louis, MO) as the standard.

Step 1. Preparation of Crude Extract -- A 3.5 kg portion of yeast wet weight was washed once in 0.1 M Tris-HCl buffer, pH 8.0, containing 0.001 M MgCl2, suspended in 0.5 lit. of 0.05 M NaCl, and stored for 18 h at 4°C. The suspension was then poured into ice-cold water and centrifuged at 15,000 g for 20 min. The supernatant was adjusted to pH 4.8 (w/v) and stored at 4°C. Sodium dodecyl sulfate (SDS) gels were prepared according to Laemmli (23). Proteins were stained with Coomassie brilliant blue R250 (24) and in SDS gels with Coomassie brilliant blue R250 (24). Gel electrophoresis was performed by the method of Hirs (25). For each gel, 200 µg of active enzyme, 2 µg of the molecular weight standards, and 2 µg of the molecular weight standards were run on a 15% polyacrylamide gel (25). The gel was stained with 0.5% Coomassie blue R250 (24) and 0.1% Coomassie brilliant blue R250 (24). Gel electrophoresis was performed by the method of Laemmli (23). 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Identification of sugars by gas-liquid chromatography. — Purified alkaline phosphatase preparations from Tunicamycin and Yeast alkaline phosphatase biosynthesis on column chromatography were extensively dialyzed against 0.1 M ammonium bicarbonate. To remove this salt, the preparations were repeatedly lyophilized. Conditions for hydrolysis, preparation of aldehyde derivatives and their analysis by gas-liquid chromatography were essentially those of Banks et al. (90) for neutral sugars and of Niedermeier and Tomana (31) for amino sugars. Both procedures included the modifications described by Hasonian et al. (32). Before hydrolysis, internal standards were added: arabinose in the case of neutral sugars and mannose for amino sugars. A column of Bio-Gel P-2 on Glass Chrom Q (Regis Chemical Co., Chicago, Ill.) was employed to separate neutral sugar derivatives; the derived amino sugars were separated with 3% Poly A 100 on Glass Chrom Q (Applied Science Laboratories, State College, Penna.). Analysis was performed with a Perkin-Elmer model 950 (Perkin-Elmer Corp., Norwalk, Conn.) or Hewlett-Packard model 400 (Hewlett-Packard, Palo Alto, Calif.), liquid chromatographs. Both instruments were equipped with hydrogen flame detectors. Derivatives were identified by their retention times relative to the internal standards, peak areas, quantitated by planimetry, were used to calculate the amount of each sugar; corrections were made for handling losses, destruction by hydrolysis, and differences in detector response.

Identification of Phosphoproteins — Protein bands with binding to alkaline phosphatase activity were isolated by the method of Niedermeier and Tomana (31). The endo H- and N-acetylglucosaminidase I (from Streptomyces griseus) was purified and assayed as previously described (31). The protein was free of 3-mannoside and protease activities. Samples of alkaline phosphatase to be treated with endo H were prepared according to Trimboli and Maloney (34). The preparations were denatured by boiling for 3 min in the presence of 0.1 M 2-mercaptoethanol and 0.2% SDS. The denatured material was incubated with endo H for 18 h at 37°C in 0.05 M Tris-HCl buffer, pH 6.0, containing 0.001 M MgCl2. Release of the carbohydrate was assayed by measuring the activity of the treated enzymes for con A.

RESULTS
Purification of Alkaline Phosphatases — A procedure for the purification of an alkaline phosphatase from yeast was developed. As shown in Table 1, a 500-fold purification was achieved with a 70% yield. The crude extract obtained in the first step contained between 90 and 95% of the activity present in totalized late log-phase cells (35) indicating that little, if any, of the enzyme in these Saccharomyces cells was present as the insoluble form described by Dottori and Berco-Pecco (4). In each chromatographic step (Fig. 1), the enzymatic activity eluted as a single peak with a recovery of 70-95%. Consequently, it is unlikely that any of the alkaline phosphatase activity in the cells was due to a second phosphatase, for example, the substrate-specific p-nitrophenylphosphatase found by Attias and Bonner (36).

Two of the chromatographic procedures we used were originally devised for the isolation of mammalian alkaline phosphatases. L-Phenylalanine-agarose was synthesized in the hope that it would be an affinity matrix for human placental alkaline phosphatase (37). It was separation achieved with this gel probably occurs chiefly through hydrophobic interaction. At the p{TAGE} of yeast alkaline phosphatase, the matrix consistently gave a 5-10-fold purification. 4-Amino-5-hydroxymethyl-2-furfuraldehyde-acid-soluble fractions have been used for affinity chromatography of calf intestinal alkaline phosphatase (38). Unfortunately, we found that 0.2% ions were required for the adsorption of Saccharomyces alkaline phosphatase; we were not able to determine the basis for this requirement. It has been reported that Zn2+ inhibits yeast alkaline phosphatase slightly (39), but, in our hands, neither inhibition nor stimulation was observed. The product we obtained using this gel was not homogenous, and improved purification could not be attained through the use of inorganic phosphate in the eluate because severe tailing occurred. Despite the suboptimal performance of the matrix which may have been caused by the availability of the ligand (39), the chromatographic step was retained as part of the procedure because it yielded a 5-fold increase in specific activity.

Properties of the Purified Enzyme
Molecular Weight — Gel filtration according to Andrews (39) suggested that the molecular weight of native alkaline phosphatase was 150,000 (Fig. 1). The result agrees favorably with the Mr values of 125,000 and 150,000 which were obtained by Attias and Bonner (36) and by Galli (77, Ph. D. thesis, Rutgers University, respectively).

Effect of pH on Stability — Yeast alkaline phosphatase, like other alkaline phosphatases (40), is stable at pH values between 7.5 and 10.0 (Fig. 5). Inactivation of the yeast enzyme at acidic pH is irreversible.

Substrate Specificity, pH Optimum, and Trypsin Phosphorylation — Alkaline phosphatase is capable of hydrolysing a wide variety of monophosphate esters (Table II). The pH for maximum activity varies with the substrate (Table II) and its concentration, the optimum becoming more alkaline as the concentration of the substrate is increased (Table III). For a series of substrates, the pH optima are inversely related to the pH values of the phosphates groups in the substrates. Such variations of optimum pH for activity are a general characteristic of alkaline phosphatases (36).

In addition to studies which are not presented in detail, we have found that Mg2+ enhances both the activity and stability of this alkaline phosphatase, most of the monophosphorylated-chitin with Tris as the acceptor. The effect of Mg2+ on enzymatic activity has been demonstrated for alkaline phosphatases from other strains of Saccharomyces, as well as for several substrate-specific phosphatases from yeast (44, 45, 46, 47) (Fig. 3). Trypsin phosphorylation in primary acceptor is a common property of alkaline phosphatases (37).

FIG. 4. Determination of the molecular weight of alkaline phosphatase by gel permeation chromatography. The apparent molecular weight of native alkaline phosphatase (50% units/mg protein) was measured by gel filtration through Sepharose 4B (conditions described in “Experimental Procedures”). The standard proteins (all from Sigma Chemical Co., St. Louis, Mo.) are: A, bovine erythrocyte carbonic anhydrase (150,000,00); B, horse liver catalase (51,000); C, bovine serum albumin (68,004); D, E, E. coli alkaline phosphatase (40,000); F, beef muscle lactate dehydrogenase (140,604); G, yeast alcohol dehydrogenase (150,000); H, relative elution volumes of standards; I, relative elution volume of yeast alkaline phosphatase.
### TABLE I

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Activity (cpm/mg protein)</th>
<th>Protein (mg)</th>
<th>Specific Activity (cpm/mg)</th>
<th>Recovery (%)</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Crude extract</td>
<td>5170</td>
<td>8.6</td>
<td>233,000</td>
<td>0.37</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>2. Streptomycin precipitation and ammonium sulfate fractionation (40 to 75% saturated)</td>
<td>1390</td>
<td>8.2</td>
<td>86,000</td>
<td>0.95</td>
<td>95</td>
<td>2.6</td>
</tr>
<tr>
<td>3. Batch adsorption to DEAE-cellulose</td>
<td>1220</td>
<td>5.7</td>
<td>3,400</td>
<td>16.8</td>
<td>44</td>
<td>45</td>
</tr>
<tr>
<td>4. Hydrophobic chromatography: L-phenylalanine-agarose</td>
<td>1720</td>
<td>4.4</td>
<td>315</td>
<td>141</td>
<td>51</td>
<td>380</td>
</tr>
<tr>
<td>5. Affinity chromatography: 4-(aminophenyl)phosphoanilinic acid-agarose</td>
<td>58</td>
<td>3.6</td>
<td>51</td>
<td>690</td>
<td>41</td>
<td>1860</td>
</tr>
<tr>
<td>6. Ion exchange chromatography: DEAE-cellulose</td>
<td>74</td>
<td>3.4</td>
<td>25</td>
<td>1560</td>
<td>40</td>
<td>3800</td>
</tr>
</tbody>
</table>

### TABLE II

Relative activity of alkaline phosphatase on various substrates and at various pH values.

The rate of hydrolysis was determined in 0.04 M sodium barbital buffer at the pH listed. Reaction mixtures contained 4.0 mM substrate and 1.0 mM MgCl₂, and were incubated at 30°C. Hydrolysis was measured by the inorganic phosphate formed.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative hydrolysis rate</th>
<th>pH 5.0</th>
<th>pH 6.5</th>
<th>pH 9.0</th>
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<tbody>
<tr>
<td>α-Nitrophenylphosphate</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>α-Naphthylphosphate</td>
<td>1.0</td>
<td>1.0</td>
<td>0.7</td>
<td>0.7</td>
</tr>
<tr>
<td>α-Glycerophosphate</td>
<td>1.2</td>
<td>0.4</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>β-Glycerophosphate</td>
<td>1.2</td>
<td>0.4</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Phosphate-phosphate</td>
<td>1.8</td>
<td>0.3</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Adenosine-5′-phosphate</td>
<td>1.2</td>
<td>0.5</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Adenosine-5′-triphosphate (ATP)</td>
<td>1.0</td>
<td>0.6</td>
<td>0.1</td>
<td>0.1</td>
</tr>
</tbody>
</table>

### TABLE III

The effect of substrate concentration on the pH for maximal activity of alkaline phosphatase.

Enzyme activity was measured as in Table II. The substrates are listed in order of increasing pH.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>pH Optimum</th>
<th>1 mmol</th>
<th>2 mmol</th>
<th>4 mmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Nitrophenylphosphate</td>
<td>8.75</td>
<td>8.90</td>
<td>&gt; 9.00</td>
<td></td>
</tr>
<tr>
<td>α-Naphthylphosphate</td>
<td>0.10</td>
<td>&gt; 8.70</td>
<td>&gt; 8.70</td>
<td></td>
</tr>
<tr>
<td>α-Glycerophosphate</td>
<td>7.65</td>
<td>8.05</td>
<td>8.15</td>
<td></td>
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<tr>
<td>Glucuron-6-phosphate</td>
<td>&gt; 7.50</td>
<td>7.81</td>
<td>8.20</td>
<td></td>
</tr>
<tr>
<td>β-Glycerophosphate</td>
<td>8.00</td>
<td>8.30</td>
<td>8.50</td>
<td></td>
</tr>
</tbody>
</table>

### REFERENCES

Glycoprotein nature of yeast alkaline phosphatase. Formation of active enzyme in the presence of tunicamycin.
H R Onishi, J S Tkacz and J O Lampen