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Inhibition of the Apparent Rate of Synthesis of the Vacuolar Glycoprotein Carboxypeptidase Y and Its Protein Antigen by Tunicamycin in *Saccharomyces cerevisiae*

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Carboxypeptidase Y from *Saccharomyces cerevisiae* contains 14% mannose, the only neutral sugar present. An antiserum can be raised in rabbits which reacts with both the protein and the sugar moieties of the enzyme. This antiserum also precipitates yeast invertase and yeast cell wall mannan. Thus carboxypeptidase Y, which is known to be localized in yeast vacuoles, is very probably a mannoprotein. Tunicamycin inhibits the apparent formation of carboxypeptidase Y to a similar extent as that of the externally localized mannoprotein, invertase. No accumulation of an inactive nonglycosylated or partly glycosylated carboxypeptidase Y occurs as determined by the immunoprecipitation technique. Tunicamycin also inhibits the apparent formation of proteinase A, whereas it does not affect the increase in the activities of a number of other enzymes. It is suggested that in the synthesis of glycoproteins there exists a regulatory link between the synthesis of their polypeptide chains and the reactions involved in their glycosylation.

Carbohydrate moieties of glycoproteins are synthesized from sugar nucleotides. The reactions involved occur in tight association with membranes and proceed in part via lipid intermediates (4, 13, 21). This has been shown in a variety of organisms, including yeasts (20, 34). Transfer of *N*-acetylglucosamine to lipid is sensitive to tunicamycin (6, 38, 39), an antibiotic from *Streptomyces lysosuperificus* (37). In yeast protoplasts, tunicamycin has been shown to inhibit mannan synthesis and secretion of invertase and of acid phosphatase (18).

Carboxypeptidase Y is located in the vacuole (10, 22). It had been first purified and characterized as a glycoprotein by Hata and co-workers (11). Its carbohydrate moiety has been shown to constitute about 15% of the enzyme by weight and to be composed largely of a neutral sugar, with a smaller amount of *N*-acetylglucosamine (1, 12, 17). It has attracted the interest of several laboratories due to its low specificity and other unique catalytic properties (3, 14, 23) and has also become the object of several studies concerned with the physiology of yeast proteinases (15).

In the present paper, the effect of tunicamycin on the formation of carboxypeptidase Y has been investigated to see whether the formation of intracellular glycoproteins is also affected by this antibiotic.

The immunoprecipitation technique was used to differentiate between possible effects of the antibiotic on the changes in the activity of the glycoprotein and on its total concentration. Since this enzyme proved to have a complex antigenic activity, the precipitation characteristics of the antisera raised against it and used throughout this study are also described in some detail.

MATERIALS AND METHODS

Organism, cultivation, and harvesting. The diploid yeast *Saccharomyces cerevisiae* X2180, isolated by R. K. Mortimer (25), was used throughout this study. The culture was maintained on YPD agar slants consisting of 2% D-glucose, 2% peptone (Difco Laboratories, Detroit, Mich.), 1% yeast extract (Merck, Darmstadt), and 2% agar-agar (Merck, Darmstadt); all values represent weight per volume. Primary cultures were grown up to the stationary phase on YPD/2 medium containing 2% D-glucose, 1% peptone, and 0.5% yeast extract at 30°C and were stored in the same way as the slants at 4°C. The cultivations were performed in conic flasks, filled 10 to 20% with the medium, on a reciprocal shaker at 80 strokes/min. Main cultures were prepared by inoculating YPD/2 medium with 0.1 to 0.5% (by volume) of the primary cultures. Tunicamycin used in the inhibition studies was kindly provided by G. Tamura of the University of Tokyo.

Growth was followed by determining either the wet weight of harvested yeast or the optical density at 578 nm in 1-cm cuvettes in an Eppendorf photometer model 1101M (Eppendorf, Hamburg). For this

latter purpose, the cultures were diluted to optical density values not exceeding 0.2.

The harvested cells were washed once with cold 50 mM potassium phosphate buffer, pH 7.0. Wet weight of the yeast was determined directly by differential weighing.

Homogenization. The yeast pellet was mixed with the cold potassium phosphate buffer, 2.5 ml/g (wet weight), and the suspension was homogenized by being passed twice through a 3/8-inch (ca. 1.0-cm) French pressure cell (Aminco, Silver Spring, Md.) at 10,000 lb/in^2. The homogenate was centrifuged for 10 min at 12,000 × *g*. The supernate obtained was referred to as crude extract.

Protein concentration in the crude extracts was determined according to Lowry et al. (24) in a total volume of 1.2 ml, using bovine serum albumin (Behringwerke, Marburg) as standard. The maximum yield of protein in the crude extracts per gram (wet weight) of harvested yeast was 60 mg.

Enzyme assays. Enzyme activities were determined by spectrophotometric methods, using a Gilford spectrophotometer model 240 (Gilford Instruments, Oberlin, Ohio). All the enzyme reactions were performed with crude extracts at 25°C unless stated otherwise. The activities of alcohol dehydrogenase and aconitase were determined according to previously described methods (5, 28). The method of Halvorson and Elias was used to determine α-glucosidase activity (9), except that the assay was performed at pH 7.0. Malate dehydrogenase was determined with 67 mM L-malate in reaction conditions given by Williamson and Corkey (41). The activities of proteinases were determined in activated crude extracts (31). For this purpose, the crude extracts were dialyzed against a 200-fold volume of distilled water in Visking dialysis tubes for 3 h at room temperature with one change of water. The dialyzed samples were buffered to pH 5.0 with 1 M sodium acetate buffer (final concentration, 40 mM), preserved with a mixture of antibiotics (10) and sodium azide (0.2 mg/ml), and incubated at 25°C. At intervals, proteinases were assayed and the maximum activities, usually found after 36 to 72 h, were determined. The activities of proteinases A and B were determined with acid-denatured hemoglobin and Azocoll, respectively. The methods and definition of activity units have been presented in detail elsewhere (30, 32). Carboxypeptidase Y was assayed according to Matern et al. (26), with *N*-acetyl-L-tyrosine ethyl ester as substrate. Triton X-100 (0.27%, reagent grade; Serva, Heidelberg) was also present. It lowered turbidity due to crude enzyme samples and also increased the esterase activity. The activity of purified carboxypeptidase Y was increased by 20% in the presence of Triton X-100. Since with the crude extracts the relationship between the reaction rate and the sample volume was not linear, activity was determined with sample volumes giving an absorbance change (ΔA) of 0.02 to 0.03/min at 340 nm. The esterase activity was calculated as micromoles of *N*-acetyl-L-tyrosine ethyl ester hydrolyzed per minute at 25°C. The contribution of proteinase B, about 10% of the total, was subtracted from the total esterase activity. It was calculated from the Azocoll hydrolyzing activity by using a ratio of 0.8 esterase unit per Azocoll unit (units/ΔA_{520}), found previously for purified proteinase B with a specific activity of 18 ΔA_{520}/min per mg (A. Hasilik, Ph.D. thesis, University of Freiburg, Freiburg, Germany, 1974).

To obtain activities of the above enzymes per milliliter of culture, the corresponding specific activities were multiplied by the maximum yield of the crude extract protein mentioned above and by the concentration of the cells in culture (grams [wet weight] per milliliter).

Invertase activity was determined with either 0.1 ml of cell suspension (this was referred to as the external activity) or with 5 μl of homogenate (uncentrifuged) to obtain the total activity. In the former case, the culture samples were centrifuged, the supernates were discarded, and the pellets were washed with 0.5 ml of 50 mM sodium acetate buffer, pH 5.0. The activity itself was measured according to Smith and Ballou (35), but the volumes were scaled down to 70% of the original ones. In one of two parallel samples, the reaction was stopped immediately by adding the reagent 3,5-dinitrosalicylate; in the other, it was stopped after a 15-min incubation with substrate at 37°C. The samples were then incubated for 5 min at 95°C and centrifuged. The ΔA_{520} values, as found between the supernate of the incubated sample and that of the control, were used to calculate the activity, which was expressed as micromoles of sucrose hydrolyzed per minute at 37°C. Both the external and total activities were calculated per milliliter of culture. In the latter case, the activity per milliliter of homogenate was multiplied by a factor of 3.42 ml/g, representing the volume of the homogenate obtained from 1 g of cells and by the wet weight (in grams) of cells harvested per 1 ml of culture.

Preparation of antisera. The antigen carboxypeptidase Y was purified to a specific activity of 120 U/mg. The purification procedure, which was analogous to that originally developed by Hata et al. (1, 11), consisted of acetone precipitation of an activated extract from baker's yeast, chromatography on hydroxyapatite and diethylaminoethyl Sephadex A-50, and gel filtration on Bio-Gel P-150. The enzyme solution containing 10 mM imidazole chloride buffer, pH 7.0, was stored at −30°C either frozen or in a solution made 50% in glycerol. The final preparation was homogeneous by the criteria of polyacrylamide disc gel electrophoresis and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The content and identity of neutral hexoses present in carboxypeptidase Y were determined by gas chromatography, using xylose as the inner standard, by Inge Fromme, Max Planck Institute of Immunobiology, Freiburg. The total content of neutral hexoses was also determined by the anthrone method (36), using mannose as standard.

For immunization, the antigen was diluted with 0.9% sodium chloride to a concentration of 1.85 mg of protein per ml and emulsified with complete Freund adjuvant (Behringwerke, Marburg). Two brown male rabbits were immunized on their backs with 220 μg of protein each; about two-thirds of each dose

was injected intracutaneously; the rest was injected subcutaneously. One rabbit received the antigen that was stored in 50% glycerol (the final concentration of glycerol in the emulsion was 5.5%), and the other one received the antigen that was stored in the absence of glycerol. Each rabbit received an additional 200, 200, and 360 μg of protein in weekly injections of emulsions prepared as above, except that complete Freund adjuvant was replaced by incomplete adjuvant. About 40 ml of blood was collected from an ear vein on the 6th, 8th, and 10th days after the last injection. A booster injection of 0.5 mg of protein was repeated 3 weeks after the last injection of the first series, and blood was collected as before. The serum obtained from the rabbit immunized with the antigen containing glycerol was termed antiserum I. There was little variation in its titer for the purified carboxypeptidase Y among the portions collected at various times (48 μg/ml of serum, on the average), and its specificity did not vary. The same was true for antiserum II, obtained from the rabbit that was immunized with the antigen without glycerol. Here the average titer was 120 μg/ml. As shown in Results, the two antisera differed in their specificity very markedly. This is why the details of the immunization procedure are presented, although we do not know whether there is any causal relationship between the mode of immunization and the specificity of the antiserum obtained. No immunoprecipitation was observed with control sera collected before immunization.

Immunoprecipitation. Antigen solution adjusted to pH 7.0 to 7.5 was mixed with 50 mM tris(hydroxymethyl)aminomethane-chloride buffer, pH 7.5, containing 2 M KCl and 2% Triton X-100 and with antiserum at a volume ratio of 1:2:1 in capped plastic vessels and was kept for 14 to 20 h at 4°C. The vessels were then centrifuged for 4 min at 12,000 × *g*. The sediments were washed three times as described by Roberts and Roberts (29). The amount of protein in the immunoprecipitate was measured by the method of Lowry et al. (24), scaled down to a total volume of 1.2 ml.

To characterize the antisera, 50-μl aliquots were tested with various antigens. A crude extract was prepared from commercial baker's yeast as described above. Yeast cell wall mannan (fraction A), isolated according to Sentandreu and Northcote (33), was kindly provided by L. Lehle. The lyophilized preparation was extracted with 0.1 M potassium phosphate buffer, pH 7.0, and the 12,000 × *g* supernatant fraction was used. Protein measurement performed as mentioned above was used to determine the concentration of mannan in the extract. Yeast invertase, the grade "standardized, analytical grade," was purchased from Serva, Heidelberg.

To determine the concentration of carboxypeptidase Y in crude extracts, the precipitation was performed with an enriched gamma globulin fraction from antiserum I. It was obtained by precipitation of the antiserum with ammonium sulfate (2 M, pH 7.0) and gel filtration of the dialyzed precipitate on a Sephadex G-200 column equilibrated with 0.5 M glycine-0.2 M tris(hydroxymethyl)aminomethane-chloride (pH 7.6)-0.01% sodium azide. The pooled active fractions were concentrated on a PM30 ultrafilter (Amicon, Lexington, Mass.) to a titer of 0.1 mg of carboxypeptidase Y (as protein) per ml. The enriched gamma globulin fraction was used in an at least twofold excess over the antigen. A calibration curve was used to quantitate the enzyme in the immunoprecipitate.

Concentration of soluble mannan in the crude extracts was determined by immunoprecipitation with 0.2 ml of antiserum II. The amount of protein in the immunoprecipitate was determined as above and calculated per milliliter of culture in the same way as enzyme activity.

RESULTS

Antigenic properties of carboxypeptidase Y. Two rabbits were immunized with purified carboxypeptidase Y. The two antisera obtained, I and II, showed typical precipitation curves with one maximum each when used to precipitate the purified carboxypeptidase Y (Fig. 1). Such a curve was also observed with the crude extract when the extract was precipitated with the antiserum I. An additional peak of precipitation was seen, however, when antiserum II was used to precipitate the crude extract (Fig. 2). Whereas the main peak of precipitation corresponded well to the amount of carboxypeptidase Y in the crude extract (about 40 μg/ml) as inferred from the esterase activity, the additional peak occurred at much lower amounts of the crude extract.

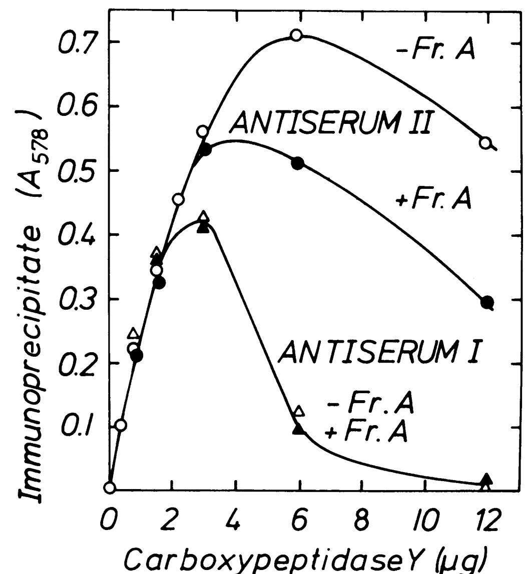

FIG. 1. *Immunoprecipitation of purified carboxypeptidase Y by rabbit antisera. The tests were performed with 50 μl of antiserum I in the absence (△) or presence (▲) of mannan fraction A (3.4 μg as protein), or with 50 μl of antiserum II in the absence (○) or presence of 3.4 μg of mannan fraction A (●).*

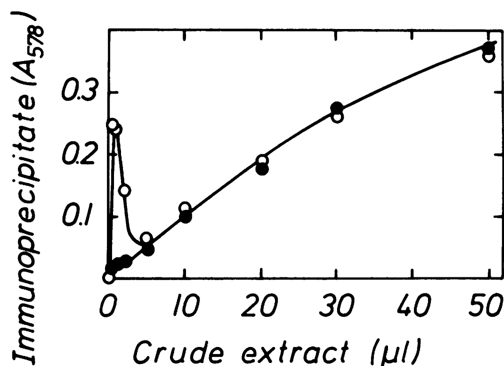

FIG. 2. *Immunoprecipitation of the crude yeast extract with rabbit antiserum prepared against carboxypeptidase Y. The tests were performed with 50 μl of antiserum II in the absence (○) or presence of 3.4 μg of mannan fraction A (●).*

Carboxypeptidase Y has been shown to contain about 3% *N*-acetylglucosamine and 14% neutral sugar (1, 11, 12, 17). Our preparation of the enzyme contained a similar amount of neutral sugar. Essentially all of it was identified as mannose by gas chromatography. A rather limited fraction of it appeared to be β-eliminable (Table 1).

TABLE 1. *Neutral sugar content of carboxypeptidase Y*[a]

Prepn	Sugar content	
	Anthrone[b]	Gas chromatography
Untreated	14.0[c]	13.5[d]
β-eliminated:		
Non-dialyzable		11.8[d]
Dialyzable	1.2	

[a] For β-elimination, the sample was treated with 0.1 N NaOH for 24 h at 25°C.

[b] Method used.

[c] Results expressed as percentage of mannose equivalents.

[d] Results expressed as percentage of mannose. Besides mannose, about 0.1% of a pentose, possibly ribose, and traces of glucose and a heptose were found. The amounts corresponded to much less than one residue of any of these sugars per molecule of carboxypeptidase Y.

Thus, carboxypeptidase Y seems to be a mannoprotein. The possibility has been considered, therefore, that antiserum II reacts with both the protein and the carbohydrate moieties of carboxypeptidase Y. In this case, it would be expected that the precipitation peak at low volumes of the crude extract obtained with antiserum II should be abolished by an excess of yeast mannan and that the titer of this antiserum for purified carboxypeptidase Y should be lowered in the presence of yeast mannan. In addition, one would predict that any other mannan- or mannoprotein-containing carbohydrate antigenic groups similar to those of carboxypeptidase Y should be precipitated by antiserum II. Indeed, the data shown in Fig. 1 and 2 demonstrate that yeast cell wall mannan, fraction A prepared according to Sentandreu and Northcote (33), suppressed the double specificity of antiserum II. Fraction A lowered the titer of this antiserum for the purified carboxypeptidase Y by about 30%, whereas it had no significant effect on the titer of antiserum I. The latter antiserum precipitated only carboxypeptidase Y, whereas antiserum II also precipitated fraction A cell wall mannan and invertase. Table 2 presents quantitative data on the maximum formation of precipitate and on the titers of the two antisera for different antigens. It can also be seen that excess of carboxypeptidase Y prevented the precipitation of either fraction A mannan or invertase by antiserum II. No invertase was precipitated by this antiserum when fraction A mannan was present in excessive amounts as expected from the antigenic similarity of their sugar components, which has been observed by Smith and Ballou (35).

Antiserum II could be rendered specific for the protein moiety of carboxypeptidase Y by precipitating its antimannan components, for example, by proper amounts of the crude extract. Antiserum II pretreated in this way had a correspondingly lower titer for the purified carboxypeptidase Y (Table 2) and showed a single-peak precipitation curve with the crude extract (not shown) that mimicked the one obtained with the untreated antiserum II in the presence of fraction A mannan (Fig. 2).

Effect of tunicamycin on the apparent rate of synthesis of carboxypeptidase Y. It has been reported that the activities of yeast proteinases including carboxypeptidase Y are higher in cells growing on minimum medium as compared with a complex one and that in both cases an increase takes place in these activities as the culture approaches the stationary phase (31). The effect of tunicamycin has been studied, therefore, in cultures that undergo transition from fermentative to oxidative metabolism. Under these conditions, a number of enzymes known or believed to be subject to catabolite repression are actively synthesized. The activities of several of these were followed to see whether the effect of tunicamycin is selective.

The limited growth of the cultures studied was little affected by tunicamycin at a concen-

TABLE 2. *Immunoprecipitation of carboxypeptidase Y, yeast cell wall mannan fraction A, and invertase by two different antisera, I and II, raised against carboxypeptidase Y*

Antigen	Addition	Antiserum I		Antiserum II	
		Titer[a]	A_{578} max[b]	Titer[a]	A_{578} max[b]
Carboxypeptidase Y	None	3	0.42	6	0.71
	None			3[c]	0.46[c]
	3.4 μg of mannan	3	0.40	4	0.55
Mannan fraction A	None	≤0.01	≤0.01	0.2	0.24
	44 μg of carboxypeptidase Y			≤0.02	≤0.02
	3.8 μg of invertase			≤0.02	≤0.02
Invertase	None	≤0.02	≤0.01	0.25	0.13
	None			≤0.04[c]	≤0.02[c]
	44 μg of carboxypeptidase Y			≤0.04	≤0.02
	3.4 μg of mannan			≤0.04	≤0.02

[a] Expressed as micrograms of antigen at the equivalence point in the precipitation test with 50 μl of antiserum.

[b] Maximum absorbance at 578 nm in the assay of the immunoprecipitated protein.

[c] Precipitation was performed with supernatant fractions of the antisera precipitated with a crude extract from commercial baker's yeast (44 mg of protein per ml of crude extract, 25 μl of crude extract per ml of antiserum).

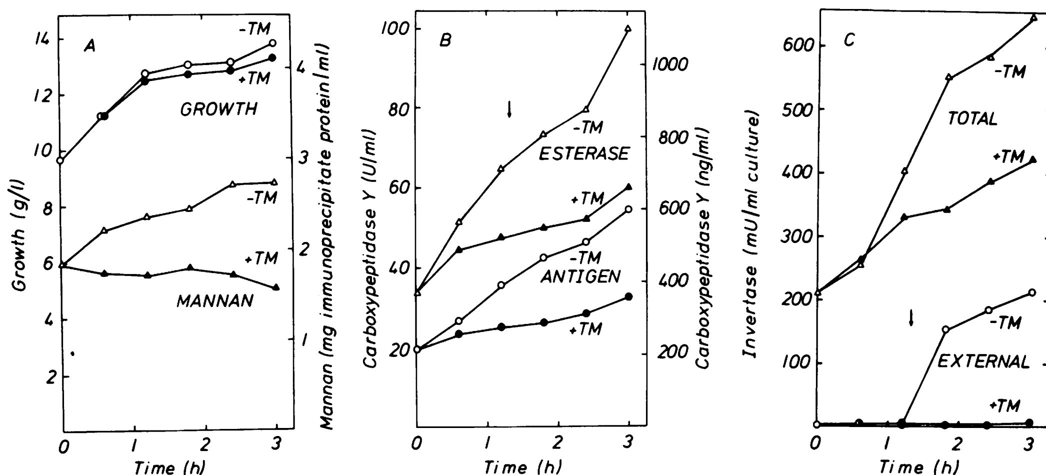

FIG. 3. *Effect of tunicamycin on the apparent synthesis of mannan and on that of several enzymes in growing yeast. Tunicamycin (6 μg/ml) was added to one aliquot of the culture at zero time. The arrow in the figure indicates the time of glucose exhaustion. The open symbols represent controls. The closed symbols show the data obtained with the culture aliquot treated with the antibiotic. (A) Growth (○, ●), and relative concentration of soluble mannan (△, ▲); (B) esterase activity of carboxypeptidase Y (△, ▲) and concentration of carboxypeptidase Y as determined by immunoprecipitation with a gamma globulin preparation specific for the protein moiety of the enzyme (○, ●); (C) external invertase (○, ●) and total invertase (△, ▲).*

tration of 6 μg/ml (Fig. 3A). The formation of mannan, immunoprecipitable from the crude extract by the double-specific antiserum II, was inhibited, however, by more than 90%. Tunicamycin also inhibited the apparent formation of carboxypeptidase Y (Fig. 3B). The inhibition of the increase in esterase activity of the enzyme was closely paralleled by that of the increase in the level of the proteinaceous antigens of this glycoprotein. It appears that there is no accumulation of carboxypeptidase Y molecules lacking the esterase activity and yet reacting with the antiserum specific for the protein moiety of this glycoprotein. Tunicamycin further caused a partial inhibition of the increase in total invertase activity, as determined with the crude cell homogenate, and a nearly complete inhibition of the development of external invertase

activity, as determined with whole cells (Fig. 3C).

In Fig. 4, cumulative increases in the activities of various enzymes expressed as percentages of the controls are compared with the cumulative increase in the wet weight of the cells. The apparent synthesis of proteinase A was inhibited to the same extent as that of carboxypeptidase Y. Like carboxypeptidase Y, proteinase A is a glycoprotein (11) and is located in the vacuole (10, 22). Interestingly, the effect of tunicamycin could be noticed already during the activation of the proteinases in the dialyzed crude extracts. The extracts were rendered turbid by adjusting their pH to 5.0 and became more or less clear during subsequent incubation at 25°C. This clearing was much less extensive in the samples from early growth stages than in those from the later stages, just as expected from their proteinase contents. It also proceeded much more slowly in the samples from tunicamycin-treated cells as compared with the corresponding controls.

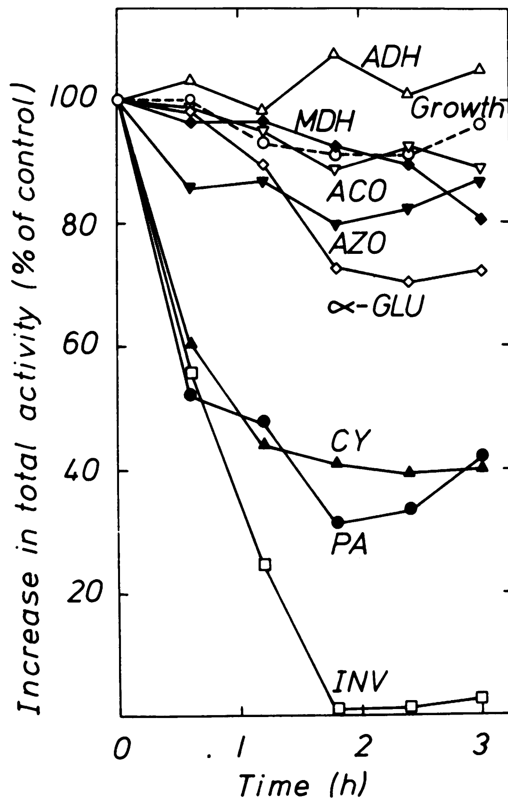

FIG. 4. *Comparison of the effect of tunicamycin on the activities of several enzymes and on growth. Cumulative increases (the differences between the activities at the times indicated on the abscissa and those at time zero) in the total activities in the inhibited culture shown are expressed as percentage of control. The data presented have been obtained in the same experiment as shown in Fig. 3. Symbols: Growth (○); alcohol dehydrogenase (△); malate dehydrogenase (◆); aconitase (▽); α-glucosidase (◇); carboxypeptidase Y esterase activity (▲); proteinase A (●); external invertase (□); proteinase B (▼).*

In contrast to carboxypeptidase Y and proteinase A, the change in the activity of proteinase B, another vacuolar hydrolase (10, 22), was not affected by tunicamycin. The appearance of the maximum proteinase B activity during activation of crude extracts, however, was delayed. This is in agreement with the mechanism proposed for activation of proteinase B in the crude extracts according to which proteinase A inactivates the inhibitor of proteinase B (32). Evidence exists that proteinase B is also a glycoprotein, but its sugar moiety seems to be built differently from that of carboxypeptidase Y (A. Hasilik, unpublished data).

There are a number of enzymes, however, the activities of which developed more or less unaffected by tunicamycin: malate dehydrogenase, aconitase, α-glucosidase, and alcohol dehydrogenase (Fig. 4). The small apparent increase in the rate of synthesis of alcohol dehydrogenase due to tunicamycin was observed in all experiments carried out.

In the presence of tunicamycin, the apparent rate of synthesis of the external invertase was considerably more inhibited than that of carboxypeptidase Y (Fig. 3). The following facts, however, have to be pointed out. In the control, the activity of carboxypeptidase Y increased steadily in the course of the experiment, whereas the formation of the external invertase was initiated only after glucose was used up. Then its activity rose very rapidly, reaching a maximum of about 15 U per g of cells in about 1 h. In the culture treated with tunicamycin the appearance of this activity was delayed for several hours, although glucose was consumed by essentially the same time as in the control. Later on, the activity started to increase slowly, reaching nearly 13 U per g of cells 8 h after the addition of the antibiotic (not shown). By varying the time of the addition of the antibiotic, the degree of the inhibition of the synthesis of external invertase was greatly changed, however. The inhibition was even less than 50% when tunicamycin was added 10 min after the initiation of the synthesis. It could also be shown that the difference in the degree of inhibition of the two enzymes is fairly small under certain conditions (Table 3). In this case, cells growing exponentially on medium YPD/2 were transferred into a similar but rather diluted medium. Within 15 min after the shift,

TABLE 3. *Effect of tunicamycin on the apparent rate of formation of the external invertase, of the esterase activity, and of the protein antigen of carboxypeptidase Y*[a]

Tunicamycin concn (μg/ml)	Rate of formation				Growth (g/liter)	
	Carboxypeptidase Y		Invertase (mU/h per ml)	Malate dehydrogenase (mU/h per ml)	Initial	After 2.5 h
	Esterase (mU/h per ml)	Protein antigen (ng/h per ml)				
0	14	150	55	90	9.3	12.3
6	8.4	84	13.6	90	9.3	12.2
	(60)	(56)	(75)	(0)		(3)

[a] The cells were grown in 2 liters of YPD/2 medium up to an optical density of 2.2, spun down, and resuspended in 0.5 liter of medium containing, per liter: 0.25 g of yeast extract, 0.5 g of peptone, 2.5 g of D-glucose, and 2 g of maltose monohydrate. Thirty minutes later, tunicamycin was added to one part of this culture. The cultures were sampled immediately before the addition of the inhibitor and then every 30 min for estimation of the external invertase activity and for preparation of crude extracts. All the activities shown changed in a nearly linear fashion over 2.5 h studied, and therefore average rates are presented. The numbers in parentheses indicate percentage of inhibition.

glucose was used up and little additional growth occurred. By this time the cells started to synthesize external invertase. Its activity increased rather slowly as compared with the experiment shown in Fig. 3. The increase was nearly linear and paralleled that in the activity of carboxypeptidase Y and malate dehydrogenase. One-half hour after the shift, tunicamycin was added to an aliquot of the culture, i.e., to cells that were actively synthesizing both carboxypeptidase Y and external invertase. Under these conditions, the synthesis of external invertase was inhibited by 75% and that of carboxypeptidase Y was inhibited by 60%.

DISCUSSION

Both the composition and the structure of the sugar moiety of the vacuolar enzyme carboxypeptidase Y resemble those of the components of the yeast cell wall. Like invertase and cell wall mannan, carboxypeptidase Y contains glucosamine and mannose, most of which is not β-eliminable. Based on a molecular weight of 61,000 to 62,000, about eight residues of glucosamine per molecule have been found (1, 17). The non-β-eliminable mannose content of 11.8% corresponds to 40 mol of mannose per mol of enzyme. It can be suggested that one molecule of carboxypeptidase Y contains four asparagine-linked oligosaccharide chains of an average composition $(N\text{-acetylglucosamine})_2$-$(\text{mannose})_{10}$. This formula resembles that of the inner core of the yeast cell wall mannan as proposed by Nakajima and Ballou (27). The analogy is also indicated by the antigenic similarity of the carbohydrate moieties of wall mannan and carboxypeptidase Y.

Sugars bound to polyprenylphosphates of the dolichol type have been shown to be intermediates in the glycosylation of glycoproteins in a number of eukaryotic cells (4, 13, 21). This is true for the formation of *N*-glycosidically (4, 16, 20, 21, 40) and *O*-glycosidically (2, 34) linked carbohydrate moieties. It is still a matter of debate, however, which class of glycoproteins is formed via polyprenols, i.e., whether these are constituents of membranes or are secretory proteins, or both. Evidence for the second possibility has come from the observation that unglycosylated protein moieties of defined glycoproteins could serve as artificial acceptors of sugars from dolichylphosphate sugars. This has been observed for the biosynthesis of immunoglobulin light chains (7) and for the yeast cell wall mannan (L. Lehle, P. Babczinski, and W. Tanner, in preparation).

Experiments by Kuo and Lampen on the inhibition by tunicamycin of the formation of external glycoproteins in yeast protoplasts (18) are pertinent to the same problem. Since it has been shown recently that in various systems the transfer of *N*-acetylglucosamine from uridine-5′-diphosphate-*N*-acetylglucosamine to the polyprenylphosphate acceptors is inhibited by tunicamycin (6, 38, 39), it is very probable that in yeast, *N*-acetylglucosamine linked to dolichylphosphate acts as an intermediate in the synthesis of external glycoproteins. Similarly, the results shown in the present paper indicate that this polyprenol derivative is involved in the synthesis of vacuolar glycoproteins as well.

It is possible that there are more similarities in the genesis of external and of vacuolar glycoproteins. In both cases it is required that a membrane be traversed by a macromolecule. Most likely this transport of macromolecules takes place at rough endoplasmic reticulum membranes and is closely associated with, and perhaps driven by, the processes participating

in the synthesis of these macromolecules (19). It has also been suggested that the syntheses of secretory and of lysosomal glycoproteins in animal cells share a number of common features (8).

Kuo and Lampen have observed that although the secretion of invertase by yeast protoplasts is stopped in the presence of tunicamycin, the activity of internal invertase does not accumulate (18). Accumulation of an inactive enzyme within the cells, however, has not been excluded in their experiments. The observation that in the case of carboxypeptidase Y, not only the increase in activity but also the apparent formation of the protein antigen is inhibited by the antibiotic (Fig. 3B and Table 3) strongly suggests that the synthesis of the protein moiety of this glycoprotein is retarded unless the glycosylation can proceed normally. It is not excluded, however, that the synthesis of the protein moiety continues unaffectedly, even if the glycosylation reactions are inhibited, and that the incompletely glycosylated glycoprotein thus formed is rapidly degraded.

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