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Role of glycosylation in secretion of yeast acid phosphatase

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The minimal glycosylation requirement for acid phosphatase secretion and activity was investigated using tunicamycin, an inhibitor of protein glycosylation, and a yeast mutant defective in the synthesis of oligosaccharide outer chains. The results obtained show that outer chain addition is not essential for secretion of active enzyme and that only 4 core chains, out of 8 normally attached to a protein subunit, are sufficient for enzyme transport to the periplasmic space. Enzyme forms with less than 4 chains were retained in membranes of endoplasmic reticulum. Secreted underglycosylated enzyme forms are partially or completely inactive.

Glycosylation; Protein secretion; Acid phosphatase; (*Saccharomyces cerevisiae*)

1. INTRODUCTION

Acid phosphatase (APase) from the yeast *Saccharomyces cerevisiae* is an extracellular glycoenzyme, which is secreted in the periplasmic space. The enzyme contains 16 *N*-glycosidically linked oligomannose-type carbohydrate chains per dimeric molecule [1]. Out of the 8 chains per enzyme subunit, 5 represent core chains with 14 mannose units and the remaining 3 chains are elongated by the addition of outer chains having on average about 48, 92 and 150 mannose units, respectively [2].

Most, if not all of the proteins assigned for secretion are glycosylated and the role of the carbohydrate moiety in protein transport and secretion has been studied using mainly tunicamycin (TM), an antibiotic which prevents asparagine-linked glycosylation [3]. The results showed that carbohydrates are not required for the secretion of most glycoproteins, but in some cases inhibition of protein glycosylation does impair export of the molecule [4]. In yeasts, it was shown that treatment of cells with TM prevents secretion of active invertase and APase [3]. In contrast, carboxypeptidase Y [5] and alkaline phosphatase [6] do not require glycosylation for their transport to the vacuoles.

In this paper the study of the role of glycosylation in secretion and activity of yeast APase was performed using TM and the *mnn9* mutant defective in the synthesis of carbohydrate outer chains [7], with the aim of determining the minimal glycosylation requirement for enzyme secretion and activity.

2. EXPERIMENTAL

S. cerevisiae strain H42 (a, *gal1*) was obtained from Dr M.E. Schweingruber and *mnn9* mutant and its parent strain, X2180, were obtained from Dr C.E. Ballou. Growth conditions and media were as described [1].

Exponential phase cells were converted to protoplasts using cytohelicase (LKB) [8]. The reaction was completed in 50–70 min, protoplasts were

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Abbreviations: APase, acid phosphatase; TM, tunicamycin; ER, endoplasmic reticulum

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harvested by centrifugation at 2000 × *g* and supernatants containing secreted APase were collected. Protoplasts were washed three times with the protoplasting buffer and lysed in 10 mM Na-acetate buffer, pH 3.8. The membrane pellet was separated from the soluble intracellular fraction by centrifugation at 25 000 × *g*, and membrane proteins were solubilized by heating at 95°C for 20 min in 1% SDS and 3% β-mercaptoethanol.

Preparation of crude cell extracts and subsequent purification of APase were performed as in [1] with the exception that in the case of the enzyme from TM-treated *mnn9* cells, ammonium sulphate precipitation was performed at 60% saturation, instead of 100%. Glycopeptides were prepared from APase as described [2].

Immunoprecipitation of APase from crude extracts of TM-treated and untreated *mnn9* cells was performed by adding 0.5, 1.0, 2.0 or 5.0 μl APase antiserum to extract aliquots (150 μl) of the same enzyme activity. After overnight incubation at 0°C, 150 μl of a suspension of *Staphylococcus aureus* cells was added, the mixture incubated for 1 h at room temperature and after centrifugation, APase activities remaining in supernatants were measured. From the data obtained, precipitation curves were constructed.

SDS electrophoresis was performed on gel slabs with a linear gradient of polyacrylamide concentrations (3–30%) according to [1] or, if gels were blotted onto nitrocellulose sheets, according to Laemmli [9]. Transfer onto nitrocellulose was done by the method of Burnette [10], and blots were probed by anti-APase serum followed by ^{125}I-protein A (Amersham) and autoradiographed.

Neutral carbohydrates were estimated by the orcinol-H_2SO_4 method [11], and *N*-acetylglucosamine was determined according to Gatt and Berman [12]. APase activity was determined as in [8].

3. RESULTS

Wild-type and *mnn9* mutant cells were grown in the presence of various concentrations of TM and, as shown in fig.1, secretion of the active APase decreased remarkably with increasing TM concentrations. Besides, it can be seen that there is no significant difference in the amount of APase activity secreted from untreated *mnn9* and wild-type H42 cells, and even a smaller difference was obtained in comparison with the *mnn9* parent strain X2180 (not shown), indicating that the addition of outer chains is not essential for enzyme secretion and activity.

The amount of secreted APase protein in the crude extract of *mnn9* cells treated with 1.0 μg TM/ml, resulting in a nearly 4-fold decrease in extracellular enzyme activity per amount of cells, was compared with that secreted from untreated cells (fig.1B, curves 1,4) by means of enzyme immunoprecipitation with APase antibodies, as described in section 2. By comparing the precipitation curves obtained, it was calculated that treated cells secreted only about 2-fold less APase protein. These results lead to the conclusion that TM treatment results in the secretion of partially or fully inactive enzyme forms.

To estimate the size of APase from *mnn9* cells treated with different TM concentrations we prepared membrane and soluble fractions, respectively, as described in section 2, separated the material by SDS electrophoresis and performed immunoblots. *mnn9* cells were used since this mutant secretes an enzyme containing only core chains, nearly uniformly sized (12–14 mannose), which enables quite accurate determination of the enzyme's molecular mass. The immunoblots of the secreted enzyme fractions and the membrane fractions are shown in fig.2. The intracellular soluble fraction did not contain APase detectable by activity measurements or immunoblotting. It can be seen (fig.2) that both the secreted and membrane-bound APases from untreated cells have a molecular mass of about 81 kDa (lanes 1,6), corresponding to a protein subunit of 59 kDa with 8 core chains of an approximate molecular mass of 2.7 kDa each. By increasing TM concentrations, molecules of the secreted enzyme, ranging from 81 kDa to the minimal molecular mass of 70 kDa appeared, which correspond to enzyme forms containing 4–8 chains (lanes 2–5). Enzyme forms having less than 4 chains, with molecular masses from 59 to 67 kDa, were retained in the membrane fraction (lanes 7–10).

The immunoblots of the membrane fraction of TM-treated wild-type cells showed that, besides the broad band of fully glycosylated APase, bands ranging from 59 to 67 kDa accumulate with increasing TM concentrations (not shown).

Secreted APase from wild-type and *mnn9* cells,

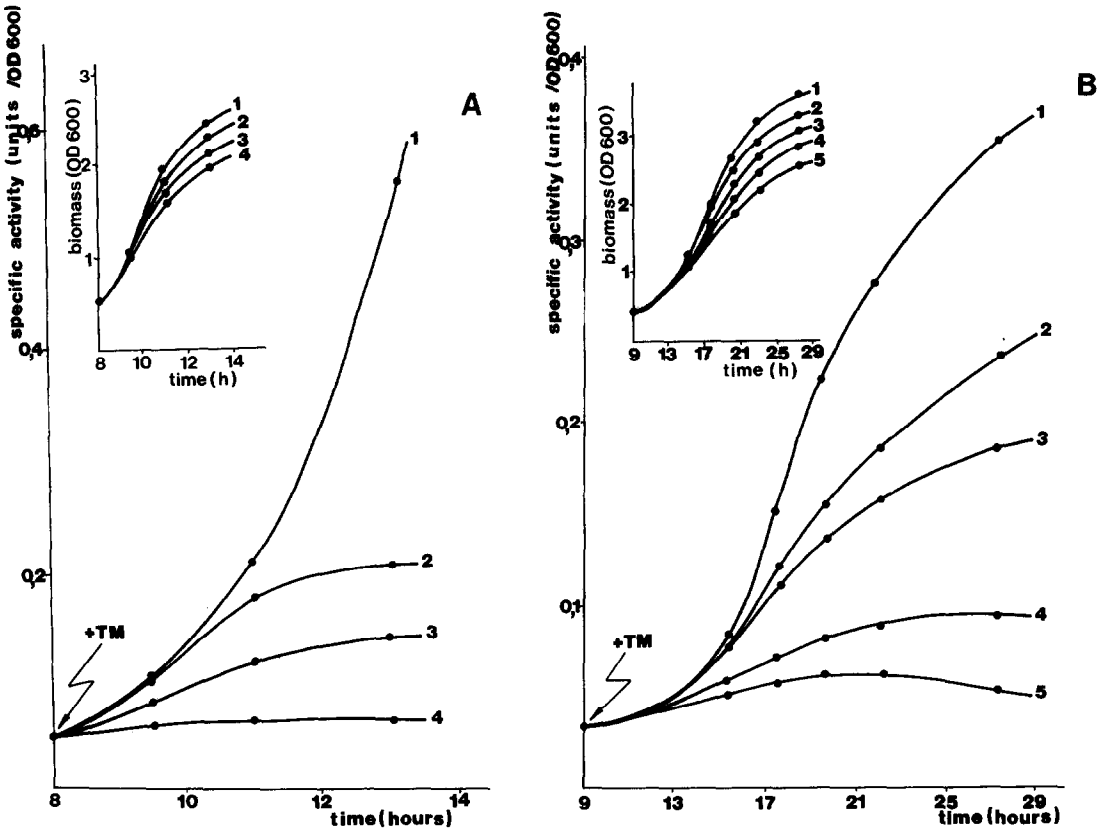

Fig.1. The effect of TM on growth (inset) and APase activity of wild-type H42 (A) and *mnn9* (B) cells. Cells were grown until the beginning of the logarithmic phase and then different TM concentrations were added to the medium. TM concentrations added were: (A) 1, 0 μg/ml; 2, 0.1 μg/ml; 3, 0.3 μg/ml; 4, 1.0 μg/ml. (B) 1, 0 μg; 2, 0.1 μg/ml; 3, 0.3 μg/ml; 4, 1.0 μg/ml; 5, 2.0 μg/ml.

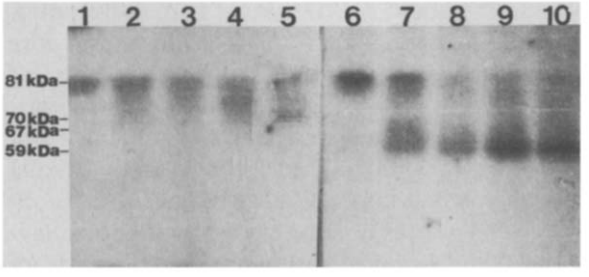

Fig.2. Immunoblots of secreted (lanes 1–5) and membrane-bound (lanes 6–10) fractions of APase from *mnn9* cells grown in media with different TM concentrations. *mnn9* cells were converted to protoplasts and secreted and membrane-bound fractions were prepared as described in section 2. TM concentrations were as indicated in the legend to fig.1B.

grown in the presence of different TM concentrations, was purified and examined by SDS electrophoresis. A very small decrease in size was observed for APase from wild-type cells treated with TM (not shown). The pattern of APase samples purified from *mnn9* cells was the same as that of the immunoblots shown in fig.2 (lanes 1–5).

Carbohydrate analysis of the purified enzymes is shown in table 1. It can be seen that the average number of carbohydrate chains decreases with increasing TM concentration and at the two highest TM concentrations a minimal average number of about 5 chains per enzyme subunit was obtained for both strains. This indicated that a further increase in TM concentration would not result in the secretion of APase with a lower number of carbohydrate chains. The mannose content of APase

Table 1

Carbohydrate content and specific activity of APase purified from yeast cells treated with different TM concentrations

TM concentration in the medium (μg/ml)	Mannose[a] (%)	*N*-Acetylglucosamine/protein subunit	Number of carbohydrate chains[b]	Spec. act. (U/mg)
Wild type				
0	100	16.4	8.20	36.1
0.1	96	13.9	6.95	25.2
0.3	95	10.2	5.10	22.6
1.0	90	9.8	4.90	11.5
mnn9 mutant				
0	100	16.4	8.20	40.5
0.1	82	13.5	6.75	26.5
0.3	76	12.3	6.15	21.9
1.0	66	10.5	5.25	12.5
2.0	60	9.9	4.95	10.1

[a] The amount of mannose in APase from untreated cells was taken as 100%

[b] Calculated on the basis of the assumption that every carbohydrate chain contains two *N*-acetylglucosamines

from TM-treated *mnn9* cells decreases correspondingly to the decrease in number of chains, which is not the case with enzymes from wild-type cells. This result can be explained if the enzyme from wild-type treated cells retained all 3 long chains which account for about 80% of the total mannose content. By comparing the gel-filtration patterns of glycopeptides prepared from the enzymes isolated from TM-treated and untreated wild-type cells it can be seen that TM treatment results only in a decrease of the last eluting fraction, representing core chains (fig.3).

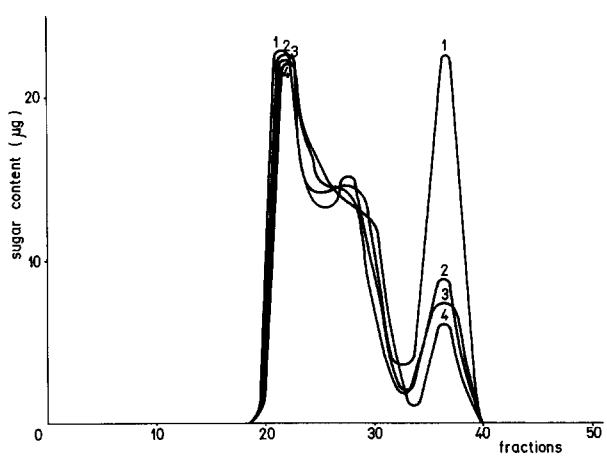

Fig.3. Gel filtration of APase glycopeptides. Glycopeptides were prepared from APase purified from wild-type H42 cells grown in the presence of different TM concentrations (indicated in table 1) as described in section 2 and separated on a Bio-Gel P-30 column (1.6 × 85 cm), with 0.1 M acetic acid as eluent.

In table 1 it is also shown that the specific activities of purified enzymes containing a decreased number of carbohydrate chains were significantly lower.

4. DISCUSSION

Schönholzer et al. [13] studied intracellular maturation and secretion of yeast APase and reported that core glycosylated enzyme accumulates in the ER in a membrane-bound form and is then transported to the Golgi apparatus where outer chains are attached and the enzyme appears in a soluble form. APase synthesized in the presence of high TM concentrations accumulates in ER membranes as an unglycosylated inactive enzyme [13,14], showing that protein glycosylation is necessary for enzyme secretion and activity. However, there were no data about the level of glycosylation required for the proper transport and activity of the enzyme.

In this paper we have demonstrated that APase synthesized in the cells treated with moderate TM concentrations has fewer carbohydrate chains than

are naturally attached. The most likely explanation for this effect is that under a slower rate of oligosaccharide transfer, due to a decreased concentration of lipid-linked oligosaccharide precursor caused by the TM inhibition [15], protein chains folded up before all oligosaccharide chains were attached and further glycosylation was hindered because of the inaccessibility of glycosylation sites on the folded protein.

The effect of the level of glycosylation on APase secretion was examined and the results presented show that outer chain addition is not necessary for secretion of soluble enzyme and that only 4 core chains, out of 8 present in the fully glycosylated protein subunit, are sufficient for enzyme transport to the periplasmic space. Enzyme forms with less than 4 chains were retained in cell membranes, presumably in the ER. On the basis of the work with secretory mutants [16] it is known that Golgi-blocked mutants accumulate invertase and APase with almost complete oligosaccharide chains, migrating on SDS electrophoresis with an apparent molecular mass from 84 to 140 kDa [13,16]. Our finding that the membrane fraction from TM-treated wild-type cells accumulates APase forms ranging in size from 59 to 67 kDa strongly indicates that underglycosylated enzyme was retained in the ER, rather than in the Golgi.

It was shown by Ballou et al. [17] that a mutation which prevents glucosylation of the lipid-linked oligosaccharide precursor reduces the efficiency of protein glycosylation. Invertase secreted from this mutant consists of a mixture of homologs with about 4–7 carbohydrate chains, in contrast to about 8–11 chains in the parent strain. These authors did not examine the accumulation and size of the intracellular membrane-bound enzyme forms in this mutant, but it is tempting to suggest that invertase forms with less than 4 chains accumulate in the ER membrane, as found here for APase.

Although the present data indicate a direct connection between APase glycosylation and secretion, the results obtained by the use of TM should be interpreted with caution, since other proteins that are essential for APase transport may require glycosylation for activity. However, the difference observed in the level of glycosylation between APase from secreted and membrane fractions of TM-treated cells clearly demonstrates that secretion of APase is directly influenced by its own glycosylation.

The role of glycosylation in transport and secretion could either be that carbohydrates are a direct signal for secretion, or alternatively, that oligosaccharide chains promote formation of a correct tertiary or quaternary structure, which was suggested to be a primary requirement for efficient transport and secretion [18]. A number of data, mostly from experiments with TM, demonstrate that carbohydrates are not an essential signal for secretion of all glycoproteins [4]. On the other hand, results have been presented showing that the protein conformation of some glycoproteins is significantly altered in the absence of glycosylation [19]. As far as APase is concerned, it was shown that the non-glycosylated enzyme, accumulated in the ER, was insoluble in 1 M KCl or in 1% Triton X-100 [14] and the same was found for underglycosylated membrane-bound forms (Mrša, unpublished), indicating protein aggregation and a strong association with the membrane. A pronounced decrease in solubility was also reported for APase deglycosylated by endo-*N*-acetylglucosaminidase H and it was shown that carbohydrates are necessary for maintaining enzyme tertiary and quaternary structure [20]. Therefore, it could be concluded that glycosylation will influence protein secretion as far as the attachment of oligosaccharide chains is required for solubility and proper protein folding.

It was previously found that most [20], or even all [21] of the APase carbohydrates can be removed in vitro without affecting enzyme activity. However, the results presented here show that secreted underglycosylated enzyme forms containing minimally 4 core chains were at least partially inactive. The specific activity of the purified underglycosylated enzyme was even lower than could be estimated on the basis of immunoprecipitation data, probably due to inactivation of the enzyme during the purification procedure. It was found that in vitro deglycosylated APase is less stable than the native one [20,21] and the same was noted for the underglycosylated enzyme (Mrša et al., unpublished). These results suggested that most, or even all of the core chains are necessary for the correct folding of APase into a fully active conformation and that a higher degree of glycosylation is required for the expression of activity than for the enzyme secretion.

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