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QUARTERLY

Evidence for the involvement of a 66 kDa membrane protein in the synthesis of sterolglucoside in *Saccharomyces cerevisiae**

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The membrane-bound sterolglucoside synthase from the yeast *Saccharomyces cerevisiae* has been solubilized by nonionic detergent, Nonidet P-40, Triton X-100, and partially purified by DEAE-cellulose column chromatography and ammonium sulfate fractionation. SDS/PAGE of the purified fraction revealed the presence of two protein bands of molecular mass 66 kDa and 54 kDa. In an attempt to identify further the polypeptide chain of sterolglucoside synthase, the partially purified enzyme was treated with [di-^{125}I]-5-[3-(*p*-azidosalicylamide)]allyl-UDPglucose, a photoactive analogue of UDPglucose, which is a substrate for this enzyme. Upon photolysis the ^{125}I-labeled probe was shown to link covalently to the 66kDa protein. The photoinsertion was competed out by the presence of unlabeled UDPglucose thus suggesting that this protein contains substrate binding site for UDPglucose.

Since photoinsertion of the probe to protein of 66 kDa correlates with the molecular mass of the protein visualized upon enzyme purification we postulate that the 66 kDa protein is involved in sterolglucoside synthesis in yeast.

Very little is known about formation of sterolglucoside (SG)[1] in the yeast *Saccharomyces cerevisiae*. It has been reported [1, 2] that upon incubation of yeast membranes with UDPglucose two glycolipids are formed. They have been tentatively identified as glucosylphosphodolichol (GPD) and sterolglucoside. Formation of these two glycolipids is probably independent since yeast mutants defective in the synthesis of GPD (*alg5* and *dpg1*) can synthesise SG to a level similar to that of the parental wild type strain [2].

Direct biochemical characterization of the enzymes involved in the synthesis of these two glycolipids has been hampered due to their extreme sensitivity to detergents [1, 2]. To obviate the problem associated with conventional purification methods, active-site-directed photoaffinity labeling of the yeast membranes with 5-azido[β-^{32}P]UDPGlc was employed. The latter studies led to the proposal that a 35-kDa membrane protein was involved in the synthesis of GPD. However, using the above photoaffinity probe it was not possible to ident-

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[1]Abbreviations: DTT, dithiothreitol; GPD, glucosylphosphodolichol; PMSF, phenylmethylsulfonyl fluoride; SG, sterolglucoside; TLC, thin-layer chromatography.

ify the protein involved in SG formation, most probably due to the lower affinity of the probe to this protein [2] as well as its low abundance (present results).

To identify the protein involved in SG formation in yeast we have constructed a highly reactive [di-^{125}I]-5-[3-(*p*-azidosalicylamide)]-allyl-UDPGlc [3] (Fig. 1), which has been subsequently used to photolabel the solubilized and partially purified enzymic protein. The results of the studies revealed a direct correlation between the extent of photolabeling of a 66 kDa membrane protein and the existence of SG synthase activity measured in the solubilized and partially purified yeast membrane fraction.

MATERIALS AND METHODS

[^{125}I]-5-[3-(*p*-Azidosalicylamide)]allyl-UDP-glucose was synthesized and iodinated according to Meikle *et al.* [3]. UDP[^{3}H]glucose (10 mCi/mmol) was obtained from New England Nuclear. Phospholipids used in the enzyme activity assays were from Sigma. All other chemicals were from commercial sources.

Yeast strain and media. Standard laboratory strain DBY 1033 was used in this study. Yeast was grown on YPG medium (0.5% yeast extract, 1% peptone and 2% glucose) to early stationary phase (A_{600} was 1–2).

Yeast membranes were prepared as described earlier [4] except that the membrane pellet was

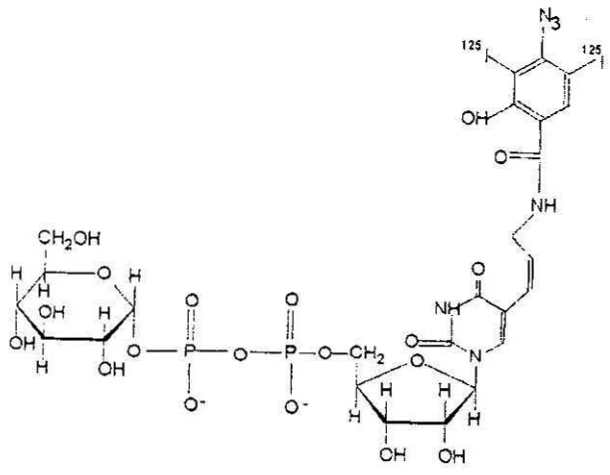

Fig. 1. Chemical structure of the photoprobe, di-[^{125}I]-5-[3-(p-azidosalicylamide)]allyl-UDPglucose. The photoprobe was synthesized and iodinated according to Meikle *et al.* [3].

washed twice with 0.15 M KCl, to remove losely bound proteins. The membranes (about 400 μg of protein) were suspended in 15 mM Hepes, pH 7.4, containing 5 mM uridine, 1mM EDTA, 20% of glycerol, 1 mM DTT, 1 mM PMSF and 0.3% of Nonidet P-40 or Triton X-100, and incubated for 30 min on ice. After centrifugation at 120000 × *g* for 45 min, the supernatant was gently shaken with Bio-beads SM-2 for 20 min to remove excess of detergent, followed by addition of glycerol to 50% concentration.

Fractionation of detergent extract containing SG-synthase activity

1. DEAE-cellulose column chromatography: about 3 mg of protein was applied to the column (6 mm × 60 mm) equilibrated with the buffer used for enzyme solubilization ("solubilization buffer") and eluted with 30 ml of the same buffer. Fractions of 1.5 ml were collected. The activity of SG synthase was determined in every second fraction after precipitation of protein with ammonium sulfate (0.8 saturation, w/v) and subsequent removal of salt by dialysis against two changes of "solubilization buffer".

2. Ammonium sulfate precipitation. The detergent extract (20 ml) was brought to 0.55 saturation of ammonium sulfate and centrifuged for 10 min at 5000 × *g*. Subsequently ammonium sulfate was added to the supernatant to 0.8 saturation and centrifuged as above. The pellet was resuspended in 1 ml of "solubilization buffer" and dialyzed as above. Determination of the phospholipid content of solubilized enzyme fraction was carried out as described by Bligh & Dyer [5] and by Rouser *et al.* [6].

Determination of the enzymic activity. About 250 μg of protein of the appropriate fraction was incubated with 0.3 μCi of UDP[^{3}H]glucose in 4 mM Tris, pH 7.4, containing 0.75 mM $MgCl_2$ and $MnCl_2$ (total vol. 100 μl) for 15 min at 30°C. Ergosterol at final concentration of 0.01% or/and the mixture of commercial phospholipids (100 μg) were added in chloroform/methanol (2:1, v/v). Organic solvent was removed by evaporation and the residue, after the addition of protein was suspended in the incubation mixture by sonication. The reaction was stopped with 2 ml of chloroform/methanol (3:2, v/v). The lipid fraction was purified by the Folch procedure as described elsewhere [2] and subjected to TLC in

the solvent system: chloroform/methanol/water (65:25:4, by vol.).

Identity of sterolglucoside was verified by TLC in the presence of SG standard [2].

Protein determination. Protein was determined by the Lowry method after quantitative precipitation with 10% trichloroacetic acid in the presence of yeast-soluble ribonucleic acid as a carrier to remove the substances interfering in the colorimetric method [7].

Photoaffinity labeling. 22 μg of protein was incubated in total volume of 50 μl for 20 s at room temperature with 0.4 μCi of [^{125}I]-5-[3-(*p*-azidosalicylamide)]allyl-UDPglucose in 4 mM Tris, pH 7.4, 0.75 mM $MgCl_2$ and 0.75 mM $MnCl_2$, 5 mM 2-mercaptoethanol, and 800 mM UDPglucose when indicated. The incubation was followed by UV radiation at 366 nm for 5 min. Protein was precipitated with 500 μl of 12.5% trichloroacetic acid. The precipitated proteins were washed with 66% ethanol, evaporated to dryness in Speed Vac and resuspended in 20 μl of the PAGE sample buffer.

Photolabeled ^{125}I-proteins were separated on SDS/PAGE and identified by autoradiography.

RESULTS

Sterolglucoside synthase in yeast membranes; solubilization and fractionation of the detergent extract

The membrane pellet was washed with 0.15 M KCl to remove loosely bound proteins. In the preliminary experiments we have established that by this procedure specific activity of the membrane-bound enzyme was increased 2–3 fold.

As it was reported earlier [1, 2] the enzyme is extremely sensitive to detergents. Out of the detergents tested (i.e., Brij 35, Emulgen 911, 1-*O*-octyl-β-D-glucopyranoside), Nonidet P-40 and Triton X-100 at a concentration of 0.3% showed reversible inhibitory effect. Removal of an excess of the detergent in the presence of Bio-beads resulted in an about 5 fold increase of the specific activity of the solubilized enzyme as compared to the membrane-bound enzyme (Fig. 2) with a 40% recovery of the total enzyme activity.

Phospholipid composition of the solubilized enzyme fraction was determined (Table 1). After the addition of commercial phospholipids at the ratio indicated in Table 1 specific activity of the enzyme increased 3–4 fold. Thus, in the subsequent attempts on enzyme solubilization, phospholipids were added to the "solubilization buffer" routinely.

The detergent extract was subjected to the ammonium sulfate precipitation and the SG synthase activity was recovered in the protein fraction precipitated between 0.55–0.80 saturation.

The activity of the membrane-bound enzyme did not require addition of the exogenous sugar acceptor (ergosterol). However after solubilization, the activity was increased two fold on addition of 0.01% of ergosterol, and after ammonium sulfate precipitation the addition of endogenous ergosterol was prerequisite.

In a parallel experiment the detergent extract has been subjected to DEAE-cellulose column chromatography [8]. The elution profile re-

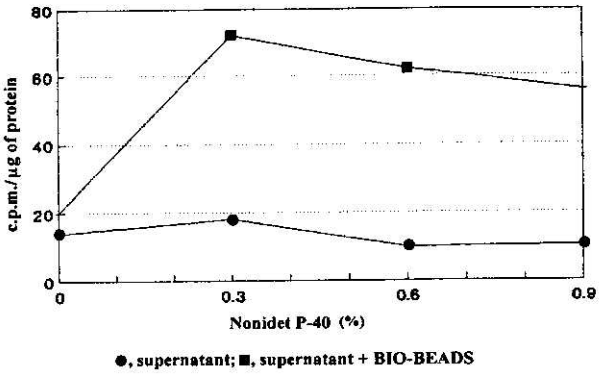

Fig. 2. Effect of removal of an excess of Nonidet P-40 on sterolglucoside synthase activity.
Yeast membranes were solubilized in the presence of Nonidet P-40. SG synthase activity was determined in the 120000 × *g* (see Methods) supernatant (lower curve) and after removal of an excess of detergent with the Bio-beads SM-2 (upper curve).

Table 1
Lipid composition of the solubilized enzyme fraction

Phospholipid	% (by weight)
Phosphatidylcholine (PC)	45
Phosphatidylethanolamine (PE)	19
Phosphatidylinositol (PI)	26
Phosphatidylserine (PS)	10

vealed two broad peaks of the activity (Fig 3). Yet, proteins of both peaks showed the same migration rate on SDS/PAGE (see Fig. 4). No conclusive explanation of this result can be offered at this moment. The fractions containing active enzyme were pooled, concentrated by ammonium sulfate precipitation (at 0.8 saturation) and after removal of salt, run on SDS/PAGE alongside with the protein fraction obtained directly from the detergent extract after ammonium sulfate precipitation at 0.55–0.8 saturation. Purification on DEAE-cellulose and ammonium sulfate precipitation of the detergent extract gave similar result, i.e. two protein bands, visualized by silver staining, of molecular mass about 66 kDa and 54 kDa (lanes 1, 2, Fig. 4). Since the degree of purification of the preparation obtained by DEAE-cellulose chromatography and direct ammonium sulfate precipitation was the same the latter method was used in the subsequent experiments.

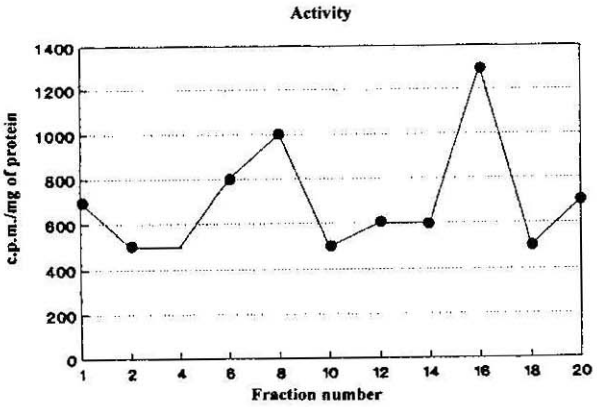

Fig. 3. DEAE-cellulose column chromatography of the solubilized membrane fraction.
Solubilized membrane proteins were applied to DEAE-cellulose column (6 mm × 60 mm) equilibrated with 15 mM Hepes, pH 7.4, containing 5 mM uridine, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 20% of glycerol and 0.3% of Nonidet P-40, and eluted with the same buffer. Fractions of 1.5 ml were collected and SG synthase activity determined in every second fraction.

Photoaffinity labeling of the solubilized and partially purified SG synthase with [^{125}I]-5-[3-(*p*-azidosalicylamide)allyl-UDP glucose

The protein fraction containing SG synthase activity, was irradiated in the presence of photoprobe. Subsequent SDS/PAGE followed by autoradiography revealed that the fraction containing SG synthase activity (lanes 6 and 7, Fig. 5) reveals the presence of at least 5 proteins binding the photoprobe upon irradiation. Since photoincorporation can be usually abolished by the presence of the enzymic substrate [9] photoaffinity labeling was carried out parallelly in the presence and absence of unlabeled UDPglucose. The protein band of molecular mass around 29 kDa is equally visible in the presence (lane 6) and absence (lane 7) of unlabeled UDPglucose suggesting that photolabeling of this protein did not compete with the substrate. This is also true for the protein of 93 kDa. Labeling of the two proteins with 45-kDa molecular mass is partially abolished by 0.8 M UDPglucose. However, as it was demonstrated earlier [2] synthesis of sterol glucoside *in vitro* is inhibited by 80% and photoaffinity labeling of the protein is abolished at much lower concentration (i.e., 0.05 and 0.1 mM) UDPglucose, respectively. Thus it seems highly unlikely that either of these proteins contains a substrate binding site for this sugar nucleotide. The only protein which is photolabeled in the absence, but not the presence, of unlabeled UDPglucose is the 66 kDa protein. In addition, this protein is detectable in the fraction of the

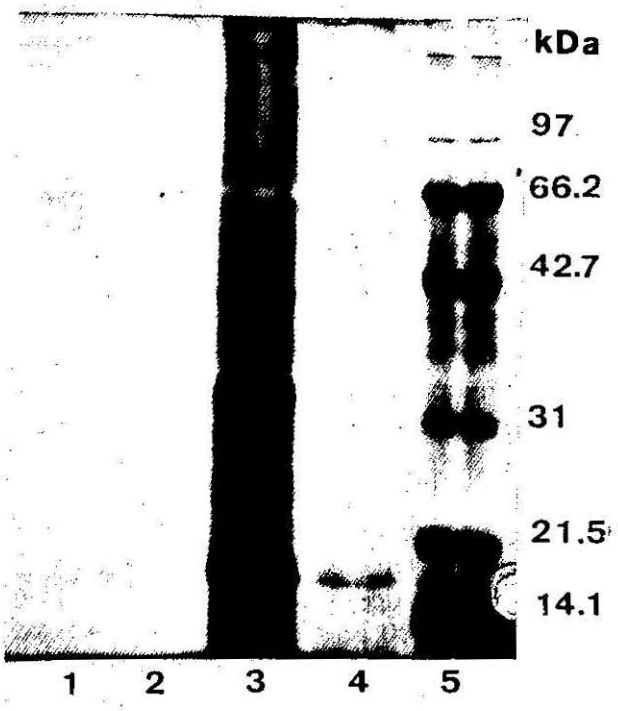

Fig. 4. SDS/PAGE of the partially purified sterolglucoside synthase.
1. DEAE-cellulose column fractions 7–9 (compare Fig. 2); 2. DEAE-celulose column fractions 15–17 (compare Fig. 2); 3. Solubilized membranes; 4. Ammonium sulfate precipitated (0.55–0.80 saturation) proteins from the solubilized membranes; 5. Protein standards. Proteins were run on 10% SDS/PAGE "mini-gels" and visualized by silver staining.

detergent extract containing SG synthase activity (lanes 3 and 7, Fig. 5). Moreover, the 66 kDa protein coincides very well with the results of SDS/ PAGE, of DEAE-cellulose column and ammonium sulfate precipitated fractions containing the SG synthase protein (Fig. 3). Taken together, the above results strongly indicate that the 66 kDa protein is involved in sterolglucoside synthesis in yeast.

DISCUSSION

Detailed biochemical characterization of the membrane-associated enzymes has been often impossible due to their rapid loss of activity after solubilization with detergent. Photoaffinity labeling is one of the approaches to improve purification and identification of glycosyltransferases involved in the synthesis of lipid-linked saccharides. 5-Azido-UDP-glucose and 5-azido-UDP-glucuronic acid have been successfully used for identification of glucosylphosphodolichol-synthase in yeast and rat liver [2, 9, 10] and UDP-glucuronyl transferase in rat liver [9]. However, this approach has also potential complications. One of them is specificity of a photoprobe. Theoretically, 5-azido-[β-^{32}P]UDPglucose used for identification of the protein involved in the synthesis of glucosylphosphodolichol in yeast, should photolabel also the protein engaged in the synthesis of sterolglucoside. However, with the use of this photoprobe it was not possible to identify conclusively the latter protein. This was most probably due to the 5 fold lower affinity of the photoprobe towards SG synthase than towards GPD synthase [2]. Thus we have taken advantage of another photoprobe, i.e. [di-^{125}I]-5-[3-(*p*-azidosalicylamide)allyl-UDPglucose which, in preliminary experiments, photolabeled more proteins in yeast membranes than the azido-derivative of UDPglucose. However, the drawback of using the probe of a higher affinity is its specificity. To minimize this complication we have partially purified SG synthase from yeast membranes and photolabeled the fractions enriched in the enzyme activity. We have established that labeling of the 66 kDa protein was abolished by unlabeled UDPGlc. In addition, a band of similar molecular mass was visualised by silver staining on SDS/PAGE of the fractions containing SG synthase activity (Fig. 3). The fact that photolabeled 66 kDa protein occurs in the fractions containing SG synthase activity allows us to conclude that this protein is involved in the synthesis of sterolglucoside in yeast.

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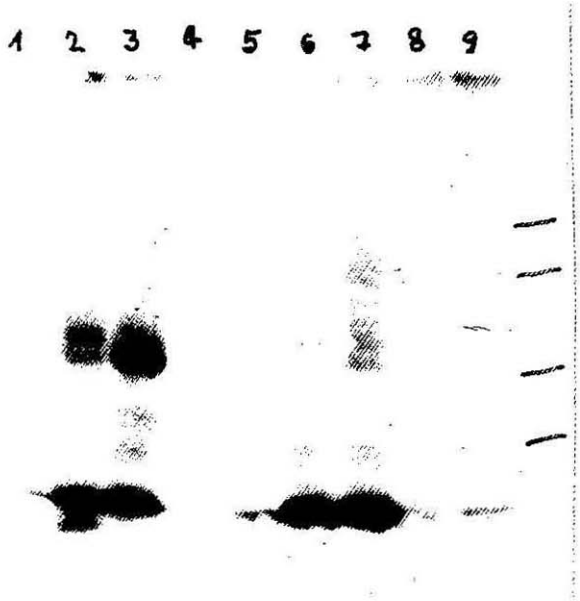

Fig. 5. SDS/PAGE of ^{125}I-photolabeled proteins of S. cerevisiae membranes.
1. Membranes incubated with unlabeled UDPglucose, control without photolysis; 2. Membranes, unlabeled UDPglucose, standard conditions of photolysis; 3. Membranes, without UDPglucose, standard conditions of photolysis; 4. Solubilized membrane proteins, unlabeled UDPglucose, photolysed; 5. Solubilized membrane proteins, without UDPglucose, photolysed; 6. Pellet after ammonium sulfate precipitation (0.55–0.80 saturation) with UDPglucose, photolysed; 7. Pellet as 6, without UDPglucose, photolysed; 8. Supernatant after ammonium sulfate precipitation with UDPglucose, photolysed; 9. Supernatant as in 8, without UDPglucose, photolysed.

REFERENCES

1. Lehle, L. (1980) Biosynthesis of the core region of yeast mannoproteins. *Eur. J. Biochem.* **109**, 589–601.
2. Palamarczyk, G., Drake, R., Haley, B. & Lennarz, W.J. (1990) Evidence that the synthesis of glucosylphosphodolichol in yeast involves a 35-kDa membrane protein. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 2666–2670.
3. Meikle, P.J., Ng, K.F., Johnson, E., Hoogenraad, N.J. & Stone, B.A. (1991) The β-glucan synthase from *Lolium multiflorum*. *J. Biol. Chem.* **266**, 22569–22581.
4. Palamarczyk, G., Lehle, L., Mańkowski, T., Chojnacki, T. & Tanner, W. (1980) Specificity of solubilized yeast glycosyl transferases for polyprenyl derivatives. *Eur. J. Biochem.* **105**, 517–523.
5. Bligh, E.G. & Dyer, W.J. (1959) A rapid method of total lipid extraction and purification. *Can. J. Biochem. Phys.* **37**, 911–918.
6. Rouser, G., Siakotos, A.N. & Fleisher, S. (1966) Quantitative analysis of phospholipids by thin layer chromatography and phosphorus analysis. *Lipids* **1**, 85–86.
7. Polacheck, I. & Cabib, E. (1981) A simple procedure for protein determination by the Lowry method in dilute solutions and in the presence of interfering substances. *Analyt. Biochem.* **117**, 311–314.
8. Forsee, T.W., Laine, R.A. & Elbein, A.D. (1974) Solubilization of a particulate UDP-glucosyl--transferase in developing cotton fibers and seeds. *Arch. Biochem. Biophys.* **161**, 248–259.
9. Drake, R.R. & Elbein, A.D. (1992) Photoaffinity labeling of glycosyltransferases. *Glycobiology* **2**, 279–284.
10. Drake, R.R., Palamarczyk, G., Haley, B. & Lennarz, W.J. (1990) Evidence for the involvement of a 35-kDa membrane protein in the synthesis of glucosylphosphoryldolichol. *Biosci. Rep.* **10**, 61–68.