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ENZYMATIC SYNTHESIS OF STERYL 3β-D-MONOGLUCOSIDES IN THE SLIME MOLD *PHYSARUM POLYCEPHALUM*

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Abstract—Microplasmodia of *Physarum polycephalum* exhibit high UDPG:sterol glucosyltransferase activity. The enzyme was purified about 28-fold by acetone precipitation and chromatography on Sephadex G-100. Its MW is 70000, pH optimum at 7.2. Apparent Km values for UDPG and sitosterol are 1.8×10^{-4}M and 5.6×10^{-6}M respectively. The enzyme is stimulated by lecithin as well as by some —SH and chelating agents. The enzyme can glucosylate a number of natural C_{27}–C_{29} sterols, however, at different rates. Sterols posessing an alkyl group at C-24 are better substrates than C_{27} sterols. The presence of a Δ^{22} double bond decreases the affinity of the sterol for the enzyme. Also the presence and localization of double bonds in the ring system exert a pronounced effect on the rate of glucosylation. Δ^5-Sterols are glucosylated at higher rate than Δ^7-sterols. Stanols and sterols with conjugated double bonds in ring B are poor substrates. 4-Methylsterols are not glucosylated at all. In contrast to UDPG:sterol glucosyltransferase present in higher plants the enzyme of *Ph. polycephalum* is weakly membrane bound and its activity is not stimulated by divalent metals and low concentrations of synthetic detergents.

INTRODUCTION

Steryl 3β-D-monoglucosides (SG) and their 6′-*O*-acyl derivatives (ASG) seem to be common constituents of higher plants [1–3]. Recently the importance of these compounds for cell metabolism has been suggested. For example, their functions as metabolically active components of plant membrane structures [4], in intercellular transport of sterols [5] or as glucose carriers through membranes [6, 7] have been considered. Enzymatic glucosylation of sterols as well as enzymatic transformation of SG into ASG has been demonstrated in cell-free preparations from several higher plants [8–11]. In a few cases the respective enzymes i.e. UDPG: sterol glucosyltransferase and complex lipid: SG acyltransferase have been partially purified and characterized [12–18].

It remains obscure whether SG and ASG are also ubiquitous in lower plants such as algae, fungi and slime molds. So far SG have been detected only in some Mycoplasma [6] and yeast species [19].

This paper presents the first evidence indicating the occurrence of UDPG: sterol glucosyltransferase—different in some respects from the enzyme present in higher plants—in Myxomycetes.

RESULTS

Formation of steryl glucosides by crude enzyme preparations

After incubation of the homogenate of *Ph. polycephalum* with UDP-glucose-[^{14}C] in the absence of any exogenous sugar acceptor 70–80% of the radioactivity was recovered in an *n*-BuOH extract. As has been shown by PC virtually all the remaining radioactivity was associated with unchanged UDP-glucose and its degradation products i.e. glucose-1-phosphate and glucose. TLC and autoradiography of the butanol extract showed that it contained only one radioactive substance with the chromatographic mobility of authentic sitosteryl 3β-D-monoglucoside. A product chromatographing with steryl monoglucoside was also formed, with yield reaching 60%, when cholesterol-[4-^{14}C] and unlabelled UDPG were used for the incubation. This shows that the enzyme in the homogenate is not specific for sitosterol but can also glucosylate other sterols.

The rate of the synthesis of the radioactive product from UDP-glucose-[-^{14}C] by the homogenate was

Table 1. Effect of EtOH, lecithin and sitosterol on the incorporation of UDP-glucose-[^{14}C] into *n*-butanol soluble products by a crude homogenate (0.35 mg protein) or Me_2CO precipitated enzyme preparation (0.4 mg protein). For details see Experimental

Compound added	Incorporation of glucose-[^{14}C] from UDPG (dpm × 10^{-3})	
	Crude homogenate	Me_2CO precipitated enzyme preparation
None (endogenous acceptors only)	58	1.2
Ethanol	62	1.5
Ethanol + lecithin	69	6.0
Ethanol + sitosterol	72	66
Ethanol + sitosterol + lecithin	86	92

slightly enhanced (20–30%) by the addition of sitosterol in the form of EtOH solution containing crude egg lecithin. Much more pronounced stimulation by exogenous sitosterol was observed with an acetone precipitated enzyme preparation (Table 1). This preparation showed very limited ability to form radioactive glucolipid in the absence of exogenous sterol—probably in the result of the partial removal of endogenous sterols. The data (Table 1) indicate that ethanol and lecithin not only facilitate the formation of micellar solutions of sitosterol but they also slightly stimulate the enzyme which is suggested by the enhancement of utilization of endogenous sterols in their presence.

The identity of the reaction product with steryl glucoside and the β-configuration of the glucosidic bond was unequivocally proved. The radioactive compound obtained after incubation of acetone precipitated enzyme with labelled UDPG and sitosterol (135 200 dpm) was acetylated and diluted with non-labelled authentic sitosteryl 3β-D-monoglucoside tetraacetate (25.5 mg). The mixture was then crystallized from aq. EtOH (5 times) without any decrease in the specific radioactivity (initial radioactivity: 5320 dpm/mg; after subsequent crystallizations: 4980, 5010, 4830, 4920, 5100 dpm/mg).

It should be mentioned that in our experiments with crude cell-free preparations the synthesis of acylated steryl glucosides (ASG) was never observed. The synthesis of these compounds has been demonstrated with homogenates of higher plant tissues [8–13] and results from acylation of SG by endogenous acyl donors. Moreover, we were unable to detect by TLC any trace of ASG in the *Ph. polycephalum* lipids. It seems therefore that in contrast to higher plants *Ph. polycephalum* does not contain acyltransferase converting SG to ASG.

It was demonstrated that the compounds exhibiting chromatographic properties of steryl glucuronosides were not synthesized on incubation of the purified enzyme with sitosterol and UDP-glucuronate-[^{14}C]. In the presence of UDP-galactose-[^{14}C] a high incorporation of ^{14}C into the SG fraction was observed. It was found, however, by means of PC, that even in this case glucose was the only radioactive product obtained upon acid hydrolysis of the steryl glycoside formed. This indicates that utilization of UDP-galactose is preceded by epimerization to UDP-glucose by UDP-galactose 4-epimerase present in the crude enzyme preparation.

Partial purification of UDPG: sterol glucosyltransferase

Determination of glucosyltransferase activity of the crude subcellular fractions obtained by differential centrifugation of the homogenate demonstrated (Table 2) that about a half of the total activity was associated with membranous structures. The remaining activity was present in the 105000 *g* supernatant. It is noteworthy that particulate fractions sedimenting within the range 600–16000 *g* showed distinctly higher specific activity (per mg protein) than the crude homogenate or 105000 *g* supernatant. This suggests that UDPG: sterol glucosyltransferase of *Ph. polycephalum* occurs in two forms: soluble and membrane-bound. However, it has been shown that repeated washing of the particulate fractions with buffer with subsequent centrifugation led to gradual solubilization of the enzyme. For example, after 5 successive washings of the most active 3000 *g* fraction about 75% of the activity originally present in this fraction was found in the 105000 *g* supernatant. It seems probable that in unbroken cells all the enzyme is weakly membrane bound and that the 'soluble' form represents a part of the enzyme solubilized during the homogenization procedure. Practically all the membrane-bound activity can be easily solubilized by acetone treatment (see Experimental). By contrast the UDPG: sterol glucosyl-transferase of higher plants is tightly membrane-bound [12, 13, 15] and can be solubilized only by treatment with relatively high concentrations of some synthetic detergents such as Triton X-100.

Based upon the above experiments a simple method of partial purification of the enzyme has been elaborated. Particulate fractions sedimenting at 600–16000 *g* were treated with acetone and further fractionated by gel filtration on Sephadex G-100 (Fig. 1). Glucosyltransferase was eluted from the column as a single peak. Combined fractions eluted between 40 and 58 ml had 28-fold higher activity than the crude homogenate. The yield (in respect to the homogenate) was about 35%.

In order to determine the MW of the enzyme the column was calibrated with blue dextran, ribonuclease, egg albumin and bovine albumin. For the glucosyltransferase a MW of approx. 70000 was obtained by interpolation. The MW of the enzyme present in higher plants is not known yet.

Partially purified enzyme demonstrated an absolute requirement for added sterol acceptor. The rate of incorporation of glucose from UDP-glucose into sitosterol glucoside was linear for approximately 30 min

Table 2. UDPG: sterol glucosyltransferase activity in the crude subcellular fractions obtained by different centrifugation of the crude homogenate of *Ph. polycephalum*

Fraction	Specific activity (nmol product/mg protein/hr)	Total activity (nmol product/g fresh microplasmodia/hr)
Crude homogenate	0.380	3.11
600 *g*	0.465	1.17
3000 *g*	0.703	0.19
8000 g	0.538	0.12
16000 *g*	0.458	0.05
105000 *g*	0.200	0.08
105000 *g* Supernatant	0.330	1.39

Incubations were carried out in the presence of exogenous sitosterol. For other details see Experimental.

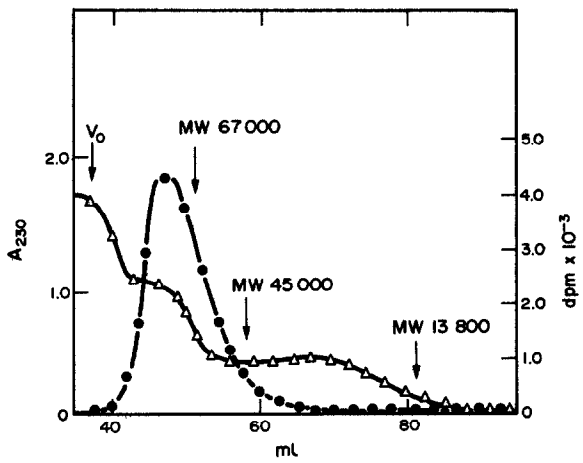

Fig. 1. Gel filtration of crude acetone precipitated enzyme preparation on Sephadex G-100. 2 ml fraction were collected and assayed for protein content (△) and UDPG: sterol glucosyltransferase activity with sitosterol as the acceptor (●). Arrows show the elution volumes of blue dextran (Vo), ribonuclease (MW 138000), egg albumin (MW 45000) and bovine albumin (fraction V; MW 67000) used for column calibration.

and was proportional to enzyme concentration. The enzyme exhibited a pH optimum at 7.2. The activity in Tris–HCl buffer was about 40% higher than in Tris–maleate or phosphate buffers. This may suggest the inhibitory effect of divalent anions on the enzyme. Apparent Michaelis constants as deduced from Lineweaver–Burk plots are 1.8×10^{-4} M for UDPG and 5.6×10^{-6} M for sitosterol.

Substrate specificity and some other properties of the glucosyltransferase

Using partially purified enzyme we have investigated the affinity for the enzyme of a number of sterols differing in the structure of the side chain and localization of double bonds in the ring system (Table 3). The results indicate a rather limited specificity of the enzyme. The rate of glucosylation was highest with sterols having a Δ^5 double bond. Sterols with Δ^7 double bond were glucosylated at a much lower rate (e.g. cholesterol–Δ^7-cholestenol; stigmasterol–α-spinasterol). A very low rate of glucosylation was observed using $\Delta^{5,7}$ sterols or 5α-cholestanol. The presence of a double bond at C-22 seemed to decrease the affinity of the sterol for the enzyme (e.g. sitosterol–stigmasterol). Also the presence of an alkyl group at C-24 in the side chain had a pronounced effect on the rate of glucosylation. Sterols possessing a methyl group at C-24 were glucosylated more readily than the corresponding sterol without such group but at lower rate than the sterol with an ethyl group (e.g. cholesterol–campesterol–sitosterol). 4-Methylsterols such as lanosterol, 24,25-dihydrolanosterol and cycloartenol were not glucosylated. It is noteworthy that the sterols glycosylated at the highest rate i.e. sitosterol and stigmasterol, were identified by Lecompte *et al.* [20] as the main sterols of *Ph. polycephalum*.

In order to compare the UDPG: sterol glucosyltransferase of *Ph. polycephalum* with the enzyme present in higher plants the effect of some reagents known as activators or inhibitors of the higher plant enzyme was studied. In contrast to the enzyme present in higher plants [13, 15, 16] glucosyltransferase of *Ph. polycephalum* was not activated by divalent metals such as Mg^{2+} and Ca^{2+}. In fact these cations slightly inhibited the enzyme at concentrations higher than 10^{-4}M. Zn^{2+} and Hg^{2+} were strongly inhibitory (50% inhibition at 5×10^{-5}M and 7×10^{-5}M respectively). No requirement for divalent metals was supported by slight stimulation in the presence of EDTA (15% at 10^{-4}M). As with the enzyme from higher plants the glucosyltransferase of *Ph. polycephalum* was inhibited by PCMB (50% inhibition at 6×10^{-4}M) and stimulated by some -SH reagents such as dithiothreitol and 2-mercaptoethanol (25% and 10% respectively at 10^{-3}M). This suggests that free -SH groups are necessary for enzyme activity. UDP and UTP strongly inhibited the enzyme (50% inhibition at 6×10^{-5}M and 7×10^{-4}M respectively). ATP had a small activating effect at low concentrations (15% at 10^{-5}M) which is in accordance with the data obtained for the enzyme isolated from higher plants [12, 13, 16]. One of the most pronounced differences in the properties of the enzymes from *Ph. polycephalum* and from higher plants was the effect of

Table 3. Effect of sterol structure on the rate of glucosylation by UDPG: sterol glucosyltransferase of *Ph. polycephalum*

Sterol added			Relative activity
Trivial name	Localization of double bond	Substituent at C-24	%
Sitosterol	Δ^5	$-C_2H_5$	100
Campesterol	Δ^5	$-CH_3$	28
Cholesterol	Δ^5	—H	23
Stigmasterol	$\Delta^{5,22}$	$-C_2H_5$	50
Δ^5-Cholestenol	Δ^7	—H	12
α-Spinasterol	$\Delta^{7,22}$	$-C_2H_5$	31
5α-Cholestanol	—	—H	8
7-Dehydrocholesterol	$\Delta^{5,7}$	—H	6
Ergosterol	$\Delta^{5,7,22}$	$-CH_3$*	11

* The configuration of the C-24 methyl group of ergosterol is opposite to that of the other C-24 alkylated sterols listed above.

All sterols were added as solutions in EtOH containing egg lecithin. Partially purified enzyme preparation was used for the incubations.

synthetic detergents on the activity. Several authors have shown that the enzyme present in higher plants is stimulated by Triton X-100 and some other detergents at relatively high concentration ranging from 0.1 to 0.5% [12, 13, 15]. The enzyme obtained from *Ph. polycephalum* was strongly inhibited by Triton X-100, Tween 20, Tween 60 and deoxycholate (50% inhibition occurred at concentrations lower than 0.05%).

DISCUSSION

It is well known that slime molds contain an unusually high level of UDPG pyrophosphorylase. This was explained by the requirement of UDPG for a very active synthesis of structural or reserve polysaccharides at some stages of the growth cycle [21, 22]. However, our experiments have shown that crude cell-free preparations of *Ph. polycephalum* use UDPG practically only for the synthesis of steryl glucosides—even in the case when only endogenous acceptors are available. If the same is true *in vivo* it must be assumed that steryl glucosides are of great importance in glucose metabolism in these organisms. Smith [6, 23] suggested that in Mycoplasma (pleuropneumonia-like organisms) steryl glucosides act as specific glucose carriers through the cell membrane. A similar function of steryl glucosides in glucose transport between different cellular compartments of *Ph. polycephalum* cannot be ruled out.

UDPG: sterol glucosyltransferase of *Ph. polycephalum* exibit many similarities with the enzyme isolated from higher plants [12, 13, 15, 16]. Both enzymes seem to be membrane-bound, require free -SH groups for activity, are stimulated by phospholipids and show optimal activity at pH 7.0–8.0. The most interesting is that the enzyme from *Ph. polycephalum* shows a very similar pattern of specificity for various sterols with that found in our previous work [15] on the properties of the enzyme isolated from *Calendula officinalis*. This may suggest that both enzymes are not only functionally but also structurally related—at least in the region of the catalytic centre. There are however also some differences such as the requirements for divalent metals and the different effects of some detergents on their activity. It seems that these properties must be re-examined with much purer enzyme preparations before any final conclusions are reached.

EXPERIMENTAL

Materials. Ph. polycephalum, strain M_3C IV was maintained in submerged, shaken cultures according to Daniel and Baldwin [24]. Microplasmodia in log phase of growth were used for enzyme preparation. All sterols used were at least 96% pure as demonstrated by GLC on SE-30 and OV-17 [15]. The commercial preparation of sitosterol contained about 12% of campesterol and traces of cholesterol.

Partial purification of UDPG: sterol glucosyltransferase. Fresh microplasmodia were homogenized with 15-fold amount of 0.1 M Tris–HCl, pH 7.2 in a Potter–Elvehjem homogenizer. The homogenate was filtered through 3 layers of cheese-cloth and successively centrifuged at 600 *g* (5 min), 3000 *g* (10 min), 8000 *g* (10 min), 16000 *g* (20 min) and 105000 *g* (1 hr). Fractions sedimenting at 600–16000 *g* were then suspended in Tris–HCl and added dropwise to a 20-fold amount of cold (−15°) Me_2CO. The precipitated protein was collected by centrifugation, washed twice with Me_2CO and dried in a vacuum (Me_2CO precipitated enzyme preparation). This material (20 mg) was suspended in 2 ml Tris–HCl, centrifuged at 105000 *g* and the supernatant applied on a Sephadex G-100 column (75 × 0.7 cm). The column was eluted with 0.1M Tris–HCl, pH 7.2 buffer containing 0.5% NaCl. 2 ml fractions were collected. All operations were carried at 0–4°.

Enzyme assay. The incubation mixtures contained in a total volume of 0.7 ml: enzyme preparation (10–400 μg protein; 50 μmol Tris–HCl, pH 7.2; UDP-glucose-[U-^{14}C] (0.05 μCi, sp. act. 313 mCi/mmol); egg lecithin (120 μg); sterol (0.3 μmol) and EtOH (0.053 ml). Sterol and lecithin was added as EtOH soln. In some cases cholesterol-[4-^{14}C] (0.1 μCi, sp. act. 53 mCi/mmol) and unlabelled UDPG (4 μmol) were used. Incubations were carried out at 30° for 10 min. The reaction was stopped by addition of 1 ml MeOH and boiling for 3 min. Subsequently 10 ml of *n*-BuOH was added and the butanol extract washed with H_2O (5 × 3 ml). Aliquots of the butanol extract were taken for radioactivity measurements or TLC.

Chromatography. TLC of steryl glucosides was carried out on Si gel with $CHCl_3$–MeOH–H_2O (128:15:4) or *n*-PrOH–conc NH_4OH–H_2O (8:1:1). In the respective solvent systems sitosteryl 3β-D-glucoside has R_f 0.35 or 0.25. SG was eluted from Si gel with MeOH. Sugars and sugar nucleotides were analysed by PC as described earlier [15].

Other methods. Radioactivity was assayed by liquid scintillation counting using toluene containing 3% PPO and 0.3% POPOP. Protein was measured according to Lowry *et al.* [25]. Steryl glucosides were hydrolysed as previously described [7].

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