Inhibition of the Expression of Cell-associated Fructosyltransferase in *Streptococcus salivarius* by Octyl β -D-Glucopyranoside

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Octyl β -D-glucopyranoside prevented the expression of cell-associated fructosyltransferase activity in *Streptococcus salivarius* ATCC 25975 grown in batch culture or incubated in non-proliferating cell suspension medium. This effect was not due to the direct inhibition of enzyme activity nor due to the loss of active enzyme into the external medium. The prevention of enzyme expression did not appear to be due to the inhibition of a general translocation mechanism for protein secretion, since fructosyltransferase activity was not detected within the cytoplasm of lysed cells grown in the presence of octyl β -D-glucopyranoside; nor was there any observed inhibition of the secretion of the extracellular enzyme glucosyltransferase. These and other observations supported the view that fructosyltransferase was not secreted across the cytoplasmic membrane in an active form before becoming associated with the cell surface.

INTRODUCTION

The fructosyltransferase activity of *Streptococcus salivarius* is essentially cell-associated, with up to 95% of the activity bound to the cell (Garszczynski & Edwards, 1973; Jacques & Wittenberger, 1981). However, it is not known whether the enzyme is an integral part of the outer surface of the cell membrane or is attached to the peptidoglycan–polysaccharide matrix of the cell wall sacculus. In the former case it could be argued that fructosyltransferase was indeed a membrane protein with its active site on the outer surface of the cytoplasmic membrane similar to the bacterial leader peptidase of *Escherichia coli* (Wolfe & Wickner, 1984). In the latter case the enzyme could be considered to be a secreted protein similar to the outer membrane proteins of Gram-negative bacteria (Ichihara *et al.*, 1982), but presumably possessing a location similar to the major cell wall protein antigens of oral streptococci (Russell *et al.*, 1983*b*).

In contrast to the cell-associated nature of fructosyltransferase, more than 99% of glucosyltransferase activity in *S. salivarius* is expressed in the external medium. By definition, therefore, glucosyltransferase is an extracellular enzyme. Studies on the mechanisms of expression of glucosyltransferase have shown that the fatty acid dodecanoic acid prevents the synthesis and secretion of this enzyme, while the non-ionic detergent Tween 80 (polyoxyethylene sorbitan mono-oleate) stimulates its production (Wittenberger *et al.*, 1978; Jacques, 1983). These effects are believed to be due in part to an alteration in the fluidity of the membrane (Jacques *et al.*, 1985). The fatty acid is presumed to intercalate with the membrane (Petit-Glatron & Chambert, 1981), while the detergent is known to enhance the number of octadecenoic acid residues present in the membrane lipids (Umesaki *et al.*, 1977; Jacques *et al.*, 1985). It is reasonable to suggest, therefore, that if fructosyltransferase were essentially a secreted protein associated with the cell-wall sacculus, then its expression on the cell surface would be modulated in a similar manner to glucosyltransferase by amphiphilic compounds. For this reason, the formation of cell-associated fructosyltransferase has been investigated in the presence of dodecanoic acid and Tween 80, and these studies have been further extended by using the non-ionic detergent octylglucoside (octyl β -D-glucopyranoside) which is known to disrupt membrane structure (Helenius *et al.*, 1979; Alonso *et al.*, 1982).

While differences in the degree of modulation of the expression of cell-associated fructosyltransferase and extracellular glucosyltransferase were apparent when cells were incubated in the presence of either dodecanoic acid or Tween 80, the differential effects of octylglucoside on the expression of these two enzymes supported the hypothesis that their mechanisms of expression differed, and that this difference was most probably associated with the final location of the two proteins relative to the cell surface.

METHODS

Chemicals. [U-glucosyl- ^{14}C]sucrose, [U-fructosyl- ^{14}C]sucrose and the liquid scintillation cocktail Aquasol-2 were purchased from New England Nuclear. Octylglucoside (octyl β -D-glucopyranoside) and chloramphenicol were purchased from Calbiochem-Behring, dodecanoic (lauric) acid from Ajax Chemicals, CM-Sephadex G25 from Pharmacia and Tween 80 (polyoxyethylene sorbitan mono-oleate) from BDH. The M-1 enzyme preparation from *Streptomyces globisporus* 1829 was kindly supplied by K. Yokogawa, Dainippon Pharmaceutical Co. Ltd, Osaka, Japan.

Organisms and growth. *Streptococcus salivarius* ATCC 25975 was stored or grown in semi-defined medium as previously described (Jacques, 1983), except that all batch cultures contained 1 mM- CaCl_2 . All experiments reported were repeated at least once and the data presented are typical of those obtained.

Non-proliferating cell suspensions. These were used to monitor the expression of cell-associated fructosyltransferase activity and extracellular glucosyltransferase activity. The suspension medium was essentially the same as that previously described except that 1 mM- CaCl_2 was routinely added (Jacques, 1983). In order to prepare *S. salivarius* free of cell-associated fructosyltransferase activity, cells from a 100 ml culture were harvested by centrifugation at mid-exponential phase (10000 g, 4 °C, 10 min), and treated for 30 min in the standard inactivation system described by Jacques & Wittenberger (1981). Following this treatment, the cells were harvested by centrifugation (10000 g, 4 °C, 10 min), washed and resuspended to 6 ml in 10 mM-potassium phosphate buffer, pH 6.5, containing 10 mM- MgSO_4 . These cells were used to prepare non-proliferating cell suspensions. The inclusion of 10 mM- Mg^{2+} in the washing buffer was essential for the subsequent production of both glucosyltransferase activities. Other ions tested fell into the sequence: none < Mg^{2+} (2 mM) < Ba^{2+} (2 mM) = Ca^{2+} (2 mM) < Mg^{2+} (10 mM; 50 mM) = Sr^{2+} (2 mM). The use of Ba^{2+} , Ca^{2+} or Sr^{2+} however, led to precipitates forming in the suspension medium.

Assay of glucosyltransferase activities. Extracellular glucosyltransferase activity and cell-associated fructosyltransferase activity were assayed as previously described, except that the assay mixture for measuring fructosyltransferase activity contained 1 mM- CaCl_2 (Jacques, 1983, 1984). One unit of enzyme activity (U) was defined as the amount of glucosyltransferase or fructosyltransferase that catalysed the incorporation of 1 μmol of the glucose or of the fructose moiety of sucrose, respectively, into 75% (v/v) ethanol-insoluble polysaccharide min^{-1} . In the case of fructosyltransferase, the measured values were multiplied by a factor of 2.2 to account for the limiting concentration of sucrose in the assay system (Jacques, 1984).

Preparation and analysis of fatty acids. The membrane-bound lipids were extracted and the methyl esters of the fatty acids prepared and analysed as reported previously (Markevics & Jacques, 1985).

Purification of the M-1 enzyme preparation. The N-acetylmuramidase enzyme, M-1 (Yokogawa *et al.*, 1975), was purified free from proteolytic activity on a CM-Sephadex G25 column (90 $\times$ 1.6 cm) using 150 mM-sodium phosphate buffer, pH 7.0, as eluant (Siegel *et al.*, 1981).

Lysis of cells. The initial rate of lysis of cells grown to early stationary phase was determined by monitoring the change in optical density at 37 °C of cell suspensions (0.70–0.95 mg dry wt ml^{-1}) in 10 mM-potassium phosphate buffer, pH 6.5, containing 10 mM- MgSO_4 , 1 mM- CaCl_2 and 20 μg purified M-1 enzyme ml^{-1} . Measurements were taken at 10 min intervals with a Corning colorimeter model 252 fitted with a 600 nm filter (Evans Electroselenium). Lysed cells were also used for measurements of intracellular fructosyltransferase activity.

RESULTS

Protein synthesis was shown to be required for the expression of cell-associated fructosyltransferase activity in non-proliferating cell suspensions after inactivation of the enzyme in the standard inactivation system (data not shown). However, the inactivation treatment not only gave variable results for the absolute amount of enzyme subsequently synthesized, but also substantially reduced the potential for the expression of both cell-associated fructosyltransferase and extracellular glucosyltransferase activities. The production of cell-associated fructosyltransferase was taken therefore as the difference between the level of activity on harvest and that after incubation for 120 min in the non-proliferating cell suspension

Fig. 1. Effect of (a) dodecanoic acid and (b) Tween 80 on the expression of cell-associated fructosyltransferase activity (■) and extracellular glucosyltransferase activity (●) in non-proliferating cell suspensions.

Table 1. Effect of chloramphenicol on the production of cell-associated fructosyltransferase and extracellular glucosyltransferase in non-proliferating cell suspensions

Cells from a 100 ml culture were harvested by centrifugation at mid-exponential phase and used to establish non-proliferating cell suspensions. Enzyme production was taken as the difference in the enzyme activities at zero time and after 120 min incubation at 37 °C.

Time (min)	Chloramphenicol (μg ml ⁻¹)	Fructosyltransferase activity [mU (mg dry wt) ⁻¹]	Glucosyltransferase activity [mU (mg dry wt) ⁻¹]
0	0	553	0
120	0	1807	300
Difference	0	1254	300
0	50	553	0
120	50	632	25
Difference	50	79	25

medium without prior treatment of the cells in the standard inactivation system. This method also measured newly expressed enzyme activities (Table 1), but the absolute values of cell-associated fructosyltransferase and extracellular glucosyltransferase were reproducible to values of 1396 ± 118 mU (mg dry wt)⁻¹ and 283 ± 36 mU (mg dry wt)⁻¹, respectively (mean $\pm$ SD).

Effect of dodecanoic acid, Tween 80 and octylglucoside on cell-associated fructosyltransferase and extracellular glucosyltransferase production in non-proliferating cell suspensions

Dodecanoic acid prevented both the expression of cell-associated fructosyltransferase and the production of extracellular glucosyltransferase (Fig. 1a). However, a higher concentration – about 110 μM – of dodecanoic acid was required to elicit a 50% inhibition of the expression of cell-associated fructosyltransferase compared with the concentration of about 50 μM required to produce an equivalent inhibition in extracellular glucosyltransferase production.

Differences in the degree of expression of the two enzymes also occurred in the presence of Tween 80. The expression of extracellular glucosyltransferase was enhanced several-fold by 0.05 μl Tween 80 ml⁻¹ while the expression of cell-associated fructosyltransferase was only stimulated by 35% in the presence of an equivalent concentration of Tween 80 (Fig. 1b).

Fig. 2

Fig. 2. Inhibition of the expression of cell-associated fructosyltransferase activity by octylglucoside in non-proliferating cell suspensions. ■, Cell-associated fructosyltransferase activity; ●, extracellular glucosyltransferase activity.

Fig. 3

Fig. 3. Differential effect of octylglucoside on the expression of cell-associated fructosyltransferase activity (■) and extracellular glucosyltransferase activity (●) in batch culture.

Octylglucoside, however, had a differential effect on the expression of the two enzymes. Increasing concentrations of octylglucoside within the range 0–5 mM prevented the expression of cell-associated fructosyltransferase activity. In contrast, extracellular glucosyltransferase production reached a maximum at 2 mM-octylglucoside, falling to a value similar to that observed in the absence of added detergent at a concentration of 5 mM (Fig. 2).

Octylglucoside in the range 0–5 mM had no effect on the activity of either enzyme when added to the assay mixture. Furthermore, the results presented in Fig. 2 implied that the cell-associated fructosyltransferase activity present in the harvested cells [683 ± 126 mU (mg dry wt)⁻¹ (mean $\pm$ SD)] was detected at the end of the 120 min incubation in the non-proliferating cell suspension medium even in the presence of 5 mM-octylglucoside. As this activity was also recovered when 50 μ g chloramphenicol ml⁻¹ was added to the suspension mixture (data not shown), the detergent appeared only to affect the expression of newly formed enzyme and not to alter the activity of that already synthesized.

Effect of octylglucoside on the expression of glycosyltransferases in batch culture

When batch cultures of *S. salivarius* were grown in the presence of 0–5 mM-octylglucoside, the degree of expression of cell-associated fructosyltransferase activity decreased with increasing concentrations of the detergent in a similar manner to that in non-proliferating cell suspensions. In contrast, extracellular glucosyltransferase production was stimulated approximately fivefold in the presence of 3–5 mM-octylglucoside (Fig. 3). In the absence of added detergent, the doubling time for *S. salivarius* was 32 ± 3 min (mean $\pm$ SD). No change in this value was observed in the presence of 1–3 mM-octylglucoside. Detectable increases in the doubling times of the cells to 40 ± 1 min and 58 ± 2 min were not observed until the concentration of octylglucoside was increased to 4 mM and 5 mM, respectively. A concentration of 10 mM-octylglucoside was bacteriostatic. Since the inhibition of the expression of cell-associated fructosyltransferase by 3 mM-octylglucoside reached 94% of the value observed at a concentration of 5 mM, it appeared that the detergent preferentially affected enzyme expression before any noticeable effects on the doubling time of the cells.

Table 2. *Fatty acid profiles of cells grown in the presence of octylglucoside*

The results are the means of duplicate experiments. An unidentified fatty acid with a retention time between C_{16:1} and C_{18:0} was present in trace amounts (<1%) in each sample.

Octyl- glucoside concn (mM)	Amount of fatty acid (%)						U/S ratio*
	Tetra- decanoic (C _{14:0})	Hexa- decanoic (C _{16:0})	Hexa- decenoic (C _{16:1})	Octa- decanoic (C _{18:0})	Octa- decenoic (C _{18:1})	Eicos- enoic (C _{20:1})	
0	4.3	38.7	10.4	5.9	32.1	8.6	1.04
1	5.5	42.8	9.1	6.6	29.1	7.0	0.82
2	6.2	42.1	8.8	7.9	26.3	8.7	0.78
3	5.2	42.1	6.9	9.5	27.5	8.9	0.76
4	5.7	41.6	7.7	12.8	22.6	9.6	0.66
5	3.3	42.6	6.1	16.4	23.8	11.9	0.61

* Ratio of unsaturated to saturated fatty acids.

No increase in fructosyltransferase activity was evident in the cell-free culture fluid (approximately 10% of the cell-associated value) when cells were grown in the presence of octylglucoside up to a concentration of 5 mM. When cells were lysed with a protease-free preparation of the *N*-acetylmuramidase M-1, no further fructosyltransferase activity was observed in the digests. For instance, in the absence of added detergent, cells with a measured cell-associated fructosyltransferase activity of 532 mU (mg dry wt)⁻¹ possessed an activity equivalent to 533 mU (mg dry wt)⁻¹ after lysis. The corresponding values for cells grown in the presence of 5 mM-octylglucoside were 61 mU (mg dry wt)⁻¹ for whole cells and 57 mU (mg dry wt)⁻¹ for the lysate. These results indicated that it was unlikely that octylglucoside was preventing either the attachment of newly secreted and active fructosyltransferase to the cell surface, or preventing transport of active preformed enzyme across the cytoplasmic membrane. The enhancement of extracellular glucosyltransferase synthesis and secretion under these circumstances lent further support to this view (Fig. 3).

It is of interest, however, that the rate of lysis by enzyme M-1 determined over a 90 min period was about threefold greater for cells grown in the presence of 5 mM-octylglucoside than for those grown in its absence (data not shown).

Reversibility of the effect of octylglucoside on cell-associated fructosyltransferase expression

A culture (50 ml) was grown in the presence of 5 mM-octylglucoside to mid-exponential phase, harvested by centrifugation (10000 g, 4 °C, 10 min) and washed with 10 ml 10 mM-potassium phosphate buffer, pH 6.5, containing 10 mM-MgSO₄. Cells equilibrated to 37 °C in 3 ml of the same buffer were used to inoculate fresh medium either free of added octylglucoside or containing 5 mM-octylglucoside. An inoculum of 1 ml cell suspension per 10 ml medium was used. At various times, samples of the cultures were removed aseptically and harvested and washed as above in the presence of 50 µg chloramphenicol ml⁻¹. Cells inoculated into medium free of octylglucoside generally remained in lag phase for 60 min. After this period exponential growth ensued, and the level of cell-associated fructosyltransferase activity increased from about 100 mU (mg dry wt)⁻¹ to about 600 mU (mg dry wt)⁻¹ after a further 90 min. This reappearance of fructosyltransferase activity was prevented by the addition of 50 µg chloramphenicol ml⁻¹ to the culture medium. A low level [about 50 mU (mg dry wt)⁻¹] of cell-associated fructosyltransferase activity was maintained throughout the 150 min incubation period by those cells reinoculated into medium containing 5 mM-octylglucoside.

*Fatty acid composition of the membrane lipids of *S. salivarius* grown in the presence and absence of octylglucoside*

Table 2 shows the results for the analyses of the fatty acid profiles of the membrane lipids of *S. salivarius* grown to early stationary phase in the presence of different concentrations of octylglucoside. The unsaturated to saturated fatty acid ratio fell as the concentration of

octylglucoside increased. This was due to an increase in octadecanoic acid at the expense of octadecenoic and hexadecenoic acids, since the level of the other unsaturated fatty acid, eicosenoic acid, slowly increased with increasing concentrations of octylglucoside. The results indicated that the membrane fatty acid composition of *S. salivarius* changed in response to the presence of octylglucoside in the culture medium, and consequently that the detergent was capable of perturbing the structure of the cytoplasmic membrane.

DISCUSSION

The problem of defining whether the fructosyltransferase of *S. salivarius* is associated with the cytoplasmic membrane or the peptidoglycan matrix with its associated polymers has been exacerbated by the inability, so far, to produce stable protoplasts free of the cell wall sacculus. Partial removal of the cell wall with the *N*-acetylmuramidase M-1, results in the release of approximately 75% of the fructosyltransferase activity from the cell (Jacques & Wittenberger, 1981). However, this release of enzyme activity is also accompanied by the release of lipid components (unpublished observations) implying that some disruption of the cytoplasmic membrane is occurring during spheroplast formation.

The present study indicated subtle differences in the effects of Tween 80 and dodecanoic acid on the expression of cell-associated fructosyltransferase compared with their effects on the truly secreted protein, glucosyltransferase. Octylglucoside, however, affected the expression of cell-associated fructosyltransferase and extracellular glucosyltransferase in a differential manner. While the altered pattern for the fatty acids of the membrane lipids indicated that octylglucoside could perturb the cytoplasmic membrane, the detergent also affected other surface properties since a significant increase in the rate of lysis of cells by enzyme M-1 was observed when cells were grown in the presence of octylglucoside. This may have been due to alterations in the peptidoglycan structure of the cell walls and/or its associated polymers (Knox *et al.*, 1979), or due to activation of autolytic activity by the detergent (Ved *et al.*, 1984). It should be borne in mind, however, that some of the enzymes required for cell wall biosynthesis are located in the cytoplasmic membrane underlying the cell wall sacculus, and that lipid carriers are required for translocation of cell wall intermediates across the membrane (Shockman & Barrett, 1983). Consequently, detergent interaction with the cytoplasmic membrane could account for an altered cell wall structure by interfering with these processes. These alternative possibilities make it difficult to determine the primary site of action of octylglucoside in preventing the surface expression of fructosyltransferase in *S. salivarius*.

Octylglucoside did not inhibit fructosyltransferase activity nor did it cause an increase in intracellular activity. It could be argued that the detergent physically prevented the expression of the enzyme on the outer surface of the membrane and thus led directly or indirectly to inhibition of further synthesis of the protein. This would imply that fructosyltransferase was a (trans)membrane protein with its active site on the outer surface of the membrane. The failure of a hybrid lipoprotein to insert into the outer membrane of *E. coli* has recently focused attention on the importance of the lipid domain in the assembly of such membrane proteins (Yu *et al.*, 1984).

Since extracellular glucosyltransferase production occurred at normal or enhanced rates under conditions where cell-associated fructosyltransferase expression was prevented by octylglucoside, one could envisage alternative explanations for the mode of action of the detergent. For instance, octylglucoside could be affecting the activation of the enzyme either by preventing its modification on association with the membrane by interfering with a mechanism similar, say, to that for the cell-association of penicillinase (Chang *et al.*, 1982; Neilsen & Lampen, 1982), or by similarly interfering with its association with components of the cell wall sacculus. A third possible explanation is that another enzyme(s) is required for activation of fructosyltransferase and that the detergent interferes with the expression of the activity of this enzyme. The existence of multiple molecular weight species of fructosyltransferase in some oral streptococci (Jacques & Wittenberger, 1981; Russell *et al.*, 1983*a*) could be explained by any of these latter alternatives. It has also been suggested that modulation of the components ($\Delta\psi$, ΔpH) of the proton motive force $\Delta\bar{\mu}_{\text{H}^+}/F$ can lead to alterations in the levels of extracellular glycosyltransferases in oral

streptococci (Keevil *et al.*, 1984; West *et al.*, 1984). Thus a possible fourth explanation for the effects of octylglucoside is that the surfactant alters the $\Delta\bar{\mu}_{H^+}/F$ or its components so as to enhance extracellular glucosyltransferase production while suppressing cell-associated fructosyltransferase expression. However, direct correlations between modulation of $\Delta\bar{\mu}_{H^+}/F$ and changes in the expression of glycosyltransferases in oral streptococci have not yet been forthcoming as no direct measurements have been made of the components of the $\Delta\bar{\mu}_{H^+}/F$ under the conditions that produce changes in the levels of the enzymes (Markevics & Jacques, 1985). Regardless of the mechanism prevailing, however, the fructosyltransferase of *S. salivarius* would not appear to be secreted in an active form across the cytoplasmic membrane in the same manner as glucosyltransferase to become subsequently associated with the cell surface.

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