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Note

Calcium dependence of the cell-associated fructosyltransferase of *Streptococcus salivarius*

NICHOLAS A. JACQUES
Institute of Dental Research, 2 Chalmers Street, Surry Hills NSW 2010 (Australia)
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The production of fructan from sucrose by the action of the fructosyltransferase(s) (FTase) of oral streptococci appears to be dependent upon metal ions, but the exact nature of this dependence has not yet been elucidated[1,2]. In the case of the purified extracellular FTase of *Streptococcus mutans*, the addition of metal ions to an active preparation had no effect on enzyme activity, although Ca^{2+} was observed[1] to prevent inactivation of the enzyme between 40 and 55°. EDTA was shown to inhibit the FTase, but this effect was reversed by a number of divalent ions, including Ca^{2+}, Sr^{2+}, Mn^{2+}, Cd^{2+}, Ni^{2+}, Cu^{2+}, or Pb^{2+}, thus only implying, amongst other possibilities, a role for a metal ion in enzyme activity[1]. In contrast, the extracted cell-associated FTase of *S. salivarius* SS2 was reported to be inhibited by both Ca^{2+} and Ni^{2+} ions.

Recently, it was shown that the cell-associated FTase of *S. salivarius* ATCC25975 was inactivated by a process mediated by Cu^{2+}. Of interest was the observation that Ca^{2+} could prevent this loss of enzyme activity[3]. The present study into the role of ions affecting the cell-associated FTase activity of *S. salivarius* has shown that measurable enzyme activity declines as cells are subcultured in medium essentially devoid of added metal ions except for Na^+, K^+, Mg^{2+}, Fe^{2+}, and Mn^{2+}. This observation has afforded proof that Ca^{2+} is a necessary cofactor for the cell-associated FTase activity of *S. salivarius* and permitted the kinetics of the enzyme to be studied in some detail. Furthermore, the Cu^{2+}-mediated inactivation of the cell-associated enzyme has been confirmed as being due to a process other than simple competition with the activating metal-ion species.

EXPERIMENTAL

Organism and medium. — *Streptococcus salivarius* ATCC25975 was used throughout these studies. Stock cultures were stored at 4° in semi-defined medium containing an excess of calcium carbonate (20 mg/mL). Transfers were made to fresh medium every month. The semi-defined medium was a modification of the strictly defined medium described by Wittenberger *et al.*[4] in which all amino acids

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except cysteine were replaced with casein hydrolysate (5 g/L) (Oxoid Ltd., London, England) and no further sodium chloride was added. The amount of each amino acid in the casein hydrolysate was known from the manufacturer's analysis. D-Glucose (25mM) served as the fermentable carbon source.

Chemicals. — [U-^{14}C]Fructose-labelled sucrose and the liquid-scintillation cocktail Aquasol-2 were purchased from New England Nuclear Corp., Boston, MA, U.S.A. Fructanase was kindly supplied by Dr. G. J. Walker. The fraction containing the enzyme had been obtained from the culture fluid of *Streptococcus mutans* OMZ176 grown in continuous culture at a dilution rate of 0.15 h^{-1} and pH 6.0 by column chromatography on hydroxylapatite. The chlorides of Mg^{2+}, Ca^{2+}, Sr^{2+}, and Ba^{2+} were used and each was of analytical-reagent grade.

Growth measurements. — *S. salivarius* was subcultured from stock suspensions into 5-mL volumes of medium in 14-mm test tubes. The cultures were incubated at 37° under anaerobic conditions (5% CO_2/95% N_2) and either subcultured with the aid of a loop every 24 h or used as an inoculum for batch cultures after 16 h. Batch cultures of *S. salivarius* were grown at 37° in screw-cap bottles containing 50 mL of the semi-defined medium. A 10% inoculum was used. Growth was monitored in 14-mm test tubes with the aid of a Corning Colorimeter model 252, using a 600-nm filter (Evans Electroselenium Ltd., Halstead, Essex, England). The dry weight of bacteria growing exponentially was linearly related to absorbance in this system, over the range 0.000–1.100. An absorbance of 1.000 was equivalent to 0.82 mg (dry wt) of cells per mL. As the cultures entered stationary phase, the cells were cooled on ice and harvested by centrifugation (12,000*g*, 4°, 10 min). After washing twice with 100-mL volumes of 10mM potassium phosphate buffer pH 6.5, the cells were resuspended to 50 mL in the same buffer.

Assay of cell-associated FTase activity. — Cell-associated FTase activity was assayed by using [U-^{14}C]fructose-labelled sucrose (1.39 mCi/mol) as previously described[3], except that the amino acid supplement was deleted from the assay mixture. The source of cell-associated FTase was generally 100 μL of a suspension of cells having a dry wt equivalent to 0.8±0.1 mg per mL. The radioactivity incorporated into the fructan was measured in a Beckman liquid-scintillation counter (model LS9000, Beckman Instruments Inc., Irvine, CA, U.S.A.). One unit of enzyme activity (U) was defined as the amount of FTase that catalyzed the incorporation of 1 μmol of the fructose moiety of sucrose into 75% ethanol-insoluble polysaccharide per min. In all cases, FTase activity was expressed as units per mg (dry wt) of cells (U/mg dry wt), as a means of indicating the amount of enzyme associated with a given cell-mass.

When, at the end of the assay, the final product was not treated with 75% ethanol but was washed instead with 10mM potassium phosphate buffer (pH 6.5) containing 10mM $MgSO_4$ (buffer A), 98% of the otherwise precipitated radioactivity was lost through the filter. By simply removing the cells by centrifugation (10,000*g*, 15 min, 4°), ~84% of the radioactivity incorporated could be recovered in the supernatant fraction, indicating that the polymer product was water-soluble.

Conversely, when the cells removed from such an assay mixture were washed in buffer A, no radioactivity could be detected in the cells, indicating that the fluoride in the assay mixture was adequately preventing the uptake and metabolism of the added sucrose. A dialysed and boiled preparation of the radioactively labelled polymer was completely hydrolysed (>93%) by a fructanase enzyme preparation from *S. mutans* OMZ176. The fructanase of *S. mutans* OMZ176 has been shown to hydrolyze both the β-(2→6) and β-(2→1) branch-linkages present in *S. salivarius* fructans of known composition[5]. As further information concerning the exact nature of the linkages in the fructan specifically produced by *S. salivarius* strain ATCC25975 was unknown, it seemed appropriate still to refer to this enzyme(s) as FTase rather than levansucrase (EC 2.4.1.10)[3].

RESULTS AND DISCUSSION

Ca^{2+} dependence of cell-associated FTase activity. — Subculturing cells of *S. salivarius* in medium devoid of Ca^{2+} at 24-h intervals for 7 days resulted in a loss of measurable, cell-associated FTase activity. When 2mM Ca^{2+} was added to the assay mixture, a 12-fold increase in detectable cell-associated FTase activity was noted. A similar increase was observed by simply growing cells in the presence of 2mM Ca^{2+}. No alteration in growth rate was apparent, irrespective of whether or not Ca^{2+} was added to the growth medium. Of the metal ions of Group II of the periodic table (Mg^{2+}, Ca^{2+}, Sr^{2+}, and Ba^{2+}), each tested up to mM concentration in the assay mixture, only Ca^{2+} activated enzyme activity. Addition of Ca^{2+} to the assay mixture at the end of the 60-min incubation at 37° failed to increase measurable enzyme activity, indicating that Ca^{2+} did not simply cause entrapment of radioactive sucrose by the polymer.

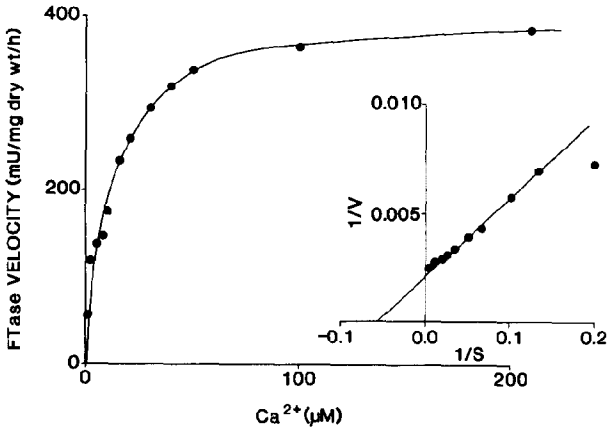

Fig. 1. Effect of Ca^{2+} on the activity of cell-associated fructosyltransferase. Cell-associated FTase was measured over the range of 0–200μM Ca^{2+} at 10mM concentration of sucrose. The insert shows the Lineweaver–Burk plot of these data.

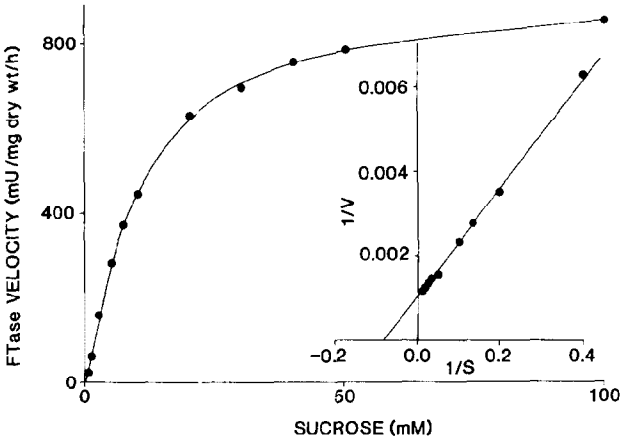

Fig. 2. Activity of cell-associated fructosyltransferase in the presence of sucrose. Cell-associated FTase was measured over the range of 0–100mM sucrose at a Ca^{2+} concentration of mM. The insert shows the Lineweaver–Burk plot of these data.

The activity of the enzyme in the presence of 10mM sucrose and increasing concentrations of calcium followed Michaelis–Menten kinetics, as shown by a typical result plotted in Fig. 1. The Lineweaver–Burk analysis of these data (insert Fig. 1) allowed the V_{max} value for the cell-associated FTase activity in the presence of 10mM sucrose to be calculated as 476 mU/mg dry wt/h in this instance or an 8-fold increase over the value of 57 mU/mg dry wt/h obtained in the absence of added Ca^{2+}. The K_m value of 17.4μM indicated the relatively high affinity of the enzyme for the divalent metal ion. The average K_m for Ca^{2+} was determined from four separate experiments and found to be 18 ±2μM. The result was in marked contrast with the reported[2] inhibition by Ca^{2+} of the solubilized cell-associated FTase of *S. salivarius* SS2.

Affinity of cell-associated FTase for sucrose in the presence of Ca^{2+}. — The effect of increasing the concentration of sucrose in the range of 0.5–100mM on the activity of cell-associated FTase in the presence of mM Ca^{2+} was determined. The rate of enzyme activity also followed Michaelis–Menten kinetics within this range of sucrose concentrations (Fig. 2). Lineweaver–Burk analysis of these data (insert Fig. 2) gave a K_m value for sucrose of 12.1mM indicating the relatively poor affinity of the enzyme for its substrate. The average K_m value obtained from three separate experiments was 12.0 ±0.1mM. Similar values of 14 and 19mM for K_m of the enzymes from *Bacillus subtilis* and *B. amyloliquefaciens* have been reported[6].

As the K_m value for sucrose is relatively high, a 1% error in determining the maximum rate of cell-associated FTase activity would require sucrose to be present at a concentration in excess of M. However, at this concentration, substrate inhibition of the enzyme was observed. Compared with the value obtained with 100mM sucrose, a 4 and 32% decrease in the calculated V_{max} for cell-associated FTase activity was observed with sucrose at a concentration of 500mM and M, respectively.

For this and other technical reasons, assays were routinely performed at a sucrose concentration of 10mM (giving rise to ~0.5 V_{max}) and a Ca^{2+} concentration of mM. When the maximum rate of activity for a sample of cell-associated FTase was required, it was calculated according to the equation

$$V_{max} = 2.2\, V_{measured}$$

which was derived from Michaelis–Menten analysis of the data, on the assumption that K_m for sucrose is 12mM.

Effect of copper on cell-associated FTase activity. — The Cu^{2+} ion has been shown to mediate in the apparent proteolytic inactivation of cell-associated FTase in *S. salivarius* ATCC25975, but not by directly affecting enzyme activity, as the solubilized form of the enzyme was not affected by Cu^{2+} ion[3]. In light of the metal-ion dependence for cell-associated FTase activity, it was of interest to show that the Cu^{2+}-mediated inactivation of the cell-associated enzyme was not due to competition by the Cu^{2+} ion for the Ca^{2+} ion activator. If Cu^{2+} were inhibiting FTase in a reversible manner, either competitively (alteration in K_m, same V_{max}) or non-competitively (same K_m decreased V_{max}), it should be possible to construct Lineweaver–Burk plots for a range of Ca^{2+} concentrations that were linear for

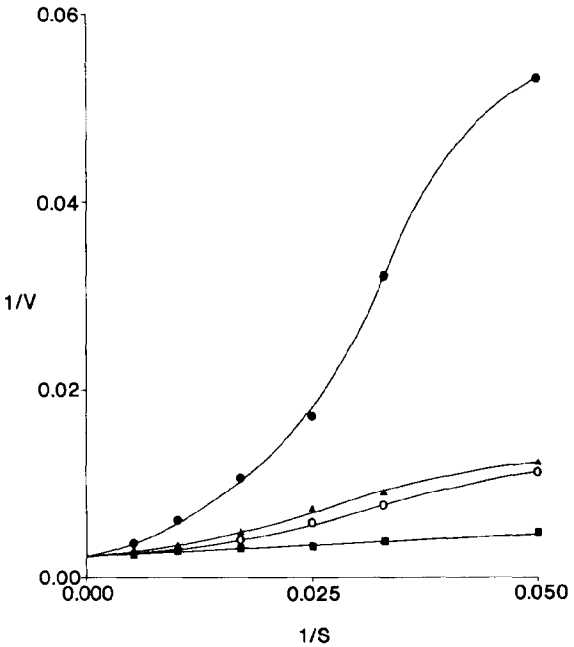

Fig. 3. Effect of Cu^{2+} on Ca^{2+}-dependent cell-associated fructosyltransferase activity. Lineweaver–Burk plots of cell-associated FTase activity were constructed for a range of Ca^{2+} concentrations at each of a number of Cu^{2+} concentrations. Sucrose was present at a concentration of 10mM. (■) no Cu^{2+}; (○) Cu^{2+} (5 μM); (▲) Cu^{2+} (10 μM); and (●) Cu^{2+} (30 μM).

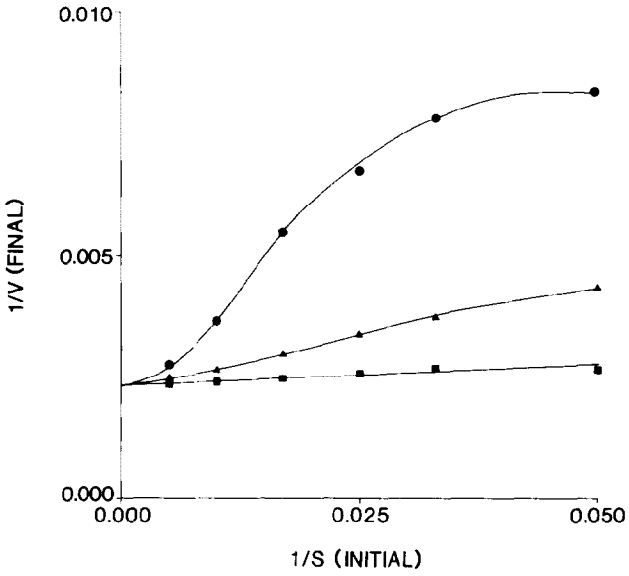

Fig. 4. Cu^{2+}-Mediated inactivation of cell-associated fructosyltransferase activity. Cell-associated FTase was incubated with non-radioactive sucrose (10mM) for 30 min in the presence of a range of Ca^{2+} concentrations [Ca^{2+} (initial)] at each of a number of Cu^{2+} concentrations. After 30 min, the Ca^{2+} level was increased to mM and radioactive sucrose added without carrier to give 1 39 mCi/mol. Incubation was continued for a further 60 min, and the amount of soluble fructan produced by each condition determined. (■) no Cu^{2+}, (▲) Cu^{2+} (5 and 10mM); (●) Cu^{2+} (30μM).

each of any number of Cu^{2+} concentrations. This was attempted with cells that possessed little measurable, cell-associated FTase activity in the absence of Ca^{2+} at Cu^{2+} concentrations of 0, 5, 10, and 30μM for each of the Ca^{2+} concentrations of 20, 30, 40, 60, 100, and 200 μM. Under these conditions, the apparent degree of inhibition afforded by each concentration of Cu^{2+} approached zero in a non-linear fashion as the Ca^{2+} concentration increased (Fig. 3), confirming that Cu^{2+} was not simply acting reversibly to inhibit the enzyme[3].

Support for the view that Cu^{2+} was a mediator in the inactivation of cell-associated FTase was obtained by modifying the foregoing experiment. The concentration of Ca^{2+} ion was increased to mM after 30 min of incubation, radioactive sucrose devoid of carrier added at this point, and the assay continued for a further 60 min. A series of curves having profiles similar to those in Fig. 3 were obtained, confirming that increasing the concentration of Ca^{2+} to a protective level[3] did not reverse the initial effects of the Cu^{2+} ion (Fig. 4). The results showed that the Cu^{2+}-mediated inactivation of cell-associated FTase was not due to competition with the Ca^{2+} ion cofactor. However Ca^{2+} (mM) could prevent Cu^{2+} inactivation of the enzyme[3], at least during the 30-min period over which inactivation was studied. This observation suggested that Ca^{2+} might also protect a site on the cell-associated enzyme that was primarily involved in the Cu^{2+}-mediated inactivation process.

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