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THE PREDOMINANTLY NONHYDROLYTIC ACTION OF ALPHA AMYLASES ON α-MALTOSYL FLUORIDE

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ABSTRACT

Crystalline alpha amylases from a number of sources utilized α-maltosyl fluoride as a glycosyl donor and acceptor at high rates (~10 to ~1550 μmol/min/mg of protein, for 30 mM substrate). All enzymes catalyzed conversion of this compound into maltooligosaccharides in preference to causing its hydrolysis Maltotetraosyl fluoride and maltooligosaccharides of d p 3 to 6+ accounted for 75–93% (by weight) of early reaction-products At a late stage, the yield of maltooligosaccharides was 2–5 times that of maltose, with chains as long as 12 D-glucosyl residues formed by one amylase (from *Asp oryzae*), which utilized α-maltosyl fluoride as a donor and as an acceptor at extremely high rates These results indicate that alpha amylases have a substantial capacity for binding two molecules of this small substrate in a distinctive way, with the C–F glycosylic bond of one and the free C-4 hydroxyl group of the other located in the region of the enzyme's catalytic groups, thereby favoring glycosylation of the suitably positioned acceptor over solvent water Hydrolysis is assumed to prevail when only a single substrate molecule or segment binds to alpha amylase with a (1→4)-α-D-glucosidic linkage or glycosylic C–F bond positioned at the catalytic center The present demonstration that glycosyl-transfer reactions can be dominantly expressed by alpha amylases, given an appropriate substrate, illustrates the inadequacy of the usual characterization of these enzymes as hydrolases that produce overwhelming hydrolysis of all substrates

INTRODUCTION

The study of enzymically catalyzed glycosylation reactions that take place without glycosidic bond-cleavage has been found to offer a unique means of gaining new insight into the scope and mechanism of carbohydrase action[1–7] An early indication of the potentiality of this approach was obtained[1] when purified alpha

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amylases from several biological sources were found to catalyze the formation of maltose and maltooligosaccharides from α-D-glucopyranosyl fluoride in preference to causing hydrolysis of this nonglycosidic substrate These results provided a much different view of the catalytic capabilities of alpha amylases than that afforded by their customary representation[8-13] as hydrolases having an overriding affinity for water and scant capacity to cause glycosyl transfer Of equal importance, considering that the observed reactions required the use of relatively high concentrations of both α-D-glucosyl fluoride and amylase, was the recognition that α-maltosyl fluoride was an obligatory intermediary in the path leading to saccharide formation Thus, it appeared likely that a more complete understanding of the catalytic capabilities of alpha amylases might be reached by using α-maltosyl fluoride as a glycosyl substrate

Initially, only very crude preparations of this previously unreported compound were available for study, and these permitted only a limited amount of information to be gathered Through their use, however, various alpha amylases were observed[2] to bring about extensive formation of maltooligosaccharides from much lower concentrations of α-maltosyl fluoride than from α-D-glucosyl fluoride These findings prompted our development of a procedure for preparing pure α-maltosyl fluoride[5] The present study deals with the utilization of the purified substrate by crystalline alpha amylases from a number of sources, specifically, with the kinds and amounts of reaction products found under different conditions, and with rates of utilization of α-maltosyl fluoride by the individual enzymes The findings are discussed with respect to the identity of the underlying chemical change effected by alpha amylases, and the nature of the factors that may determine whether or not water enters reactions catalyzed by these enzymes A preliminary account of the findings has been presented[14]

RESULTS AND DISCUSSION

The present work provides evidence that highly purified alpha amylases have a substantial capacity to utilize α-maltosyl fluoride both as a donor and as an acceptor substrate A finding of particular significance is that these enzymes catalyze transfer reactions dominantly, even with the substrate at low concentration In one set of experiments, for example, α-maltosyl fluoride (5 6 mM) was subjected to the action of different alpha amylase preparations in concentrations sufficient to convert the greater part (73–97%) of the substrate into products in 20 min at 30° Components of the digests were recovered essentially quantitatively following paper chromatography, and their yields determined as D-glucose As shown in Table I, maltooligosaccharides containing from three to six D-glucose residues were produced in abundance from the α-maltosyl fluoride by all six alpha amylases, their combined yield exceeded that of maltose in all but one digest It is evident that, under these experimental conditions (where the ratio of water to that of the fluoro sugar was 10,000 1), nearly all of the alpha amylases catalyzed glycosyl transfer-reactions to a greater extent than hydrolysis

More-detailed information on the action of the various alpha amylases on α-

TABLE I

ACTION OF CRYSTALLINE ALPHA AMYLASES ON 5 6 mM α-MALTOSYL FLUORIDE

Components recovered from digests	*Yields of components from digests with different amylases (as μmol of* D-*glucose)*[a]					
	B subtilis amyloliquefaciens *(100 μg/ml)*	B subtilis *heat stable (100 μg/ml)*	B subtilis biotechus nagase *(100 μg/ml)*	*Hog pancreas (8 μg/ml)*	B subtilis amylosaccharlticus *(2 μg/ml)*	Asp oryzae *(1 ug/ml)*
Maltotriose through maltohexaose	3 6	3 4	3 2	3 6	2 6	2 1
Maltose	0 7	0 7	0 7	1 2	2 4	3 5
Residual α-maltosyl fluoride	1 3	1 2	1 6	1 2	0 4	0 2

[a]Mixtures of α-maltosyl fluoride (5 6 mM) and of alpha amylase (as listed) in 0 025M acetate buffer (pH 5 6) were incubated for 20 min at 30° Components from 0 54 ml of each digest (3 0 μmol substrate) were recovered following chromatography, and determined as D-glucose[15]

maltosyl fluoride was gained through a series of experiments in which enzymic digests were analyzed and compared at early and late stages of substrate utilization Multiple, ascending, preparative chromatography in 6 4 3 1-butanol–pyridine–water was used to provide for the clear separation and recovery of individual maltooligosaccharides up to the hexaose

Also, a higher concentration (30 mM) of substrate was employed in order to provide adequate amounts of individual, initially formed products for qualitative and quantitative comparison with products recovered at a late stage

Table II shows the composition and overall characteristics of the six paired digests examined It is evident from the total (combined) yields of all reaction products and the amount of residual α-maltosyl fluoride recovered, that 90% or more of the carbohydrate present in each digest was recovered for study, also, that the extent of substrate utilization was from 6–15% in the early-stage mixtures, and from 77–95% in those of the late stage Moreover, in the case of the early-stage (20 min) digests, the amounts of enzyme used and of total products recovered permit estimation of specific initial rates of "overall utilization" of 30mM α-maltosyl fluoride by the various alpha amylase preparations These rates, which measure the combined usage of the substrate as a glycosyl donor and as a glycosyl acceptor, were approximately 10 μmol/min/mg for the liquefying alpha amylases from the three *B subtilis* strains, ~45 and ~160 μmol/min/mg, respectively, for the pancreatic and saccharifying *B subtilis* amylases, and ~1550 μmol/min/mg for the amylase from *Asp oryzae* Despite the wide range of velocities found among the enzymes, the high rates observed in all instances attest to the substantial catalytic activity of these endo-glucanases for this substrate containing only two D-glucose residues

TABLE II

ACTION OF ALPHA AMYLASES ON 30 mM α-MALTOSYL FLUORIDE OVERALL CHARACTERIZATION OF EARLY- AND LATE-STAGE DIGESTS

Crystalline alpha amylase preparation	*Early-stage digests*[a]				*Late-stage digests*[b]			
	Alpha amylase content (μg)	*Total products*[c] *(μmol)*	*Residual substrate*[c] *(μmol)*	*Substrate used (%)*	*Alpha amylase content (μg)*	*Total products*[c] *(μmol)*	*Residual substrate*[c] *(μmol)*	*Substrate used (%)*
B subtilis var *amyloliquefaciens*	6 4	2 8	20 2	12	12	4 7	1 0	82
B subtilis (heat-stable enzyme)	8	3 2	18 5	15	12	4 8	1 0	83
B subtilis var *biotechus nagase*	8	3 1	18 7	14	12	4 8	1 2	80
Hog pancreas	0 8	1 5	22 6	6	3 7	5 2	0 6	90
B subtilis var *amylosacchariticus*	0 3	2 0	22 0	8	0 75	5 4	0 3	95
Aspergillus oryzae	0 05	3 1	19 7	14	0 10	4 2	1 3	77

[a]Test mixtures (400 μL) comprised α-maltosyl fluoride (12 μmol) and alpha amylase (as listed) in 0 01M acetate buffer (pH 5 6), incubated for 20 min at 30° [b]Test mixtures (100 μL) comprised α-maltosyl fluoride (3 μmol) and alpha amylase (as listed) in 0 08M acetate buffer (pH 5 6), incubated for 1 h at 30° [c]Quantity recovered by preparative chromatography, determined and expressed as D-glucose See Fig 1 for kinds and proportions of individual reaction-products recovered from each digest

The types and proportions of individual reaction products present in the foregoing digests by the different alpha amylases are shown in Fig 1 Attention is directed to one unusual product (X) detected in chromatograms of early-stage digests as a spot, located between maltose and maltotriose, that stained extremely slowly with silver nitrate This product was examined following its isolation in small (0 3–1 mg) amounts from early digests with several different alpha amylases It is a non-reducing compound which, when hydrolyzed (10 min, 100°) with 0 01M sulfuric acid, is converted wholly into maltotetraose and hydrogen fluoride Beta amylase, which hydrolyzes α-maltosyl fluoride at the C–F glycosylic bond[5], catalyzed the complete hydrolysis of X to maltose and hydrogen fluoride Analyses showed 0 85 mol of fluoride for each 4 mol of D-glucose Taken together, the data strongly suggest that compound X is maltotetraosyl fluoride

The striking finding (Fig 1) is the high proportion of compound X present in the initial stage of utilization of α-maltosyl fluoride by the various alpha amylases This amounted to 50% of the weight of all reaction products in four instances, 20 and 30% in two others With one exception, the yield of maltose was low (7–10%), whereas that of higher maltooligosaccharides accounted for 40–60% of all products The *Asp oryzae* amylase produced a preponderant amount of compound X, but yielded slightly more maltose than higher oligosaccharides. It is evident that, initially,

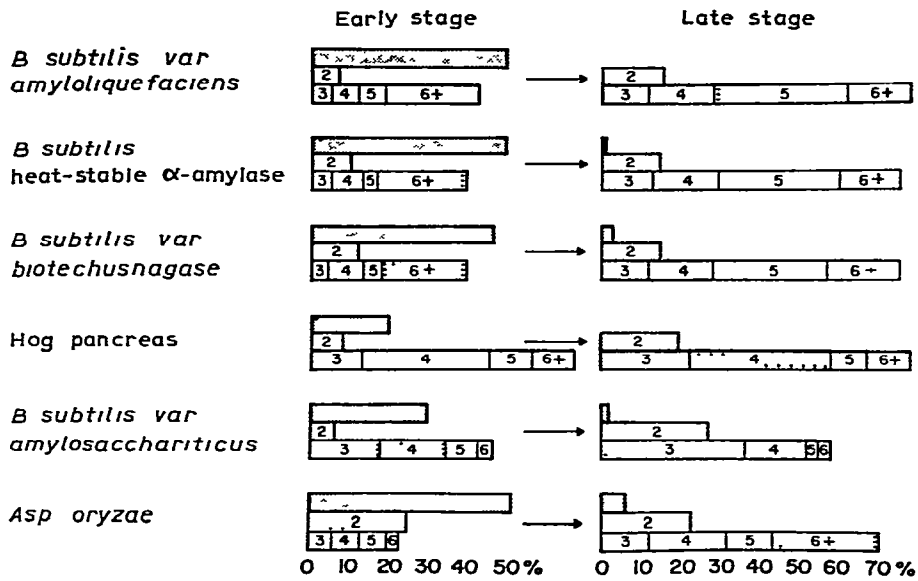

Fig 1 Product patterns at early and late stages of digestion of 30 mM α-maltosyl fluoride by different alpha amylases Product recoveries were from chromatograms developed with 6 4 3 1-butanol–pyridine–water Shaded bars represent a nonreducing product, X, presumed to be maltotetraosyl fluoride, eluted from the 3–4-cm region separating maltose and maltotriose Numbered bars 2 represents maltose, 3, 4, 5, 6+, maltotriose through maltohexaose and beyond Stippled bars indicate the preponderant maltooligosaccharide product(s) in the digest The length of each bar corresponds to the yield, as % by weight, of all products recovered* The extent of substrate utilization in each digest is given in Table II

most of the alpha amylases preferentially catalyzed maltosyl transfer from α-maltosyl fluoride to the C-4 hydroxyl group of a second molecule of maltosyl fluoride, rather than to water, even though the latter was available as an acceptor at more than 1800 times the molar concentration of the maltosyl fluoride

By the late stage of digestion, little or no product X (that is, maltotetraosyl fluoride) remained Evidently, it was used as an intermediate in the formation of maltooligosaccharides having a degree of polymerization (d p) of three or higher At late conversion, the yield of such maltooligosaccharides was 2–5 times that of maltose with all of the alpha amylases tested A possible role for maltose as an acceptor in the formation of these oligosaccharides is not excluded

As illustrated in Fig 1, several different patterns of products were formed from α-maltosyl fluoride by the various types of amylases tested The formation of distinctive product-patterns upon hydrolysis of starch or maltooligosaccharides by alpha amylases of different origins has been well established[10 12 16 17] In the present experiments, the identity of the most prominent maltooligosaccharide product

*Although not shown in the diagram, small proportions (3–7%) of D-glucose and D-glucosyl fluoride were recovered from both of the digests of α-maltosyl fluoride by the saccharifying amylase of *B subtilis* var *amylosaccharlticus*

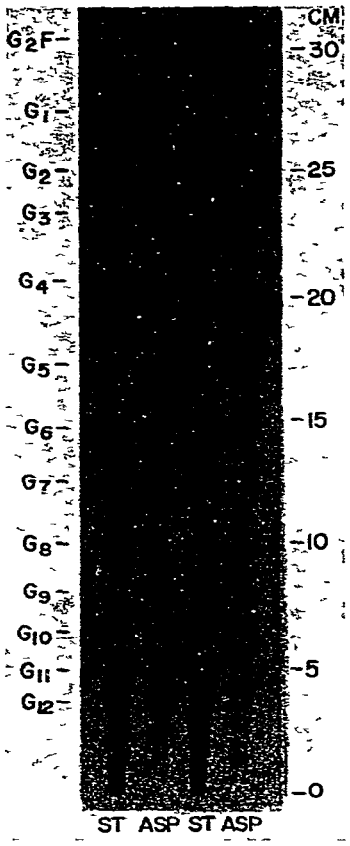

Fig 2 Ascending chromatogram showing the series of oligosaccharides produced by the action of 1 μg of crystalline *Aspergillus oryzae* alpha amylase/mL on 30mM α-maltosyl fluoride (ASP), compared with a partial hydrolyzate of short-chain amylose as standard (ST)

tended to change as digestion proceeded Maltohexaose, for example, was the initially dominant maltooligosaccharide in reactions catalyzed by the three bacterial liquefying amylases, later, maltopentaose was preponderant The most unusual change was the development of late-state prominence of higher maltooligosaccharides in the *Asp oryzae* digest.

Fig 2 shows the chromatogram of a late-stage digest of 30mM α-maltosyl fluoride by 1 μg of the mold enzyme/mL, compared with partly hydrolyzed amylose as the standard With the high-resolution solvent-system[18] used, the enzymically produced oligosaccharides may be monitored to approximately d p 12 This demonstration of the ability of the *Asp oryzae* alpha amylase to catalyze glycosylation reactions in which water is kept out of a succession of steps is completely out of keeping with the traditional view of alpha amylase action as being essentially entirely hydrolytic The progressive accumulation of higher oligosaccharides in the late stage would indicate that the enzyme catalyzes the formation of such oligosaccharides faster than it catalyzes their degradation

As already noted (Table II), the mold enzyme was found to catalyze the "overall utilization" of 30mM α-maltosyl fluoride at the rate of ~1550 μmol/min/mg, an extremely high value relative to the stated activity of this crystalline amylase for hydrolysis of starch (120 μmol of maltose/min/mg) Measurements of the rate of hydrolysis of 1% soluble starch (at pH 5 6 and 37°) for the particular preparation used in the present study gave a somewhat lower value, 88 μmol of maltose formed/min/mg Further information on the relative activity of the two substrates was obtained by determining the initial rates of fluoride ion release from α-maltosyl fluoride as catalyzed by the enzyme A series of buffered (pH 5 6) digests containing 12–180mM substrate and 0 2 μg *Asp oryzae* amylase/ml was incubated for 20 min at 30°, and then assayed for free fluoride by the use of a specific probe for fluoride ion Less than 15% of the C–F glycosylic bonds of the substrate were broken under the digest conditions, allowing initial reaction rates (v = μmol of fluoride anion released/min/mg of protein) to be determined for the different concentrations (S) of substrate The results provided a linear curve for the relationship $1/v$ versus $1/S$ Calculations by a least-squares method[19] gave K_m = 34mM α-maltosyl fluoride, and V_{max} = 1560 μmol of fluoride released/min/mg Of particular interest was the experimental finding that the rate of cleavage of the C–F glycosylic bond of 30mM α-maltosyl fluoride was 790 μmol/min/mg (730 μmol/min/mg if calculated on the basis of the kinetic constants) The speed of usage of 30mM α-maltosyl fluoride as a donor substrate was, thus, about one-half that found for its "overall utilization" under identical reaction-conditions (that is, ~1550 μmol/min/mg, Table II) The difference (~760 μmol/min/mg) provides a rough measure of the initial rate at which 30mM α-maltosyl fluoride was used as a glycosyl acceptor by the *Asp oryzae* amylase This high rate of use as an acceptor is in accord with the finding that brief incubation of α-maltosyl fluoride with a low concentration of the enzyme afforded a sufficient amount of transfer product X for structural examination

In the case of pancreatic amylase, whose starch-hydrolyzing capacity is known to depend on the presence of chloride[17,20], a comparison was made of the utilization rates of α-maltosyl fluoride in paired reaction-mixtures differing only in their chloride content Catalysis of C–F bond-cleavage by the enzyme was found to proceed approximately twice as fast in digests containing 10mM sodium chloride as in digests having less than 0 3mM salt, thus providing further evidence that the catalyst is, in fact, the alpha amylase itself

We envisage that the major, initial interaction between alpha amylases and α-maltosyl fluoride is one in which two molecules of substrate are bound to the enzyme is such a way that the C–F glycosylic bond of one, and the C-4 hydroxyl group of the other, are positioned in the region of the enzyme's catalytically active groups Presumably, the properly positioned 4-hydroxyl group would compete effectively with water as the glycosyl acceptor, so that glycosyl transfer would occur Interaction providing hydrolysis of α-maltosyl fluoride to maltose would occur to a lesser extent We further envisage that successive glycosyl transfer reactions would occur in which newly formed maltotetraosyl fluoride, maltohexaosyl fluoride, and so on, would

serve as co-substrates with α-maltosyl fluoride (and perhaps with each other), pair-binding to enzyme in the orientations already noted Maltose, too, may participate as an acceptor in such reactions At some point in the chain-elongation process, newly formed molecules would reach sufficient length (varying with the type of amylase) to bind more effectively in another mode namely. with the D-glucose residues of one molecule occupying both donor and acceptor sites of the enzyme, and with a (1→4)-α-D-glucosidic linkage positioned in the region of the catalytic groups Hydrolysis would then be expected to produce an even- or uneven-numbered maltooligosaccharide fragment, or maltose

With bacterial amylases of the liquefying type, where maltohexaose was found to be the dominant maltooligosaccharide in the first stages of reaction, the polymerization of α-maltosyl fluoride must have involved several successive glycosyl-transfer steps It would seem likely that maltooctaosyl fluoride or a higher homolog would have been produced before appreciable binding could occur in the mode leading to hydrolysis and maltohexaose formation Maltooligosaccharide chains comprising fewer than seven D-glucosyl residues have been found to be relatively insusceptible to hydrolysis by bacterial, liquefying amylases[21 22]

In sum, the results obtained with α-maltosyl fluoride extend those reported earlier[1] with α-D-glucopyranosyl fluoride They clearly demonstrate that alpha amylases are able to catalyze glycosylation reactions *in preference to hydrolysis* when acting on substrates that bind to enzyme primarily in a paired fashion as donor and acceptor Yet, with such substrates as starch or glycogen, which mainly bind with a single chain positioned across the catalytic center, the same enzymes catalyze hydrolysis* As either type of reaction may be dominantly expressed, it is evident that the usual description of alpha amylases as hydrolases[11] (implying the ability to catalyze overwhelming hydrolysis of all substrates), is inadequate The adoption of a broader view of alpha amylases, for instance as glycosylases or catalysts of glycosyl–proton interchange[1 2], is necessary in order to account for their demonstrated capabilities Present findings illustrate, finally, how the study of productive glycosylation reactions that occur without glycosidic-bond cleavage may be used to gain fresh insight into the catalytic capacities of carbohydrases, even when these have supposedly been well defined on the basis of extensive studies made with glycosidically linked substrates

EXPERIMENTAL

Reaction components — α-Maltosyl fluoride was prepared from analytically pure, crystalline hepta-*O*-acetyl-α-maltosyl fluoride by deacetylation, and purified on columns of silica gel 60, as recently described[5] The compound produced a single

*A sizable proportion of nonhydrolytic reactions has been found to occur, however, when glycogen is acted upon by *B amyloliquefaciens* and hog-pancreas alpha amylases in the presence of a suitable acceptor (for example, *p*-nitrophenyl β-D-glucopyranoside)[23]

spot on chromatograms, and migrated at R_{Glc} 1 15 in the solvent systems used Solutions were made in cold, acetate buffers (pH 5 6) of appropriate ionic strength immediately prior to use

Alpha amylase preparations from six different sources were examined The twice-crystallized enzyme from hog pancreas (Type 1-A, Sigma Chemical Co) was a suspension in half-saturated sodium chloride and 3mM calcium chloride, stock solutions of 1 mg/mL were prepared by centrifuging known volumes of the suspension at 8000*g* for 15 min and dissolving the sediment in cold, distilled water The other alpha amylases were dry solids that were dissolved in ice-cold, distilled water to give similar stock-solutions Liquefying alpha amylase from *Bacillus subtilis* var *amylo-liquefaciens* (Fukumoto) was a twice-recrystallized product from Seikagaku Kogyo Co , Tokyo, Japan, found to be homogeneous on ultracentrifugation[23] The temperature resistant, liquefying alpha amylase from *B subtilis* was a twice crystallized preparation (Daiwa Kasei Co , Ltd , Osaka, Japan) specified as being homogeneous on gel filtration Crystalline, liquefying alpha amylase from *B subtilis* var *biotechus nagase* was kindly furnished by Dr. T Komaki of Nagase and Co , Ltd , Osaka, Japan Twice-crystallized, saccharifying alpha amylase from *B subtilis* var *amylo-sacchariticus* (Fukumoto) was the product of Seikagaku Kogyo Co Three-times crystallized alpha amylase from *Aspergillus oryzae* (Sankyo Co , Ltd , Tokyo, Japan) was specified as being homogeneous by electrophoretic and ultracentrifugal analysis, and as having hydrolytic activity for soluble starch (at pH 5 5 and 37°) of 120 μmol reducing sugar released (as maltose)/min/mg

Patterns of action of different alpha amylases on 5 6 and 30mM α-maltosyl fluoride — Incubated mixtures containing α-maltosyl fluoride and alpha amylase as well as concurrently incubated, control mixtures of substrate and buffer, were applied as 20-cm bands on Whatman No 1 paper A standard of partly hydrolyzed amylose (see later), providing a uniform series of linear maltooligosaccharides, was spotted 1 cm from the ends of each band Digests of 5 6mM α-maltosyl fluoride were chromatographed by using a single ascent in 6 4 3 1-butanol–pyridine–water Early- and late-stage digests of 30mM substrate were subjected to two 22-h ascents in the same solvent system to provide wide separation of the lower maltooligosaccharides Carbohydrate components of each mixture were located by staining strips cut from the ends of bands, including the maltooligosaccharide standards A silver nitrate dipping-method[24] was used, with the strips being hung in air for 10 min following application of the sodium hydroxide reagent, to allow detection of nonreducing compounds The individual components were quantitatively eluted from sections of the 18-cm center panel by use of 1 1 methanol–water Duplicate aliquots of the centrifuged eluates were dried under vacuum at 55° in a Rotary Evapo Mix (Buchler Instruments Co) and analyzed for total D-glucose content The phenol–sulfuric acid method[15] was used, with precise timing of steps and with concurrent measurements made in duplicate on each of a series of eight D-glucose standards Correction was made for traces of carbohydrate eluted from corresponding sections of control chromatograms

For demonstrating the range of oligosaccharides finally produced from α-maltosyl fluoride by the action of crystalline, *A. oryzae* alpha amylase, a mixture of 30mM substrate and 1 μg enzyme/mL in 0 05M acetate buffer (pH 5 6) was incubated for 1 h at 30° Chromatograms of 15-μL samples were developed in 35·39·26 1-butanol–pyridine–water[18] (two 22-h ascents), and stained with silver nitrate[24] Chromatographed concurrently, as a standard, was a partial hydrolyzate of short-chain amylose. This was prepared by incubating 4 mL of a 5% solution of d p 19 amylose (EX-1, Hayashibara Laboratories, Okayama, Japan) with 2 μg/mL of *B subtilis* liquefying alpha amylase at pH 5 6 for 15 min at 30°, after heat inactivation, D-glucose was added to a concentration of 0 25 mg/mL

Fluoride determination — Measurements of free fluoride (in the presence or absence of maltosyl fluoride) were made with the aid of a specific fluoride-ion probe (Orion specific ion meter, Model 407A, and combination fluoride electrode, Model 96–09) Test samples and sodium fluoride standards were diluted with an equal volume of a solution comprising M sodium acetate buffer (pH 5 2), M sodium chloride, and 0 4% 1,4-cyclohexanebis(dinitrilotetraacetic acid) monohydrate, and then poured into 5-mL polyethylene beakers Meter readings were recorded 45 sec after immersion of the electrode in the solution under test

Identification of product X — Duplicate mixtures (0 40 mL) of 30mM α-maltosyl fluoride and 0 125 μg *Asp oryzae* alpha amylase/mL in 0 02M acetate buffer (pH 5 6) were incubated for 20 min at 30° Each was chromatographed as a 20-cm band in 6 4 3 1-butanol–pyridine–water (three 22-h ascents) Product X, located with the aid of stained guide-strips, was eluted with methanol, dried samples were obtained from 50- and 10-mL portions of eluate by removing solvent in a vacuum evaporator The larger sample was hydrolyzed in 6 mL of 0 01M sulfuric acid for 10 min at 100°. Analyses showed that the hydrolyzate contained 0 186 μmol fluoride anion/mL, and 0 877 μmol of total carbohydrate (as D-glucose)/mL[15], that is, 0 85 mol of fluoride for each 4 mol of D-glucose Product X, from 10 mL of eluate, was dissolved in 210 μL of water One 75-μL portion was treated with 75 μL of 0 02M sulfuric acid (10 min, 100°), another was incubated with 75 μL of a solution containing 30 units of α-glucosidase-free beta amylase[25] for 4 h at 30° Samples (15 μL) of the unhydrolyzed, acid-hydrolyzed, and enzyme-hydrolyzed product were chromatographed, together with maltooligosaccharide standards, using two 22-h ascents in 6 4 3 1-butanol–pyridine–water On staining with silver nitrate, unhydrolyzed X gave a single, very slowly staining spot 11 5 cm from the origin, midway between maltose (14 3 cm) and maltotriose (9 3 cm) The acid hydrolyzate yielded a single spot at 5 5 cm identical in migration with authentic maltotetraose (5 5 cm) and faster than maltopentaose (3 6 cm) The hydrolyzate with beta amylase yielded a single spot at 14 3 cm, identical in migration with maltose Similar results were obtained with product X recovered from early-stage digests of α-maltosyl fluoride by alpha amylases from hog pancreas and from *B subtilis* var *amyloliquefaciens*.

Identification of maltooligosaccharides — Oligosaccharides recovered from digests of α-maltosyl fluoride by each amylase were tentatively identified by their

chromatographic mobility and staining behavior versus maltooligosaccharide standards In addition, a 100-μL sample of a late-stage digest prepared with each amylase was treated with 40 units of α-D-glucosidase-free beta amylase[25], and the mixture incubated for 4 h at 30° In all cases, D-glucose and maltose were the only products detected by chromatography

Initial rates of action of Asp oryzae *alpha amylase* — A series of mixtures, containing 12–180mM α-maltosyl fluoride and 0 2 μg of *Asp oryzae* alpha amylase/mL (in 0 05M acetate buffer, pH 5 6) was incubated for 20 min at 30°, and then analyzed for free fluoride content Initial rates of 408, 490, 542, 790, 949, and 1362 μmol/min/mg of protein were found for 12, 15, 20, 30, 60, and 180mM substrate, respectively

Mixtures containing 1% of soluble potato starch of dextrose equivalent 0 5 (Mallinckrodt), and 0 25, 0 50, or 1 μg of *Asp oryzae* alpha amylase/mL in 0 05M acetate buffer (pH 5 6), were incubated for 10 min at 37° Analyses of reducing-sugar content (as maltose) were made by the Nelson–Somogyi procedure[26,27] standardized with solutions of maltose monohydrate An initial velocity of 88 μmol/min/mg of protein was found in each case

Effect of chloride on the action of pancreatic amylase on α-maltosyl fluoride. — Initial rates of cleavage of the C–F bond of α-maltosyl fluoride were determined on paired mixtures containing 30mM substrate, 10 μg of hog pancreatic amylase/mL, and 0 05M acetate buffer (pH 5 6), but differing in chloride content After incubation for 20 min at 30°, the mixtures were analyzed for concentration of free fluoride Specific initial rates of 10 7 and 22 7 μmol of fluoride released/min/mg of protein were found for mixtures containing less than 0 3mM and 10mM chloride, respectively

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NOTE ADDED IN PROOF

α-Maltotetraosyl fluoride has recently been synthesized chemically (E J Hehre and D S Genghof, unpublished results) and found to have properties, including chromatographic mobility, in accord with those reported herein for compound X

REFERENCES

1 E J Hehre, D S Genghof, and G Okada, *Arch Biochem Biophys*, 142 (1971) 382–393
2 E J Hehre, G Okada, and D S Genghof, *Adv Chem Ser*, 117 (1973) 309–333
3 E J Hehre, D S Genghof, H Sternlicht, and C F Brewer, *Biochemistry*, 16 (1977) 1780–1787
4 E J Hehre, *Abstr Pap Am Chem Soc Meet*, 174 (1977) CARB-73
5 D S Genghof, C F Brewer, and E J Hehre, *Carbohydr Res*, 61 (1978) 291–299

6 M BROCKHAUS AND J LEHMANN, *Carbohydr. Res*, 53 (1977) 21–31
7 J. LEHMANN AND B ZEIGER, *Carbohydr Res*, 58 (1977) 73–78
8 E H FISHER AND E. A STEIN, *Enzymes*, 2nd edn, 4 (1960) 313, 333
9 M A JERMYN, *Rev Pure Appl Chem*, 11 (1961) 92–116
10 W J WHELAN, *Methods Carbohydr. Chem*, 4 (1964) 252
11 INTERNATIONAL UNION OF BIOCHEMISTRY, *Enzyme Nomenclature*, Elsevier, 1965, p 136
12 C T GREENWOOD AND E A MILNE, *Adv Carbohydr Chem*, 23 (1968) 281–366
13 J A THOMA, J E SPRADLIN, AND S DYGERT, *Enzymes*, 3rd edn, 5 (1971) 115–189
14 G OKADA, D S GENGHOF, AND E J HEHRE, *Abstr Int Symp Carbohydr Chem, VIIIth, Kyoto (Japan)*, 1976, p 69
15 M DUBOIS, L A GILLES, J K HAMILTON, P A REBERS, AND F SMITH, *Anal Chem*, 28 (1956) 350–356
16 R. BIRD AND R H HOPKINS, *Biochem J*, 56 (1954) 86–99
17 D FRENCH, *MTP Int Rev Sci, Biochem Ser One*, 5 (1975) 267, 309
18 J. A. THOMA AND D. FRENCH, *Anal Chem*, 29 (1957) 1645–1648
19 I H SEGAL, *Enzyme Kinetics*, Wiley–Interscience, 1975, pp 943–944
20 H C SHERMAN, M L CALDWELL, AND M ADAMS, *J Am Chem Soc*, 56 (1928) 2535–2537
21 J ROBYT AND D FRENCH, *Arch Biochem Biophys*, 100 (1963) 451–467
22 S OKADA, S KITAHATA, M HIGASHIHARA, AND J FUKUMOTO, *Agric Biol Chem*, 33 (1969) 900–906
23 M TAKESHITA AND E J HEHRE, *Arch Biochem Biophys*, 169 (1975) 627–637.
24 W E TREVELYAN, D P PROCTER, AND J S HARRISON, *Nature*, 166 (1950) 444–445
25 J J MARSHALL AND W J WHELAN, *Anal Biochem*, 52 (1973) 642–646
26 N NELSON, *J Biol Chem*, 153 (1944) 375–380
27 M SOMOGYI, *J Biol Chem*, 195 (1952) 19–23