

A novel α -glucosidase from the acidophilic archaeon *Ferroplasma acidiphilum* strain Y with high transglycosylation activity and an unusual catalytic nucleophile

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Ferroplasma acidiphilum strain Y (DSM 12658), a ferrous iron-oxidizing, acidophilic and mesophilic archaeon, was found to produce a membrane-bound α -glucosidase (α GluFa) showing no significant similarity to any of the known glycoside hydrolases classified in different families and having an unusual catalytic site consisting of a threonine and a histidine residue. The highest α -glucosidase activity was found at low pH, 2.4–3.5, and the substrate preference order was: sucrose > maltose > maltotriose \gg maltotetraose \gg malto-oligosaccharides from maltopentaose to maltoheptaose \gg soluble starch (k_{cat}/K_m was 293.0, 197.0, 18.8, 0.3 and 0.02 s⁻¹ · mM⁻¹ respectively). The enzyme was able to transfer glucosyl groups from maltose as donor, to produce exclusively maltotriose (up to 300 g/l). Chemical modification and

electrospray ionization MS analysis of 5-fluoro- α -D-glucopyranosyl-enzyme derivatives, coupled with site-directed mutagenesis, strongly suggested that the putative catalytic nucleophile in this enzyme is Thr²¹². Iron was found to be essential for enzyme activity and integrity, and His³⁹⁰ was shown to be essential for iron binding. These results suggest that the metalloenzyme α GluFa is a new member of the glycosyl hydrolase family that uses a novel mechanism for sugar glycosylation and/or transglycosylation.

Key words: α -glucosidase, catalytic nucleophile, *Ferroplasma acidiphilum*, glucosyl transferase, glycosyl hydrolase, transglycosylation.

INTRODUCTION

Extremophilic microorganisms capable of thriving in harsh environments are widely distributed in Nature and have become the subject of intense investigation in recent years [1–7]. They produce unique stress-tolerant enzymes, extremozymes, involved in metabolic and cellular adaptation to the prevailing extreme environmental conditions. These tolerances and preferences (e.g. thermophily, psychrophily, acidophily, alkaliphily and halophily) confer upon extremozymes particular advantages for many industrial applications [8,9]. We have recently reported the isolation and characterization of *Ferroplasma acidiphilum* strain Y, an archaeon that grows under extremely acidic conditions (pH range of growth 1.3–2.2), oxidizes ferrous iron as its sole energy source and fixes inorganic carbon as the sole source of carbon [10]. We have also demonstrated that five intracellular and membrane-bound enzymes cloned from *F. acidiphilum* strain Y had optimum pH values much lower than the mean intracellular pH value of 5.6 (O. V. Golyshina, P. N. Golyshin, K. N. Timmis and M. Ferrer, unpublished work).

The present study focuses on glycosidases, including amylases, α -glucosidases, glucoamylases, pullulanases and cyclodextrin glucosyltransferases, enzymes that catalyse the hydrolysis of glycosidic bonds via a general acid catalysis involving a proton donor and a nucleophile/base [11]. In all cases, the carboxylic side chains of glutamic and aspartic residues are involved in catalysis. α -

Glucosidases (EC 3.2.1.20; α -D-glucoside glucohydrolases) catalyse the liberation of glucose from non-reducing ends of short oligosaccharide substrates [12]. Some α -glucosidases preferentially hydrolyse α -linked di-, oligo- and/or polyglucans, while others prefer heterogeneous substrates such as sucrose and aryl glucosides [13]. They also mediate transglycosylation reactions, activities (e.g. those from buckwheat [13], *Aspergillus niger* [14], *Bacillus stearothermophilus* or brewer's yeast [15]) that are exploited in biotechnology to produce food oligosaccharides [16,17] or to conjugate sugars with biologically useful materials [18]. In the present study, we describe a membrane-bound α -glucosidase from *F. acidiphilum* strain Y, which shows no significant similarity to other known glycoside hydrolases classified in different families and that, unusually, has a catalytic centre involving threonine and histidine residues.

MATERIALS AND METHODS

Full details of all experimental methods are given in the Supplementary Materials and methods section at <http://www.BiochemJ.org/bj/391/bj3910269add.htm>.

Materials and strains of microorganisms

F. acidiphilum strain Y (DSMZ 12658) and *Escherichia coli* strains (i) XL1-Blue MRF⁺ (Stratagene, La Jolla, CA, U.S.A.), for

Abbreviations used: iPr₂P-F, di-isopropyl fluorophosphate ('DFP'); EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodi-imide; ESI-MS, electrospray ionization MS; 5F α GlcF, 5-fluoro- α -D-glucopyranosyl fluoride; GH, glycoside hydrolase; ICP-MS, inductively coupled plasma MS; IPTG, isopropyl β -D-thiogalactoside; LB, Luria-Bertani; PCMPs, *p*-chloromercuriphenylsulphonic acid; TNM, tetranitromethane; α GluFa, α -glucosidase from *Ferroplasma acidiphilum* strain Y^T.

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The nucleotide sequence data reported will appear in DDBJ, EMBL, GenBank[®] and GSDN Nucleotide Sequence Databases under the accession number AJ717661.

library construction and screening, (ii) XL0LR (Stratagene), for expression of the α -glucosidase from phagemids, and (iii) DH5 α , for site-directed mutagenesis and expression of mutant enzymes (Invitrogen, Carlsbad, CA, U.S.A.), were maintained and cultivated, if not mentioned otherwise, according to the manufacturer's instructions and the standard methods described previously [10,19]. In some cases, additions of 1 g/l sucrose, maltose or glucose were also made to cultures of *F. acidiphilum* grown in the medium 9K. F α GlcF (5-fluoro- α -D-glucopyranosyl fluoride) was synthesized as described by McCarter and Withers [20]. DNA restriction and modification enzymes were from New England Biolabs (Beverly, MA, U.S.A.).

Cloning, expression of α gluFa from *F. acidiphilum* strain Y^T and purification of the recombinant protein

An expression library of the *F. acidiphilum* genome was established in the bacteriophage lambda ZAP vector using the ZAP Express kit (Stratagene), and the library was used to infect XL1-Blue MRF' cells, which were plated in NZY soft agar containing 2% (w/v) sucrose and 10 μ M FeCl₂ over a bottom layer of NZY agar [19] also containing sucrose and FeCl₂. The 22.5 cm \times 22.5 cm plates containing approx. 10 000 phage clones were incubated overnight and then overlaid with 50 ml of iodine solution (Sigma). Positive clones exhibiting a violet halo were picked and purified by serial dilution. The pBKGLuFa phagemid was generated from one of the selected phage colonies by the helper phage excision procedure (Stratagene) and transferred to *E. coli* XL0LR cells. The complete nucleotide sequence of the DNA fragment, coding for the α gluFa enzyme described in the present study has been deposited in DDBJ, EMBL, GenBank[®] and GSDB Nucleotide Sequence Databases under the accession number AJ717661.

For the expression of α gluFa, *E. coli* cells containing pBKGLuFa were grown at 37 °C in LB (Luria–Bertani) medium containing 50 μ g of kanamycin/ml and 10 μ M FeCl₂. When the absorbance A_{600} reached 1.0, IPTG (isopropyl β -D-thiogalactoside) was added to a final concentration of 1 mM to induce expression. Cells were harvested 3 h after induction, and resuspended in buffer A (10 mM sodium citrate buffer, pH 3.0) containing one protease inhibitor cocktail tablet (Roche) and 10 μ g/ml DNase I grade II (Roche), incubated on ice for 30–45 min and then sonicated for a total of 4 min. The soluble fraction was separated from insoluble debris by centrifugation (10 000 g, 30 min and 4 °C), dialysed overnight against buffer A, concentrated to 1 ml by ultrafiltration on a Centricon YM-10 membrane (Amicon, Millipore, Billerica, MA, U.S.A.), and the α GluFa (α -glucosidase from *F. acidiphilum* strain Y^T) was purified as follows. The sample was applied to a HiPrep 16/10 SP XL (Amersham Biosciences, Little Chalfont, U.K.) column, which was washed with buffer A and subsequently eluted with a linear gradient of NaCl (total volume, 200 ml; 0–0.2 M). Active fractions were pooled and dialysed against 50 mM sodium citrate (pH 3.0) and 1 M (NH₄)₂SO₄, concentrated to 1 ml on a Centricon YM-10 membrane and filtered using a 0.22 μ m filter. The α GluFa-containing fractions were loaded on to a Resource 15PHE hydrophobic chromatography column (PE 4.6/100) previously equilibrated with the same buffer. After washing with the equilibration buffer [50 mM sodium citrate, pH 3.0 and 1 M (NH₄)₂SO₄], α GluFa was eluted with a linear gradient of (NH₄)₂SO₄ (total volume 25 ml; 1.0–0 M). The eluted enzyme was dialysed against buffer A overnight, concentrated to 1 ml by ultrafiltration and applied on to a Superose 12 HR 10/30 gel-filtration column pre-equilibrated with 10 mM sodium citrate (pH 3.0) and 150 mM NaCl. Separation was performed at 4 °C at a flow rate of 0.5 ml/min. The puri-

fied recombinant α -glucosidase was dialysed against buffer A overnight and stored at –20 °C at a concentration of 10 mg/ml until use.

Hydrolytic assays

Unless otherwise indicated, the hydrolytic activity towards sucrose, starch, amylose, amylopectin, pullulan and dextrin was determined by measuring the release of reducing sugars from 1% (w/v) substrate solutions using the dinitrosalicylic acid method [21]; hydrolytic activity towards kojibiose, nigeriose, isomaltose, iso-maltotriose, trehalose and malto-oligosaccharides from maltotetraose to maltoheptaose was measured by HPLC analysis (see below) in reaction mixtures containing the substrate (1%, w/v). Reactions were stopped by heating for 15 min at 80 °C. Activity towards *p*-nitrophenyl- α/β -D-glucopyranoside (2 mM) was measured spectrophotometrically in a UV/visible spectrophotometer by following the increase in absorbance at 346 nm (ϵ_{346} 4800 M⁻¹·cm⁻¹). Unless otherwise indicated, hydrolytic activities were routinely measured by incubating the purified enzyme (5 μ g/ml) with a substrate at various concentrations in 100 mM sodium citrate buffer (pH 3.0) at 50 °C for an assay time of 30 min. All values were determined in triplicate and corrected for autohydrolysis of the substrate. One enzyme unit was defined as the amount of enzyme liberating 1 μ mol of glucose (or equivalent reducing sugar) or *p*-nitrophenol per min. The standard assay used in the present study was performed at 50 °C in 100 mM sodium citrate buffer (pH 3.0) and 1% sucrose as substrate.

Kinetic parameters (k_{cat} and K_m) were determined at 50 °C in 100 mM sodium citrate buffer (pH 3.0). Substrate concentration was varied in the range 0.1–20.0 mM and the activity was measured as described above. Kinetic parameters were calculated by fitting the initial rate values to the Hanes–Woolf transformation of the Michaelis–Menten equation.

Transglycosylation assay and HPLC conditions

The transglycosylation assay was carried out at 50 °C in 0.2 M sodium citrate buffer (pH 3.0) containing 5 μ g of purified α GluFa/ml and 600 g/l maltose. Aliquots were taken at intervals for a 180 min period, heated at 80 °C for 15 min, diluted 1:5 (v/v) with water, centrifuged and then filtered using Ultrafree-MC filter (0.45 μ m) devices (Millipore). Substrate–product analyses were carried out by HPLC using a 4.6 mm \times 250 mm Lichrospher-NH₂ column (Merck, Darmstadt, Germany) and acetonitrile/water (75:25, v/v) as the mobile phase at 0.7 ml/min and a refractive index detector (Varian, Basel, Switzerland). The column was kept constant at 25 °C. Integration was carried out using the Millennium software (Waters).

Inactivation kinetics

The enzyme (final concentration, 0.1 mg/ml) was incubated with compounds to be tested as inhibitors over the concentration range 0–10.0 mM at 50 °C in 100 mM sodium citrate buffer (pH 3.0). Aliquots (50 μ l) were withdrawn at time intervals, chilled on ice and analysed by the standard α -glucosidase assay. Residual activity was expressed as the percentage of the control value obtained without the addition of chemical. Values for the inactivation rate constants (k_i) and the dissociation constants for the inactivators (K_i) were determined by fitting to the equation

$$K_{obs} = k_i[I]/(K_i + [I]) \quad (1)$$

where K_{obs} is the measured rate constant and [I] is the concentration of the inhibitor.

Chemical modification of α GluFa

α -Glucosidase was subjected to modification of specific amino acids with PCMPS (*p*-chloromercuriphenylsulphonic acid), iPr_2P -F (di-isopropyl fluorophosphate; 'DFP'), TNM (tetranitromethane) and EDC {1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide}, using standard methods [22–24]. Unless otherwise indicated, chemical modifications were routinely performed by incubating purified recombinant α GluFa (100 μ g) either in 10 mM sodium citrate buffer (pH 3.0) (for PCMPS, iPr_2P -F and TNM) or in 10 mM Mes buffer (pH 4.0) containing 10 mM KCl (for EDC), with the corresponding chemical. Activity measurements were done both immediately and after 30 min of incubation. In all cases, residual activity after modification was measured immediately, in three independent assays, and expressed as percentage of the control value obtained without the addition of the inhibitor. Full details are given in the Supplementary Materials and methods section (<http://www.BiochemJ.org/bj/391/bj3910269add.htm>).

Construction of α GluFa protein variants containing Glu, Asp and His mutations

Point mutations were introduced into the phagemid pBK α GluFa using the QuikChange SDM procedure (Stratagene) with the oligonucleotide pairs synthesized at Sigma-Genosys (Pampisford, Cambs., U.K.). Eight Glu residues at positions 54, 258, 311, 426, 450, 452, 516 and 531 were replaced by Gln residues (α GluFa E/Q), 17 Asp residues at positions 40, 125, 172, 179, 185, 202, 250, 276, 334, 346, 391, 397, 428, 476, 502, 504 and 510 were replaced by Gln (α GluFa D/Q), and eight His residues at positions 9, 41, 47, 243, 275, 325, 377 and 390 were replaced by Ala (α GluFa H/A). The oligonucleotides used for mutagenesis are listed in Supplemental Table S1 at <http://www.BiochemJ.org/bj/391/bj3910269add.htm>. The resulting mutated plasmids were transformed into *E. coli* DH5 α electrocompetent cells (Invitrogen), which were plated on to LB agar supplemented with 50 μ g/ml kanamycin. Mutations were confirmed by DNA sequencing using the sequencing primers 5'-AACTCATTATATATATTG-AATC-3' (CH4) and 5'-ATTAGTGTTCATGACGGTAAA-3' (CH1158). Mutant proteins were purified using the same method as for the wild-type protein.

Detection of the catalytic nucleophile

To identify the catalytic nucleophile of *F. acidiphilum* α -glucosidase, the enzyme was labelled with 5F α GlcF and then subjected to proteolysis and ESI-MS (electrospray ionization MS) to identify labelled peptides, as described by Lee et al. [25], although with small modifications. Briefly, a stock solution of the enzyme (50 μ l; 10 mg/ml) was incubated with 5F α GlcF (50 μ l; 10 mM) at 37°C for 5 min. The sample was diluted with 200 μ l of 50 mM phosphate buffer (pH 2.0) and incubated with 15 μ l of pepsin (1 mg/ml) for 15 min at room temperature (25°C). The sample was then quickly frozen and analysed immediately upon thawing. A control sample not treated with 5F α GlcF was prepared according to the same procedure. Mass spectra were recorded on a VG AutoSpec spectrometer equipped with an ESI ion source. ESI-MS in the negative mode was performed in a QTOF (quadrupole/time-of-flight) mass spectrometer. Neon served as the collision gas for high-energy collision-induced dissociation. Peptides were separated by reversed-phase HPLC using a C18 column (4.6 mm \times 150 mm; Análisis Vínicos, Tomelloso, Spain) and a refractive index detector (Spectra-Physics, San Jose, CA, U.S.A.) and eluted with a gradient of 0–60% eluting solvent [0.045% trifluoroacetic acid/80% (v/v) acetonitrile in water] for 60 min at a flow rate of 100 μ l/min. The temperature of the

column was kept constant at 25°C. Total ion chromatograms of the labelled and unlabelled enzyme digests were compared to find the fraction containing the labelled peptide fragments, which were collected and analysed by MS/MS fragmentation analysis.

CD and ICP-MS (inductively coupled plasma MS)

CD spectra of α GluFa at a concentration of 10 mg/ml and pH in the range 1.0–7.0 were measured with a Jasco J-720 spectropolarimeter equipped with a constant-temperature cell holder (40°C), and 0.1 cm cell. Spectra were measured in the following buffers (100 mM): sodium citrate (pH 1.0–4.5), sodium acetate (pH 4.5–5.5), Mes (pH 5.5–7.0) and Hepes (pH 7.0). When necessary, the enzyme solution was incubated with 1 mM EDTA before absorption spectra were recorded. The metal ion content of α GluFa variants was determined using a PerkinElmer Life Sciences ICP mass spectrometer (model PE ELAN 6100 DRC). The metal content was determined by dilution of 50 μ g of enzyme with 5 ml of 0.5% (v/v) HNO₃ to digest the protein and release the metal ions, and this solution was used without any further manipulation.

RESULTS AND DISCUSSION

Cloning of *F. acidiphilum* α gluFa gene and sequence comparison

An *F. acidiphilum* genome expression library was generated in *E. coli* using the phage lambda ZAP vector (see the Materials and methods section). Activity-based screening of the library led to the detection of three different clones with α -glucosidase activity. One, designated α GluFa, was studied further after re-cloning its gene in the phagemid vector. Although the level of expression of the recombinant protein was approx. 1.4% (w/w) after induction with 1 mM IPTG, α -glucosidase activity was undetectable in freeze-dried cell extracts (<0.01 unit/mg, using sucrose or maltose as substrate). High enzymatic activities were, however, obtained by supplementation of the growth medium with Fe(II) at concentrations exceeding 10 μ M and extensive dialysis of cell extracts against 10 mM sodium citrate buffer at pH 3.0. α GluFa was purified to homogeneity, as described in the Materials and methods section, from a cell-free extract of *E. coli* grown in LB medium supplemented with 100 μ M FeCl₂ and 50 μ g of kanamycin/ml. We obtained 6.2 mg of pure enzyme from 1 litre of culture, which had specific activities of 99, 65, 42 and 0.15 units/mg of protein for the hydrolysis of maltose, sucrose, maltotriose and maltotetraose respectively at 50°C and pH 3.0 in 10 mM sodium citrate buffer.

The α gluFa α -glucosidase gene derived from the phagemid clone pBK α GluFa (GenBank® accession no. AJ717661) encodes a protein of 531 amino acids, having a theoretical molecular mass of 57300 Da and a deduced pI of 6.42. The sequence of the cloned fragment showed no convincing BlastP hits in the SwissProt/TrEMBL and NCBIInr databases, and showed no significant similarity to sequences of known glycosyl hydrolases. Moreover, α GluFa does not show any significant homology to the members of the GH (glycoside hydrolase) family GH 13 [the α -amylase family, formed by a large group of homologous (β/α)₈-barrel proteins that degrade starch, such as α -amylases, α -glucosidases, pullulanases and isoamylases] and GH 31 (comprising most α -glucosidases) of Coutinho and Henrissat [26] (Carbohydrate-Active Enzymes server: <http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html>).

Biochemical characterization

The α gluFa gene was expressed in *E. coli* XL0LR and the activity of its purified recombinant product with various substrates was

Table 1 Kinetic parameters of α -glucosidase from *F. acidiphilum* for hydrolysis of sugar-based substrates

Activity was measured at 50 °C in 100 mM sodium citrate buffer (pH 3.0).

Substrate	k_{cat} (s ⁻¹)	K_m (mM)	k_{cat}/K_m (s ⁻¹ · mM ⁻¹)
Sucrose [α -D-Glc-(1 → 2)- α -D-Fru]	94.0 ± 11.0	0.32 ± 0.06	293.0 ± 44.0
Maltose [α -D-Glc-(1 → 4)-D-Glc]	126.0 ± 8.0	0.64 ± 0.09	197.0 ± 20.0
Maltotriose [α -D-Glc-(1 → 4)-D-Glc-(1 → 4)-D-Glc]	13.0 ± 1.1	0.69 ± 0.08	18.8 ± 1.8
Maltotetraose [α -D-Glc-(1 → 4)-D-Glc-(1 → 4)-D-Glc-(1 → 4)-D-Glc]	2.8 ± 0.1	9.30 ± 0.40	0.3 ± 0.1
<i>p</i> -Nitrophenyl α -D-glucopyranoside	22.5 ± 3.5	5.00 ± 0.10	4.5 ± 0.6

determined. The molecular mass of α GluFa was estimated to be 57 000 Da on the basis of relative mobility on the SDS and native polyacrylamide gels (results not shown).

Kinetic parameters

The kinetic constants (k_{cat} , K_m and k_{cat}/K_m) of the purified recombinant *F. acidiphilum* α GluFa for sugar-based substrates were calculated. As shown in Table 1, the substrate preferences of the α GluFa were: sucrose > maltose ≫ maltotriose ≫ *p*-nitrophenyl- α -D-glucopyranoside ≫ maltotetraose ≫ malto-oligosaccharides from maltopentaose to maltoheptaose (k_{cat}/K_m values of 293.0, 197.0, 18.8, 0.3 and 0.02 s⁻¹ · mM⁻¹ respectively). Other glycosides, such as soluble starch, pullulan, kojibiose, nigeriose, isomaltose, iso-maltotriose, trehalose and *p*-nitrophenyl- β -D-glucopyranoside, were not substrates of α GluFa.

Cation requirement

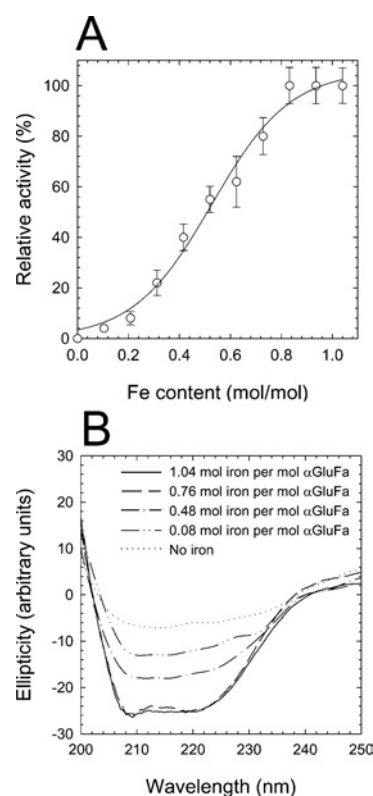
α GluFa was shown to contain 1.04 ± 0.08 mol of iron/mol of α GluFa (ICP-MS analysis). Alternative bivalent cations Ni(II), Co(II), Mn(II), Ca(II) and Mg(II) were not detected in α GluFa and did not stimulate α -glucosidase activity. Dialysis of α GluFa against a solution containing 1 mM EDTA or EGTA to remove iron inactivated the enzyme (Figure 1A). The structural integrity of α -glucosidase was examined by CD spectroscopy. As shown in Figure 1(B), α GluFa exhibited minima at approx. 210–220 nm, characteristic of β -sheet structure. However, when α GluFa was depleted of iron through the action of a chelator, the CD spectrum changed to resemble those of denatured proteins (Figure 1B). This suggests that iron plays a significant functional role in the structural stability of the enzyme, its local or general conformation and thereby its activity.

Effect of pH and temperature

The optimum pH of the purified α GluFa measured with sucrose as substrate was 2.4–3.5 (Figure 2), although it retains > 92 % activity at pH 2.0 and > 74 % at pH 1.5. No measurable activity was detected at pH values above 7.0 and below 1.0. At 50 °C, α -glucosidase was highly stable at pH 2.4–3.5 ($t_{1/2}$ = 34 min at pH 2.5; $t_{1/2}$ = 9.5 min at pH 6.0). As shown in Figure 2, the molar ellipticity at 210 nm, and thus the helical structure content of α GluFa, was maximum at the pH where maximal activity was achieved; however, at pH values < 2.0 or > 5.0, the enzyme started to unfold. The highest specific activity of the enzyme was observed to be at 55–60 °C for sucrose hydrolysis, and its thermostability was < 5 min at 65 °C, 32 min at 60 °C, 57 min at 55 °C, 190 min at 50 °C and > 400 min below 50 °C. No measurable activity was detected above 85 °C.

α -Glucosidase from *F. acidiphilum* transfers glucosyl groups from maltose to produce maltotriose selectively

The formation of a glycosidic bond (transglycosylation) and its hydrolysis are two variants of the same catalytic process [27]. This

**Figure 1** Dependence of α GluFa activity on iron content

(A) Influence of iron removal on α GluFa activity. Purified enzyme (50 μ M in 100 mM citrate buffer, pH 3.0) was incubated with 1 mM EDTA. At different time intervals, two aliquots from the mixture were taken. One of these was used to determine the ability to hydrolyse sucrose using the standard methods, and the second one was used to determine the content of iron by ICP-MS. Hydrolytic activity was measured in 100 mM sodium citrate buffer (pH 3.0) using sucrose as substrate at 50 °C, and is expressed as a percentage of that found in the absence of EDTA. (B) CD of α GluFa. The secondary-structure CD was measured at wavelengths between 200 and 250 nm. The CD spectra were measured for recombinant (—) and EDTA-dialysed α GluFa (50 μ M) in 100 mM sodium citrate buffer (pH 3.0) for 6, 11 and 24 h (corresponding to 0.76, 0.48 and 0.08 mol of iron/mol of α GluFa respectively). The metal ion content of α GluFa was determined using a PerkinElmer Life Sciences ICP-MS before CD was recorded.

duality allows the use of sugar hydrolytic enzymes in synthetic processes [15]. Although previously characterized glycosidases (EC 3.2) are being applied for synthetic purposes, their applications are often limited by low yields and poor regioselectivity [28,29]. Thus most α -glucosidases transfer glucose units to the 6-OH group of the acceptor, yielding products such as isomaltose or panose, although transfer to other hydroxy groups (2-OH, 3-OH and 4-OH) also usually takes place [12–15]. As a result, a mixture of oligosaccharides consisting of α -1,3, α -1,4 and α -1,6 linkages is produced [30]. To assess whether α GluFa could

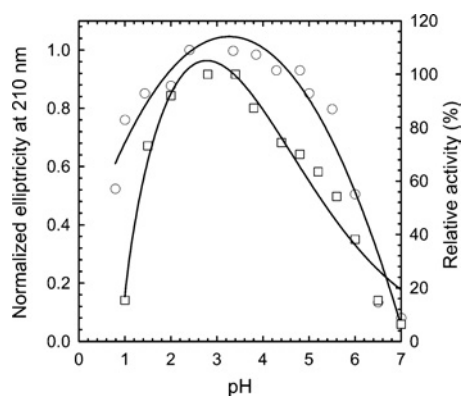


Figure 2 Effect of pH on activity and peptide ellipticity of α GluFa

The optimal pH for enzyme activity (\square ; right axis) was measured by the dinitrosalicylic acid method at 50 °C using sucrose as substrate. Secondary structure by far-UV CD was monitored by measuring the molar ellipticity at 210 nm (\circ ; left axis). The following 100 mM buffers were used: citrate (pH 0.8–4.5), acetate (pH 4.5–5.5), Mes (pH 5.5–7.0) and Hepes (pH 7.0).

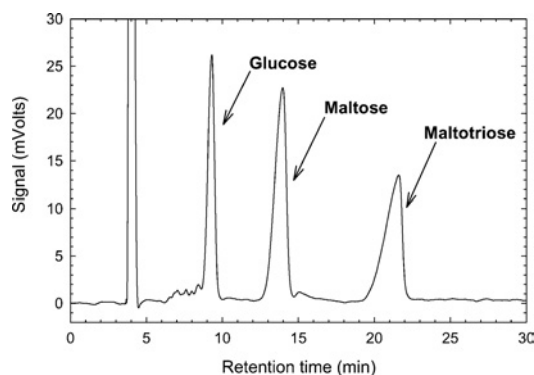


Figure 3 HPLC chromatogram of the reaction mixture in the transglycosylation assay

Conditions: 600 g/l maltose in 0.2 M sodium citrate buffer (pH 3.0) and 5 μ g of purified α GluFa/ml, at 40 °C.

be useful for the synthesis of oligosaccharides, its transglycosylation capability was investigated. We found that α GluFa catalyses the transglycosylation of glucosyl moieties of maltose, selectively producing the trisaccharide maltotriose (Figure 3; also see Supplemental Figure S2 at <http://www.BiochemJ.org/bj/391/bj3910269add.htm>). Also see Supplemental Table S2, which shows the composition of the reaction mixture over time. In the presence of 5 μ g of α GluFa/ml and 600 g/l maltose, the transferase reaction yielded more than 300 g/l maltotriose after 3 h. The transglycosylation ratio was defined as the percentage of maltose that undergoes a transferase reaction rather than a hydrolysis, and was calculated on the basis of the molarities of glucose and maltotriose. As shown in Supplemental Table S2 (<http://www.BiochemJ.org/bj/391/bj3910269add.htm>), the values were very high (> 70%). Interestingly, the transglycosylation ratio was not affected in the course of the reaction (which is normally the case when the concentration of maltose decreases). A selective production of high yields of maltotriose (up to 300 g/l) in the reaction at pH 3.0 with α GluFa suggests that *F. acidiphilum* α -glucosidase has restrictive binding properties. In this regard, Mala et al. [15] suggested that an enzyme having a low capacity to synthesize a tetrasaccharide will also have a low capacity to hydrolyse it, since the conditions to form enzyme–substrate complexes should be the same for both reaction directions. In the case of α GluFa,

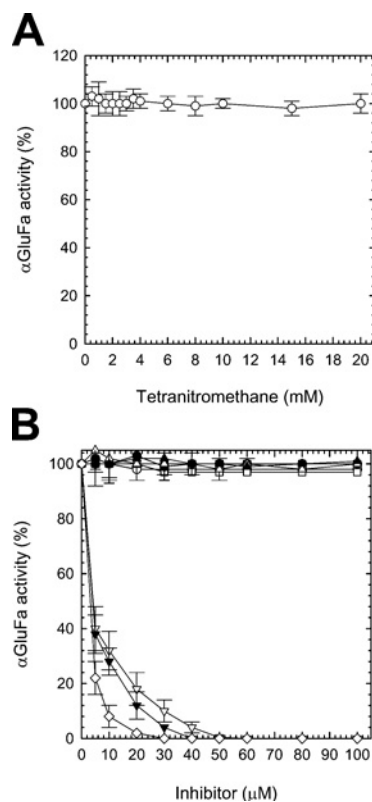


Figure 4 Inhibition of α GluFa

Purified α -glucosidase was incubated with increasing concentrations of (A) TNM (\circ) and (B) *N*-ethylmaleimide (\blacksquare), iodoacetate (\square), *p*-chloromercuribenzoate (\blacktriangle), PCMPS (\triangle), tosylphenylalanylchloromethane (\blacktriangledown), diethyl pyrocarbonate (\blacklozenge), *iPr*₂*P*-F (\bullet), PMSF (\circ) and 5F α GlcF (\diamond). Upon incubation, the aliquots were withdrawn and analysed using the standard α -glucosidase assay (see the Materials and methods section). Residual activity was expressed as a percentage of the control value obtained without the addition of inhibitory chemicals.

its hydrolytic activity decreased markedly from maltose to maltotriose and from maltotriose to maltotetraose (Table 1), which suggests limitations for binding of longer oligosaccharides, which in turn enable the selective synthesis of trisaccharides (i.e. maltotriose) even at low or moderate maltose concentrations. This fact highlights the transglycosylation capability of α GluFa even at low or moderate maltose concentrations.

Inactivation by active-site inhibitors and chemical modification

To probe the residues essential for catalysis, α GluFa was incubated with various inhibitors and its hydrolytic activity was determined (Figure 4). We initially studied chemical modification of α GluFa by EDC/nucleophile modification. Since carbodiimide reacts with the carboxylic groups of aspartic and glutamic residues and the phenolic groups of tyrosine residues, it leads to severe inactivation in glycosidases, α -amylases and cyclodextrin glucoamylases [24]. Despite the fact that we employed a large excess of EDC compared with enzyme (3000:1) to achieve a high level of modification [24], no inactivation of α GluFa was observed ($k_{\text{cat}}/K_m = 285 \pm 41$ and $293 \pm 44 \text{ s}^{-1} \cdot \text{mM}^{-1}$ for the treated and untreated enzymes respectively). Nitration of α GluFa with the tyrosine-specific reagent TNM also had no effect on α -glucosidase activity (Figure 4A). These results suggest that α GluFa-mediated hydrolysis does not involve CO₂H groups of Asp, Glu or Tyr residues.

α GluFa was also resistant to cysteine-specific reagents, such as *N*-ethylmaleimide, iodoacetate, *p*-chloromercuribenzoate and

PCMPs (Figure 4B), and sequence analysis revealed that α GluFa does not possess any cysteine residue. α GluFa was also not affected by serine hydrolase inhibitors, such as *iPr*₂*P*-F and PMSF. On the other hand, the enzyme was strongly inhibited by histidine-specific inhibitors, such as tosylphenylalanylchloromethane ('TPCK') and diethyl pyrocarbonate (Figure 4B), which caused a marked concentration-dependent decrease of enzyme activity with $K_i = 4.2 \pm 0.12$ and $2.9 \pm 0.10 \mu\text{M}$ respectively at pH 3.0; this result strongly suggested that histidine residues are involved in the catalytic function of the enzyme [31–33]. α GluFa was also strongly inhibited by the reversible inhibitor 5F α GlcF, which inhibited immediately upon addition and maintained the same level of inhibition over time. A similar observation has also been reported for an α -glucosidase from *A. niger* [25]. An apparent K_i value of $3.1 \pm 0.10 \mu\text{M}$ was determined in a second set of experiments that addressed 5F α GlcF as a competitive inhibitor [25]. The presence of glucose and maltose, the end-products of the hydrolysis of sucrose, maltose and maltotriose, did not affect the hydrolytic activity of the enzyme (at least at concentrations ≤ 0.2 M).

Glu and Asp residues are not catalytic residues in α GluFa

α GluFa contains eight Glu and 17 Asp residues, one of which might in principle mediate sugar hydrolysis. To confirm that none of them is involved in catalysis, as suggested by the carbodi-imide modification, single mutants were constructed in which each of the glutamic and aspartic residues were separately replaced by Gln (E/Q and D/Q respectively). The mutated proteins were expressed in *E. coli* DH5 α , purified, and the kinetic constants k_{cat}/K_m of these enzymes with sucrose as substrate were determined. As shown in Supplemental Figure S1 (<http://www.BiochemJ.org/bj/391/bj3910269add.htm>), similar hydrolytic patterns were observed for the wild-type and α GluFa E/Q and D/Q variants (k_{cat}/K_m values from 229 ± 37 to $305 \pm 50 \text{ s}^{-1} \cdot \text{mM}^{-1}$). We also assayed by HPLC the apparent specific activities of the mutant variants with maltose as substrate. As shown in Supplemental Figure S1 (<http://www.BiochemJ.org/bj/391/bj3910269add.htm>), the differences among variants and wild-type enzymes were more pronounced with maltose as substrate compared with sucrose as substrate, although none of the mutated proteins was inactive [minimum specific activity: 129 units/mg for D391Q (Asp³⁹¹ \rightarrow Gln) mutant]. This suggests that neither glutamic nor aspartic residues are catalytic residues in α GluFa and, therefore, that its active site structure differs substantially from known glycosyl hydrolases (proof awaits three-dimensional structure determination, which is currently in progress), whose sugar-binding sites involve the carboxylic side chains of glutamic and aspartic residues [11].

Identification of Thr²¹² as a catalytic nucleophile

To identify the catalytic nucleophile, 80 μg of α GluFa was incubated with the inhibitor 5F α GlcF. A sample of α -glucosidase either untreated (Figure 5A) or treated for 5 min with 5F α GlcF (1.6 mM; Figure 5B) was digested with pepsin at pH 2.0 and loaded on to a C18 HPLC column connected to an ESI mass spectrometer, and comparative mass spectra were obtained. ESI-MS analysis revealed the presence of a peptide of m/z 756.62 in the labelled α -glucosidase. The putative labelled peptide was isolated by reversed-phase HPLC and subjected to ESI-MS/MS fragmentation analysis (Figure 5C), which revealed a fragment of 575.6 Da, corresponding to the mass difference between the mass of the peptide, 756.62 Da, and the mass of the 5F α GlcF label, 181 Da. Additionally, eight prominent peaks of m/z 159.1, 273.9, 286.1, 387.2, 501.3, 554.9, 558.6 and 568.5 were detected

(Figure 5C). Interpretation of the spectrum indicated that the labelled peptide corresponded to peptide sequence WVTNG, which corresponds to amino acids 210–214 of the protein. Of these amino acids, only threonine and asparagine residues are putative nucleophilic candidates. To identify the nucleophilic residue unambiguously, site-directed mutagenesis was used to replace Thr²¹² and Asn²¹³ by Gln. Replacement of Thr²¹² by Gln (T212Q) resulted in a 600-fold reduction in activity (k_{cat}/K_m values decreased from 293 to $0.5 \text{ s}^{-1} \cdot \text{mM}^{-1}$). Activity could be partially restored by the addition of an external nucleophile, i.e. formate (98 mM formate restored up to 90% original activity). No inactivation of α GluFa occurred through replacement of Asn²¹³ by Gln ($k_{\text{cat}}/K_m = 293 \pm 44$ and $270 \pm 41 \text{ s}^{-1} \cdot \text{mM}^{-1}$ for the native and modified enzymes respectively). These results suggest that Thr²¹² is essential for α -glucosidase activity and is the nucleophile of the enzyme. Moreover, the far-UV spectra of the T212Q (Supplemental Figure S3 at <http://www.BiochemJ.org/bj/391/bj3910269add.htm>) and N213Q (results not shown) variants were very similar to that of the wild-type enzyme, suggesting that the mutations did not introduce significant changes in secondary structure in the enzyme.

His³⁹⁰ is essential for iron binding

Treatment of α GluFa with histidine-specific reagents resulted in a substantial reduction of α -glucosidase activity. To determine which amino acid(s) is(are) important for α -glucosidase activity, we replaced all eight histidine residues in the protein, His⁹, His⁴¹, His⁴⁷, His²⁴³, His²⁷⁵, His³²⁵, His³⁷⁷ and His³⁹⁰, by an alanine residue, through site-directed mutagenesis, and analysed the α -glucosidase activity, iron content and secondary structures of the variants produced (Table 2). Complete loss of activity was observed in the variant enzyme with His³⁹⁰ replaced by Ala. Mutations at His⁴⁷, His²⁴³ and His³²⁵ only slightly affected the hydrolytic activity, whereas mutations at His⁹, His⁴¹, His²⁷⁵ and His³⁷⁷ had no effect. The iron content of the His³⁹⁰ variant was significantly lower than that of the wild-type enzyme, and mutations at His²⁴³ and His⁴⁷ caused a partial loss of the iron. The lower iron content directly correlated with decreased α -glucosidase activity. Furthermore, the secondary structure of the His⁹, His⁴¹, His²⁷⁵ and His³⁷⁷ mutant proteins was very similar (according to their molar ellipticity) to that of the wild-type enzyme, whereas secondary structure of H390A (see also Supplemental Figure S3 at <http://www.BiochemJ.org/bj/391/bj3910269add.htm>) and, to a lesser extent, that of the H47A and H243A variants were significantly different.

We further compared the secondary-structure content of single mutants T212G and H390A and double mutants T212G/H390A (all of them inactive) with that of the wild-type protein. As shown in Supplemental Figure S3 (<http://www.BiochemJ.org/bj/391/bj3910269add.htm>), only proteins containing mutations at His³⁹⁰ exhibited significant alterations in secondary structure. This demonstrated conclusively that iron elimination in His \rightarrow Ala variants, rather than the amino acid mutation itself, is responsible for structural perturbation and loss of activity, whereas Thr²¹² is directly implicated in catalysis, being essential for α -glucosidase activity.

Taken together, these studies indicate that iron plays an essential role in the structural integrity of α GluFa, that His³⁹⁰ is the ligand for iron binding/co-ordination in the enzyme and that Thr²¹² is the nucleophile in its catalytic activity. This, in turn, suggests that α GluFa is a novel α -glucosidase with a novel catalytic mechanism for sugar glycosylation and transglycosylation in which the formation of a glycosyl-enzyme intermediate is initiated by nucleophilic attack on the carbonyl carbon of the sugar by the hydroxy group of the catalytic threonine residue, rather than by

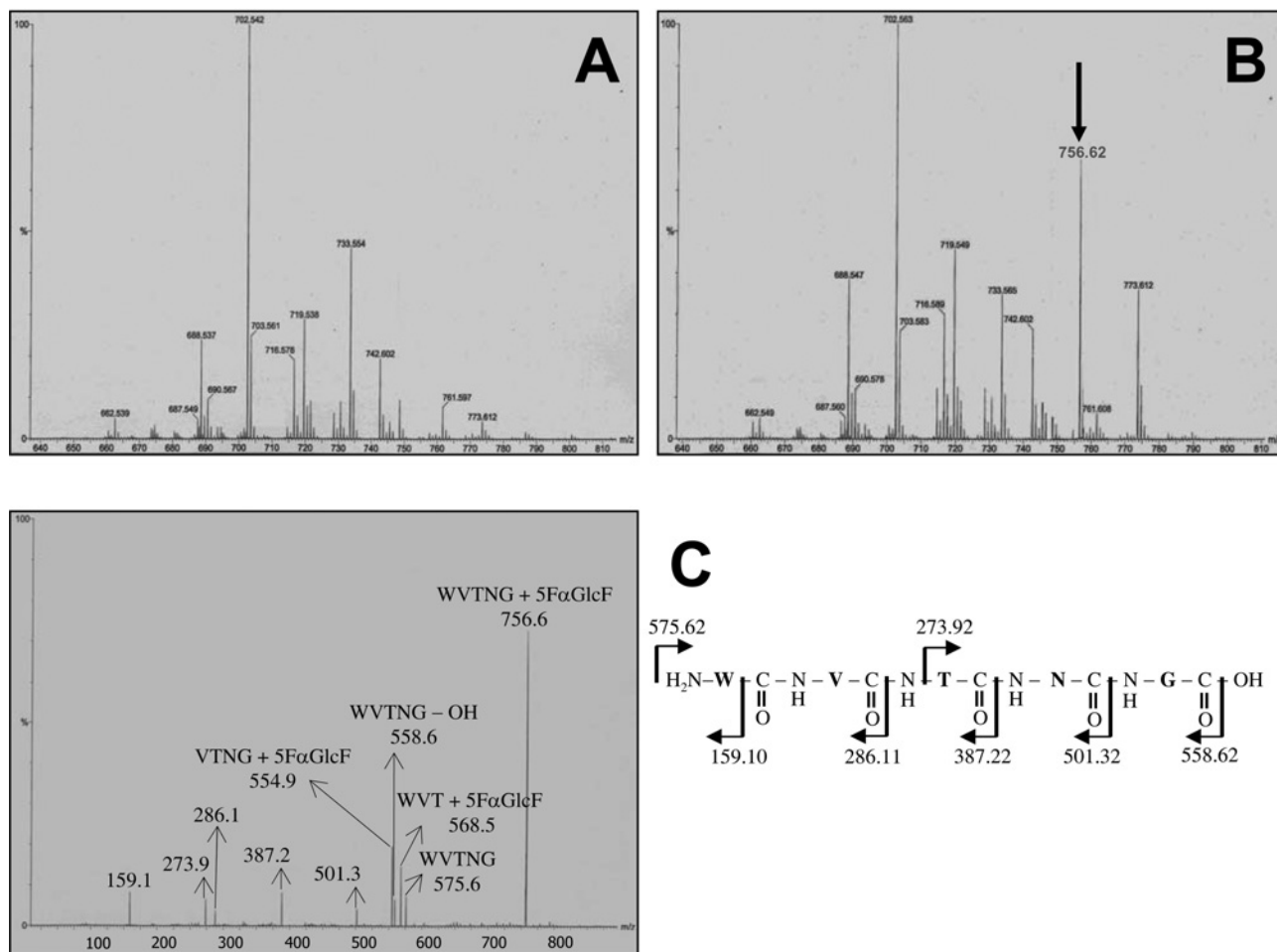


Figure 5 Identification of catalytic residues by labelling with 5F α GlcF and MS/MS analysis

Comparative mass spectra of (A) unlabelled and (B) labelled *F. acidiphilum* α -glucosidase. The arrow indicates the peptide unique to the peptic digest of labelled enzyme. Only the fragments of m/z ranging from 640 to 810 are shown. No differences were observed above m/z 810 and below m/z 640. (C) MS/MS spectrum of the 5F α GlcF labelled peptide (m/z 756.620) and an interpretation of the spectrum.

Table 2 Catalytic specificity (k_{cat}/K_m), iron content and molar ellipticity of wild-type α -glucosidase and His \rightarrow Ala mutants from *F. acidiphilum*

Mutant	k_{cat}/K_m ($s^{-1} \cdot mM^{-1}$)*	Iron content (mol/mol)	Molar ellipticity at 210 nm (%)
Wild-type	293.0 ± 44.0	1.04 ± 0.08	100
H9A	302.0 ± 38.0	1.00 ± 0.09	100
H41A	312.0 ± 33.0	0.96 ± 0.08	100
H47A	220.0 ± 31.0	0.86 ± 0.08	88
H243A	227.0 ± 32.0	0.55 ± 0.07	65
H275A	297.0 ± 35.0	1.04 ± 0.08	100
H325A	296.0 ± 37.0	1.00 ± 0.08	100
H277A	291.0 ± 27.0	0.99 ± 0.06	102
H390A	5.4 ± 0.4	0.05 ± 10^{-3}	2

* Measured at 50 °C in 100 mM sodium citrate buffer (pH 3.0) using sucrose as substrate.

the general acid/base mechanism, mediated by glutamic and/or aspartic residue (as in all glycosidases) or tyrosine residue (in sialidases) [27,34]. The nucleophile water, or probably the iron-interacting water molecules at His³⁹⁰, then probably attack the glycosyl-enzyme intermediate to form the product, analogous to the zinc-water molecule interaction in metalloproteases [35,36].

Although the formation of such intermediates has been proposed previously for hen's-egg white lysozyme, several α -amylases, cyclodextrin glucanotransferases and *B. subtilis* xylanase, in all these cases the nucleophile and the acid/base catalysts were the carboxylic chains of glutamic and aspartic residues [37]. Moreover, although threonine has been found to be the active-site nucleophile in other hydrolases, such as glycosylasparaginase, dipeptidyl peptidase IV, γ -glutamyltranspeptidase and threonine phosphatases [38–44], in these systems the reactive nucleophile threonine requires activation via deprotonation by histidine or interaction with another threonine residue. Clearly, structural analysis of *F. acidiphilum* α -glucosidase is needed to confirm these conclusions, although, so far, we have not been able to obtain crystals that are stable within the pH range 2.4–3.5. Nevertheless, the present study opens up new perspectives for the investigation of structural adaptation of proteins to extreme conditions and novel structural features in the glycoside hydrolase superfamily.

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