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Catalytic role of the calcium ion in GH97 inverting glycoside hydrolase

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ABSTRACT

The role of calcium ion in the active site of the inverting glycoside hydrolase family 97 enzyme, *Bt*GH97a, was investigated through structural and kinetic studies. The calcium ion was likely directly involved in the catalytic reaction. The pH dependence of k_{cat}/K_m values in the presence or absence of calcium ion indicated that the calcium ion lowered the pK_a of the base catalyst. The significant decreases in k_{cat}/K_m for hydrolysis of substrates with basic leaving groups in the absence of calcium ion confirmed that the calcium ion facilitated the leaving group departure.

1. Introduction

Several glycoside hydrolases require the participation of a metal ion for catalysis. For instance, *exo*-mannosidases belonging to glycoside hydrolase families (GH) 38, 47, and 92 require a divalent metal ion, such as zinc or calcium. The metal ion bridges the C2 and C3 oxygen atoms and is associated with the stabilization of the transition state [1]. GH4 glycoside hydrolases which employ an unique redox-elimination mechanism to cleave the glycosidic linkage require a divalent metal ion for catalysis [2]. The divalent ion helps to stabilize the enediolate intermediate, which arises during redox-elimination. GH2 β-galactosidase uses a magnesium ion for catalysis. The magnesium ion makes direct contact with the acid/base catalyst and thus functions in tuning the pK_a of the acid/base catalyst [3,4].

GH97 is an unique family that contains an inverting α-glucoside hydrolase (*Bt*GH97a) and a retaining α-galactoside hydrolase (*Bt*GH97b) [5–7]. Both enzymes contain one calcium ion in the active site which plays an important role in the catalysis of both enzymes, likely orienting functional residues and/or acting as a Lewis acid. Indeed, incubation with EDTA causes substantial reduction of their catalytic activity [5,6]. The calcium ion in the ligand-free inverting *Bt*GH97a is coordinated by four glutamate residues (Glu194, Glu508, Glu526, and Glu532) and three water molecules (Fig. 1A). Three water molecules are replaced by the O2 of the valienamine unit, the O3 and N4B of the 4-amino-4,6-dideoxy α-D-glucose unit in the acarbose-complex form (Fig. 1B). The interaction with N4B is of interest because N4B is the pseudo-scissile bond atom in acarbose and the calcium ion seems to be directly involved in catalysis. Among the amino-acid residues coordinated with the calcium ion, Glu508 and Glu532 are closely involved with the catalytic reaction. The inverting glycoside hydrolase possesses two functional groups, which act as catalytic acid and base, in the active site. The catalytic acid transfers the proton to the oxygen atom in the scissile glycosidic linkage and promotes the departure of the substrate leaving group. The catalytic base activates a catalytic water molecule to abstract the proton, with the water molecule attacking on the anomeric carbon of the substrate. The carboxy group of Glu532 is then poised to act as the catalytic acid. The side chain of Glu508 interacts with the water molecule in position for an in-line attack at the anomeric center together with the carboxy group of Glu439, both functioning as catalytic bases in the

Abbreviations: BSA, bovine serum albumin; 2C4NPG, 2-chloro-4-nitrophenyl α-D-glucopyranoside; 4C2NPG, 4-chloro-2-nitrophenyl α-D-glucopyranoside; 2,4DNPG, 2,4-dinitrophenyl α-D-glucopyranoside; 3,4DNPG, 3,4-dinitrophenyl α-D-glucopyranoside; EDTA, ethylenediamine-*N,N,N′,N′*-tetraacetic acid; GH, glycoside hydrolase family; MNPG, *m*-nitrophenyl α-D-glucopyranoside; PNPG, *p*-nitrophenyl α-D-glucopyranoside

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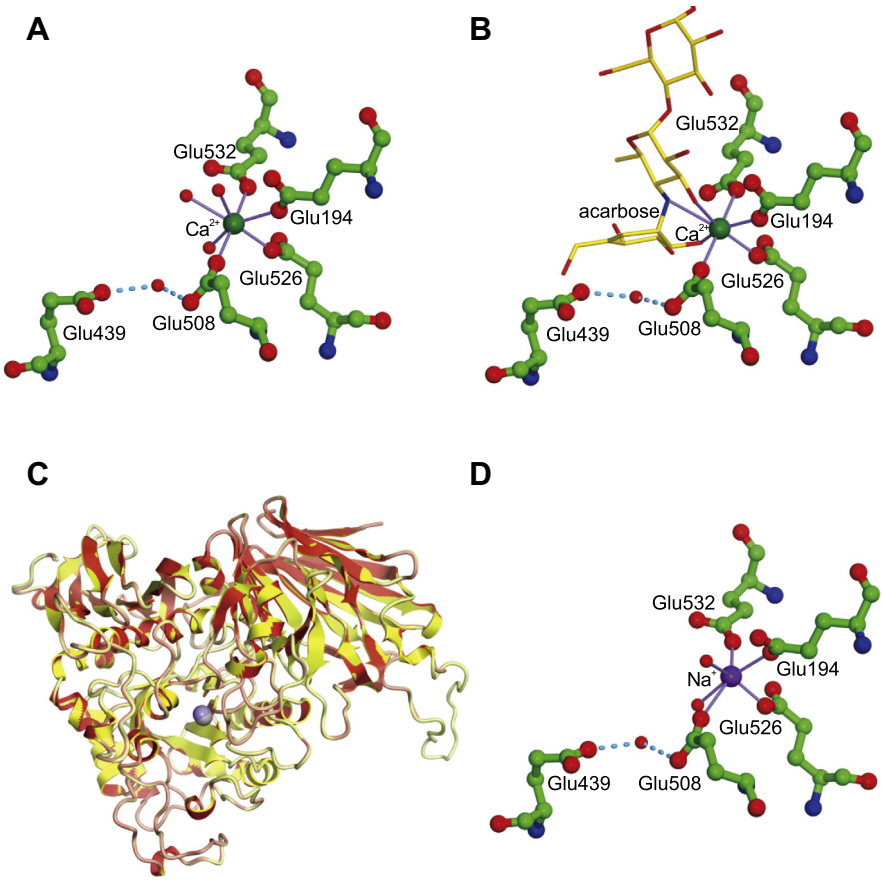

Fig. 1. Structural comparison of *Bt*GH97a and EDTA-treated *Bt*GH97a. The calcium ion binding sites, native *Bt*GH97a (A; PDB ID, 2D73), and acarbose-complex *Bt*GH97a (B; PDB ID, 2ZQ0). (C) Overall structure of the EDTA-treated *Bt*GH97a (red) with native *Bt*GH97a (yellow). (D) The sodium ion binding in the EDTA-treated *Bt*GH97a. Figures were created using CueMol 2 (http://cuemol.sourceforge.jp/).

inverting mechanism. The retaining α-galactosidase, *Bt*GH97b, contains a calcium ion at the same position as *Bt*GH97a [7]. It has three equivalent carboxy groups, corresponding to Glu194, Glu526 and Glu532, and four water molecules, which are associated with the calcium binding. Since the enzyme does not have a residue equivalent to Glu508, one water molecule of the four is located at the same position as the carboxy group of Glu508, and makes a hydrogen bond to the residue functioning as the nucleophile catalyst in the retaining mechanism.

The purpose of this study was to elucidate the role of the calcium ion in the catalysis of the inverting *Bt*GH97a using kinetic studies and analysis of the crystal structure of EDTA-treated *Bt*GH97a. These studies revealed that the calcium ion participates in the catalysis differently from what was previously observed for other GHs, as it stabilizes the deprotonated state of the base catalyst and assists in the departure of the substrate leaving group.

2. Materials and methods

2.1. Materials

p-Nitrophenyl α-D-glucopyranoside (PNPG) was purchased from Nacalai Tesque (Kyoto, Japan). Aryl glucosides, 2,4-dinitrophenyl, 2-chloro-4-nitrophenyl, and 4-chloro-2-nitrophenyl α-D-glucopyranosides (2,4DNPG, 2C4NPG, and 4C2NPG) were synthesized according to a published procedure [8]. Other aryl glucosides, 3,4-dinitrophenyl and *m*-nitrophenyl α-D-glucopyranosides (3,4DNPG and MNPG, respectively), were synthesized from 1,2,3,4,6-penta-*O*-acetyl-D-glucopyranose and the corresponding phenol derivative with trifluoromethane sulfonic acid as a catalyst. Under a nitrogen atmosphere, trifluoromethane sulfonic acid (5.5 mmol) was added to a mixture of 1,2,3,4,6-penta-*O*-acetyl-D-glucopyranose (5.5 mmol) and the desired phenol (11 mmol) in 10 mL of dichloromethane. The mixture was stirred for 3 h at room temperature, and then dry pyridine (4 mL) and acetic anhydride (0.95 mL) were added and stirred for 1 h at room temperature. Sodium acetate trihydrate (1.8 g) was added to the mixture and it was stirred vigorously for several minutes. Saturated $NaHCO_3$ solution was added and the organic layer was extracted with dichloromethane, washed with ice-cold water, and dried over Na_2SO_4. Pyridine was removed by co-evaporation with toluene and the product was purified by column chromatography, performed on 40–50 μm silica gel (2:1 hexane/ethyl acetate for *m*-nitrophenyl 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranoside, 3:2 hexane/ethyl acetate for 3,4-dinitrophenyl 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranoside). The ^1H NMR spectra of all aryl per-*O*-acetyl-glucosides were identical to published data [8,9]. Deacetylation of 2,4-dinitrophenyl, 2-chloro-4-nitrophenyl, and 3,4-dinitrophenyl 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosides were performed by the acetyl chloride method [8] and 4-chloro-2-nitrophenyl and *m*-nitrophenyl 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosides were deacetylated with sodium methoxide in dry methanol.

2.2. Enzyme purification

The genes encoding the wild-type *Bt*GH97a and the E532Q mutant were amplified from previously constructed plasmid DNAs [6] by PCR. Each resultant fragment was ligated into the pET23d