Identification of the Catalytic Residues of Bifunctional Glycogen Debranching Enzyme*

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Eukaryotic glycogen debranching enzyme (GDE) possesses two different catalytic activities (oligo-1,4–1,4-glucantransferase/amylo-1,6-glucosidase) on a single polypeptide chain. To elucidate the structure-function relationship of GDE, the catalytic residues of yeast GDE were determined by site-directed mutagenesis. Asp-535, Glu-564, and Asp-670 on the N-terminal half and Asp-1086 and Asp-1147 on the C-terminal half were chosen by the multiple sequence alignment or the comparison of hydrophobic cluster architectures among related enzymes. The five mutant enzymes, D535N, E564Q, D670N, D1086N, and D1147N were constructed. The mutant enzymes showed the same purification profiles as that of wild-type enzyme on β-CD-Sepharose-6B affinity chromatography. All the mutant enzymes possessed either transferase activity or glucosidase activity. Three mutants, D535N, E564Q, and D670N, lost transferase activity but retained glucosidase activity. In contrast, D1086N and D1147N lost glucosidase activity but retained transferase activity. Furthermore, the kinetic parameters of each mutant enzyme exhibiting either the glucosidase activity or transferase activity did not vary markedly from the activities exhibited by the wild-type enzyme. These results strongly indicate that the two activities of GDE, transferase and glucosidase, are independent and located at different sites on the polypeptide chain.

Glycogen debranching enzyme (GDE)1 is a bifunctional enzyme exhibiting both transferase (oligo-1,4–1,4-glucantransferase, EC 2.4.1.25) and glucosidase (amylo-1,6-glucosidase, EC 3.2.1.33) activities (1). Both enzymes reside on a single polypeptide chain (2, 3), and each has its own distinct catalytic activity. GDE is a key enzyme in the carbohydrate metabolism of mammalian cells and yeast. GDE along with phosphorylase allows the complete degradation of glycogen and the release of glucose-1-phosphate and glucose. A maltosyl or maltotriosyl residue from the branched chain of glycogen is first transferred to the main chain by the transferase to expose the α-1,6-glucosyl stub that is in turn hydrolyzed by the glucosidase, thus allowing glycogen phosphorylase to degrade the linearized α-(1,4) polymer. Genetic deficiency of the enzyme in human Type III glycogen storage disease (GSD-III or Cori’s disease) is characterized by hepatomegaly, hypoglycemia, variable myopathy, and cardiomyopathy (4, 5).

GDE has been purified and characterized from rabbit (2, 3) and yeast (6, 7). Inhibitors specifically affecting either the transferase or the glucosidase activity provided evidence that each of the two activities occur at distinct catalytic sites (8). Liu et al. (9) showed that the transferase activity was irreversibly inactivated by carbodiimide in the presence of amines without affecting the glucosidase activity and concluded the existence of two distinct active sites, although the locations were not defined. The amino acid sequence analysis of rabbit GDE indicated that the N-terminal half may encompass the transferase activity, leaving the glucosidase activity at the C-terminal half (10).

Several facts point to the resemblance of yeast GDE to the mammalian GDEs. The yeast, human, and rabbit GDEs have 1536, 1515, and 1555 amino acid residues, respectively, as deduced from nucleotide sequences (10–12). The yeast GDE N-terminal half also possesses four conserved sequences in the α-amylase family, and the C-terminal half displays about 50% identity with the C-terminal half of other mammalian GDEs. Foremost, yeast GDE exhibits the same transferase and glucosidase action toward glycogen and branched cyclodextrins as those of mammalian GDEs (7). Therefore, yeast GDE can serve as a model for the eukaryotic GDE for elucidating the structure-function relationship.

To determine the location of the active sites of both the transferase and glucosidase activities, we constructed several mutant yeast GDEs by site-directed mutagenesis. From the analysis of the enzymatic activities, the amino acid residues involved in the catalysis of the transferase and glucosidase of yeast GDE were identified.

EXPERIMENTAL PROCEDURES

Chemicals—Maltopentaose and 6-O-α-glucosyl cyclomaltoheptaose (Glc-β-CD) were purchased from WAKO Chemicals. 6-O-α-Maltotetraose cyclomaltoheptaose (6-O-α-glucosyl-β-cyclodextrin); Glc-β-CD, 6-O-α-glucosyl cyclomaltoheptaose (6-O-α-glucosyl-β-cyclodextrin); HCA, hydrophobic cluster analysis; HPLC, high performance liquid chromatography.

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¶ The abbreviations used are: GDE, glycogen debranching enzyme; glucosidase, amylo-1,6-glucosidase; transferase, oligo-1,4–1,4-glucontransferase; TAA, Taka-amylase A; CGTase, cyclodextrin glycosyltransferase; β-CD, cyclomaltoheptaose (β-cyclodextrin); Glc-β-CD, 6-O-α-glucosyl cyclomaltoheptaose (6-O-α-glucosyl-β-cyclodextrin); Glc-β-CD, 6-O-α-maltotetraose cyclomaltoheptaose (6-O-α-maltotetraose-β-cyclodextrin); HCA, hydrophobic cluster analysis; HPLC, high performance liquid chromatography.

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The amino acids were numbered from the N-terminal end. Conserved residues are in bold. Asterisks indicate the target amino acid residues on the yeast GDE for site-directed mutagenesis. TAA (Taka-amylase, \(\alpha\)-amylase from Aspergillus oryzae), CGTase (cycloextrin glycosyltransferase from Bacillus circulans), H-GDE (Human muscle glycogen debranching enzyme), R-GDE (Rabbit muscle glycogen debranching enzyme), Y-GDE (glycogen debranching enzyme from Saccharomyces cerevisiae D-546).

| Enzyme | Consensus sequences |
|--------|-------------------|
| TAA    | 111–GMYLVDDVAVNH  |
| CGT    | 128–GIKIVDFADPHHN |
| H-GDE  | 192–NVICITDDYVYNH |
| R-GDE  | 232–NVLCTDDVYVYNH |
| Y-GDE  | 218–NMLSLIDIVFVNH |

| Consensus sequences | Glu-164 (Asp-535) | Glu-255 (Glu-564) | Asp-670 |
|---------------------|-------------------|-------------------|--------|
| I                   | 201–DGIRIDTV      | 262–YGIYEDLV     | 289–LGFVYENHD |
| II                  | 224–DGIRDAYV      | 253–FFGDSNWL     | 320–QYFTDHD |
| III                 | 504–QGVNLDCN      | 534–YVAELFT      | 602–ALFMDTHTD |
| IV                  | 544–QGVNLDCN      | 574–YVAELFT      | 642–ALFMDTHTD |

![FIG. 1. Hydrophobic cluster analysis (HCA) plots of oligo-1,6-glucosidase from R. cereus and the C-terminal half of yeast GDE.](http://www.jbc.org/)

**TABLE I**

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|--------|-------------------|
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**RESULTS**

**Analysis of the Amino Acid Sequences for the Prediction of Amino Acids Essential for GDE Activity—As shown in Table I, alignment of the amino acid sequences of human muscle (12), rabbit muscle (10), and yeast (11) GDE revealed the presence of four consensus sequences commonly found in the \(\alpha\)-amylase family or glycosyl hydrolase family 13 (17) on the N-terminal half. It has been suggested that the consensus sequences II, III, and IV in the \(\alpha\)-amylase family contain three conserved acidic amino acids (two Asp and one Glu) (18). Jespersen et al. (19) suggested that the corresponding carboxyl residues in human GDE are involved in the transferase activity. Therefore, amino acid residues, Asp-535, Glu-564, and Asp-670, which are located in the consensus sequences II, III, and IV of yeast GDE, respectively, were chosen as the target for site-directed mutagenesis to determine their role in transferase activity. Liu et al. (10) suggested that the C-terminal half might...
enzymes were highly expressed and have the same elution mutations without any second-site mutations. All the mutant enzyme.

Among the acidic amino acids, Asp-1086 and Asp-1147 of yeast GDE showed similar patterns in the HCA plot as Asp-206 in the conserved region II and Glu-230 in the conserved region III, (Fig. 1). Fur-the conserved region II and Glu-230 in the conserved region III, the two catalytic residues of oligo-1,6-glucosidase (Fig. 1). Further.

The transferase-deficient mutants were at the same level as that of the wild-type GDE. Therefore, we then compared the kinetic parameters of each mutant and wild-type enzyme.

Using Glc-β-CD as a substrate, the glucosidase activity of GDE was also analyzed by HPLC. The transferase-deficient mutant enzymes D535N, E564Q, and D670N indeed gave hydrolysis products of glucose and β-CD from the substrate Glc-β-CD similar to the wild-type enzyme (Fig. 3a), whereas the glucosidase-deficient mutant enzymes D1086N and D1147N failed to hydroyze the substrate Glc-β-CD (Fig. 3b).

The HPLC analyses indicate that the mutants retained either the transferase or the glucosidase activity (Table II), depending on the site of mutation each possessed. However, the question remains whether the retained activity of each mutant varied from that of the wild-type GDE. Therefore, we compared the kinetic parameters of each mutant and wild-type enzyme.

Kinetic Parameters of Mutant GDEs—The glucosidase activity of the transferase-deficient mutants, D535N, E564Q, and D670N, was measured using Glc-β-CD as a substrate, whereas the transferase activity of the glucosidase-deficient mutants, D1086N and D1147N, was measured using maltopentaose as a substrate (see “Experimental Procedures”). As shown in Table III, kinetic parameters for the glucosidase activity of the transferase-deficient mutants were at the same level as that of the wild-type enzyme. In addition, $K_m$ values for the transferase...
activity of glucosidase-deficient mutants were also similar to that of wild-type enzyme (D1086N, D1147N, wild-type: 10.0, 10.6, 10.8 mM, respectively). These results indicated that each mutant GDE catalyzes its respective reaction similar to the wild-type, without being influenced by the loss of other functions.

**DISCUSSION**

The five mutants D555N, E564Q, D670N, D1086N, and D1147N were produced to discriminate the individuality of the two catalytic sites of GDE as earlier proposed (8–11). The identical purification profiles of these mutant enzymes on β-CD-Sepharose-6B affinity column chromatography with the wild-type enzyme indicate that the mutant GDEs have the same binding affinity to the wild-type enzyme, suggesting that the GDE transferase is an N-terminal half. (Fig. 4). Because the catalytic mechanism and located at the N-terminal half and the glucosidase at the C-terminal half. (Fig. 4). Because the catalytic mechanism and structural conformation of the active sites of cyclodextrin glucanotransferase, CGTase (23–25). The Asp-229 of CGTase and Asp-549 of rabbit GDE had been confirmed to work as a catalytic nucleophile of the transferase (26, 27). Because Asp-535 of yeast GDE corresponds to Asp-229 of CGTase and Asp-549 of rabbit GDE in the amino acid sequence alignment (Table I), the possibility exists that Asp-535 may play the same role in transferase action. It has been suggested that the GDE transferase is an α-retaining glucosidase (9) and likewise a CGTase. Therefore, the transferase of yeast GDE may employ a double displacement mechanism to process α-linked glucose polymers as demonstrated by CGTase (28).

Spectrochemical analysis of the glucosidase reaction revealed that the yeast GDE hydrolyzed the α-1,6-glucosidic linkage and released a β-anomer of glucose from Glc-β-CD, an inverted configuration of the expected product (data not shown). Similarly, such an α-inverting mechanism was also observed with the glucosidase of rabbit GDE (9). In the case of the α-inverting glucosidase belonging to the glycosyl hydrolase family 15 (29), the combination of differential labeling and site-directed mutagenesis or the crystal structure analysis identified two carboxyl residues of glucoamylase as the catalytic acid/catalytic base (30–32). Hence, Asp-1086 and Asp-1147 of the yeast GDE located at the C-terminal half may act as a general acid catalyst or general base catalyst for the glucosidase reaction. However, structural conformation of the active sites may differ between the glucosidase of yeast GDE and glucoamylase, because while the glucoamylase was inactivated by the modification induced by the inhibitor of water-soluble carbodiimide (33–35), the glucosidase of yeast GDE was not affected (data not shown).

In this study, we clearly demonstrated that the transferase and glucosidase of yeast GDE are well discriminated at two distinct sites of a single polypeptide chain, the transferase located at the N-terminal half and the glucosidase at the C-terminal half. (Fig. 4). Because the catalytic mechanism and primary structural characteristics of the transferase of yeast GDE is similar to the enzymes belonging to the α-amylase family, the N-terminal half of the yeast GDE protein may also be folded in the form of an α/β-barrel structure. On the other hand, though glucosidase at the C-terminal half of yeast GDE exhibited a catalytic mechanism (α-inverting) in good agree-
ment with that of glucoamylase, their primary structural characteristics indicate a possible difference of structural conformation between the two types of glucosidases. This study thereby provides clear evidence of the distinctiveness of the active sites of the transferase and glucosidase of yeast GDE and may serve as a basis for the in depth understanding of the structure-function relationship of bifunctional glycogen debranching enzymes.

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J. Biol. Chem. 2001, 276:28824-28828.
doi: 10.1074/jbc.M102192200 originally published online May 25, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M102192200

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