The functional role of glutamine-280 and threonine-282 in human α-galactosidase

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Received 22 July 1994; revised 31 October 1994; accepted 24 November 1994

Abstract

Our previous study on chimeric mutants of α-galactosidase suggested that two peptide regions encoded by exons 1–2 and 6 of the enzyme gene contribute to substrate recognition (Ishii, S. et al. (1994) Biochim. Biophys. Acta 1204, 265–270). In this study, we constructed five single amino acid substitutions for functional analysis of the amino acid residues around glutamine-279, the mutation site detected in an atypical Fabry disease patient. Two mutants, Q280S (Gln280 → Ser; CAA → TCA) and T282A (Thr282 → Ala; ACT → GCT), showed increased $K_m$ and decreased thermostability as compared with normal enzyme. Circular dichroism spectrum was not modified. An additional chimeric mutation in the exon 1–2 region by substitution with the homologous sequence of α-N-acetylgalactosaminidase cDNA restored catalytic activity and thermostability in both mutants. These data indicated the functional significance of glutamine-280 and threonine-282 for expressing the activity and stability of α-galactosidase molecule, and also the presence of an intramolecular interaction between the two peptide regions encoded by exons 1–2 and 6.

Keywords: α-Galactosidase; Amino acid substitution; Thermostability; Chimeric mutation

1. Introduction

Human α-galactosidase (α-Gal) is a lysosomal hydrolase responsible for the catabolism of glycoconjugates with terminal α-galactosidic moiety [1]. The accumulation of glycosphingolipids, mainly globotriaosylceramide, has been observed in tissues from patients with Fabry disease, an X-linked recessive disorder caused by a deficiency of this enzyme. The gene structure for α-Gal has been reported [2–4], but the functional structure of the enzyme protein is poorly understood.

The mutations in classic Fabry disease have been detected in highly scattered regions of the α-Gal gene [5–14], but the four mutations for a late-onset atypical variant of this disease are single base substitutions localized in the upstream region of exon 6 (Q279E, M296V, and R301Q) or at a glycosylation site (N215S) [14]. Recently, we characterized a gene expression product for one of them, Q279E, and suggested that the mutation site is closely associated with the galactose recognition site [15].

Another approach to the active site of α-Gal using chimeric mutants showed a functional significance of the region around glutamine-279. In a previous study [16], we constructed three mutants of α-Gal by chimeric substitution with the corresponding sequences of α-N-acetylgalactosaminidase (α-NAGA), which has a high amino acid sequence homology (47%) with that of α-Gal [17,18]. One of the chimeric mutants CMB6, comprising the #245–272 α-NAGA sequence (corresponding to the #259–286 α-Gal sequence), acquired an increase in $K_m$ and a decrease in specific activity.

The purpose of the present study was to identify the amino acid residues responsible for the α-Gal activity in the region around the amino acid glutamine-279.

2. Materials and methods

2.1. Mutagenesis and subcloning

Seven amino acid substitutions had been introduced simultaneously in the CMB6 mutation described in our previous study [16] (Fig. 1). In this study, we prepared five individual amino acid substitutions instead, around one
mutation site in the exon 6 region, which had been detected in an atypical Fabry patient. Base substitutions were introduced with an oligonucleotide-directed in vitro mutagenesis kit (Amersham, Buckinghamshire, UK): W277L (Trp277 → Leu; TGG → TTG), N278E (Asn278 → Gin; AAT → GAG), Q280S (Gln280 → Ser; CAA → TCA), V281R (Val281 → Arg; GAA → CGA) and T282A (Thr282 → Ala; ACT → GCT). The replicative form DNA of the mutant M13 vector was isolated and digested with BglII and KpnI. The resulting 490-bp fragment was purified and inserted into the pAcYM1 expression vector, containing α-Gal cDNA (pAcGal) [16] in place of the corresponding fragment.

In some experiments, the chimeric substitution CMB12 was added to the Q280S and T282A mutants (CMB12-Q280S and CMB12-T282A), with the BglII-KpnI fragment in the pAcYM1 expression vector, containing chimeric mutant cDNA (pAcCMB12) [16]. The nucleotide sequence was confirmed by the dideoxy method using a 7-DEAZA sequencing kit (Takara Shuzo, Kyoto, Japan).

2.2. Expression and purification of mutant enzymes

A recombinant baculovirus was prepared by simultaneous transfection of Sf9 cells with pAcGal comprising the mutant DNA sequence and wild-type virus DNA (AcNPV). The recombinant virus with the mutant α-Gal sequence was selected from the culture medium by the limiting dilution method. Subsequently, Sf9 cells were infected with the recombinant virus for 18 h, and cultured in the serum-free Grace’s medium for 6 days. The expressed mutant enzyme was collected from the culture medium, and purified by concanavalin A-Sepharose and Mono Q (Pharmacia LKB Biotechnology, Uppsala, Sweden) column chromatography as described previously [16].

2.3. Enzyme assay and protein determination

α-Gal activity was assayed with an artificial substrate, 4MU-α-Gal (Nacalai Tasque, Kyoto, Japan) [19]. The reaction mixture, containing 5.0 mM 4MU-α-Gal in 0.1 M sodium citrate buffer (pH 4.6) was incubated at 37°C for 6 min. The reaction was terminated with an excess amount of 0.1 M glycine buffer (pH 10.7). Protein concentration was monitored by 280 nm absorbance, with the extinction coefficient calculated for the α-Gal expressed in insect cells as described previously [16].

2.4. Characterization of mutant enzyme

The circular dichroism (CD) spectrum was obtained with the Jasco J-600 spectropolarimeter (Japan Spectroscopic, Tokyo, Japan). Each sample was suspended in 10 mM sodium acetate buffer (pH 5.0), and the enzyme protein concentration was adjusted to 10 μg/ml.

SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli [20]. The molecular weight of the native form protein was estimated according to the method of Andrews [21] by gel filtration on a column of Superose 12 (Pharmacia LKB Biotechnology) equilibrated with 25 mM 2-(N-morpholino)ethanesulfonic acid (MES)-NaOH buffer (pH 6.0), containing 0.1 M NaCl. Molecular weight markers for gel filtration were: aldolase (160 kDa), bovine serum albumin (67 kDa), egg albumin (45 kDa), and cytochrome c (12 kDa) (Serva, Heidelberg, Germany).

3. Results

3.1. Single amino acid substitutions

The baculovirus expression products in Sf9 cells were purified as 46-kDa single bands on SDS-polyacrylamide gel electrophoresis for all five mutants (data not shown).
Kinetic properties of mutant enzymes

<table>
<thead>
<tr>
<th></th>
<th>$K_m$ (mM)</th>
<th>$V_{max}$ (μmol/min per mg)</th>
<th>$V_{max}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>W277L</td>
<td>2.4</td>
<td>69.2</td>
<td>28.8</td>
</tr>
<tr>
<td>N278E</td>
<td>2.2</td>
<td>66.7</td>
<td>30.3</td>
</tr>
<tr>
<td>Q280S</td>
<td>5.3</td>
<td>48.6</td>
<td>9.2</td>
</tr>
<tr>
<td>V281R</td>
<td>3.0</td>
<td>65.2</td>
<td>21.7</td>
</tr>
<tr>
<td>T282A</td>
<td>5.0</td>
<td>45.1</td>
<td>9.0</td>
</tr>
<tr>
<td>Normal</td>
<td>2.2</td>
<td>46.8</td>
<td>21.3</td>
</tr>
</tbody>
</table>

Substrate: 4MU-α-Gal. Concentrations: 1–8 mM. * * Enzyme activity was assayed in 0.1 M sodium citrate buffer (pH 4.6). Each value is the mean of three separate determinations.

Kinetic properties are summarized in Table 1. $K_m$ increased more than 2-fold for Q280S and T282A. The catalytic efficiency expressed as $V_{max}/K_m$ toward 4MU-α-Gal was decreased only in these two mutants.

Significant difference was not observed in the CD spectrum between the mutants (Q280S, T282A) and normal α-Gal (Fig. 2). Superose 12 column chromatography suggested that they were monomeric in solution (data not shown).

### 3.2. Combined mutants

The chimeric substitution in CMB12 did not affect the stability of enzyme activity (Fig. 3). Single amino acid substitutions in Q280S and T282A resulted in a marked decrease in thermostability. An additional chimeric substitution restored the thermostability at acidic pH, but showed different effects at neutral pH: decrease in Q280S, and no effect in T282A.

Both Q280S and T282A mutations showed a reduced enzyme activity at acidic pH, which was restored to the normal level by the chimeric substitution CMB12 in both CMB12-Q280S and CMB12-T282A (Fig. 4), although the chimeric mutation CMB12 itself resulted in a decrease of catalytic activity [16]. All mutants, except T282A, were stable under the assay condition in this study: 37°C for 6 min at pH 4.0. The stability of the T282A activity was slightly affected at acidic pH.

$K_m$ was 2-fold high in the CMB12 mutant, and an additional single amino acid substitution did not affect the value in the CMB12-Q280S mutant. However, it increased further up to the 3.5-fold higher level in the CMB12-T282A mutant (Fig. 5). The combined mutants also showed an increase in $V_{max}$, 2-fold, as compared to that for CMB12. As a result, the catalytic efficiency $V_{max}/K_m$ became 2-fold high for CMB12-Q280S, as compared with that for CMB12, and became similar to that for normal α-Gal.

### 4. Discussion

The data in our present study clearly demonstrated the significance of glutamine-280 and threonine-282 for catalytic activity and thermostability of α-Gal. A substitution for each amino acid (Q280S and T282A) resulted in abnormalities of physicochemical and kinetic properties to the same degree as CMB6. Three other amino acid substitutions (W277L, N278E, and V281R) did not affect the enzyme activity.
The catalytic activity may have been slightly affected at low pH below 4.5 in T282A due to a decreased thermostability under the acidic condition. This effect was less prominent under the experimental conditions at pH 4.6 in this in vitro study. CD spectrum for these two mutant proteins indicated that there was no drastic conformational change in the molecule. We assume that glutamine-280 and threonine-282 are located in or near the active site of the enzyme α-Gal.

The tertiary structure of either α-Gal or α-NAGA is not known, but we suggested in a previous study [16] that at least two peptide regions #43-87 and #259-286 in α-Gal, and probably #29-73 and #245-272 in α-NAGA, comprised the recognition site of α-linked galactose in a synthetic substrate 4MU-α-Gal. The chimeric mutant CMB126 showed a low affinity to α-linked galactose, at the same level as α-NAGA. This low affinity can be explained by a replacement of threonine with alanine at the amino acid residue 282 in α-Gal, and 268 in α-NAGA, based on our observation that CMB12-T282A had a markedly high $K_m$ with more stability than T282A.

A stable intramolecular interaction was suggested between chimeric mutant CMB12 region and amino acid substitution regions (Q280S and T282A) in this study, as shown for the interaction between two chimeric regions [16]. $V_{max}$ and thermostability increased in Q280S and T282A after addition of the CMB12 mutation, which contained 17 amino acid substitutions. The site for this stabilization has not been identified at present.

Acknowledgements

This work was supported by grants from the Ministry of Education, Science and Culture of Japan, the Ministry of Health and Welfare of Japan, Yamanouchi Foundation for Research on Metabolic Disorders, and the Naito Foundation.

References


