Human Lysosomal α-Glucosidase

CHARACTERIZATION OF THE CATALYTIC SITE*

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The substrate analogue conduritol B epoxide (CBE) is demonstrated to be an active site-directed inhibitor of human lysosomal α-glucosidase. A competitive mode of inhibition is obtained with glycogen as natural and 4-methylumbelliferyl-α-β-glucopyranoside as artificial substrate. The inactivation of the enzyme is time and concentration dependent and results in the covalent binding of CBE. Catalytic activity is required for binding to occur. CBE-labeled peptides containing the catalytic residue of lysosomal α-glucosidase were isolated and identified by microsequencing and amino acid analysis. The peptides appeared to originate from a protein domain which is highly conserved among α-amylases, maltase, glucoamylases, and transglucosylases. Based on the sequence similarity and the mechanism of CBE binding, Asp-518 is predicted to be the essential carboxylate in the active site of lysosomal α-glucosidase. The functional importance of Asp-518 and other residues around the catalytic site was studied by expression of in vitro mutagenized α-glucosidase cDNA in transiently transfected COS cells. Substitution of Asp-518 by Glu-513 is shown to interfere with the posttranslational modification and the intracellular transport of the α-glucosidase precursor. The residues Trp-519 and Asp-518 are demonstrated to be critical for catalytic function.

The lysosomal enzyme α-glucosidase (EC 3.2.1.3) hydrolyzes at low pH (pH 4–5) both α-1,4- and α-1,6 linkages in the natural substrates glycogen, maltose and isomaltose (Jeffrey et al., 1970a, 1970b). Deficiency of this enzyme in human and animal species results in glycogenosis type I1 or Pompe’s disease, a recessively inherited lysosomal glycogen storage disorder (Hers, 1963).

Information on the primary structure of lysosomal α-glucosidase has been obtained via molecular cloning and analysis of cDNA and genomic sequences (Hoeftsloot et al., 1988, 1990a, 1990b; Martiniuk et al. 1990). The cDNA codes for a protein of 952 amino acids with an apparent molecular mass of 110 kDa. The seven potential glycosylation sites of the precursor are all used, and some of the carbohydrate chains are phosphorylated (Hasilik and Neufeld, 1980; Reuser et al., 1985) In addition to glycosylation and phosphorylation, the maturation of α-glucosidase involves proteolytic processing at both the amino- and the carboxyl-terminal ends. It results in the formation of two lysosomal species of 76 and 70 kDa (Reuser et al., 1985; Oude Elferink et al., 1988; Hoeftsloot et al., 1988). A molecular species of 95 kDa has been identified as a processing intermediate.

With respect to the functional characteristics of lysosomal α-glucosidase, it was informative to discover a remarkable sequence similarity with the disaccharidases sucrase and isomaltase. Structural conservation of the active site of the three enzymes seemed likely because of overlapping substrate specificities (Hunziker et al., 1986; Hoeftsloot et al., 1988). With this in mind we have tested whether conduritol B epoxide (CBE), an active site-directed inhibitor of sucrase and isomaltase (Quaroni et al. 1974) could be used to label the catalytic residue of lysosomal α-glucosidase.

CBE is a well known inhibitor of β-glucosidase (Grabowski et al., 1986; Dinur et al., 1986), and its mechanism of action has been described in detail (Legler, 1973; Grabowski et al., 1984). The compound acts as substrate analogue and binds covalently to a carboxylate. Aspartate and glutamate are the preferential catalytic residues (Herrchen and Legler, 1984; Dinur et al., 1986). Sucrese and isomaltase are among the few α-glucosidases shown to be sensitive to inhibition by CBE (Legler, 1973; Quaroni et al., 1974). The inhibitor was shown to bind to isomaltase and sucrase in a ratio of 1 mol of inhibitor/1 mol of enzyme, and more specifically to the β-carboxyl group of the aspartic acid residues at positions 505 and 1249, respectively (Quaroni and Semenza, 1976; Hunziker et al., 1986).

In the present study we have used CBE to identify the catalytic site of lysosomal α-glucosidase, and we have studied the role of potentially important amino acid residues in the catalytic site region by expressing mutagenized α-glucosidase cDNA in mammalian cells.

MATERIALS AND METHODS

Biochemical Assays—Lysosomal α-glucosidase was purified from human placenta as described previously (Reuser et al., 1985). The enzymatic activity was measured with 4-methylumbelliferyl-α-β-glucopyranoside (4-MU) (Reuser et al., 1978) and with glycogen (Koster et al., 1972). Protein concentrations were determined according to Lowry et al. (1951).

1. M. M. P. Hermans, unpublished results.
2. The abbreviations used are: CBE, conduritol B epoxide; 4-MU, 4-methylumbelliferyl-α-β-glucopyranoside; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography.
The kinetics of enzyme inhibition by CBE was analyzed using 4-
MU and glycogen as substrates in 0.2 M sodium acetate, pH 4.3, with 150 and 50 ng of purified placental α-glucosidase, respectively.

To study binding of CBE to human placental α-glucosidase, the inhibitor was added to 10 μg of active or heat-inactivated enzyme in a final concentration of 10 mM, in 20 mM sodium acetate, pH 4.3. Heat inactivation was performed for 10 min at 100 °C. After incubation with the different molecular forms of the protein were separated by SDS-
PAGE (Laemmli, 1970), and stained with Coomassie Brilliant Blue.

Isolation of CBE-labeled Peptides—Placental α-glucosidase was incubated with CBE (1 μmol/50 μg of protein) in 20 mM sodium acetate, pH 6.3, for 7 h at 37 °C, and then digested against 100 mM ammonium bicarbonate, pH 8.5. The protein was digested with trypsin-t-1-1-tryosyl-2-phenylalanyl chloromethyl ketone (Cooper Biomedical) for 24 h at 37 °C. The peptides were recovered by lyophilization and dissolved in 50% acetonitrile and 0.05% trifluoro-
acetic acid. The samples were subjected to reverse-phase HPLC by using a RP-8 column (Merck). The peptides were eluted with a 0–100% acetonitrile gradient, at a flow rate of 0.5 ml/min. Fractions were collected and the selected peptides were recovered by lyophi-
lisation. An aliquot of each sample was used to determine the amino acid sequence of the peptide. The remainder was divided and dissolved in 100 mM ammonium bicarbonate, pH 8.5, for incubation with chymotrypsin (24 h at 37 °C) or in 25 mM ammonium bicarbonate, pH 7.8, for digestion with V8 protease (16 h at 25 °C followed by 4 h at 37 °C). The digests were subjected to reverse-phase HPLC as described above. Isolated peptides were sequenced on an Applied Biosystems Sequencer (477A) on line with a phenylthiohydantoin analyzer (Biosystems Sequencer (477A)). The oligonucleo-
tides used in this study were synthesized on an Applied Biosystems 381A DNA synthesizer and are listed in Table I.

Construction of Mutants—Site-directed mutagenesis was carried out using the Muta-Gen™ in vitro Mutagenesis Kit from Bio-Rad based on the method developed by Kunkel (1985). The oligonucleo-
tides used in this study were synthesized on an Applied Biosystems 381A DNA synthesizer and are listed in Table I.

The full-length human lysosomal α-glucosidase cDNA (Hoefsloot et al., 1988) cloned into M13mp19 was used to transform Escherichia coli C3326 (dut'+'). Single-stranded DNA containing uracil resi-
dues was isolated from phages to serve as template in the mutagenesis reaction. The phosphorylated oligonucleotides were annealed to the template. Extension took place with TdT. DNA polymerase and ligation with T4 DNA ligase. Twelve plaitites were selected and phages were spotted onto nitrocellulose filters with a slot-blot apparatus. To identify mutants the filter was hybridized at room temperature with the °P-labeled oligonucleotide used for mutagenesis and was washed at two to three degrees below the estimated melting temperature (Wallace et al., 1981). Mutant cDNAs were cloned in the eukaryotic expression vector pSGS (Green et al., 1988). The presence of the mutation was verified by double-stranded DNA sequencing with the sequencing™ sequencing kit according to the instructions of the manufacturer (Pharmacia LKB Biotechnology Inc.) using as primers oligonucleotides complementary to the cDNA.

**TABLE I**

| Oligonucleotides used for mutagenesis | 3' | 5' |
|--------------------------------------|----|----|
| D518 | 5' AGTGGATGATGATCAGAAGCG | 3' |
| G518 | 5' AGTGGATGATGATCAGAAGCG | 3' |
| N518 | 5' AGTGGATGATGATCAGAAGCG | 3' |
| E518 | 5' AGTGGATGATGATCAGAAGCG | 3' |
| R516 | 5' GCCCTGGAGGCCCATGATG | 3' |
| E513 | 5' GCCCTGGAGGCCCATGATG | 3' |

**RESULTS**

Binding of CBE to Lysosomal α-Glucosidase—The inhibition of lysosomal α-glucosidase by CBE was studied. Saturable first order kinetics of inactivation were observed, which indicates that a transient reaction intermediate is formed prior to the covalent binding of enzyme and inhibitor (Fig. 1). The Lineweaver-Burk plots show a competitive mode of inhibition. The 1/[S] intercept provides the -1/Kₐ (1+[I]/Kᵢ) values, in which [I] represents the inhibitor concentration. The calculated Kᵢ value is 11 mM with 4-MU and 7 mg/ml with glycogen as substrate.

The covalent binding of CBE to lysosomal α-glucosidase is further illustrated in Fig. 2. A 7-h incubation of the enzyme with the inhibitor at 37 °C resulted in a mobility shift in SDS-
PAGE. A minor part of the enzyme molecules remained unmodified. The mobility shift was not observed when the enzyme was inactivated prior to incubation with CBE (Fig. 2). This illustrates that the observed effect is not solely caused by the presence of CBE in the sample mixture but is actually due to the binding of CBE via a catalytic reaction.

Isolation of CBE-labeled Peptides—To identify the catalytic, CBE-binding residue of lysosomal α-glucosidase, the enzyme was incubated with the inhibitor and subsequently digested

**FIG. 1** Inhibition of human placental lysosomal α-glucosi-
dase by CBE (Lineweaver-Burk plots). The enzymatic activity was measured with 4-methylumbelliferyl-α-D-glucopyranoside (A) and with glycogen (B). CBE was added in final concentrations of 0 mM (+), 5 mM (▲) and 10 mM (■).
Catalytic Site of Lysosomal α-Glucosidase

FIG. 2. The effect of CBE binding on the SDS-PAGE mobility of placental lysosomal α-glucosidase. Active and heat-inactivated placental α-glucosidase were incubated with 10 mM CBE for 7 h. The different samples were compared by SDS-PAGE. The gel was stained with Coomassie Brilliant Blue. M: molecular weight markers.

FIG. 3. HPLC elution profiles of tryptic peptides from lysosomal α-glucosidase. Human placental α-glucosidase was digested with trypsin before (A) or after (B) inhibition with CBE. The resulting peptides were separated by HPLC using a RP-8 column. x axis, elution time; y axis, absorbance (220 nm).

with trypsin. The tryptic peptides were separated by reverse-phase HPLC (Fig. 3B). An equal portion of enzyme was subjected to the same procedure without CBE treatment (Fig. 3A). The elution profiles appeared to differ in only two aspects. The elution profile of the tryptic peptides derived from CBE-inactivated enzyme (Fig. 3B) versus active enzyme (Fig. 3A) contained an extra peak (marked B) and the area of peak A was diminished. The peptides A and B from Fig. 3B were isolated, and the sequences of the first seven amino acids were determined by gas-phase microsequencing. Both peptides had the same NH₂-terminal sequence, corresponding to position 480-486 of the previously determined amino acid sequence of lysosomal α-glucosidase (Fig. 4). The appearance of peptide B in addition to A in the tryptic digest is apparently caused by the binding of CBE. Based on the amino acid sequence of lysosomal α-glucosidase and the potential cleavage sites for trypsin, the peptides A and B are expected to end at amino acid residue 527.

To zoom in on the region containing the catalytic residue, the tryptic peptides A and B (Fig. 3B) were further digested with either V-8 protease or with chymotrypsin, and the resulting peptides were analyzed by reverse-phase HPLC. Figs. 5 and 6 show the distinct elution profiles caused by CBE binding. The amino acid sequences of peptides C and D derived by V-8 protease digestion of peptide A and B, respectively, were the same (Table II). Thus, the difference in elution time of peptides C and D was attributed to the binding of CBE to peptide D. The amino acid sequence of both peptides starts

FIG. 4. Partial amino acid sequence of lysosomal α-glucosidase (Hoefsloot et al., 1988). The predicted cleavage sites for trypsin (T), V-8 protease (V), and chymotrypsin (C) are indicated.

FIG. 5. HPLC elution profiles of V-8 protease peptides from lysosomal α-glucosidase. The tryptic peptides indicated in Fig. 3B with A and B were isolated and digested with V-8 protease. The resulting peptides were separated as described in Fig. 3. The essential part of the elution profile obtained after digestion of peptide A is shown in the left panel (marked A). The elution profile obtained after digestion of peptide B is illustrated in the right panel (marked B). x axis, elution time; y axis, absorbance (220 nm).

FIG. 6. HPLC elution profiles of chymotryptic peptides from lysosomal α-glucosidase. The tryptic peptides indicated in Fig. 3B with A and B were isolated and digested with chymotrypsin. The resulting peptides were separated as described in Fig. 3. The essential part of the elution profile obtained after digestion of peptide A is shown in the left panel (marked A). The elution profile obtained after digestion of peptide B is illustrated in the right panel (marked B). x axis, elution time; y axis, absorbance (220 nm).
at phenylalanine residue 506 and terminates theoretically at asparagine 520. Also the chymotrypsin fragments E (derived from A) and F and G (derived from B) were isolated. Sequence data were only obtained from peptide F, but more than 4 residues could not be determined due to material wash out at each consecutive sequencing step (Table II). Peptide F starts at position 513 (Fig. 4). To further characterize peptide F, the amino acid composition was determined and compared with the composition as deduced from the cDNA sequence (Table III). The molar concentration of Glx was set at 1. The concentration of some of the amino acid residues was clearly underestimated. But in combination with the sequence data of peptide F this analysis indicated that the chymotrypsin fragment containing the CBE-binding residue starts at Asp-513 and extends to Phe-525. Notably, this implies that chymotrypsin failed to cleave at the potential cleavage site between Trp-516 and Ile-517 (Wilkinson, 1986). The occurrence of alanine could not be explained.

In conclusion, the combined sequence data indicate that the catalytic site of lysosomal α-glucosidase is situated between residue Phe-512 and Glu-521. The sequence of this peptide is strongly conserved among lysosomal α-glucosidase, sucrase, and isomaltase and includes only two carboxylates, i.e. at position 513 and 518.

Construction and Expression of Active Site Mutants—In vitro mutagenesis was employed to further characterize the role of specific amino acid residues in the catalytic site domain of lysosomal α-glucosidase. To this end the previously cloned 3.6-kilobase α-glucosidase cDNA was inserted in the EcoRI site of M13mp19, and specific mutations were introduced with oligonucleotides (see Table I). Based on the results described above and the sequence similarity of lysosomal α-glucosidase, sucrase, isomaltase, and related enzymes (see “Discussion”) the following amino acid residues were chosen to be modified. The aspartic acid residue at position 518 was substituted by glutamic acid, leaving the carboxylate intact. More drastic changes were the substitutions of Asp-518 by asparagine or glycine and when tryptophan was replaced by asparagine, a residue present at that position in related proteins.

The effect of the mutations was studied by measuring the activity of lysosomal α-glucosidase in transiently transfected COS cells with the artificial 4-MU substrate and with the natural substrate glycogen. As a negative control, COS cells were transfected with a bacterial β-galactosidase construct. The results are presented in Table IV. Transfection with the wild-type cDNA resulted in a significant increase of activity for both substrates. In contrast, no increase of activity was measured after transfection with the mutant cDNA constructs. The mutant enzymes were apparently catalytically deficient. As expected, reversion of Gly-518 to Asp-518 restored the wild-type phenotype. The effect of the different mutations was also evaluated by measuring the α-glucosidase activity secreted by the cells. Also the secreted mutant enzymes appeared to be catalytically deficient (Table IV).

To verify that mutant proteins were actually produced by COS cells, lysosomal α-glucosidase was immunoprecipitated from cell homogenates and culture media and analyzed by Western blotting (Fig. 7). Three forms of lysosomal α-glucosidase were detectable in COS cells transfected with wild-type cDNA; a precursor with a molecular mass of 110 kDa, a processing intermediate of 95 kDa and a mature enzyme of 76 kDa (Fig. 7A). A second mature component of 70 kDa present in human placenta was not detectable in the transfected COS cells. The wild-type 110-kDa precursor of α-glucosidase was secreted in the medium (Fig. 7B). None of the substitutions of Asp-518 or Trp-516 appeared to affect the synthesis and posttranslational modification of lysosomal α-glucosidase, and the precursor was in each case normally secreted. However, the maturation of the precursor was completely blocked by substitution of Asp-513 by Glu-513 and secretion did not occur. Furthermore, it was observed that each single amino acid substitution leading to an alteration of charge resulted in a change of electrophoretic mobility (Fig. 7, A and B). This effect was seen when aspartic acid was replaced by asparagine or glycine and when tryptophan was substituted by arginine. Back mutation of Gly-518 to Asp-518 restored the wild-type mobility.

In summary, the results obtained with the active site mutants indicate that the tryptophan residue at position 516 and the aspartic acid residue at position 518 are important for the catalytic function of lysosomal α-glucosidase. The function of the aspartic acid residue at position 513 is more difficult to assess since the substitution by glutamic acid interferes with the maturation process.

Binding of CBE to Active Site Mutants—It was shown in Fig. 2 that catalytic activity is required for binding of CBE.

| Table II | Amino acid sequences of chymotrypsin and V8 protease peptides |
|----------|---------------------------------------------------------------|
| Peptide C | F H D Q V X F D X M |
| Peptide D | F H X Q V X F D G M |
| Peptide F | D G M X I |

* X, unidentifiable amino acid residue.

| Table III | Amino acid composition of peptide F obtained by chymotrypsin cleavage of tryptic peptide B |
|-----------|------------------------------------------------------------------------------------------|
| Amino acids | Estimated from % composition | Deduced from cDNA sequence |
| Asx | 4.4 | 4 |
| Glx* | 1.0 | 1 |
| Ser | 0.3 | 1 |
| Gly | 1.4 | 1 |
| Pro | 0.5 | 1 |
| Met | 0.6 | 2 |
| Ile | 0.7 | 1 |
| Phe | 0.8 | 1 |
| Trp | ND | 1 |
| Ala | 1.5 | 0 |

* Glx has been taken as 1.
* ND, not determined.

| Table IV | Catalytic activity of active site mutants of lysosomal α-glucosidase in transiently transfected COS cells measured 90 h after transfection |
|----------|----------------------------------------------------------------------------------------------------------------------------------|
| Type of mutation | Cells* | Medium* | Cells* |
| Wild type | 313.7 | 61.2 | 1070 |
| D518 → N518 | 18.5 | 1.1 | 38 |
| D518 → E518 | 23.2 | 0.8 | 142 |
| D518 → G518 | 25.0 | 1.2 | 85 |
| D518 → G518 → D518 | 300.4 | 67.9 | 2736 |
| W516 → R518 | 28.3 | 0.9 | 133 |
| W518 → E513 | 70.8 | 1.4 | ND |
| β-Gal construct | 34.6 | 0.8 | 290 |

* The activity is expressed as nanomoles 4-MU/milligram protein/hour.
* The activity is expressed as nanomoles of glucose/milligram protein/hour.
* ND, not determined.
Catalytic Site of Lysosomal α-Glucosidase

Two different approaches were taken to identify the catalytic site residues of human lysosomal α-glucosidase. The substrate analogue and active site-directed inhibitor conduritol B epoxide was used to label the essential carboxylate. In this way the catalytic residue was found to be located in a peptide extending from Asp-513 to Asn-520. The second approach involved site-directed mutagenesis to establish the identity of the presumed catalytic residue more definitively.

We demonstrate that CBE inhibits the activity of lysosomal α-glucosidase toward 4-MU-α-D-glucopyranoside and isomaltase in a competitive manner. The inactivation is time and concentration dependent and follows Michaelis-Menten kinetics. Using the artificial 4-MU substrate, a $K_i$ of 11 mM was measured. A much lower dissociation constant of 0.17 mM has been reported by Grabowski et al. (1986) who studied the effect of CBE on the hydrolysis of 4-MU-α-D-glucopyranoside by human glucocerebrosidase (β-glucosidase). Other studies point out that CBE has a higher affinity for β- than for α-glucosidases (Legler, 1973; Quaroni and Semenza, 1976; Legler and Harder, 1978; Bause and Legler, 1980).

The reaction mechanism has been studied in detail (Legler, 1973; Braun et al., 1977). It is assumed that a protonated epoxide is formed as a transient intermediate before the epoxide ring of CBE is opened by a carboxylate (Asp or Glu) and a covalent ester bond is established.

The existence of a covalent bond between CBE and lysosomal α-glucosidase is demonstrated by the stability of the bond under strongly denaturing conditions such as used for SDS-PAGE. The mobility shift caused by CBE binding did not occur when heat-inactivated α-glucosidase or catalytically defective mutants were incubated with CBE (Figs. 2 and 8). Thus, catalytic activity is essential for CBE binding. From the fact that the electrophoretic mobility of the wild-type intracellular 110-kDa precursor is not altered by CBE, we conclude that this precursor is catalytically inactive.

The covalent attachment of CBE to the catalytic site residue enabled us to isolate the peptide to which CBE was bound. The HPLC profiles obtained after tryptic digestion of native and CBE-inactivated α-glucosidase appeared to differ by the position of a single peptide. This peptide was identified by microsequencing and was found to start at residue Val-480. The primary sequence deduced from the cloned cDNA predicts that it ends at Arg-527. It includes a region of α-glucosidase which is highly conserved in suscrose and isomaltase, and it contains several aspartic acid and glutamic acid residues which are common binding sites for CBE. By subsequent digestion of this tryptic peptide with V8 protease and with chymotrypsin, the number of potential binding sites could be reduced to two, i.e. Asp-513 and Asp-518. The region containing both these residues is almost identical in lysosomal α-glucosidase and in intestinal sucrase and isomaltase (Fig. 9). For rabbit sucrase and isomaltase it was shown that CBE binds to the

**DISCUSSION**

Therefore, all active site mutants should lack CBE binding capacity. This assumption was verified (Fig. 8). As expected, the electrophoretic mobility shift due to CBE binding was only observed for the catalytically active wild-type enzyme. Interestingly, the binding of CBE appeared to be restricted to the 95- and the 76-kDa processed forms of lysosomal α-glucosidase.

FIG. 7. Processing of wild-type and mutant lysosomal α-glucosidase in COS cells. Lysosomal α-glucosidase was immunoprecipitated from transiently transfected COS cells and from the culture medium 90 h after transfection and analyzed by Western blotting after SDS-PAGE. The molecular mass of the wild-type lysosomal α-glucosidase species is indicated. A, cells. B, media.

FIG. 8. The effect of CBE binding on the SDS-PAGE mobility of wild-type and mutant lysosomal α-glucosidase in COS cells. Lysosomal α-glucosidase was immunoprecipitated from transiently transfected COS cells and from the culture medium 90 h after transfection and incubated with 10 mM CBE for 7 h. The different samples were compared by SDS-PAGE. The molecular mass of the wild-type lysosomal α-glucosidase species is indicated.

FIG. 9. Amino acid homology around the catalytic site of rabbit sucrase and isomaltase. The amino acid sequences of lysosomal α-glucosidase (Hoefsloot et al., 1988), human isomaltase (Green et al., 1987), and rabbit sucrase and isomaltase (Hunziker et al., 1986) are aligned. The essential carboxylates of rabbit sucrase and isomaltase are underlined (Quaroni and Semenza, 1976).
β-carboxy group of the aspartic acid residues Asp-505 (isosmaltase) and Asp-1249 (sucrase) in mutually homologous positions. By analogy we expect residue Asp-518 to be the CBE-binding catalytic residue of lysosomal α-glucosidase. Binding of CBE to Asp-518 can possibly explain why chymotrypsin failed to cleave after Trp-516 (one of the favored cleavage sites), whereas it does hydrolyze the peptide bond between Phe-512 and Asp-513. Steric hindrance may be the cause.

To obtain more direct information on the role of potentially important residues in the catalytic site region, amino acid substitutions were made by site-directed mutagenesis at the positions Asp-513, Trp-516, and Asp-518. The nature of all 3 amino acid residues turned out to be critical for catalytic function. One of the most subtle changes, the substitution of Asp-513 by Glu-513, had the most dramatic effect. Both the posttranslational modification and the intracellular transport of α-glucosidase were blocked. The mutant precursor appeared to be synthesized normally but was neither secreted nor converted to mature enzyme. The reason remains obscure as the predicted secondary structure (Chou and Fasman, 1978) and the surface probability (Emini et al., 1985) of the wild-type and the mutant protein do not differ significantly.

The substitutions of Trp-516 by Arg-516 and of Asp-518 by Glu-518, Asn-518, or Gly-518 were more informative, since these mutations did not interfere with enzyme maturation. Considering the mechanism of inactivation (Legler, 1973), the loss of activity caused by the isosteric substitution of Asp-518 by Asn-518 and by the alteration of Asp-518 to Gly-518 can be explained by the loss of the essential carboxyl group. However, it was somewhat unexpected that even the minor change from Asp-518 to Glu-518 deprived α-glucosidase from its catalytic function. This suggests that the spatial conformation of the β-carboxyl group at position Asp-518 is of crucial importance. Elongation of the side chain may prevent substrate binding by steric hindrance. Alternatively, the loss of activity may be caused by the higher pKα value of glutamic acid compared to aspartic acid which might prevent glutamic acid to act as catalytic base at the enzyme’s optimal pH of 4.3. Also, the substitution of Trp-516 (aromatic) by Arg-516 (basic) leads to loss of catalytic function. However, it is virtually excluded that the enzyme inactivation is caused by the covalent binding of CBE to this particular residue. Tryptophan has a non-polar side chain and practically no acidic or basic properties. The inactivation is more likely due to the higher pKα value of glutamic acid compared to aspartic acid which might prevent glutamic acid from acting as catalytic base at the enzyme’s optimal pH of 4.3.

In conclusion, our results strongly indicate that Asp-518 is the catalytic base of lysosomal α-glucosidase. A second aspartic or glutamic acid residue is expected to act as proton donor. This hypothesis is in line with the recently published data of Onodera et al. (1989) showing that rabbit lysosomal α-glucosidase has only one catalytic center with two ionizable groups with pKα values of 3.6 and 6.1, respectively.

Acknowledgements—We would like to thank Prof. Dr. Hans J. Reuser for continuous support, Dr. Lies Hoefsloot and Marianne Hoogeveen for technical advice, Dr. Andre Hoogeveen for his contribution to the HPLC analyses, Alvin Chan for technical assistance, Tom de Vries Lentrich for photography, and Jeannette Lokker for preparing the manuscript. We are grateful to Prof. Dr. Gunter Legler for his critical comments and his expert advice concerning the reaction mechanism of CBE. Prof. Dr. Giorgio Semenza and Dr. Hans Wacker are acknowledged for exchanging information and material.

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