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The diabetogenic antibiotic streptozotocin modifies the tryptic digest pattern for peptides of the enzyme O-GlcNAc-selective N-acetyl-β-D-glucosaminidase that contain amino acid residues essential for enzymatic activity

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Abbreviations:
PBS, phosphate buffered saline
STZ, streptozotocin
O-GlcNAcase, O-GlcNAc-selective N-acetyl-β-D-glucosaminidase
OGT, O-GlcNAc transferase
GlcNAc, N-acetylglucosamine
MNU, methylnitrosourea
PUGNAc, O-(2-acetamido-2-deoxy-D-glucopyranosylide) amino-N-phenylcarbamate

ABSTRACT

Streptozotocin (STZ) inhibits O-GlcNAc-selective N-acetyl-β-D-glucosaminidase (O-GlcNAcase), the enzyme that removes O-GlcNAc from proteins. The active site of the enzyme was recently proposed to include aspartates 174, 175, and 177, with STZ inhibition via a transition state analog. We explored the effect of STZ on the tryptic peptide digest pattern of O-GlcNAcase. LC/MS/MS analysis demonstrated that STZ modified two areas of the enzyme. One peptide, LGCFEIAK (894–901), in a C-terminal region previously proposed to possess O-GlcNAcase activity, was methylated by STZ. Another peptide, EYEIEFIYIASPGLDITFSNPK (128–149), was detected only after treatment with STZ and was in an N-terminal region, overlapping a glutamate-rich area containing an adjacent phenylalanine residue. No covalent modification of this peptide could be demonstrated. Detection of this peptide after treatment with STZ was accompanied by the simultaneous inability to detect the nearby peptide KLDQVSQFGCR (157–167), which contains a cysteine residue recently shown to be essential for enzymatic activity. To determine which of the first two peptides might also be important for O-GlcNAcase activity, site-specific mutagenesis was performed. Mutation of the N-terminal phenylalanine and serine residues resulted in almost complete inhibition of activity. In contrast, mutation of conserved C-terminal glycine and cysteine residues caused little inhibition of enzymatic activity. Together, these data extend the region of the active site N-terminally and give independent evidence to support the idea that STZ inhibits O-GlcNAcase through formation of a transition state analog that resides in the active site of the enzyme and in doing so alters its conformation and ensuing tryptic digest pattern.

1. Introduction

A number of nuclear and cytoplasmic proteins are covalently modified by the attachment of a single monosaccharide N-acetylglucosamine (GlcNAc) to either serine or threonine, a reaction catalyzed by the enzyme O-GlcNAc transferase (OGT) [1–6]. In the pancreatic β-cell, which is uniquely enriched in OGT [7,8], this enzyme links the extracellular

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glucose level to intracellular protein O-glycosylation [9–11]. The enzyme that removes O-GlcNAc from proteins, O-GlcNAc-selective N-acetyl-β-D-glucosaminidase (O-GlcNAcase) [12,13], is also present in β-cells [14]. We have previously shown that the diabetogenic toxin streptozotocin (STZ), an analog of N-acetylglucosamine (GlcNAc) [15], irreversibly inhibits the activity of O-GlcNAcase [7,14]. Interestingly, islet O-GlcNAcase was found to be particularly sensitive to STZ [14]. Thus, the high level of expression of OGT in pancreatic β-cells combined with increased sensitivity of islet O-GlcNAcase to STZ appears to explain the selective β-cell toxicity of STZ [16].

Subsequently, the gene coding for O-GlcNAcase has gained significant further attention as a candidate gene for type 2 diabetes [17]. A single nucleotide polymorphism (SNP) in this gene was recently shown to be highly correlated with type 2 diabetes in humans [17]. Very recently, the active site of the enzyme was proposed by two independent groups to include aspartate residues at positions 174, 175, and 177, and the mechanism of action of STZ proposed to be through formation of a transition state analog [18–21]. In light of these observations, we further explored the possible mechanism by which STZ inhibits O-GlcNAcase, focusing on the effects of STZ on the tryptic peptide digest of the protein and additional regions of the enzyme that might be important for enzymatic activity.

2. Materials and methods

2.1. Recombinant O-GlcNAcase cloning and expression

The entire gene for human O-GlcNAcase with a His_{10} epitope tag inserted upstream was subcloned into pCRScript, then pET16b (Novagen) using *Bam*HI and *Nde*I. The new construct, called pWA28, was used to transform *Escherichia coli* BL21 (DE3) pLysS (Novagen). After overnight incubation on selective agar at 37 °C, isolated colonies were picked and used as inoculum for Terrific Broth (12 g Bacto tryptone, 24 g Bacto yeast extract, 4 ml glycerol, 0.17 M KH_2PO_4, and 0.72 M K_2HPO_4 per liter), supplemented with 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. After overnight incubation at 37 °C, 200 rpm, 1 ml of the culture was diluted 1:250 into fresh Terrific Broth supplemented with 100 μg/ml ampicillin and incubated at 37 °C with shaking at 200 rpm. When the OD_{600} reached 0.400, IPTG was added to a final concentration of 1.0 mM. After 3 h, cells were harvested, and the pellet was stored at −80 °C until needed.

2.2. Purification of recombinant O-GlcNAcase

A 5 g cell pellet was resuspended in 12 ml B-PER (Bacterial Protein Extraction Reagent, Pierce Chemical Co.) supplemented with 10 μg/ml lysozyme and 10 μg/ml DNase. The cell suspension was sonicated for 2 min with interspersed cooling cycles and centrifuged at 12,000 rpm for 30 min at 4 °C to pellet unbroken cells and debris. The supernatant was mixed with an equal volume of 2× binding buffer (80 mM phosphate, 1.0 M NaCl, and 12 M urea, pH 7.40) and passed through a 0.45 μm filter. The lysate was added to a 5.0 ml Ni^{2+} column and eluted with a linear gradient of elution buffer (40 mM phosphate, 0.5 M NaCl, 6 M urea and 0.5 M imidazole) that increased from 0 to 100% over 60 min. Fractions were analyzed for O-GlcNAcase protein by SDS-PAGE, combined for dialysis overnight against 20 mM sodium phosphate, and after quantification, stored at −80 °C until needed.

2.3. Production and affinity purification of anti-O-GlcNAcase antibodies

Four peptides corresponding to the human O-GlcNAcase amino acid sequence were synthesized (Anaspec, Inc.). Terminal cysteine residues were added at the time of synthesis to allow directional conjugation to carrier proteins and chromatography beads. Chicken egg albumin (Sigma) was activated for conjugation with a 10-fold molar excess of Sulpho-SMCC (Pierce). Free SMCC was removed from Carrier-SMCC conjugates by chromatography through sephadex G-50 equilibrated in PBS. The first peak was collected, and protein concentration determined by measuring absorbance at 280 nm. Peptides were conjugated to activated carriers with a 10-fold molar excess of peptide by incubation at room temperature for 2 h. Four rabbits per antigen were immunized every 21 days with 100 μg peptide carrier conjugates. Animals were bled 10 days after booster injections beginning after the third injection. All antisera were tested via Western blotting against recombinant O-GlcNAcase protein and found to be approximately equally reactive. As a result, antisera were pooled. Afterward, anti-O-GlcNAcase polyclonal antibody was affinity purified. For affinity column preparation, 5 mg of peptide were conjugated to 5 ml of hydrated activated thiol sepharose 4b beads (Amersham) in a total volume of 10 ml 0.1 M Tris–HCl, 0.2 M NaCl, pH 7.50. The conjugation reaction was incubated overnight at 4 °C. The column was washed thoroughly before being loaded with sera. Approximately 10 ml of rabbit sera was mixed with an equal volume PBS, incubated with the affinity beads overnight at 4 °C, washed extensively, and eluted with low pH buffer (60% 0.6 M acetic acid, 40% 1× PBS). The eluate was immediately neutralized with 2 M Tris base and dialyzed four times with 1 l PBS. The purified antibody was then concentrated with an Amicon stir cell concentrator (YM30 membrane) and the yield determined by absorbance at 280 nm.

2.4. Western blotting

Samples were loaded onto SDS-polyacrylamide gels. Colored molecular weight markers were run on each gel. Proteins were separated for 1 h at 175 V at room temperature and transferred to ECL nitrocellulose paper (Amersham) for 1 h (100 V, 4 °C). Nitrocellulose blots were blocked for 1 h at room temperature in TBS-casein blocking buffer (Pierce) containing 0.1% Tween-20. After blocking, blots were probed with HRP-labelled anti-O-GlcNAcase antibody (1 μg/ml) in blocking buffer for 1 h at room temperature. Blots were washed three times (10 min each) with TBST (10 mM Tris pH 7.40, 150 mM NaCl, 0.1% Tween-20). After washing, blots were developed with ECL reagent (Amersham). After air-drying, blots were exposed to Bio-Max X-ray film (Kodak).