Modification of substrate-binding site of glutamyl endopeptidase from Bacillus intermedius


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Glutamyl endopeptidases (GEPs) are serine proteases belonging to the chymotrypsin structural family. Although the family as a whole has been described in detail, the molecular mechanism underlying strict substrate specificity of GEPs remains unclear. The most popular hypothesis attributes the key role in recognition of the charged substrates by GEPs to the conserved amino acid His213 (chymotrypsin numbering system). In order to test the role of this residue in the substrate specificity, we obtained a GEP from Bacillus intermedius with an amino acid substitution (His213→Thr) and studied its catalytic properties. Such modification proved not to affect the primary specificity of the enzyme. The introduced substitution had little effect on the Michaelis constant (K_m increased 4.9 times) but considerably affected the catalytic constant (k_cat decreased 615 times). The obtained data suggest that the conserved His213 residue in Bacillus GEPs is not a key element determining their primary substrate specificity.

Keywords: charge recognition/glutamyl endopeptidase/serine protease/site-specific mutagenesis/substrate specificity

Introduction

Chymotrypsin-like serine proteases have been described in detail in terms of both their structure and biochemical properties. Enzymes of this family can be divided into four groups by their substrate specificity: they can cleave polypeptide chains after positively charged amino acids (trypsins), large hydrophobic residues (chymotrypsins), small neutral residues (elastases), or hydrolyze the bonds formed by alpha-carboxyl groups of dicarboxylic amino acids [glutamyl endopeptidases (GEPs)]. Such preference variability combined with structural similarity between the molecules and a lot of various data available on these proteins made them a convenient model for studying the mechanisms underlying substrate specificity. Many publications explore this system (Perona and Craik, 1995, 1997; Perona et al., 1995; Hedstrom, 1996; Lesk and Fordham, 1996; Dang et al., 1997) including rational design of trypsin-derived enzymes with chymotryptic specificity (Hedstrom et al., 1992, 1994a,b; Kurth et al., 1997).

GEPs are the group of chymotrypsin-like proteases least explored in functional terms, although the first and most popular enzyme with such specificity (V8 protease from Staphylococcus aureus) was discovered over 30 years ago (Drapeau et al., 1972). Studies on microbial and viral enzymes of this group were covered in a series of publications describing isolation and enzymatic properties of GEPs (Niido et al., 1990; Kitadokoro et al., 1993; Leschinskaya et al., 1997; Demidyuk et al., 1997), sequencing of the proteins and their genes (Kakudo et al., 1992; Svendsen and Breddam, 1992; Rebrikov et al., 1999), analysis of the three-dimensional structure of the enzymes by X-ray diffraction techniques (Nienaber et al., 1993; Cavarelli et al., 1997; Kuranova et al., 1999; Barrette-Ng et al., 2002; Meijers et al., 2004), molecular modeling of protein structure (Barbosa et al., 1996), and their directed modification (Snijder et al., 1996; Stennicke et al., 1996; Rago et al., 2000). At the same time, the molecular mechanism underlying strict substrate specificity of GEPs remains unclear.

By analogy with trypsin (Graf et al., 1987) it was proposed that a compensator of the substrate charge should be present at the S1 site of GEPs. Comparison of amino acid sequences of GEPs (Barbosa et al., 1996; Rebrikov et al., 1999; Ziebuhr et al., 2000), analysis of the available X-ray crystal structures (Nienaber et al., 1993; Barrette-Ng et al., 2002; Meijers et al., 2004), and structural modeling of GEP molecules (Barbosa et al., 1996) disclosed a structurally conserved residue His213 (chymotrypsin numbering system) localized in the S1 binding site. This residue is the main candidate for the role of the negative charge compensator, since no other basic residues common for all GEPs exist in the S1 binding site. At the same time, alternative hypotheses were proposed for certain enzymes (Barbosa et al., 1996; Rago et al., 2000; Meijers et al., 2004).

Previously, we have found and described a secretory GEP from Bacillus intermedius (BIGEP), the corresponding gene has been cloned, expressed in Bacillus subtilis cells, and sequenced (EMBL accession number Y15136) (Rebrikov et al., 1999). Recently, we determined the three-dimensional structure of the enzyme (Meijers et al., 2004). His186 residue corresponding to the His213 was localized in the enzyme structure. In this paper we report generation of a BIGEP with a mutation at His213 and the effect of this mutation on the substrate specificity of the enzyme.

Materials and methods

Materials

Oligonucleotides (Litech, Russia), tris-(hydroxymethyl)amino-methane (Tris), 2-(N-morpholino)ethanesulfonic acid (MES), azocasein, oxidized insulin B-chain (Sigma, USA), Coomassie Brilliant Blue G-250 (Loba, Austria), bromophenol blue, glycine, ammonium persulfate, Coomassie Brilliant Blue R-250, bovine IgG (Reanal, Hungary), Tli DNA polymerase, T4 DNA ligase, restriction endonucleases and the corresponding
buffers, as well as acrylamide, bisacrylamide (Promega, USA), LE agarose (USB, USA), yeast extract, tryptone, casamino acids, and L-tryptophan (Difco, USA).

The substrates of GEPs, N-benzyloxycarbonyl-L-glutamyl-p-nitroanilide (Z-E-pNA) and N-benzyloxycarbonyl-L-alanyl-L-alanyl-L-leucyl-L-glutamyl-p-nitroanilide (Z-AALE-pNA), were synthesized as described elsewhere (Milgotina et al., 2001). The peptide substrates L-tryptophan and benzoyl-L-arginyl-p-nitroanilide (Bz-R-pNA) were kindly provided by G.N. Rudenskaya (Chemical Faculty, Moscow State University, Russia).

_Bacillus subtilis_ strain AJ73 was kindly provided by Dr Yu. Jomantas (State Research Institute of Genetics and Selection of Industrial Microorganisms, Russia).

**General methods**

Polyclonal antiserum against BIGEP was prepared by three consecutive subcutaneous immunizations of a rabbit with pure enzyme solution (1 mg total protein). The antiserum was pre-exhausted with crude extract of _Escherichia coli_ and purified by affinity chromatography on immobilized BIGEP.

Transformation of _B. subtilis_ AJ73 cells with plasmid DNA was carried out as described by Anagnostopoulos and Spizizen (1961).

N-terminal amino acid sequences were determined by automated Edman degradation using a Model 816 Protein Sequencer (Knauer, Germany) equipped with a Model 120A PTH Analyzer (Applied Biosystems, USA).

Optical density was measured on a Model 2550 Microplate Reader (Bio-Rad, USA) and a UV-2100 spectrophotometer (Shimadzu, Japan).

Mass spectra were recorded on a Vision 2000 TOF mass spectrometer using matrix-assisted laser desorption/ionization (ThermoBioanalysis, UK).

Polymerase chain reaction (PCR) was performed using a DNA Tertsik MS2 Multichannel Amplifier (DNA-Technology, Russia).

Protein electrophoresis was performed in vertical 12.5% polyacrylamide gels with 0.1% sodium dodecyl sulphate (Laemmli, 1970). Bovine serum albumin (66 kDa), albumin from chicken egg white (45 kDa), glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle (36 kDa), carbonic anhydrase from human erythrocytes (29 kDa), trypsinogen from bovine pancreas (24 kDa), and trypsin inhibitor from soybean (20 kDa) (Sigma, USA) were used as molecular weight standards.

Protein concentration was assayed after Bradford (1976) with modifications (Gasparov and Degtiar’, 1994) using IgG as a standard.

Nucleic acids were separated by horizontal electrophoresis in 0.8–2% agarose gel using 89 mM Tris–borate buffer with 2 mM EDTA, pH 7.6. DNA was visualized by staining of electrophoretic gels in 1 μg/ml ethidium bromide for 5–30 min.

Total proteolytic activity was evaluated by azocasein cleavage (Charney and Tomarelli, 1947). Briefly, 100 μl of 1% of azocasein in 20 mM Tris–HCl buffer, pH 8.5, were incubated with 50 μl of the enzyme for 15–60 min at 37°C. The reaction was stopped by adding 200 μl of 10% solution of trichloroacetic acid. After centrifugation at 9500 g for 5 min, 250 μl of the supernatant were mixed with 50 μl of 5 M NaOH and the absorbance was measured at 450 nm. The activity unit was defined as the amount of enzyme that changed optical density by 1 AU per minute.

Determination of the specific enzyme activity was carried out using Z-AALE-pNA as a substrate. One milliliter of the substrate solution (0.4 mM) in 20 mM Tris–HCl buffer, pH 8.5, was incubated with 20 μl of the enzyme solution for 50 min at 37°C. After termination of the reaction by adding 0.5 ml of 1 M sodium acetate buffer (pH 4.7), the absorbance was measured at 410 nm. The activity unit was defined as the amount of enzyme that released 1 μM p-nitroaniline per minute.

The activity assay with Glp-AAL-pNA and Bz-R-pNA as substrates followed the protocol of Revina et al. (1989).

**Construction of plasmid vector pICE**

The pICE vector was constructed by cloning the erythromycin resistance gene extracted from pCB22 vector (Sorokin et al., 1986) into _Bgl_II and _EcoRI_ sites of the p58.21 vector (Rebrikov et al., 1999) (Figure 1).

**Mutation construction**

The mutation was introduced in the BIGEP gene using PCR. The reaction proceeded in 25 μl of 10 mM Tris–HCl buffer, pH 9, containing 50 mM KCl, 0.1% Triton X-100, and 2.5 mM MgCl₂ in the presence of deoxynucleoside triphosphates (200 μM each), 10 pmol primers, and 1 U _Tli_ DNA polymerase.

At the first stage, a fragment of the GEP gene carrying two nucleotide substitutions corresponding to the amino acid substitution H186-T was synthesized using 50 ng of p58.21 template DNA. One of the primers used (T213, CCCTGCCTAGTAAATCCCAACG) was designed to introduce the nucleotide substitutions (underlined), while the other one (ERV, CCGCTCCTATCCGTACAG) was designed to provide for an _EcoRI_ site in the resulting DNA fragment.

The following amplification program was used: 94°C for 4 min; 25 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min. The reaction product was eluted from 1.5% agarose gel after electrophoresis. The sample obtained was used as a megaprimer at the subsequent stage of mutagenesis.

At the second stage, we obtained the fragment of DNA for cloning into _EcoRV_ and _ BamHI_ sites of pICE vector. The p58.21 vector was used as a template while BHI oligonucleotide (GAGCTATACGATTCGAC) designed to introduce a _BamHI_ site into the resulting product was used as a primer. The product of the first stage served as a second primer. The following amplification program was used: 94°C for 4 min; 25 cycles of 94°C for 4 min, 50°C for 2 min and 72°C for 1 min.

At the third stage, this product was reamplified to produce preparative quantities of it using the BHI and _ERV_ primers.

The following amplification program was used: 94°C for 4 min; 25 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 1 min.

The resulting fragment was introduced into _EcoRV_ and _BamHI_ sites of pICE vector (Figure 1). The plasmid obtained was used to transform a _B. subtilis_ laboratory strain AJ73. The transformed bacteria were cultivated on L-agar plates with 10 mg/ml chloramphenicol. The clones unable to grow on a solid medium with 10 mg/ml erythromycin were selected among the obtained clones and their ability to form a zone of hydrolysis on milk agar plates was tested.

The selected clones were cultivated in tubes with LB liquid medium and the centrifuged biomass resuspended in water (1 μl per 1 mg biomass) was incubated in a boiling water
bath for 3–5 min. The suspension was centrifuged (10 000 g, 10 min) and 1 µl was used in the amplification reaction similar to the third stage. The clones yielding a product of the size similar to that of the p58.21 vector (933 bp) were used in the subsequent experiments.

The point mutations were confirmed on an ABI 373A DNA Sequencer (Applied Biosystems, USA) using ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, USA). The ERV and BHI oligonucleotides were used as primers.

The plasmid carrying a modified GEP gene was called pT213.

Purification of the modified and wild-type BIGEP produced by the recombinant B. subtilis strain

*Bacillus subtilis* strain AJ73 (pT213) was cultivated in 250 ml Erlenmeyer flasks containing L-broth medium and 10 mg/ml chloramphenicol with vigorous agitation for 48 h. The cells were removed by centrifugation. Ammonium sulfate was added to the culture broth to 70% saturation and it was incubated at +4°C for 12 h. After centrifugation (10 000 g, 15 min), the pellet was dissolved in 20 mM MES-NaOH, pH 5.8. The solution obtained was loaded into an Econo-Pac High S cartridge, 1 ml (Bio-Rad, USA) equilibrated with the same buffer. The enzyme was eluted by a linear gradient of NaCl (0–0.25 M) in 30 ml of the same buffer at a flow rate of 0.7 ml/min. The presence of the target protein in the eluate was confirmed by double radial immunodiffusion in 1% agarose gel in 0.1 M borate buffer containing 0.5 M NaCl, pH 7.9, at +4°C using rabbit antibodies against wild-type GEP from *B. intermedius*.

The fractions corresponding to the precipitation zones were concentrated by ultrafiltration using a PTGC membrane (Millipore, USA) and loaded into a Superdex 75 HR 10/30 (Amersham Pharmacia Biotech, USA) column equilibrated with 150 mM NaCl in 20 mM Tris–HCl, pH 8.3. The enzyme was eluted with the same buffer at a flow rate of 1 ml/min.

Wild-type BIGEP produced by the recombinant *B. subtilis* strain was purified as described elsewhere (Rebrikov et al., 1999).

**Determination of the thermal stability of the modified BIGEP**

Enzyme aliquots (60 µg/ml in 20 mM Tris–HCl, pH 8.5) were incubated at 50°C for 150 min and immediately placed on ice. The residual activity was determined by Z-AALE-pNA hydrolysis as described above and compared with the activity of the enzyme not exposed to heat treatment.

**Determination of the catalytic parameters with Z-AALE-pNA as a substrate**

In order to test time-related changes in accumulation of the enzymatic reaction product, 30 µl of enzyme solution (10 and 60 µg/ml for the modified and wild-type enzymes, respectively) were added to 670 µl of Z-AALE-pNA solution in 20 mM Tris–HCl, pH 8.5. The reaction mixture was quickly stirred and the changes in optical density at 410 nm were monitored for 5–60 min at a constant 37°C. The substrate concentration in the reaction mixture was 0.015–2.5 mM. Various concentrations of Z-AALE-pNA were prepared from stock solutions in N,N-dimethylformamide. The concentration of N,N-dimethylformamide in the reaction mixture did not exceed 4% (v/v). The delay of monitoring was at most 10 s after the enzyme introduction. The experiment was repeated three to four times for each substrate concentration. The concentration of free p-nitroaniline was calculated using 8800 M cm⁻¹ as the molar extinction at 410 nm (Erlanger et al., 1961). The kinetic parameters were determined from the initial rates of hydrolysis using the Lineweaver-Burk plot.

**Substrate specificity on oxidized insulin B-chain**

The enzyme solution (1 µl, 170 µg/ml) was added to 15 µl of oxidized insulin B-chain (1 mg/ml) in 5 mM Tris–HCl, pH 8.2 (for mass spectrometry) or 50 mM N-methylmorpholine–HCl, pH 8.2 (for sequencing) (at a 1/600 enzyme/substrate molar ratio).
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Fig. 2. MALDI–TOF mass spectrum of oxidized insulin B-chain cleavage products by BIGEP with amino acid substitution His186–Thr.

Results

Construction and expression of mutant GEP from B. intermedius

The data obtained by Snijder et al. (1996) and Stennicke et al. (1996) demonstrated an extraordinary sensitivity of GEPs to the kind of amino acid at position 213. That is why we realized the importance of selecting an amino acid for replacing His186(213) in BIGEP (chymotrypsin numbering system in parentheses). Analysis of the primary and three-dimensional structures of serine proteases (Barbosa et al., 1996; Lesk and Fordham, 1996; Ziebuhr et al., 2000; Barrette-Ng et al., 2002; Meijers et al., 2004) demonstrated structural similarity of the 208–214 region (chymotrypsin numbering system) in all proteases of this group and, in the case of GEPs, its involvement in formation of the S1 binding site. Note that the average deviation of the Ca atom in residue 213 is as low as 0.564 Å for 13 three-dimensional structures of chymotrypsin-like serine proteases of mammals, bacteria, and viruses (Lesk and Fordham, 1996). Since these 13 enzymes without GEP activity have Thr in five cases, Val in six cases, Leu and Met in one case each at position 213, we considered Thr and Val as promising native-like substitutions for His213. However, the latter was declined considering the data obtained by Stennicke et al. (1996).

BIGEP gene with two nucleotide substitutions corresponding to the amino acid substitution His186(213)–Thr (chymotrypsin numbering system in parentheses) was generated by PCR. The mutant gene structure has been confirmed by sequencing. The modified gene in pT213 was expressed in B. subtilis laboratory strain AJ73. This plasmid differed from the previously obtained p58.21 vector (Rebrikov et al., 1999) only by the two above-mentioned nucleotide substitutions corresponding to the amino acid substitution His186–Thr. BIGEP gene expression was governed by its own regulatory elements. The protein was released to the extracellular space due to the presence of its own leader sequence within the coding region. Production of active mutant BIGEP has been confirmed by the ability of pT213-transformed cell colonies to form zones of hydrolysis on milk agar plates (in contrast to the recipient strain). In addition, double radial immunodiffusion demonstrated the interaction between the culture broth and antibodies against wild-type BIGEP.

Electrophoretically homogeneous His186(213)–Thr mutant protein was isolated at 500 µg/l culture broth. According to SDS–PAGE, the molecular weight of the mutant protein was 23 kDa and coincided with that of the wild-type enzyme.

The N-terminal sequence of the modified enzyme (VVIIGD) corresponded to the previously determined sequence of the wild-type protein (Rebrikov et al., 1999).

The His186(213)–Thr mutant remained completely active after incubation at 50°C for 150 min. In addition, no notable decrease in the activity was observed after storage of the modified enzyme at 4°C for 10 months.

Cleavage of proteinase substrates by the mutant enzyme

The mutant BIGEP was able to hydrolyze azocasein with a specific activity of 0.6 U/mg (specific activity of the wild-type enzyme is 36.8 U/mg) and Z-AALE-pNA (0.16 U/mg), a specific substrate of GEPs, but not specific substrates of chymotrypsin (Glp-AAL-pNA) and trypsin (Bz-R-pNA).

Cleavage of oxidized insulin B-chain by the mutant enzyme

Substrate specificity of the mutant BIGEP was evaluated by cleavage of oxidized insulin B-chain. Mass spectrometry demonstrated formation of only two fragments of oxidized insulin B-chain with molecular weights of 1531 and 1983 Da under the influence of the mutant enzyme (Figure 2). N-terminal sequencing of the peptide cleavage products in the reaction mixture without their separation demonstrated the appearance of the only additional amino acid sequence Ala–Leu–Tyr. These data confirmed that the only hydrolyzed bond in the oxidized insulin B-chain was Glu13–Ala14 (Figure 3).

Description of the catalytic properties of the wild-type and mutant BIGEP

The catalytic properties of the wild-type and mutant BIGEP were studied by cleavage of Z-AALE-pNA, a specific substrate of GEPs. The His186(213)–Thr substitution proved to increase $K_m$ by 4.9 times and to decrease $k_{cat}$ by 615 times relative to the wild-type protein. The obtained data are given in Table I. Standard deviations of the presented $K_m$ and $k_{cat}$ values were below 10%.

Discussion

The most interesting aspect of GEP activity is the mechanism underlying their strict substrate specificity for cleavage of bonds formed by $\alpha$-carboxyl groups of negatively charged...
residues. It seems clear that the S1 binding site should carry a compensatory residue of opposite charge to recognize the substrate charge. The existence of such a system has been experimentally confirmed in some cases. For instance, Asp189 serves as a compensatory residue in trypsin that is structurally similar to BIGEP (Graf et al., 1987), while it is a pair of residues Arg179 and Arg341 in Asp-specific interleukin-1β converting enzyme (Thornberry and Molineaux, 1995). However, no conserved strong basic residue in the S1 subsite has been revealed for bacterial GEPs (Nienaber et al., 1993; Barbosa et al., 1996; Demidiuk and Kostrov, 1999).

At the same time, the crystal structure of GEPs (Nienaber et al., 1993; Cavarelli et al., 1997; Barrette-Ng et al., 2002, Meijers et al., 2004), molecular modeling of the active sites of these proteins (Barbosa et al., 1996), and experiments on directed modification of GEP from Streptomyces griseus (Stennicke et al., 1996) and Glu-specific arterivirus Nsp4 3C-like serine protease (Snijder et al., 1996) allow us to consider His213 (chymotrypsin numbering system) localized in the S1 site as an essential residue for functioning of these enzymes. This residue is a common structural feature of microbial and viral GEPs; it can carry a positive charge and was considered as the most probable key element for recognition of substrate negative charge by bacterial GEPs (Nienaber et al., 1993; Barbosa et al., 1996). Although there may be other compensators of substrate charge, they are not universal which implies different functional mechanisms in various GEPs (Barbosa et al., 1996).

Previously, we cloned and sequenced the GEP gene from B. intermedius (BIGEP) (Rebrivik et al., 1999). The gene was expressed under control of its own regulatory elements in the cells of B. subtilis laboratory strain AJ73. Here we used this experimental model to directly test the hypothesis about the key role of His213 in recognition of substrate negative charge by directed mutagenesis.

No GEPs with an amino acid substitution at position 213 have been described previously. At the same time, introduction of the corresponding nucleotide substitutions in GEP genes has been reported. Snijder et al. (1996) studied Glu-specific arterivirus Nsp4 3C-like serine protease. ORF1a sequence of equine arteritis virus (EAV) including the region corresponding to non-structural protein 4 (Nsp4) was cloned and expressed in RK-13 cells. A set of mutant genes including substitutions corresponding to His1198(213)–Leu/Arg/Tyr mutations (chymotrypsin numbering system in parentheses) demonstrated a completely abolished processing of ORF1a protein at the Nsp4/S (Glu–Ser) and Nsp5/S (Glu–Gly) function. These data confirm the important role of His1198(213) (chymotrypsin numbering system in parentheses) in the functioning of this enzyme but do not confirm that this residue determines its specificity. Stennicke et al. (1996) used glutamic acid specific protease from S. griseus (SGPE) as a model. They used an expression system quite similar to that used in this work; the protease was synthesized in B. subtilis cells as a secretory protein. Similar to the above-mentioned publication, His213–Ala/Gly/Lys/Asn/Arg/Ser/Val mutations were introduced, which completely inhibited synthesis of the protein. This can be due to disturbed processing or severe changes in protein structure. These data confirm the significance of His213 for enzyme functioning but neither confirm nor reject that His213 can determine the primary specificity of the enzyme.

We introduced the mutations responsible for point amino acid substitution His186(213)–Thr (chymotrypsin numbering system in parentheses) into BIGEP gene. The structure of the modified gene was confirmed by sequencing. An electropheretically homogeneous mutant enzyme was isolated and its processing site proved to be identical to that of the wild-type protein. Thus, we obtained the first GEP with a mutation at position 213.

The pattern of hydrolytic cleavage of oxidized insulin B-chain demonstrated that the mutant enzyme hydrolyzes the only Glu13–Ala14 bond (Figures 2 and 3). This indicates that the histidine residue at position 186(213) is not responsible for the primary specificity of BIGEP activity.

The kinetics of the chromogenic substrate Z-AALE-pNa cleavage by the modified BIGEP suggests that modification of the protein at position 186(213) is substantial for efficient catalysis. The introduced substitution had relatively little effect on the Michaelis constant ($K_m$ increased 4.9 times) but considerably affected the catalytic constant ($k_{cat}$ decreased 615 times) (Table I). It was shown that the mutant proteinase is stable in experiment conditions and therefore the change of $k_{cat}$ is not correlated with the reduction of the active enzyme quantity during incubation.

The data obtained allow us to propose that the conserved His213 of bacterial GEPs is not a key element responsible for their primary substrate specificity (at least in the case of Bacillus GEPs). However, this residue may be substantial for precise positioning of the cleaved bond relative to the nucleophile, oxygen of the hydroxyl group of serine in the catalytic site.

Acknowledgements

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References


Table I. $K_m$ and $k_{cat}$ values for Z-AALE-pNa cleavage by the wild-type and modified BIGEP

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<th>$K_m$ (mM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (s$^{-1}$ M$^{-1}$)</th>
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<tr>
<td>Wild-type BIGEP</td>
<td>0.67</td>
<td>16.6</td>
<td>24776</td>
</tr>
<tr>
<td>Modified BIGEP (H186-T)</td>
<td>3.3</td>
<td>0.027</td>
<td>8.2</td>
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