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Antibody-induced oligomerization and activation of an engineered reporter enzyme

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Summary

Our objective is to produce a protein biosensor (or molecular switch) that is specifically activated in solution by a monoclonal antibody. Many effector-dependent enzymes have evolved in nature, but the introduction of a novel regulatory mechanism into a normally unregulated enzyme poses a difficult design problem. We used site-saturation mutagenesis and screening to generate antibody-activated variants of the reporter enzyme beta-glucuronidase (GUS). The specific activity of the purified epitope-tagged GUS variant was increased by up to ~500-fold by the addition an equimolar concentration of a monoclonal antibody. This molecular switch is modular in design, so it can easily be re-engineered for the detection of other peptide-specific antibodies. Such antibody-activated reporters could someday enable point-of-care serological assays for the rapid detection of infectious diseases.

Keywords

biosensor; molecular switch; high throughput screen; induced dimerization; reporter enzyme

Introduction

The capacity to regulate enzymes at will would be useful for point-of-care serological diagnostics¹, protein therapies^{2; 3}, and synthetic biology⁴. The activities of many natural proteins are regulated by post-translational modification or non-covalent interactions with an effector molecule. Such regulated enzymes will hereafter be called molecular switches or protein biosensors. The textbook example of allostery is hemoglobin, a tetrameric protein that binds oxygen in a cooperative manner. The binding of O₂ to one subunit leads to conformational changes that increase the O₂-affinity of other subunits within the holoenzyme. Hemoglobin is thus a sensor and a carrier of oxygen. Allosteric proteins are fairly common in nature⁵, but are relatively difficult to design.

Our goal is to design molecular switches that are activated by arbitrarily designated analyte molecules. Our designs are based upon the *Escherichia coli* β -glucuronidase (GUS) because its catalytic activity can easily be detected with commercially available synthetic substrates. We chose a monoclonal antibody as a model analyte because an antibody-activated reporter enzyme would enable rapid serological assays. The conversion of any unregulated reporter

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enzyme into an antibody-activated molecular switch is a difficult protein engineering problem. GUS and other reporter enzymes (including beta-galactosidase, alkaline phosphatase and beta-lactamase) are normally unregulated, and thus are constitutively active as long as they are properly folded. The fusion of a peptide epitope to the amino or carboxy terminus of a reporter enzyme generally allows it to bind monoclonal antibodies, but these binding events rarely affect the enzyme's intrinsic catalytic activity.

First generation protein sensors were produced by inserting peptide epitopes into permissive sites within reporter enzymes beta-galactosidase⁶, alkaline phosphatase⁷, and beta-lactamase⁸. This approach generally produces catalytically compromised enzymes that are activated up to 4-fold by antibody binding⁹. These switches are thought to be activated through allosteric mechanisms¹⁰, but it remains difficult to predict whether the insertion of any peptide epitope within a particular position of a protein will impart the desired allosteric properties.

High throughput screening enables the systematic evaluation of large numbers of chimeric proteins. Guntas and Ostermeier randomly inserted the gene encoding the maltose-binding protein (MBP) within the TEM-1 beta-lactamase gene. They expressed the resulting library of MBP-beta-lactamase chimeras in a population of *E. coli*, and screened for clones that exhibit maltose-dependent beta-lactamase activity. The enzyme activities were only modestly increased (<2-fold) by maltose¹¹, although subsequent refinements to the approach have produced beta-lactamase variants that are significantly more responsive (>100-fold activation)¹². This labor-intensive library construction and high throughput screening process would have to be recapitulated to produce sensors of analytes other than maltose. Here we demonstrate a relatively simple but effective strategy for the design of antibody-activated molecular switches.

Results

Rationale

Our strategy was to mutate GUS, a homotetramer¹³, so that the resulting variant formed an inactive but conformationally stable dimer. We were not certain which mutation combinations would accomplish this feat, so we used site-saturation mutagenesis to randomize two residues in the "short" subunit interface (Figure 1, blue and green balls). Each monomer contains an active-site close to this interface (yellow balls), so the dissociation of the tetramer into dimers would likely compromise enzyme activity. GUS mutants with diminished activity (hypomorphs) were identified in a high throughput histological screen. The hypomorphic alleles were purified and subcloned into a vector that fused them to the GST gene. The GST protein tends to dimerize proteins to which it is fused^{14 15}; a second histological screen was used to identify GST-GUS fusions with restored GUS activity.

GST-dependent GUS mutants

A previous study showed that the mutation of particular residues in the short subunit interface of GUS improved the thermostability of the tetramer¹⁶. These results suggest that subunit interactions across the short interface are essential for activity. We employed site saturation mutagenesis to randomize two GUS codons (516 and 517) within the parental *his₆-gusA*-pET28a+ vector (Figure 1). Phosphorylated primers containing the degenerate NNK (where K = T or G) sequence at the two selected positions were used to PCR amplify the entire plasmid. The PCR product was purified, self-ligated and used to transform *E. coli* strain DH5Δlac(DE3)^{17; 18}.

After the transformation, ~2000 clones were grown on LB-agar plates containing kanamycin (25 μg/mL) and chloramphenicol (34 μg/mL) and the colonies adsorbed onto nitrocellulose filters. The filter-bound colonies were transferred to similar plates that also contained 0.5 mM

isopropyl-beta-D-thiogalactopyranoside (IPTG, to induce over-expression of the plasmid-borne *gusA* gene) and the histochemical GUS substrate 5-bromo-4-chloro-3-indolyl beta-D-glucuronide (X-gluc, 80 mg/L). After overnight incubation at 37° C, the colonies that did not exhibit detectable GUS activity (the majority) were scraped from the plates and propagated together in liquid culture. The corresponding plasmids were isolated, and their *gusA* alleles were subcloned into expression vector pET42a+ so that they were fused to the Glutathione S-transferase (GST) gene. The GST protein forms dimers¹⁹ and causes proteins to which it is fused to dimerize as well^{14; 15}. The resulting library of GST-GUS variants was screened as described above for clones with restored GUS activity. Each clone with GUS activity was re-streaked onto LB-kan/chl agar plates containing IPTG and X-gluc; the resulting colonies were visualized to confirm that their phenotypes were genetically stable.

Ex vivo function

Eight GST-GUS variants consistently exhibited activity in reactions with X-gluc. The corresponding *gusA* alleles were sequenced, and different mutations at residues 516 and 517 were observed (Table 1). Two mutants include an ATG stop codon at position 516, but were expressed in an amber suppressor strain DH5Δlac(DE3) and are thus likely to encode E516. One variant (M516K, Y517E) was isolated twice and was most active when fused to GST. It was first subcloned back into the original vector (pET28a+), thus replacing the N-terminal GST tag with a six histidine tag.

The GST-M516K/Y517E GUS and his₆-M516K/Y517E GUS proteins were separately expressed in *E. coli* and purified to homogeneity using either glutathione or immobilized metal affinity chromatography. Each purified protein was separately reacted with 1 mM para-nitrophenyl-B-D-glucuronide (pNP-gluc) in 50 mM Tris-HCl buffer (pH 7.6), and the formation of the para-nitrophenol (pNP) product was followed at 405 nm in a UV/vis spectrophotometer. The GST-M516K/Y517E GUS (100 nM) consistently showed higher specific activity (8.4 mAbs/min) than equimolar concentrations of his₆-M516K/Y517E GUS (0.8 mAbs/min). In contrast, the tagged but otherwise wild-type his₆-GUS and GST-GUS proteins exhibited similar specific activity (data not shown).

Antibody-dependent GUS variant

While the GST-dependent GUS variant could be adapted for a detection assay *in vivo*, our objective was to design a modular molecular switch that can be activated *in vitro* by an intact antibody. We chose the anti-hemagglutinin (anti-HA) monoclonal antibody as a model analyte to show that oligomerization and activation could be induced by an intermolecular effector. We inserted DNA sequence encoding the HA epitope (YPYDVPDYA) and a ten amino acid spacer immediately upstream of the M516K/Y517E *gusA* gene. The his₆-HA-M516K/Y517E GUS fusion protein was expressed in *E. coli* and purified to homogeneity by virtue of its his₆ tag; the yield was similar to that of the his₆-wild type GUS protein (~10 mg/L culture).

Varying quantities of purified his₆-HA-M516K/Y517E GUS protein (25 – 200 nM) were reacted with 1 mM pNP-gluc in the presence or absence of saturating (~100 nM) anti-HA monoclonal antibody (typical plot shown in Figure 2). The antibody increased the specific activity of his₆-HA-M516K/Y517E GUS by a factor of 17–300 fold, depending upon the concentration of enzyme (Table 2, Supplemental Table 1). The antibody-dependent increase in activity was inversely proportional to the concentration of enzyme, most likely due to a residual tendency toward self-oligomerization. We also employed a fluorogenic substrate, 4-methylumbelliferyl-beta-D-glucuronide (MUG)²⁰, in order to improve the sensitivity and observed GUS activation within the assay. We reacted his₆-HA-M516K/Y517E GUS (10 – 200 nM) with 1 mM MUG in 50 mM Tris-HCl buffer (pH 7.6) and monitored the formation of fluorescent methylumbelliferone product in a spectrofluorometer. The specific activity of

the minimum detectable concentration of his₆-HA-M516K/Y517E GUS (10 nM) increased ~500-fold upon addition of the anti-HA antibody (Table 2 and Supplementary Table 1). Gel filtration analysis of the his₆-HA-M516K/Y517E GUS (~116 kDa) and his₆-HA-wild-type GUS (~235 kDa) suggests that the mutant forms an inactive dimer as predicted (Figure 3). Both proteins also eluted at the void volume, suggesting a tendency to aggregate.

As a control, the his₆-HA-wild-type-GUS was expressed, purified and reacted with the HA antibody. As expected, the antibody had no detectable effect upon enzyme activity (data not shown). To confirm that the antibody specificity of the sensor could be switched, we used site-directed insertion mutagenesis to replace sequence encoding the HA epitope with that encoding c-myc (EQKLISEEDL). The his₆-myc-M516K/Y517E GUS protein was expressed, purified and reacted with 1 mM pNP-gluc in the presence or absence of the anti-myc monoclonal antibody (Figure 4). The antibody increased the specific activity of the his₆-myc-M516K/Y517E GUS (100 nM) 9-fold. In contrast, the anti-HA monoclonal antibody had no effect, ruling out the possibility that some other factor within the anti-HA antibody sample was non-specifically activating GUS.

Kinetic properties of the antibody-dependent GUS variant

The steady state kinetic parameters (k_{cat} and K_M) of the his₆-wild-type GUS and antibody-bound his₆-HA-M516K/Y517E GUS in reactions with pNP-gluc at 37° C were determined. The his₆-wild-type GUS reaction data readily fit Michaelis-Menten equation (Figure 5a), which suggests that the active sites are independent. In contrast, the his₆-HA-M516K/Y517E GUS reaction data best fit the Hill equation, where n , the degree of cooperativity, was 3.7 ± 0.5 (Figure 5b). The data did not fit the Michaelis-Menten equation at all. This result suggests that the antibody-bound his₆-HA-M516K/Y517E GUS binds the pNP-gluc substrate cooperatively. In other words, both the antibody and the substrate are required for maximum enzyme activity. This catalytic efficiency (k_{cat}/K_M) of the activated mutant is ~9% of the wild-type value, demonstrating that GUS-based biosensors are potentially active enough for rapid point-of-care diagnostic assays.

Discussion

We used site-saturation mutagenesis in conjunction with negative and positive histochemical screens to generate a GUS mutant that can be activated either by fusion to the dimeric GST protein or by non-covalent interaction with anti-HA (or anti-myc) monoclonal antibodies. The mutagenesis and screening strategies were designed to identify mutants that form inactive dimers, but retain a latent capacity to form active tetramers. It remains formally possible that the activation occurs through an allosteric mechanism¹⁰, but several lines of evidence favor the induced oligomerization hypothesis. Gel filtration analysis showed that his₆-M516K/Y517E GUS is mostly dimeric, and his₆-wild-type GUS is mostly tetrameric (Figure 3).

The correlation between enzyme activity and tetramerization is easy to rationalize. Active site residues R562 and E557²¹ are in the “short” subunit interface (blue and green balls in Figure 1). We propose that the non-conservative M516K and Y517E mutations disrupt the association of monomers across the short interface. The energetic cost of burying these two charged residues in the mostly hydrophobic interface (plus the usual entropic cost of oligomerization) exceeds the remaining binding energy. The active-sites within the unbound mutant dimers likely adopt an alternative conformation that is not conducive for catalysis. The binding energy of the bivalent monoclonal antibody helps overcome the energetic cost of burying those charged residues, thereby shifting the equilibrium toward the tetramer.

The antibody-bound M516K/Y517E GUS displays two unusual kinetic traits that are consistent with the induced oligomerization mechanism. First, the antibody/enzyme complex exhibits

very cooperative substrate-binding; the Hill coefficient is similar to that of hemoglobin (Figure 5b). Previous workers have similarly shown that mutations in the subunit interface of glutathione reductase²², and aspartate receptor²³ can produce mutants that bind their substrate cooperatively. Second, a pronounced ~10 minute lag occurs before the enzyme-catalyzed hydrolysis of pNP-gluc enters the steady-state (Figure 2); pre-incubation of our sensors with antibody does not affect the lag (data not shown). We propose that the mutant active-site begins in a non-productive conformation, and that antibody-binding is not sufficient to stabilize the optimal configuration of the dimers for catalysis. Substrate-binding is cooperative because it shifts the equilibrium of the tethered dimer pair towards the properly assembled tetramers with productively folded active sites. Slow conformational changes on this time-scale have previously been observed in mutant proteins²⁴.

The sensitivity of a biosensor is defined by the dependence of its activity upon the concentration of analyte. We estimate that ~100 nM of an anti-HA antibody was required for maximum activation of 10 nM his₆-HA-M516K/Y517E GUS. We do not know why a molar excess of antibody is required, but can propose three non-mutually exclusive explanations. First, the commercially available antibody that we employed might not bind the epitope tightly ($K_D > 10$ nM). A different anti-HA monoclonal antibody (17/9) exhibited an IC_{50} of 3.5×10^{-7} M in reactions with the HA tag peptide, and an IC_{50} of 1×10^{-8} M with a longer peptide that includes the HA tag²⁵. Different antibodies, however, exhibit different binding affinities.

Second, the his₆-HA-M516K/Y517E GUS protein might not display the HA epitope efficiently. The structure of the wild-type *E. coli* protein has not been solved. Our mutagenesis strategy was guided by a homology model based upon the human GUS structure¹³, but the sequences of the N- and C-termini of these homologues is highly divergent. The bilateral symmetry of the GUS tetramer makes it possible to display epitopes on geometrically permissive surface loops. Crystal structures of intact IgG antibodies²⁶ suggest that antigens up to 70 Angstroms apart could still be bound by a single bivalent antibody molecule. Third, the M516K/Y517E mutation combination was selected for responsiveness (i.e. ~500-fold activation by antibody) rather than for sensitivity. Mutations in the subunit interface that are less deleterious would likely create more sensitive (and less responsive) sensors. The optimization of these parameters should enable molecular switches suitable for the detection of antibodies (and possibly other multimeric protein analytes) within clinical samples.

Our design strategy could be used to convert multimeric proteins other than GUS into molecular switches. Most reporter proteins, including beta-galactosidase, alkaline phosphatase and glucose oxidase, and ~35% of all intracellular proteins²⁷ require oligomerization for activity. Previous workers have mutated residues in the subunit interfaces of alkaline phosphatase²⁸ and GST²⁹. In both studies, the size and charge of the substituted amino acid caused shifts in the monomer-dimer equilibrium of these proteins. We identified several different mutation combinations that favored the formation inactive GUS dimers (Table 1). The most GST-dependent variant (M617K, Y157E) contained charged residues that are energetically costly to bury. Site-directed mutagenesis could be used to introduce analogous amino acid replacements into almost any oligomeric enzyme.

The current standard in immunological assays is the Enzyme-Linked ImmunoSorbent assays (ELISAs). In this labor-intensive technique, antibodies that recognize pathogen markers are adsorbed to 96 well plates. Samples are reacted with the plate-bound antibodies; unbound molecules are washed away. The antibody-antigen complexes are reacted with a second detection antibody that recognizes a separate epitope of the antigen. The second antibody is usually conjugated to a reporter enzyme. The complexes are washed to remove unbound reporter enzyme, then reacted with a colorimetric substrate. Our sensor reacts with an antibody and a fluorescent substrate in solution without any washing steps, and therefore represents a

simpler solution to the antigen detection problem. The development of switches with different activities (outputs) could someday enable multiplex serological assays.

Materials and Methods

Materials

Expression vectors pET28a+ and pET42a+ were from Novagen (Madison, WI). *E. coli* strain BL21 (DE3) Gold/pLysS was from Stratagene (La Jolla, CA); DH5Δlac(DE3) was described previously²¹. The oligonucleotides were synthesized by IDT (Coralville, IA); the pNP-gluc and MUG were from Sigma-Aldrich, and the X-gluc was from Gold Biotechnology (both St. Louis, MO). The mouse monoclonal anti-HA and anti-myc antibodies were from Covance (Princeton, NJ). The BigDye 3.1 DNA sequencing and GeneAmp XL Long PCR kits were from Applied Biosystems (Foster City, CA)

Site-saturation mutagenesis and screening

The *gusA* codons encoding amino acids 516 and 517 (within expression vector his6-*gusA*-pET28a+) were randomized by whole plasmid PCR³⁰ using the following 5' phosphorylated degenerate primers 5'-GGGCTGCACTCCANNKNNKACCGACATGTGGA-3' and 5'-GGCTAACGTATCCACGCCGTATTCGGTG-3'. The library was then generated by the purification, self-ligation and transformation of strain DH5Δlac(DE3) as previously described¹⁷. The his6-*gusA* clones exhibiting no GUS activity were subcloned into expression vector pET42a+ (immediately downstream of the GST gene) using the restriction enzymes Nco I and Hind III.

Insertion of epitope tags

The most GST-dependent *gusA* allele (M516K/Y517E) was subcloned back into pET28a+ (immediately downstream of the his6 tag sequence) using the restriction enzymes NcoI and HindIII. We had previously generated the HA epitope within the unstructured region of p53³¹; DNA sequences from this construct encoding the HA epitope and a ten amino acid p53 linker to the 5' end of M516K/Y517E *gusA* (his6-HA-linker-*gusA*) through the following overlap PCR procedure. The HA tag was PCR amplified from HA-p53-delta68-pET28a+³¹ using primers 5'-TTGGGGTTTCTACAGGACGTAACATATGTCTGGGAGCTTCATCTGGACCTGGG-3' (p53rev196/5'*gusA*) and 5'-GAGTCTCGATCCCGCGAAATTAATACGA-3' (5'*pET*). The 5' end of the his6-M516K/Y517E *gusA*-pET28a+ was amplified with 5'-CCCAGGTCCAGATGAAGCTCCCAGACATATGTTACGTCCTGTAGAAACCCCA A (5'*gusA*/p53rev196) and 5'-GCTCAGCGGTGGCAGCAGCCAACCTC-3' (GC 3'*pET*). Each PCR product was purified with a Qiagen silica spin-column as directed by the manufacturer. The two purified PCR products (50 ng each) were recombined and PCR amplified in a third PCR using the 5'-GAGTCTCGATCCCGCGAAATTAATACGA-3' (5'*pET*) and 5'-GCTCAGCGGTGGCAGCAGCCAACCTC-3' (GC 3'*pET*) primers. The full-length his6-HA-M516K/Y517E *gusA* PCR product was purified with a Qiagen silica spin-column and cloned back into the his6-M516K/Y517E *gusA*-pET28a+ plasmid using restriction enzymes Msc I and Nco I. The resulting clones were sequenced with the 5'*pET* primer to confirm the presence of the HA epitope tag. After this stage of cloning, there remained 45 amino acids between the 6-his tag and the HA epitope tag.

The upstream region of p53 was removed again using whole plasmid PCR with the phosphorylated primers 5'-TATCCGTATGATGTGCCGGATTATGCG (p53HA*gusA*) and 5'-CATATGGCTGCCGCGCGCACCA (p53HA*gusA*rev). The resulting his6-HA-linker-*gusA* construct was sequenced in its entirety to confirm that no undesired random mutations were introduced during the PCR amplification steps. The myc tag was inserted by using his6-

HA-M516K/Y517E *gusA*-pET28a+ as a template with the primers AATCAGTTTCTGTTCCATATGGCTGCCGCGCGGCACCAG (*myc*_{gusArev}) AGCGAAGAAGATCTGACTGAAGACCCAGGTCCAGATGAAGC (*myc*_{gusAout}) in a whole plasmid PCR as described above. DNA sequencing showed that the resulting product encoded the *myc* epitope instead of the HA epitope.

Sequencing

The *gusA* alleles were sequenced using the Applied Biosystems BigDye protocol at the Center for Fundamental and Applied Molecular Evolution (Emory University). The 3' end of every *gusA* allele in this paper was sequenced using the primer GC 3'pET. *his*₆-M516K/Y517E *gusA*-pET28a+, GST-M516K/Y517E *gusA*-pET42a+, and *his*₆-HA-M516K/Y517E *gusA*-pET28a+ were sequenced in their entirety, using the following additional primers: 5' pET, 5'-GCCATTTGAAGCCGATGTCACGCCG-3' (*gusA* 360), 5'-GGACTTTGCAAGTGGTGAATCCGCAC-3' (*gusA* 720), and 5'-CTGCTGCTGTCTCGCTTTAACCTCTCT-3' (*gusA* 1080).

Protein purification – GST and IMAC

The *his*₆-tagged GUS variants were chemically transformed into BL21(DE3)Gold/pLysS. The variants were expressed at room temperature overnight and lysed using sonication after suspension in 50 mM Tris, pH = 7.6 and 0.5 M NaCl. They were then purified as described¹⁷. The GST-tagged GUS variants were purified from the DH5Δlac(DE3) strain according to the manufacturer's instructions (Novagen). All proteins used in this study were >95% pure as judged by SDS-polyacrylamide gel electrophoresis. The concentrations of each purified protein were quantified using the Bradford protein assay (Bio-Rad, Hercules, CA). Molar concentrations were based on the number of GUS active-sites (molecular weight 70 kDa).

Enzyme assays

The purified GUS proteins (molecular weight = 68,200) were diluted to 25 nM to 200 nM and reacted with 1 mM pNP-gluc and either 0.5 μL of the anti-HA monoclonal antibody, anti-*myc* antibody or distilled H₂O in 50 mM Tris, pH 7.6, in a 96 well microplate (Nunc). Based upon estimates by the manufacturer (Covance), we calculate that the final concentration of antibody (molecular weight = 150,000) is ~100 nM. The reactions were monitored in a Thermo LabSystems Multiskan microplate spectrophotometer at 37° C for one hour, and the initial activities were calculated from the slopes of the lines after the lag phase. For the fluorescent assays, 1 mM of 4-methylumbelliferyl-β-D-glucuronide (MUG) was added to 10 – 200 nM of protein in 50 mM Tris, pH 7.6. The reaction was monitored in a fluorometer at 37° C for thirty minutes or until the substrate was depleted.

To determine the steady-state kinetic parameters, *his*₆-wild-type GUS (10 nM) was reacted with 10–800 μM pNP-gluc in 50 mM Tris (pH 7.6); all reactions were conducted in triplicate in a 1 mL (1 cm path length) polystyrene cuvette (Fisher Science). The formation of the para-nitrophenol (pNP) product at 37° C was followed in a Shimadzu UV-1601 spectrophotometer. The absorption extinction coefficient of p-nitrophenol (pNP) at 405 nm under these conditions is 16.64 mM⁻¹ cm⁻¹. The *his*₆-HA-M516K/Y517E GUS (100 nM) was mixed with approximately equimolar anti-HA antibody and 10 – 500 μM pNP-gluc in a microfuge tube (again, in triplicate). The reactants (175 μL) were transferred to a small quartz cuvette (0.3 cm × 0.3 cm × 27 mm), and product formation at 37° C was monitored in the spectrophotometer. The initial velocity values (excluding the lag exhibited by the antibody-bound *his*₆-HA-M516K/Y517E GUS, and taking into account the smaller volume and cuvette path length) were fit to the Michaelis-Menton and Hill equations

$$v_i = \frac{V_{\max} [S]^n}{K_M^n + [S]^n}$$

(where n is the Hill coefficient, a measure of cooperativity) by the non linear least squares algorithm of Kaleidagraph 3.0.5 (Adelbeck software, Reading, PA).

Gel Filtration

The purified his₆-M516K/Y517E GUS and his₆-wild-type GUS proteins were separately run on a Pharmacia Superdex 200 GL 10/300 column equilibrated with 50 mM Tris-HCl (pH 7.6), 150 mM NaCl and eluted at a flow-rate of 0.5 mL/min. The elution profiles of these proteins were compared to those of molecular weight standards (Bio-Rad). The molecular weights of the mutant and wild type GUS were determined by extrapolation of the standard curve generated by gel filtration of the Bio-Rad standards.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Crystal structure of human β -glucuronidase¹³

β -glucuronidase is normally a homotetramer; two monomers are shown (blue and green). Residues in the “short” interface (blue and green space filling balls) normally interact with their counterparts in other monomers (not shown). Catalytic residues (E413, E504, Y468, *E. coli* protein numbering) are close to the short interface (yellow balls). The two amino acids mutated in this study are shown in red and labeled M516K/Y517E. The GST protein, HA epitope and myc epitopes were separately fused to the N-terminus (orange) to induce oligomerization.

Figure 2. Activity assays of his₆-HA-M516K/Y517E GUS

The purified his₆-HA-M516K/Y517E GUS protein (50 nM) was reacted with 1 mM pNP-gluc and either 0.5 μ L of the anti-HA monoclonal antibody (filled squares) or distilled H₂O (empty squares) in 50 mM Tris, pH 7.6, in a 96 well microplate. The reactions were monitored at 405 nm in a spectrophotometer at 37° C for one hour, and the initial activities (Supplemental Table 1) were calculated from the slopes of the lines after the lag phase.

Figure 3. Gel filtration of his₆-HA-wild-type GUS and his₆-HA-M516K/Y517E GUS

The purified his₆-HA-M516K/Y517E GUS and his₆-HA-wild-type GUS proteins were separately run on a Pharmacia Superdex 200 GL 10/300 column equilibrated with 50 mM Tris-HCl (pH 7.6), 150 mM NaCl and eluted at a flow-rate of 0.5 mL/min. The elution profiles of these proteins were compared to those of molecular weight standards (Bio-Rad). The molecular weights of the mutant and wild type GUS were determined by extrapolation of the standard curve generated by gel filtration of the Bio-Rad standards.

Figure 4. Activity assays of his₆-myc-M516K/Y517E GUS

The purified his₆-myc-M516K/Y517E GUS protein (100 nM) was reacted with 1 mM pNP-gluc and either 2.5 μ L of distilled H₂O, the anti-myc monoclonal antibody, or the anti-HA monoclonal antibody in 50 mM Tris, pH 7.6, in a 1 ml cuvette. The reactions were monitored at 405 nm in a spectrophotometer at 37° C for one hour, and the initial activities were calculated from the slopes of the lines after the lag phase (Table 2, Supplementary Table 1).

Figure 5. Substrate dependent kinetics of his₆-wild-type GUS and his₆-HA-M516K/Y517E GUS (a) The his₆-tagged wild-type (empty triangles, 10 nM in 1 mL) or (b) HA-M516K/Y517E GUS bound to an equimolar concentration anti-HA monoclonal antibody (filled squares, 100 nM in 175 μ L) were separately reacted with 10 μ M - 800 μ M pNP-gluc in 50 mM Tris (pH 7.6). The formation of the pNP product was followed in a spectrophotometer. The steady-state kinetic parameters were determined by fitting the initial velocity values to the Michaelis-Menten and Hill equations (Table 3).

Table 1

GST-dependent GUS variants

Clone	Codon 516	Codon 517
wild-type	Met	Tyr
1-22	Gln	Thr
1-26	Lys	Trp
1-29	Met	Gly
2-10	Stop (Gln) ^A	Tyr
2-17	Lys	Glu
2-24	Lys	Glu
3-7	Stop (Gln) ^A	Tyr
3-18	Ala	Val

^AThe nucleotide sequence for this position was TAG, but all alleles were expressed in *E. coli* amber suppression strain DH5Δlac(DE3), so the protein likely contains a glutamine at this position.

Table 2Activation of his₆-HA-M516K/Y517E GUS by anti-HA antibody

Substrate	4-methylumbelliferyl- β -D-glucuronide	pNP- β -D-glucuronide
Enzyme	fold activation	fold activation
10 nM	490 $\pm$ 90	ND
25 nM	400 $\pm$ 100	ND
50 nM	200 $\pm$ 100	300 $\pm$ 170
100 nM	90 $\pm$ 20	360 $\pm$ 180
200 nM	25 $\pm$ 7	100 $\pm$ 70

Table 3

Steady state kinetic parameters

Enzyme	Model	k_{cat} (sec ⁻¹)	K_M (μM)	Hill coeff.
wild-type	Michaelis-Menten	196 ± 8	340 ± 30	----
wild-type	Hill	181 ± 17	291 ± 58	1.1 ± 0.1
activated mutant	Michaelis-Menten	----	----	----
activated mutant	Hill	13.7 ± 1.0	280 ± 16	3.7 ± 0.5