Site directed mutagenesis of glycine-286 in recombinant urate oxidase results in a loss of uric acid functionality.

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Abstract:

Multiple sequence analysis of urate oxidase isoforms shows significant amino acid conservation. This implies that these residues are significant to the structure of the enzyme, the functionality of the enzyme, or both. Site directed mutagenesis at a fully conserved residue would likely result in decreased activity of the enzyme. This is especially likely for glycine. Therefore, it is unlikely that urate oxidase can maintain specificity in its structure if glycine is mutated to another amino acid. To test this we will isolate the gene encoding for urate oxidase from *Bacillus subtilis* and alter one nucleotide such that alanine is coded for at position 286. Subsequently, the altered gene will be transformed into a new host organism for translation. We will then compare the activity of the mutant and a control urate oxidase by means of an enzymatic assay.

Specific Aims

The primary objective of this experiment is to determine the extent of specificity of glycine-286 in the urate oxidase enzyme. That is, to what end will the mutation from glycine-286 to alanine-286 alter the rate of catalysis.

The secondary objective is to determine the function of the glycine-286 with regards to enzyme structure and function as a whole. This aim is more open ended and any data collected from this experiment will lend itself to some determination of the relationship between the wild type residue and the structure of the enzyme.

Background and Scope

Urate oxidase is a homotetrameric enzyme responsible for ring separation of purine rings, specifically uric acid degradation to allantoin.[1] This protein is not expressed in humans and thus the build up of uric acid, hyperuricemia, is fairly common in individuals who cannot excrete uric acid as quickly as they produce it. This can lead to gout and several complications occurring during chemotherapy, typically tumor lysis syndrome.[2] Modern treatments derive from either uricosuric medications, uric acid synthesis inhibitors, or various recombinant urate oxidase preparations. Uricosuric medications increase the rate of excretion of uric acid, but with impared kidney function this is not effective. Allopurinol is effective at reducing the concentration of uric acid by inhibiting the function of xanthine oxidase, which converts xanthine to uric acid.[1] That being said, some patients have shown hypersensitivity in response to treatment with Allopurinol.[1] Fasturtec was the first recombinant urate oxidase treatment for both gout and tumor lysis syndrome; however, its limited activity and frequent immunoreactivity in patients greatly reduces its usability.[3] Conjugating the recombinant enzyme with a fatty acid and serum albumin has proven to be a breakthrough for successful treatment. Krystexxa was the first to do this and has increased the half life and activity of the treatment, while also decreasing adverse reactions in patients.[2] This medication used the increased stability of the conjugated system and a

recombinant baboon urate oxidase, which more closely resembles what remains of the gene in the human genome.[2] Further enzyme improvements are still desired as Krystexxa is difficult to produce and thus extremely expensive.[2]

The use of recombinant urate oxidases continues to represent an appealing and realistic means of treating gout and tumor lysis syndrome. Research that pushes the boundaries of our understanding of urate oxidase can help with development of future treatment programs and are expected to continue to elicit interest and generate funding. This experiment in particular will focus on our understanding of the structure-function relationship in urate oxidase of *Bacillus subtilis,* a potential model organism for future treatment plans. *Bacillus subtilis* is an attractive alternative to the partial baboon urate oxidase mentioned above due to its lower cost, ease of use, and lack of ethical concerns. Glycine is a compelling amino acid to mutate because it is likely to cause a significant change in protein structure. The change in activity after the mutation in the pucL gene - pucL codes for urate oxidase synthesis in *Bacillus subtilis* - could lend itself to further study of mutations at this residue or in the isoform of the protein.

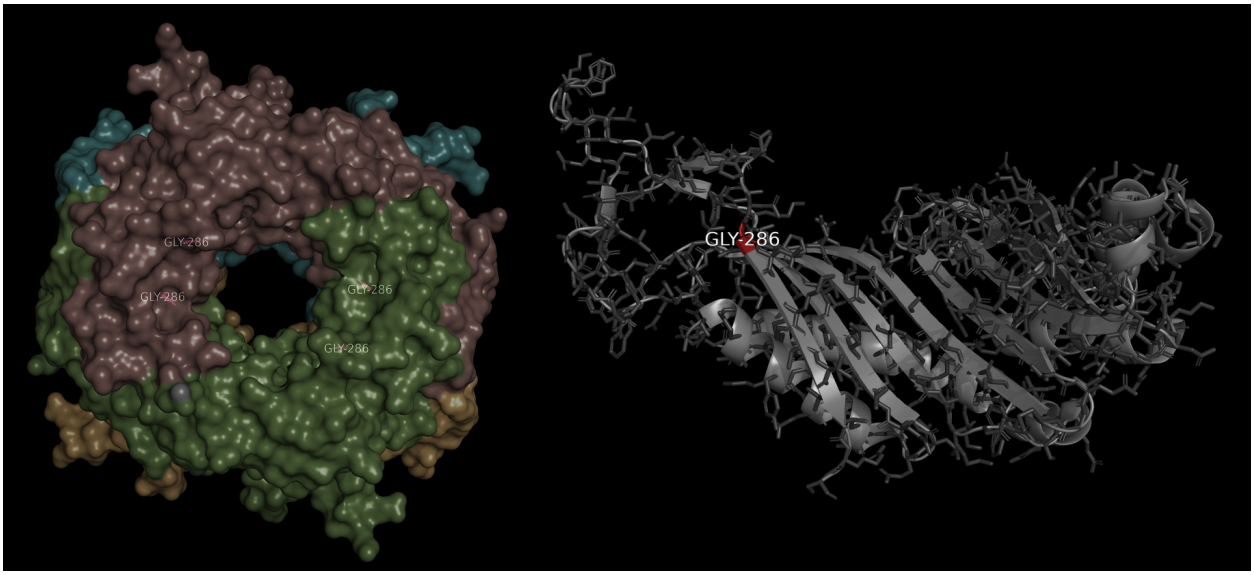
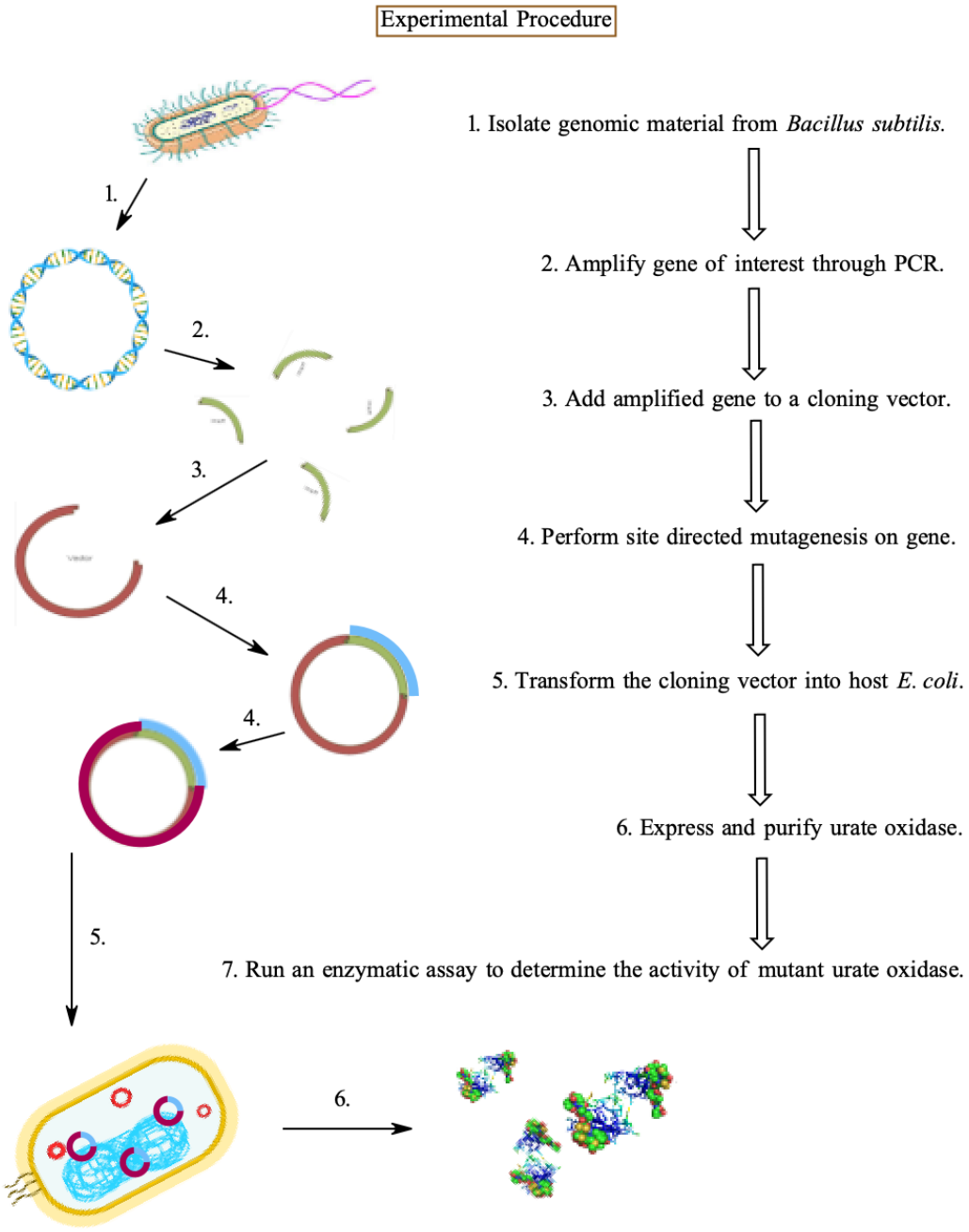


Figure 1
Figure (left) gives a structural interpretation of the full urate oxidase enzyme (obtained via x-ray crystallography). Figure (right) highlights the residue of interest in one monomeric subunit of urate oxidase (obtained by x-ray crystallography).

Experimental Methods and Materials



1. Isolate genomic DNA from *Bacillus subtilis* ATCC 6633.

Biochemical materials required for this step:

- *Bacillus subtilis* ATCC 6633
- Ribonuclease
- Tris-saturated phenol
- Chloroform

Procedure for this step:
This procedure for the acquisition of genomic DNA in bacillus subtilis was described elsewhere and was the inspiration for this interpretation.[4] Start by centrifuging 1 mL of cell suspension at 8,000 g for 120 seconds, then remove the supernatant from the top of the solution. Next, wash the cells with 400 µL of STE buffer solution. The STE buffer should consist of 100 mM NaCl, 10 mM Tris/HCl, and 1.0 mM EDTA at a pH of 8.0. Wash with 400 µL of STE buffer a second time and centrifuge a second time under the same conditions this further cleans the supernatant. After this the remaining pellets are suspended in a 200 µL TE buffer, which consists of 10 mM Tris/HCl and 1 mM EDTA at a pH of 8.0. Subsequently, add 100 µL of Tris-saturated phenol at pH 8.0, and then lyse the cells by vortex-mixing the centrifuge tube for 60 s. To separate the organic phase the sample should then be centrifuged at 13,000 g and 4 ℃ for 300 seconds. Transfer 160 µL of the upper aqueous phase to a 1.5 mL centrifuge tube. Add 40 µL of TE buffer and mix in 100 µL of chloroform. Centrifuge again at 13,000 g and 4 °C for 300 seconds. If a white interface remains, centrifuge under the same conditions until the lysate contains no white interface. Remove the upper 160 µL aqueous surface once more to a clean 1.5 mL centrifuge tube. Finally add 40 µL of TE buffer and 5 µL of ribonuclease and incubate at 37 °C for 10 mins to remove erroneous RNA. Add 100 µL of chloroform and centrifuge one last time at 13,000 g and 4 °C for another 300 seconds. Remove the upper 160 µL aqueous surface and place in a new tube for storage at -20 °C or immediate use.

2. Perform PCR on the gene of interest, pucL, to purify and amplify it for insertion into a cloning vector.

Biochemical materials required for this step:
- DNA isolate from *Bacillus subtilis*
- Taq DNA polymerase
- Deoxynucleoside triphosphates
- 2 engineered primers:
    - (5′-GCTCGAGGGCTTTCAGGCTCCGACAT-3′) (Reverse)
    - (5′-CGGATCCATGTTCACAATGGATGACCTG -3′) (Forward)
- ThermoPol buffer

The forward primer above contains a BamHI site and a start codon, whilst the reverse primer contains a XhoI site.[5] These restriction sites are key to the selective addition of this gene into our vector later in the experiment. It is important to start this step by dethawing any frozen PCR components on ice. This should ensure relatively slow and constant temperature increase. Once everything is thawed, prepare a thermocycler tube for a 50 µL solution. To the tube add 5 µL of 10X ThermoPol buffer, 1 µL of 10 mM dNTPs, 1 µL of both 10 µM primers, 0.25 µL of Taq DNA polymerase, approximately 25 ng of the isolated template DNA, and fill to 50 µL with DI water (approximately 45 µL). For reference, ThermoPol 1 X contains 20mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, and 0.1% Triton® X-100 at a pH of 8.8.[6] Spin down the buffer to incorporate all ingredients thoroughly. Run in the thermocycler for 30 cycles of 60 seconds at 94 °C, 45 seconds at 50 °C, and 90 seconds at 72 °C.[5] At this point we should have a relatively pure pucL gene which we can add to an appropriate cloning vector.

3. Add the amplified gene to the cloning vector.

Biochemical materials required for this step:

- pET-21a vector
- XhoI restriction enzyme
- BamHI restriction enzyme
- T4 DNA Ligase
- ATP solution

To start this process it is necessary to treat 60 ng of pET-21a cloning vectors with both BamHI and XhoI restriction enzymes such that the correct sites are available for ligation. Treat 2-3 µL of PCR product with the restriction enzymes as well. This is typically done after incubating the enzymes at 37 °C in acetate or chloride buffers according to their instructions. Incorporate the solutions of the cloning vector (2 µL) and the gene of interest (3 µL) with 1 µL of T4 DNA Ligase that has been diluted with ligase buffer, 1 µL of 10 mM ATP, 2 µL of 100 mM DTT, 2 µL of 10X ligase buffer, and fill to 20 µL total with DI water in a centrifuge tube.[7] The 10X Ligase buffer is at pH 7.6 and contains: 200 mM Tris-HCl, 100 mM $MgCl_2$, and 250 µg/mL of acetylated BSA.[7] Stir the solution with a pipette tip and incubate at 16 °C overnight. After this we should have our gene of interest placed on a cloning vector which we can then perform site directed mutagenesis on.

4. Add primer designed to alter codon to express alanine mutant and start a control group

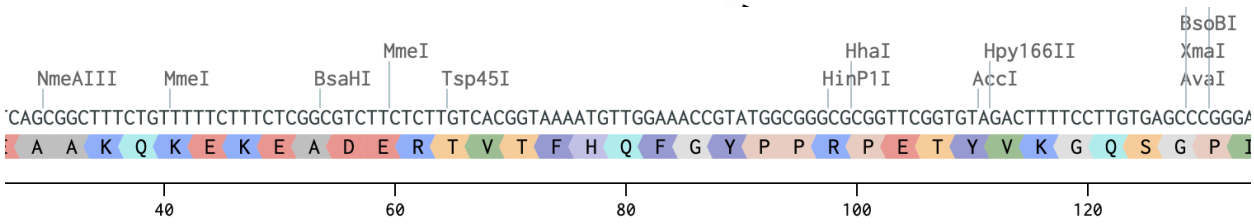


Figure 2
This figure highlights the residue of interest, and was the basis for the design of the mutation inducing primer. The primer is complementary to the sequence above and replaces what would be the second guanine with a cytosine.

Biochemical materials required for this step:

- Mutation primer: (5'-CGCCCGGCCATA**GCT**TTCCAACATTTT-3')
- High Fidelity DNA polymerase
- Deoxynucleoside triphosphates
- T4 DNA Ligase

Separate the 20 µL of cloning vectors into 2 10 µL solutions in two separate centrifuge tubes. One of these will be a control group and the other will be mutated. Set the control group to the side and let cool because the control group has a vector ready to be transformed by our host cell. Heat the mutation sample to 95 °C, to denature the plasmids into two single strands and to denature previously used proteins, and add the mutation primer in excess, approximately 500 -

1,000 ng. Cool down to 75 °C and add 0.5 µL of 10 times diluted high fidelity DNA polymerase. At this point the primers will add to one of the separated single strands and the DNA polymerase will begin synthesizing new DNA along the single strand. This effectively recreates the vector we just separated; however, we have successfully made the one nucleotide, and thus one amino acid substitution. Let cool to 25 °C and add the 0.5 µL of T4 DNA Ligase to complete reconstruction of the double stranded cloning vector.

5. Transform the cloning vector into host *E. coli*

Biochemical materials required for this step:

- 2 X One Shot BL21 Star™ (DE3) pLysS *E. coli*
- LB and SOC growth media
- Ampicillin

Now we are ready to express the wild type and mutated recombinant proteins. The selected competent cell was engineered for increased efficiency in expression. Start by thawing two *E. coli* vials in ice water. Add 2 µL of control group cloning vectors to one vial and 2 µL of mutant cloning vectors to the other vial. Incubate the vials in ice water for 30 minutes.[8] Afterwards, shock the vials by placing them in the 42 °C water bath for 30 seconds.[8] When 30 seconds has elapsed return vials to the ice water bath.[8] Add 250 µL of room temperature SOC for bacterial growth to each vial.[8] Secure vials in a shaking incubator and shake at 37 °C for 1 hour at 225 rpm. Prepare two LB plates with 6 mL of LB medium and 100 µg/mL of ampicillin.[5] The ampicillin is important because it selectively removes *E. coli* that have not transformed one of our vectors. This is because these vectors express ampicillin resistance. Create about ten colonies from the control group on the first plate and ten more colonies from the mutated group on the second plate.

6. Recombinant enzyme expression and purification

Biochemical materials required for this step:

- IPTG
- Q-Sepharose beads
- Ammonium sulfate
- Sephadex G-50 beads

We first need to produce sufficient recombinant protein. To do this add bacteria from four colonies on the mutant plate to 100 mL of LB growth medium containing 1mM IPTG in a beaker. Let incubate for four hours. Repeat this process for the control group. Harvest test groups separately in a centrifuge at 5,000 rpm and 4 °C. Separate the cell pellet and add 2 mL of buffer. This buffer is meant to stabilize protein structures and is composed of 10 mM imidazole, 50 mM NaH_2PO_4, and 300 mM NaCl at pH of 8.0. Sonicate both groups with 10 cycles of 5 second pulses at 65 W. Next centrifuge the suspensions once more for 12 min at 13,000 rpm. Add 5 mL of 0.02 M Tris-HCl buffer at pH 8.5 and dialyze for 12 hours against 2 L of 0.02 M Tris-HCl at pH 8.5.[9] Prepare a 2.5 x 7 cm Q-Sepharose column by equilibrating it with 20 mM of Tris-HCl at pH 8.5 and then washing it with three bed volumes of the same buffer.[9] Set the flow rate to 60 mL/hour and add the cell supernatant. Elute the proteins with a linear NaCl gradient from 0 to

0.5 M in the Tris-HCl buffer.[9] Determine fractionation using the UV-Vis spectrum of pure urate oxidase. Concentrate the fractions by slowly adding ammonium sulfate at 70% saturation while in an ice bath.[10] Let the mixture rest overnight and add the precipitate into a new Tris-HCl buffer. Prepare a 1.5 x 45 cm Sephadex G-50 column in the same manner as the previous column aside from the salt gradient and set the flow rate to 0.6 mL/min.[9] Precipitate and concentrate the urate oxidase once again. Note that it may be desirable to use a dye or protein marker to assist in visualizing the movement of the urate oxidase, but this is not necessary here.

7. Run a urate oxidase enzyme assay to determine the efficiency of the mutant urate oxidase

Biochemical materials required for this step:

- Horseradish Peroxidase
- Uric acid
- O-dianisidine
- Common buffer salts and solutions

Urate oxidase reacts with uric acid and produces hydrogen peroxide. This hydrogen peroxide production can be linked to the production of oxidized o-dianisidine by creating a coupled enzymatic assay with horseradish peroxidase and our purified urate oxidases. In a test tube add 10 µL of purified mutated enzyme and 10 mL of the same Tris-HCl buffer solution from step 6. Prepare a 10 mL reagent solution containing 10 mg uric acid, excess o-dianisidine (50 mg), and 10 µL of horseradish peroxidase concentrate. Make sure the pH does not decrease below 8.0, this is when urate oxidase activity is altered, and add the reagent solution to the urate oxidase solution. Make sure to cover reaction tubes as o-dianisidine reacts with light. After 120 seconds add 2 mL 2 M HCl to kill all enzymes and measure the absorbance at 446 nm. Repeat this procedure for 4 min, 6 min, 8 min, and 10 min. Repeat for the control protein and compare the quantity of o-dianisidine produced over time.

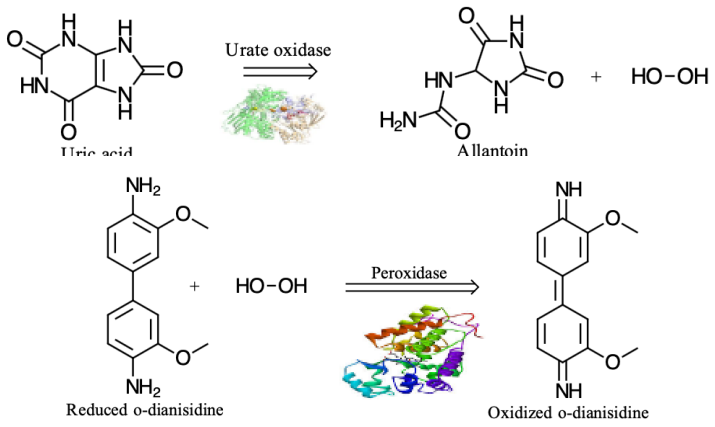


Figure 3
This figure displays the coupled enzymatic reaction where uric acid consumption is measured by the production of reduced o-dianisidine

Proposed Outcomes

It is likely that site directed mutagenesis at glycine-286 will result in loss of specificity of the enzyme. This means that very little if any enzyme functionality will remain after the point mutation. Glycine is often quite significant in that it is essential for its ability to fit in sharp corners of protein secondary structures. Even alanine, which is the next smallest amino acid, is unlikely to allow for the same flexibility in structure. A theoretical graph showing the absorbance readings from the coupled enzymatic assay is shown below.

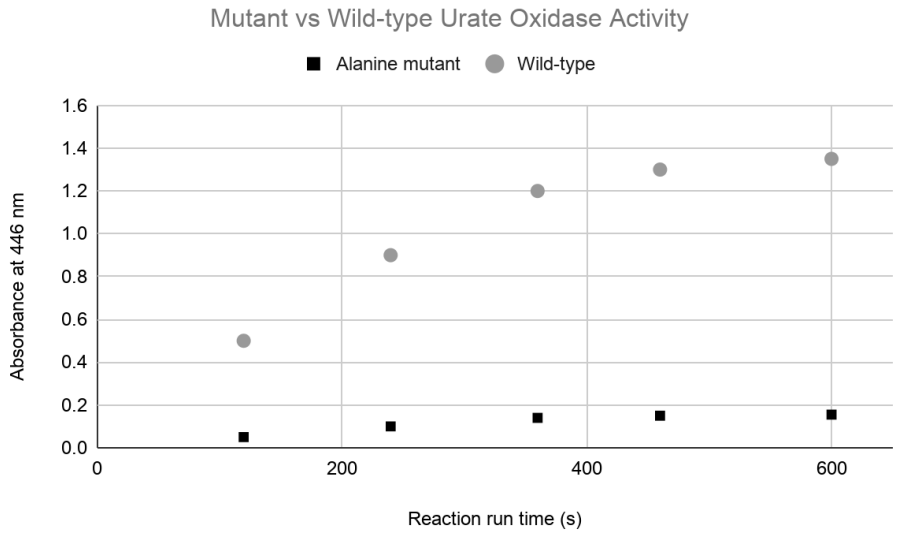


Figure 4
This figure is a reasonable theoretical comparison of wild-type and alanine mutant enzyme product formation over time. This is perhaps a liberal estimation because it is also very likely that the described mutation would lead to complete loss of enzyme activity.

Alternative Approaches

An alternative approach to these proposed methods could be to combine steps 2-4 such that amplification and mutation occur during the same step. This would require primers at the site of interest that change the codon as described above, but also allow for DNA polymerase to initiate near this site. This would be challenging because the gene of interest, pucL, is 1485 base pairs long and the amino acid is not particularly close to the beginning or the end of the sequence. Perhaps two primers complementary to each strand could be added to the codon site whilst marking the original strands for degradation.
The most promising alternative hypothesis involves mutagenesis of glycine-286 to serine-286 by altering the first guanine in the primer to adenine instead of altering the second nucleotide in the primer, as such: 5’-CGCCCGGCCATA**AGT**TTCCAACATTTT-3’
This is presumably the next most likely mutation to confer specificity with the wildtype protein because serine is the next most structurally similar amino acid to glycine. Serine does have a hydroxyl group that could lead to interactions with the nearby asparagine-285 or asparagine-254, either of which could alter the structure of the enzyme.

Experiment Materials and Budget

Biochemical materials:

- *Bacillus subtilis* ATCC 6633 for our source of urate oxidase
- Ribonuclease for RNA degradation
- Tris-saturated phenol for RNA degradation
- Chloroform for genomic DNA extraction
- Taq DNA polymerase for PCR amplification
- Deoxynucleoside triphosphates for PCR amplification
- 2 engineered primers: for PCR amplification
    - (5′-GCTCGAGGGCTTTCAGGCTCCGACAT-3′) (Reverse)
    - (5′-CGGATCCATGTTCACAATGGATGACCTG -3′) (Forward)
- ThermoPol buffer for PCR amplification
- pET-21a vector for ligation of urate oxidase gene
- XhoI restriction enzyme for separation of the cloning vector
- BamHI restriction enzyme for separation of the cloning vector
- T4 DNA Ligase for the recombination of the vector and DNA
- ATP solution for human DNA ligase enzyme activity
- Mutation primer: (5’-CGCCCGGCCATA**GCT**TTCCAACATTTT-3’) for desired mutation
- High Fidelity DNA polymerase for site directed mutagenesis
- 2 X One Shot BL21 Star™ (DE3) pLysS *E. coli* for expression of urate oxidase
- LB and SOC growth media for the growth of our *E. coli*
- Ampicillin for the extermination of non transformed *E. coli*
- IPTG for initiating the LAC operon and thus gene expression
- Q-Sepharose beads for ion exchange chromatography
- Sephadex G-50 beads for size exclusion chromatography
- Horseradish Peroxidase for our coupled enzymatic assay
- Uric acid for our coupled enzymatic assay
- O-dianisidine for our coupled enzymatic assay

Total biochemical materials cost = 26 x $100 = $2,600

Apparatuses:

- Centrifuge and centrifuge tubes for preparation and purification of DNA and proteins
- Vortex mixer for mixing DNA during preparation phase
- Freezer/Refrigerator for storing and incubating at low temperature
- Thermocycler and thermocycler tubes for performing PCR
- Hot plate for a constant high temperature water baths
- Sonicator for cell extraction
- 2.5 x 7 cm column for protein purification
- 1.5 x 45 cm column for protein purification
- UV-Vis spectrometer for final absorbance measurements
- Pumps capable of being programmed to produce a linear salt gradient for ion exchange chromatography

Total apparatus cost = 10 x $1,000 = $10,000

Total labor cost = wage x number of hours in lab = $13/hour x 25 hours = $325

Total cost of the experiment = cost of materials + cost of machines + cost of labor = $12,935

References

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