Investigations of an Amino Acid in the Active Site of MTHFS

Laboratory Manual for Biochemistry II

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Introduction: MTHFS

The enzyme 5,10-methenyltetrahydrofolate synthetase (MTHFS) is involved in regulation of folate pools. Folates shuttle one-carbon units in various oxidation states between molecules in biological systems. Folates are ubiquitous in eukaryotes and bacteria, but only occur in some archaea, which use other cofactors to shuttle one-carbon units.

As shown in Figure 1, MTHFS catalyzes the conversion of 5-formyltetrahydrofolate (also called 5-formylTHF or folinic acid) to 5,10-methenyltetrahydrofolate, coupled with conversion of ATP to ADP. This equilibrium is sufficiently far to the right (thanks to coupling of ATP hydrolysis) that the reverse reaction is not significant. 5-formyltetrahydrofolate is produced by a different enzyme, serine-hydroxymethyltransferase (SHMT), as a side reaction. 5-formyltetrahydrofolate is considered to be a "storage form" of folate, as it does not itself serve as a one-carbon donor or acceptor, but must be converted to a different form prior to use.

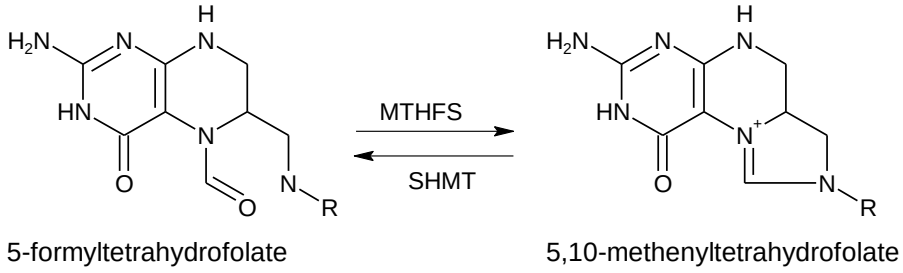


Figure 1. The reaction catalyzed by MTHFS.

The substrate of MTHFS, 5-formyltetrahydrofolate, is of interest because it is used in several pharmacological treatments, under the drug name leucovorin. As chemotherapy (anticancer) treatment, 5-formyltetrahydrofolate is administered along with fluorouracil. Fluorouracil is a mechanism-based inhibitor of thymidylate synthesis, ultimately blocking DNA synthesis. The 5-formyltetrahydrofolate serves to increase overall folate pools, increasing the cell's attempted biosynthesis of thymidine nucleotides (which requires a one-carbon unit from 5,10-methylene tetrahydrofolate), augmenting the effect of the 5-fluorocil. A second use of leucovorin is as a rescue agent after the administration of methotrexate. Methotrexate has toxic effects on fast-growing cells, making it useful against some cancers and immune system hyperactivity (as in rheumatoid arthritis). Methotrexate inhibits dihydrofolate reductase (DHFR) which converts folates (from dietary folic acid) to tetrahydrofolate. Thus, cells end up starved for folates. As a rescue agent, leucovorin (folinic acid) is administered to provide an alternate source of active folates, requiring the activity of MTHFS for this conversion.

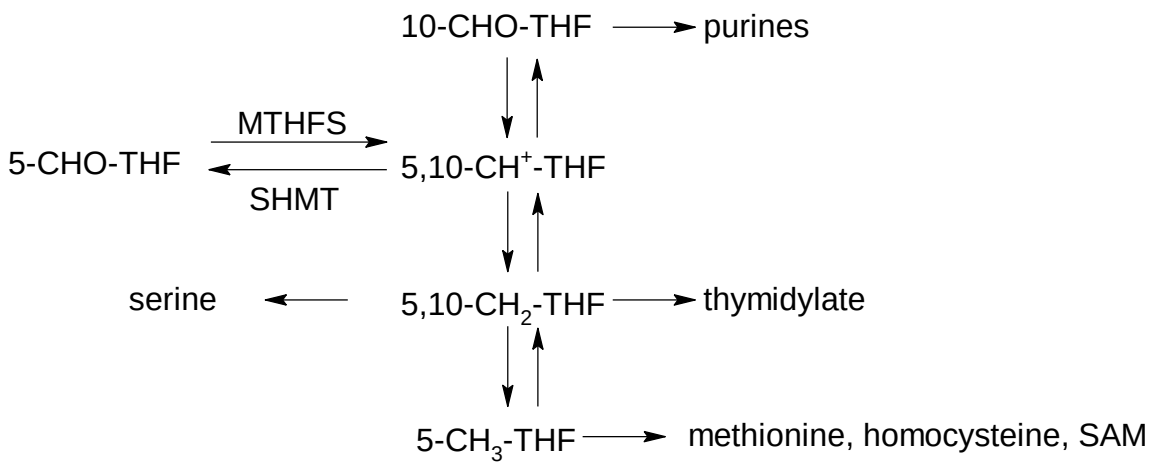


Figure 2. Various forms of tetrahydrofolate (THF) and their uses in the cell.

The overall goal for this semester-long laboratory project is to determine whether a specific amino acid residue at a position of your choosing in MTHFS is important for the binding of ATP. We will start by making a hypothesis about an amino acid known to be in the MTHFS binding site. We will create a protein in which the one amino acid of interest has been changed to alanine. Since the alanine sidechain is small, non-polar, and unable to participate in hydrogen bonding, alanine is a common choice for investigating the role of a single amino acid residue in a protein. Unfortunately, there is no easy way to take an existing protein sample and convert one of its amino acid residues to another residue. In principle, we could produce our altered protein by chemical synthesis, but that process is expensive and inefficient. Instead, new proteins are typically produced by changing the DNA that encodes them, and using an organism (or reconstituted cell-free system) to carry out transcription and translation for us. This process is typically more efficient than total synthesis, can produce far more protein than isolating the protein from a natural source, and can be used to produce mutant proteins, including our alanine-containing MTHFS mutant.

For our work, we will use *E. coli* (a common bacterium found in the human gut) to make our proteins. *E. coli* are easy to grow on a variety of media and grow relatively quickly (dividing every half hour or so), so it isn't hard to get a whole lot of them, and the methods of getting them to make foreign proteins are well-developed. To get *E. coli* to make a lot of desired protein (instead of just making their normal proteins, in sensible amounts), we provide them with a gene (a piece of DNA) that encodes our protein. Typically we use a circular piece of DNA, called a plasmid, to maintain a gene in *E. coli*. This plasmid doesn't just contain our gene; it also contains a gene that will give the *E. coli* resistance to an antibiotic. This gives us a way to select for only the *E. coli* that contain our plasmid. Those *E. coli* that contain our plasmid can grow in the presence of antibiotics, *E. coli* that have lost (or have not taken up) the plasmid cannot.

Thus, although you will start week 1 by looking at the protein crystal structure, you will be in “DNA land” for the following four weeks, as you alter the existing MTHFS gene to switch one codon from your amino acid of interest to alanine. Once you’ve made your mutation at the DNA level, you’ll put your modified gene sequence (on a plasmid) into *E. coli* bacterial cells, and persuade them to produce your mutant

MTHFS for you. Then you'll spend a few weeks isolating and purifying the protein, followed (finally!) by kinetic characterization of your mutant protein.

A note about thinking and labelling: As you work through the semester, be sure to stop and think about what's in each sample. Do you have DNA, or cells containing your DNA, or protein at each step? You'll want to pay attention to what you record in your notebook as well. You'll want to avoid telling your instructor that your DNA has activity or that your protein has been sequenced.

Week 1: Choose an amino acid and design primers

Purpose

To select an amino acid likely to be important for the binding of ATP in the active site of 5,10-methenyltetrahydrofolate synthetase (MTHFS), and to design primers to mutate the codon of that amino acid to the codon for alanine.

Introduction

The overall goal for this semester-long laboratory project is to determine which MTHFS amino acids are important for the binding of ATP. You will begin that project today by selecting an amino acid to study. In future weeks, you will change the MTHFS gene, insert that new gene into bacteria, produce and purify mutant enzyme, and finally study that enzyme using kinetics.

The ortholog (species-specific form) of MTHFS you will study this term comes from the *Mycoplasma pneumoniae*, the causative agent of walking pneumonia. This specific form of the enzyme has been crystalized in the presence of its substrates, and an x-ray crystal structure has solved for that crystal. This crystal structure is like a three dimensional molecular model, and you can use it to determine distances between amino acid side chains and ATP. You will use a program called PyMOL to display and manipulate the structure to measure these distances. In selecting an amino acid, please remember that most of chemistry can be explained through the attraction or repulsion of charges and the larger the charges, the larger the effect. The most important amino acids for binding are likely to be those that can hydrogen bond with or have ionic interactions with the ATP.

Once you have selected an amino acid to study, you will need to design primers to change the codon for that amino acid to the codon for adenine. Those primers will be relatively long and an exact match to the wild-type MTHFS gene except for one or two nucleotides. If we do not already have your primers in the freezer from a previous year, we will order them from a vendor, and they will be available next week.

Materials/Equipment

Laptop computers with PyMOL (v1.3)
Three button mice (highly recommended)
Internet access

Protocol

Learn how to use PyMOL and select an amino acid likely to contribute to the binding of ATP
Note that these directions are for Windows. If you are using a different operating system, you may need to modify them. You can ask your instructor for help, but remember that he or she is a biochemist, not a software engineer.

1. Open a browser and point it at www.rcsb.org. This will bring up the Protein Data Bank. This web site contains coordinates generated from tens of thousands of crystal structures of proteins.
2. In the search box at the top, type 1U3G. This is the code for a crystal structure of MTHFS bound to folinic acid, ADP, and phosphate. While ATP is a substrate for this enzyme, these crystals contain ADP and phosphate (the hydrolysis products). If we suppose that ADP and phosphate

bind where the ATP would, this cocrystal structure will give us a good idea what the enzyme might look like with the two substrates, ATP and folinic acid, bound.

3. A page will come up that gives details about the structure and references the publication in which it is described. At the right side of the page, click the triangle next to "Download Files".
4. This expands a list of formats that can be used to download the coordinates from the crystal structure. Click on "PDB Format". In Windows, this will show the file download on the bottom of your window. Click the caret (^) and select "Show in folder". Drag the file onto the desktop to make it easy to find.
5. Minimize your browser and open PyMOL using the start menu (Start>Program Files>PyMOL>PyMOL).
6. Two windows will open in PyMOL. One is labeled "The PyMOL Molecular Graphics System" (white) and the other is labeled "PyMOL Viewer" (black).
7. We will now load the coordinates file for the structure of MTHFS into PyMOL. In the white window, select File>Open. Use the window that opens to search for and select the 1U3G file (the MTHFS coordinates) that you downloaded in step 4.
8. The black window should now display a green stick representation of the crystal structure.
9. On the right side of the black window will be "all" and "1U3G" followed by the letters A, S, H, L, and C. Click "all" and "1U3G" such that "all" has a black background and "1U3G" has a grey background. This will display the "1U3G" data. The letters A, S, H, L, and C are menu boxes that correspond to action, show, hide, label, and color.
10. For fun, let's display MTHFS in a ribbon diagram. Click **H** (hide) and select **everything**. Click **S** (show) and select **cartoon**. Click **C** (color), then select **chain**, then select **chainbows**. Isn't that gorgeous? Remember that the spirals are alpha-helices and the arrows are beta strands (parts of beta-sheets).
11. This is a good place to practice rotating, moving, and zooming your structure. You can rotate by holding the left mouse button and dragging the mouse. You move by holding the middle mouse button and dragging and you zoom by holding the right mouse button and dragging. Play around with moving, zooming and rotating your structure.
12. There are multiple ways to display this protein. These are under the **S** button. Click **H** and select **everything** to clear this display. Next, click **S** and select a new view (spheres, mesh, ribbon, etc…) to see what it looks like. Repeat this process a few times to get a feel for what types of display are possible.
13. Ok, enough fun, let's get back to work. We need to find and interesting amino acid to study in MTHFS. The closer an amino acid is to the substrates (folinic acid, ADP, and P_i) in the active site, the more likely it will play an important role in the activity of the enzyme. Let's highlight each substrate in a different color to make it easier to see which amino acids are in close proximity. This will be easiest to do if we make separate groups for each molecule in the structure (MTHFS, folinic acid, ADP, and P_i).
14. Click on the S (select) at the bottom right corner of the black screen. This will bring up a scroll bar at the top of the black screen with the amino acid sequence for MTHFS as well as symbols for the other molecules in the structure. THF is folinic acid and MG is magnesium.
15. Select all the amino acids in MTHFS by highlighting by holding and dragging the mouse. You will have to release the mouse, move the scroll bar, and drag again several times to get the entire sequence highlighted. Note that a new gray bar "sele" for your selection has appeared at the upper right hand side of the screen. Once you have selected the entire protein sequence, rename this bar to "MTHFS" by clicking **A** (action), selecting **rename sequence**, and typing in MTHFS.

16. Repeat this process for the phosphate, the ADP, the folinic acid, and the Mg ion. You should now have made categories for the five separate molecules/ions in the structure.
17. Color and display each molecule how you like. However, you may find it easier to identify specific atoms if you color by element. You can modify a given molecule by clicking on its menu bar (A, H, S, L, and C). Play around with the structure and try to pick out an amino acid that you think is important for binding ATP in the active site. You know how to select this amino acid using the select button at the bottom right of the window. You may pick any amino acid you like (except R115 because it was the example in our prelab lecture). Come up with a hypothesis for how the amino acid binds the ATP. Be specific! Which atoms and what types of interactions are involved?
18. Once you have chosen your amino acid, you need to gather two types of data for your lab report. The first is you need to measure distances between relevant atoms in the structure. Next you should save some pictures of your structure that illustrate why you picked the given amino acid for study.
19. For your lab report, calculate the distances between the relevant atoms in your structure. For example, if you hypothesize that the positive charge on a lysine side chain holds the phosphate in place through an electrostatic interaction, then measure the distance between the closest hydrogen on the lysine side chain and the closest oxygen on the phosphate. This is the closest that you could expect the positive charge on the lysine and the negative charge on the oxygen to approach each other. The closer these two charges are to each other, the stronger the interaction. You can display hydrogens by typing "h_add" without the quotation marks on the command line at the bottom of the viewer (black) window.
20. To determine the distance between atoms, start by clicking the "selecting" text (green) in the lower right corner of the black window until it displays "atoms" in red. Color, display, and orient your structure so that you can clearly see the two atoms between which you would like to know the distance. Make sure that none of the atoms in your structure are selected. Then select the two atoms of interest by ctrl middle mouse clicking each. Then type "dist" into the dialog box in the white window and hit return. This will create a new object that will display as a line between your atoms with a number in the middle. The number is the distance between those atoms in angstroms. Your atoms should be within 5 angstroms of one another for the interactions to be strong enough to be relevant.
21. Develop a hypothesis for how your chosen amino acid helps to bind ATP in the active site of the enzyme. Be specific: what atoms are interacting? What molecules are those atoms a part of? What is the specific interaction (dipole-dipole, hydrogen bond, ionic, a combination of these) between the atoms of interest? You will need this specific hypothesis for your laboratory report.
22. Finally, save a few pictures for your lab report. Color, display, and orient your structure so as to best support your argument for choosing your amino acid. Save the picture by clicking file>save image as>PNG in the white window. Name and save your file to the desktop. Email it to yourself or grab it from a flash drive before you log out.

Design primers to change the codon for your amino acid to the codon for alanine

1. Find the codon for your amino acid in the gene sequence for MTHFS in the DNA sequence given in Appendix A1.
2. Use a codon table to determine how you will change the wild-type codon to the codon for alanine. Alanine has four codons. Choose the codon that is closest to the wild-type sequence.
3. You will be designing primers for the top and bottom strands. For each of these primers, start with the 3 nucleotides of the codon of interest and add 15 nucleotides (not codons) of the gene

sequence on to each end. If your selected amino acid is near the N or C terminal ends of the protein, you may need to include plasmid sequence as part of your primer. The entire plasmid, with the MTHFS gene in red, is given in Appendix A2.

4. Check the melting temperature of your sequences using the New England Biolabs Tm Calculator (http://tmcalculator.neb.com/#!/). You will use Phusion DNA polymerase next week with the High-Fidelity (HF buffer). The concentration of your primers will be roughly 230 nanomolar. You want your primers to have a melting temperature of at least 58°C. If your melting temperature is lower, add some more bases to one of the ends of your primer (or both).
5. Your final forward and reverse (top and bottom) primers should have the following characteristics.
    a. Both primers should be within one degree of each other in melting temperature.
    b. Primers should have a melting temperature of 58°C or higher.
    c. Primers should have two or more GCs at the 3' end if possible. This clamps down the ends since GC base pairs have 3 hydrogen bonds and AT base pairs only have 2. This is especially important at the 3' end of the primer because that is where DNA polymerase will start. If you do not have two or more G or C nucleotides at the 3' end of a primer, try subtracting or adding a few nucleotides until you do. Make sure to recalculate the melting temperature of any primers you modify.
6. When you think you have your primers designed, have them approved by your instructor. Then send your instructor an electronic copy of the primer sequences, your intended mutation, and the calculated melting temperatures so that he or she can order them if necessary.

Final sample check and cleanup

You don't have samples yet. Make sure to record all data in your notebook including primer sequences, melting temperatures, amino acid to be changed, your hypothesis, and important distances you measured. Place the file containing the picture of your amino acid interacting with ATP in a safe place. You will need it for your next lab report and for the last lab report of the semester.

You will be expected to keep a notebook in the format given in Appendix B for the remainder of the semester.

Week 2: Changing the codon for your chosen amino acid to the codon for alanine

Purpose

To create a mutant plasmid in which the codon for your selected amino acid is changed to alanine.

Introduction

Last week, you used a cocrystal structure of 5,10-methenyltetrahydrofolate synthetase (MTHFS) and a program called PyMOL to select an amino acid that you hypothesized to be important in the binding of ATP. You also designed primers to change the codon of your selected amino acid to the codon for alanine. This week, you will carry out a reaction to make that change as well as a positive control reaction. We have never observed a faulty negative control reaction for this procedure, so we will omit it to save the cost of reagents this week and gel space next week.

The technique you will use this week is called site-directed mutagenesis. If successful, it will produce a new plasmid where one or two base pairs have been changed in the MTHFS gene relative to wild type. The technique requires a plasmid containing the wild type MTHFS gene (the template), the primers you designed last week, deoxynucleoside triphosphates, a DNA polymerase, and a buffer to keep the DNA polymerase active. The primers you designed contain the mutated codon and they will become part of the new plasmid. This is how the codon is changed in the products.

For those of you who have previously carried out PCR, this process will seem familiar, and in fact, it uses the same reagents and equipment. However, this technique does not involve actual PCR. In PCR, the products of one cycle can serve as templates for the next cycle; this is why products increase geometrically with cycle count in PCR. In this site-directed mutagenesis protocol, only the original, wild-type plasmid can serve as the template in each cycle. Thus, products increase linearly with cycle count. The mechanism behind this is fascinating, but it is beyond the scope of this laboratory. If you are interested in an explanation, please ask your instructor.

As the product of your site-directed mutagenesis reaction should have only one or two nucleotides changed, it should be the same size as the wild-type MTHFS plasmid (5841 bp). This reaction can be tricky; it doesn't always work for students. Make sure that you are very careful in your pipetting and that you do not forget to add any reagents. To help evaluate the cause of any reaction failures, you will also run a positive control. This control uses the wild-type MTHFS plasmid as a template, but its primers are designed to amplify only a portion of that plasmid. This reaction is PCR, produces a 1000 bp product, and it is very reliable. When troubleshooting, its success eliminates many possible sources of error.

After your reactions are complete, you will have two types of plasmid DNA in your tube. One will be the original wild-type template, and the other will contain a mutated MTHFS gene. Next week, you will transform bacteria with your products. The wild-type template is supercoiled and transforms with much greater efficiency than your mutated plasmid, which will be linear. If you do not remove the wild-type template, most of your transformed bacteria will contain the wild-type MTHFS gene instead of the mutation. To eliminate this possibility, you will digest the template plasmid using an enzyme called DpnI. This enzyme will hydrolyze DNA that is methylated. Many bacteria methylate their DNA shortly after replication as part of mismatch repair; we will learn more about this in lecture in the last third of the term. Since your wild-type template was isolated from bacteria, it will have methyl groups. Your site-directed mutagenesis reaction did not contain the necessary enzymes to add methyl groups, so your

mutated plasmid products have none. Thus DpnI will digest your wild-type template while not affecting your mutated plasmid products.

Materials/Equipment

Microcentrifuge tubes (sterile)
Microcentrifuge racks
Microcentrifuge
Adjustable pipettes (10 μL, 200 μL, 1000 μL)
Pipette tips (sterile)
200 μL PCR tubes (or whatever size fits your thermocycler)
Ice baths
Phusion DNA polymerase (2U/μL)
5 x Phusion DNA polymerase buffer (HF)
Forward and reverse primers specific to each student's mutation (1 μg/μL)
Control forward and reverse primers (1 μg/μL)
Plasmid template containing the wild-type MTHFS gene (25 ng/μL)
10 mM dNTP mix (2.5 mM each dNTP, CAS numbers: 74299-50-6, 3770-58-9, 3624-46-2, 74299-51-7)
Sterile, deionized water
DpnI (20 U/μL)
Thermocycler

Safety

There are multiple hazards associated with this laboratory. Students are required to wear goggles, gloves, and lab coats for all experiments. Long hair must also always be tied back. PCR reagents may cause skin or eye irritation. The thermocycler lid and heating block will be hot enough to cause burns while the program is running.

Protocol

Site-directed mutagenesis

1. Prepare two reactions in the 200 μL PCR tubes. One reaction will use primers specific to your mutation and the other reaction will use the control primers (positive control). When working on your calculations, remember that you cannot accurately pipette less than 1 μL. You may need to dilute a stock for accurate pipetting. Make sure you label your tubes before starting!
    - X μL of 5x reaction buffer (where X is the amount that will make it 1x in the final volume)
    - 25 ng of wild-type MTHFS plasmid template (stock is 25 ng/μL)
    - 125 ng of the forward primer (stock is 1 μg/μL)
    - 125 ng of the reverse primer (stock is 1 μg/μL)
    - 1 μL of 10 mM dNTP mix (2.5 mM each dNTP)
    - Enough sterile, deionized water to make a 50 μL reaction
2. Vortex or flick vigorously with a finger to mix. Centrifuge the tube briefly at the highest setting to bring all liquid to the bottom of the tube.
3. Your instructor will add the polymerase: 0.5 μL of Phusion DNA polymerase.
4. Mix and centrifuge again.
5. Place in thermocycler.

Once all students have added their samples to the thermocycler, your instructor will start it. The program is as follows:

98°C for 30 sec (once)

98°C for 30 sec 55°C for 1 min 72°C for 12 min	These three steps are repeated 16 times

72°C for 12 min (once)

This program will take much longer than the laboratory period. Your instructor will remove the reactions and place them in the freezer later tonight or early tomorrow morning.

DpnI digest

1. Sometime before the next laboratory period, pull your reactions out of the freezer and thaw them.
2. Remove the DpnI tube from the freezer and keep it on ice. Add 0.5 μL DpnI to each of your reaction tubes. Pipette directly into the thawed reaction mixture to make sure the enzyme is not on the side of your pipette tip. Put the DpnI tube back in the freezer.
3. Mix and centrifuge the tubes
4. Incubate the reactions at 37°C for 1-3 hours depending on what is convenient for your schedule.
5. Place the reactions back in the freezer.

Final sample check and cleanup

At the end of this experiment, you should have two tubes in the Biochemistry II box in the freezer: Your site-directed mutagenesis reaction and the positive control reaction. All leftover materials may be disposed of in the sink or wastepaper baskets as appropriate. As instructed above, the stock tube of DpnI should be placed back in the freezer immediately after use.

Week 3: Transformation of bacteria with mutated plasmid DNA

Purpose

To put the plasmid DNA you modified last week into bacteria. You will also run an agarose gel to verify that your site-directed mutagenesis reaction was successful.

Introduction

Last week, you attempted to change the codon for an amino acid of interest to the codon for alanine using a technique called site-directed mutagenesis. That codon was part of the 5,10-methenyltetrahydrofolate synthetase (MTHFS) gene that is part of a plasmid. A successful site-directed mutagenesis reaction will have produced a linear double-stranded DNA product from that plasmid. Today, you will transform that "linear plasmid" into a bacterial strain that is well suited to take up, repair, and replicate plasmid DNA. XL1-Blue is a strain of *E. coli* that satisfies these criteria; it is deficient in endonucleases (enzymes that cut DNA) and enzymes involved in DNA recombination. Mutations and deletions of these enzymes help to prevent the fragmentation of your plasmid DNA and the removal or addition of DNA sequences. Once the XL1-Blue strain has been transformed with your DNA, the bacteria will ligate the ends of your "linear plasmid" to produce a circular plasmid that can be replicated in the cell. You will use these transformed bacteria to "grow" a relatively large amount of mutated plasmid DNA next week.

The XL1-Blue cells you will transform have been made *competent*, which means that they can take up foreign DNA, by a process that involves slow growth at room temperature followed by washes with calcium chloride. You will use a heat-shock protocol to get these cells to take up your plasmid. How this process works at the cellular level is not well understood; it has been developed empirically. Since you don't know the mechanism of transformation, you need to follow the directions exactly since you cannot predict the effects of changes to the protocol.

Once you have transformed the competent cells with your plasmid, you will apply them to LB-agarose plates to grow overnight. In addition to the MTHFS gene, your plasmid contains a gene that codes for an enzyme that makes the bacteria resistant to the antibiotic kanamycin (see Appendix A3 for a plasmid map). More specifically, the gene codes for neomycin phosphotransferase II, an enzyme that will phosphorylate kanamycin and inactivate it. You only want bacteria that have taken up your plasmid to grow on the LB-agarose plates. To select for these bacteria, kanamycin has been added to those plates. Only bacteria that have been transformed with your plasmid will have the kanamycin resistance gene, and only these bacteria will be able to grow on the plates. Other bacteria will be killed by the kanamycin.

Good scientists validate their work whenever it is feasible. In addition to transforming competent cells, you will also run an agarose gel to evaluate the success of your site-directed mutagenesis reactions last week. While you used a PCR-like protocol, successful site-directed mutagenesis amplifies DNA in a linear manner, not geometrically like real PCR. This makes it difficult to detect your products by gel electrophoresis. However, there is a side product in site-directed mutagenesis that is amplified geometrically. There is a very strong correlation between this side product and successful site-directed mutagenesis. This product is similar in size to your linearized plasmid, and it is what you will be looking for on the agarose gel.

Last semester, we discussed the theory behind agarose gels, and you ran one. Please refer to the lecture notes (they are still posted) for a molecular description of how agarose gel electrophoresis

works. Briefly, DNA has two negative charges for each base pair. An electric field is applied across an agarose gel that has been loaded with DNA samples. Being highly negatively charged, the DNA moves towards the positively charged electrode. This movement is through a "mesh" of agarose. Larger molecules take more time to navigate this "mesh" than smaller molecules; this results in larger molecules moving more slowly than smaller molecules. We detect the DNA in the gels through the use of a molecule that fluoresces when it is bound to DNA. This compound, SYBR Safe, was added to the gels when they were poured.

Materials/Equipment

14 mL Falcon tubes (Corning, REF 35059, sterile, 3 per student)
Microcentrifuge tubes
Microcentrifuge racks
Microcentrifuge
9" glass Pasteur pipettes
Bunsen burner
Sparker/lighter
Ice bath
37 degree Celsius shaker bath
42 degree Celsius bath
37 degree Celsius incubator
Competent XL1-Blue cells prepared using the method of Inoue (200 μL per student)
Test tubes with LB media (sterile, 3 mL per student)
LB agar plates containing 50 μg/mL kanamycin (3 per student)
Agarose gels (5 students per gel, assumes 13 well combs, SYBR Safe)
Agarose gel systems
Power supplies
Trans-illuminator
Gel loading dye (6x)
DNA ladder (2-log ladder or similar)
1x TAE buffer (made with DI/RO water)

Safety

There are multiple hazards associated with this laboratory. Students are required to wear goggles, gloves, and lab coats for all experiments. Long hair must also always be tied back.

Physical dangers include Bunsen burners that are used to sterilize glass and inoculating loops at several points and the risk of electric shock during electrophoresis.

The lab strains of *E. coli* in use in these experiments are non-pathogenic, but we will reduce the risk of spreading them or their antibiotic resistance plasmids by treating any materials that contact cells either with bleach or by autoclaving.

The agarose gels have SYBR Safe stain already incorporated in them. SYBR Safe DNA gel stain is flammable and contains components that may be absorbed through the skin.

Trans-illuminators shine high energy blue or UV light on agarose gels, causing the stain in them to fluoresce. You should not look directly at a blue light trans-illuminator without a filter, and you should avoid both eye and skin exposure to light from a UV trans-illuminator.

Protocol

Transformation of competent XL1-Blue cells.
Adapted from Inoue H., Nojima H., Okayama H. Gene. 1990; 96(1): 23-8.

1. Label three 14mL Falcon tubes and place them on ice to chill for a couple minutes. Then aliquot 50 μL of freshly thawed (but not warmed) competent cells to each Falcon tube, being careful to deliver the cells to the bottom of the tube.
2. Add 2μL of the transforming DNA (from last week's lab) to the competent cells in the Falcon tubes. Swirl the tubes gently several times to mix their contents. Set up a positive control for each transformation experiment (a tube of competent bacteria that receives 1μL of supercoiled plasmid with a kanamycin resistance gene). Also set up a negative control where you add 2μL of your DpnI-treated positive control from last week.* Store the tubes on ice for 30 minutes. While these samples incubate, prepare the samples for your gel and load them (protocol below). You may incubate these cells on ice for more than 30 minutes if necessary.

   *Your positive control from last week should have produced a 1000 base pair fragment of DNA that does not contain a gene for kanamycin resistance. This fragment was made from a plasmid template that did contain the kanamycin gene; however, the primers were designed to avoid this gene. This is a control for your sterile technique and for whether or not your DpnI digest destroyed the plasmid template for this control. If everything has works correctly, would you expect to have colonies on the agar plate associated with this control? If the opposite of your predicted result occurs, how would you explain it?

3. Transfer the tubes to a rack placed in a preheated 42°C circulating water bath. Store the tubes in the rack for exactly 90 seconds. Do not shake the tubes. Heat shock is a crucial step. It is very important that the cells be raised to exactly the right temperature at the correct rate. The incubation times and temperatures given here have been worked out using Falcon tubes. Other types of tubes will not necessarily yield equivalent results.
4. Rapidly transfer the tubes to an ice bath. Allow the cells to cool for 1-2 minutes.
5. Add 800 μL of LB medium to each tube. Transfer the tubes to a shaking incubator set at 37°C. Incubate the cultures for 45 minutes to allow the bacteria to recover and to express antibiotic resistance encoded by the plasmid. To maximize the efficiency of transformation, gently agitate (<225 cycles/minute) the cells during the recovery period.
6. Transfer the appropriate volume (200 μL for a full plate) of transformed competent cells onto LB agar plates containing the appropriate antibiotic. Use a Bunsen burner to sterilize and bend a Pasteur pipet. When the pipet has cooled to room temperature, spread the transformed cells gently over the surface of the agar plate. Repeat with both of your control transformations.
7. Store the plates at room temperature until the liquid has been absorbed.
8. Invert the plates and place them in the incubator at 37°C. Transformed colonies should appear in 12-16 hours.

The next day

9. You or a lab colleague need to come in and move your plates to the refrigerator. It doesn't matter what time as long as it is the next day.

Agarose gel electrophoresis

1. Through agarose gel electrophoresis, you will be analyzing your site-directed mutagenesis reaction and your positive control from last week. Add 10 μL of each to a separate, appropriately labeled microcentrifuge tube. Add 2 μL of 6x gel loading buffer to each tube, pipetting directly into the previously added sample. Vortex the samples briefly, and then centrifuge them briefly at the highest setting to bring all the liquid to the bottom of the tube.
2. Carefully load 10 μL of each gel sample onto an agarose gel. Make sure to leave the leftmost lane open for a molecular weight marker, and load your samples in adjacent lanes. Indicate the locations of the samples in your notebook.
3. If you are the last one to load a gel, alert your instructor so he or she can start it. The gel will be run at 100 volts.
4. Near the end of the lab period, your instructor will take you to the gel imaging system to discuss and image the gel. Make sure to write down the number of the photo; it will be uploaded to the website.

Final sample check and cleanup

At the end of the laboratory, you should have placed three plates (inverted) in the 37 degree Celsius incubator. Place the tube containing remaining site-directed mutagenesis sample (not the blue gel sample) in the Biochemistry II box in the -20 degree Celsius freezer. All items that touched bacteria should be placed in the autoclave buckets on the bench, except the bent Pasteur pipettes you used to spread cells on plates. These should be put in a large beaker with bleach. Your instructor will add them to the broken glass box later. All other materials can be poured down the drain or placed in the wastebasket as appropriate.

Week 4: Isolation of mutated plasmid DNA from bacteria

Purpose

To isolate the plasmid DNA containing your 5,10-methenyltetrahydrofolate (MTHFS) gene from the *E.coli* strain XL1-blue. The isolated plasmid will be sent out for DNA sequencing to verify that the codon of interest has been mutated to the codon for alanine.

Introduction

Last week, you transformed the products of a site-directed mutagenesis reaction into a bacterial cell line that is good at repairing and producing DNA. You can think about those bacteria as little factories, and this week, you will grow them to produce a relatively large amount of plasmid DNA. If your reactions were successful last week, then the plasmid you isolate will have the codon of interest mutated to the codon for alanine.

The process of isolating plasmid DNA is commonplace, and there are many vendors who sell kits. These kits provide all the reagents needed to lyse the bacteria and purify the plasmid DNA from the various other components of the cell. Bacteria are generally lysed with a combination of a detergent and base. Acid is then added to neutralize the base. This changes the charge on many of components of the cells, and they precipitate out of solution; the plasmid DNA remains in the supernatant at this step.

The supernatant is then applied to a silica column (it looks more like a filter) in the presence of chaotropic salts, salts that cause DNA to denature; under these conditions, the DNA binds tightly to the column. The salts and other cellular materials are washed away through the use an ethanol containing solution; DNA is not soluble in ethanol. Finally, the plasmid DNA is eluted through the use of an aqueous buffer.

You will quantitate the concentration of plasmid DNA through the use of UV/visible spectroscopy. The nitrogenous bases of DNA are aromatic, and as a group, they have a maximum absorbance near 260 nm. For most quantitations, biochemists assume that an absorbance of 1 at 260nm results from a double-stranded DNA concentration of 50 μg/mL. You can use this as a conversion factor (1 Abs = 50 μg/mL) when you calculate your DNA concentration at the end of this laboratory exercise.

Materials/Equipment

Microcentrifuge tubes (sterile)
Microcentrifuge racks
Microcentrifuge
Adjustable pipettes (10 μL, 200 μL, 1000 μL)
Pipette tips (sterile)
Heat block set to 90°C
Plasmid purification kit (Wizard Plus SV Minipreps DNA Purification System from Promega)
NanoDrop UV/visible spectrometer
Vacuum concentrator
Sterile 90°C water
Test tubes with 10 mL LB media (one per student)
Kanamycin stock solution (50 mg/mL, 10 μL per student, CAS 3847-27-6)
Inoculation loop
Bunsen burner and striker

Bleach (commercial, 5%)

Safety

Kanamycin monosulfate is a reproductive toxin.

Bleach is a skin irritant and causes serious eye damage. It will also bleach your clothing.

The DNA isolation kit contains components that are allergens, reproductive toxins, skin and respiratory irritants, damaging to the eyes, and are toxic upon ingestion.

Protocol

The day before our normal laboratory meeting

1. Sterilize your work bench with disinfectant, and set up a Bunsen burner.
2. Remove your mutant plasmid transformation plate from the refrigerator and place it on your sterile work space.
3. Appropriately label a test tube containing 10 mL of LB media.
4. Using sterile technique, add 10 μL of kanamycin stock solution to the test tube.
5. Using sterile technique, transfer a colony from your plate to the 10 mL of LB media.
    a. Flame the inoculation loop.
    b. Remove the cover on your test tube and flame the test tube lip.
    c. Touch your inoculation loop to a blank space on your plate to make sure it is not too hot to pick up a colony.
    d. Scrape off a colony from your plate.
    e. Place the end of the loop in the test tube and gently bang it around.
    f. Place the top back on your test tube.
    g. Flame the inoculation loop to sterilize it.
6. Place the test tube in the 37°C shaker bath with rapid shaking. Double check the bath is shaking when you leave the room.
7. Place your agar plate back in the refrigerator.

During the laboratory period (adapted from Promega protocol FB004)
Lysing cells and separating the plasmid containing supernatant

1. Remove your overnight culture from the shaker bath.
2. Using sterile technique, pipette 1.5 mL of overnight culture into each of two microcentrifuge tubes (3 mL total between the tubes).
3. Centrifuge both tubes on the highest setting for five minutes.
4. Discard the supernatant from each tube using first a 1000 μL pipette to get most of it, followed by a 100 μL pipette to get the last bit. You will have better control with the smaller pipette. Discard supernatants into a small beaker with bleach.
5. Resuspend both pellets in the same 250 μL of Cell Resuspension Solution from the kit. To accomplish this, resuspend the first pellet in 250 μL of Cell Resuspension Solution, and then pipette that 250 μL (with bacteria in it) onto the second pellet and resuspend it. You may use the vortex at this step. <u>DO NOT</u> resuspend each pellet in a separate aliquot of 250 μL.
6. Add 250 μL of Cell Lysis Solution to your resuspended cells. Invert the sample 4 times to mix. DO NOT vortex or roughly mix as this will break the genomic DNA into small pieces. It is easy to separate large genomic DNA from small plasmids; it is not easy to separate small pieces of genomic DNA from small plasmids.

7. Add 10 μL of the Alkaline Protease Solution. Invert 4 times, and incubate at room temperature for 5 minutes.
8. Add 350 μL of the Neutralization Solution, and invert 4 times.
9. Centrifuge on the highest setting for 10 minutes.

Binding plasmid DNA to the spin columns

10. Insert the spin column into a 2 mL collection tube (no cap).
11. Pipette the supernatant from step 9 into the spin column. Take care to not draw any solid into the pipette.
12. Centrifuge at the highest setting for 1 minute. Discard the liquid in the collection tube down the drain.

Washing the plasmid DNA that is now bound to the spin column

13. Add 750 μL of Wash Solution to the spin column. Centrifuge on the highest setting for 1 minute. Discard the liquid in the collection tube down the drain.
14. Add 250 μL of Wash Solution to the spin column. Centrifuge on the highest setting for 1 minute. Discard the liquid in the collection tube down the drain.
15. To remove any remaining Wash Solution, centrifuge on the highest setting for 2 minutes.

Elute the bound DNA from the spin column

16. Transfer the spin column to a sterile 1.5 mL microcentrifuge tube to collect your elution.
17. Add 100 μL of sterile, 90^{o}C water to the center of the spin column.
18. Centrifuge on the highest setting for 1 minute.
19. Briefly vortex the solution that elutes from the spin column to ensure it is homogenous. Label the tube with your mutation (example: E55A), your initials, and DNA.

Quantitate the concentration of your plasmid DNA

20. Use the NanoDrop UV/visible spectrometer to determine the absorbance of your plasmid DNA sample at 260 nm.
21. Use the conversion factor (1 absorbance unit = 50 μg/mL double stranded DNA) and your absorbance at 260 nm to calculate the concentration of your DNA in μg/mL.

Prepare a sample of your plasmid to be sent off for DNA sequencing

22. You will send 1 μg of plasmid DNA for sequencing. Determine the volume of DNA you must send using the concentration calculated in step 21 above.
23. Pipette that volume into a 200 μL PCR tube. If you do not have enough DNA, pipette all but 10 μL into the PCR tube. Be certain you are not shipping out all of your plasmid DNA. Look at the signup sheet provided, and pick a letter that hasn't been used. Label your tube with that letter, and fill out the rest of the signup sheet with your mutation, your name, and the concentration of your DNA.
24. Place your labeled, PCR tube in the rack provided. Your instructor will dry these samples and mail them for sequencing.

Final sample check and cleanup

At the end of the laboratory, you should have placed an appropriately-labeled PCR tube with sample plasmid in the rack to be sent out for sequencing. Your remaining plasmid DNA should be placed in the Biochemistry II box in the -20 degree Celsius freezer for next week's experiment. Add an equal volume

of bleach to the remains of your overnight culture and let it sit for a minute or two. Then pour it down the drain along with all other bleached samples. All other materials can be poured down the drain or placed in the wastebasket as appropriate.

Week 5: Analysis of DNA sequencing data and transformation of bacteria with mutant DNA

Purpose

To verify that your site-directed mutagenesis reactions mutated the MTHFS gene and to transform a bacterial strain that is good at making proteins with that gene.

Introduction

When carrying out a series of experiments, scientists don't just assume their reactions worked; they verify them as often as possible. Last week, you prepared DNA samples to sequenced by a vendor. This week you will analyze the results to see if your site-directed mutagenesis reaction from Week 2 was successful. If it was, then the codon from your chosen amino acid will be changed to the codon for alanine.

Once you have verified that you successfully mutated the wild type MTHFS gene, you will transform the plasmid containing it into a bacterium that is good at producing proteins, the *E. coli* strain BL21 (DE3). This strain is missing a protease that can hydrolyze foreign proteins (Lon protease). It also lacks a membrane protease that degrades proteins outside the cell (OmpT). If they were to be present, either of these proteases could lead to reduced protein yields.

In addition to missing proteases, BL21 (DE3) has had the gene for T7 RNA polymerase inserted into its genome (from λDE3 phage). This polymerase recognizes the T7 promoter in the lac operon on your MTHFS plasmid and is necessary for the transcription of your mutant gene. See Appendix A3 for a map of the plasmid containing your mutant gene.

Finally, our particular BL21 (DE3) cells have been transformed with a helper plasmid that contains the genes for several tRNAs that are rare in *E. coli*. While almost all organisms use the same codon table, most amino acids have more than one codon, and each organism uses specific codons to different extents. Specific to our situation, *M. pneumoniae*, the organism that your wild type MTHFS came from, uses particular arginine and isoleucine codons that are rarely used in *E. coli*. Since these codons are rare in *E. coli*, it does not naturally produce the corresponding tRNAs in abundance. To compensate for this, our BL21 (DE3) cells contain the helper plasmid pSJS1244 that has the genes to express these two tRNAs. To select for bacteria that contain this plasmid, it also contains a gene for spectinomycin resistance. This is why the agar plates will contain spectinomycin in addition to kanamycin for this transformation.

Materials/Equipment

14 mL Falcon tubes (Corning, REF 35059, sterile, 3 per student)
Microcentrifuge tubes
Microcentrifuge racks
Microcentrifuge
9" glass Pasteur pipettes
Bunsen burner
Sparker/lighter
Ice bath
37 degree Celsius shaker bath
42 degree Celsius bath
37 degree Celsius incubator

Competent Rosetta 2 (DE3) or BL21 (DE3)/pSJS1244 cells prepared using the method of Inoue (200 μL per student)
Test tubes with LB media (sterile, 3 mL per student)
LB agar plates containing 50 μg/mL kanamycin (CAS 3847-27-6) and 30 μg/mL spectinomycin (CAS 1695-77-8) (3 per student)
Files from DNA sequencing

Safety

There are no new hazards this week. As always, wear googles, a lab coat, and gloves. Tie long hair back and take care not to burn yourself with the Bunsen burner or on hot glass. Continue to work carefully when handling bacterial cells, bleach, and kanamycin.

Protocol

Analysis of DNA sequencing

1. Download and install Chromas Lite (the free one). http://www.technelysium.com.au/chromas_lite.html
2. Open the chromatogram file of interest in Chromas Lite.
3. Scroll to the region of the chromatogram that contains the codon of interest. Use the MTHFS amino acid and nucleotide sequence (Appendix A1) for reference. Note that sequencing did not start at the beginning of the MTHFS gene; the numbers in Appendix A1 will not match the numbers on the chromatogram. Look for DNA sequence matching the nucleotide sequence given in A1, then follow it to find the codon you attempted to change.
4. Take a screen shot of the region of interest (print screen on a PC).
5. Paste the screen shot into another program (such as PowerPoint), and clearly label the codon that has been changed.
6. Discuss the results with your instructor before proceeding to the transformation.

Transformation of competent cells.

Adapted from Inoue H., Nojima H., Okayama H. Gene. 1990; 96(1): 23-8.

10. Add the transforming DNA (2 μL to 50 μL of competent cells) to the competent cells in chilled Falcon tubes. Swirl the tubes gently several times to mix their contents. Set up a positive control for each transformation experiment (a tube of competent bacteria that receives 1 microliter of supercoiled plasmid with a kanamycin resistance gene). Also set up a negative control in which you add 2 microliters of water instead of DNA. Store the tubes on ice for 30 minutes.
11. Transfer the tubes to a rack placed in a preheated 42°C circulating water bath. Store the tubes in the rack for exactly 90 seconds. Do not shake the tubes. Heat shock is a crucial step. It is very important that the cells be raised to exactly the right temperature at the correct rate. The incubation times and temperatures given here have been worked out using Falcon tubes. Other types of tubes will not necessarily yield equivalent results.
12. Rapidly transfer the tubes to an ice bath. Allow the cells to cool for 1-2 minutes.
13. Add 800 μL of LB medium to each tube. Transfer the tubes to a shaking incubator set at 37°C. Incubate the cultures for 45 minutes to allow the bacteria to recover and to express antibiotic resistance encoded by the plasmid. To maximize the efficiency of transformation, gently agitate (<225 cycles/minute) the cells during the recovery period.
14. Transfer the appropriate volume (200 μL for a full plate) of transformed competent cells onto LB agar containing both kanamycin and spectinomycin antibiotics. Use a Bunsen burner to sterilize and bend a Pasteur pipet. When the pipet has cooled to room temperature, spread the

transformed cells gently over the surface of the agar plate. Repeat with both of your control transformations.

15. Store the plates at room temperature until the liquid has been absorbed.
16. Invert the plates and place them in the incubator at 37°C. Transformed colonies should appear in 12-16 hours.

The next day

17. You or a lab colleague need to come in and move your plates to the refrigerator. It doesn't matter what time as long as it is the next day.

Final sample check and cleanup

At the end of the laboratory, you should have placed three plates (inverted) in the 37 degree Celsius incubator. Place the tube containing remaining plasmid with your mutant MTHFS gene in the Biochemistry II box in the -20 degree Celsius freezer. All items that touched bacteria should be placed in the autoclave buckets on the bench, except the bent Pasteur pipettes you used to spread cells on plates. These should be put in a large beaker with bleach. Your instructor will add them to the broken glass box later. All other materials can be poured down the drain or placed in the wastebasket as appropriate.

Week 6: Induction of protein expression

Purpose

To overexpress your mutant MTHFS gene in the *E. coli* host cells, making mutant protein in much larger than normal amounts.

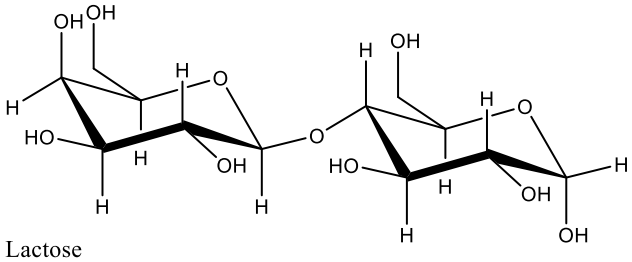
Introduction

With the advent of molecular cloning, it has become possible to copy a gene sequence from one organism and place it in another. This is very useful to biochemists because it makes it far easier for us to obtain specific proteins in large quantities. Our specific ortholog (species specific gene) of 5,10-methenyltetrahydrofolate synthetase (MTHFS) comes from *Mycoplasma pneumoniae*, the bacterium that causes walking pneumonia. It is undesirable to use this bacterium to produce mutants of MTHFS because it could make researchers sick, and because it is very slow growing. Fortunately for us, the Kim lab at Berkeley [Chen, S.; Shin, D.-H.; Pufan, R.; Kim, R.; Kim, S.-H. Proteins: Struct., Funct. Bioinf. 2004, 56 (4), 839-843.] has used molecular cloning techniques to copy the MTHFS gene from *M. pneumoniae* and place it in the plasmid you were given in Week 2. You have since modified that gene to change the codon for an amino acid to the codon for alanine, and last week, you transformed that new plasmid into the *E. coli* strain BL21(DE3)/pSJS1244. This strain has been modified to optimize it for expressing large amounts of protein.

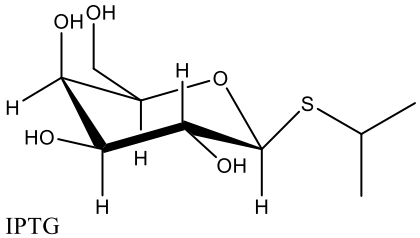
The expression of mutant MTHFS gene in your plasmid is under the control of the lac promoter (part of the lac operon). As we have discussed in class, genes regulated by the lac promoter are expressed when lactose is present. Lactose binds to the lac repressor (a protein). The lac repressor changes shape and falls off the lac promoter (a DNA sequence). The lac promoter is then unobstructed, and the genes of interest are then transcribed by T7 RNA polymerase.

Lactose is consumed by bacteria; in the laboratory, we would need to add it in the middle of the night to keep our BL21(DE3) bacteria expressing MTHFS. To avoid this, we use a lactose mimic (similar shape and charge distribution as lactose) to turn the lac promoter on. Our mimic is isopropyl β-D-1-thiogalactopyranoside (IPTG). As shown in the figure below, this molecule “looks like” lactose, but cannot be metabolized by the bacteria; thus we only need to add it once.

We cannot, however, add IPTG to our bacterial cultures right after inoculation. Induction (turning on) of the lac promoter redirects much of the energy of the cell away from cell division and towards producing your mutant MTHFS. Each bacterium represents a tiny factory that is making your mutant MTHFS for you; you want to have as many factories as possible before instructing them to stop dividing and start making MTHFS. Thus, you will grow your cultures to a high density of bacteria before adding IPTG. You will monitor the bacterial density using visible spectroscopy. When you measure an absorbance of 0.8-1 at 600 nm (orange/red light), this means that you have lots of bacteria, but not so many that they have started dying from lack nutrients. It is at this density of bacteria that you will add IPTG to overexpress your mutant MTHFS.



Lactose



IPTG

Materials/Equipment

Microcentrifuge tubes
Microcentrifuge racks
Microcentrifuge
Test tube racks
50 mL centrifuge tubes (sterile)
Large, refrigerated centrifuge
Adjustable pipettes (10 μL, 200 μL, 1000 μL)
Pipette tips (sterile)
Test tubes containing 10 mL of LB media (sterile, 1 per student plus a few extra)
Flasks containing 100 mL LB media (sterile, 1 per student plus a few extra)
50 mg/mL kanamycin (CAS 25389-94-0) solution in water (120 μL per student)
30 mg/mL spectinomycin (CAS 1695-77-8) solution in water (120 μL per student)
1 M IPTG (CAS 367-93-1) in water (120 μL per student)
Spec-20 or other visible spectroscopy system
Cuvettes
Bleach
37 degree Celsius shaker bath
Room temperature shaker

Safety

Please note that this protocol takes place over three consecutive days. Remember that you cannot work in lab alone; there must always be a faculty member or another student with you. As always, wear goggles, gloves, and a lab coat in the laboratory. IPTG is a suspected carcinogen.

Protocol

Day 1: Preferably in the late afternoon or evening

1. Using sterile technique, add 10 μL of kanamycin stock solution and 10 μL of spectinomycin stock solution to 10 mL of LB media in a test tube. (Be sure to get the experimental plate from your

transformation of BL21(DE3)/pSJS1244 with your MTHFS plasmid, not an older plate with the XL1-Blue strain and not a control plate.)

2. Continuing your sterile technique, inoculate the test tube with a colony from your transformation agar plate from the previous week.
3. Place your tube in the 37 degree Celsius shaker bath with vigorous shaking overnight. Make sure the incubator is shaking when you leave the laboratory.

Day 2: Preferably in the early or late morning

1. Using sterile technique, add 100 μL of kanamycin stock solution and 100 μL of spectinomycin stock solution to 100 mL of LB media in a flask.
2. Remove your overnight culture from the 37 degree Celsius shaker. Make sure to turn the shaker back on once you have your tube.
3. Agitate the test tube to ensure the bacteria are distributed evenly. Using sterile technique, add 1 mL of the overnight culture to the flask. Label and place your test tube in the refrigerator; you might need it later if you drop or spill the flask.
4. Place the flask in the 37 degree Celsius shaker with vigorous shaking.
5. Allow the culture to shake for 4 hours. Then, using sterile technique, remove 600 μL of the culture from the flask and add it to a cuvette. Measure the absorbance of the sample at 600 nm. If the absorbance is between 0.8 and 1, proceed to the IPTG addition (step 7). Note that if the culture does not look at all cloudy to you, you can skip taking the absorbance until it does. Always make sure to turn the shaker back on immediately after adding or removing your flask.
6. Place the flask back in the shaker. Use the absorbance you measured to estimate how long to wait. These bacteria divide (and thus the absorbance should double) every 30 minutes. Keep monitoring samples until the culture in the flask has an absorbance between 0.8 and 1.
7. Using sterile technique, remove 1 mL of culture from your flask and add it to a microcentrifuge tube. Centrifuge that tube on the highest speed for two minutes. Remove the supernatant and discard it in a beaker with bleach. The pellet at the bottom tube is your uninduced sample. Label and place the microcentrifuge tube in the Biochemistry II box in the -20 degrees Celsius freezer.
8. Using sterile technique, add 100 μL of IPTG stock to your flask.
9. Place the flask on the room temperature shaker with vigorous shaking.

Day 3: Preferably in the morning

10. Remove your flask from the room temperature shaker. Make sure to turn the shaker back on. From here on, you will not be growing bacteria so contamination of your sample is no longer a substantial issue. You no longer need to use sterile technique, but you do need to avoid contaminating other surfaces with your sample.
11. Remove 1 mL of sample from the flask and add it to a microcentrifuge tube. Centrifuge that tube on the highest speed for two minutes. Remove the supernatant and discard in a beaker with bleach. The pellet at the bottom tube is your induced sample. Label and place the microcentrifuge tube in the Biochemistry II box in the -20 degree Celsius freezer.
12. Carefully pour the remaining cell culture into two 50 mL centrifuge tubes. Place the bottoms of the tubes on the bench, squat down so they are at eye level, and make sure they have exactly the same amount of liquid in each.
13. Centrifuge tubes at maximum speed in the refrigerated, tabletop centrifuge for 20 minutes at 4 degrees Celsius. Make sure that the tubes are directly across from one another in the rotor.

14. Remove tubes from the centrifuge. Pour supernatants off into a beaker with an equal volume of bleach. Place tubes upside down on a small stack of paper towels for one minute. Cap and label tubes. Place them in the appropriate bag in the -20 degree freezer.

Final sample check and cleanup

At the end of the laboratory exercise, you should have uninduced and induced pellets in microcentrifuge tubes in the Biochemistry II -20 degree Celsius freezer. You should also have two 50 mL centrifuge tubes with pellets in that same freezer.

Remove your 10 mL culture from Day 1 from the refrigerator. Add an equal volume of bleach to it, making sure the bleach touches all sides. Let it sit for a few minutes; then pour it down the drain. Wash the test tube out with soap and water, and place it in the drying rack. Rinse the flasks with bleach, and then wash them with soap and water. Throw all disposable items that have touched bacterial culture in the autoclave buckets. All other materials can go down the drain or in the waste baskets as appropriate. Do not throw out antibiotic or IPTG stocks; your fellow students may still need these.

Week 7: Protein extraction using the freeze-thaw technique

Purpose

To extract your 5,10-methenyltetrahydrofolate synthetase (MTHFS) mutant in a soluble, folded form from cell pellets.

Introduction

While there are some systems where proteins are exported directly from bacterial cells into the growth media, overexpressed proteins are extracted from cell pellets in most cases. Depending on the stability of the protein and the equipment available, there are multiple techniques that can be used to accomplish this goal. These include lysis with detergent solutions, sonication, French press, and freeze-thaw extraction. Detergents are molecules with a hydrophobic end and a water soluble end (they are amphipathic). These molecules have similar properties to membrane lipids; thus, they can insert into and disrupt the plasma membranes of bacteria, allowing proteins to escape into the surrounding solution. In sonication, bacteria are subjected to high-frequency sound waves that disrupt the membranes. A French press uses high pressure to force bacteria through a tiny hole; the shear forces that result break open cells and allow proteins to leak out. Detergent solutions and sonication can lyse cells quickly and cheaply; however, previous experiments have demonstrated that these techniques are ineffective at producing soluble and active MTHFS. French press has been reported to work; however, it is low in throughput and the equipment is expensive.

You will be extracting your MTHFS mutant using a technique descriptively called freeze-thaw. Bacterial membranes are disrupted by the freezing and thawing of the cells. When the cells freeze, ice crystals form and the cell expands; when the cells thaw, they contract. This repeated expansion and contraction of the cells leads to breaks in the membrane and soluble proteins leak out. This method is cheap and very gentle; it is useful when there is concern about proteins unfolding. However, it is a slow method; it takes a great amount of time for cells to gently thaw.

Materials/Equipment

Microcentrifuge tubes
Microcentrifuge racks
Microcentrifuge
Adjustable pipettes (200 μL, 1000 μL)
Pipette tips
Liquid nitrogen (in a Dewar flask)
Wet ice bath
Ice cold 1 x phosphate buffered saline (PBS)
Cold (ice) temperature shaker bath

Safety

Liquid nitrogen can cause cryogenic burns and frostbite. Don't put your fingers in it, not even with gloves on.

If your liquid nitrogen is in a Dewar, handle with care! These containers are insulated by having vacuum between two layers of glass. If dropped or tipped over, they can implode, sending glass flying multiple feet in all directions. Handle with care, and always wear your safety goggles, lab coat, and gloves.

Protocol

1. Place centrifuge tubes containing cell pellets in liquid nitrogen for five minutes. You needn't immerse the whole tube, but the pellet should be below the surface of the cryogen.
2. Move tubes to a wet ice bath (a mixture of ice and liquid water). Allow pellets to thaw until they are freely flowing. They should not be chunky, and no part of the pellet should be stuck to the tube. This can take up 2 hours for the first thaw. It is considerably quicker for subsequent thaws. Gently tap the bottoms of the tubes on the lab bench to encourage them to flow.
3. Repeat steps 1 and 2 two times (three freeze-thaw cycles in all).
4. Suspend each pellet in 0.5 mL of ice cold PBS. Consolidate both pellets in the same 50 mL centrifuge tube.
5. Gently shake the centrifuge tube on ice for 45 minutes.
6. Transfer the suspended pellet to one or more 1.5 mL microcentrifuge tubes and centrifuge on the highest setting for 5 minutes.
7. Use a pipette to transfer all supernatant to a clean microcentrifuge tube with a clear label, and place the tube in the Biochemistry II box in the refrigerator.

Final sample check and cleanup

You should have placed the final supernatant from your freeze-thaw extract in the refrigerator. Pipette tips and dirty centrifuge tubes should be placed in autoclave waste.

Week 8: Protein purification using immobilized metal affinity chromatography

Purpose

To separate your 5,10-methenyltetrahydrofolate synthetase (MTHFS) mutant from other cellular debris (proteins, lipids, nucleic acids, and carbohydrates).

Introduction

Immobilized metal affinity chromatography (IMAC) works on the principle that certain amino acid side chains can reversibly bind to metal ions. A particularly common form of this technique involves the binding of a poly-histidine tag to an immobilized cobalt ion. To enable this purification, molecular cloning techniques are used to fuse a six histidine sequence to the gene product (protein) that is to be isolated. Cell extracts containing this protein are poured over a resin containing immobilized cobalt. Proteins with a poly-histidine tag bind the resin, and all other proteins are washed off the resin under mild conditions. This technique is particularly effective as the number of natural proteins that contain a six histidine sequence is few to none.

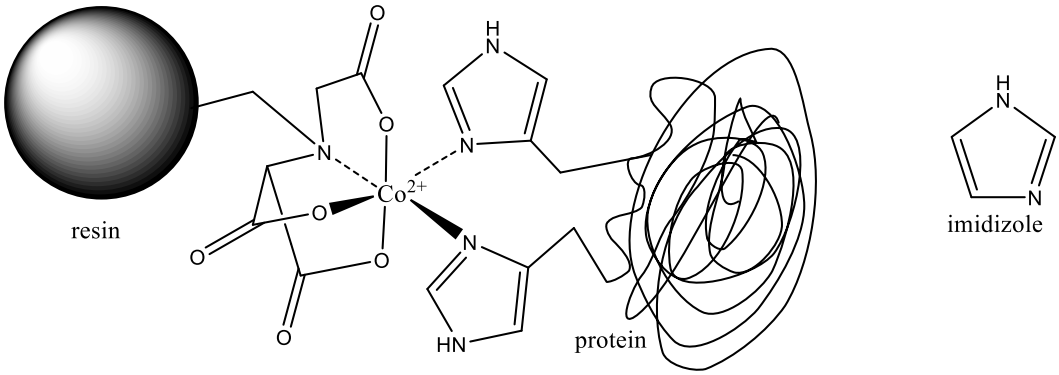


Figure: Immobilized metal affinity resin with bound protein (left). Only two of the six histidines from the poly-histidine tag are shown. Imidazole (right) can displace the poly-histidine tag at high concentrations.

After the non-specific proteins (those without the poly-histidine tag) are washed from the resin, the specific protein is eluted with imidazole. Imidazole has a very similar structure to that of the side chain of histidine. Imidazole is added to the resin at a concentration much higher than that of the bound protein. As such, the imidazole molecules replace the histidines surrounding the cobalt ions and displace the protein from the resin.

Materials/Equipment

Microcentrifuge tubes
Microcentrifuge racks
Microcentrifuge
Adjustable pipettes (200 μL, 1000 μL)
Pipette tips
Packing tape
Room temperature shaker
Talon® Superflow™ metal affinity resin (200 μL per student)
Wash buffer (50 mM HEPES pH 7.0, 10 mM imidazole, 300 mM NaCl) (10 mL per student)
Elution buffer (50 mM HEPES pH 7.0, 300 mM imidazole, 300 mM NaCl) (1.2 mL per student)

Safety

Imidazole (CAS 288-32-4) can cause skin, eye, and respiratory irritation. Always wear goggles, gloves, and lab coats.

Procedure

Prepare resin

1. Mix the Talon® Superflow™ metal affinity resin with vigorous shaking. Quickly pipet 200 μL of the resin into a 1.5 mL microcentrifuge tube.
2. Spin down the resin at 700 G for 30 seconds. Look at the markings on the side of the tube. If you have roughly 100 μL of actual resin, go on to step 3. If not, add some more resin until you have 100 μL.
3. Carefully remove the supernatant from your resin. In all steps, err on the side of leaving supernatant behind as opposed to removing resin.
4. Add 1 mL of wash buffer (50mM HEPES pH 7.0, 10 mM imidazole, 300 mM NaCl) to your resin. Mix by inverting several times. Centrifuge the resin at 700G for 2 minutes. Remove the supernatant.
5. Repeat step 4.

Apply cell extract to resin

6. Add 1 mL to 1.5 mL of your freeze-thaw extract to the resin. Make sure to record the amount you have added and to save at least 20 μL for a future polyacrylamide gel. Tape your microcentrifuge tube to the shaker and shake at a medium to high rate for 20 minutes at room temperature.
7. Centrifuge the tube at 700 G for 5 minutes.

Wash proteins without histidine tag from resin

8. Add 1 mL of wash buffer to the resin and shake as in #6 for 10 minutes. Centrifuge at 700 G for 5 minutes and remove supernatant.
9. Repeat step 8 two times.

Elute histidine tagged proteins from resin

10. Add 300 μL of elution buffer (50mM HEPES pH 7.0, 300 mM imidazole, 300 mM NaCl) to your resin. Incubate at room temperature for 10 minutes with shaking. Centrifuge resin at 700 G for 5 minutes. Remove and save supernatant in the Biochem II box in the refrigerator. This supernatant sample should contain most of your protein.
11. Add an additional 300 μL of elution buffer (50mM HEPES pH 7.0, 300 mM imidazole, 300 mM NaCl) to your resin. Incubate at room temperature for 10 minutes with shaking. Centrifuge resin at 700 G for 5 minutes. Remove and save supernatant as in step 10.

Final sample check and cleanup

You should have saved the supernatant from steps 10 and 11 along with at least 20 μL of your freeze-thaw extract from the previous week. These three samples should be in the refrigerator. Discard all other tubes and tips in the trash; all liquids may be disposed of in the sink.

Week 9: Protein purification using size-exclusion (gel-filtration) chromatography

Purpose

To remove the imidazole from the 5,10-methenyltetrahydrofolate synthetase (MTHFS) mutant that you isolated last week.

Introduction

In the previous purification, you eluted your protein from an immobilized metal affinity resin with a high concentration of imidazole. Imidazole is not a compound that is normally found in cells. It may interfere with your future kinetic characterization of MTHFS, and it must be removed. Fortunately, imidazole is a small molecule and is readily separated from proteins by size-exclusion chromatography.

Size-exclusion chromatography (otherwise known as gel-filtration chromatography) uses a stationary phase that is made up of small beads that have channels (or caves) in them. Molecules that are small enough to fit into these channels, and thus spend time in them, devote a greater fraction of their time to the stationary phase than big molecules. In all chromatography, molecules that spend a greater fraction of their time in the stationary phase move more slowly than molecules that spend a smaller fraction. Thus, large molecules like proteins will move much more quickly through a size-exclusion resin than small molecules, like imidazole. Please note that elution order in size-exclusion chromatography is the opposite of gel electrophoresis: the larger molecules move faster than smaller molecules.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a method that separates proteins according to their sizes. All electrophoresis is based on charged molecules moving in an electric field. As you know, some amino acid side chains are charged at near-physiological pH. These side chains give the protein an overall charge that can be positive or negative. This can be problematic as we want all our proteins to move in the same direction in the electric field (toward the positive electrode). We overcome this difficulty by adding sodium dodecyl sulfate (SDS) to our protein samples (in the Laemmli buffer, see below) and to the polyacrylamide gels. Dodecyl sulfate (the anion of SDS) has a negatively-charged end of the molecule and a hydrophobic end (it is amphipathic). The hydrophobic end associates with hydrophobic amino acids; approximately one SDS binds to the protein for every two amino acids. Since dodecyl sulfate has a negative charge this results in a total negative charge that is roughly equal to the number of amino acids divided by two. This amount of charge overwhelms the original charge of the proteins, making them all highly negative. The SDS also disrupts the tertiary structure of the protein and causes it to unfold. Thus, all proteins will be unfolded and move toward the positive electrode through the gel based on their molecular weight, not their native shape.

Materials/Equipment

Pasteur pipettes
Cotton
Microcentrifuge tubes
Microcentrifuge racks
Microcentrifuge
Adjustable pipettes (200 μL, 1000 μL)
Pipette tips
Equipment stands with clamps
Sephadex G-25 size exclusion resin (swollen before lab according the manufacturer's directions)
50 mM HEPES pH7.0, 30 mM NaCl (10 mL per student)

SDS-polyacrylamide gels (one 10 cm x 10 cm gel per three students)
Polyacryamide gel systems
Power supplies
3 x Laemmli buffer (contains SDS and 2-mercaptoethanol)
1 x SDS-PAGE running buffer
EZ-run™ Prestained *Rec* Protein Ladder (ThermoFisher Scientific)
GelCode Blue® (ThermoFisher Scientific)
Plastic containers with lids to stain gels
Room temperature shaking platform
Gel imaging system (camera on tripod)

Safety

Many of the hazards in this sequence are associated with gel electrophoresis. Outside of the risk of electric shock, there are several dangerous chemicals. Sodium dodecylsulfate (SDS), 2-mercaptoethanol (βME), and acrylamide are toxic if swallowed or inhaled. Acrylamide (the monomer used to make the gels) is also toxic on skin contact, a skin allergen, an eye irritant, and is a suspected reproductive toxin and carcinogen. βME is toxic on skin contact, a skin irritant, an allergen, and causes serious eye damage. SDS is a skin and respiratory irritant and causes serious eye damage. Gel Code Blue (used to stain polyacrylamide gels) is toxic on ingestion and skin contact, a skin irritant, and causes serious eye damage. As always, wear goggles, lab coats, and gloves in the lab.

Procedure

Size-exclusion chromatography

1. Obtain a Pasteur pipette. Roll a very small tuft of cotton into a loose ball. Place the ball of cotton into the pipette and push it down to the neck using another pipette. Mount the pipette vertically using an equipment stand and clamp.
2. Transfer 1 mL of Sephadex G-25 size exclusion resin to the Pasteur pipette. Allow water to drain. If you have made a good cotton ball, the water should drain through the pipette without the loss of any resin. Keep adding slurry to the pipette until you get a column of resin that is 4.25 cm in length from where the neck of the pipette begins to narrow.
3. Once you have 4.25 cm of resin, add 5 x 1mL of 50 mM HEPES (pH7.0), 30 mM NaCl to the column in multiple additions. Add new buffer as soon as possible; the weight of the buffer will cause your column to drip more quickly.
4. Prepare five 1.5 mL microcentrifuge tubes in a rack. Label the tubes numbers 1 through 5.
5. Add 300 μL of your metal affinity-purified protein to the top of the column and immediately collect the 300 μL of liquid that elutes from the other end in tube 1. As soon as you have collected this sample, move it to an ice bath to minimize protein degradation. For this and all subsequent additions, make sure to allow the column to stop dripping before adding the next 300 μL of buffer.
6. Add 300 μL of 50 mM HEPES (pH7.0), 30 mM NaCl to the top of the column and immediately collect the 300 μL of liquid that elutes from the other end in tube 2.
7. Repeat step 6 three more times and fill tubes 3, 4, and 5.
8. Your protein should be most concentrated in tube 3. Additional protein will be present in tube 4.
9. Use the NanoDrop UV/vis spectrometer to determine the concentration of protein in tube 3 (elution 3). If you have protein in this sample (there is a peak at 280 nm), use this sample for

your gel. Record the value at 280 nm and at 400 nm in your notebook. You will use these values to calculate the concentration of protein in your lab report. While your gel runs, collect UV/vis data for the rest of your size exclusion elutions.

SDS-polyacrylamide gel electrophoresis

1. You will run three samples on a SDS-polyacrylamide gel: Your induced protein pellet, your freeze-thaw extract, and your most concentrated fraction from size-exclusion chromatography (mostly likely fraction 3).
2. To prepare your induced protein pellet, add 20 μL of Laemmli buffer to the pellet and vortex on high for 1 minute. To prepare the freeze-thaw extract and size-exclusion elution, add 3 μL of Laemmli to 10 μL of each sample in separate tubes. Mix briefly.
3. Briefly centrifuge all three samples to bring liquid to the bottom of the tube. Place samples on the 95 degree Celsius heat block for 10 minutes.
4. Remove samples from the heat block. Briefly centrifuge to bring liquid to the bottom of the tubes, mix briefly, and briefly centrifuge again.
5. If not already present, ask your instructor to load the molecular weight marker on the gel.
6. Load 2 μL of your induced protein pellet sample and 10 μL of each of your freeze-thaw extract and size-exclusion samples in separate lanes on the gel. Three students must share each gel so make sure to load your samples in adjacent lanes. If you cannot pipette your induced protein pellet sample (it has a snotty consistency), ask your instructor for to demonstrate how to sonicate that sample.
7. If you are the last student to load samples on the gel, notify your instructor that the gel is ready to be started.
8. Once the gel has run an appropriate amount of time as judged by the molecular weight maker (about an hour), it is ready for staining.
9. Your instructor will demonstrate how to stop the gel, remove it from the electrophoresis system, and separate the glass plates. Place the gel in a thin layer of GelCode Blue® in plastic container.
10. Label the container with a unique name that all students in your gel group will recognize. Gently shake or rock the container overnight at room temperature.
11. The next day (days later is okay too), pour off the used GelCode Blue® into an appropriate waste container. Hold the gel down with your gloved fingers to ensure it does not "pour" off as well.
12. Add a 1 cm deep layer of tap water to your container and gently shake the gel at room temperature for 15 minutes.
13. Carefully pour the water down the drain. Take an image of the gel using the camera and tripod.

Final sample check and cleanup

You should save all elutions containing significant protein (based on your UV/vis measurements) from the size-exclusion chromatography purification. These go in the Biochemistry II box in the refrigerator. Your packed, size-exclusion column should be disposed in the broken glass box. Except for the gel stain (see above), all other materials can go in the trash or down the sink as appropriate.

Weeks 10-12: Kinetic analysis of your 5,10-methenyltetrahydrofolate synthetase (MTHFS) mutant.

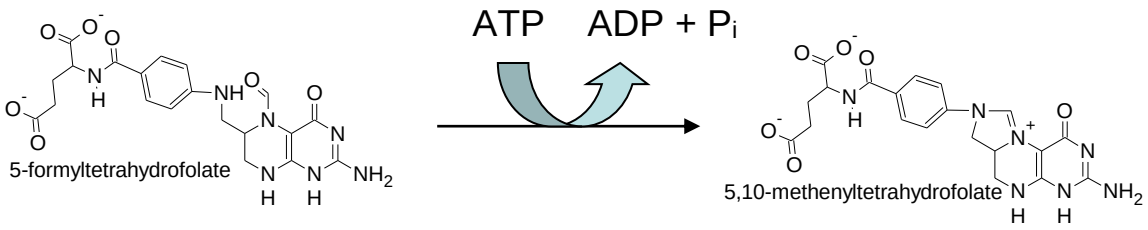
Purpose

The purpose of the next three weeks of lab is to determine if your MTHFS mutant is active, and if it is, to find its K_m for ATP and its k_{cat}.

Introduction

The goal of this term's project has been to investigate the importance of specific amino acids in the catalytic activity and binding of ATP by the enzyme MTHFS. To accomplish this goal, we have created several site-directed mutants of this protein where each mutant has a particular amino acid changed to alanine. If changing an amino acid significantly changes the activity or binding properties of the enzyme, the original amino acid is important for the function of the enzyme. To test how a particular mutation changes the enzyme, we will use initial rate data and the Michaelis-Menten equation to determine K_m (essentially a binding constant) and k_{cat} (turnover number). For a review of how to determine K_m and k_{cat} from initial rate data, please see sections 7.1 and 7.2 in your book.

The reaction catalyzed by MTHFS is given below.



In this reaction, 5-formyltetrahydrofolate (folinic acid) is reduced to 5,10-methenyltetrahydrofolate along with the hydrolysis of ATP to ADP. While MTHFS has two substrates, we will only determine a K_m for ATP. k_{cat} is the maximal turnover rate for the enzyme and thus this value is the same for all substrates. We will use our data for determining the K_m for ATP to calculate the k_{cat} of the mutant MTHFS.

To determine the K_m for ATP we will conduct pseudo-first order kinetics. In these experiments, the concentration of ATP is varied so that the ATP K_m can be determined while the concentration of folinic acid is held constant at a high concentration compared to the K_m for folinic acid.

5,10-methenyltetrahydrofolate (the product) absorbs light at 356 nm while 5-formyltetrahydrofolate (the reactant) does not. ATP, ADP, and phosphate also do not absorb 356 nm light. This makes it easy for us to monitor the rate of reaction with the UV/visible spectrometer. As the concentration of 5,10-methenyltetrahydrofolate increases, the absorbance at 356 nm increases. The extinction coefficient for 5,10-methenyltetrahydrofolate at 356 nm is known, and we can use it to convert our absorbance values into concentrations.

Materials/Equipment

Microcentrifuge tubes
Microcentrifuge racks
Microcentrifuge
Adjustable pipettes (10 μL, 200 μL, 1000 μL)
Pipette tips
Spec-20 or other UV/visible spectrometer capable of measuring absorbance at 356 nm
Cuvettes
Ice buckets
Fume hood
Laptop computers to enter and evaluate data in real time
Purified wild-type MTHFS as a control and for students with inactive mutants
0.5 M MES pH 6.0 (CAS 4432-31-9)
1.0 M NaCl (CAS 7647-14-5)
1.0 M $MgCl_2$ (CAS 7791-18-6)
10% Triton-X 100 (v/v) (CAS 9002-93-1)
10% β-mercaptoethanol (v/v) (CAS 60-24-2)
100-300 mM ATP (CAS 987-65-5)
5-10 mM folinic acid (CAS 58-05-9)

Safety

Folinic acid is a skin, eye and respiratory irritant as well as an allergen. Triton X-100 is a skin irritant, toxic on ingestion, and can cause serious eye damage.

β-mercaptoethanol (βME) is toxic on skin contact, a skin irritant, an allergen, and causes serious eye damage. It also smells bad, so handle the 10% stock solution in the fume hood. Leave all tips and pipettes that interacted with 10% β-mercaptoethanol in the hood.

As always, wear goggles, lab coats, and gloves in the laboratory.

Protocol

Unlike the other experiments you ran this semester, you will not be given a specific protocol for this one; you will need to design your own. As you will need time to determine an acceptable protocol, you have been given three laboratory periods to complete this exercise. You did determine a K_m and k_{cat} for lactate dehydrogenase last semester, and the overall method used can be applied here.

Almost all enzymes require some sort of buffer for optimal reactivity. Your reactions will be carried out under the following conditions:

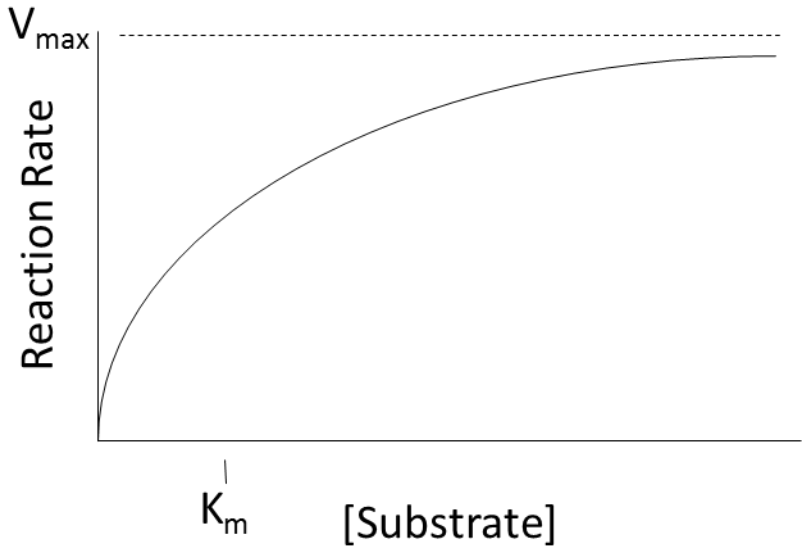
50 mM MES pH 6.0
30 mM NaCl
1 mM $MgCl_2$
0.1% Triton-X 100
0.01% β-mercaptoethanol
pH 7.0
1 mM ATP (you will vary this concentration)
100 μM folinic acid

If you need to dilute your enzyme, do not use water. The following solution will allow you to dilute your enzyme without a loss of activity:

50 mM MES pH 6.0
30 mM NaCl
0.1% Triton-X 100
0.01% β-mercaptoethanol

Your K_m determination will involve 3 steps.

1. Run initial experiments at 1 mM ATP to determine a dilution of enzyme. A good dilution will give you a straight line for the 90 seconds or so of data you will collect. This straight line needs to have a slope that is significantly greater than a control reaction with no enzyme.
2. Run a series of experiments with one reaction at progressively doubling or halving ATP concentrations. You want the rates (slopes) for these reactions to form a graph like that below so that you can estimate the K_m for this reaction.



3. Once you have an estimate of the K_m, run 15 reactions. These reactions will represent measurements at 5 different concentrations of ATP in triplicate. Your ATP concentration should be 0.25xK_m, 0.5xK_m, 1xK_m, 2xK_m, and 4xK_m. You will create a Lineweaver-Burk plot using this data and use it to determine K_m and k_{cat} as we learned last semester.

Final sample check and cleanup

At the end of any laboratory period, make sure that you have returned your purified enzyme to the Biochemistry II box in the refrigerator. For your final laboratory period, make sure to show your instructor your final Lineweaver-Burk plot before leaving. Do not throw out any stocks. Leave all tips and pipettes that interacted with 10% β-mercaptoethanol in the hood. Your instructor will dispose of them later. All other materials may be poured down the drain or thrown in the waste basket as appropriate.

Appendix A1: Gene and protein sequence of MTHFS

MTHFS protein and DNA sequence

```
 M   D   K   N   A   L   R   K   Q   I   L   Q   K   R   M   A   L   S      18
ATG GAC AAA AAT GCC TTA AGA AAA CAA ATT CTG CAA AAA AGA ATG GCA TTA AGT     54

 T   I   E   K   S   H   L   D   Q   K   I   N   Q   K   L   V   A   F      36
ACC ATT GAA AAA AGT CAC TTA GAT CAA AAG ATT AAT CAA AAA TTA GTT GCT TTT    108

 L   T   P   K   P   C   I   K   T   I   A   L   Y   E   P   I   K   N      54
TTA ACT CCT AAG CCA TGC ATT AAA ACA ATT GCA CTT TAT GAA CCC ATT AAA AAT    162

 E   V   T   F   V   D   F   F   F   E   F   L   K   I   N   Q   I   R      72
GAG GTT ACT TTT GTT GAC TTC TTC TTT GAG TTT TTA AAG ATT AAC CAA ATA AGA    216

 A   V   Y   P   K   V   I   S   D   T   E   I   I   F   I   D   Q   E      90
GCT GTT TAC CCC AAG GTA ATA AGT GAT ACC GAA ATT ATC TTT ATT GAT CAG GAG    270

 T   N   T   F   E   P   N   Q   I   D   C   F   L   I   P   L   V   G     108
ACA AAT ACA TTC GAA CCT AAT CAA ATA GAC TGC TTC CTA ATA CCA TTG GTA GGC    324

 F   N   K   D   N   Y   R   L   G   F   G   K   G   Y   Y   D   R   Y     126
TTT AAT AAA GAC AAT TAC CGT CTA GGC TTT GGC AAG GGC TAT TAT GAC CGT TAT    378

 L   M   Q   L   T   R   Q   Q   P   K   I   G   I   A   Y   S   F   Q     144
TTA ATG CAA TTA ACT AGA CAA CAA CCT AAA ATA GGA ATA GCG TAC AGT TTC CAA    432

 K   G   D   F   L   A   D   P   W   D   V   Q   L   D   L   I   I   N     162
AAA GGT GAT TTT TTA GCC GAT CCT TGG GAT GTA CAA CTA GAC TTA ATT ATT AAT    486

 D   E   *                                                                 165
GAT GAA TAA                                                               495
```

Appendix A2: Plasmid sequence of pSKB3-MTHFS

Plasmid Sequence with MTHFS gene inserted. In RED is the coding sequence for MTHFS.

TGGCGAATGGGACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCC
GCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATTTAGTG
CTTTACGGCACCTCGACCCCAAAAAACTTGATTAGGGTGATGGTTCACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTCGCCCTTTGACGTTGGA
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CACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGC
TACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTCCTTCT
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CGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCTGATGCGGTATTTTCTCCTTACGCATCTGTGCGGTATTTCACACCGCATATATGGT
GCACTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCAGTATACACTCCGCTATCGCTACGTGACTGGGTCATGGCTGCGCCCCGACACCCG
CCAACACCCGCTGACGCGCCCTGACGGGCTTGTCTGCTCCCGGCATCCGCTTACAGACAAGCTGTGACCGTCTCCGGGAGCTGCATGTGTCAGAGGT
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CAGCTCGTTGAGTTTCTCCAGAAGCGTTAATGTCTGGCTTCTGATAAAGCGGGCCATGTTAAGGGCGGTTTTTTCCTGTTTGGTCACTGATGCCTCC
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AACCAGTAAGGCAACCCCGCCAGCCTAGCCGGGTCCTCAACGACAGGAGCACGATCATGCGCACCCGTGGGGCCGCCATGCCGGCGATAATGGCCTG
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GCGACGGCGCGTGCAGGGCCAGACTGGAGGTGGCAACGCCAATCAGCAACGACTGTTTGCCCGCCAGTTGTTGTGCCACGCGGTTGGGAATGTAATT
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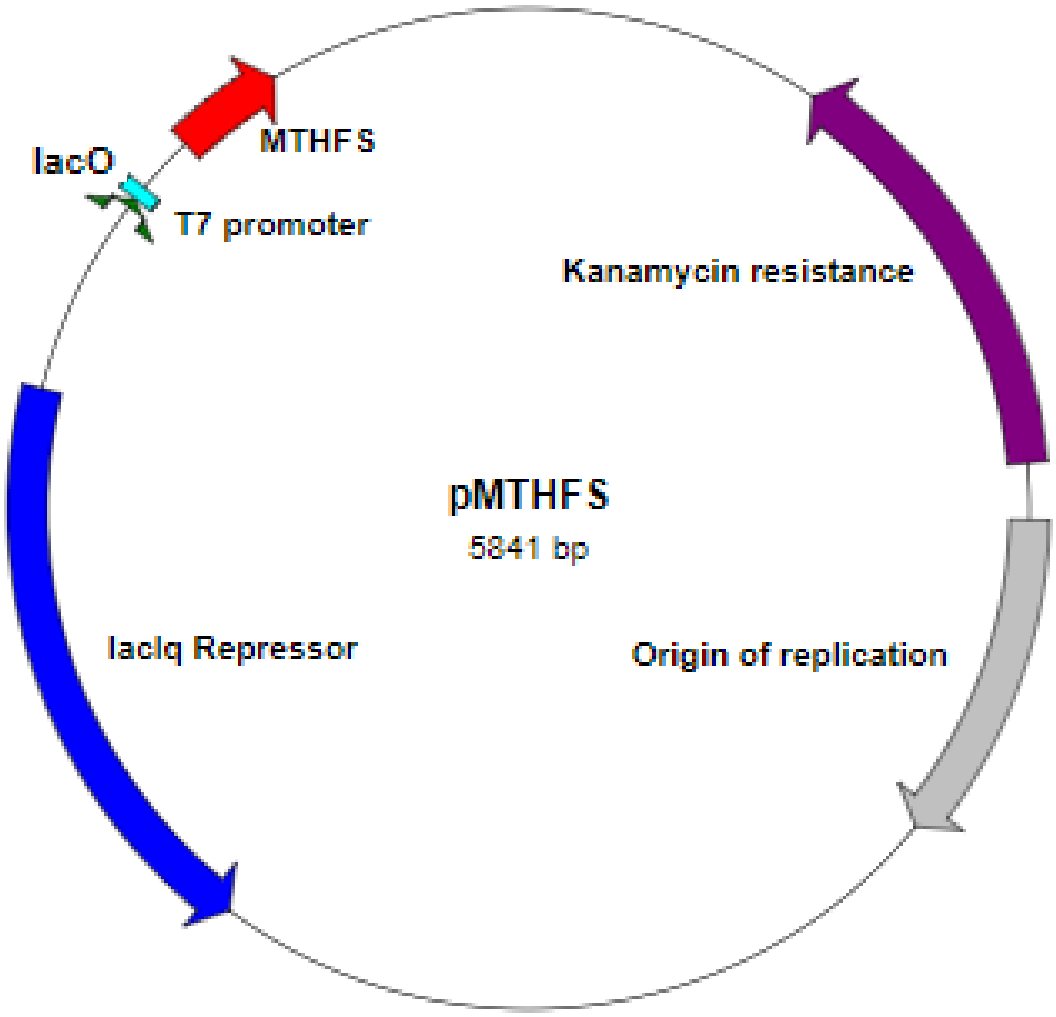
GCATACTCTGCGACATCGTATAACGTTACTGGTTTCACATTCACCACCCTGAATTGACTCTCTTCCGGGCGCTATCATGCCATACCGCGAAAGGTTT
TGCGCCATTCGATGGTGTCCGGGATCTCGACGCTCTCCCTTATGCGACTCCTGCATTAGGAAGCAGCCCAGTAGTAGGTTGAGGCCGTTGAGCACCG
CCGCCGCAAGGAATGGTGCATGCAAGGAGATGGCGCCCAACAGTCCCCCGGCCACGGGGCCTGCCACCATACCCACGCCGAAACAAGCGCTCATGAG
CCCGAAGTGGCGAGCCCGATCTTCCCCATCGGTGATGTCGGCGATATAGGCGCCAGCAACCGCACCTGTGGCGCCGGTGATGCCGGCCACGATGCGT
CCGGCGTAGAGGATCGAGATCTCGATCCCGCGAAATTAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTT
GTTTAACTTTAAGAAGGAGATATACCATGGGCAGCAGCCATCATCATCATCATCACGATTACGATATCCCAACGACCGAAAACCTTTACTTCCAGGG
CCATATGGACAAAAATGCCTTAAGAAAACAAATTCTGCAAAAAAGAATGGCATTAAGTACCATTGAAAAAAGTCACTTAGATCAAAAGATTAATCAA
AAATTAGTTGCTTTTTTAACTCCTAAGCCATGCATTAAAACAATTGCACTTTATGAACCCATTAAAAATGAGGTTACTTTTGTTGACTTCTTCTTTG
AGTTTTTAAAGATTAACCAAATAAGAGCTGTTTACCCCAAGGTAATAAGTGATACCGAAATTATCTTTATTGATCAGGAGACAAATACATTCGAACC
TAATCAAATAGACTGCTTCCTAATACCATTGGTAGGCTTTAATAAAGACAATTACCGTCTAGGCTTTGGCAAGGGCTATTATGACCGTTATTTAATG
CAATTAACTAGACAACAACCTAAAATAGGAATAGCGTACAGTTTCCAAAAAGGTGATTTTTTAGCCGATCCTTGGGATGTACAACTAGACTTAATTA
TTAATGATGAATAAGATCCGAATTCGAGCTCCGTCGACAAGCTTGCGGCCGCACTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAA
AGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTG
AAAGGAGGAACTATATCCGGAT

Appendix A3: Plasmid map for pSKB3-MTHFS



Appendix B: Notebook information

Scientists need to keep accurate records of their work. These records assist them in "remembering" exactly how previous experiments were carried out. These records are also important to assist their colleagues in repeating or extending their work. In addition, laboratory notebooks will be used in court cases (to help establish first inventor) where patents are being disputed. For all these reasons, it is important for you to keep a detailed laboratory notebook.

Some simple guidelines for keeping your laboratory notebook are given below. I have attached an example of a laboratory notebook (with comments) as well.

Guidelines for good laboratory notebooks

1. In general, your lab book should contain sufficient detail that one of your peers could pick it up and use it as a guide to repeat your experiments.
2. All laboratory notebooks must be bound. No loose leaf or three ring binders are allowed.
3. Laboratory notebooks must be written in indelible ink. No pencils or erasable ink are allowed. Errors in your notebook are to be crossed out with a single line. This indicates that there was an error, but allows you or someone else to see what you originally wrote.
4. Put your name and the year on the front cover of your notebook.
5. Any pictures or data to be inserted into a laboratory notebook must be affixed with double sided tape. This tape will rip the page if you try to remove or replace the data.
6. Leave the first few pages of your notebook empty for a table of contents.
7. All notebook pages must have a page number.
8. All experiments must have a descriptive title. This title should be referenced with a page number in the index at the front of your notebook.
9. The title page of each experiment should have a date. If an experiment takes more than one day, update the date as you write up the experiment.
10. All samples must be clearly labeled in the notebook using at least your initials and a page number. If multiple samples are generated in the space of a notebook page, add additional identifiers to make them unique.

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PAGE NUMBERS ON EVERY PAGE

8-19-05

DATE AT BEGINNING OF EXPERIMENT

TITLE AT BEGINNING OF EXPERIMENT. TITLE AND PAGE # SHOULD BE INDEXED IN FRONT OF LAB BOOK

Talon Purification ~~and desalting~~ of freeze thaw extract from page 154.

1. Talon resin was obtained from the refrigerator, swirled to resuspend, and 400 λ was pipetted into a 1.5 mL microcentrifuge tube.

NUMBERED STEPS FOR EASIER READING

2. Microcentrifuge tube was spun down at 700g for 1 min. Supernatant from tube was discarded. Using the gradations on the tube, about 100 λ of spun down resin is present.

3. 1mL of wash buffer (50mM HEPES pH 7.0, 0.3M NaCl, 10mM Imidazole) was added to the resin. Resin was resuspended by inverting the tube several times and then spun down for 2min at 700g. Supernatant was discarded.

4. ~~Step 2~~ Step 3 was repeated. 156

5. 1mL of sample "TWJ 2005 154 FT RIIS" was applied to the resin. Tube was taped to the shaker platform in lab and shaken at a setting of 90 for 32min at room temperature. Sample was then spun down at 700g for 5minutes. Supernatant was carefully removed and saved.
Sample label = "TWJ 2005 156 flow"

A SINGLE LINE CROSSING OUT MISTAKES

6. 1mL of wash buffer was ~~added~~ to the sample. The tube was taped to the shaker ~~bath~~ platform and shaken at a setting of 90 for 14 min. The supernatant was removed and saved.
Sample name = "TWJ 2005 p156 14a"

Sample spun at 700g for 5min. →

7. Step 6 was repeated. Shake time was 12 min instead of

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8. 300λ of elution buffer (50mM HEPES pH 7.0, 0.3M NaCl, 300mM Imidazole) was added to the resin. Tube was taped to the shaker platform and shaken at a setting of 90 for 11min. Sample was spun down at 700g for 5min. Supernatant was removed and saved.
Sample label = "TWJ 2005 p157 E1"

SAMPLE NAMES CLEARLY LABELED

9. Step 8 was repeated except shaking was for 14min.
Sample label = "TWJ 2005 p157 E2"

10. Gel was run with original sample "TWJ2005 pg154 FT R115" and with sample from Step 8, "TWJ 2005 p157 E1". 2λ of gel loading buffer was added to 10λ of each sample. Samples were then mixed and boiled for 10min. Gel was prepared as described on page 57.

DATA AFFIXED TO NOTEBOOK USING DOUBLE SIDED TAPE

Sample #	Sample
1	TWJ2005 pg154 FT R115
2	TWJ2005 pg157 E1

Appendix C: Lab report directions

General Laboratory Report Format

This document describes the general format for each of your four formal laboratory reports. In addition to these general directions, specific directions for each report may be found below. Make sure to follow the general and specific directions for each report.

The primary purpose of writing laboratory reports in this class is for you to reinforce and demonstrate your understanding of the techniques you learn in lab. Most of your lab grade will be based on your ability to demonstrate your understanding in these reports.

Post-college career, you will be required to write documents in very specific formats. There are real world penalties for not following the required formats. This is particularly true in science. Consequences could include: Not getting a grant, annoying your boss and not getting promoted, having your paper rejected, having the drug you developed rejected by the FDA, and others. To help train you to write scientific documents, your laboratory report must adhere to the following format. Part of your grade will be your ability to follow this format.

In addition to format constraints, you will often be faced with situations where there is a maximum length allowed for your document. In order to practice this, your reports will be limited to 3 pages in length. You may choose spacing and margins as you like. Please do not use font smaller than 10 pt.

The entire report should be written in your own words. If you use quotation marks to directly quote something, your instructor will assume you did not understand the concept in question. If you copy something directly without putting it in quotes and/or referencing it, that is plagiarism and a violation of the Academic Integrity Policy. Your report should contain the following sections.

1. Introduction: This section should include a description of the question/s that were studied and/or products that you were trying to make. It should also explain how these questions or products figure into the overall project and why they are important. In addition, this section should describe the theory behind the techniques used.
2. Experimental: This section should include a brief description of the techniques and methods used to gather the data in the experiment. There should be enough detail here that someone could repeat the experiment with your experimental section.
3. Data: All the data that were collected in the experiment. This should include tables, graphs, and pictures from the experiment.
4. Calculations: A detailed description of every calculation used to analyze the data. This is where you show your work. You may hand write this section if you like.
5. Results and conclusions: This is where you describe the answers you found to the questions you discussed in the introduction. If the focus of the report is a product, this is where you describe the proof that you did or did not successfully make it. Make sure to reference the data you collected to justify your conclusions. You should also discuss any sources of error here.

One final note: In any writing, you should keep your audience in mind and endeavor to make the document flow such that it guides your reader to the information you choose. Your instructor is your audience for these reports. When writing them, you should be thinking, “How can I most clearly express my understanding to my instructor in the limited space provided?” A portion of your laboratory grade will be dedicated to how easily the required information can be read from your reports.

Specific Directions for Lab Report 1

Reports are the same format as last term. In addition to the standard items described in the General Laboratory Report Format directions above, please make sure to include the items listed below in your report. As always, make it fit in 3 pages. Your calculation section is not part of your page count.

Introduction

1. Describe the enzyme we are studying and why it is important. Give at least two reasons.
2. Give a two or three sentence description of how we will study this enzyme. This is a brief overview of the entire project for this semester.
3. Describe which amino acid you are changing, what you changed it to, and why you chose that amino acid. Include a picture from the modeling we carried out with the program PyMol here.
4. Briefly describe the process used to change the amino acid in lab. How does it work? Why did you do it? Focus mostly on the parts of the process completed so far.

No Experimental Section

Data

5. Include a picture of your gel with the molecular markers and your samples clearly labeled. Indicate on the picture the sizes of all bands for at least one of the markers. Information about the molecular weight marker can be found at this link (https://www.neb.com/products/n3200-2-log-dna-ladder-01-100-kb).
6. Estimate the size of your DNA products from PCR from the gel. If you had no visible products, choose someone else's and estimate their size. Clearly state whose results you are using.
7. Assuming that the amount of DNA in each of the bands of the molecular weight markers is 25 ng, estimate the total mass of DNA in your PCR sample. Please remember that your PCR sample contained 50 microliters and you didn't load all of this on the gel. Make sure to include your calculations for this (even if they are really easy) on your calculations page.
8. Construct a table that describes how many colonies you observed on each plate after transformation. Go look at your plates and estimate the number of colonies (an exact count is not necessary).

Results and Conclusions

9. Did your PCR reaction work? If it did not work (or it was weak PCR) discuss sources of possible error using your control reaction, your experimental reaction, the molecular weight marker, and the rest of the class' reactions. Possible sources of error: The polymerase was bad, the dNTPs were bad, individual student error, the thermocycler was broken, or the gel was bad. Make sure to directly address each of these possible errors; give each its own sentence even if it seems repetitive. As appropriate, identify the most likely source of error given the data you have from lab. You should use what you know about other students' results as part of this analysis.
10. Did your transformation work? If it did not work, suggest sources of error. Use your PCR results from the gel and your control plates to evaluate your sources of error. Specifically discuss the evidence for or against experimental error on your part, quality of your PCR products, bad DpnI, and bad competent cells. Make sure to directly address each of these possible errors; give each its own sentence even if it seems repetitive. Again, identify the most likely source of error given the data you have from lab. You should use what you know about other students' results as part of this analysis.

Specific Directions for Lab Report 2

In addition to the standard items described in the General Laboratory Report Format directions above, please make sure to include the items listed below in your report. As always, make it fit in 3 pages. Your calculation section is not part of your page count.

Where items are duplicated from an old report, do not copy and paste. Rewrite them in your own words without looking at the old report.

Introduction

1. Include one or two sentences telling what MTHFS is and why it is important.
2. Draw out a figure showing the overall reaction catalyzed by MTHFS. Include structures and names in your figure. Draw the structures yourself using Chemdraw, a similar program, or by hand.
3. Explain how the work described in this lab report fits into the overall project.
4. Describe the theory behind the DNA purification. Just focus on the spin columns here. What is going on in each step? Where is the DNA in each step?
5. Describe how UV/vis spectroscopy of DNA works.
6. Describe how DNA sequencing works.

Experimental

7. Include all details of the experiment here. You may reference the lab manual (specifically) but make sure any deviations or details missing from the manual are described here. Someone should be able to repeat your experiment using your lab report.

Data

8. Include a copy of your UV/vis spectrum here. Make a table with the absorbance of your sample at 260, 280, and 400 nm.
   Practice good graphing technique. Good technique clearly shows your reader what is important in your data.
   a. Give your graph a title and label the axes.
   b. Make sure your graph has a figure caption.
   c. Only graph the region between 200 and 400 nm. The absorbance at 260 nm is most important in the graph. Make sure this peak is at least 2/3 the height of your y-scale. If this means that absorbance values below 260 nm run off the top of your graph, that is OK.
9. Include the concentration of your stock DNA here. Make sure to show your calculations for this in the calculations section.
10. How many microliters of DNA did you send off for sequencing? Show calculations for this in your calculations section.
11. Include a table with estimated number of colonies for each of the various transformations.
12. Include the region of the sequencing chromatogram that shows that your mutation has been made. (This is a screenshot that you have annotated.) Clearly label this section to show that the mutation was made. Give the original sequence and say what amino acid codon used to be there.

Results and Conclusions

13. Did you transform bacteria with DNA containing your mutation last week, or did you transform them with something else? Use your sequencing data to answer this question. If your data are inconclusive, explain why.

14. Did your transformation work? Use the data from your experiment to explain why it did or did not. If your data are inconclusive, explain why.
15. If something did not work in your experiment, create a hypothesis to explain your result that is consistent with the data.

Specific Directions for Lab Report 3

In addition to the standard items described in the General Laboratory Report Format directions above, please make sure to include the items listed below in your report. As always, make it fit in 3 pages. Your calculation section is not part of your page count.

Introduction

1. Include one or two sentences telling what MTHFS is and why it is important. Do not cut and paste this from your previous report.
2. Draw out a figure showing the overall reaction catalyzed by MTHFS. Include structures and names in your figure. You may cut and paste this from your previous report. Do reference this figure in your text.
3. Describe how the work in this lab report fits into the overall project.
4. Explain the theory behind your induction of bacteria to produce MTHFS. As always, explain what is going on at the molecular level for this description and those below. Hint: Lac operon.
5. Explain the theory behind freeze thaw extraction of proteins from bacteria.
6. Describe the theory behind immobilized metal affinity chromatography purification of MTHFS.
7. Describe the theory behind size exclusion chromatography purification of MTHFS.

Experimental

No experimental section for this report.

Data

8. List the mutant you were purifying and your yield (concentration in mg/mL) based on UV/vis. Units are important here.
9. Include a picture of your gel with molecular weight makers labeled. Label each lane and indicate where you think MTHFS is (or should be) on the gel. We used the EZ-Run Prestained *Rec* Protein Ladder from Fisher in class. Here is a link with an image of this molecular weight marker (https://www.fishersci.com/shop/products/fisher-bioreagents-ez-run-prestained-i-rec-i-protein-ladder-3/p-3016448#). The red band on the website image will be the band on your gel that is a slightly different color than the others. It has been stained with blue, so it no longer looks bright red.

Results and Conclusions

10. Did you successfully purify MTHFS? Explain your answer using your data. How do your data indicate purification (or lack thereof)? You only have two samples, but you can use both of them to address this.
11. Do the data in this lab report prove that you have purified a mutant of MTHFS? If not describe one other possibility consistent with your data. Hint: The key term in the first sentence of this item is *mutant*. Do your gel data prove you have purified a mutant of MTHFS and not something else? The molecular weight of MTHFS is roughly 22.2 kiloDaltons.

12. If something did not work in your experiment, create a hypothesis to explain your result that is consistent with the data. Make sure to discuss how the data support your hypothesis.

Specific Directions for Lab Report 4

Introduction

1. State the hypothesis for your mutant. Use a PyMOL picture to help describe your hypothesis. . You can use the picture from your original report. You might be using a different mutant and hypothesis than was in your original report. If you needed to switch to someone else's mutant, you may use their picture. Make sure to cite them if you use their picture.

Data

1. Items 3 through 5 below will be for wild type if your mutant had no activity. Your results section will discuss your purified mutant regardless of which you used to determine K_m and k_{cat}.
2. <u>If you have separate data for an inactive mutant</u>, include that data in graphs of concentration of product vs. time. You should have two of these graphs.
3. For the K_m and k_{cat} determination, include copies of graphs of all your kinetic data. These graphs should be concentration of product (not absorbance) vs. time. Do this on five separate graphs, one for each concentration (three sets of data per graph). I'll be looking at these to see if your slopes are straight and to see if your replicate slopes are in good agreement with one another. There should be three separate slopes on each graph. Make sure to include the equations for each line on each graph (I cannot give you credit for your calculations without it). The extinction coefficient for 5,10-methenyltetrahydrofolate at 356 nm is 25100 $M^{-1}cm^{-1}$.
4. Include a Lineweaver Burk plot of your data. Make sure to include the equation for your line on this graph (I cannot give you credit for your calculations without it). <u>Do not average the points for each replicate</u>. I want to see the variability in the data.
5. Include the K_m and k_{cat} for your data. Make sure the units for these are molar and s^{-1}, respectively. If you used wild type enzyme, assume the stock had a concentration of enzyme that was 1.0 mg/mL. The molecular weight of MTHFS is approximately 22200 g/mole.

Calculations: Don't forget to include all your calculations.

Results and Conclusions

6. What do your measurements say about the role of your selected amino acid in MTHFS? Use the activity of your mutant for this discussion. Also include your original hypothesis for your mutant.
7. Explain any sources of error in the experiment.