

BL4820 BASIC BIOCHEMICAL TECHNIQUES

Lecture 4 - Glutamate Oxaloacetic Acid Transaminase (GOT) Purification -- Expt 3 Part A

Read in Text: p. 69-75

Lab Expt #3 - 1st Part in 4th Week - Expt #10 p. 109-113

What you will do in Lab: The purification of Glutamate Oxaloacetate Transaminase (GOT) from pig heart will be done over a 3 week period. **BE SURE TO SAVE SAMPLES AT EVERY STEP OF THE GOT PURIFICATION SO COMPLETE ANALYSIS CAN BE DONE ON ALL PARTS OF THE EXPERIMENT.** The following scheme will be used for the purification:

- **Week #4 Lab** - Crude extract GOT Assays, GDH contamination assay, Heat denaturation and preparation of Fraction I (Centrifuged crude extract called FI) and Fraction II (centrifuged heated crude extract - called FII).
- **Week #5 Lab** - Ammonium sulfate precipitation and preparation from FII of week #4 of the ammonium sulfate fractions - ASI is first precipitate, ASII is second precipitate containing the bulk of GOT which will be dialyzed, and ASIII the supernatant of the second ammonium sulfate precipitation step. Carry out Folin/Lowry protein assays on FI and FII.
- **Week #6 Lab** - Ion exchange (carboxyl methyl cellulose or CMC) chromatography on dialyzed ASII is done and fractions collected are assayed for GOT activity and absorbance at 280 nm for protein. Folin/Lowry protein assays on ASI, ASII, ASII after dialysis and ASIII are done. The peak fraction CMC fraction with the most GOT activity is saved for analysis by gel electrophoresis in the Week # 7 and 8 labs.

What you will turn in for the Lab Report: All data collected during the purification will need to be reported in tables and graphics. It is extremely important to make sure that you get all the data (from both your own work and that of your partners) so that a complete report can be written at the end of the 3 weeks of experiments.

General Introduction: All protein/enzyme purifications begin with a mixture of proteins and the objective of purifying one of these proteins/enzymes away from all the others in the mixture. To achieve this end you need 2 things - 1) a way to tell how much of the protein/enzyme of interest is present in the mixture and we call that the assay, which just means test method, for the protein/enzyme of interest; and 2) an assay for total protein such as the Folin/Lowry assay. To purify the protein you apply a series of steps which separates proteins and at each step assay for the protein/enzyme of interest and total protein. **Your goal is to improve the specific activity of the enzyme of interest and specific activity is defined as the ratio of your activity assay for the enzyme of interest divided by the total protein.** So as the purification proceeds through each step, the specific activity should increase. At the same time, you try to use efficient steps in

the purification so that you are able to retain a high yield. Usually, no matter how efficient your purification steps are you lose some of the enzyme of interest and so your yield goes down.

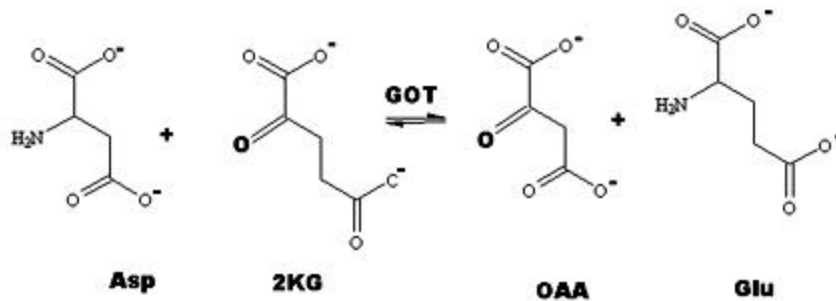
An Important Definition:

$$\text{Specific Activity} = \frac{\text{Enzyme Activity}}{\text{Total Protein}}$$

A key goal in enzyme purification is to increase Specific Activity to the highest value possible!

1. Glutamate Oxaloacetate Transaminase (GOT)

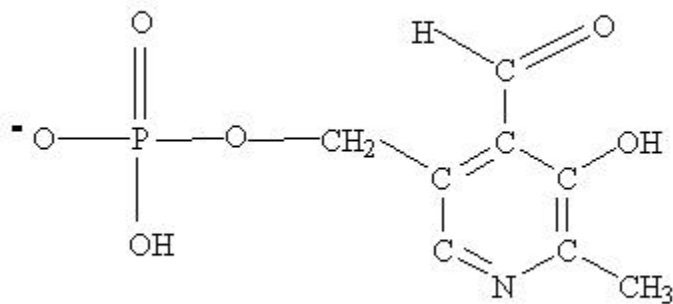
Glutamate Oxaloacetate Transaminase (GOT) catalyzes the transfer of an amino group from an amino acid (Glu) to a 2-keto-acid to generate a new amino acid and the residual 2-keto-acid of the donor amino acid:



Since the reaction catalyzed by GOT is completely reversible, I illustrate it here with Asp (an amino acid) as the donor of the amino group which will be transferred to the acceptor 2-keto group of the 2-keto-glutarate, which we sometimes call alpha-keto-glutarate. I wrote it this way since the official name of GOT is actually Aspartate Transaminase ([EC 2.6.1.1](#)). It is worth noting that the name "transaminase" can be replaced by "aminotransferase", which is useful to know since the class 2 enzymes are all called transferases. The Enzyme Commission number ([EC 2.6.1.1](#)) is a way to link to a Web page with properties of all enzymes which are known. At the web page for EC 2.6.1.1 you can find a list of properties of GOT and links to other databases with information on GOT including amino acid sequences and other data.

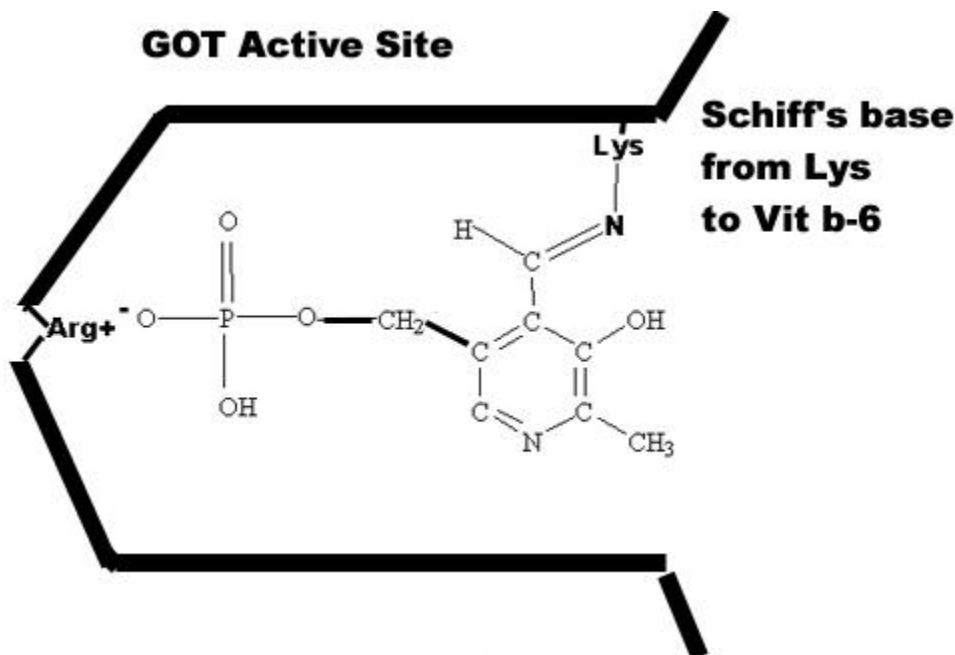
Transaminase, including GOT, are dependent on the cofactor pyridoxal phosphate, which has vitamin b-6 as its core:

Pyridoxal Phosphate



Vitamin B₆

This cofactor provides an aldehyde group to the enzyme, which is not available among the side chains of the 20 amino acids found in proteins. The phosphate group is added to the vitamin b-6 in our food and food supplements after it is ingested. The phosphate group provides a way to bind the cofactor to the enzyme via a strong ionic interaction. The aldehyde group readily reacts with primary amines like the alpha-amino groups of amino acids. This process activates the amino group so that it can be cleaved by water. This releases the keto-acid core of the amino acid and leaves the amino group on the enzyme. Now the acceptor keto-acid binds and reacts with the activated amino group to form the new amino acid. To illustrate how the resting enzyme looks, an amino group of an active site Lys's side chain amino group is shown bound to the cofactor in the GOT active site:

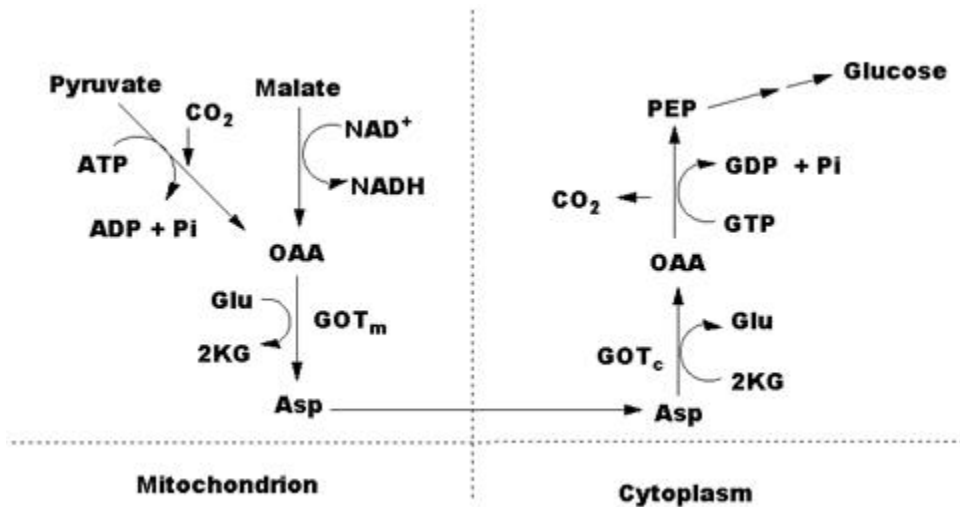


This resting form of GOT is ready for accepting the amino group of a donor amino acid (Glu or Asp), after the Schiff's base has been cleaved with water. The active site of the GOT also has amino acid side chains controlling the amino acids which bind and will act as donors of the alpha-amino group. In other words, the active site controls the substrate specificity of

transaminases.

2. GOT Physiological Function

Transaminases are involved in the cell in the formation of amino acids for which the cell can synthesize the 2-keto-acid. There are many different transaminases in the cell which basically differ only in substrate specificity. So you may find it very interesting that there are actually 2 forms of GOT in the typical mammalian cell such as those like the pig heart we are extracting the GOT from in this expt. Why are there iso-enzymes of GOT in the cell? (iso-enzyme means that there are 2 or more enzymes with the same substrate specificity but differ in amino acid sequence and in cell location). The existence of GOT iso-enzyme forms has to do with the cellular function of GOT being involved carbon compound transport:

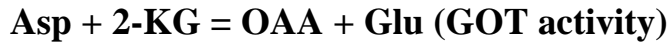


This graphic illustrates the role of the 2 forms of GOT, one in the mitochondria and one in the cytosol. The pathway shown is for synthesis of sugar (glucose) to replenish and keep your blood sugar at a normal level when there has been no recent intake of carbohydrates in your food. The pathway is called gluconeogenesis (see Fig. 10-3 in text). Here the mitochondrial GOT runs in the direction were are assaying it this lab, while the cytoplasmic GOT runs in the opposite direction to provide OAA (oxaloacetate) for synthesis of PEP (phosphoenolpyruvate) in the cytoplasm. PEP is the key intermediate for the synthesis of glucose, which can be exported to the blood from liver or heart cells. The origin of pyruvate in the mitochondrion is most likely amino acids resulting from the breakdown of proteins during fasting or between carbohydrate containing meals.

3. GOT Enzyme Activity Assay

To assay the activity of GOT we will use a different type of enzyme assay from that used in Expt #2 to assay the phosphatase, which was an end-point type of assay. To assay GOT activity we will use a coupled assay and monitor it continuously:

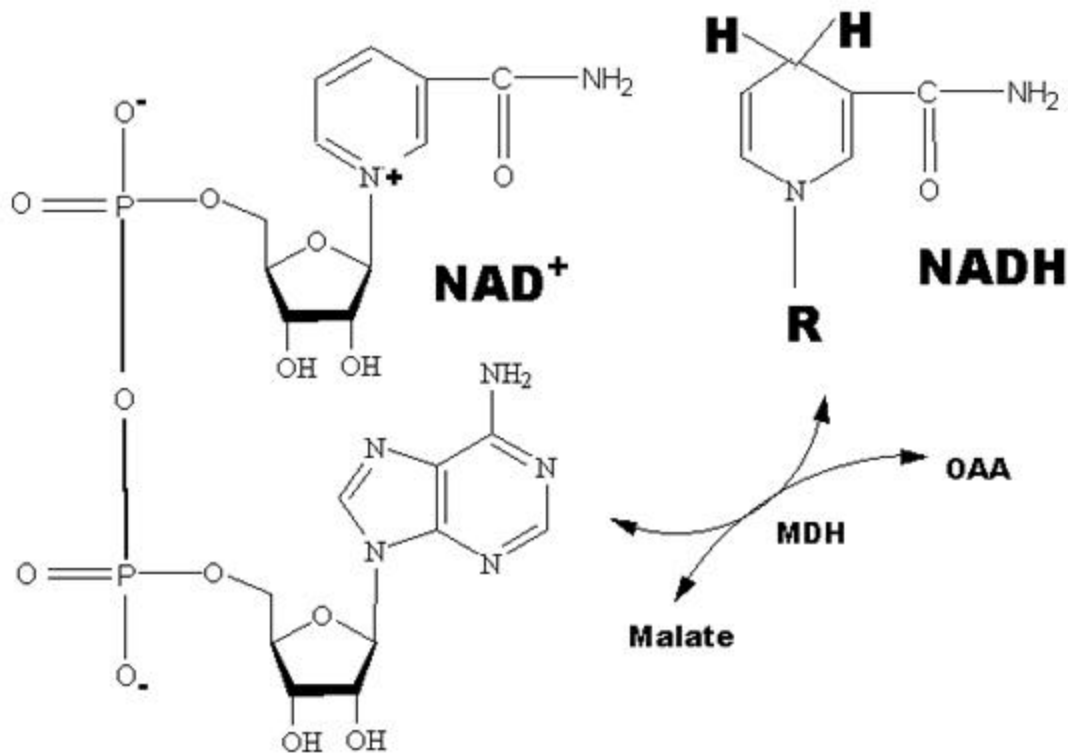
1.



2.



MDH (malate dehydrogenase) is added in excess so that it will not be limiting. This allows you to measure the activity of GOT since its activity level will limit the supply of OAA for the MDH activity. Thus, the OAA produced in the GOT catalyzed reaction will react with an equal amount of NADH. We will monitor the change in NADH using a spectrophotometer. The structure of NADH is shown below:



NADH is the reduced form of this cellular cofactor. When it donates its 2 electrons to OAA for its reduction to malate, NAD⁺ is formed. We can follow this process since NADH and NAD⁺ differ in spectral properties. NADH with its reduced pyridine ring (shown in the upper right corner of the above graphic) absorbs light at 340 nm, while NAD⁺ has the oxidized ring normally found in pyridine and lacks absorbance at 340 nm. So as NADH is converted to NAD⁺ during the MDH catalyzed reaction where the rate limiting OAA is converted to malate, the A-340 nm decreases. You can follow the course of the reaction by monitoring the change in A-340 during the reaction.

You will assay GOT by following the A-340 nm change with time, which will shown as a digital

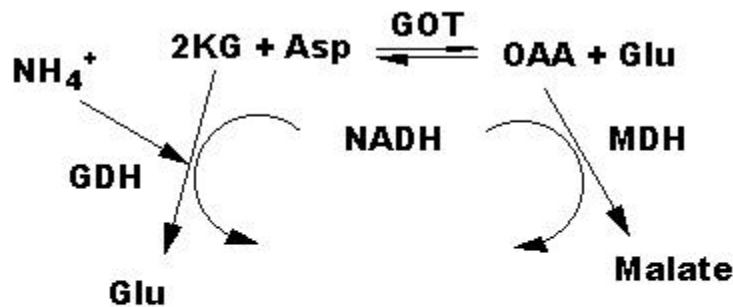
graphic directly on the screen of your spectrophotometers in the lab. Thus, you will **not** need to do the procedure described in the text involving taking time points and estimating the initial and final concentration of NADH (as discussed on p. 112 and Fig. 10-2). I will discuss in the next lecture how we will convert the A-340 changes with time or absorbance rates for GOT to enzyme activity measured in terms of μmol OAA produced per min.

4. Interference with GOT Activity Assay by Glutamate Dehydrogenase (GDH)

One problem with this method of assaying GOT is that other enzymes in the pig heart extract may also catalyze the oxidation of NADH. Therefore, you will assay for glutamate dehydrogenase activity (GDH):



This assay will be done by omitting Asp from the standard GOT assay and monitoring A-340 nm. Here is what may go on in your standard GOT assay if there is some ammonium ion in your pig heart extract which gets into the assay:



Side-Reaction Causing some extra Oxidation of NADH

Since you are following the oxidation of NADH by monitoring change in A-340 nm, if there is ammonium ion in your extract, the side reaction shown on the left side of the graphic above will cause extra NADH oxidation in addition to that catalyzed by GOT.

5. GOT Purification Steps Done in Week #4

Pig Heart extract will be prepared by the TA at the beginning of the lab session this week. You will use this extract to prepare a heated extract with part of the crude pig heart extract and with a small part of it you will prepare a centrifuged crude extract. With the unheated centrifuged crude pig heart extract (called Fraction I or F-I) you will determine how much GOT activity was present before you carried out the heating step. After heating the extract and centrifuging (called Fraction II or F-II), you will determine how much GOT activity remains and so you can figure out how much activity you lost during the heating step. Be sure to save samples of F-I and F-II

for protein assays you will do in the next lab. The bulk of F-II is saved for processing in the lab next week.

Be careful during the heating of the extract since the water bath will be near boiling. Also be careful not to over heat your extract since this is a key step in retaining enzyme activity.

Normally, you would not heat an enzyme to purify it because heat denatures most enzymes. But by adding the substrate 2-KG during heating, the GOT is protected from denaturation. Other proteins in the pig heart extract are denatured and precipitate so that GOT is purified by the heating step.

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