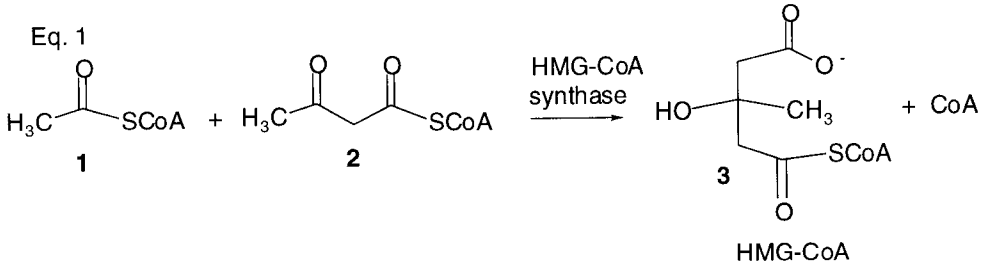
Exam 3 Chemistry 5.50

1 (50 points). 3-Hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase catalyzes the formation of a key intermediate in ketogenesis and cholesterogenesis. The reaction is shown in Eq. 1 in which acetyl-CoA (**1**) reacts with acetoacetyl-CoA (**2**) to produce 3-hydroxy-3-methylglutaryl-CoA (**3**).

Eq. 1



The following experiments have been carried out in an effort to propose a mechanism for this reaction.

i. Isotopic labeling experiments with the CoA moiety of acetyl-CoA (**1**) or acetoacetyl-CoA (**2**) indicate that the CoA in HMG-CoA is dervived from **2**.

ii. Incubation of the enzyme with a 50 fold excess of [^{14}C]-acetyl-CoA (**1**) at 0° C for 5 min followed by isolation of the protein by Sephadex chromatography and scintillation counting of the fractions eluted from the column, indicated that 1 equivalent of radioactivity migrated with the protein. Denaturation of the protein in acid followed by analysis on SDS-PAGE indicated that the label remained attached to the protein.

iii. If the protein isolated from the Sephadex chromatography experiment described in ii was immediately incubated with acetoacetyl-CoA (**2**), HMG-CoA was generated quantitatively. However, if the radiolabeled protein was allowed to sit for 2 hours prior to addition of acetoacetyl-CoA (**2**), only acetate was isolated.

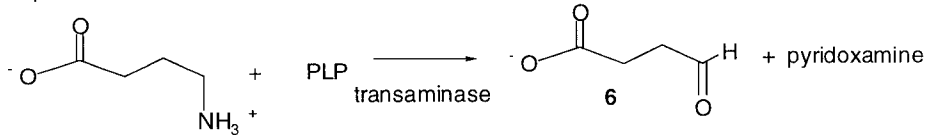
Questions (50 points):
1 (25 points). Based on this information propose a mechanism by which **1** and **2** generate **3**. Clearly show how each piece of information is accommodated by your proposal.

2 (13 points). Nature has in general chosen to use thiol esters rather than oxygen esters for this type of carbon carbon bond forming reaction. Why?

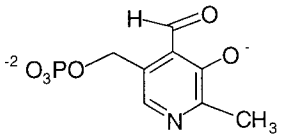
3 (12 points). Which of the two substrates (**1** or **2**) would you label and with which isotope to investigate the mechanism of carbon-carbon bond formation? How would you carry out an experiment to test your mechanistic hypothesis for this reaction and what might you predict the results to be?

2. γ-Aminobutyric acid (GABA) amino transaminase (GABA-AT) catalyzes the conversion of γ-aminobutyric acid to **6** as shown in Eq. 2.

Eq. 2



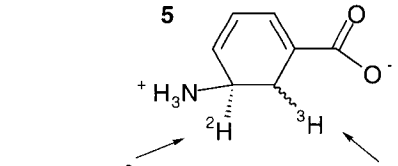
PLP is pyridoxal phosphate **4**



Note: α ketoglutarate then reacts with the pyridoxamine to convert it back to the active form of the cofactor and generate glutamate.

Gabaculine (5-amino-1,3-cyclohexadienylcarboxylic acid, **5**) a naturally occurring neurotoxin isolated from *Streptomyces* has been shown to be a mechanism based irreversible inhibitor of this enzyme. When **5** is administered to mice, the brain transaminase is rapidly inactivated and the brain levels of the neurotransmitter γ-aminobutyric acid (GABA) rise 20 fold. You are given the following information about the inhibition by **5**.

i. Incubation of the enzyme with [4-^{2}H]-gabaculine resulted in an isotope effect k_H/k_D of 2.3 on the rate of enzyme inactivation.



ii. Incubation of the enzyme with [3-^{3}H]-gabaculine resulted in ^3H_2O during the enzyme inactivation.

iii. When ^{14}C labeled gabaculine was incubated with the enzyme, radiolabel was associated with the protein concomitant with inactivation. Denaturation of the radiolabeled enzyme, however, resulted in complete loss of the radiolabel from the protein.

Questions (50 points). 1 (5 points). How is pyridoxal phosphate bound to this protein and all PLP requiring enzymes?

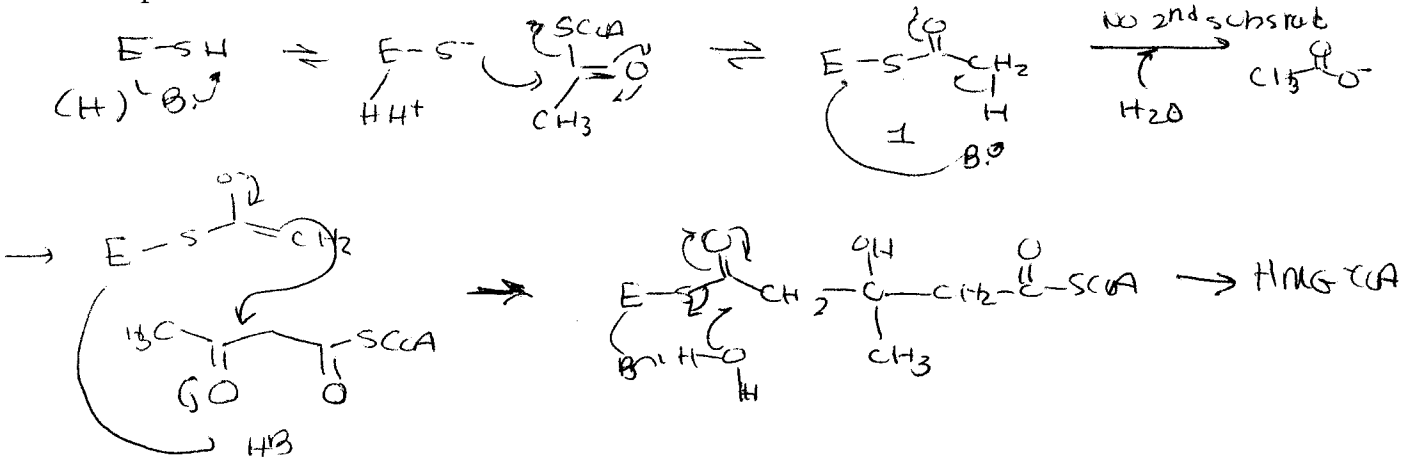
2 (15 points). Propose a mechanism for the first half reaction of the normal transaminase reaction.

3 (30 points). Propose a mechanism for the inactivation of this enzyme by gabaculine. Show how each piece of data (i through iii) is accounted for by your mechanism.

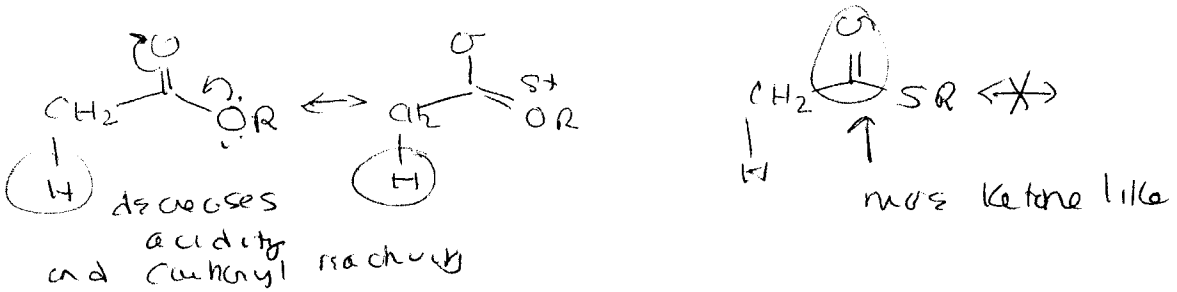
Answers Exam 3, Chem 5.50

Problem 1. This is an example of a Claisen reaction for the formation of a C-C bond. The fact that the CoA from **2** ends up in the product requires that **1**, acetylCoA is the substrate that is activated for C-C bond formation. The Sephadex data tells you that acetylCoA by itself is capable of forming a tight or more likely, a covalent intermediate with the protein and that it is stoichiometric with enzyme. (recall fatty acid biosynthesis) When the protein is denatured in acid, the label still is attached to the protein. This data tells you two things. 1. The linkage is covalent and not just tight binding. 2. The covalent linkage is stable to acid and this eliminates a number of amino acid side chains from consideration as being involved in this linkage. Histidine, aspartate, glutamate linkages are acid labile, as is the linkage with tyrosine. Since you find out in section iii that native protein sitting at neutral pH for several hours results in acetate production, this observation eliminates lysine as a covalent catalyst. You cannot distinguish between serine and cysteine. In this particular case cysteine is involved in covalent catalysis, but you cannot unambiguously establish this from the information given. The data in iii tells you that this covalent intermediate generated from acetylCoA is chemically competent as product is generated on addition of **2**. If you measured the rate of the reaction, you could also tell if the reaction was kinetically competent. As noted above, one also knows from this data that the covalent linkage to the acetyl group is chemically labile.

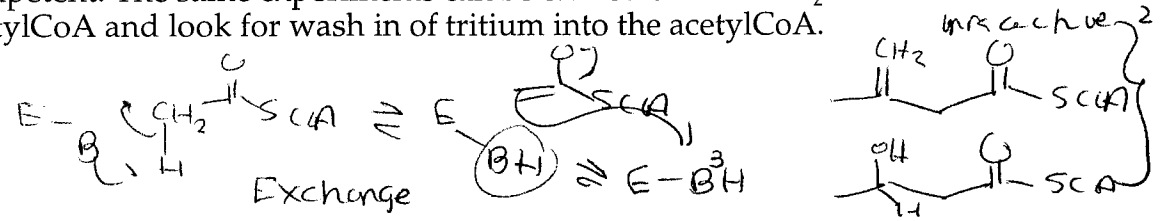
Proposed mechanism is shown below that accounts for the above information:



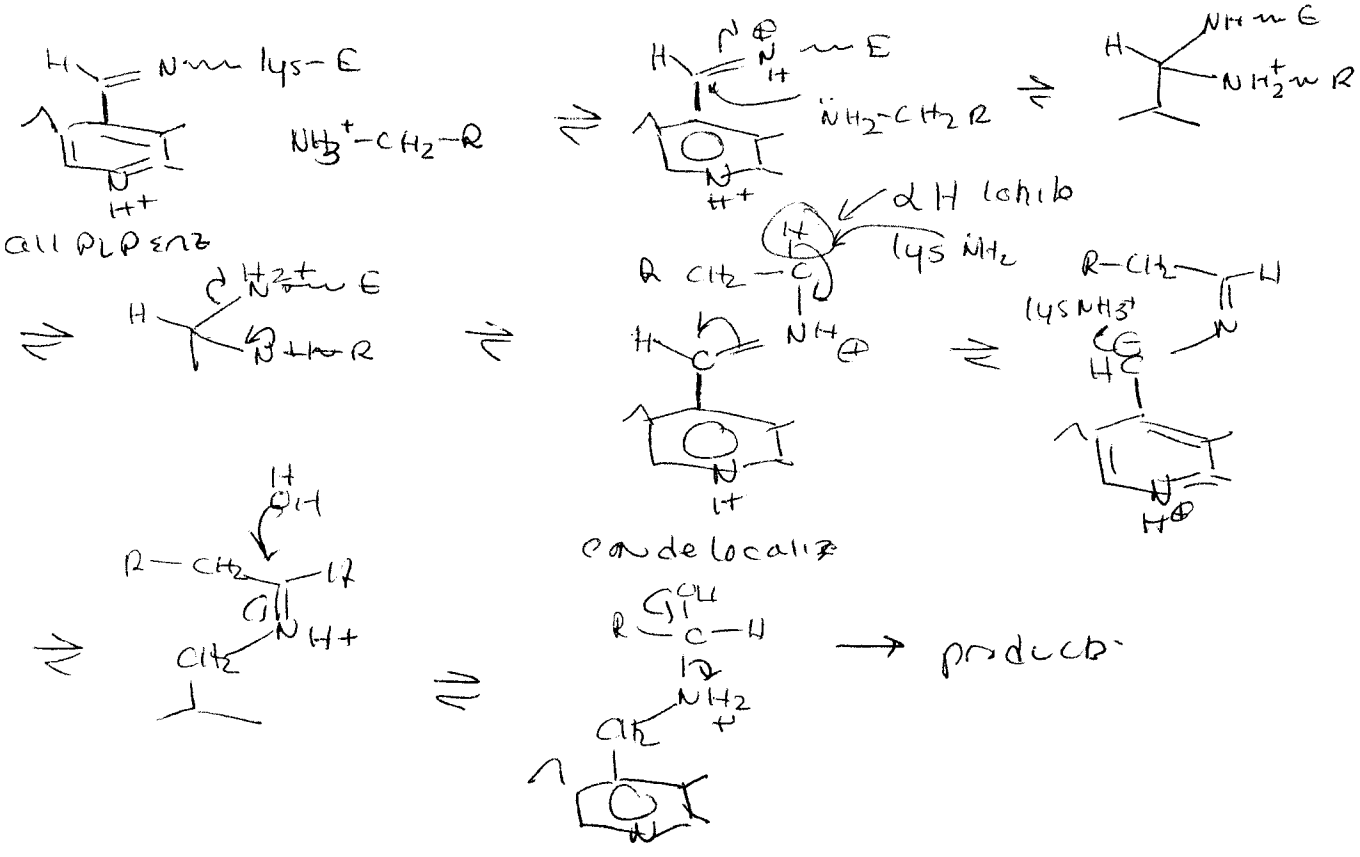
2. AcetylCoA and other thiol esters are widely used in biology rather than oxygen esters for two reasons, both relating to the ability of non-bonded electrons from oxygen of the ester to energetically overlap with the π electrons of the carbonyl group as shown below. Overlap is energetically much less favorable with S. This effect decreases the electrophilicity of the carbonyl (activates it for nucleophilic attack) and enhances the acidity of the α hydrogen that needs to be removed to generate a C-C bond. In the latter case (S), it is easier to stabilize the unstable enolate.



3. You would label acetylCoA to study the mechanism as revealed in part 1. A number of experiments are possible each with its pros and cons. You could place deuterium in the α position. If you chose to add 3 deuteriums, remember that if you see an isotope effect, it represents a combination of primary and secondary effects. You may or may not see a Vmax effect, depending on which step or steps are rate limiting. If you are looking for a V/K effect, that is if C-H bond cleavage occurs before the first irreversible step, then it is easier to use tritium. Measuring a selection with deuterium would require a mass spectrometer and isolation of the starting material and product as a function of extent of reaction. Many of you suggested use of tritium in the α position and to look for exchange in the absence of the second substrate. If you see tritiated water, and the rate is fast enough (kinetic competence), it suggests a carbanion intermediate. If the rate of washout is slow, then perhaps you need to use an unreactive substrate analog to alter the conformation and perhaps enhance the exchange rate (substrate synergism). Two examples of unreactive substrate analogs are shown below. If one sees no exchange, then the results are mechanistically uninformative, as the active site may be very sequestered. The same is true if the exchange rate is not kinetically competent. The same experiments can be carried out with ^3H_2O and unlabeled acetylCoA and look for wash in of tritium into the acetylCoA.



Problem 2. The reaction shown in Eq. 2 is a transamination reaction and follows all of the basic rules we learned in class as shown below. The first step is transimination with the lysine imine (reactivity enhanced relative to the aldehyde by protonation of the imine) in the active site. The second step is removal of the more acidic α hydrogen. The third step is hydrolysis of the ketimine to give pyridoxamine and the aldehyde.



For question 3, you need to recognize that gabaculine looks just like the normal substrate GABA. Thus the first two steps of the reaction are identical to the normal reaction. The removal of the α hydrogen is supported by the observed isotope effect of 2.3. This is an apparent isotope effect. Remember in the steady state, the observed rates result from contributions from a number of first order rate constants that can mask the intrinsic effect. The loss of ^3H to water tells you that this proton becomes labilized during the inactivation process. The standard way to activate the β position in PLP systems, is to first activate the α position (a general rule for PLP dependent systems). The driving force for this reaction would be aromatizaton of the ring. The data in iii tells you initially that you have a very tight binding or covalently bound inhibitor. Denaturation releases the inhibitor, suggest a non-covalent interaction, but one could also have a chemically labile linkage that is modified on denaturation. The mechanism shown below can be accommodated within these constraints.