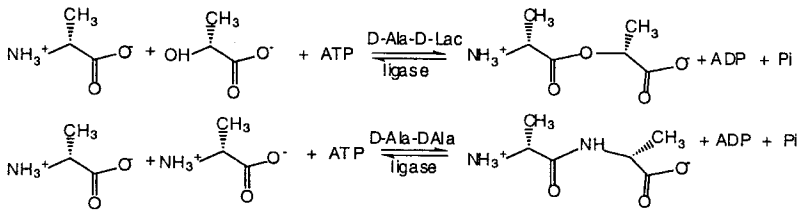
Problem Set 4 Chem. 550 Oct 14 due the 21

Bacterial cell wall biosynthesis has been an attractive target for antibacterial drugs including the vancomycin family of glycopeptide antibiotics. One of the mechanisms of the vancomycins is that they interfere with the processing of the peptidoglycan (PG) intermediates bearing D-alanine-D-alanine (D-Ala-D-Ala) termini. Covalent crosslinking of adjacent peptide strands in the PG layer provides mechanical strength to the cell wall. The transpeptidase enzymes makes the crosslinks with displacement of the terminal D-alanine. The vancomycins bind to the D-alanine-D-alanine PG and block the transpeptidase activity and hence crosslinking. Decreased crosslinking is one of the mechanisms of antibacterial activity. Problem 1 involves one mechanism of resistance to vancomycins. Understanding the resistance mechanism has allowed design of inhibitors of the enzymes responsible for resistance which then given with vancomycin, potentiate its effect.

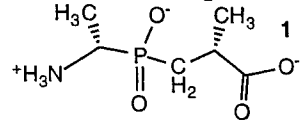
1. Clinically significant resistance to the antibiotic vancomycin in life-threatening infections by vancomycin resistant enterococci arises by reprogramming the PG termini of the enterococcal cell wall. The normal D-Ala-D-Ala dipeptide termini which have high affinity sites for vancomycin binding (see cartoon in Figure 1) are replaced by a D-Ala-D-Lac , depsipeptide termini. [D-Lac is lactate and a depsipeptide has an ester rather than an amide linkage, see eq. 1 below] Vancomycin binds D-Ala-D-Lac one thousand fold less tightly than D-Ala-D-Ala. A key molecular determinant of vancomycin resistance is thus the switch in specificity between a dipeptide ligase and a depsipeptide ligase. These ligases make the dipeptide or the depsipeptide that is then added directly to the growing peptide within the peptidoglycan. The structures of dipeptide ligase, D-Ala-D-Ala ligase (pdb 1IOW), and the depsipeptide ligase,D-Ala-D-Lac ligase (pdb 1EHI), have recently been solved and have been placed in the 5.50 folder under problem set 4. These enzymes catalyze the reversible reactions shown in Eq. 1.

Eq 1



The turnover number for D-Ala-D-Ala ligase is 650 min^{-1} and the turnover for D-Ala-D-lactate ligase is 44 min^{-1}. What is most remarkable given what you have learned about proteases is the ability of each of these enzymes to discriminate between the alcohol and the amino group of their respective substrates. The proteins are structurally homologous and members of the ATP grasp superfamily of proteins. You are given the following information about D-Ala-Dala ligase and D-

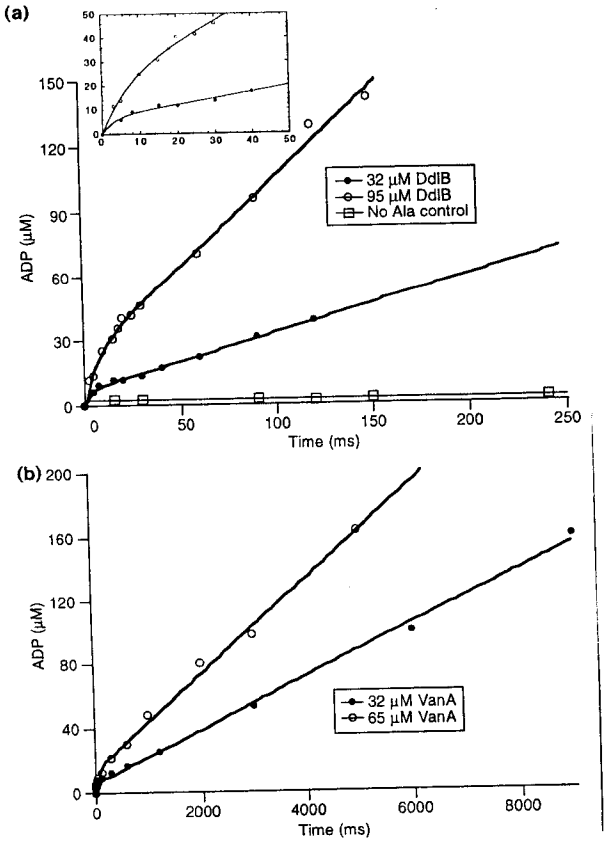
Ala-D-Lac ligase. Both proteins were crystallized in the presence of MgATP and compound **1** and the coordinates have been deposited in 1IOWpdb and 1EHI.pdb (see 5.50 folder).



The role of MgATP in this reaction has been examined using two methods we have discussed in class. 1. Rapid quench studies were carried out using either acid or EDTA (ethylenediamine tetraacetic acid) to quench the reaction. The amount of [^{32}P]-ADP was monitored as a function of time as shown in Figure 1a and b. The reactions were carried out as follows: For each time point, enzyme (32 or 65 μM) in 100 mM buffer, 10 mM $MgCl_2$ and 10 mM KCl were loaded into one syringe and [α^{32}P]ATP plus D-alanine and/or D-lactate in buffer were loaded into the second syringe. A computer-controlled motor pushed the plungers of the syringes into their barrels that forced the reactants together. The reaction mixtures were incubated for 3 ms to 9s and then quenched with either 500 mM EDTA or 2N HCl. The protein was removed and the supernatant was analyzed for [^{32}P]-ADP. The results of a typical experiment with D-Ala-D-Ala ligase and D-Ala-D-lactate ligase are shown in Figures 1a and 1b, respectively. The data in these figures were analyzed using equations analogous to those discussed in class for chymotrypsin.

$$E + \text{D-Ala} + \text{D-Lac} + \text{ATP} \xrightarrow{k_1} [E\bullet(\text{an intermdiate})\bullet \text{D-ala}\bullet\text{ADP} \xrightarrow{k_2}$$

$$E + \text{D-Ala-D-Lac} + \text{ADP} + \text{Pi}$$



Rapid quench analysis of ADP burst in the forward direction. **(a)** Effect of enzyme concentration on the burst amplitude and linear rate of D-Ala–D-Ala synthesis by DdlB. Assays contained 250 μM ATP, DdlB, 30 mM D-Ala, 10 mM $MgCl_2$, 10 mM KCl, and 100 mM HEPES, pH 7.8, at 30°C and were quenched with EDTA. Using 32 μM total DdlB, the burst rate is 18,000 min^{-1}, the linear rate is 620 min^{-1} (compared to the the steady-state rate of 658 min^{-1} from the lactate dehydrogenase/pyruvate kinase coupled assay), and the burst amplitude is 26% of the active enzyme concentration. The reaction with 95 μM total DdlB has a burst rate of 7900 min^{-1}, a linear rate of 650 min^{-1} (steady state rate of 658 min^{-1}), and a burst amplitude of 30% of the active DdlB concentration. **(b)** D-Ala–D-Lac synthesis by VanA. Assays contained VanA, 500 μM ATP, 10 mM $MgCl_2$, 10 mM KCl, amino acids and 100 mM MES, pH 6.2, at 30°C and were quenched with EDTA. Open circles, 65 μM VanA, 40 mM D-Ala, and 40 mM D-Lac; filled circles 32 μM VanA, 30 mM D-Ala and 30 mM D-Lac. Using 32 μM total VanA, the burst rate is 3,780 min^{-1}, the linear rate is 44 min^{-1} (steady-state rate of 43 min^{-1}), and the burst amplitude is 25% of the active enzyme concentration. The reaction with 95 μM total VanA has a burst rate of 1100 min^{-1}, a linear rate of 39 min^{-1} (steady-state rate of 40 min^{-1}), and a burst amplitude of 30% of the active VanA concentration.

$$Y = Ae^{-Bt} + L[E]t$$

where burst rate:

$$B = k_1 + k_2$$

and linear rate:

$$L = \frac{k_1 k_2}{k_1 + k_2}$$

and burst magnitude:

$$A = [E]\left(\frac{k_1}{k_1 + k_2}\right)^2 \left(\frac{1}{1 + K_m/[S]}\right)^2$$

2. [^{17}O, ^{18}O]-ATPγS made chiral by virtue of ^{17}O, ^{18}O was incubated with D-Ala-D-Lac ligase in the presence of D-Ala and D-Lac. The reaction was monitored by ^{31}P NMR and upon completion, the inorganic thiophosphate was isolated and subjected to analysis by the method of Trentham and Webb discussed in class. The results are shown in Figure 2.

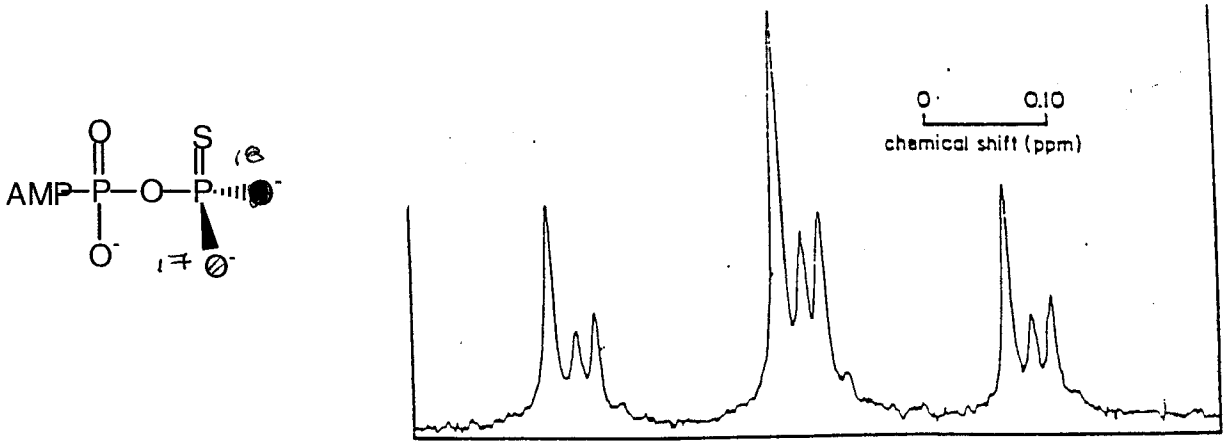


Figure 2 ^{31}P NMR spectra ATPβS derived from the inorganic thiophosphate from chiral [^{17}O, ^{18}O] ATPγS

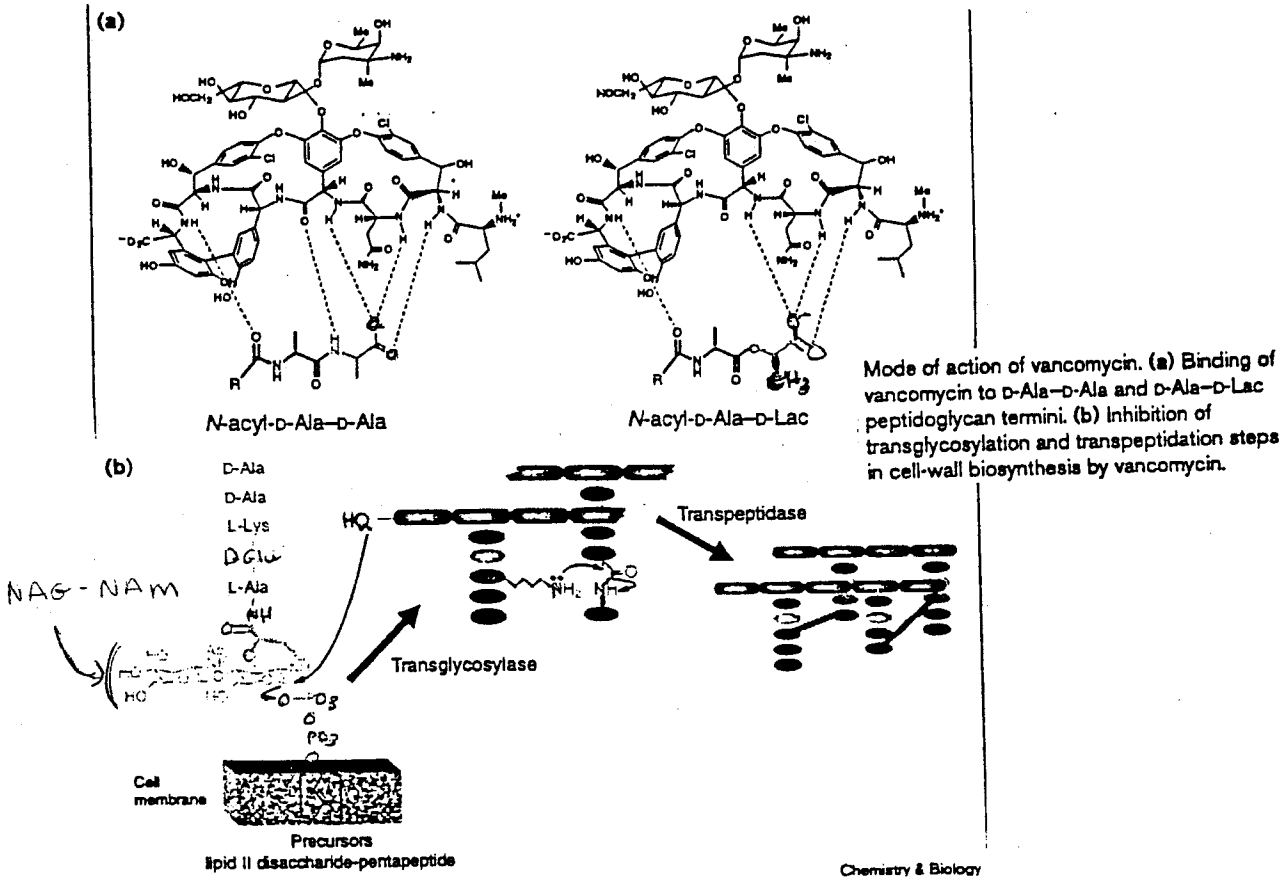
Questions:

1. Go to the Chemistry 5.50 web site and examine the structures of D-Ala-D-Lac ligase and D-Ala-D-Ala ligase. As you did in the previous problem sets draw the substrate or substrate analogs that you observe in the active site of each of these proteins and all the residues with which they interact. Label the amino acid side chains and indicate the distances between these side chains that may play an important role in catalysis and those of the substrate. What surprises have you observed in the active sites of each of these enzymes? Does this give you insight into the chemical

mechanism of this reaction? If you cannot answer the question at this time, return to this question after answering questions 2 through 4.

2. What are several possible conclusions that can you drawn from the rapid chemical quench data described in Figure 1? Does the structure help you distinguish between these options? If so, how?
3. What conclusions can you draw from the stereochemical experiment? Explain why the data are so messy and why it is important to start with both diastereomers of [^{17}O, ^{18}O]-ATPγS. Does the answer to this question, help limit the number of options from the rapid chemical quench data? Why?
4. Given the available information, propose a mechanism for D-Ala-D-Lac ligase and for D-Ala-D-Ala ligase.

Vancomycin and Cell Wall biosynthesis



Mode of action of vancomycin. (a) Binding of vancomycin to D-Ala–D-Ala and D-Ala–D-Lac peptidoglycan termini. (b) Inhibition of transglycosylation and transpeptidation steps in cell-wall biosynthesis by vancomycin.