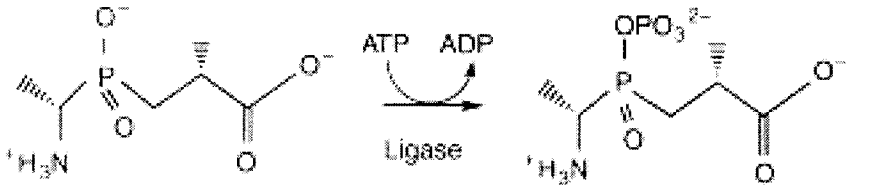
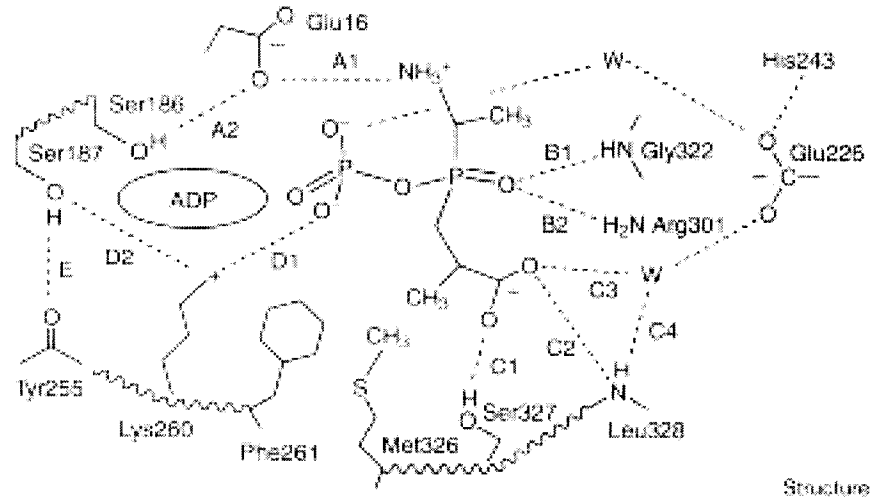
1. Activation of carboxylic acids (poor leaving groups) for amide or ester formation utilizes either one of two strategies. ATP either phosphorylates or adenylates (attaches AMP) the oxygen of the carboxylate. The equations describing the reactions of the two ligases, tell you that the carboxylate of D-alanine is potentially activated in both cases through the formation of a phosphoanydride. The x-ray structures of both ligases with analog 1 bound in the active site have been solved. The figures below are taken directly from Structure (2000) 8, 463-470. The converstion of 1 to 2 is proposed to a be a mimic of the normal reaction in which ATP phosphorylates the carboxylate of the D-Ala.



1 2 Structure

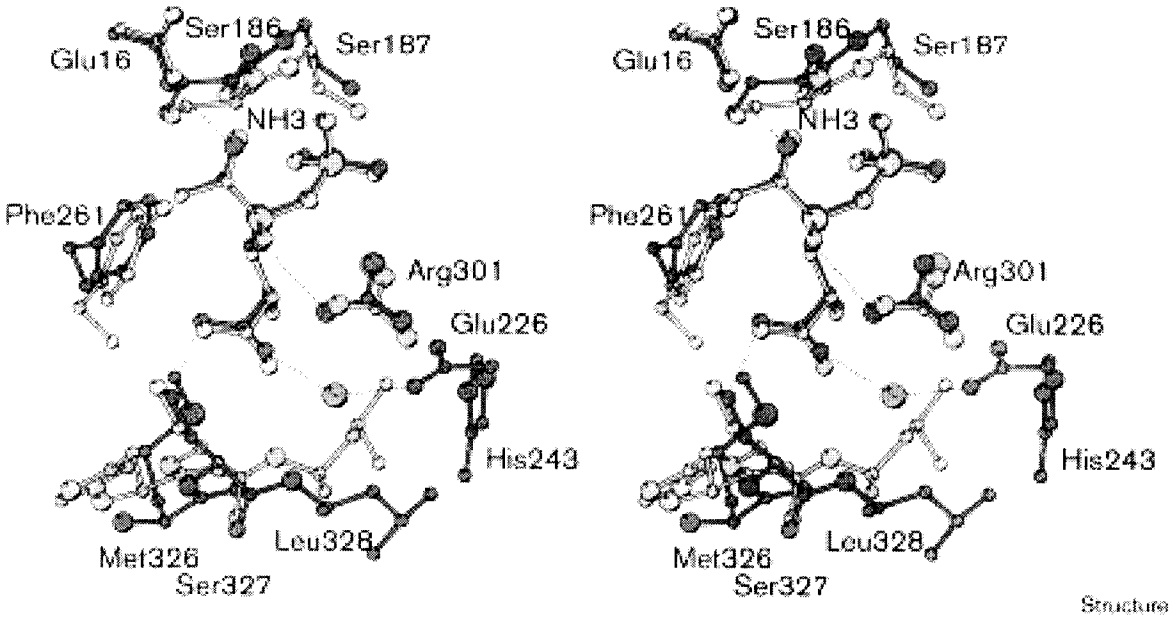
Both the phosphorylated inhibitor and ADP are bound in the active site. The inhibitor is very tightly but not covalently bound in the active site. It has a half life of 24 h in the active site! An excellent review has been published in Chemistry and Biology 7, R 109 to 19 (2000). Both Ligases are members of a superfamily of proteins called the ATP grasp superfamily. They all have the same structure and they all phosphorylate carboxylates with Mg^{+2} ATP.



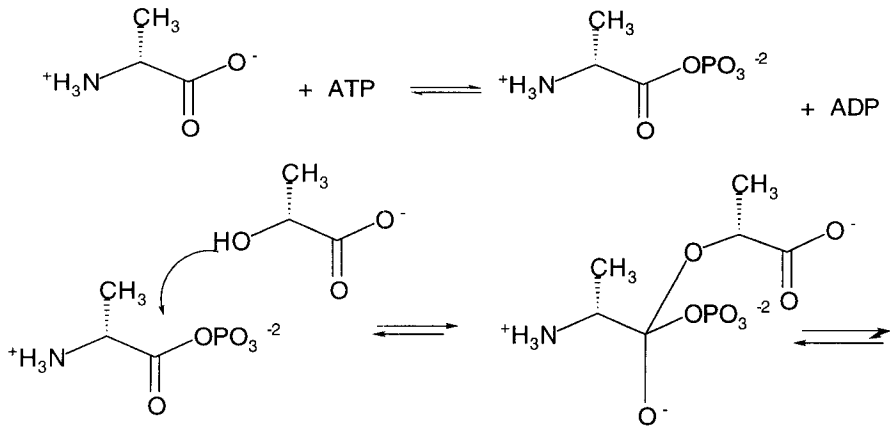
The distances of the indicated residues to the inhibitor are summarized in the Table taken from the Structure papers on both Ligases.

Distance	LmDdl2	DdlB-Y216F	DdlB
A1	2.9	3.0	2.8
A2	2.8	2.8	2.6
B1	3.1	3.0	3.2
B2	3.0	3.0	3.1
C1	2.6	2.6	2.3
C2	4.0	3.1	2.8
C3	2.8	2.5	2.4
C4	3.3	—	—
D1	2.7	3.0	2.8
D2	3.1	2.9	3.4
E	2.9	2.8	2.5

Below is an overlay of the phosphorylated inhibitor bound in the active site of both the ester and the amide ligase.



The following mechanism is the simplest one that can be proposed to accommodate the available data thus far.



2. The data in Figure 1 are very similar to those observed with chymotrypsin, the double mutant in the tyrosine phosphatase discussed in class, and the glycosyltransferase in problem set 3. The data for both enzymes suggests that in the first turnover ADP is released faster than all subsequent turnovers. There are many explanations for this observation. One explanation, given the structural data is that ATP phosphorylates alanine in both cases to give the phosphoanhydride of alanine and ADP. ATP could also phosphorylate the enzyme, as in the case of the tyrosine phosphatase and release ADP and then the phosphorylated enzyme could phosphorylate the alanine. Note this result would have different stereochemical consequences. Alternatively, there may be no covalent intermediate (enzyme bound or substrate bound) and the rate limiting step could be product release or a conformational change. In the case of both enzymes the size of the burst is not stoichiometric with enzyme, telling you that k1 and k2 both play a role in the rate limiting step in the first turnover. In addition, some of you may have noted that the rate constant for the burst varied with concentration of enzyme, while the steady state rate did not. Neither rate constant should vary, the rates should be proportional to the concentration of your catalyst. In the original paper, no explanation was provided for the rate constant difference in the burst.

The structures of both enzymes support a mechanism in which the ATP activates the carboxylate oxygen to make it a better leaving group via formation of a phosphoanhydride.

An aside based on recent studies in class: While you had not yet been introduced to exchange reactions at the time of this problem set, these methods could also be used to provide evidence for intermediates. The mechanism has been studied in detail by both types of exchange reactions. A positional isotope exchange study was carried out in which the non-bridging oxygen between the β and γ position of the phosphates in ATP was ^{18}O labeled and the reaction was incubated in the absence of the second substrate, lactate. As a function of time the recovered ATP could be examined for scrambling between the bridge and non-bridge positions for 18O at the β phosphate. This experiment cannot be carried out with D-Ala, D-ala ligase unless the Km for binding of the second alanine is much higher than the binding for the first Ala. A second type of exchange reaction requires product dissociation. Using ATP, alanine and [^{32}P]-ADP, [^{32}P]-ADP can be incorporated into ATP in the absence of lactate. Both types of

experiments have been carried out on the Ester Ligase. Both experiments revealed exchange, that is bridge, non bridge scrambling of oxygens in the case of PIX exchange reaction and ^{32}P incorporation into ATP in the case of second exchange experiment. The rates of exchange, however, in both of these reactions were substantially lower than the overall rate of the reaction and therefore, while the studies suggested the presence of an intermediate, the kinetic competence was not demonstrated.

3. Stereochemical studies on phosphoryl transfer, as discussed in class, are very informative mechanistically. If one observes inversion of configuration then one has an in line attack with no intermediate. If one observes retention of configuration, then one has two in line attacks, each with inversion, that leads to an overall retention of configuration. Thus a stereochemical experiment could potentially be informative. The mechanism involving a phosphoanhydride intermediate predicts inversion of configuration as the second step, nucleophilic attack occurs on the carbonyl and not the phosphate and hence the stereochemistry of the P group is not altered. A single in line attack of the carboxylate oxygen of the alanine on the γ-phosphate of chirally labeled ATPγS results in the inversion. The method of analysis of the inorganic thiophosphate isolated from the reaction mixture used the procedure developed by Trentham and Webb described in class. The analysis reveals that the reaction did in fact go with inversion of configuration supporting the phosphoanhydride mechanism. [The 17O obliterates (broadens all of the signals in the P NMR and the 18O causes a perturbation on the P chemical shift that is related to the bond order of the P-O bond. The messiness of the NMR spectrum relates to the method of analysis that yields ATP without any label, the inability to make 100% chiral starting material and the wash-out of label during the extended transformation of Pi into ATP so that the stereochemistry can be analyzed. Remember that this analysis requires a knowledge of the stereochemistry of each transformation.

4. One possible mechanism, the one favored for all ATP grasp superfamily members is shown above.