Problem Set 4 (C-C bond formation, phosphoryl transfer reactions and the role of ATP)

1. Chemists can use the same strategies as nature to make new carbon-carbon bonds stereospecifically using enzymes such as aldolases. One particularly useful enzyme catalyzes the reaction shown in Eq 1, where R (electrophile) can be highly diversified. This enzyme uses 2-keto-3-deoxy-6-phosphogalactonate as a substrate. The enzymes have a TIM barrel (α8β8) structure shown in Fig 1A. A blow up of a product in the active site of this barrel with a constellation of residues that might be involved in catalysis is shown in Fig 1B.

Eq 1
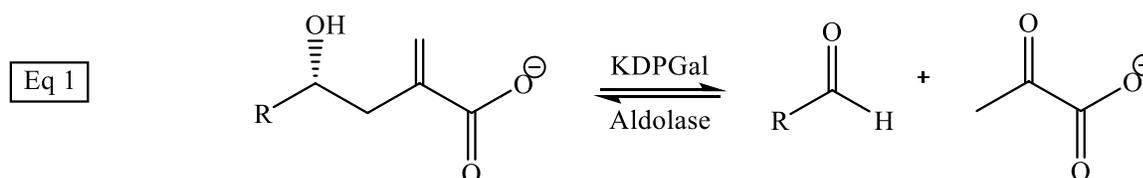
Electrophile	Nucleophile	KDPGal aldolase V_{rel}
D-Glyceraldehyde	Pyruvate	100
2-Pyridinecarboxaldehyde	Pyruvate	+++
D-Ribose	Pyruvate	+++
Chloroacetaldehyde	Pyruvate	++
D-Erithrose	Pyruvate	+++
L-Erithrose	Pyruvate	+
D-Threose	Pyruvate	+
L-Threose	Pyruvate	+++
Glycoaldehyde	Pyruvate	++++

A   B
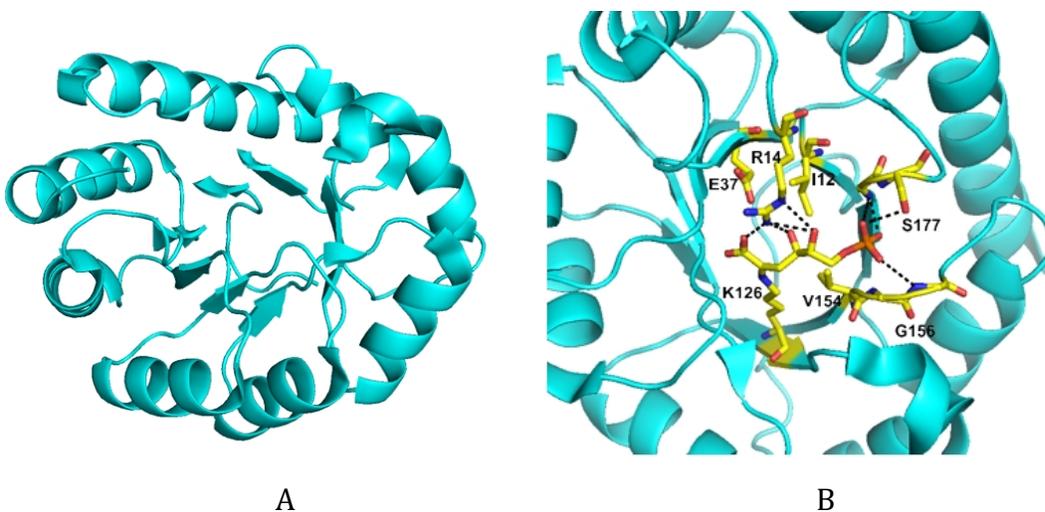
Figure 1. A ribbon diagram of the aldolase (A) and a close up of the active site (B) including the bound substrate.

Questions:
1) What does the structure tell you about the type of aldolase involved in the reaction in Eq1?
The structure is a type I aldolase which uses catalytic lysine to activate the substrate to form an imine which then enhances the acidity of the proton at Cα to facilitate formation of an enamine.
What does it tell you about the residues that might be involved in catalysis? You saw in class with the class I FBP aldolase that it utilizes an active site glutamate to deprotonate the OH to facilitate C-C bond cleavage and perhaps enamine formation. What the investigators are interested in is how the enzyme controls the stereoselectivity of the reaction. They suggested by looking at the structure that V154 might play an important role. They also suggested that the phosphate of the substrate interacts with S177 and G156. Note in Figure 1B above, the dotted lines indicate H bonding interactions. To use this enzyme in a practical fashion, it is important that R in Eq 1 can be just about anything, that it is as non-specific as possible, but that it retains its ability to add enamine to only one face of the RCHO.

2) Using the structural information, draw a mechanism (curly arrows using not AH or B for acids and bases, but amino acid side chains) by which the C-C bond can be made or cleaved.
The following is a mechanism proposed for a Class I KDPG aldolase from a thermophilic organism where many intermediates along the reaction coordinate have been trapped. The robustness of many enzymes isolated thermophiles make them attractive for practical engineering. This proposed mechanism was taken from Bioorganic and Medicinal Chemistry 14 (2006) 3002-10. As in the case I gave in the power point presentation in class on C-C bond formation as an example, this group was also able to trap a carbinolamine and an imine. What is interesting and also observed in the case of a completely different aldolase is the importance of conserved waters in many of the structures. Thus these authors have used waters to help get the different groups in the appropriate protonation states. One water remains bound during the entire catalytic cycle and appears to shuttle protons between the glutamate and the substrate. The second water is the result of dehydration of the carbinolamine and serves as a nucleophilic water for hydrolysis of the Schiff base. Of course from the structural data above, you could not possibly propose a mechanism as detailed as indicated below. However, in this type of aldolase, it is striking how K, E, and waters appear over and over again. The role of specific waters in the active sites of enzymes has been under-appreciated. Again note that you must deprotonate the K to make it a good nucleophile, and the glutamate (a general base catalyst) does not do this directly, as you might draw from available information, but through a water. Similarly, once the glutamate becomes protonated, it now functions as a general acid catalyst. We have now seen many times one group can function as GBC and then GAC, indicating a conservation of the required amino acids in the active site.
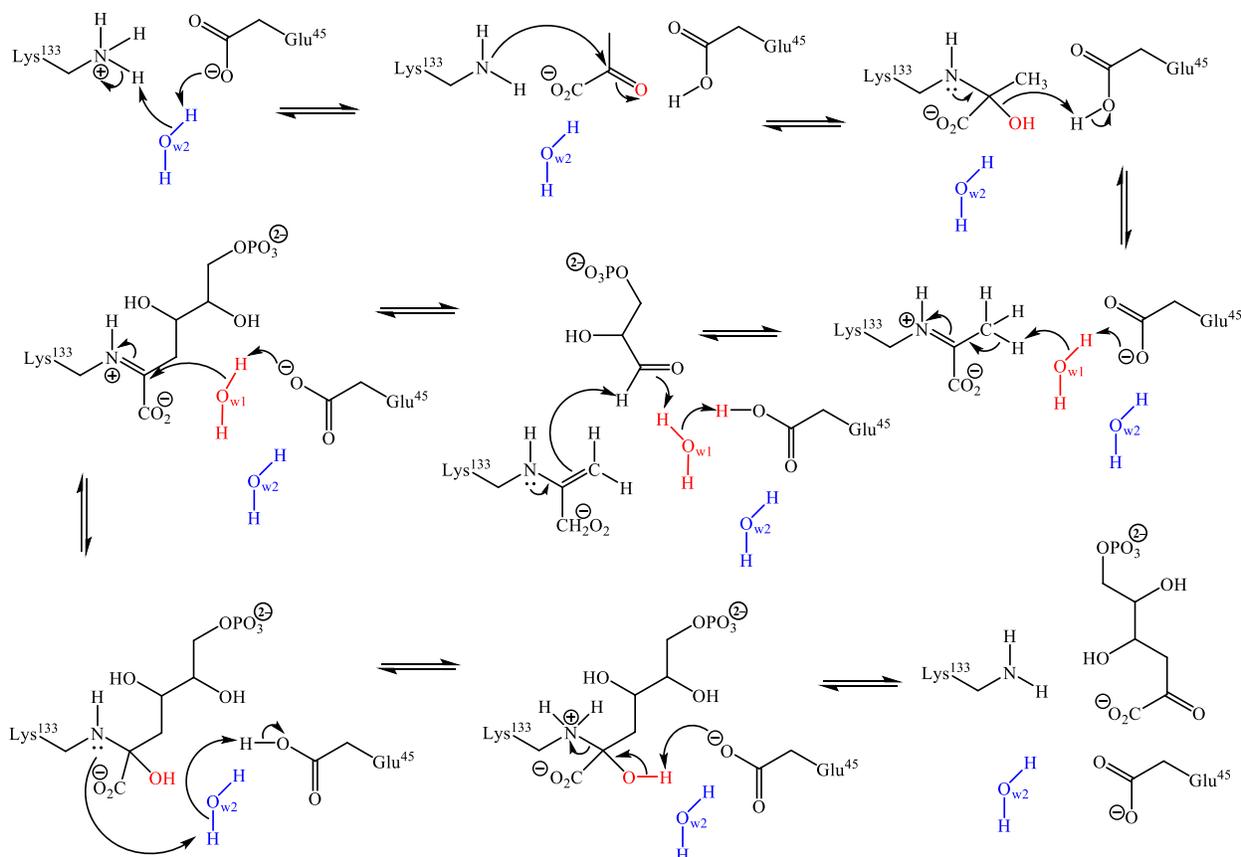
Courtesy of Elsevier. Used with permission.

3) Looking at the structure in Fig 1B and the bound substrate, can you rationalize the specificity of the enzyme suggested by the data in the Table above?

The enzyme appears to be rather non-specific for the RCOH, however, the +++, in place of k_{cat}/K_m is not very informative. One needs k_{cat}/K_m values. What is interesting is that you get turnover without any phosphate in the aldehyde. However, if you looks at the rate of the glyceraldehyde 3-P (GAP), a natural substrate, and compare it with the same aldehyde with the phosphate removed, the rate is 1% of GAP. Thus the phosphate does make a difference.

2. Under physiological conditions, HMG-CoA synthase catalyzes the conversion of acetyl-CoA and acetoacetyl-CoA to 3-hydroxy-3-methylglutaryl-CoA shown in (Eq 2). The back reaction (conversion of HMG-CoA back to acetylCoA and acetoacetyl-CoA) can occur in the test tube. HMG-CoA synthase is the second step in the pathway that makes the 5-carbon fragments required to make lanosterol and cholesterol. In patients with type I diabetes, this enzyme is overactivated and the excess HMG-CoA produced is shuttled into ketone bodies (acetoacetate and acetone) which will be discussed in the second half of the course. Studies have shown that the synthase that catalyzes this reaction has an essential cysteine (C111) in its active site. In Figure 2 is a diagram depicting the x-ray crystal structure of the synthase incubated with HMG-CoA.
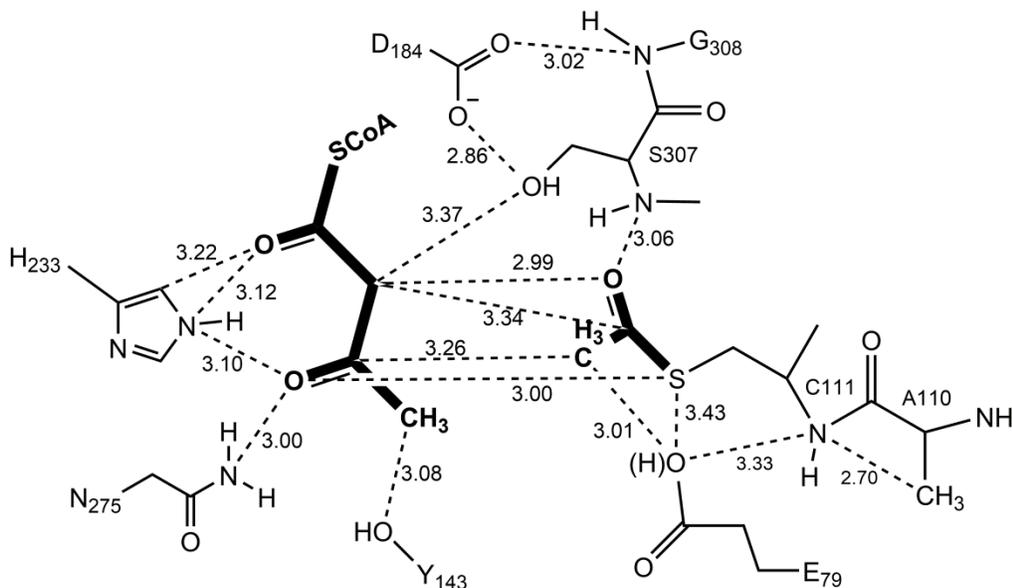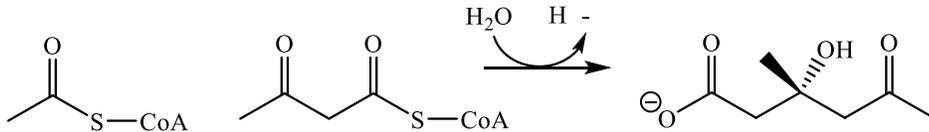
Figure 2. The structure (with distances in Å) resulting from incubation of HMG-CoA with synthase which took several weeks to crystallize prior to the solution of the structure.

Questions:
1. The acetylCoA ends up as the -CH₂CO₂⁻ moiety in HMG-CoA. Propose a mechanism (curly arrows and if possible specific residues that might be involved) for C-C bond formation and CoA hydrolysis in this reaction that uses the insight you can obtain from the structure (Figure 2).
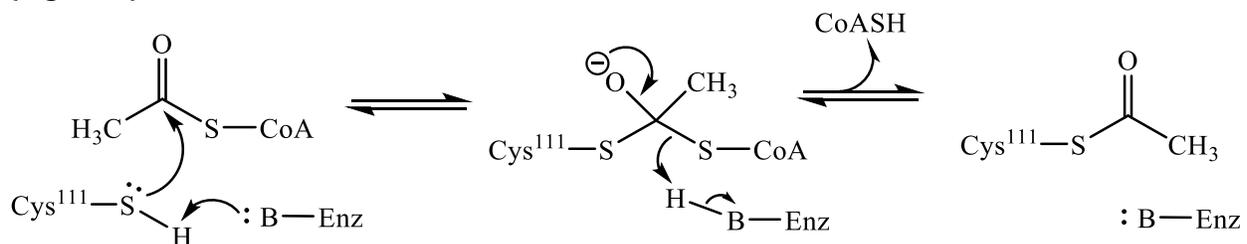
The structure in Fig 2 shows that Cys111 is acylated. It gives little information whether the His233, for example, could be close enough to this cysteine to facilitate formation of the thiolate and imidazolium (protonated histidine). However, in enzymatic reactions that use cysteine in covalent catalysis, His is often used to deprotonate it.
Remember you CANNOT SEE protons in X-ray structures in general. Thus their presence is based in the investigator's intuition. In the second step, E79 is used as a general base

catalyst. What is missing in the Eq above and in Eq B below is the H-bonding interaction (likely from an amide N-H to the carbonyl of the thioester which initially helps the substrate to bind, but then stabilizes, by shorter H bonds, the transition state or high energy tetrahedral intermediate of the reaction. Once you form the enolate, it attacks the carbonyl of acetoacetylCoA using the protonated His to polarize the carbonyl and to transfer a proton. In Eq C, you now need to hydrolize the thioester, a reaction catalyzed by the enzyme. One needs a GBC to activate the water, some way to stabilize the tetrahedral intermediate or transition state. The thiol is the best leaving group, but this step could also be catalyzed. This mechanism shows yet againthe importance of covalent and general acid/base catalysis.

B

C
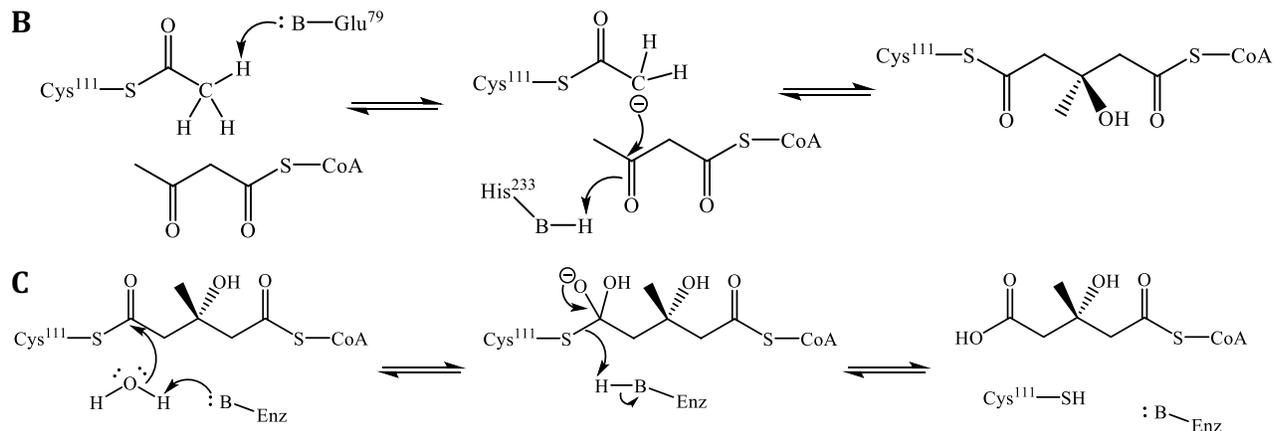
2) You have now encountered carbonyl chemistry in the mechanism of peptide bond formation by serine proteases and in the mechanisms of aldol and Claisen reactions. In each case you have learned that these reactions involve tetrahedral intermediates (high energy) or transitions states. Include these states in your mechanism in part 1, if you have not already done so. How would this type of intermediate be stabilized by the enzyme? The answer to this question is addressed above. Without additional information and numerous structures one can only propose mechanisms.

3) In class we discussed that enzymes are able to catalyze rates of reactions by 10^6 to 10^{15} times the rates of the uncatalyzed reactions. We learned about three distinct types of mechanisms that can contribute to this acceleration. Given your mechanism and the structure in Figure 2, propose mechanisms for rate acceleration with HMG-CoA synthase. Covalent catalysis and general acid and base catalysis are clear. How binding energy could be used in catalysis could be postulated. This energy is used to align the substrates and groups involved in catalysis to within a few tenth of angstroms. It could be used to stabilize the transition state, by making "better" or shorter H bonds than exist in the ground state. You need to desolvate your substrates on binding etc.

4) In Eq 2 and in metabolism in general, thioesters are made from coenzymeA. What is the structure of CoA? What would you expect the difference in chemical reactivity to be between thioesters of CoA and $HSCH_2CH_2NHCOCH_3$? Why?
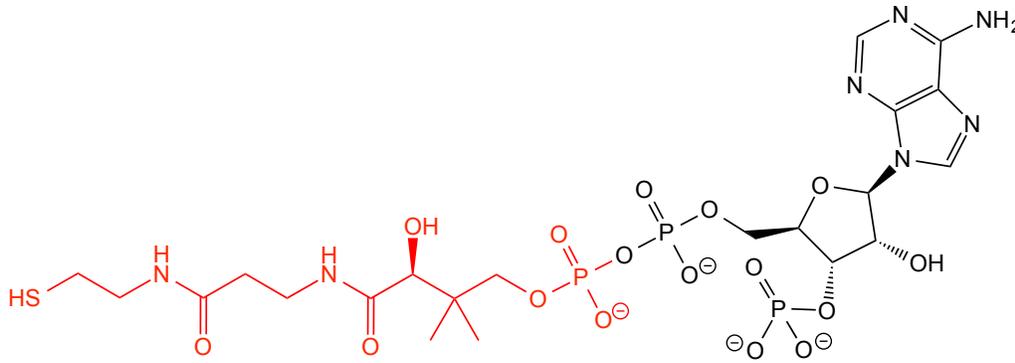
I would expect NO difference in chemical reactivity as the business end of the molecule is the thioester. The CoA might provide some binding energy, but the long arm is used to insert an acylated substrate into a deeply buried active site via a tunnel. In the case of fatty acid synthases tht we will discuss in the second half of the course, this type of arm allows movement into many active sites in a single mega (2.3 million Da) complex. All organisms use CoA, thus a role other than chemistry is important.

5) Rationalize why Nature uses thioesters rather than oxygen esters.
Thioesters, due to the resonance effects shown below, are much less stable than oxygen esters: their carbonyls are more activated for nucleophilic attack and their α hydrogens are more acidic.
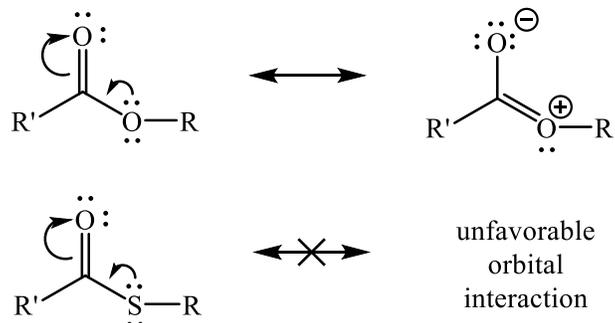
The non-bonded electrons from the O in the ester overlap favorably energetically with the pi electrons of the carbonyl. In the case of S, this is not true: orbitals are energetically mismatched. Thus S acts more like a CH_2R (ketone). Similar arguments can be made about stabilization of the enolate. In both esters and thioesters, the pK_a of the α H is not very acidic, but it is more acidic with thioesters than oxygen esters (18 vs 22).

3. AcetylCoA has a large free energy of hydrolysis:
$$\text{Eq 3.} \quad CH_3COSCoA + H_2O \rightarrow CH_3CO_2^{\ominus} + HS\text{-}CoA \quad \Delta G^{\circ\prime} = -31.5 \text{ kj/mol}$$
Nature uses ATP to make $CH_3COSCoA$.
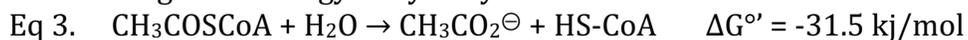
Questions:
1) If the sulfur S in Acetyl-CoA structure is replaced by O to make an oxygen ester, what would you expect its free energy of hydrolysis to be in comparison with AcetylCoA: more exergonic, the same, or less exergonic? Provide a chemical rational for your choice.

I would expect the ester bond to have a much lower free energy of hydrolysis. The thioester is more activated for hydrolysis than oxygen esters for reasons noted above. The product of the reaction in both cases is acetate. Thiolate is a better leaving group than the alkoxide.

2) Given the large free energy of hydrolysis for acetylCoA, its synthesis must use the energy currency in the cell, specifically ATP, to convert acetate into this molecule. Propose two distinct ways in which ATP could be used to activate acetate so that the thioester could be formed. Show the two mechanisms (curved arrows). Why is Mg^{2+} essential for this process given what you know about the properties of ATP?

One of the important roles of ATP is to phosphorylate or adenylate carboxylates of substrates [RCO_2^-], converting the OH into a better leaving group and activating the carbonyl for nucleophilic attack. Both phosphorylation and adenylation, if the charges are appropriately neutralized in the active site of the enzyme, provide a much better leaving group than OH^-. Furthermore, in contrast with esters where the carbonyl is made less reactive via resonance, in the mixed anhydrides the electrons delocalize over the P or the AMP, and thus the carbonyl is more activated for attack.

NH$_2$

Ad   Ad

+ A   +

carboxylic
acid   acyl-AMP intermediate   thio
                                                                ester

HO   OH
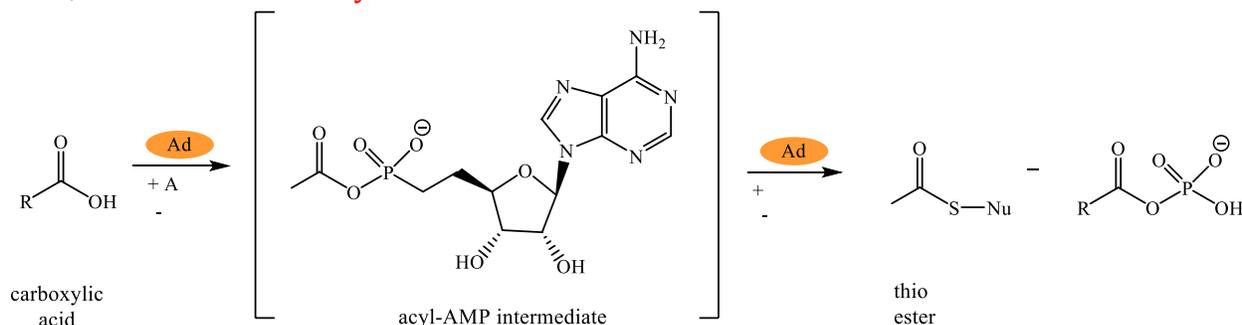
Mg^{2+} plays an important role in charge neutralization. Ligands to Mg^{2+} rapidly exchange, and the Mg^{2+} can change coordination during turnover. Initially it can reside in a coordination environment to facilitate the initial attack on the alpha or gamma P of ATP, and then it changes coordination environment to make ADP or PPi a better leaving group.

3. In one of your mechanisms, one of the products of the reaction can be further hydrolyzed to help drive the reaction of thioester formation to the right. Show how and why this could occur. Hint: Look in your book Table 14-4 "Standard Free Energies of Phosphate Hydrolysis of some interesting biological molecules" to help you answer this question.
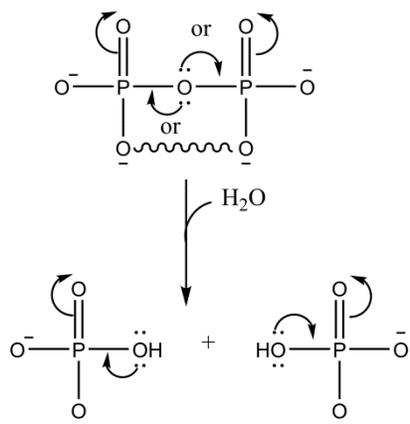
From Voet and Voet, the resonance structures and charge-charge repulsions between phosphoryl groups decrease the stability of the phosphoanhydride relative to the products. The free energy of hydrolysis of PPi is -33.5 kJ/mol and is very similar to ATP. The arguments are similar. Thus cells can drive a reaction toward completion by having inorganic pyrophosphatase, an enzyme that catalyzes this hydrolysis.

or

or

H$_2$O

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