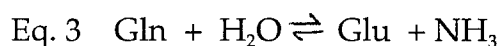
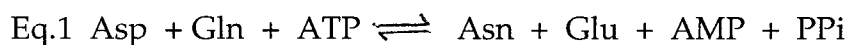


Exam 2 Chemistry 5.50

Problem 1 (70 points) In many biosynthetic pathways (purine and pyrimidine biosynthesis for example) glutamine provides the N as "NH₃" to make the purine and pyrimidine bases. Nature has evolved two different protein families to deliver ammonia from glutamine. They are mechanistically similar, and members of each family are structurally homologous. The two families, however, have structurally distinct folds. The enzyme asparagine synthetase uses glutamine to provide the ammonia required to form asparagine from aspartic acid (Eq. 1). This enzyme is a member of a superfamily of enzymes called glutamine amidotransferases. In addition to the reaction shown in Eq. 1, asparagine synthetase catalyzes two additional reactions shown in Eqs. 2 and 3.



The reactions shown Eq. 2 and that in Eq. 3 occur at separate and spatially distinct sites (Figure 1). A molecular tunnel connects these sites that are separated by 19 Å.

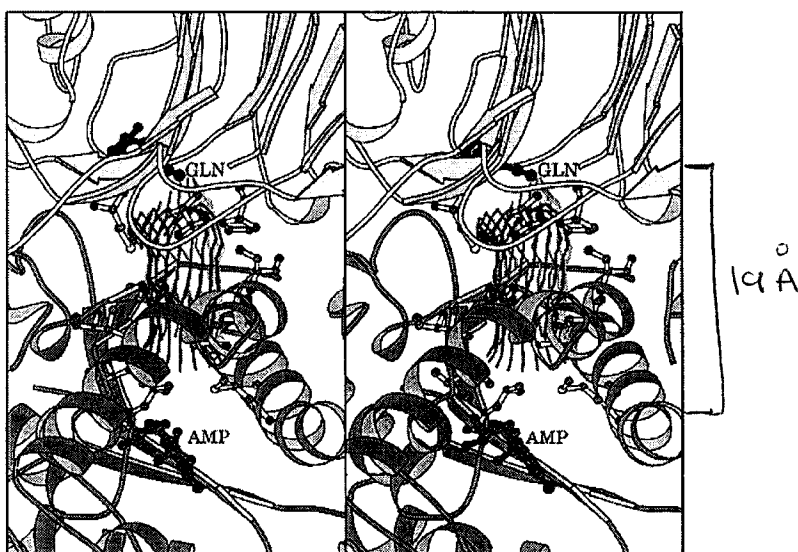
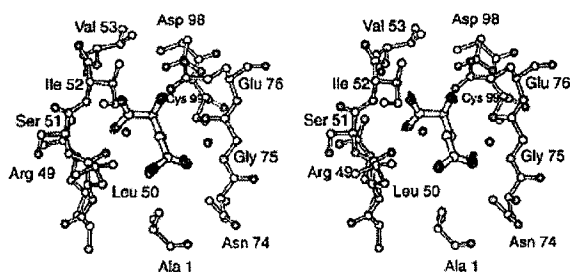
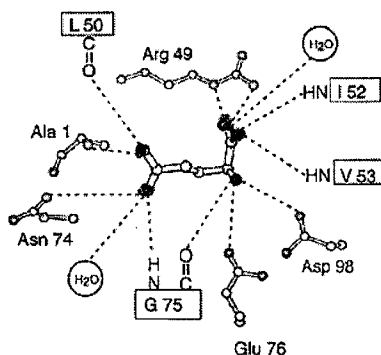


Figure 1 The two active sites of asparagine synthetase. The glutamine binding site shows GLN in a ball and stick rendition and the asparagine synthesis site shows AMP in a ball and stick rendition. The wire mesh is the putative tunnel between the two active sites. Reaction is prevented in the glutamine binding site by replacing cysteine at the 1 position (N-terminus of the protein) with alanine.



(a)



(b)

Figure 2. (a) A blow up of the active site where the glutamine reaction occurs. To prevent reaction, Cysteine 1 has been replaced by an alanine. The glutamine binding domain is at the N-terminus of the protein and you are looking at all residues within 5 Å of the glutamine binding site. Note that (b) is a rotated view of (a). Note, red is for oxygen and blue is for nitrogen.

You are given the following information about asparagine synthetase.

The two domains of the asparagine synthetase can be studied separately. A natural product 6-diazo-5-oxonorleucine (DON, shown in Figure 3A) is a potent inhibitor of the glutaminase activity. Incubation of [¹⁴C]-DON with asparagine synthetase followed by removal of an aliquot to assay for glutaminase activity is shown in Figure 3B. If the same inactivation study is carried out in the absence of ATP, but in the presence of aspartic acid and ADPNP (Figure 3A), the results are shown in Figure 3C. The glutaminase activity has also been shown to be eliminated in the presence of ICH₂CONH₂. The rate of inactivation as a function of pH has been determined and is shown in Figure 4.

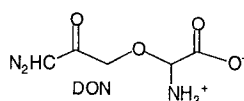
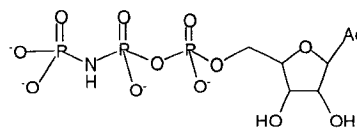


Fig 3A



ADPNP, a non-hydrolysable ATP analog

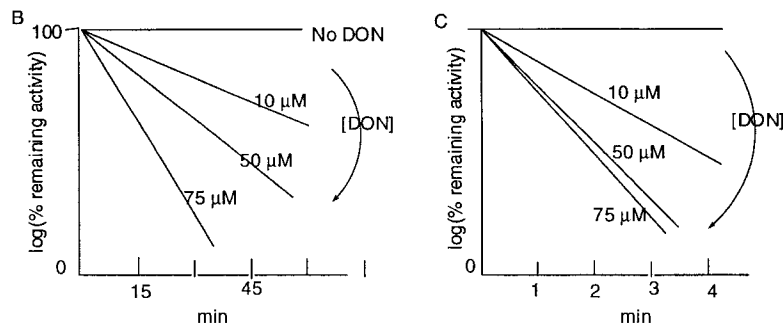


Figure 3 3A Structure of DON and ADPNP; 3B Results of [¹⁴C]-DON incubated with asparagine synthase and then assayed for activity. 3C [¹⁴C]-DON incubated with aspartic acid and ADPNP and asparagine synthetase and assayed for activity.

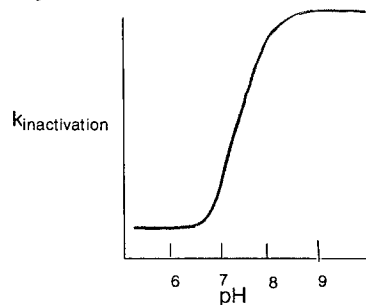


Figure 4 pH dependence of the Inactivation of the glutaminase activity of asparagine Synthetase by iodoacetamide.

Questions: (40 points)

- 1(10 points). Propose a mechanism for the inhibition of the glutaminase activity by DON.
- 2 (10 points). Propose an explanation for the differences between the inactivation data in Figures 2B and 2C.
- 3 (5 points). What does the data in Figure 4 tell you about the glutaminase activity?
- 4 (5 points). What does the structure in Figure 2 tell you about the glutaminase activity? What groups might be involved in catalysis?
- 5 (10). Propose a mechanism to account for the glutaminase activity.

As noted in Figure 1 there appears to be a tunnel between the glutamine and the aspartate/Mg²⁺ATP binding sites. The residues with side chains pointing into the tunnel are Met120, Ile142, Ile143, L232, Met329, Ser346, Ala388, Met392, Ser393, Ala399, Val401. All of these residues are conserved if one does a Clustal W alignment of 25 asparagine synthetase sequences from the genome sequencing projects.

Question (10 points)

1. (3 points) What does the conservation of the tunnel residues tell you? 2. (7 points) Are these the types of conserved residues that you would expect given the putative role of the tunnel? Why?

The second domain (C-terminus) binds aspartate and Mg^{2+} ATP. The chemistry of this domain has been examined. The following experiments have been carried out. When ^{18}O -aspartate (Figure 5A) was incubated with ATP and glutamine, the products AMP and P_i were isolated and examined by ^{31}P NMR. The results are shown in Figure 5B. Chirally labeled [^{16}O , ^{18}O]ATP γ S was incubated with aspartate and glutamine and the thiophosphate was isolated. The thiophosphate was subjected to the method of Trentham and Webb for analysis of the stereochemistry as discussed in class (see last page of the test).

Questions: (20 points)

1. (5 points). What does the data in Figure 5B tell you about the role of ATP in the asparagine synthetase reaction? Explain how the data in Figure 5B leads you to this conclusion. Show the proposed chemical transformation. 2. (8 points) The stereochemical experiment described above cannot work as described. How would you rectify the situation so that stereochemical information could be obtained from the experiment? 3. (7 points) For the stereochemical analysis of Trentham and Webb to work, what are the two known experimental facts that will allow one to interpret the results of the ^{31}P NMR data?

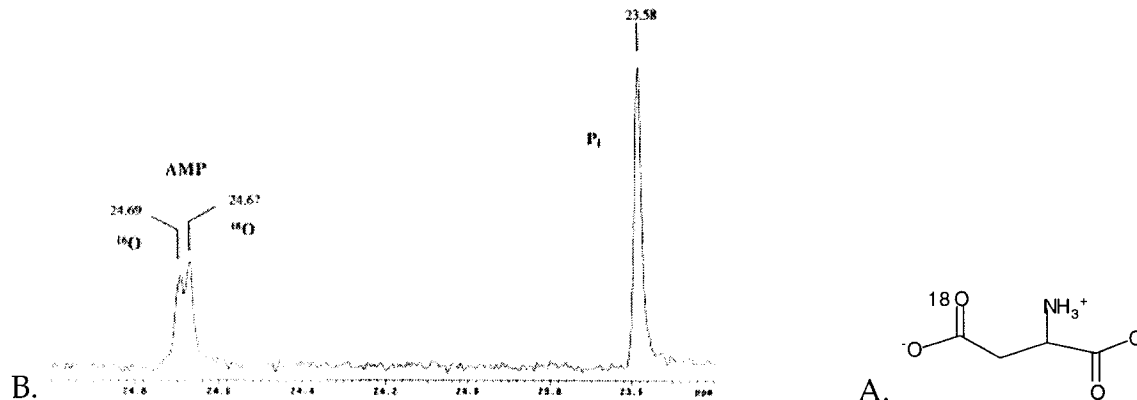


Figure 5. A. The structure of [^{18}O]-aspartate. B. The ^{31}P NMR spectrum of the products of the reaction of [^{18}O]-aspartate, ATP and glutamine with asparagine synthetase.

Problem 2 (30 points)

Renin is an endoprotease that has been a major target of drug companies in the design of new antihypertensive agents. Early studies on the substrate specificity of this protein, changing one amino acid at a time, suggested that the enzyme has a preference for aromatic and hydrophobic amino acids in the P1 and P2 binding sites. Compound **1** (Figure 6) has been shown to be an excellent substrate for this enzyme with a K_m of 10^{-6} M and a k_{cat} of $50s^{-1}$. Many studies have been carried out on this protein in an effort to define the catalytic mechanism, which would assist in rational design of potent inhibitors. You are given the following information about this system.

a. Incubation of varying concentrations of renin with saturating amounts of substrate **1** and analysis of product, p-nitrophenol, using stopped-flow Vis spectroscopy gave the results shown in Figure 7. b. Compound **2** (Figure 6 is a potent inhibitor of the enzyme).

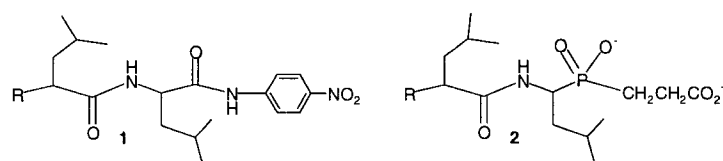


Figure 6. The substrate (**1**) and the inhibitor (**2**) for renin. The K_i for **2** is 20 nM.

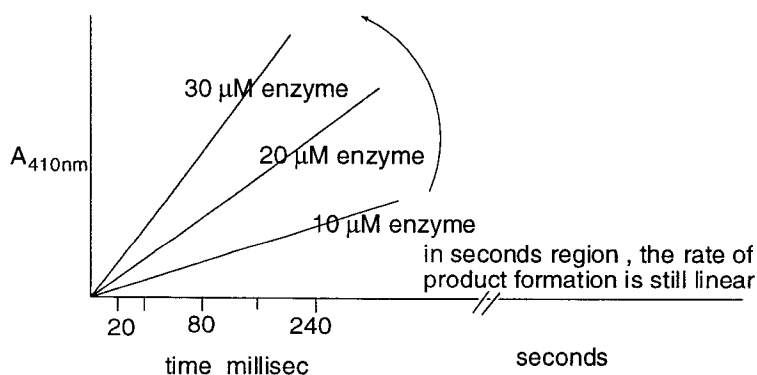


Figure 7. Stopped Flow kinetics experiments monitoring the rate of release of p-nitrophenol as a function of renin concentration.

Questions (30 points)

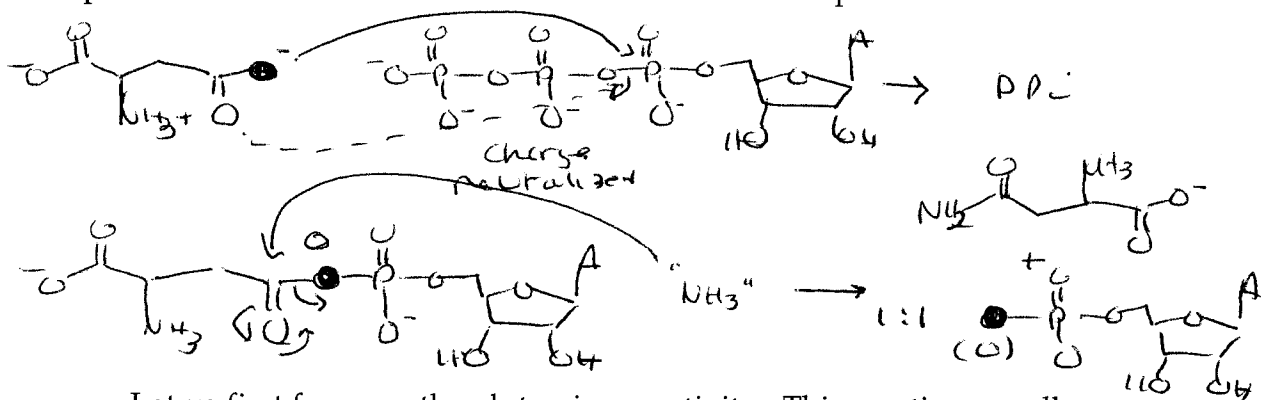
1. (15 points). What does the kinetic data in Figure 7 tell you about the mechanism of renin? 2. (15 points) What does the potent inhibition of renin by **2** tell you about the mechanism? Does it allow you to distinguish between cysteine and serine proteases and zinc and aspartate proteases? Why?

Answers Exam 2

1. This problem represents a joining of reaction mechanisms analogous to the cysteine proteases discussed in class and the phosphoryl transfer reactions given in problem set 4. The methods covered to examine the mechanism were structure and site directed mutagenesis, use of mechanism based-time dependent inactivators and competitive inhibitors and use of group selective reagents. In addition the use of stereochemistry and analysis using ^{31}P NMR and the ^{18}O perturbation on the P chemical shift were used to examine mechanism.

Asparagine synthetase uses ATP to activate the carboxylate of aspartate by adenylation, for nucleophilic attack. This activation is similar to the activation in D-ala-D-ala ligase using ATP. In this case activation was via phosphorylation. Both of these mechanisms are widely used in biochemical transformations. The asparagine synthase catalyzes several reactions in addition to Eq. 1. Both of these reactions proceed at a reduced rate relative to the normal reaction, but have been mechanistically informative.

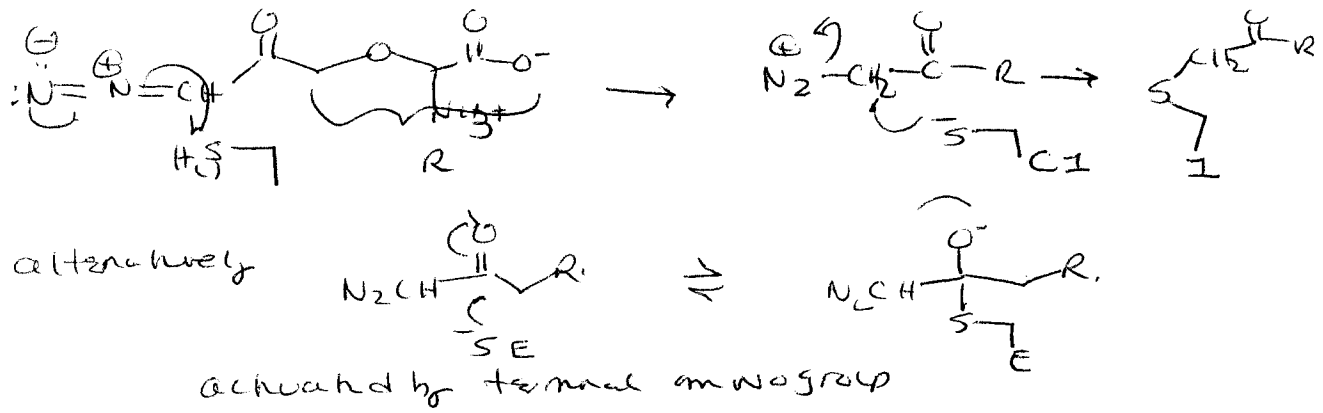
Proposed mechanism for the transformation shown in eq. 1



Let us first focus on the glutaminase activity. This reaction usually proceeds at 1% the rate of the normal reaction when the other substrates are present. This is an example of substrate synergism and is not surprising in that you would not want to release ammonia unless the aspartate is activated to form asparagine. Formation of glutamine also requires ATP and thus if you had high levels of glutaminase activity, you would be wasting energy by ATP hydrolysis. You were given a number of pieces of information about the glutaminase activity to help you think about a mechanism for ammonia release. First you were given the structure. The glutamine binding site is 19 Å from the site where aspartate is activated. A tunnel connects the two sites. In all glutamine requiring enzymes examined to date this turns out to be the case. Why nature might have chosen this strategy will be discussed subsequently. The structure (Figure 2) was obtained with a mutant in which the N-terminal cysteine was replaced with an alanine. This result and the closeness of the alanine to the glutamine, tells you that cysteine most probably plays an essential role in the chemistry on glutamine. If you look at this chemistry, you are hydrolyzing a peptide bond, as we have previously discussed with cysteine proteases. Thus one option could be that the cysteine is involved in covalent catalysis. Other options are also possible, but it is a good bet looking at the active site, that cysteine must be invoked in the catalytic mechanism.

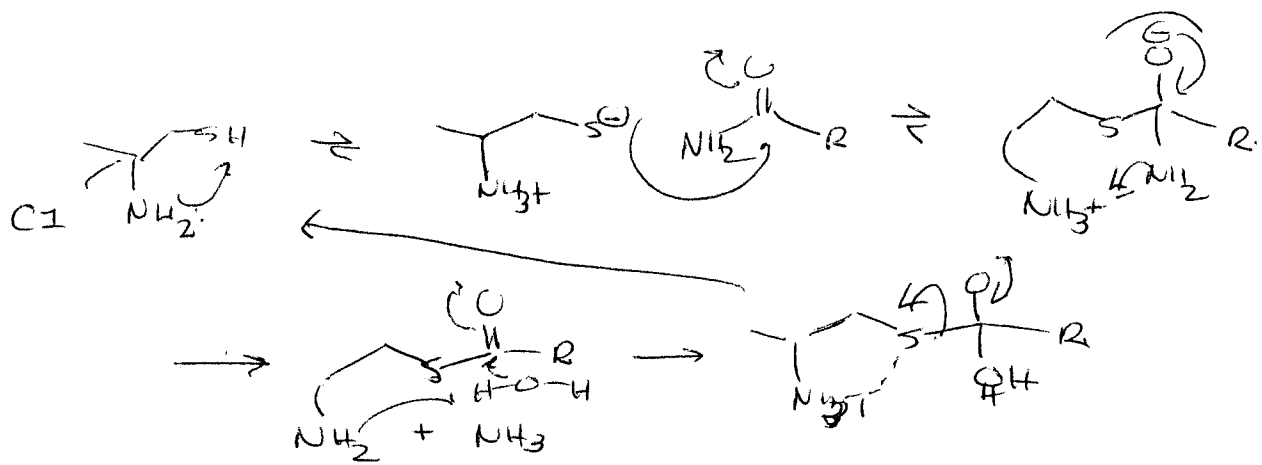
The second piece of information is that DON is an inhibitor and the data in Figure 3 tells you that inhibition is time dependent, suggesting irreversibility and potentially covalent modification. Recall we discussed in class aldehydes

and trifluorinated ketones as inactivators of cysteine proteases. The experiments are carried out by incubation of DON with enzyme and as a function of time diluting a small aliquot of the inactivation mixture 10^3 fold into an assay mixture containing gln, ATP and aspartate. The control shows you that no activity is lost over the time of the experiment. Dilution into the assay mixture, removes the inhibition by ADPNP. What you notice in Figure 3B and C is that the inactivation is potentiated in the presence of aspartate and a non-hydrolyzable ATP analog. This suggests substrate synergism. Ammonia will not be released from the glutamine binding site until the other substates are bound and ready to react. The inactivation rate is much faster when aspartate and ADPNP is present. This inhibitor is known to work as is shown below. Other mechanisms are possible. Many of you drew tetrahedral intermediates, analogous to the mechanisms shown for cysteine proteases. In both cases one has covalent modification of the active site, irreversible inhibition, under the assay conditions. Tying up more active sites results in the time dependent inhibition observed.



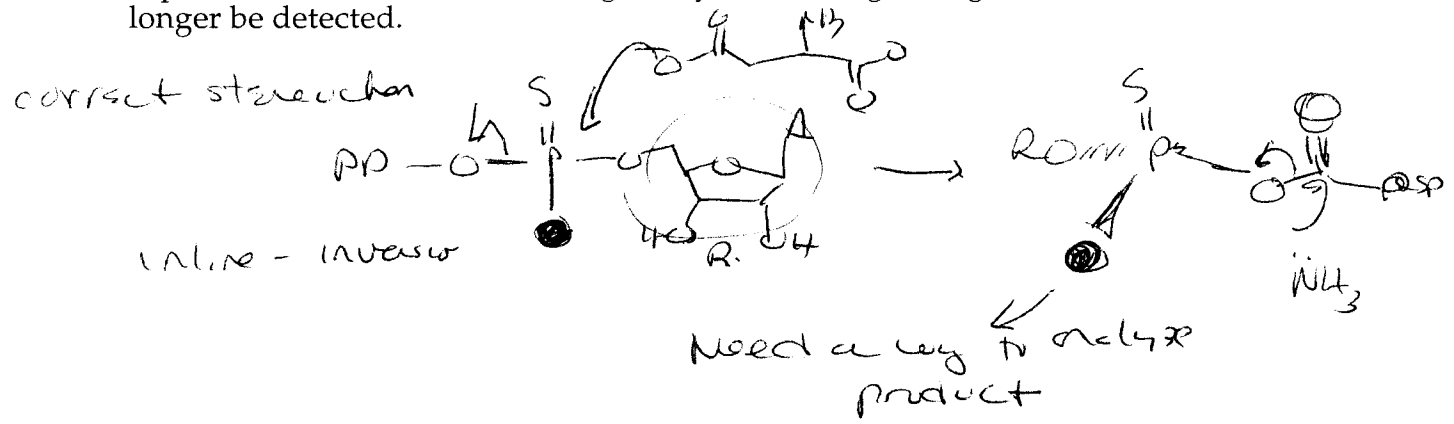
An additional piece of evidence for the importance of cysteine comes from the use of the group selective reagent, iodoacetamide. Iodoacetamide is a group selective reagent for cysteine, as discussed in class for tyrosine phosphatases. The inactivation rate is increased when some group with a pKa of about 7.5 is deprotonated. The pKa, can be perturbed by the environment. Inactivation by cysteine is one possibility. This could be easily experimentally tested by using radiolabeled iodoacetamide and isolating the modified protein, degradation with a protease, isolation of labeled peptides and sequencing to show the label is attached to the N-terminal cysteine.

Thus all of this data supports a typical cysteine protease mechanism except that there is no histidine in the active site to help deprotonate the cysteine and activate it for nucleophilic attack. It turns out that the amino group at the N-terminus of a protein, has a lower pKa than the amines of lysines and these groups have been found in a number of cases to act as general base catalysts. This was a subtlety of the reaction mechanism that you may or may not have picked up on. The only other groups in the active site that could possibly serve this function would be the carboxylates. Alternatively the pKa of cysteine could be perturbed by the active site environment, however, it is not obvious from the structure which groups would be involved in this perturbation. All of this data could result in a mechanism shown below.



A second mechanistic issue is how the ammonia gets from one active site to the second, 19 Å removed. Diffusion is rapid and much faster than the overall rate of the reaction. Nature wants to avoid protonation of the ammonia to an NH_4^+ as now the species is no longer chemically reactive. The hypothesis is that the tunnel is important and hydrophobic to avoid protonation. If ammonia diffused out of the glutamine binding site into solution, it would immediately be protonated and then would need general base catalysis to activate it for attack on the phosphoanhydride. An experiment to show that ammonia never gets into solution is to use ^{15}N labeled glutamine and show that even in the presence of ^{14}N labeled ammonia in solution, all the nitrogen in asparagine at early times in the reaction is ^{15}N labeled. This experiment works as the ammonia reaction is much slower than the glutamine dependent reaction. Conservation of residues also suggests the importance of the tunnel for the overall reaction.

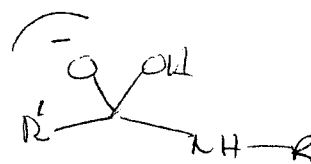
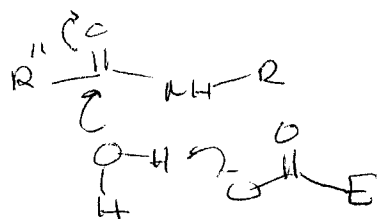
The role of ATP is further clarified by the experiment using ^{18}O aspartate. As only one of the aspartate oxygens are labeled and they are equivalent chemically, the product contains a 1:1 mixture of ^{16}O and ^{18}O as evidenced by the chemical shift perturbation in Figure 5A. Thus the oxygen of aspartate attacks the α phosphate and displaces PPi . The attack would be in-line with inversion of configuration if no covalent intermediate were involved. Thus a stereochemical experiment yielding inversion of configuration would be informative. In any case the phosphate that should have been made chiral is the α and not the γ phosphate. Analysis of the AMP product could use ^{31}P NMR methods, but the details would be different from those of Trentham and Webb for Pi . One would also need to use labeled [^{17}O or ^{18}O] carboxylate of the aspartate to generate a chiral product. Analysis of the product could occur using NMR or mass spectroscopy. Analysis of the NMR spectrum is dependent on the phenomenological observation that the chemical shift of the P signal is altered 0.02 ppm by substitution of the ^{18}O isotope of oxygen for ^{16}O and that the perturbation is related to the bond order. In addition if ^{17}O is used in your experiment, it obliterates the P signal, by broadening the signal so that it can no longer be detected.



Problem 2. The second problem involves some protease, renin. Not much information is provided. Renin is a member of the aspartate protease family. First you are given a substrate **1**. Hydrolysis yields a colored product that can be monitored as a function of time. The time scale on the graph in Figure 7 and the method stopped-flow spectroscopy suggests that you are monitoring the first turnover of the enzyme and subsequent turnovers (millisec to sec). You can assume that the experiment has been carried out at sufficient concentrations of enzyme that the first turnover can be monitored. The concentration of enzyme used in the experiments depends on the extinction coefficient of the anilide released. In contrast to the reaction of chymotrypsin with esters, the mutant tyrosine phosphatase with para nitrophenylphosphate and the glycosidase in problem set 3 with a para nitrophenol glycoside, no burst of product is observed in the first turnover. This result is similar to the results observed with chymotrypsin with peptide substrates and the wt-phosphatase discussed in class. The results simply infer that some step subsequent to release of the anilide is not rate limiting and hence no burst is observed. This could mean, as in the case of chymotrypsin with peptides that k_2 and not k_3 is the slow step. It could also mean that there is no covalent intermediate.

In support of the later interpretation is the potent inhibition of the enzyme by **2**. This is putative transition state analog (binding energy used to bind, since no chemistry can occur). With serine and cysteine proteases that involve covalent catalysis a tetrahedral intermediate requires a group from the enzyme, serine or cysteine. With zinc and aspartate proteases that do not involve covalent catalysis the tetrahedral intermediate generated with water, similar to the phosphate tetrahedral structure. Thus one interpretation of the minimal amount of data is that this enzyme is a zinc or aspartate protease involving no covalent intermediates.

Mech non-covalent interme.



catalyst +



I would be

with a covalent intermediate - sterically hindered to bind

