BOGDAN FEDELES: Hi, and welcome to 5.07 biochemistry online. I'm Dr. Bogdan Fedeles. Let's metabolize some problems. Now, I have here problem two of problem set three, which is an excellent exercise about the mechanism of inhibition of enzymes, specifically proteases.

Now, this deals with the same protease from problem one of this problem set, which is interleukin converting enzyme, or ICE. Therefore, it's best that you familiarize yourself with the mechanism of action of this enzyme, ICE, by solving problem 1 and then continuing with this video, here.

As you found out by solving problem one, ICE is a cysteine protease. It features cysteine and a histidine in its active site. The mechanism starts by the histidine acting as a general base and deprotonates the cysteine SH group. The thiolate anion then can attack the substrate, forming first, a tetrahedral intermediate.

When this tetrahedral intermediate collapses, it cleaves the peptide bond and accomplishes the chemical reaction of the protease. Then, the newly formed thioester is hydrolyzed to release the other half of the product. Let's take a look at the mechanism.

Here is our peptide substrate. And this is the peptide bond that's going to be cleaved by protease. As we mentioned, we have a cysteine in the active site and we have a histidine that's just going to function as a general base. I'm going to denote it as B.

In the first step, the histidine is going to deprotonate the cysteine. The cysteine is then going to attack the carbonyl of our peptide bond, and the electrons are going to go to the oxygen, and form a tetrahedral intermediate.

Here, notice the histidine is going to be protonated, and the oxygen is going to have a negative charge. In the next step, this tetrahedral intermediate is going to fall apart by breaking the peptide bond that the protease is supposed to cleave. There is the thioester with the cysteine in the active site and one half of our product is going to be released, here. Once again, the histidine is going to be deprotonated at this step.

In the second step, the thioester we just formed in the active site will be hydrolyzed by a water molecule, which will be activated by the same histidine in the active site. Here is our water molecule. So, the histidine is going to deprotonate the water, activating it for attack on the carbonyl, forming once again, a tetrahedral intermediate.

I have a negative charge here and a positive charge on the histidine. Finally, this tetrahedral intermediate will collapse, restoring the cysteine in the active site, which can be reprotonated by the protonated histidine, and releasing the second half of our substrate, here.

So there you have it. Now we restored the active site with the cysteine and the histidine. And this is the second half of our peptide that we cleaved. So notice the carboxyl side is right here and the amine side was released a little bit earlier.

The mechanism that you just saw is very similar to the serine protease mechanism that is described in the book and in the lecture notes. Notice, every time we form a tetrahedral intermediate, this is probably stabilized in an oxyanion hole formed by some of the residues on the backbone of the enzyme.

Now let's take a look at a couple of strategies for inhibiting a cysteine protease like ICE. This problem is proposing two strategies. One involves an aldehyde inhibitor, the other involving an acyl methyl ketone inhibitor. Let's take a look.

Here is the structure of a proposed aldehyde inhibitor. Notice here, this is a aspartate residue, or aspartate looking residue, which together with the other couple of amino acids, forms the recognition portion of the inhibitor that we're allowing to bind the protease. The R group is going to be an aldehyde, which will be crucial for actually inhibiting the enzyme.

Question one of this problem is asking us to propose a mechanism by which the aldehyde inhibitor works. We're given an important clue that this is a mechanism based inhibitor. A mechanism based inhibitor means that the inhibitor binds in the same fashion as the normal substrate of the enzyme. Therefore, after we have reviewed the mechanism of the cysteine protease and remembering some of our carbonyl chemistry, we should be able to propose the following chemical reaction.

Here is the active site of our protease. This is the cysteine and this is the histidine, which we're denoting as a general base, B. And here's our aldehyde inhibitor, which I'm going to just show

the aldehydic group, right here.

Since the R group next to this aldehyde resembles very closely the natural substrate of the enzyme, this aldehyde group will be positioned in the same place where we would normally find the peptide bond that will be cleaved by the enzyme.

Therefore, this thiolate group, once it forms, will be in great position to react with the aldehyde and form a tetrahedral intermediate. Therefore, the histidine deprotonates the cysteine, and the cysteine can then attack the carbonyl to form a tetrahedral intermediate. Therefore, we get this tetrahedral intermediate and a protonated histidine base.

Now normally, the reaction would proceed from here to form a thioester, but because this is an aldehyde, the reaction stops here, and that's how the enzyme will be inhibited because we have now bound this inhibitor. We'll have it covalently bound in the active site to this cysteine.

An interesting observation, which you can't really tell from the problem, when people looked at the x-ray structure of this tetrahedral intermediate, they noticed that the negative charge on the oxygen is not, in fact, stabilized in the oxyanion hole that would stabilize such tetrahedral intermediates for the normal reaction.

The ability of the aldehyde group to react with the cysteine in the active site and form a covalent bond can readily explain why the molecule would function as an inhibitor. Nevertheless, the reaction between the aldehyde and the nucleophile, the thiolate, is readily reversible. So whenever the inhibitor is in its carbonyl form, it can potentially fall off from the active site of the enzyme.

Therefore, how good of an inhibitor this molecule is will depend on how tight it binds to the enzyme, and not necessarily on the fact that it forms a covalent bond in the active site. This is an example of a reversible inhibitor, even though it forms a covalent bond with the enzyme. Therefore, its ability to inhibit an enzyme will depend on the relative concentration between the inhibitor and the natural substrate of the enzyme.

Let's remember the Michaelis-Menten equation written for a reversible inhibitor. As you recall, we have an enzyme reacting with a substrate. We have k1 and k minus 1 the rate constant to form the enzyme substrate complex, which then with k2, is going to form the product and reform the enzyme.

But the inhibitor will react with the enzyme in the absence of the substrate in an equilibrium,

forming an enzyme inhibitor complex which does not lead to any product. The constant of this equilibrium, I'm going to call it Ki, and this is the dissociation constant of the enzyme inhibitor complex.

As you have seen in the notes, the rate, taking into account the inhibition constant here, the rate v is going to be Vmax times the concentration of the substrate over Km. This is Michaelis constant for the enzyme. Times 1 plus concentration of inhibitor over Ki plus concentration of substrate, s.

Now, this equation tells us exactly how the rate is going to change as we increase or decrease the concentration of the inhibitor. Notice here, that this term is always greater than 1 because concentration and Ki are going to be positive numbers. So this is 1 plus something positive. It's always going to be greater than 1, therefore, the denominator is going to be bigger than if we had Km times 1 plus s.

So in the absence of the inhibitor, the denominator is going to be Km plus s. Therefore, when we add the inhibitor, this denominator gets bigger, and therefore the whole fraction gets smaller. We get a smaller rate. This is the basis for why the inhibitor will inhibit the enzyme, and therefore the rate of the reaction is going to be smaller.

To see this graphically, we can write the reciprocal of the equation and look at the Lineweaver-Burk plot. The reciprocal of the equation is going to be 1/v equals-- and if we crunch the numbers, going to come up with Km/Vmax times 1 plus I/Ki times 1/s plus 1/Vmax. Let's plot this.

I'm going to have 1/v and here I'm going to have 1/s. Now, if the concentration of substrate is really, really high, 1/s is going to be almost zero. So at the limit, when 1/s is zero, then we should get v equals Vmax. So therefore, let's say here it's 1/Vmax, and therefore without any inhibitor, we're going to get a line that looks like this.

Now, as we're adding an inhibitor, this slope-- that is the coefficient or 1/s-- is going to be increasing. Therefore, we should get higher slopes. The higher the concentration of I, the bigger the slope. So it's going to look like this. So as concentration of I increases, the slope of this graph will increase.

Notice however, that all these lines, even though correspond to slower rates, as the concentration of substrate increases-- that is 1/s gets closer to 0-- they will all converge to the

same Vmax. This is the key feature of a competitive inhibition because the substrate in high quantities can out compete the inhibitor.

Nevertheless, this mechanism only applies when the binding of an inhibitor to the enzyme is fast and reversible. If the binding is not reversible, obviously the enzyme will be inactivated forever, and then we will see a time dependent inactivation of the enzyme. The same phenomenon will happen if the reverse reaction, that is the dissociation of the inhibitor from the enzyme, is a slow process as well. All these considerations form a comprehensive answer for part one of the problem.

Question two asked us to provide several reasons for which aldehyde inhibitors are not actually desirable as therapeutics. Once again, we have to think about the carbonyl chemistry. We saw here that aldehydes can react very well with nucleophiles such as thiols and thiolates. But aldehydes in solution can react with water and form what we call geminal diols. That look like this.

Therefore, the effective concentration of the aldehyde in solution will be diminished because of this equilibrium, and that inhibitor might not be efficient at that lower concentration. Another consideration involves the oxidation of aldehydes.

These could be enzymatically or even non-enzymatically oxidized to form carboxylic acids or carboxylates. These would obviously not be very reactive and any inhibitor that gets oxidized will stop being an inhibitor.

Additionally, owing to their reactivity, aldehyde group could react with many other biomolecules. Think about the amino acid side chains. Many of them are actually, in fact, capable of reacting with aldehydes. This will also diminish the effective concentration of the inhibitor and it may even cause side effects. Therefore taking all these into considerations, aldehydes may not be a great solution for therapeutics.

Question 3 is asking us to propose a mechanism by which the second kind of inhibitor, the acyloxymethyl ketone is inhibiting the protease. As you see here, the acyloxymethyl ketone is actually very similar to the aldehyde inhibitor.

Notice this group is exactly the same as the carbonyl group of the aldehyde, but instead of having just the hydrogen, we have a methylene next to a aryloxy or acyloxy group. This, as you know, is a very good leaving group and provides a second reactive site, this methyl group

here, that can react with the enzyme.

The second kind of inhibitor is in fact very similar to the tosyl, phenyl, chloro ketone inhibitor of serine proteases, which is discussed at length in the lecture notes and in the book. These inhibitors fall in a general class of alpha substituted ketones and they feature two reactive sites. One is the carbonyl and the other one is the methylene group, which has attached a good leaving group.

Now, here is a general form of a alpha substituted ketone. Here is the ketone, here is the methylene group attached to a good leaving group, which I'm going to denote x. Now, here are the residues in the active site. Here is the cysteine with the thiol group, and here is the histidine, which I'm going to draw out. Histidine, all right.

So as we saw before, the reaction will start by the histidine acting as a general base. The histidine deprotonates the cysteine, which then can attack the ketone carbonyl. This leads to the formation of a tetrahedral intermediate. And we have a positive charge, here on the histidine, and a negative charge on the O minus, here.

Now, this tetrahedral intermediate presumably will be stabilized in an oxyanion hole, as you guys have seen before with some kind of hydrogen bonds to the backbone of the protein. Now, in the next step, this is something that you can't really anticipate or know without doing some experiments. But it turns out this O minus is a good SN2 nucleophile to displace the good leaving group, x.

So we're going to have an S2 reaction, This O minus attacks the carbon, and then the x takes the electrons and leaves. What we're going to form here is an epoxide. All right, so this is the epoxide and we still have our protonated histidine here. And there's a positive charge here, and of course, the x group is taking its electrons and leaves as an anion.

Now, in the next step, because this is sitting actually close enough to the epoxide, the epoxide is going to get protonated. This takes the proton from the histidine. So now we have a protonated epoxide, and the histidine now is in its deeper native form, and the epoxide has a positive charge.

Now, because we have a protonated epoxide, this acts as a very good leaving group, and this carbon becomes very susceptible for an SN2 reaction. And it turns out, the histidine, this nitrogen, is a good enough nucleophile to react in an SN2 type reaction. And in fact, the

reaction is helped by this other nitrogen. I should have used a different color.

So what we're getting out here is a covalent bond between the histidine and the alpha position of the original alpha substituted ketone. Now, this reaction will not be, in fact, reversible. So we should just say one arrow only. So there we have our histidine. Now it's covalently attached to our inhibitor and this step is, in fact, irreversible. So this prevents the inhibitor from ever dissociating once this reaction has taken place.

Now, even though this tetrahedral intermediate around the carbonyl carbon, it can fall apart as we saw before, reforming the carbonyl and the cysteine thiol, the covalent bond to the histidine remains. And this is, in fact, the reason for which these inhibitors are irreversible and will show a time dependent inhibition.

Regardless of the more complicated mechanistic detail I just showed you, the take home message here is that when using an alpha substituted ketone, the end result will be a covalent bond between the enzyme and the inhibitor that is not reversible. Therefore, we expect to see a time dependent inactivation of the enzyme. The more enzyme is being taken out of the reaction by the inhibitor, the slower the overall reaction will be until there is no more enzyme left to catalyze the reaction.

The problem also provides some kinetic data, which, in fact, supports the time dependent inhibition features of the second kind of inhibitor. In this figure, we see on the y-axis, the concentration of the product that is formed, and on the x-axis we see the time.

Now, if we look at the dark circles, which is the control reaction in which we have some substrate, but no inhibitor, we see that the product is produced and increases linearly with time. However, once we add the inhibitor at a certain concentration, and this would be the dark squares, then we see that the amount of product increases for a while, but then it grows slower and slower until it eventually stops.

Now at this point, if we add an excess of substrate, we see that the reaction does not restart, meaning the entire amount of enzyme has been inactivated, and this inactivation is irreversible. However, we're also provided this additional piece of the data. If we run the reaction in an excess of substrate, we see here the open circles, the amount of product produced increases linearly with time.

But if we add the same amount of inhibitor, these open squares actually are on top or right

next to the open circles on this line, which says that the inhibitor has virtually no effect at this level of concentration. Which tells us that the inhibitor is in fact competing with the substrate and we will have an excess of the substrate. The inhibitor does not get a chance to bind and inhibit the enzyme, at least within this interval of time.

Now this way of plotting kinetic data is perhaps a little misleading because the amount of product that we obtained from the reaction does not reflect how much of the enzyme gets inhibited. We want, in fact, to look at the percentage of enzyme activity that is remaining after a given amount of time.

Therefore, if we were to plot percentage enzyme activity versus time, for a controlled reaction we expect the reaction to proceed, and the enzyme to stay just as active at any given point in time, so we will see a straight line. However, when we add an inhibitor, which shows a time dependent inhibition, then the percentage enzyme activity that remains at every point in time will be decreasing.

And in fact, will be decreasing to the point that it reaches the maximum of slope for the maximum amount of inhibitor present in the reaction mixture. So this line represents the fastest that the enzyme can be completely inactivated by an inhibitor. It will be governed by the binding affinity of the inhibitor to the enzyme.

This answers the third and last question of this problem. I hope you enjoyed this exploration of the various strategies by which inhibitors can inhibit proteases. This problem highlights, in fact, the importance of understanding the mechanism of action of enzymes. Only then we can begin to design therapeutically useful inhibitors.