Lecture 20B • 06/01/12

l-Aspartic acid: pKa1 = 2.09, pKa2 = 9.82, pKa3 = 3.86

pKa1 and pKa2 always refer to the acid group and the main amino group that all amino acids would have. The side-chain pKa is listed next [as pKa3]. If we only had the main carboxylic acid and amino groups ... we have this thing called the pI, which is the pH at which you reach the isoelectric point, which is where we have the maximum amount of neutral from of an amino acid. It might make sense that, if we didn't have this third functional group, it should be the average of the two pKa values. What would happen if we did have pH = 2.09? We'd have half of this amino acid in its fully-protonated form, where the amino group that we protonated and the two carboxylic acids still have their protons, and we have half of it where the first amino acid, with the lower pKa value which is the stronger acid, 50% of that acid would be dissociated. It turns out that,for this particular amino acid, the next time there's a switchover that occurs, is at we're at 3.86, which means we have half of the singly-deprotonated amino acid, half of the doubly-deprotonated [amino acid]. At pH = 2.09, we'll have 50/50 mix of the fully-protonated form and the singly-deprotonated form. Notice that this first form is cationic, where the next form is technically neutral. If we get to the point where we have pH 3.86, we'll again have a 50/50 mix; what will it be? Of that same neutral form and, now, we have the anionic form.

Where this amino acid would differ from others is we continue to raise the pH, which means make it more and more basic, there would be one more proton that would come off, and that would come off that positively-charged nitrogen. If the idea of the pI is where you're maximizing the neutral form, at the lower pH, 2.09, that's the lower end at which 50% would be in the neutral form; if we increase the pH to 3.86, that's the high end at which half of it would be in its neutral form, so between those two pH values, you'd reach your maximum amount of the neutral form. That's why for compounds like this that have two acid groups, the pI is the average of the pKas of just the acid groups. We could go through this same kind of story with a compound that has two amino groups, and we would find a similar situation there. The maximum amount of the neutral form occurs when you are in a pH in between the pKas of the two amino groups. If you have the simple case of a regular amino acid that has no ionizable side chains, it is the average of the only two pKa values that are there that gives you the pH at which you would have the maximum of the neutral form of the molecule.

For l-aspartic acid, about 50% of the amino acid is in its neutral form at pH = 2.09 and pH = 3.86. It will therefore reach its maximum amount of the neutral form in between those two pH values. That's how we come up with, in this case, the pI is the average of those two acid pKas.

What do we do with this? Let's pick another amino acid. [2.98 for average of pKas] How about a simple one like glycine. What is the R group for lysine? Just an H. [pKa 1 = 2.34, pKa 2 = 9.60, pI = 5.97] What's the significance of this? Imagine we had a solution of both aspartic acid and glycine. Let's say we chose to force the solution to have a pH of 4. If the pH is 4, will aspartic acid be more in its anionic, neutral, or cationic form? Cationic? It has to do with that this pI value means. pI is the pH at which you have the maximum of the neutral form of the amino acid. If we go to higher pH, are we moving towards a more or less acidic solution? Less acidic, so we could also say more hydroxide around. If we had more hydroxide around to react, what's going to happen to this compound? It's going to get deprotonated. If at 2.98 you're at the maximum amount of the neutral form, then if you go to higher pH, that means you're going to have more the anionic form, the basic form, of aspartic acid. But, if the pI for glycine, for example, is 5.97, if we were at pH 4, we would be too acidic to have the maximum neutral form of glycine, so glycine is going to have more of its structure in the cationic or acidic form.

Imagine a solution contains both glycine and l-aspartic aid. At pH 4, a larger proportion of l-aspartic acid will be in its anionic form, since the solution is more basic than the pI. Conversely, glycine will have a larger proportion of its cationic form, since the solution is more acidic than the pI. Imagine we had this: that we plunk a bit of solution onto an appropriate substrate, and we hook up positive and negative terminals to it. Let's say that it's a solution at pH 4, so this is exactly the situation described above – two amino acids mixed together at a particular pH. Turn the power on, what's going to happen? The glycine will go to the negative terminal, and the aspartic acid's going to go the positive terminal. Why? Because at pH 4, more aspartic acid is negatively charged, so it's going to move towards the positie terminus; opposite charges attract. Then, the glycine, which is in its cationic form, is going to move towards the negative terminus. This is the process of electrophoresis – using pH to preferentially force certain compounds into differently charged forms, so they can be separated on the basis of charge. In this case, the amino acids can be separated by charge, since at pH 4 one molecule will predominate in its cationic form, the other in its anionic form.

A solution of an amino acid is not something that can be visualized; the amino acid solutions themselves are [either clear or slightly yellow in color, but in other words there's nothing remarkable about one versus the other that we would easily be able to separate them just on sight]. All of the amino acids [except one?] all react with a compound called ninhydrin, which is written in two forms. It is a compound that exists in equilibrium between its hydrate and its ketone forms. [why is it that the middle carbonyl is the one that's reactive?] It is the middle carbonyl that reacts with any amino acid to form exactly the same product. [mechanism]

The first step is that you undergo protonate-open-attack-deprotonate, protonate-open-attack-deprotonate, or a variation thereof, to get an imine. Simplifying a little bit, this is amino acid; under the right conditions, that amino acid can become deprotonated – or it may already be deprotonated, because amino acids self-neutralize, so may be in its zwitterionic form to begin with. We end up with something where, buried inside of here, is an imine. It turns out, for whatever reason, that this particular form of imine is reactive. Having a carboxylate turns out to be reactive; it can undergo decarboxylation. When it does, there's an isomerization that occurs. Notice at this point we've lost stereocenter for the R group. Even though we just made an imine earlier, remember that imine formation is reversible, so in solution, we could imagine ... I'll call it reverse mode, protonate-open-attack-deprotonate, protonate-open-attack-deprotonate, to open up an imine, which will get us an amine. Notice, in that hydrolysis, there's no more R group.

We've made this amino group. There's still ninhydrin around in solution. After another imine formation, and a slight bit of tautomerization, we end up with the following – effectively, two ninhydrins stuck together. This is called [Ruheman's purple] – bright purple color. After you've done your electrophoresis, you could spray that paper with this ninhydrin solution. It'll react with the amino acids and spot them purple wherever they might be. That's the visualization aspect. Development would be by applying an electric current; visualization would be by using this ninhydrin. [mechanism simplification][summary]

Sequencing – to determine the order and kind of amino acids in a protein; protein is another word for polypeptide. Let's say that we're given a particular amino acid chain. To keep the argument generalized, let's say that I've disguised the names of these amino acids [to protect the innocent].[fake single-letter abbreviation] If I wanted to classify this as the type of peptide that this molecule is, what would I call it? A tetrapeptide, meaning there's four amino acids. If these were the valid one-letter abbreviations, then if I wanted to represent in abbreviation form this tetrapeptide, what would it be called? How would I name this tetrapeptide? You just name off each amino acid? From amino to the acid. The term 'amino acid' itself will tell you which order to list these in. I'll call this the tetra peptide – alpha, beta, gamma, delta. How do I determine which amino acid is in which order? Chemically, how would I do it? that's a slightly more complicated subject. [mechanism of oxazolinine] If I didn't care, at this point, about preserving the order of the amino acids, how could I split all of these amino acids apart from each other? Hydrolysis. What would be a good set of conditions? Water in strong acid will lots of heat for lots of time. [24 hours in H+] We'll get the four individual amino acids.

Here's what we could do. This is called ion-exchange chromatography. [functionalized styrene copolymer] The material that's in one of these ion-exchange columns is very similar [to the Merrifield resin], where you make a co-polymer of plain styrene along with p-vinyl[benzene]sulfonic acid, the double bond analog of tosic acid. If you were to take these two compounds with the appropriate initiator, you can end up with another one of these co-polymers, where every once in a while, you have one of these sulfonic acid groups. What if you then reacted this with sodium hydroxide? Sulfonic acids as strong acids, which means even though this might have a negative charge on it, it's still going to be pretty much a neutral compound, neutral in terms of pH; that's because a sulfonate, which is the type of ion we have here, just like p-toluenesulfonate, is the conjugate base of avery strong acid. If you were varying the pH of the solution flowing past this kind of polymer, then you could reprotonate it for force it to stay deprotonated. Similarly, if you have amino acids where you were changing the pH, you would be changing the proportion of them that are in cationic form and anionic form. Why does that matter? The sodium ion could easily be displaced by the positive charge of one of these amino acids, at least temporarily.

Here's what the experimental set-up would look like for what I'm going to be describing. This piece of glassware is the column. Generally, at the bottom it is filled by sand or some other kind of inert material so that the bottom level of the column if perfectly level, it's flat. You then fill that column up with the separatory phase. In this case, since we're talking about ion-exchange chorimatography, it'll be filled with this ion-exchange resin. You then load the column by taking an amino acid soup, you could say – taking the protein chain, you put it into whatever acid you're going to chew it up wtih, let it sit, elta ll those amide bonds get hydrolyzed. Take the mixture of it and put it just at the very top layer of that column. This is what the system would look like initially. You could do a gravity column, which is where you just hope that the solvent is somehow going to fiter through on it's own; generally, not practical, so generally you have to add solvent then you put an air source to force the solvent through. You never let the solvent level drop below the level of the column, because in order for the separation to be clean, you want a consistent density of the resin all the way through the column, and you want a flat bottom and top, so that things exit at the same time – at least, if you have one layer that flows through. The idea is that, at a particular pH, all the different amino acids will have slightly different proportions of their cationic and anionic forms As they pass through the resin, some of them get attached to the resin, take more time to come off the column. This amount of time is called the retention time. Since the amino acids don't change – there's the set of 20ish that we look for – this has been developed and repeated so many times that there's a protocol for it; if you just follow that protocol, you'll get all the amino acids that come off, one at a time. You figure our what time they come off the column; that time, the elution time, the retention time, corresponds to the identity of the amino acid itself. You do this process. After some time, your mixture will split into bands, which you might not see at this point. There is this visualization method of using ninhydrin, but you lose the identity of the amino acid. If you're trying to separate the amino acids, it wouldn't make sense to remove the difference between them while trying to separate them, so you let them come through and off the column first, without being visualized, but you have a ninhydrin feed that you add right afterwards.

Once a polypeptide has been fully hydrolyzed, the amino acid soup is added to the top of an ion-exchange column. As solvent is forced through the column, some of the amino acids move more slowly through that column, because they're having heavy ionic interactions with the ion-exchange resin. Since this experiment has been reproduced billions of times, a protocol has been established [to] identify the amino acid by how long it takes to elute, which means come off the column. This time is referred to as the retention time or the elution time. There is a quantitative measure that we'll be able to take besides time. [Beer's law] It's a way of being able to establish concentration based on absorbance, based on spectroscopy. We're going to use a similar trick here. A lot of these separation materials operate off of polarity as well. There is what is known as size-exclusion chromatography. Size would have a less important factor in this case.

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pI = 2.98

– For l-aspartic acid, ~50% of the amino acid is in its neutral form @ pH= 2.09 and pH = 3.86. The maximum quantity of the neutral form will therefore exist between those two pH values –> pI = (pKa1 + pKa2) / 2

Glycine: pKa1 = 2.34, pKa2 = 9.60, pI = 5.97

Imagine a sol'n contains both glycine & l-aspartic acid. @ pH 4, a larger proportion of l-aspartic acid will be in its anionic form, since the sol'n is more basic than the pI. Conversely, glycine will have a larger proportion of its cationic form, since the so'n is more acidic than the pI. In this case, the amino acids can be separated by charge, since @ pH 4 one molecule will predominate in its cationic form, the other in its anionic form. –> Electrophoresis

Ninhydrin

Sequencing – determining the order and kind of amino acids in a protein (polypeptide)

Ion-exchange chromatography

Once a polypeptide has been fully hydrolyzed, the amino acid "soup" is added to the top of an ion-exchange column. As solvent is forced through the column, some of the amino acids travel more slowly because they are found in greater proportion in their cationic form, so they interact heavily with the ion exchange resin. A protocol has been established to identify an amino acid by the amount of time it take to elute (come off the column) –> retention time or elution time.

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Structures

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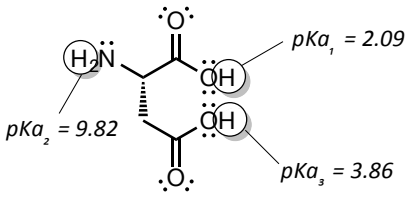

L-aspartic acid

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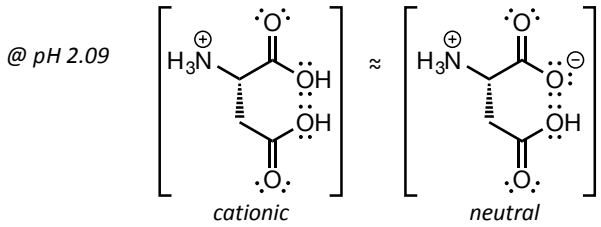

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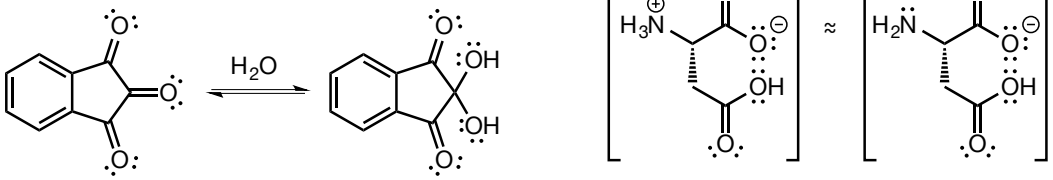

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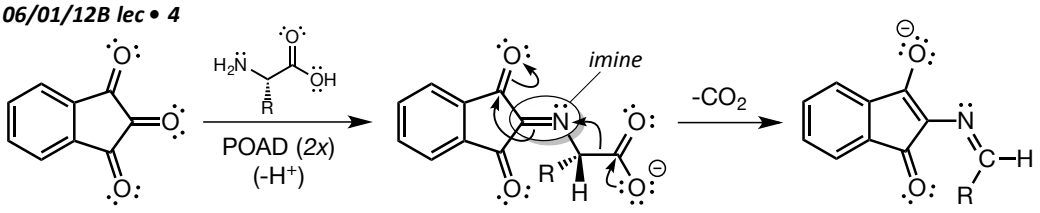

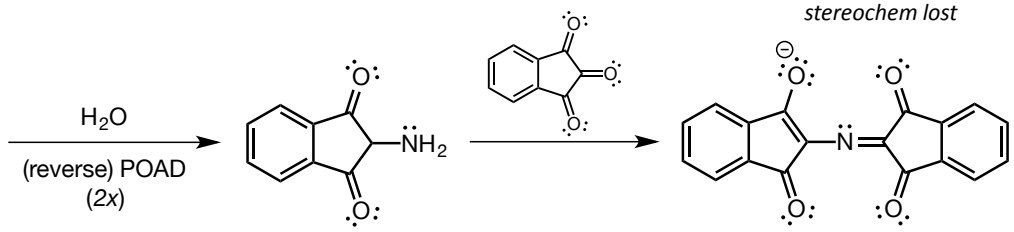

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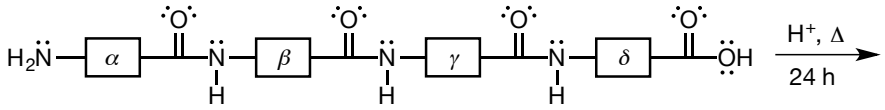

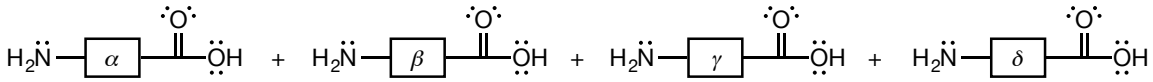

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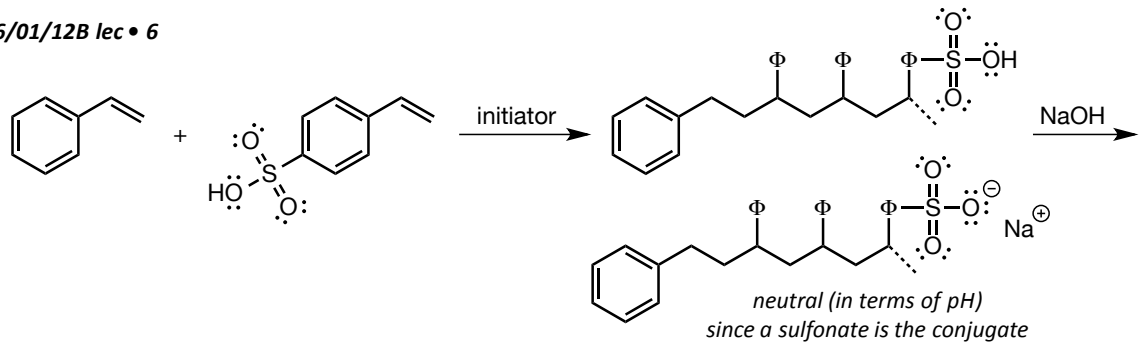

neutral (in terms of pH)
since a sulfonate is the conjugate
base of a very strong acid