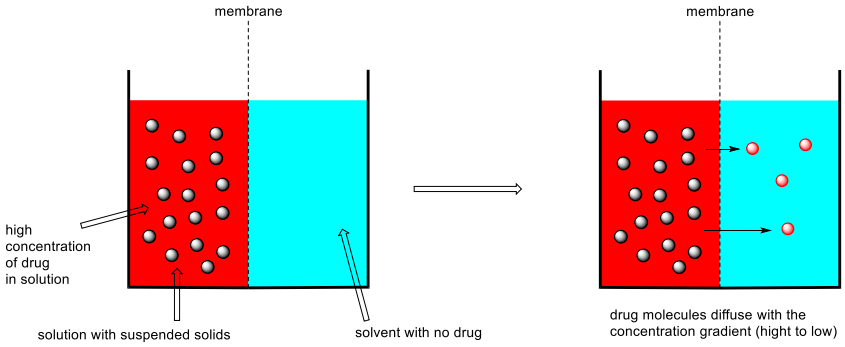
BPS 2110
Assignment 8 Answers

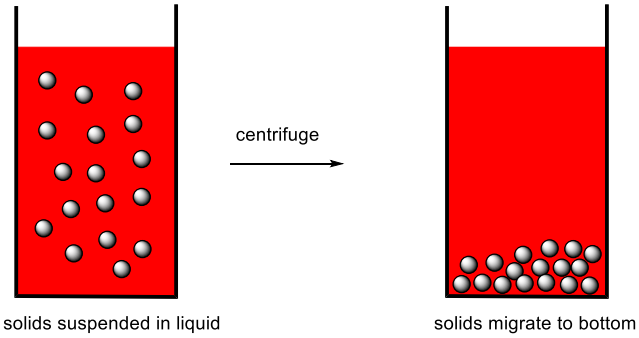
1. Insulin is a hormone produced in the pancreas.
    a. What function does insulin regulate in the body?
    Regulates metabolism of fats, carbohydrates and proteins
    b. What are the effects of high and low insulin concentrations?
    High insulin causes low blood sugar (glucose is converted into glycogen)
    Low insulin causes conversion of glycogen into glucose raising blood sugar amounts; begins the catabolism of proteins
    c. What is the approximate basal concentration of insulin in a human?
    10^{-10} M
    d. Briefly describe how insulin was discovered.
    Pancreatic ducts in dogs were tied off. This caused necrosis of most pancreatic cells, leaving the Islets of Langerhans behind. Insulin extract was obtained from these. Purification was easier this way without the extra cells.
    e. What were the original sources of commercial animal-derived insulin?
    Pancreases were harvested as a by-product of meat production. Most common animals used were cows, pigs, horses and fish.

2. What were the common impurities found in animal-source insulin and how did they affect the use of the drug?
    Proinsulin – allergenic. Over time diabetics would become allergic to the insulin source animal and would have to switch to a different animal source.
    Glucagon
    Somatostatin
    Proteases – these would slowly destroy other proteins including insulin. Their presence limited the shelf life of insulin.

3. The typical purification of protein-based drugs involves some common steps. Describe the method and the rationale behind each of the processes listed below.
    a. Homogenization
    Break open the organ and/or cells containing the protein to be extracted. Blender, sonication, osmotic shock
    b. Clarification (2 methods)
    Separate the liquid fraction of a biological isolate from the solid fraction
    Dialysis uses a semi-permeable membrane, placed between the mixture and a buffer solution. Membrane allows the solutes to pass through while keeping the solid fraction behind. Solutes pass from the high concentration side (mixture) to the low concentration side of the membrane.



Centrifugation uses centripedal force to create an artificial gravity. This causes solids in suspension to sink quickly to the bottom of the container where they form a pellet. The liquid is then decanted from the solid.



c. Isoelectric precipitation

Cause certain proteins to precipitate from solution, leaving other proteins and molecules behind in solution by changing the ionization of the molecules. Changing the pH of the solution changes the ionization state of proteins in the solution, according to the pattern of ionizable groups on amino acid side chains. If the net number of +ve charges and –ve charges on a protein become equal, the protein is at the isoelectric point, is now neutral and insoluble in water. It precipitates leaving other substances behind in solution.

d. Denaturing precipitation

Cause certain proteins to precipitate from solution, leaving other proteins and molecules behind in solution by changing the conformation of the molecules. Can be done in several ways. In general, conditions are imposed that cause the proteins in solution to change their overall conformation (denature). This changes the solubility properties of proteins, causing some to precipitate, while others remain in solution.

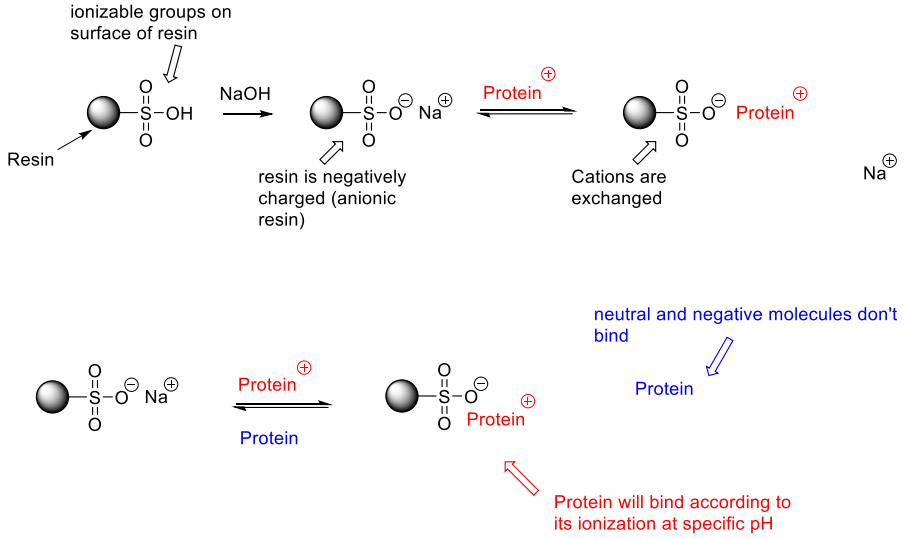
Changing the pH of the solution changes the ionization state of proteins in the solution, according to the pattern of ionizable groups on amino acid side chains. The change in ionization changes the pattern of non-bonding interactions that maintain the conformation.

Adding alcohols or other non-polar solvents disrupts the Van der Waals interactions in the center of proteins. This may expose electrostatic interactions, or hydrogen bonding interactions in the center of the protein structure to water, weakening these interactions and causing the protein to change shape.

Heating the solution increases molecular motions in proteins. If the protein flexes enough to allow water to penetrate into the non-polar portions of the protein, this may allow the water to disrupt the electrostatic interactions, or hydrogen bonding interactions in the center of the protein structure, weakening these interactions and causing the protein to change shape.

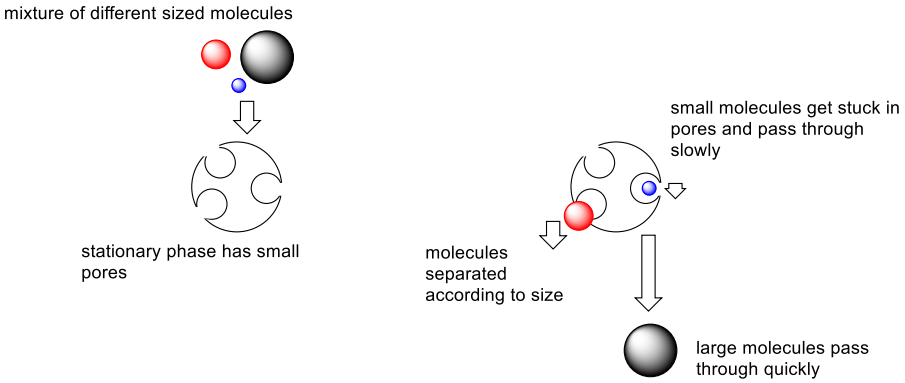
e. Ion exchange chromatography

Pass a mixture in solution through an ion exchange column using a buffer at a specific pH. The pH sets the ionization state of each protein in the mix.
The solid phase on the column is covered in ionized groups (+ve or –ve) which attract oppositely charged proteins to the solid. Neutral molecules or molecules with a charge that matches the solid phase are not attracted strongly and pass quickly through the column. Molecules with charges that are opposite to the charges on the resin pass slowly, with a speed that is determined by their overall charge.



f. Size exclusion chromatography

Pass a mixture in solution through a size exclusion gel. The gel contains pores of a set size. Small molecules fit inside the pores and get "stuck" in the pores. These pass through the gel very slowly. Very large molecules do not fit in the pores and can pass through the gel very quickly. Intermediate sized molecules get partly stuck and pass through at a speed determined by how much they get "snagged" by the pores.



g. Recrystallization
Dissolve a mixture in a minimum of hot solvent. The solvent should dissolve impurities well. The solvent should dissolve the desired molecules well at high temperature and poorly at low temperature.
After dissolving, the solution is allowed to cool. Crystals of the desired molecule precipitate from solution, impurities remain in solution. Method works well if the desired molecules form the bulk of the mixture. Not very effective if the desired stuff is a minor component in the mixture.

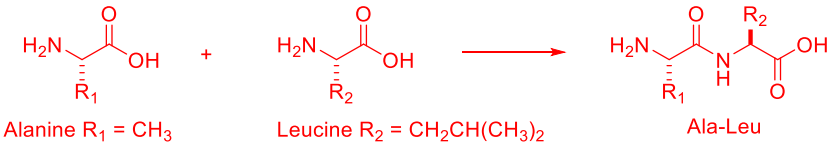
h. Lyophilization
Freeze a solution containing the solute to be solidified. Remove the frozen solvent under reduced pressure, while keeping the mixture frozen.
Maintains the structure of many proteins by keeping a one-molecule thick layer of water on the surface of the protein molecules.

4. What is meant by HPLC and why does this method provide better quality separations than other forms of chromatography?
High Pressure Liquid Chromatography.
Uses solid phase with very small particle size. This increases the effective surface area of the solid phase, increasing the number of molecular interactions involved in the separation. Requires high pressure to force the liquid between the very small spaces between particles.

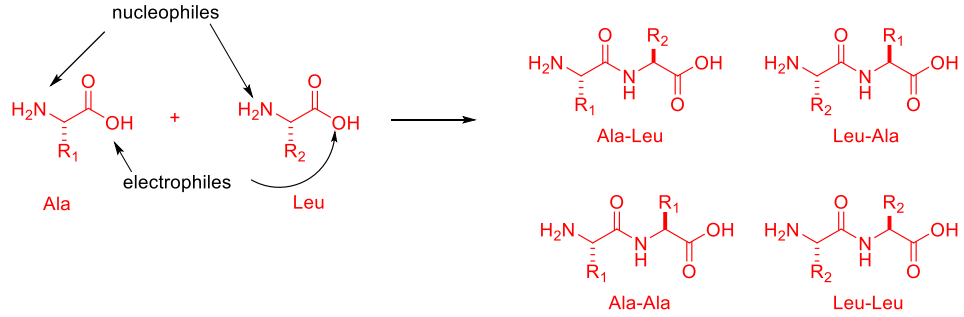
5. Describe the structure of insulin
   a. What is the general structure of this protein?
   Two peptide chains joined by disulfide bonds
   Both chains are mostly in the α helix conformation.
   b. How many amino acids does the protein contain?
   A chain has 21 amino acids
   B chain has 30 amino acids.
   c. How many disulfide bridges are found in this protein?
   Three. A chain has one intrachain disulfide bond. There are 2 interchain disulfide bonds

6. The chemical synthesis of biomolecules is most efficiently carried out using solid phase synthesis (SPS).
   a. What is the general idea behind SPS?
   One of the components in the reaction is attached to a solid phase. For purification, filtration is all that is required to separate the product from the other impurities.
   b. Why is SPS so effective in making biomolecules?
   Biomolecules are very large and require lots of chemical reactions to make. Each reaction requires purification. The products of these reactions have very similar chemical properties, and can be very difficult to purify. Simply filtering reduces the complexity of the synthesis.

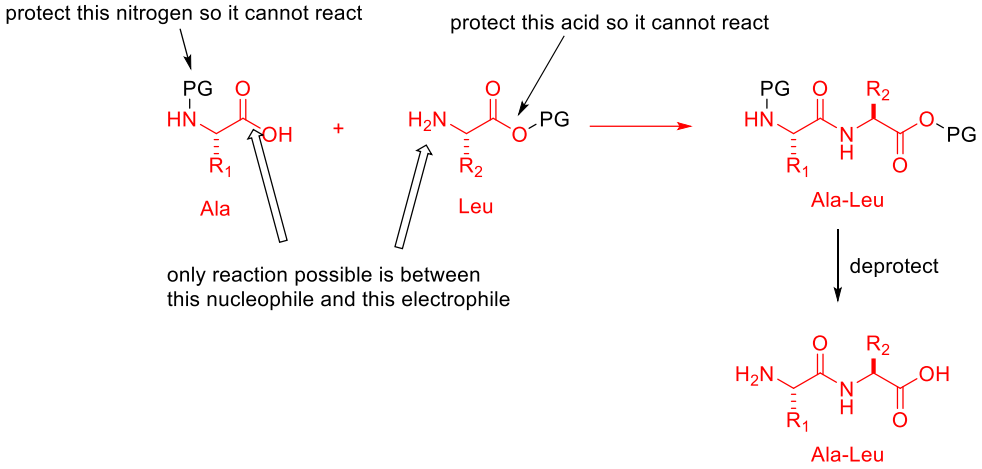
7. A chemist wishes to prepare the dipeptide by connecting an alanine to a leucine using a coupling agent.
    a. Write out the equation for the overall transformation.



    b. If she dissolves equal amounts of the two amino acids together, and adds the coupling agent, what products will be obtained?

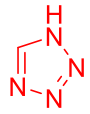


    c. Assuming she wants the amino acids connected in the order Ala-Lys, what would be the appropriate protecting group strategy to use?



    d. If she is using the method of solid-phase synthesis, which amino acid should be attached to the resin first?
    In peptide SPS the C-terminus is normally linked to the solid phase. Therefore the leucine should be attached first

8. Oligonucleotides are made using SPS according to procedures that were partly developed in Canada.
    a. What are the steps in coupling nucleotides together and what reagents are used in each step?

Coupling uses tetrazole 

Capping uses acetic anhydride Ac_2O

Oxidation uses iodine in water I_2 in H_2O

Deprotection uses acid in water H_3O^+

b. Why is it necessary to perform a capping operation after each nucleotide is added?
If trace amounts of uncoupled DNA chains are left behind they will react in subsequent couplings. This will create a mixture of several DNA sequences (desired one and several "deletion" sequences). This mixture will be very hard to purify (DNA strands all have same physical properties). By capping the unreacted 5'-OH's you prevent the formation of the other DNA chains.

c. How is the final deprotection and removal from the resin performed?
Add NH_3. This removes all the protecting groups from phosphate and also removes the DNA from the resin.

d. DNA polymerase enzymes add nucleotides using the 3'-OH, while DNA synthesizers add nucleotides using the 5'-OH. Why is the 3'-OH not used for machine-based synthesis?
5'-OH is a primary alcohol. 3'-OH is a secondary alcohol. Primary alcohols are less hindered and therefore are better nucleophiles than secondary ones. When performing a chemical reasction, will get better results by using the best nucleophile in displacement reactions.

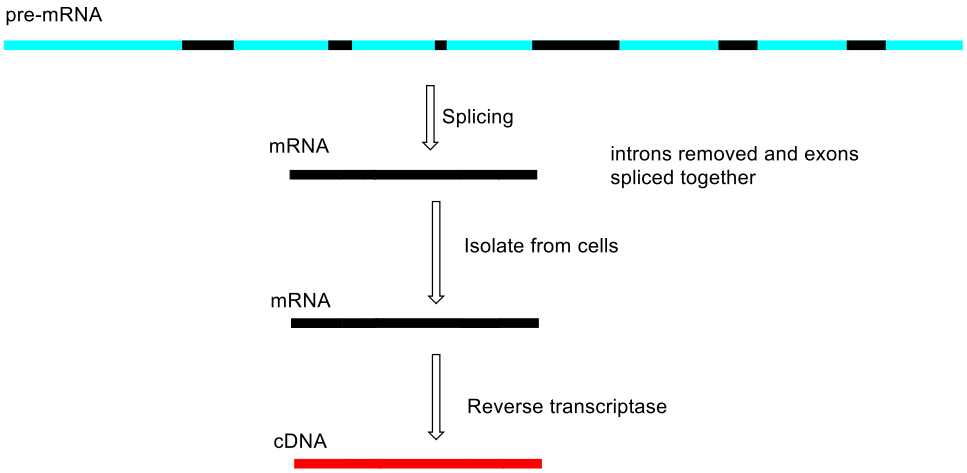
e. What does this method of making DNA mean for the researcher planning to synthesize DNA?
Nucleotides are added in the opposite order on a DNA synthesizer than they are by a polymeraze enzyme.

9. What are the six basic steps to follow when making a recombinant protein?
   - isolate the DNA for the protein you want to make
   - insert the DNA into a vector
   - insert the vector into cells
   - isolate the cells that express the protein you want
   - grow these cells in quantity
   - isolate and purify the protein

10. Eukaryote genes have some differences to prokaryote genes.

a. Why is it sometime necessary to use a DNA synthesizer when making recombinant eukaryote proteins using a prokaryote?
Eukaryote DNA contains introns that do not code for the desired protein. Must remove the introns and splice the exons before placing DNA into bacteria (bacteria do not use introns). Hard to splice DNA, easier to just make a piece that has the sequence you want.
b. What other method can be used to make DNA for this purpose?
Make cDNA from mRNA
c. Describe in general the method listed in part b.

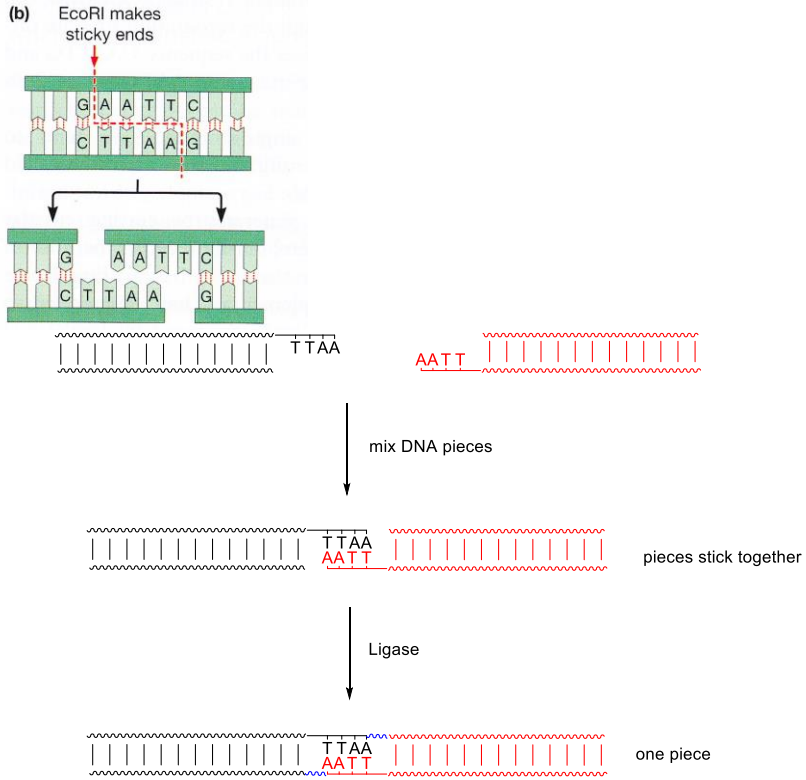
Isolate all the mature mRNA from cells. Purify the mRNA to isolate the desired mRNA. Use reverse transcriptase to convert mRNA into DNA.



11. What are the two most common types of vectors used in recombinant work?
Plasmids and viruses.

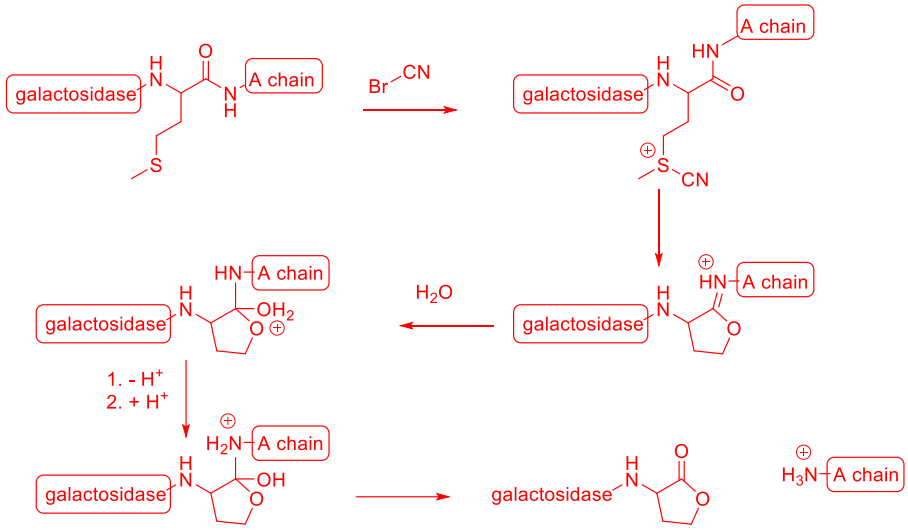
12. Plasmids are a common vector used for recombinant work.
a. What is a plasmid?
Autonomously replicating minichromosome. Small piece of circular DNA found in bacteria.
b. What type of plasmid is preferred and why?
Episomes. These tend to have amplified copy numbers inside bacteria. This results in higher amounts of protein being produced.
c. What general features are found on modern plasmids used in recombination?
Antibiotic resistance gene. Color-producing enzyme. Polylinker.
d. What is the role of the features listed in part c?
Antibiotic resistance gene used to purify transformed bacteria. Place the vectors in bacteria. Some bacteria take up the vectors some do not. Grow bacteria on medium containing antibiotic. Only those bacteria that take up the vector survive.
Color producing enzyme. Only those bacteria that take up and express the vector produce color. Use for easy identification of transformed cells.
Polylinker is special stretch of DNA containing multiple different restriction sites. Allows the plasmid to be used with lots of different restriction enzymes.

13. Special enzymes are used to splice and manipulate DNA during recombination.
    a. What type of enzyme is used to cut DNA?
       Restriction enzymes or restriction nucleases
    b. What are the two types of ends that are produced by these enzymes?
       Blunt and sticky
    c. Which type of end in part b is most useful for recombinant work and how do these ends help DNA splicing?
       Sticky

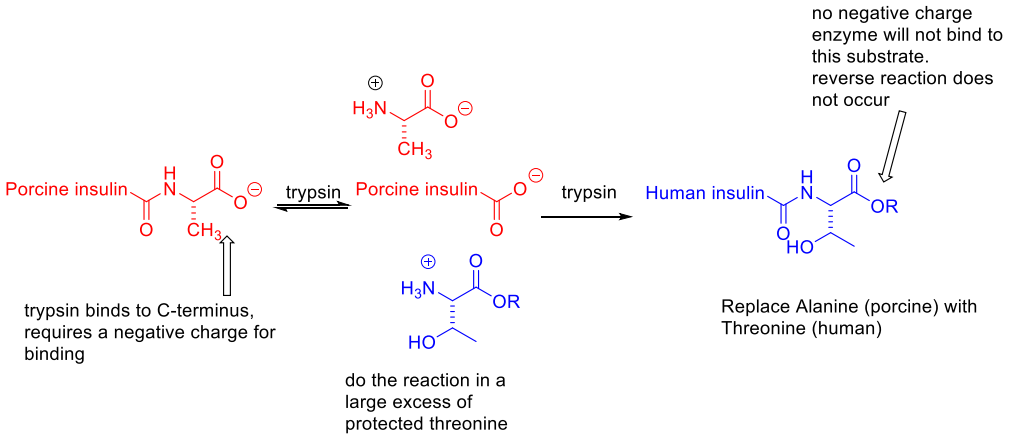


    d. What type of enzyme is used to splice pieces of DNA together?
       Ligase

14. Provide a mechanism to show how cyanogen bromide can be used to cut protein chains apart.



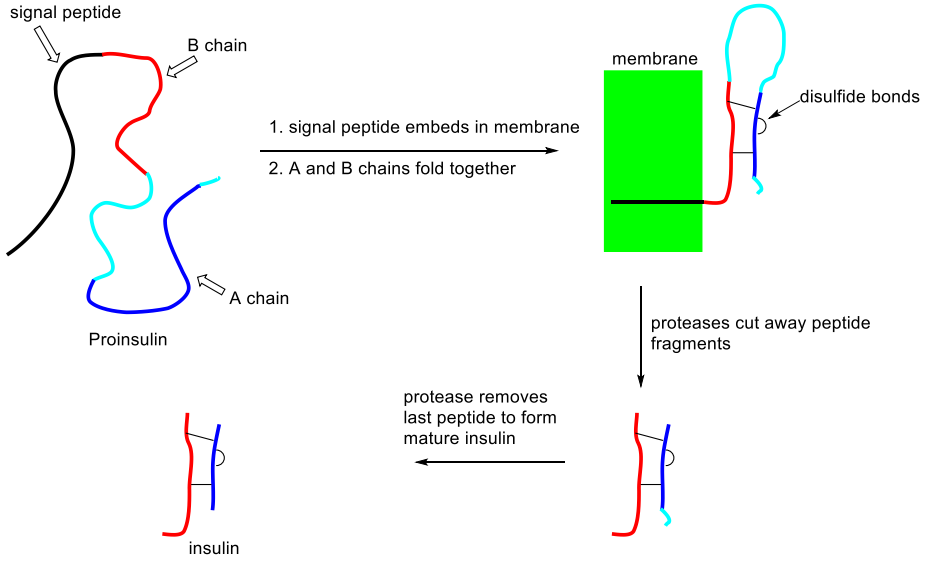
15. During the early 1980's, researchers developed a semi-synthetic route to make human insulin on large scale.
    a. What was the raw material for this semi-synthetic route and why was this source chosen?
    Porcine insulin. Available in large quantities. Only 1 amino acid different from human insulin.
    b. What enzyme was used to carry out the transformation?
    Porcine trypsin
    c. What kind of reaction did this enzyme normally carry out in the body?
    Remove the C-terminal amino acid from proteins. Enzyme has special affinity for C-terminal amino acids in which the 2nd-last amino acid is lysine.
    d. Describe the reaction that was used to semi-synthesize human insulin.



    e. What general chemical principle controlled the direction of the reaction and how did this apply?
    LeChatelier's principle.
    f. How did the engineers ensure that the product of the reaction was not further transformed, and how did this modification work?
    The threonine was added as an ester. This gave a final product which was not charged at the C-terminus. Trypsin will only bind to peptides that contain a –

ve charge at the C-terminus, and therefore would not bind to the product peptide. The reverse reaction was therefore not possible.

16. Show how insulin is normally biosynthesized in the body.



17. Insulin works, but is not always very "drug-like".
    a. What is the major issue associated with insulin administration that contributes to this?
    Concentration difference between normal blood levels and amounts in commercial drug (10^{-10} M and 10^{-3} M)
    b. How is this issue addressed by the method of administration (2 methods)?
- Subcutaneous injection into the fat layer under the skin. Drug precipitates out and then slowly dissolves resulting in low concentration.
- Use wearable pump to inject very small volumes of drug continuously.
    c. Prior to 1985, how did the chemical properties of insulin help to solve this challenge?

Zinc crystals of insulin form hexamers which dissolve slowly in water.

18. Lispro was the first fast-acting insulin to be developed.
    a. What changes were made to the protein to achieve this?
    Switch the order of 2 amino acids in the protein (Pro-Lys switched to Lys-Pro)
    b. What was the basis for making these changes?
    IGF-1 has a similar amino acid sequence to insulin, and dissolves quickly in water. IGF-1 has the order of the 2 amino acids as Lys-Pro
    c. How did the changes lead to a faster acting drug?
    Disrupts the oligomerization of zinc crystals. This speeds up dissolution.

19. Aspart is the latest fast-acting insulin to be developed.
    a. What changes were made to the protein to achieve this?
    Proline changed to Aspartic acid. This changes a residue that is normally neutral into one with a –ve charge.

b. How did the changes lead to a faster acting drug?
Charge repulsion between molecules in the crystal speeds dissolution.

20. Glargine was the first slow-acting insulin to be developed.
    a. What changes were made to the protein to achieve this?
    2 arginines added to C-terminal of B chain. C terminal asparagine replaced by glycine on A chain..
    b. How did the changes lead to a longer-lasting drug?
    Arginines raise the isoelectric point from 5.4 to 7.2. The new isoelectric point is close to 7.4, making the protein less water soluble at physiological pH.
    c. What was the main goal of this action?
    Maintain more consistent basal concentration

21. Detemir is another slow-acting insulin.
    a. What changes were made to the protein to achieve this?
    Remove last amino acid from B chain, attach a palmitic acid to 2nd last amino acid
    b. How did the changes lead to a longer-lasting drug?
    Reduced water solubility and increase lipid solubility. Get slower dissolution into blood.
    c. What was the main goal of this action?
    Maintain more consistent basal concentration