(1) Describe the function of the following compounds in chemical sequencing, synthesis and modification:

(a) Dithiothreitol

(b) Dicyclohexyl carbodiimide

(c) t-butyloxycarbonyl chloride

(d) Cyanogen Bromide

(2) Protein primary sequences can be determined by translating gene sequences using the universal code. What are the advantages and limitation of this method of determining protein primary sequences?

(3) Consider the following sequence:

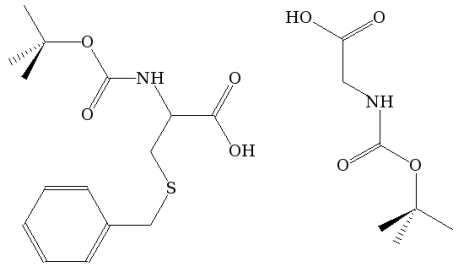
MGVYIVDHFT GNYALNEDKD VVAYEHAPKS LDDFVEEALK VEAGEVPAAP

List the expected fragments resulting from a limited proteolytic digest using trypsin.

(4) You have just determined the chemical sequence of a protein. Describe an experimental procedure to locate disulfide bonds, if they are present.

(5) Describe all steps in the synthesis of Lys-Ala. (include solvent system changes and washes)

(6) Consider the starting materials below; Boc-Gly on the left and Boc, S-benzyl-Cys on the right. Each starting material was treated with anhydrous trifluoroacetic acid The Gly containing product was then treated with dicylcohexyl carbodimide and then mixed with the Cys containing product in triethylamine. Draw the dipeptide products of this liquid phase reaction?



(7) Skeletal muscle contraction is regulated by the action of the troponin complex in response to Ca^{2+} signals. SDS-PAGE discontinuous electrophoresis reveals three bands with molecular weights of 36 kD, 24 kD and 18 kD. Describe experiments to address the following: (10 marks)

(a) What is the stoichiometry of the troponin complex? (ie. How many subunits of each polypeptide are present in the complex)

(b) What are the subunit interactions within the troponin complex? (ie. Which subunits bind to one another)

(c) Does the conformation of the troponin complex change in the presence of Ca^{2+}?

(8) You wish to synthesize the following peptide using chemical (solid phase) methods.

C H E M I S T R Y (or in three letter code - Cys His Glu Met Ile Ser Thr Arg Tyr)

Assuming each synthetic step has a 90% yield, how many moles of peptide will be synthesized if you start with 1.0 μmoles of Tyr covalently attached to the solid support?

(9) Can an unknown protein with a pI of 9.0 be visualized using native PAGE? Explain?

(10) The product of one cycle of Edman degradation is a PTH-amino acid. (5 marks)

(a) How are PTH-amino acids identified after a cycle of Edman degradation?
(b) If the PTH-amino acid does not correspond to any of the twenty common amino acids, what would be a reasonable explanation? How might you identify the unknown amino acid?

(11) (Western blots are among the most sensitive methods for detecting small quantities of proteins. Why is this method significantly more sensitive than the Coomassie Brilliant Blue stain? (4 marks)

(12) You are planning to chemically sequence a novel protein and perform N-terminal (Edman's reagent) and C-terminal End Group (carboxypeptidase A) experiments. You identify a single C-terminal residue (Ala) but do not detect any N-terminal End Group labelling. You know all of your reagents are working perfectly. Give a probable explanation for this result.

(13) In the 1960's, Anfinson performed a series of experiments that demonstrate the primary sequence of polypeptides contain the information necessary to fold into their native state. Explain Anfinson's experiments and results.

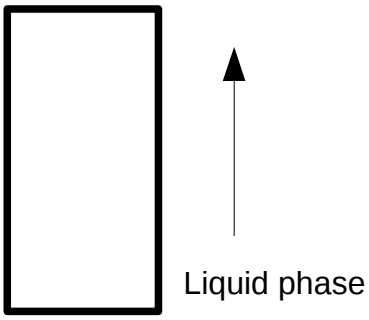
(14) What evidence exists that surface residues have only a minor role in protein folding?

(15) You have step-eluted a 5 mL protein sample from an ion exchange chromatography column using a solution containing 0.1 M NaAcetate (pH 5.0) and 0.5 M KCl. You now want to change the KCl concentration of the solution by dialysis. Design a reasonable dialysis experiment(s) that results in a protein sample in 0.01 M NaAcetate (pH 5.0) and 0.1 M KCl.

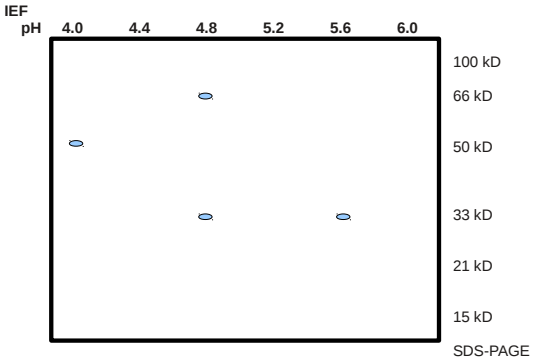
(16) Provide short answers to the following questions.

(a) What is the purpose of the 'fragmentation step' in chemical sequencing of proteins?
(b) What are semi-synthetic proteins?
(c) What are the advantages of capillary electrophoresis?
(d) What is the difference between hydrophobic interaction and reverse phase chromatography?

(17) Predict how a mixture of the amino acids Val, Gly and Glu would separate using Thin Layer Chromatography and a relatively non polar (eg. water:ketone) liquid phase. Mark and label the relative postion of each amino acid on the following diagram.



(18) Consider the following 2D gel separation of an unknown protein mixture. The isoelectric focussing dimension was run under native conditions in the pH range 4-6 followed by an SDS-PAGE separation in the vertical dimension.

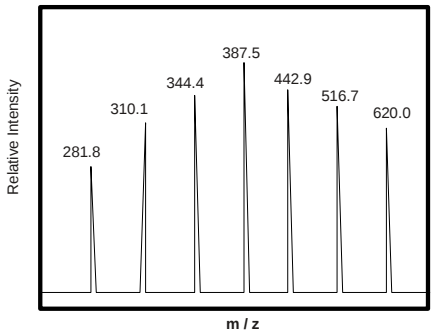


a) How many proteins are present in the unknown protein mixture?
b) Assume an identical unknown protein mixture is subjected to size exclusion chromatography and produces a single peak with an apparant molecular mass of 100 kD. What do we know about the size and subunit composition of each protein in the mixture?

(19) For each of the following questions, identify the products that would be produced upon completion of the chemical reaction(s). (Note: No need to draw the structures)

(a) Boc-Ala mixed with Val in the presence of anhydrous acid (eg. trifluoroacetic acid) in the liquid phase.
(b) Phenylisothiocyanate, Ala and Boc-Lys in the presence of a mild base in the liquid phase?
(c) Phenylthiohydantoin-Ala and Boc-Lys in the presence of a strong acid in the liquid phase?

(20) The following is raw data from a portion of an electrospray ionization mass spectrogram of a short polypeptide. Calculate the mass of the short polypeptide.



Answers:

1a – Dithiothreitol is a reducing agent used break potential disulfide bonds between and within subunit of multi subunit proteins. Covalent links between and within subunits interfere with the separation of subunits and subsequent fragmentation reactions.

1b – Thermodynamically, peptide bond formation is an unfavorable or endothermic process. DCCD reacts with carboxylate residues generating an improved leaving group and renders the overall process exothermic. This allows peptide bond formation to rapidly proceed to completion.

1c - t-butyloxycarbonyl chloride (Boc) is an N-terminal protecting group that forms an amine ester with the α-amino group in the presence of mild base and is removed by anhydrous acid. Protecting groups, such as Boc, are used to direct and control peptide synthesis.

1d – Cyanogen bromide is used in the chemical fragmentation of polypeptides. It specifically cleaves peptide bonds following Met residues.

2 – Translating gene sequences is cheap, rapid and accurate. Translating gene sequences cannot yield information about multi subunit proteins, disulfide bridges, post-transcriptional or post-translational modifications.

3 -

MGVYIVDHFT GNYALNEDKD VVAYEHAPKS LDDFVEEALK VEAGEVPAAP

Limited proteolysis means the reactions have not gone to completion and thus all possible products must be listed (including uncut starting material). The are 10 fragments listed below:

MGVYIVDHFT GNYALNEDK

MGVYIVDHFT GNYALNEDKD VVAYEHAPK

MGVYIVDHFT GNYALNEDKD VVAYEHAPKS LDDFVEEALK

MGVYIVDHFT GNYALNEDKD VVAYEHAPKS LDDFVEEALK VEAGEVPAAP

D VVAYEHAPK

D VVAYEHAPKS LDDFVEEALK

D VVAYEHAPKS LDDFVEEALK VEAGEVPAAP

S LDDFVEEALK

S LDDFVEEALK VEAGEVPAAP

VEAGEVPAAP

4 – Repeating the initial separation and fragmentation in the absence of reducing agents will preserve any disulfide bonds that may exist. Comparing the separated fragmentation products in the presence and absence of reducing agents will reveal intersubunit disulfides and may reveal intrasubunit disulfides. Given Cys is a relatively rare residue, in most cases, the residues involved in the disulfide bond can be unambiguously determined.

5 – (a) Couple Boc-L-ala to a suitable resin (eg. chloromethyl polystyrene) in mild base; Rinse

(b) Deprotect Boc-L-ala-resin using anhydrous acid; Rinse

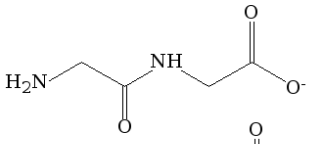
(c) Activate Boc-L-lys (side chain blocked) with DCCD; Rinse

(d) Couple Activate Boc-L-lys (side chain blocke) to L-ala-resin; Rinse

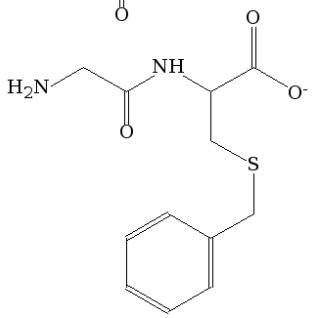
(e) Deprotect Boc-L-lys-L-ala-resin and release from resin using strong acid; Rinse

(f) product L-lys-L-ala is separated from incomplete products (chromatography)

Note: After each of the steps "a" through "d", the solid support must be rinsed or washed to remove the unreacted products.



6 – The products are Gly-Gly and Gly-S-benzyl-Cys. The anhydrous acid step removes the Boc protecting group from each amino terminus. The DCCD treatment activates the carboxylate of Gly. In triethylamine, the carboxylate of Gly will react with any available amino terminus (ie. Gly and S-benzyl-Cys)



7a – In order to answer this question we need two pieces of information:

(1) the molecular weight of the troponin complex

The molecular mass can be determined by several methods of which the most straightforward is Size Exclusion Chromatography (SEC) and comparison to a standard curve of molecular weights (eg. V_e/V_o vs log Mw). Alternative methods include analytical ultracentrifugation (not discussed in class), native PAGE (relatively inaccurate) and mass spectrometry (more difficult for complexes).

(2) the relative amounts of each subunit in the complex

If the mass of the troponin complex is small you may be able to deduce the subunit composition from the known subunit molecular masses. For example, if the complex is 96 kDa it must contain 1 x 36 kD, 1 x 24 kD and 2 x 18 kD.

If the mass of the troponin complex is large, you are unlikely to be able to deduce the composition from the known subunit molecular masses. In these cases, you will need to determine the relative amounts of each subunit in the complex.

The relative amounts of each subunit can also be determined by several methods. The most accurate is likely to be end group analysis as all PTH-amino acids have a virtually identical extinction coefficient which facilitates accurate comparisons of relative amounts. This involves the separation of reduced, denatured complex followed by treatment with Edman's reagent (PITC) and/or carboxypeptidases.

Alternative methods include separating and purifying the subunits in the complex followed by accurate determination of subunit concentrations (eg. Bradford, UV spectroscopy, etc.). Success would be dependent upon the ability to separate and purify the subunits without losses.

7b – In order to answer this question we need to separate the subunits and then conduct a series of binding assays.

Separation can be accomplished by reducing and denaturing the sample prior to a chromatography step (IEC, RP or even SEC). Once separated, the denaturant must be removed prior to the binding assay.

Again, there are many potential methods for carrying out the binding assay. The most straightforward is native PAGE. Here each of the subunits would be run individually (as a control or reference) and as pairs (eg. A + B, A + C, B + C). Comparison of the native PAGE bands to molecular weight standards would allow the detection of homomeric subunit interactions (eg. A + A, B + B, C + C). The formation of a novel band(s) of higher Molecular weight in the lanes containing subunit pairs would be indicative of a subunit interaction.

Alternative methods would include SEC and affinity chromatography. SEC would use the exact same set of samples (eg. Individually and as subunit pairs) and is conceptually identical to the native PAGE example and has the advantage of detecting homomeric subunit interactions with greater accuracy. Affinity chromatography would involve linking each subunit individually to a separate sample of resin (ie. three columns). Subsequently, each subunit would be passed down the column and retention would indicate a subunit interaction. (Note: for the affinity chromatography method, non-covalent homomeric interaction would have to broken (eg. denaturant) prior to testing for self-aggregation.

7c – To answer this question we require a method capable of detecting a signal arising from a binding induced conformational change.

In practice, spectroscopic methods are uniquely suited for this task and inevitably involve the comparison of the spectroscopic signal in the absence and presence of the ligand. Alternative potential methods include native PAGE.

Spectroscopic methods including Circular Dichroism (detects secondary structure content) and NMR H/D exchange (detects surface accessible H's) directly detect structural features of the protein and are the best bet for detecting a conformational change. UV and/or fluorescence spectroscopy are also possible though they detect the electronic environment of the chromophore as oppossed to a structural feature (eg. Ca^{2+} binding near the chromophore can produce a signal change that may not be due to a conformational change).

Native PAGE in the presence and absence of ligand will only work if there is a large conformational change that affects the overall shape of the troponin complex and therefore its relative mobility. In the case of a small or local conformational change the migration properties of the relatively large troponin complex are unlikely to be altered.

(Final Note: Complete X-ray or NMR structures in the presence or absence of Ca^{2+} are exceeding difficult to determine and would not be a cost- or time-effective means to answering the above question. Further, it is important to note that a structure may suggest but does not prove a conformational change occurs ... after all they are just a picture).

8 – Starting with Tyr attached to the solid support, we must perform 8 coupling and 8 deblocking reactions in order to complete the synthesis. If each step has a yield of 90%, the yield for the overall reaction will be

0.90^{16} = overall yield (expressed as a fraction)

and the moles of peptide synthesized would be

0.90^{16} x 1.0 μmoles = moles of peptide synthesized

Note: if you actually calculate the number, the answer is 0.19 μmoles

9- Yes.

Using normal native PAGE buffers (pH 8.8), the net charge of the unknown protein is slightly positive and it will not enter the gel, unless the electrodes are switched (anode at top). In order to increase the net positive charge and allow the protein to move within the gel, low pH buffers are required.

10a- Purified PTH-amino acids are run on a Reverse Phase or Ion Exchange chromatography column and their retention time is compared to those of standard, known PTH-amino acids. Identical retention times correspond to identical residues.

10b- Since proteins are composed of the 20 common amino acids, the most reasonable explanation is a post-translationally modified residue. Since most post-translationally modified residues have been characterized, these chromatographic retention time of these 'mystery' residues are compared to those of the known post-translationally modified residues. Alternatively, techniques such as mass spectroscopy, NMR, etc. can be used to identify the unknown residue.

11- Coomassie stain binds stoichometrically to proteins and the maximal signal depends upon the quantity of protein. Western blots immobilize the protein sample to prevent diffusion and use enzyme-linked or radiolabelled antibodies to generate a signal. Thus, the maximum signal is a function of substrate quantity or time which can be readily altered.

12- The Edman reagent typically reacts with free amino groups under alkaline condition and will hydrolyze amino terminal peptides in the presence of anhydrous acid. The most likely explanation is that either the free α-amino group or the peptide bond is modified and unreactive.

Extra information - Both types of post-translational modifications are known to occur, though the modification of the amino group is far more common. For example, pyroglutamyl residues are found at the amino terminus of many eucaryotic proteins and do not react with the Edman reagent.

13- Anfinson worked with RNase A, a small protein with four disulfide bridges that hydrolyzes RNA. Anfinson showed the following:

a – RNase A retains full activity when all disulfides are fully reduced

b – RNase A has no activity when fully denatured and all activity is restored when renatured in the presence of reducing agents

- Once folded, RNase A forms the correct four disulfides in the presence of oxidizing agents

c – RNase A has no activity when fully denatured and recovers ~ 1% activity when renatured in following treatment with oxidizing agents

- Approximately 1% of RNase A molecules form the correct four disulfides when treated with oxidizing agents while denatured

Together, these experiments indicate disulfide bond formation is random in the presence of denaturants while only the correct disulfides are formed in the absence of denaturants. Consequently, RNase A must adopt its native fold in the absence of denaturants.

14- Several lines of evidence exist:

(i) The mutation rates of surface residues are far higher than those for residues in the hydrophobic core.
(ii) Systematic chemical modification of surface residues does not affect protein folding.
(iii) Denaturants typically interact with residues of the hydrophobic core and not surface residues.

15 – You need to reduce the concentartion of NaAcetate by a factor of 10 and KCl by a factor of 5. Consequently, the minimum volume of dialysate will be determined by the NaAcetate dilution using a solution without NaAcetate.

(5 mL)(0.1 M) + (x mL)(0 M) = (5+x mL) (0.01 M)

0.5 mL M = 0.05 mL M + 0.01x mL M

x = 0.45 mL / 0.01 = 45 mL

Knowing that a 45 mL solution containing no NaAcetate will yield the correct final NaAcetate concentration, we can calculate the concentration of KCl as follows:

(5 mL)(0.5 M) + (45 mL)(y M) = (50 mL) (0.1 M)

2.5 mL M + 45y mL M = 5 mL M

y = 2.5 mL M/45 mL = 0.056 M

Thus we need to dialyze our protein against 45 mL of 56 mM KCl.

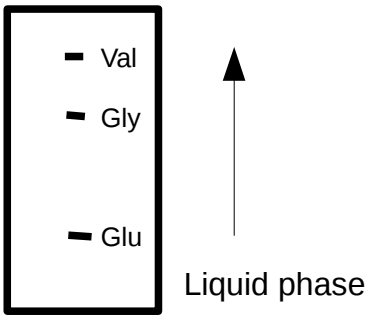
16a – Cleave polypeptide into fragments sufficiently small to sequence (<50 residues)

16b – Proteins composed of chemically and biologically synthesized fragments. Requires the production of a truncated or cleaved biologically synthesized fragment.

16c – Primary advantages are (i) increased heat dissipation which allows for higher voltages and faster separations, and (ii) diffusion and convective mixing are limited by the narrow capillary and will produce sharper peaks which give larger signals (ie. improved resolution)

16d – The degree of resin substitution with non-polar groups. HIC resins are lightly substituted and generally interact with hydrophobic patches of native, folded proteins. RP resins are densely substituted and behave as though a non-polar organic solvent coats the bead. Proteins enter the non-polar organic solvent layer where they denature.

17 –



The non-polar Val residue migrates furthest while the polar charge Glu residue hardly moves. The Gly (intermediate character between non-polar and polar uncharged) has an intermediate migration though it will be closer to Val.

18a – At minimum, there are 3 protein samples (at pI 4, 4.8 and 5.6) though there may be 4 (we do not know if the 2 bands at pI 4.8 are one protein or two).

18b – Since the IEF was under native conditions there are at least 3 proteins. Knowing that each of these proteins must have a molecular mass of 100 kD from the SEC, it is clear there are only three proteins with the following composition.

pI 4.0 band would correspond to a dimer (50 kD x 2 = 100 kD)
pI 5.6 band would correspond to a trimer (33 kD x 3 = 99 kD)
pI 4.8 band would correspond to a heterodimer. (66 kD + 33 kD = 99 kD)

(Note: Given the resolution and errors associated with SEC, 100 kD and 99 kD cannot be distinguished from one another)

(19) For each of the following questions, identify the products that would be produced upon completion of the chemical reaction(s).

(a) Anhydrous acid will 'deblock' the Boc-Ala producing both Ala and Boc (actually the breakdown products isobutylene and CO_2). Val is unreactive in anhydrous acid. Upon complete reaction, Val, Ala and Boc breakdown products would be present.

(b) In mild base, coupling type reactions can occur. Phenylisothiocyanate (PITC) will add to available amines, producing Phenylthiocarbamyl-Ala (PTC-Ala) and to a lesser extent Boc-Lys-PTC (side chain amine). While a small quantity of peptide bond formation occurs in the absence of DCCD, this reaction is exceedingly slow given the large energy barrier (E_a). Consequently, PITC will react with available amines and detectable quantities of peptide bond formation will be prevented. Upon complete reaction, PTC-Ala and Boc-Lys-PTC are produced.

(c) Strong acid will 'deblock' Boc-Lys producing Lys and Boc-breakdown products and leaving phenylthiohydantoin-Ala unchanged.

(20) Choose any two adjacent peaks in the mass spectrogram (typically choose strong peaks as they are measured with the greatest accuracy).

Peak 1 m/z = 387.5 Peak 2 m/z = 344.4

Clearly Peak 2 has the larger charge as its m/z ratio is smaller than Peak 1, and we can set up our two basic equations.

(a) $387.5 = (M + z)/(z)$

(b) $344.4 = (M + z + 1)/(z + 1)$

Equation (a) can be rearranged to $z = M / 386.5 = 0.00259\ M$ and substituted into equation (b) yielding

$344.4 = (M + 0.00259\ M + 1) / (0.00259\ M) + 1$. Solving for M yields

$0.892\ M + 344.4 = 1.00259\ M + 1$

$343.4 = 0.111\ M$

$M = 3094$ D