CHAPTER 3
Proteins

Molecular Biology of the Cell, Fifth Edition

Questions

3-1 The table lists the average strengths of noncovalent bonds.

	strength in vacuum (kcal/mol)	strength in water (kcal/mol)
ionic	80	3
hydrogen	4	1
Van der Waals attraction (per atom)	0.1	0.1

Table Q3-1

Protein Lock binds to protein Key in a reaction with $\Delta G° = -14$ kcal/mol. When these proteins bind, 350 $Å^2$ of the surface of Lock is closely apposed to the same area on the surface of Key, so that water is excluded.

A. Estimate how much of the binding energy the van der Waals interactions contribute. (For this calculation, assume that an average atom has a surface area of 7 $Å^2$.)

B. If hydrogen bonds contribute the remaining binding energy between amino acids, estimate how many hydrogen bonds form between Lock and Key. Does it depend on whether the hydrogen-bonded amino acids occur within the buried surface or elsewhere in the proteins? Explain.

3-2 Consider a protein X that is known to exist in two slightly different conformations, called X1 and X2. Proteins A, B, and C are ligands that can bind to protein X. The sites on X required for binding to A, B, and C are located in different regions of the protein. On the basis of experimental evidence of protein–protein complexes, you devise the hypothetical binding reaction scheme shown in Figure Q3-2. According to the hypothesis, which pairs of binding sites on X exhibit negative coupling (negative linkage)? Which pairs exhibit positive coupling (positive linkage)? Does the addition of a high concentration of protein A increase or decrease the affinity of X for B? Of X for C?

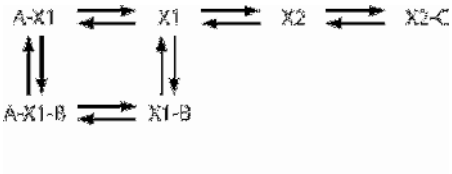


Figure Q3-2

3-3 You are working in a laboratory trying to understand how cells change shape in response to treatment with a particular chemical. On treatment, normal wild-type cells convert from a flattened irregular shape to a rounded, nearly spherical shape. Previously, another student in the laborator identified a mutant that remained flat after treatment. The gene that encodes the normal protein is called *Flat1* and the mutant gene is designated *flat1*$^{-}$. To identify additional genes responsible for this behavior, you mutagenize *flat1*$^{-}$ cells and look for those that can respond normally to stimulation. You identify cells that contain a "suppressor" mutation in a second gene, called *supr1*$^{-}$, that restores the wild-type behavior to cells with the *flat1*$^{-}$ mutation. Further genetic manipulation and DNA sequencing reveals the data shown in the table below.

Initially, you are surprised to find that cells containing a mutation in *supr1*$^{-}$ and a normal copy of *Flat1* have the same mutant behavior as cells containing only a *flat1*$^{-}$ mutation. However, when you examine the nature of the amino acids that are changed in the mutants, you instantly suggest a hypothesis to account for the observations. What is your hypothesis? What biochemical experiment with purified proteins will test your hypothesis? What biochemical results would support the hypothesis?

	shape before treatment	shape after treatment	mutations
Flat1 and Supr1 (wild-type)	flat	round	none
flat1^{-} and Supr1 (original mutant)	flat	flat	Lys to Asp change
Flat1 and supr1^{-}	flat	flat	Glu to Lys change
flat1^{-} and supr1^{-} (suppressed mutant)	flat	round	Lys to Asp change in Flat1 and Glu to Lys change in Supr1
deleted Flat1 and deleted Supr1	flat	flat	no Flat1 or Supr1 protein at all

Table Q3-3

3-4 The Src protein kinase is composed of several domains that interact to control the behavior of the protein. Figure Q3-4 (A and B) shows the structure of the repressed conformation of Src. In 1979, long before the x-ray crystal structure of Src was known, scientists used a technique called partial proteolysis to explore the structure of Src. Treatment with small amounts of the V8 protease cleaved Src at a single location to generate two protein fragments. The addition of more V8 protease caused further cleavage of one of the fragments, but in both cases kinase activity was maintained. Figure Q3-4C shows a protein gel from this experiment.

The proteolytic fragments are detected by their radioactivity because the Src protein contained radioactive atoms; in some reactions Src was radiolabelled throughout its length ("all") and in others the radiolabel was only at the N-terminal end ("end").

A. Indicate on the ribbon diagram where you think cleavage first occurs to generate the 34 kD and 26 kD fragments. Circle and label the regions corresponding to each fragment.

B. Which fragment contains the kinase domain? On the basis of the regulation of Src explained in the textbook, would you have expected this fragment to be active as a kinase, as observed? If so, do you expect the kinase activity be greater than, the same as, or less than that of the intact protein? Explain.

C. Dephosphorylation, mutation, or deletion of a particular tyrosine residue in the C-terminal tail of the protein leads to increased activity of the kinase and promotes cancerous transformation of cells. Do you expect the proteolytic fragment containing the kinase domain to be regulated strongly by phosphorylation of the key tyrosine in the C-terminal tail? Explain.

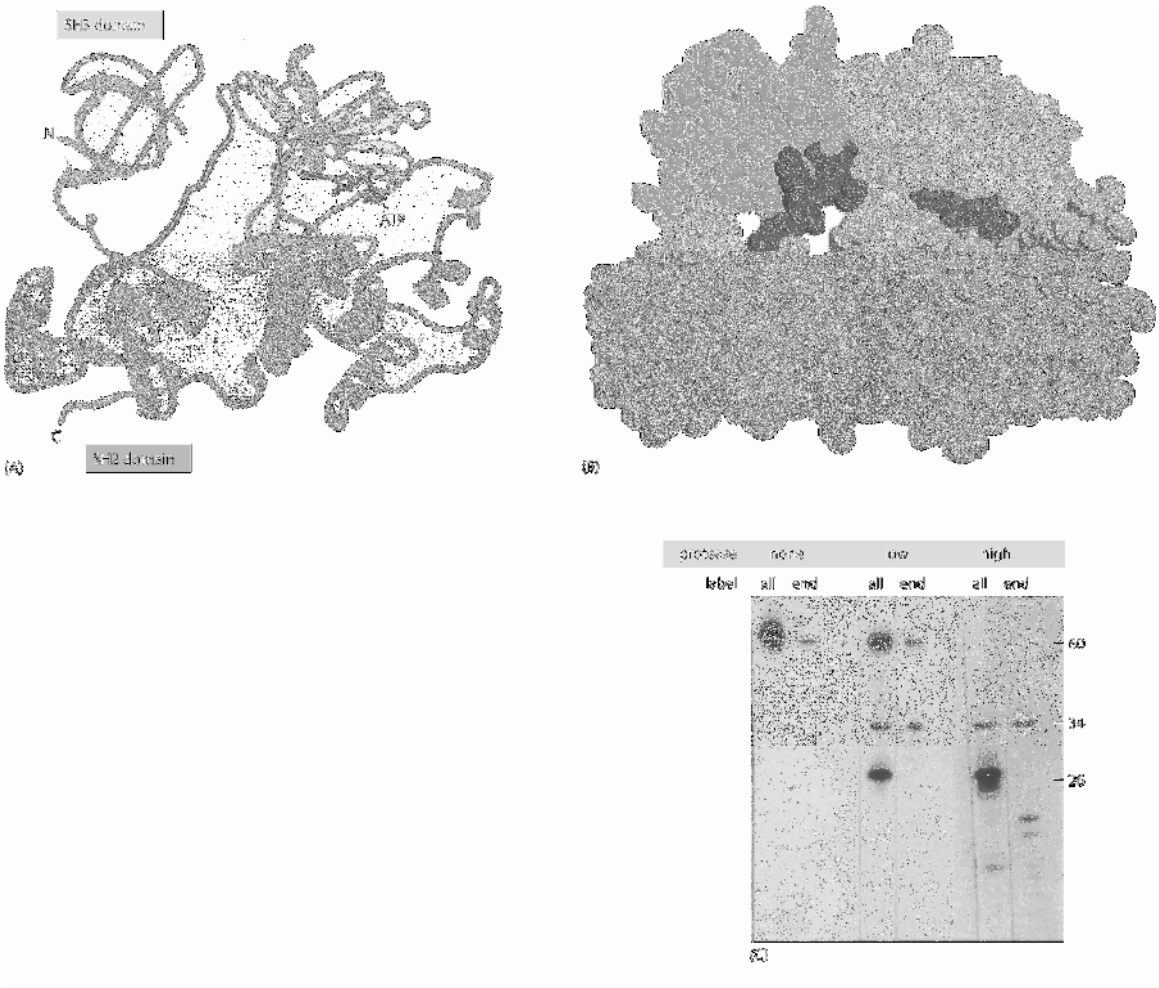


Figure Q3-4

3-5 Antibodies raised against proteins of interest are useful tools for biomedical research, because they allow the visualization or isolation of individual proteins from a complex mixture of different proteins. A Western blot (WB) is one method of visualizing proteins: all proteins are extracted from cells, the proteins are denatured and separated by size using electrophoresis through a gel, and the protein of interest is detected by binding to an antibody that is linked to a fluorescent or luminescent molecule.

Immunofluorescence (IF) provides a way to visualize a particular protein within a cell using microscopy: the cell is "fixed" (killed and preserved intact), flooded with an antibody that binds a protein of interest, and washed free of unbound antibody. Because the antibody is also bound to a fluorescent molecule, the subcellular location and intensity of the fluorescence indicates the location and concentration of the protein of interest.

Unfortunately, many antibodies that work well for WB are not useful for IF. Consider an antibody that yields an intense signal located at the expected position on a WB. When the WB is performed with cells lacking the protein of interest, the intense signal is absent but there remain a few faint signals from non-specific proteins of the wrong size. Yet this antibody is not useful for IF because in IF the wild-type cells and the cells lacking the protein of interest look the same. Suggest two possible explanations for the observation that the antibody is more specific in a WB than in an IF experiment; note that at least five explanations are possible.

3-6 Immunoprecipitation (IP) is commonly used in biomedical research to examine whether proteins interact physically with each other. You perform an IP procedure to determine if a protein called Elm interacts with proteins called Oak and Red. First, you link an Elm-specific antibody to small beads that settle out of solution readily. Second, you grind up cells, extract the soluble proteins, add the beads, and incubate for 2 hours. Third, you centrifuge the mixture to separate the beads from the unbound proteins. Fourth, you wash the beads three times to remove weakly bound proteins; to do this, you add a large volume of buffer to the beads, incubate for 5 minutes, then remove the buffer. Finally, you add detergent to the beads to denature and dissociate all proteins before separating them on a gel and revealing them with additional specific antibodies. Because you do not have antibodies against Oak and Red, you genetically engineer cells in which both of these proteins are fused to a small protein handle or tag called HA, for which you purchase specific antibodies. Your gel is shown in Figure Q3-6.

A. Your lab partner Chris looks at your gel and says that you have evidence that Elm binds directly to Oak and to Red. Do you agree? Is there a plausible alternative interpretation of your data?

B. Chris says that your data demonstrate that Elm, Oak, and Red form a three-protein complex. Do you agree? Explain.

C. Assume that you have solid evidence that Elm binds directly to Oak and it also binds directly to Red. Chris says that Elm has higher affinity for Red

(has a lower K_d) than it has for Oak. Do you agree? Explain, with reference to an equation for K_d.

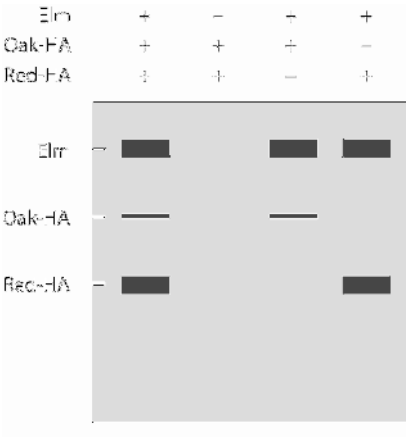


Figure Q3-6

3-7 By computational analysis of genome sequences, you discover a new protein family with members from many different eucaryotic and procaryotic organisms, but nothing is known about the functions of these proteins. You suspect that these proteins may share a previously unknown protein fold and thus you wish to determine the x-ray crystal structure of a member of this family. Because the most challenging steps in structural determination are the production of very pure, concentrated protein and the formation of crystals in which protein molecules pack closely together in a uniform conformation, you need to choose carefully which member of the family to use for structural experiments. You decide to use a procaryotic protein, because it is easier to get large amounts of protein. You narrow the choice to family members from the model organism *E. coli*, the pathogenic (disease-causing) organism *Yersinia pestis*, the thermophilic (heat loving) organism *Thermus aquaticus*, and the halophilic (salt loving) organism *Haloquadratum walsbyi*. You will overexpress one of these proteins in *E. coli* from recombinant DNA. Which one is likely to be easier to purify and to be more energetically stable and conformationally uniform under the crystallization conditions? Explain.

3-8 Members of the Mitogen-Activated Protein Kinase (MAPK) family are essential for many responses to the extracellular environment. A MAPK becomes active after it becomes phosphorylated by a second kinase. These MAPKs contain a "docking site" distant from the active site that binds to conserved 10–15 amino acid "docking motifs" found in a variety of proteins (see Figure Q3-8). Docking motifs are found in proteins that bind MAPKs (scaffold proteins), proteins that are substrates of MAPKs, proteins that activate MAPKs by phosphorylating them (MAPK kinases), and proteins that inactivate MAPKs by dephosphorylating them (phosphatases). The kinetic properties of a particular MAPK were measured with a small peptide substrate that contains only the preferred phosphorylation site.

The K_m is 300 nM and the k_{cat} is 20/sec. Do you expect the k_{cat} for the natural protein substrate to be higher or lower than that for the peptide substrate? What about the K_m? Explain, with reference to the reaction shown in panel A below:

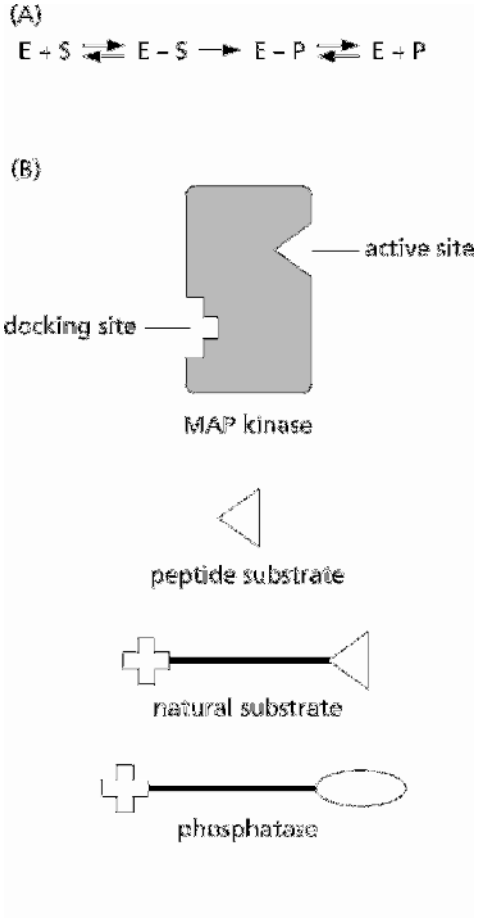


Figure Q3-8

Answers

3-1

A. In this simplified scheme, approximately 10 kcal/mol of the binding energy is contributed by van der Waals interactions: $(2 \times 350\ Å^2)$ (0.1 kcal/mol atom)/(7 $Å^2$/atom) = 10 kcal/mol.

B. The information given is not sufficient to estimate how many hydrogen bonds form between Lock and Key to account for the remaining 4 kcal/mol of binding energy. The number of hydrogen bonds needed to account for the remaining binding energy depends on whether the amino acids involved are sequestered away from or exposed to water. If the hydrogen-bonded amino acids are buried, then a single bond can account for the entire 4 kcal/mol of binding energy. If they are exposed, then about four hydrogen bonds are needed. If the amino acids are partly exposed, then perhaps two or three hydrogen bonds are needed.

3-2 The A/C and the B/C pairs exhibit negative linkage. The A/B pair exhibits positive linkage. Addition of a high concentration of A increases the affinity of X for B because it stabilizes X in the X1 conformation, which has an increased affinity for both A and B, pulling the equilibrium toward more X1 and less X2. Addition of a high concentration of A decreases the affinity of X for C because A pulls X into the X1 form, reducing the amount of the X2 conformation by mass action, and X2 has a higher affinity for C than does X1.

3-3 The hypothesis is that the Flat1 protein binds to the Supr1 protein, and this dimerization is required for the change in cell shape. Furthermore, this interaction depends on an ionic bond between a positively charged amino acid, lysine, on Flat1 and a negatively charged amino acid, glutamate, on Supr1. When an amino acid is mutated to another with the opposite charge the ionic interaction is destroyed, but when both amino acids are switched in their electrical charge the interaction is restored. To test this hypothesis, you can perform biochemical binding measurements between the wild-type and mutant proteins. The hypothesis predicts that purified Flat1 and Supr1 will bind each other when both are wild-type or both are mutant, but not when one is wild-type and the other is mutant.

3-4

A. See Figure A3-4.

B. The 26 kD C-terminal fragment contains the kinase domain that uses ATP as an enzymatic substrate. Yes, from the description of Src regulation in the textbook, this fragment is expected to be active as a kinase, as was observed. The kinase activity of the kinase domain is expected to be greater than that of the intact protein, because in the intact protein the N-terminal portion of the protein inhibits the kinase activity (in other words, it is an inhibitory regulatory domain).

C. The fragment with the kinase domain and the C-terminal tail (the 26 kD fragment) will not be regulated strongly by the phosphorylation of this tail. In the intact protein, the tail phosphorylated on tyrosine inhibits the kinase activity by binding to the SH2 domain in the N-terminal half of the protein; in the kinase domain fragment alone, there is no SH2 domain to interact with the C-terminal tail and inhibit the kinase activity (see Figure m3-68/3-69 in the textbook).

References:

Collett MS, Erikson E & Erikson RL (1979) Structural analysis of the avian sarcoma virus transforming protein: sites of phosphorylation. *J. Virol.* 29, 770–781.

Brugge JS, Darrow D (1984) Analysis of the catalytic domain of phosphotransferase activity of two avian sarcoma virus-transforming proteins. *J. Biol. Chem.* 259, 4550–4557.

MacAuley A, Cooper JA (1989) Structural differences between repressed and derepressed forms of p60c-src. *Mol. Cell. Biol.* 9, 2648–2656.

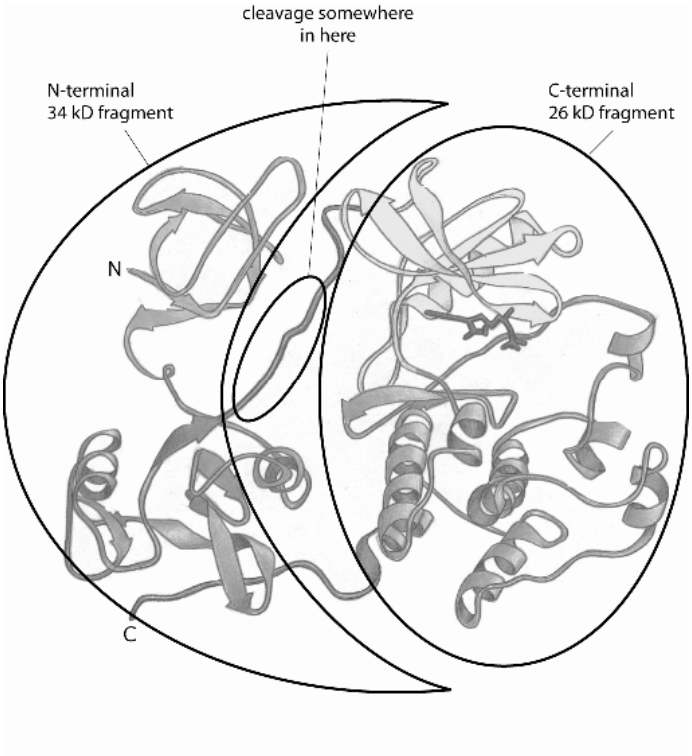


Figure A3-4

3-5 The answer should contain any two of several plausible explanations, similar to these: (1) A WB has the advantage of separating proteins by size so that cross-reacting proteins are often physically separated from the protein of interest. (2) The antibody may bind only to the unfolded state of the protein of interest, and the fixation used on the intact cells may not have unfolded the region that binds to the antibody. (3) The antibody may bind strongly to both the folded and the

unfolded states of the protein of interest, but bind only the folded state of non-specific cross-reacting proteins. Because the WB procedure denatures proteins, it will not give a strong signal for the non-specific cross-reacting proteins. (4) The specific protein of interest may be spread throughout the cells, whereas one or more non-specific cross-reacting proteins may be concentrated in a subcellular milieu. In this case, the IF signal from the localized cross-reacting protein will appear more intense than the signal from the non-localized protein of interest. (5) The protein of interest inside cells may bind to a protein partner or undergo a conformational change that blocks or competes with binding of the antibody, thus reducing the signal from the protein of interest under folded conditions. In this case, when the proteins are denatured and separated by size for the WB, the protein of interest becomes able to bind the antibody.

3-6

A. No, I don't agree. These data alone are unable to rule out the possibility that Elm binds to an unnamed protein that in turn binds to Oak, and that there is an analogous bridging protein for Red. Note that the data do rule out the possibility that Oak is needed to bridge the interaction between Elm and Red, and the possibility that the Elm–Oak interaction occurs only through bridging by Red.

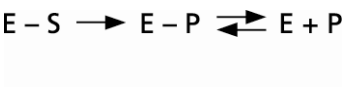
B. No, I don't agree. Chris's explanation is possible, but the data are also consistent with the possibility that Elm forms two different kinds of complexes, one containing Oak and the other containing Red.

C. No, I don't agree. Chris may be correct, but the data are consistent with the possibility that the affinity (the K_d) of Elm for Red is identical to that for Oak. Note that $K_d = ([\text{Elm}]\,[\text{ligand}])/[\text{Elm–ligand}] = k_{off}/k_{on}$. The experiment measures the amount of Elm–ligand complex that remains at the end of the immunoprecipitation procedure. From the equation, there are at least four possible explanations for how the K_d and [Elm] might be the same for Oak and Red, yet yield different [Elm–ligand] measurements. First, the k_{off} (and k_{on}) of Oak may be higher than that for Red; because the washing steps took so long, you may have lost much of the Elm–Oak complex that is present in cells. Second, cells might have much more Red than Oak, which pushes the formation of more Elm–Red complex by mass action. Third, the antibody against Elm may interfere with binding of Elm to Oak but not with binding of Elm to Red. Finally, one Elm molecule may bind a large complex of Red molecules but bind only a single molecule of Oak.

3-7 The protein from the thermophilic organism is most likely to have the desired properties. Proteins in these organisms have evolved to be stable and functional at high temperatures. In other words, the $\Delta G°$ of folding in these organisms is much greater than for organisms that live at moderate temperatures. There are two advantages to selecting the protein from the thermophilic organism. First, in the purification procedure, if you heat the protein extract to a temperature near boiling, all of the *E. coli* proteins will be denatured and will aggregate (just as a

gelatinous goo becomes solid when you make a hard-boiled egg), but the thermophilic protein will remain soluble. Second, the thermophilic protein will probably be more stable and more uniform at moderate temperatures than a protein evolved to be flexible, dynamic, and functional at those moderate temperatures.

3-8 The k_{cat} is likely to be somewhat decreased for the protein relative to the peptide. Recall that the k_{cat} is a composite measurement of two steps:



The docking interaction is unlikely to have a strong effect on the conformation of the active site because it serves to tether both positive and negative regulators to the MAPK, so the conversion of E-S to E-P is likely to be similar for both substrates. However, the docking interaction is likely to make the dissociation of E-P slower for the protein than for the peptide. In contrast, the K_m will be significantly lower for the protein than for the peptide, primarily because the affinity of the MAPK for the protein will be much greater as a result of the binding of the docking site to the docking motif, reflected in a much lower K_d. Consider the equation $K_m = (k_{off}+k_{cat})/k_{on} = K_d + k_{cat}/k_{on}$. For the protein relative to the peptide, K_d and k_{cat} will be lower and k_{on} will be higher or the same, so K_m will decrease substantially.