Proteins Have Stable Equilibrium Conformations

NATIVE AND DENATURED STATES ARE STABLE STATES OF PROTEINS

An important feature of proteins is their *stable equilibrium* conformations. A protein has a given native structure under given native conditions, and it has a given denatured structure under given denaturing conditions. That structure does not depend on the kinetic process by which the protein reached that state. So, a protein that was folded on a ribosome in a cell is in the same state as a protein that was refolded from an unfolded structure in a test tube, as long as it is under the same conditions. That means that protein properties can usually be expressed by equilibrium thermodynamics, without the need for pathway information. There are some exceptions, however. The structures of protein crystals or aggregates sometimes do depend on the initial conditions or how fast they were formed. Here, we explore some stable states of proteins and the forces that stabilize them.

Protein stability is important for several reasons. First, it matters to the cell. A cell maintains *proteostasis* (the folding homeostasis of the full set of all its proteins, its proteome). Cells have developed sophisticated mechanisms for maintaining and regulating protein stability and conformational equilibria in the face of protein degradation or of misfolding and aggregation. Mechanisms include the use of chaperones (proteins that help other proteins fold), proteases, the proteasome, and control of protein synthesis and degradation rates. The stabilities of proteins are factors in amyloid and other diseases, aging, and cancer. Second, protein stability gives insights into the physical forces of protein folding. Third, when developing biotechnological therapeutics (protein drugs), it is essential to formulate solution conditions that manage protein folding, degradation, aggregation, solubility, crystallization, and fibrillization.

Under biological conditions, a protein is typically *folded*, or *native*.[1] Under harsher conditions—in acids or bases, at high temperatures, or in chemical denaturants—a protein can be *unfolded* or *denatured*. Changes in protein structure are either *reversible* or *irreversible*. Reversibility means that if you perturb a protein away from its initial state—even through a large perturbation—and you then re-establish the initial conditions, the protein will return to its initial state. The advantage of finding conditions for reversibility is that you can interpret the results using the powerful tools of equilibrium thermodynamics.

Some transformations of proteins are irreversible. High temperatures can covalently degrade proteins, for example, by hydrolyzing the amide side-chain groups of glutamine and asparagine, removing the NH_2 group and converting the remaining side chain into glutamic acid or aspartic acid. Protein backbones can be covalently degraded by proteases. Covalent degradation is irreversible because lowering the temperature or removing the protease after covalent bonds have been broken does not return the protein to its original structure.

At high concentrations, proteins may crystallize or aggregate, either reversibly or irreversibly. Irreversible aggregation among proteins may arise either from covalent bonding between proteins, for example through disulfide bond formation, or when chains become so highly entangled that they cannot disengage from each other on the experimental timescale. In this chapter, we focus on reversible processes.

Anfinsen's Hypothesis: Native States Are Thermodynamically Stable

Until the 1960s, a key question was whether proteins could fold and unfold reversibly. Previously, protein science had been hindered by an inability to purify proteins. Experimentalists had often inadvertently studied irreproducible processes such as aggregation. It had not been clear that protein structures were thermodynamically stable states of matter. Experiments on bovine pancreatic ribonuclease A by Christian B Anfinsen in the early 1960s gave the first proof that folding was reversible and that native states were thermodynamically stable [1]. Anfinsen broke the native disulfide bonds, denatured the proteins, then reestablished native conditions, and found that the protein refolded correctly. At that time, disulfide bonding was the method of choice because disulfides were uniquely trappable and identifiable. His work, for which he was awarded the 1972 Nobel Prize in Chemistry, showed that the native structure of a protein could be fully encoded within its amino acid sequence (that is, it is thermodynamically stable), and thus successful folding does not require a special processing history or kinetic sequence of events. On the other hand, protein fold-

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[1] Throughout this book, we use the terms *native* and *folded* interchangeably, and we use *unfolded* and *denatured* interchangeably. We use the terms *conformation* and *configuration* interchangeably, consistent with standard usage in statistical mechanics. We use the term "state" in a sense that is macroscopic, not microscopic. A state corresponds to a signal that you can see in some experiment. Fluorescence or circular dichroism can distinguish the native state (N) from the denatured (D) state, for example. Therefore, by our definition, a "state" encompasses an *ensemble* of microscopic chain conformations—often a huge number of such *microstates*.
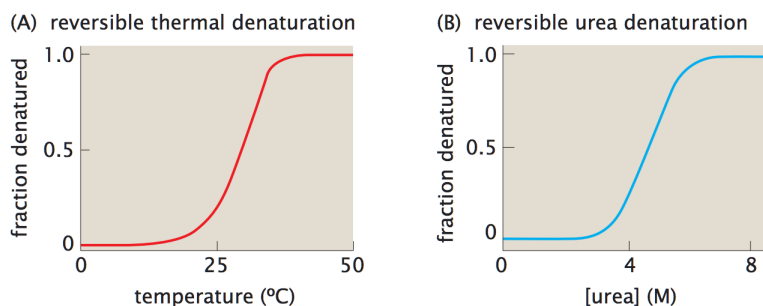
(A) reversible thermal denaturation

(B) reversible urea denaturation

ing in the cell can be assisted by other molecules. For example, some proteins are known to undergo *co-translational folding*; their folding happens as they are being released by the ribosome. And, the folding of many proteins is often facilitated *in vivo* by the class of proteins called *chaperones*, which help other proteins fold inside cells and prevent their misfolding and aggregation. Nevertheless, many small single-domain proteins are routinely unfolded and refold reversibly in test tubes, without biological helper molecules. Hence, native structures of proteins are stable equilibrium states of matter.

The Basic Experiment of Protein Stability Is Equilibrium Denaturation

The basic experiment that measures protein stability is equilibrium denaturation. You make up a series of different protein solutions, $1, 2, \ldots, M$, having different amounts of denaturing agent $x_1, x_2, \ldots, x_M$. By "denaturing agent," we mean either temperature or a chemical, such as guanidinium hydrochloride (GuHCl), urea, alcohol, or acids or bases, for example. Then, for each particular solution, having denaturing agent in amount x, you measure (typically by some form of spectroscopy) the proportion $f_N(x)$ of native protein molecules and the proportion $f_D(x) = 1 - f_N$ of denatured protein molecules (assuming you observe only the two states, a common situation). Increasing the denaturing agent increases the population of D relative to N. In the absence of denaturing agent, the protein is fully native (except for fluctuations). In high concentrations of denaturing agent, the protein is denatured. **Figure 3.1A** shows denaturation by high temperatures; Figure 3.1B shows denaturation by high concentrations of urea. Such plots, called *melting* or *denaturation* curves, are typically sigmoidal in shape, as a function of denaturing agent x. At the midpoint, a small change in denaturant concentration shifts the distribution of protein conformations. In this sense, protein denaturation is a miniaturized version of a phase transition in a larger system; for example, a small change in temperature, at the right temperature, causes water to boil or freeze.

To get insight from a denaturation profile, you need to convert it into a quantity called the *free energy*,[2] *G*. In order to compute the *folding free*

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[2]There are two types of free energy: the Gibbs free energy G and the Helmholtz free energy F. F differs from G in how pressure–volume effects are treated. Pressure–volume changes, which can be large for gases or gas–liquid systems, are typically small for protein solutions, so the enthalpy H and internal energy U are interchangeable, $H \approx U$, and the Gibbs free energy $G(T, P, N) = H - TS \approx F(T, V, N) = U - TS$ is

Figure 3.2 Free energies of folding can be determined from denaturation curves using Equations 3.1 and 3.2 (A and B). The free-energy dependence on denaturants such as urea or GuHCl is linear (C), so ΔG_{fold} in the absence of denaturant can be extrapolated. In contrast, the temperature dependence (D) is curved.
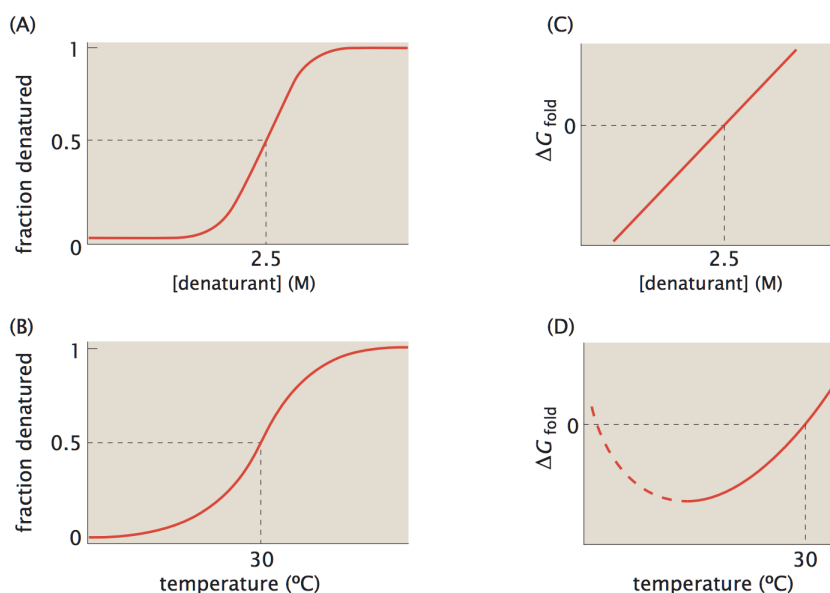
energy, $\Delta G_{fold} = G_N - G_D$ for the folding of a protein from its denatured state (D) to its native state (N), define a folding *equilibrium constant K* as

$$K = \frac{f_N}{f_D},\qquad(3.1)$$

where f_N and f_D are the fractions of native and denatured states. You can express the free energy of folding in terms of K as

$$\Delta G_{fold} = -RT\ln K,\qquad(3.2)$$

where T is the absolute temperature (in Kelvin) and R is the gas constant. For unfolding, you have $\Delta G_{unfold} = -\Delta G_{fold}$.[3] Next, we describe how to use ΔG_{fold} to get insights into the driving forces of folding.

Figure 3.2A illustrates chemical denaturation, and Figure 3.2C shows $\Delta G_{fold}(c)$, the dependence of the free energy of folding on the concentration c of GuHCl denaturant. $\Delta G_{fold}(c)$ is typically a linear function of c. The slope of $\Delta G_{fold}(c)$ versus c is called the *m-value*.

Another way to denature proteins is by heating them (see Figure 3.2B). $\Delta G_{fold}(T)$ is usually a curved function of temperature T (see Figure 3.2D). You can measure the properties of proteins as a function of temperature in a *calorimeter*. A differential scanning calorimeter applies a series of different temperatures to a protein solution at equilibrium. A calorimeter measures the heat taken up or given off by the protein solution at each temperature. The heat absorption in protein unfolding rises to a maximum with increasing temperature, then decreases (Figure 3.3). The temperature, T_m, at which the heat

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interchangeable with the Helmholtz free energy. Experimental studies usually report the free energy G and enthalpy H.

[3] $R = 8.314\,\mathrm{J\,mol^{-1}\,K^{-1}}$) is the gas constant. $R = kN_{avo}$, where k is Boltzmann's constant, $1.38 \times 10^{-23}\,\mathrm{J\,K^{-1}}$ per molecule, and $N_{avo} = 6.022 \times 10^{23}$ molecules $\mathrm{mol^{-1}}$. At room temperature ($T = 300$ K $= 27°$C), the product RT is approximately $0.6\,\mathrm{kcal\,mol^{-1}}$ using $R = 1.987 \times 10^{-3}\,\mathrm{kcal\,mol^{-1}\,K^{-1}}$). (Note the conversion 1 cal $= 4.184$ J.)
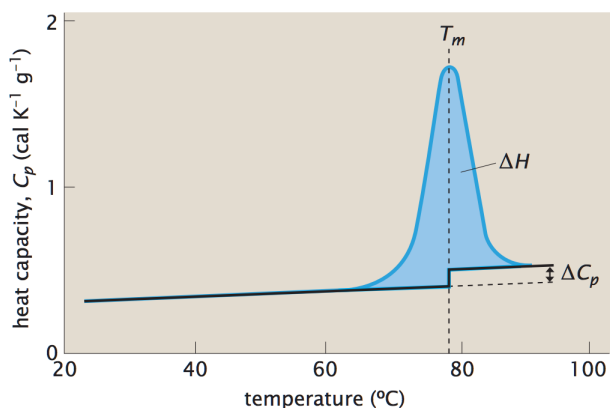
Figure 3.3 Calorimetry measures the heat capacity of protein unfolding with temperature. Increasing the temperature of a protein solution leads to a peak in the heat capacity, indicating that energy is absorbed as the protein unfolds. T_m is the temperature of the peak of the transition. ΔC_p is the heat capacity of unfolding, measured at constant pressure. It is the difference between the pre-transition and post-transition baselines. ΔH, the calorimetric enthalpy of unfolding, is given by the area under the peak, extrapolating to T_m from the baselines. (Adapted from PL Privalov and NN Khechinachvili. *J Mol Biol.* 86(3):665–84, 1974. With permission from Elsevier.)

absorption is maximal—called the *melting temperature* or *denaturation temperature*—is the midpoint of the denaturation transition of the protein. You want to know the *excess* heat capacity of the unfolding transition: the heat capacity change from unfolding minus the heat capacity change of the pure solvent. As a practical matter, you can obtain ΔC_p at the denaturation point by either subtracting the sloping baseline or integrating to get the enthalpy and finding the slope, $\Delta C_p = dH/dT_m$.

Calorimetry experiments show that protein denaturation resembles a *melting process*. At its melting point, a material's energy and entropy both increase sharply with temperature, as bonds break. Similarly, at the midpoint temperature of thermal denaturation, a protein's energy and entropy increase. The increased energy indicates that some intrachain interactions are broken, and the increased entropy indicates that the system gains conformational freedom. To explore this more deeply, we will now develop models. To do this, we need the language of thermodynamics and statistical mechanics, which we review later. But first, let's see how to convert from experimental denaturation curves to free energies. From the temperature dependence of $\Delta G_{fold}(T)$, you can get two component quantities: the change in the enthalpy upon folding, $\Delta H_{fold}(T)$, and the change in the entropy upon folding, $\Delta S_{fold}(T)$, through the thermodynamic relationship [2]

$$\Delta G_{fold} = \Delta H_{fold} - T\Delta S_{fold}. \qquad (3.3)$$

The curves $\Delta G_{fold}(c)$ and $\Delta G_{fold}(T)$ give insights into the forces that drive protein conformational changes.

Stabilities and Structures Give Insights about the Driving Forces of Folding

To understand the forces that stabilize native proteins, first look at native structures. Native structures are compact. And their cores are mostly hydrophobic. This indicates the importance of hydrophobic interactions in a folded protein. Look at secondary structures, α-helices and β-sheets. A key feature is their hydrogen bonding. Also, proteins tend to be well packed, indicating the importance of van der Waals interactions. Some native proteins have *salt bridges*, where a positively charged atom is situated near a negatively charged atom, indicating

local
interaction

nonlocal
interaction
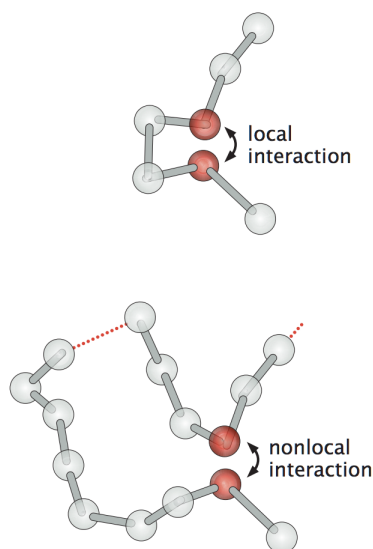
Figure 3.4 Interactions are defined as local or nonlocal, based on their separation in the chain sequence.

possible stabilization by an electrostatic attraction. But to fully interpret protein stabilities, you need more than just what you see in native structures. Chain entropies, which can be large, are not observable from structures alone. You can get insights into entropies by using statistical mechanical models.

As a matter of terminology, let's distinguish *local* from *nonlocal* interactions (Figure 3.4). Local interactions are those between close neighboring amino acids in the chain sequence, such as in helices and turns. Nonlocal interactions are interactions that occur between amino acids farther apart in the chain sequence, such as in β-sheets. Nonlocal interactions are formed, for example, when two oil-like amino acids displace water to come into contact with each other. Protein stability comes from both types of interactions. Local versus nonlocal is terminology that refers to the *chain separation* between the contacting monomers, to be distinguished from *short-ranged versus long-ranged*, which refers to the dependence on the distance through space between the interacting monomers. Coulombic interactions are long-ranged through space (the energy depends on distance r as $1/r$) and van der Waals attractions are short-ranged through space (depending on $1/r^6$), for example, irrespective of the chain separation. Here is a brief summary of the types of noncovalent intrachain interactions in proteins.

Hydrophobic interactions are important for folding

The *hydrophobic effect* refers to the tendency of oil and water to separate. About half of the 20 types of amino acid side chains have oil-like, or nonpolar, character. In its native structure, a protein's nonpolar amino acids tend to be buried within its core, implying that folding is driven, at least partly, by the tendency of a protein's nonpolar amino acids to hide from water. Here are two indications of the importance of hydrophobic interactions in protein folding: (1) proteins are unfolded by solvents, such as GuHCl, urea, alcohols, and surfactants, which weaken the hydrophobic driving force for folding, and (2) there is a large positive heat capacity of unfolding, $\Delta C_{p,\text{unfold}} > 0$, for typical small proteins. A large positive heat capacity is a signature of the hydrophobic effect in simple systems. A characteristic fingerprint of hydrophobic interactions is that the transfer of nonpolar molecules such as benzene, toluene, or alkanes from their pure liquid state to water increases the heat capacity, $\Delta C_p = C_{p,\text{with solute}} - C_{p,\text{without solute}}$ (Table 3.1).

Table 3.1 The transfer of nonpolar molecules into water at 23°C is unfavorable (the free energy is positive), dominated by the entropy, and associated with a positive change in heat capacity

Substance	Surface area (Å^2)	ΔG^{hyd} (kcal mol^{-1})	ΔH^{hyd} (kcal mol^{-1})	ΔS^{hyd} ($\text{cal K}^{-1}\,\text{mol}^{-1}$)	ΔC_p^{hyd} ($\text{cal K}^{-1}\,\text{mol}^{-1}$)
Benzene	240	4.64	0.497	−13.88	53.8
Toluene	275	5.45	0.413	−16.9	62.9
Ethylbenzene	291	6.26	0.483	−19.4	76.0
Cyclohexane	273	6.74	−0.024	−22.7	86.0
Pentane	272	6.86	−0.478	−24.47	95.6
Hexane	282	7.77	0.00	−26.08	105.0

(Adapted from RL Baldwin. *Proc Natl Acad Sci USA*, 83:8069, 1986; PL Privalov and SJ Gill. *Pure Appl Chem*, 61:1097, 1989.)

(A) interface scale
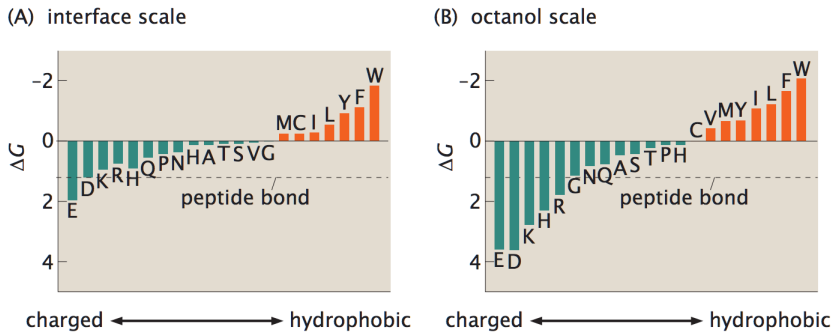
(B) octanol scale

Figure 3.5 Two hydrophobicity scales for amino acids. A 5-mer peptide has one of the 20 different amino acids in the middle position. The peptide is partitioned from water into (A) a lipid bilayer interface or (B) a bulk octanol medium. The *orange* bars show the (hydrophobic) residues that favor the oil-like environment relative to water, and the *green* bars show the (charged and polar) residues that favor being in water. The horizontal *dashed* line indicates the contribution of the peptide bond to all amino acids. Histidine (H) is shown twice, for its charged and uncharged forms (the charged form is on the left). (A, data from WC Wimley and SH White. *Nature Struct Biol*, 3:842, 1996; B, data from WC Wimley, TP Creamer, and SH White. *Biochemistry*, 35:5109–5124, 1996.)

The hydrophobic effect is a consequence of water–water hydrogen bonding. Roughly speaking, hydrogen bonding between two water molecules is strong, so water molecules tend to configure around solutes in ways that maximize their hydrogen bonding with other water molecules. Said differently, two nonpolar molecules that are put into water will associate with each other to minimize their exposure to water, which maximizes water–water hydrogen bonding. *Hydrophobicity scales* express approximately the relative tendencies of different types of molecules to partition from water into oil-like environments. Two such scales for amino acids are shown in Figure 3.5. There are many different hydrophobicity scales for amino acids, depending on the type of oil and the conditions of measurement. In general, molecules that register as hydrophobic on one scale tend to register as hydrophobic on the other scales, but there are variations among scales. Figure 3.6 illustrates one application of hydrophobicity scales; it shows that strings of hydrophobic amino acids in a protein sequence can identify the membrane-spanning parts of membrane proteins.

Hydrogen bonds stabilize native protein structures

A *hydrogen bond* occurs when a hydrogen-bond donating group, such as an amide, $-N-H$, shares its hydrogen with an accepting group, such as a carbonyl oxygen, $O=C-$, in this case leading to $-N-H\cdots O=C-$. Hydrogen bonding is extensive in native protein structures, mainly among backbone carbonyls and amides, particularly in α-helices and β-sheets. Mutational studies, as well as experiments using *osmolytes* such

(A)

(B)
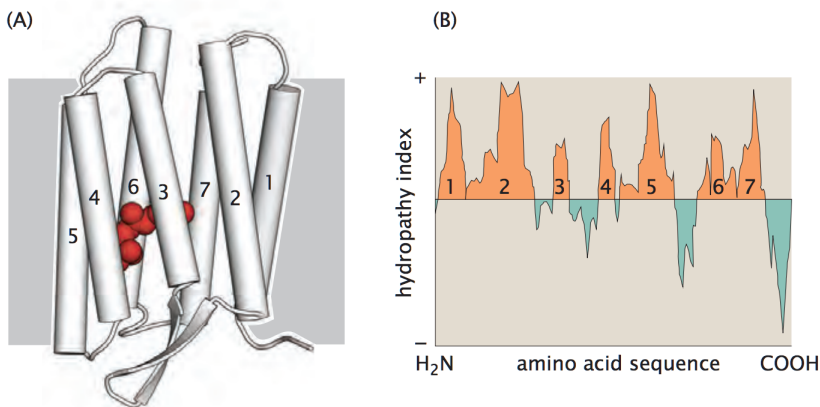
Figure 3.6 A hydropathy plot identifies the hydrophobic stretches of sequence that define membrane-spanning regions of a membrane protein. A hydropathy plot uses a hydrophobicity scale to show the hydrophobicity as a function of sequence position. *Orange* indicates hydrophobic regions, while *green* indicates polar or charged regions. (A) Bacteriorhodopsin has seven membrane-spanning α-helices corresponding to (B) seven *orange* peaks in the hydropathy plot. (Adapted from D Eisenberg. *Annu Rev Biochem*, 53:595–623, 1984. Modified with permission from the Annual Review.)

as trimethylamine *N*-oxide (TMAO), indicate that a hydrogen bond contributes between 0.3 and 1.5 kcal mol^{-1} to protein stability, although it is doubtful that there is such a thing as an "average" hydrogen bond in proteins. Strengths of hydrogen bonds vary substantially depending on their environments. In vacuum, a hydrogen bond can be 5 to 10 kcal mol^{-1}. In proteins, "hydrogen bond strength" refers to the difference between the free energy of the hydrogen bond in the native protein and that of the hydrogen bond in the denatured state, usually of the protein with its surrounding water molecules. Hydrogen bonds have significant electrostatic character. So, they are stronger in oil-like environments (like the interior of a protein), where the dielectric constant is low (estimated to be 2–12), than in waterlike environments, where the dielectric constant is high (around 80).

van der Waals interactions contribute to tight packing in proteins

Amino acids pack tightly within native protein cores. Tight packing results mainly from van der Waals interactions. These are (1) *short-ranged attractions* that draw atoms together and (2) even *shorter-ranged repulsions* that prevent two atoms from occupying the same space. van der Waals attractions and repulsions are largely responsible for the close steric fitting together of amino acids in protein cores. Mutational studies show that the tendency to fill space efficiently is about as strong as the hydrophobic interaction. For example, adding a methyl group to an empty cavity stabilizes a protein by about 0.9 kcal mol^{-1}.

Close packing is not difficult to achieve. Shake up nuts and bolts in a jar, and they'll pack well too. Interestingly, the fraction of space filled inside a protein is around 74%, which is about the same as for the maximum-density packing of identical spheres. How can a protein interior be so densely packed? It turns out that by mixing together small and large and irregular objects, you can sometimes pack space even more tightly than 74% by filling the nooks and crannies effectively. Think about how marbles can fill the small spaces between well-packed bowling balls, for example. Similarly, amino acids can fill space well too, because of their different sizes and shapes. In the text that follows, we describe how these various types of forces contribute to protein stability.

To understand how these forces contribute to protein folding and unfolding, we start from a few basic premises. First, proteins are chain molecules. Chain molecules can adopt many different conformations because different backbone torsional states typically have similar energies, separated by small barriers. Proteins denature, at least in part, because there are a much larger number of denatured conformations than native ones. That is, proteins gain chain entropy by unfolding to a large ensemble of denatured conformations. Second, native structures are stabilized by intrachain interactions, which can include van der Waals, hydrogen bonding, electrostatic, and hydrophobic interactions.

Third, side-chain interactions play a different role than backbone interactions in the folding code. Backbone interactions cannot explain a folding code since all proteins have the same backbone atoms: lysozyme folds differently than ribonuclease because of their different side-chain sequences. So, to explain the protein folding code, we focus on the hydrophobic interactions that explain the hydrophobic

cores of proteins. Charge interactions are also encoded in side chains, but there are relatively few charge–charge interactions in most proteins, and they tend to be located on a protein's surface, essentially in water, where they interact weakly. Charge interactions are described in more detail in Appendix 3A and in Chapter 9. Fourth, the sharpness of protein folding transitions is a nearly universal feature of globular proteins.

Before discussing the physics of folding, we first give a brief review of statistical mechanics, mainly the *Boltzmann distribution law* [2].

STATISTICAL MECHANICS IS THE LANGUAGE FOR DESCRIBING PROTEIN STABILITIES

Statistical mechanics says that you can obtain the free energy in terms of a microscopic description of the system, called the *partition function Q*, through the relation

$$G = -RT \ln Q. \tag{3.4}$$

The partition function is the sum of the relative *statistical weights* over all $j = 1, 2, 3, \ldots$ microscopic states accessible to the system:

$$Q = \sum_{j=1}^{s} \omega(\epsilon_j) e^{-\beta \epsilon_j}, \tag{3.5}$$

where ϵ_j is the energy of state j, $\beta = (RT)^{-1}$, and $\omega(\epsilon_j)$ is the *density of states*, that is, the number of distinct microscopic configurations that have a given energy ϵ_j. In this way, $\omega(\epsilon_j)$ is the count of the number of states having that particular energy, and is called the *degeneracy* of that state. The quantity $e^{-\beta \epsilon_j}$ is called the *Boltzmann factor* for state j.

To make a statistical mechanical model, you must first know the microscopic states of the system, their energy levels ϵ_j, and their densities $\omega_j = \omega(\epsilon_j)$. Then you can compute the probability p_j of any state j using the Boltzmann distribution law:

$$p_j = \frac{\omega(\epsilon_j) e^{-\beta \epsilon_j}}{Q}. \tag{3.6}$$

The power of statistical mechanics is that it provides a way to express physically observable properties. Experiments usually reflect the average properties of large numbers of molecules. So, to compare with experiments, we want to compute *ensemble averages* of quantities such as the energy or the fraction of protein molecules folded. For a property A with a value of A_j in state j, the ensemble average is given by

$$\langle A \rangle = \sum_j A_j p_j, \tag{3.7}$$

so that the value for each state is weighted by its Boltzmann probability p_j.

In this chapter, we combine the Boltzmann distribution law with some simple models to explore the equilibrium states of proteins. Why are proteins compact and globular, with hydrophobic cores? What dictates a protein's native secondary structures? What is the folding code? That is, what intermolecular interactions can explain how a tertiary structure is encoded in an amino acid sequence? Why do proteins denature at high temperatures, in denaturants, or in acidic or basic solutions?

(A) square lattice, $z = 3$
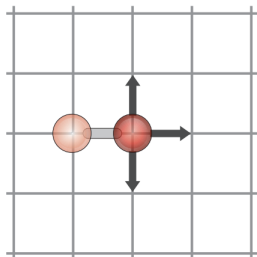
(B) cubic lattice, $z = 5$
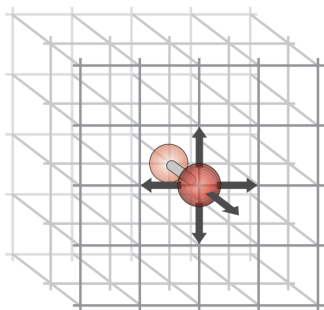
Figure 3.7 The basic unit step used in lattice models of proteins. Chain conformations are restricted by chain self-avoidance and lattice geometry. (A) On the 2D square lattice, after the first step is taken, the maximum number of possible directions for the next step is $z = 3$ (shown as arrows), because the chain cannot double back on itself. (B) On the 3D cubic lattice, $z = 5$.

Why do some proteins also denature at low temperatures (a process called *cold denaturation*)? We begin with the *HP model*, arguably the simplest model of protein folding.

Why Do Proteins Fold and Unfold? The HP Model

Under native conditions, proteins fold because many of the chain's amino acids are more strongly attracted to each other than they are to water. Under denaturing conditions, proteins unfold: (1) to increase the chain's conformational entropy and (2) because denaturing solvents effectively weaken the interactions between amino acids that hold the native protein together. As a simple model, let's represent a protein molecule as a string of monomer beads on a lattice (**Figure 3.7**). Each monomer is a single bead centered on one lattice site such that no lattice site can have more than one monomer. The beads are linked together by rigid covalent bonds. Consider a short chain having just six beads. The chain can adopt different possible configurations on a 2D square lattice. The *conformational space* is the set of all the viable configurations of the chain. This is often referred to as the set of *self-avoiding walks* because the chain can only follow bond directions that match the lattice edges and self-reversals are not permitted.

In this *HP model*, the 20 different types of amino acids are approximated using a simple *binary code* of just two monomer types: hydrophobic (H) or polar (P). A contact is defined when two monomers are adjacent to each other in space but not adjacent in the sequence. Whenever two H monomers form a noncovalent contact (that is, an *HH contact*), there is a favorable interaction energy $\epsilon_0 < 0$; all other types of contacts are assumed to have an energy of zero [3].

As an example, consider the 6-mer sequence in which monomers 1, 4, and 6 are hydrophobic (H) and the rest are polar (P). Each of the 36 lattice configurations shown in **Figure 3.8** is called a microscopic state, or a *microstate*. Microstates are the finest-grained description a model provides of its accessible states. The microstates live on different rungs of an *energy ladder*, depending on how many hydrophobic contacts they make. You can collect microstates together to define *macrostates* in whatever ways are convenient or useful or experimentally measurable. For example, in Figure 3.8, the three different energy levels provide a natural definition of three macrostates: the native state N is the collection of all microstates having two hydrophobic contacts (there is only one such microstate, for this HP sequence); the intermediate state I is the collection of all microstates having one HH contact (there are seven such microstates for this HP sequence); and the denatured state D is the collection of all the other 28 microstates (those states having no HH contacts). There is an inherent symmetry to many of these possible walks, but because we are concerned only with the contacts between pairs of beads, we are free to redefine our origin and consider only unique walks. Thus, in the enumeration of unique walks, one proceeds by considering only conformations that make a first step down and only make a step to the right after some previous step to the left. The single folded conformation has a lower energy than the unfolded conformations, so the single microstate occupies the lowest rung of the energy ladder. The lowest-energy level is called the *ground state*. The next higher rung is called the first *excited state*, and the highest rung for this model is called the second excited state. The
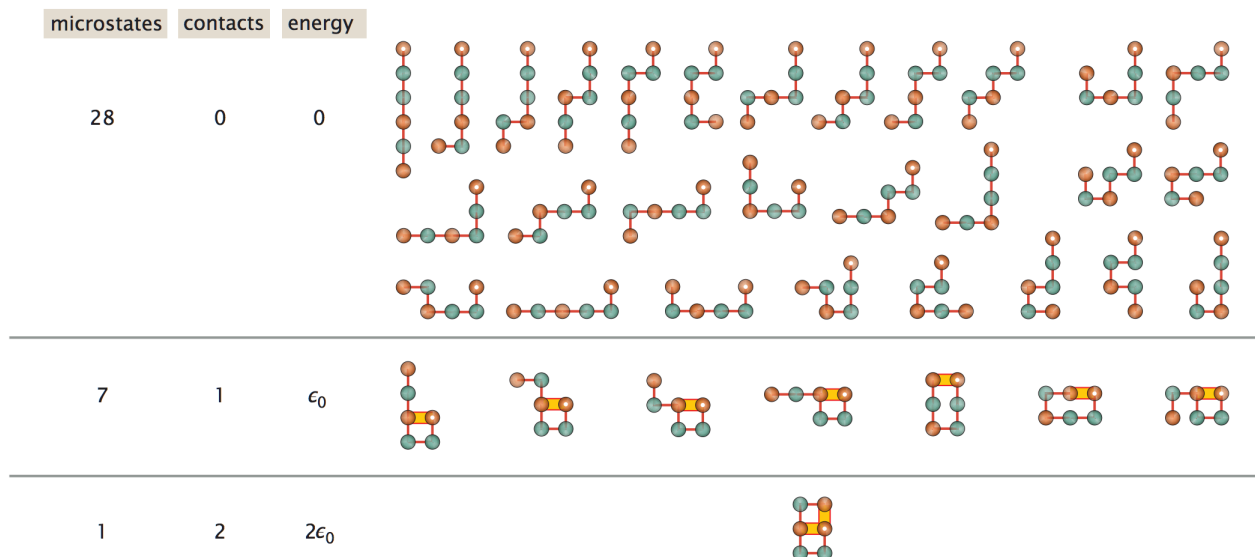
Figure 3.8 **Energy ladder of a six-bead model sequence: HPPHPH.** This six-bead binary sequence model has 1 low-energy conformation with two HH contacts, 7 conformations with one HH contact, and 28 conformations with no HH contacts. Hydrophobic residues are *orange* circles, polar residues are *green*, and HH contacts are indicated by *orange* bands. A *white* dot indicates the first bead of the chain. Note the geometrically clustered *orange* hydrophobic cluster of three in the lowest-energy form. ϵ_0 is a negative number, and so this state with $2\epsilon_0$ energy is the most energetically favorable state. (From KA Dill and S Bromberg. *Molecular Driving Forces: Statistical Thermodynamics in Biology, Chemistry, Physics and Nanotechnology*. 2nd ed. Garland Science, New York, 2011.)

degeneracies, that is, the numbers of microstates in each macrostate, are 1, 7, and 28, respectively.

According to Equation 3.5 the partition function Q, summing over all the weights for the states of this model HP sequence, is

$$Q = 28 + 7e^{-\epsilon_0/RT} + e^{-2\epsilon_0/RT} = 28 + 7x + x^2, \qquad (3.8)$$

where we have simplified the notation by using $x = e^{-\epsilon_0/RT}$. According to Equation 3.6 the populations of the folded, intermediate, and unfolded states are

$$p_N = x^2/Q,$$
$$p_I = 7x/Q, \qquad (3.9)$$
$$p_D = 28/Q.$$

Here is how you apply statistical mechanics. First, you specify the contact energy ϵ_0 and the temperature T. Then you compute the populations, which are given by Equation 3.9 for this model. Finally, you test your model predictions against experiments. Using Equation 3.7 for a property A that has the value A_D in the denatured state, A_I in the intermediate state, and A_N in the native state, the ensemble average is computed from the populations as

$$\langle A \rangle = \sum_j A_j p_j = A_D p_D + A_I p_I + A_N p_N. \qquad (3.10)$$

For example, the average energy is

$$\langle \epsilon \rangle = 0 p_D + \epsilon_0 p_I + 2\epsilon_0 p_N. \qquad (3.11)$$
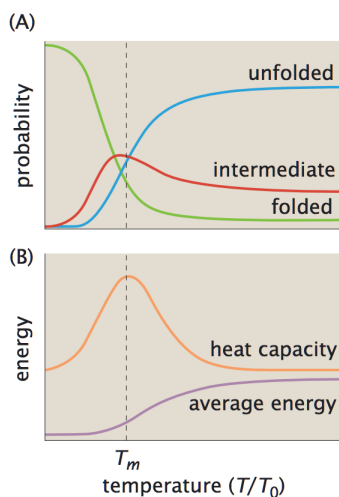
With this approach, you can calculate the averages and variances of many types of properties—the radius of the chain, amounts of secondary structure, the probabilities of any particular chain contacts that might be of interest, or the end-to-end distance, for example—as functions of temperature. For instance, using a thermodynamic relationship, you can compute the heat capacity as $d\langle\epsilon\rangle/dT$. Another property of interest is the free-energy difference between any of the three states. For example, the free energy of folding, $\Delta G_{\text{fold}} = G_N - G_D$, is

$$\Delta G_{\text{fold}} = -RT\ln\left(\frac{p_N}{p_D}\right) = 2\epsilon_0 + RT\ln 28. \tag{3.12}$$

The model predicts a *folding transition* between the protein's ensemble of open conformations and its single lowest-energy native state. The transition happens where $p_N = p_D$, that is, where $\Delta G_{\text{fold}} = 0$. So, you can compute the midpoint temperature of the folding transition for this model using Equation 3.12:

$$T_m = \frac{-2\epsilon_0}{R\ln 28}. \tag{3.13}$$

Figure 3.9 shows the predicted populations of N, I, and D as functions of temperature. At low temperatures, the folded state is the most populated (most stable). At high temperatures, the model protein is unfolded. Temperature controls the balance. At low temperatures, the chain folds because the HH "sticking energy," which drives the system into the folded state, contributes more to ΔG_{fold} than the chain entropy does. At high temperatures, the chain entropy, which drives the system into the unfolded state, contributes more to ΔG_{fold} than the HH sticking energy does. The denaturation curve is sigmoidal because thermal energies at low temperatures are not sufficient to break bonds, thermal energies at intermediate temperatures break HH bonds, and at high temperatures, no further bond breakage happens because the bonds are already all broken. Consistent with experiments, this behavior leads to a peak in the heat capacity, which is, by definition, just the point at which the incremental energy absorption versus temperature is maximal.

In this model, there are three states: N, I, and D. This model sets the stage to discuss protein-folding *cooperativity* in Chapter 4. But, alternatively, you are free to collect together and label states in whatever ways are convenient for your problem of interest. For example, if your experiment measures only native and non-native states, then you could define the population of non-native molecules as $p_{\text{non-native}} = p_I + p_D$.

(A)

(B)

Figure 3.9 Heating denatures the model sequence HPPHPH. (A) Relative populations p_N of folded (2 HH contacts), p_I of intermediate (1 HH) and p_D of unfolded states (0 HH contacts) versus temperature using Equations 3.8 and 3.9. (B) Average energy $\langle\epsilon\rangle = 2p_N\epsilon_0 + p_I\epsilon_0 + 0p_D$ (*purple*). Heat capacity $= d\langle\epsilon\rangle/dT$ (*orange*). A fixed HH sticking energy ϵ_0 defines a constant $T_0 = \epsilon_0/R$, which has units of temperature, so T/T_0 on the x-axis is a dimensionless temperature scale.

Proteins Have a Folding Code

This toy lattice model shows the nature of the *protein folding code*, that is, how different amino acid sequences can encode the folding of chain molecules into different specific native structures. In the HP model, a protein folds to a compact state with a hydrophobic core because such structures maximize the number of HH pairings among the amino acids. The sequence HPPHPH encodes one particular folded conformation at low temperatures: its single native structure is the conformation having the maximal number of HH contacts. For a different HP sequence, the maximization of HH contacts will lead to a

different native structure. (Some sequences, however, do not encode a unique fold; for example, the all-P sequence PPPPPP does not fold at all.) This model shows that a simple *binary code* (that is, sequence of hydrophobic and polar monomers) is sufficient to cause a chain molecule to adopt a single compact conformation from among a large space of alternative possible conformations.

Evidence that the protein folding code is dominated by the binary HP patterning comes from the experiments of Kamtekar et al. [4]. In those experiments, Hecht and coworkers randomized the sequence of amino acids in a four-helix-bundle protein, subject only to the constraint that interior residues must be H and exterior residues must be P. They found that these HP sequences all folded stably into the four-helical-bundle structure, indicating the importance of the HP sequence pattern in specifying the native fold.

This simple binary-code lattice model captures the basic ingredients of folding: namely, that a chain has many non-native conformations and only one native conformation, and that conformations cannot violate *excluded volume* (that is, two amino acids cannot occupy the same space) (see Figures 3.7 and 3.8.) This and other types of models have shown that for many (but not all!) properties of proteins, atomic details are not needed. In fact, details are sometimes more distracting than helpful. For example, where entropies are important, as in folding, it is usually more crucial that a model be able to count conformations correctly over the whole conformational space than capture high-resolution atomic detail. Moreover, lattice models are also useful in "theoretical experiments" in which principles are explored by turning interactions on and off; you can explore entropies in such control experiments since all possible states can be enumerated. Of course, for other properties, other details can be important.

However, if folding is dominated by the HP code, how do we explain the prevalence of secondary structures, like helices and sheets? To explain secondary structures, we need two additional factors. First, hydrogen bonding, a signature of secondary structures, must play a role. But, helices and β-hairpins, when covalently snipped out of proteins and put by themselves into water, usually don't form very stable secondary structures. So, hydrogen bonding alone is not sufficient to explain the stabilities of short helices and sheets in native proteins. Tertiary forces must help to stabilize these forms. Next we describe how chain compactness stabilizes secondary structures, and how compactness reduces the number of conformations.

The Collapse of the Chain Helps Stabilize Secondary Structures

When a polymer chain is confined within a small volume, it will preferentially populate secondary structures, such as α-helices and β-sheets. This is a simple geometric consequence of confining any 1D rope-like object in a tight space at high density. The only systematic and regular way to pack a chain or a rope into a small space is to use helix-like or sheet-like conformations (Figure 3.10). Think of the lines of people in an airport security line. The lines run back and forth in regular patterns, resembling a squared-up β-sheet in 2D; there are no other regular arrangements that can pack a line of people into a tight space. Look at Figure 3.8; you see that only 4 of the 36 possible configurations would fit into a 2×3 compact lattice, and they have more secondary structures than the others. Figure 3.10 shows results for 41 different

Figure 3.10 Increasing the chain compactness increases the secondary structure content.
Polymer chains can fill tight spaces only if they form regular structures such as helices and sheets. The number of conformational microstates diminishes in going from the largest to smallest confining space. Plotted here is the relative compactness of the unfolded state versus secondary structure. Compactness is measured by the inverse of the Stokes radius and secondary structure by circular dichroism. *Red* circles represent proteins that were studied in fully unfolded states; *blue* circles represent proteins studied in their stable partially unfolded states. (Adapted from VN Uversky and AL Fink. *FEBS Lett*, 515:79–83, 2002.)
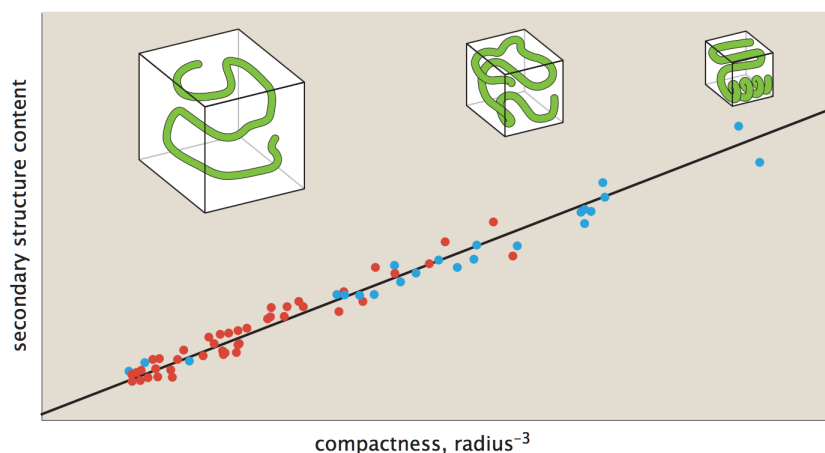

proteins, each under a condition that causes it to have some intermediate compactness (the inverse of the average volume occupied, that is, of the radius cubed), neither fully unfolded nor fully folded. It shows that the amount of secondary structure a protein adopts is proportional to the chain's compactness. So, secondary structures in proteins are partially stabilized by cooperation with the hydrophobic interactions that stabilize the compact structures.

Protein Folding Energy Landscapes Are Funnel-Shaped

A protein folding *energy landscape* illustrates the balance between a folding protein's interactions and its chain entropy. An energy landscape is a mathematical expression of an energy or free energy as a function of the various *degrees of freedom*, that is, of the conformational options that are available to the molecule. Even for the simple models discussed previously, an energy landscape is complex and of high dimensionality. There are two main types of cartoons that help to visualize such mathematical functions. One way to visualize energy landscapes is to show the *density of states* versus the energy $n\epsilon_0$, where $n = 0$ defines the denatured state, $n = 1$ the intermediate state, and $n = 2$ the native state (see Figure 3.8). For this HP sequence, we have 28 microstates for $n = 0$, 7 microstates for $n = 1$, and 1 microstate for $n = 2$ (**Figure 3.11A**). Turn this plot on its side to see the funnel shape
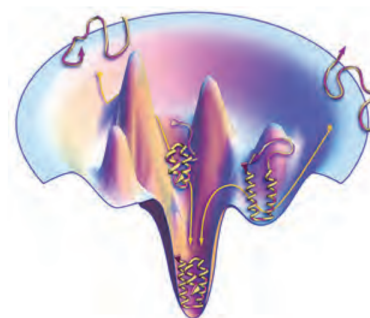
Figure 3.11 An energy landscape funnel describes the density of states. (A) The density of states for our HPPHPH 6-mer. (B) Now, rotate the axes of (A) to see the energy landscape. It has a funnel shape. There are many conformations having high energy (few HH contacts), so these states have high conformational entropy. There are few conformations having low energy (more HH contacts), so these are low-entropy states. The native (N) state has the maximum number of HH contacts in this model.
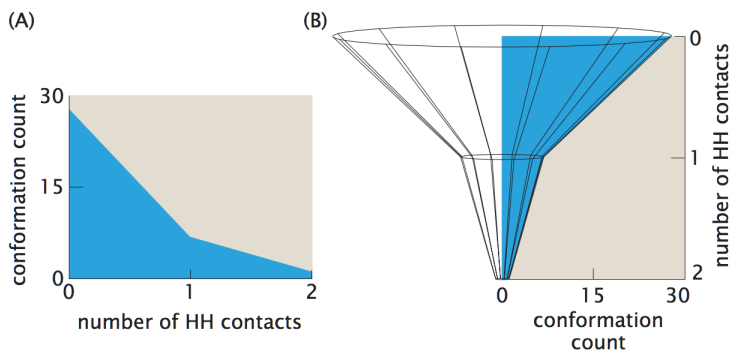
of *n* versus the density of states. Figure 3.11B shows that the number of states is large at the top (there are many open conformations having high energy), smaller in the middle (representing the fewer different states of intermediate energy), and fewest at the bottom (representing the small number of states that have the lowest possible energy). While the exact shape of this curve will depend on the monomer sequence and the type of model used to represent proteins, the basic physical principle of the funnel shape of the energy landscape applies to all proteins in any model—namely that there are always more conformations of high energy than of low energy.

There is also another type of diagram for visualizing an energy landscape. In this case, we plot the free energy of the chain as a function of the many different degrees of freedom (bond angles, bond lengths, etc.). Figure 3.11 shows a two-dimensional version of this type of high-dimensional energy landscape. Such pictures sometimes show landscapes with bumps and wiggles: in such cases, the independent variable that is being represented is not the single density-of-states value (as in Figure 3.11), but rather one of the large number of possible actual conformational coordinates (as in **Figure 3.12**). Such pictures provide a way of thinking about trajectories from some particular chain conformation to another, indicating, metaphorically, how the skiers reach the bottom (native state) of a mountainside that has trees, bumps, gulleys, and other obstacles.

Figure 3.12 Another type of energy landscape also shows the funnel shape for protein folding. This type of cartoon energy landscape represents the free energy (*y*-axis) versus the chain degrees of freedom (*x*-axis). Like Figure 3.11, it shows the many unfolded states at the top and the few native-like states at the bottom. But this landscape also shows schematically the hills and valleys in energy of the different chain conformations. (From KA Dill and JL MacCallum. *Science*, 338:1042–1046, 2012. Reprinted with permission from AAAS.)

SIMPLE PROTEINS DENATURE WITH TWO-STATE THERMODYNAMICS

Now, let's explore a different simple model—called the *two-state model*—for describing experiments on the folding stabilities of small globular single-domain proteins. To explain the term "two-state," imagine that you could reach into a protein solution and pull out protein molecules, one at a time. For materials such as proteins that undergo two-state transitions, at the denaturation midpoint (where the fractions are equal, $f_N = f_D = \frac{1}{2}$ and therefore $\Delta G = 0$), you would find that half of the protein molecules are fully folded and half are fully unfolded. The alternative is that you might find *intermediate states*; that is, some individual molecules would be *partially folded*. Small proteins usually have two stable states and no partially folded equilibrium intermediates. Processes are also called *cooperative* if they undergo two-state transitions.

You can determine the cooperativity from calorimetry (see Figure 3.3). You use two measurements. First, calorimetry experiments give a quantity called the *calorimetric enthalpy*, ΔH_{cal}. This direct measurement requires no model-based interpretation. Second, you measure two other quantities from a calorimetry experiment: T_m is the melting midpoint temperature and $\Delta C_{p,max}$ is the peak of the heat capacity (after subtracting the baseline value). Now, combining the latter two quantities with a *model assumption that the system in your calorimeter has a two-state transition* gives a prediction of the *van't Hoff enthalpy*, $\Delta H_{vH} = 2T_m\Delta C_{p,max}$. Therefore, if these two enthalpies are equal, $\Delta H_{vH} = \Delta H_{cal}$, this means that the transition is two-state, involving no intermediates. Such data provide one form of evidence that typical small single-domain globular proteins undergo two-state folding transitions. Another indication that a transition is two-state is when two different experimental quantities, such as

circular dichroism measurements of secondary structure and ultraviolet spectroscopy measurements of the burial of tryptophan groups, have denaturation curves that superimpose on each other. If those curves do not superimpose, it indicates the presence of intermediates. We explore the physical origins of protein-folding cooperativity in Chapter 4.

There is extensive experimental data on the thermal stabilities for reversible folding and unfolding of small proteins: the free energies $\Delta G_{fold} = G_D - G_N$, enthalpies ΔH, entropies ΔS, and heat capacities, ΔC_p. These thermal quantities mainly depend just on the number N of amino acids in the chain. So far, extensive studies have shown no other strong dependence of the thermal properties of folding stability. Stability does not appear to depend on the amount of secondary structure or types of tertiary structure, or numbers of hydrophobic amino acids or hydrogen bonds, or counts of salt-bridging ion pairs, for example. This is remarkable because other important properties of proteins—such as their native structures and biochemical mechanisms—can depend strongly on such details. This simplicity allows us to readily capture the observed dependencies of folding stability on temperature, denaturants, pH, salts, and the effects of protein confinement within tight spaces. In the next section, we assume that proteins fold with two-state cooperativity. In Chapter 4, we explore the physical basis of two-state cooperativity.

We begin with the chain entropy. As illustrated in **Figure 3.13**, for a chain having N amino acids, the number of conformers in the denatured state will be approximately $Q = z^N$, where z is the number of different conformations per amino acid. So, we approximate the chain entropy in the denatured state as

$$S_D = R \ln Q = NR \ln z. \tag{3.14}$$

For a 2D square lattice, you would use $z = 3$ (see Figure 3.7). For a 3D simple cubic lattice, you would use $z = 5$. These are the numbers of lattice step directions a bond can take from a given lattice site to a neighboring site without landing on top of the preceding bond at that site (but not accounting for other longer-range overlaps).

Next, let's represent the energetics of folding using a *transfer model*: in the folding process, an amino acid is transferred from being solvated in water to being buried in the protein core. Then the folding free energy will be approximately

$$\Delta G_{fold}(N) = N(g + RT \ln z), \tag{3.15}$$

where $g < 0$ is the free energy of transferring an amino acid from water into a hydrophobic-core environment (resembling ϵ_0 in our previous lattice model (see Figure 3.8). The main points embodied in Equation 3.15 are that (i) the total contact free energy and the entropy of folding for the whole protein should scale linearly with the number of residues, (ii) the native structure is stabilized by the residue–residue attractions captured in g (note that g is negative), and (iii) the chain entropy opposes collapse.

$Q_{denatured} = 5^{100}$

$N (\sim 100)$

$z = 5$
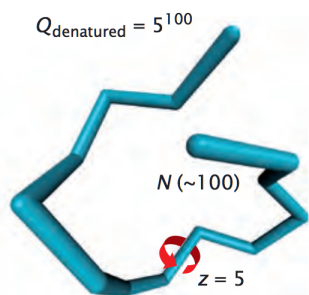
Figure 3.13 To compute the chain entropy, count the conformations and take the logarithm.
z represents the number of rotational conformers around each peptide bond and N is the total number of amino acids. So, in a simple approximation, the folding from the denatured state $(Q_D = z^N)$ into the native state $(Q_N = 1)$ has a chain entropy decrease of $\Delta S_{fold} = S_N - S_D = -NR \ln z$.

Protein Stability Depends Linearly on Denaturant Concentration

Chemical agents such as guanidine hydrocholoride and urea tend to denature native proteins. Other chemical agents, such as glycerol, sugars, some salts, TMAO, and sarcosine, tend to have the opposite effect; they stabilize native proteins. Added denaturants cause a water solution to be a more favorable environment for amino acids, helping to unfold proteins. Added stabilizer compounds cause a water solution to be a less favorable environment for amino acids, driving proteins more strongly toward their native structures. We can use Equation 3.15 to describe how chemical agents affect protein stability.

In the transfer model, adding denaturants or stabilizers linearly weakens or strengthens the interactions experienced by each residue (with their surroundings):

$$g(c) = g_0 + m_1 c. \qquad (3.16)$$

In the absence of denaturant ($c = 0$), the free energy of dissociation of a residue from its neighbors in the protein is g_0. The reason for the linear dependence on denaturant concentration, $m_1 c$, is that increasing the amount of denaturant in the solution increases proportionally to the amount of denaturant in the first solvation shell around each nonpolar solute molecule (or solvent-exposed residue in our case), decreasing proportionally to the solute–solute attraction. So, you can think of denaturant molecules as being distributed randomly in every available space in solution, including at sites in the first shells around nonpolar solutes (called a *mean-field* approximation). Substituting Equation 3.16 into Equation 3.15 predicts a linear dependence of the folding free energy on denaturant concentration,

$$\Delta G_{\text{fold}}(c) = N(RT \ln z + g_0 + m_1 c). \qquad (3.17)$$

Equation 3.17 predicts that the slope, $m = Nm_1$, called the *m-value*, of $\Delta G_{\text{fold}}(c)$ versus c is a product of the increased surface area of monomers exposed upon denaturation multiplied by the free energy per unit surface area. Figure 3.14 shows that the experimentally observed *m*-values increase linearly with chain length N, as predicted.

For denaturing agents, $m_1 > 0$; that is, increasing the concentration of the chemical in solution decreases the protein's stability. On the other hand, for chemical agents that stabilize proteins, $m_1 < 0$ in this case, increasing the chemical concentration stabilizes the folded protein. Stabilization can arise from different physical causes. Some stabilizers act to increase the polarity of an aqueous solution. Others stabilize by excluded volume: such agents occupy space in an otherwise inert way,
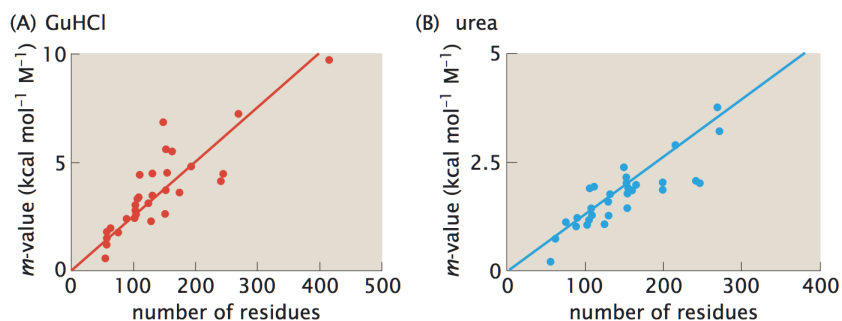
Figure 3.14 **The denaturant *m*-value depends linearly on chain length.** Denaturation by GuHCl (A) or urea (B) shows that the *m*-values depend linearly on the chain length N as indicated by Equation 3.17 [5]. (Data from JK Myers, CN Pace, and JM Scholtz. *Protein Sci*, 4:2138–2148, 1995.)

(A)

(B)
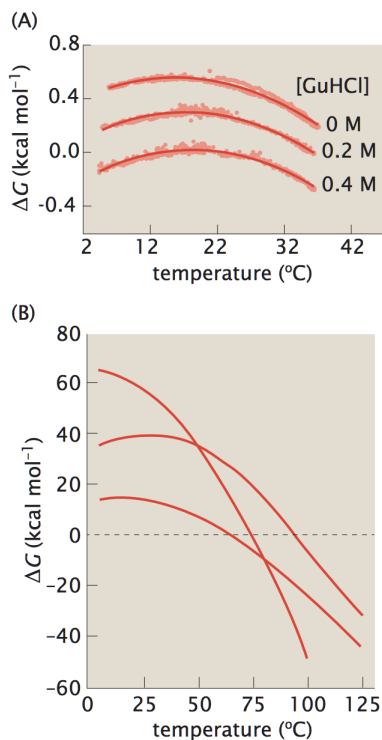
Figure 3.15 Protein folding free energies depend on temperature. (A) Denaturation curves of yeast ferricytochrome *c*. In this experiment, denaturation is also assisted by added guanidine hydrocholoride, so that the downward curvature on the left is observable. This illustrates *cold denaturation*, a temperature *below which* the protein will denature. (B) Unfolding free energies of three different proteins, indicating their unfolding at high temperatures. (A, from CJ Nelson, MJ LaConte, and BE Bowler. *J Am Chem Soc*, 123:7453–7454, 2001. With permission from American Chemical Society; B, from GI Makhatadze and PL Privalov. *Adv Protein Chem*, 47:307–425, 1995. With permission from Elsevier.)

restricting the conformations that the denatured protein could adopt, therefore effectively stabilizing the native state. For example, polysaccharide chains are sometimes covalently linked onto biotechnologically important proteins, such as erythropoetin (EPO), to give stable solution formulations. Next, let's consider how protein stabilities depend on temperature.

Protein Stability Is a Nonlinear Function of Temperature

Figure 3.2 and **Figure 3.15** show that protein stability is not a linear function of temperature. Extensive experiments on the transfer of amino acids from water into oil-like media show that the residue-residue interaction free energy, which is g in our simple transfer model, depends on temperature.

When two nonpolar groups dissociate in water, there is a large positive change in the heat capacity per amino acid, Δc_p, that is approximately independent of temperature. To capture experimental data on model compound transfer data requires three parameters: Δc_p; T_h, the temperature at which the enthalpy of transfer is zero; and T_s, the temperature at which the entropy of transfer is zero. Model-compound transfer experiments can be modeled as

$$\Delta g(T) = \Delta h - T\Delta s = \Delta c_p(T - T_h) - T\Delta c_p \ln\left(\frac{T}{T_s}\right). \qquad (3.18)$$

Now, by substituting Equation 3.18 into Equation 3.15 and using $g = g_0 + \Delta g(T)$, our folding model gives

$$\Delta G_{\text{fold}}(T) = N\left[RT\ln z + g_0 + \Delta c_p(T - T_h) - T\Delta c_p \ln\left(\frac{T}{T_s}\right)\right]. \qquad (3.19)$$

Equation 3.19 predicts that $\Delta G_{\text{fold}}(T)$ is a curved function of temperature. It predicts two denaturation temperatures, that is, points at which $\Delta G_{\text{fold}} = 0$. One denaturation midpoint occurs at a high temperature: above that point, the conformational entropy dominates the free energy, and the chain unfolds, as shown also in the HP lattice model from before. Proteins can also be denatured, in principle, at low temperatures. Cold denaturation is a peculiarity of hydrophobic interactions in water. At low temperatures, a protein would unfold because the HH interactions become weaker as temperature is lowered (but this is often obscured by the freezing point of water).

This transfer model predicts that that the entropy, enthalpy, heat capacity, and free energy of protein folding should depend linearly on the chain length N, consistent with the experiments shown in **Figure 3.16** [6].

Proteins having stronger intramolecular attractions have higher denaturation temperatures. The denaturation temperature T_m of a protein reflects its balance of enthalpy and entropy. T_m is the temperature at which the free energy of folding is zero,

$$\Delta G_{\text{fold}}(T_m) = N[g(T_m) + RT_m \ln z] = 0, \qquad (3.20)$$

so

$$T_m = \frac{-g(T_m)}{R\ln z}. \qquad (3.21)$$

(A)

(B)

(C)
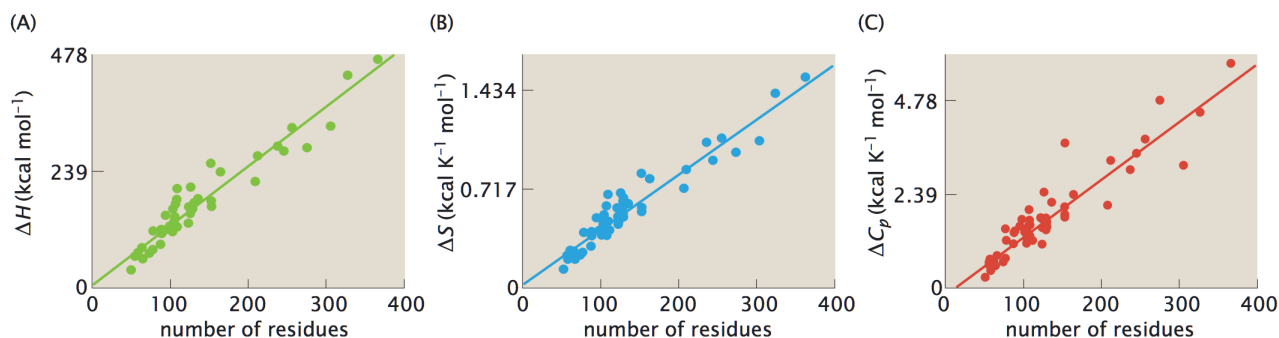
Figure 3.16 Thermal properties of proteins depend linearly on chain length. (A) ΔH, enthalpy of unfolding at 373.5 K, (B) ΔS, entropy of unfolding at 385 K, and (C) ΔC_p, temperature-independent heat capacity of unfolding, determined for 59 proteins, are plotted as functions of chain length N. The lines are linear regressions. (Adapted from AD Robertson and KP Murphy. *Chem Rev*, 97:1251–1267, 1997.)

Recalling that $g < 0$ (residue–residue attractions are favorable in the absence of denaturant), you see that stronger attractions lead to a higher T_m. As an example, if you substitute the value $z = 7.54$ for an average (ideal) protein [5], and a typical melting temperature, say, $T_m = 353\,\text{K}$, you find that $g(T_m) = -1.43\,\text{kcal mol}^{-1}$.

Folding is driven by a small difference between a large chain entropy that opposes folding and a large residue–residue contact free energy that favors folding. At T_m, the net folding free energy is zero, $\Delta G_m = 0$. At other temperatures (such as room temperature or physiological temperatures), a protein's stability is small: ΔG is usually around 5–20 kcal mol^{-1}. This small stability may be important biologically because, if proteins were too stable, they might be unable to respond to changes in the environment, or to undergo conformational changes that are essential for functioning and recycling of amino acids.

PROTEINS TEND TO UNFOLD IN ACIDIC OR BASIC SOLUTIONS

Proteins can denature if they are in acidic or basic solutions. This is because such solutions cause proteins to have a net charge. Proteins become more positively charged in acid solutions. Proteins become more negatively charged in basic solutions. The consequence is that if a protein has a net charge on it (either positive or negative), there will be net charge repulsions among pairs of charged residues. Those charge repulsions tend to unfold proteins because unfolding relieves the high charge density in the folded state.

The charges on proteins come from the acidic amino acids (glutamic acid and aspartic acid) or the basic amino acids (arginine, lysine, and histidine). For example, the protonation of an acidic side chain can be expressed as the following equilibrium:

$$HA \xrightarrow{K_a} H^+ + A^-, \qquad (3.22)$$

where A represents an acidic group, such as an aspartic or glutamic acid side chain, H$^+$ is a dissociated proton and HA is the protonated uncharged form. If the pH of the solution around an acidic side chain is higher than about 4.1, these acidic side chains will have a net negative charge because they give up their protons to the surrounding
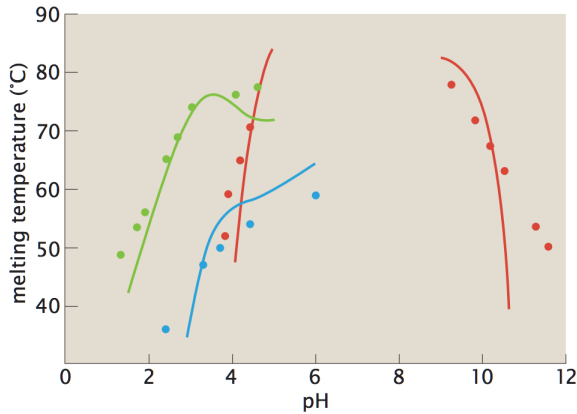
Table 3.2 Typical pK_a values of ionizable groups in proteins. These are intrinsic values these groups would have in water. They can be changed depending on their surroundings in the protein

Group	Acid		Base	pK_a
C-terminal carboxyl group: Aspartic acid Glutamic acid		⇌		3.1 4.1 4.1
Histidine*		⇌		6.0
N-terminal amino group		⇌		8.0
Cysteine		⇌		8.1
Tyrosine		⇌		10.9
Lysine		⇌		10.8
Arginine		⇌		12.5

* Histidine is the only amino acid that has a pK_a in the common range of experiments. So, in some locations in protein structures it exists in charged form and in others it is uncharged.

solution. Or, if the pH of the solution is lower than about 10, the basic amino acids will have a net positive charge because they take up protons from the solution. Histidine can also have a net charge if the pH is below about 6 (Table 3.2). So, if the pH of a solution is sufficiently high or low, a given protein can have a net charge, due to net charges on whatever numbers of these types of side chains it has. If the protein has a sufficiently high net charge, those charges will repel each other, causing an expansion of the chain, denaturing the protein. This is how acids or bases denature proteins. A simple model is given in Appendix 3A.

Two important consequences follow from these electrostatic contributions to protein folding stability. First, Figure 3.17 shows that a protein's melting (denaturation) temperature, a quantity that reflects

the protein's folding stability, will be reduced in acidic or basic solutions.

Second, if a protein has a net charge (that is, the protein is not at its *isoelectric point*), adding salt to the solution will typically stabilize the folded state. (Adding salt increases the charge shielding or κ in Equation 3.A.10.) The salt molecules swarm around the protein, shielding charges, thus weakening the electrostatic repulsions among the fixed charges on the protein.

Proteins that have a large net charge and low hydrophobic content usually lack ordered structure (**Figure 3.18**) [7].

Now, we go beyond the simple idea that all z^N denatured conformations are equivalent to each other. Understanding some properties of proteins requires that we take into account that some denatured-state conformations can have different radii and different free energies than others. The denatured state is an ensemble of conformations.

A DENATURED STATE IS A DISTRIBUTION OF CONFORMATIONS

In this section, we make three points about the denatured states of proteins. First, a denatured state is a broad distribution of microstates. Second, the denatured state distribution can shift its average size under different denaturing conditions. Third, denatured chains that are located in confined or restricted spaces have different properties than those that are unrestricted. Let's first consider the distribution of the different end-to-end distances r of the denatured protein. Modeled as a random-flight polymer chain, the free energy of a denatured conformation is [2]

$$\frac{G_D}{RT} = -N \ln z + \frac{3}{2}\left(\frac{r}{r_0}\right)^2 + \frac{aN^2 v}{r^3}, \quad (3.23)$$

where $r_0 = Nb^2$ is the average end-to-end distance of a random-flight or freely jointed chain of N links, each of length b [2]. The second term in Equation 3.23 expresses the *elastic energy*, the basis for the retractive force in rubber bands, which arises because the end-to-end distance of
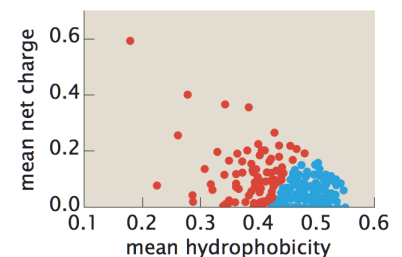
a freely jointed polymer chain follows a Gaussian distribution about r_0:

$$G_{\text{elastic}} = -TS_{\text{elastic}} = -RT \ln\left(e^{-3r^2/(2r_0^2)}\right) = \frac{3}{2}RT\left(\frac{r}{r_0}\right)^2. \qquad (3.24)$$

According to Equation 3.24, random-flight polymer chains act like Hooke's-law springs, in which the free energy has a square-law dependence on end-to-end distance r.

The third term in Equation 3.23 expresses the fact that chains prefer to be either expanded or compact, depending on nonlocal solvent-mediated interactions based on the so-called Flory approximation [8]. If intramolecular attractions are favorable, the chain will collapse. But, if interactions with solvent are more favorable, the chain will populate open expanded conformations. v is the volume of a chain residue. Nv/r^3 is the volume fraction of space that is occupied by the residues, or the probability of neighboring a given residue, which, multiplied by N gives the expected number of residue–residue contacts per residue. a is an interaction energy between a pair of residues ($a = 1 - g_0(T)$ in the model given previously). When $a < 0$ (poor-solvent conditions), residues are attracted to each other strongly, and the third term favors chain collapse. When $a > 0$ (good-solvent conditions), residues prefer to interact with the solvent, and the third term favors chain expansion.

The Radius of the Denatured Chain Grows as $N^{0.6}$

Different proteins will have different denatured-state radii r_{D}, depending on N, the number of amino acids in the chain. To find the approximate radius of the denatured state, r_{D}, use Equation 3.23 to compute the radius that minimizes the free energy,

$$\left.\frac{d}{dr}\left(\frac{G_{\text{D}}}{RT}\right)\right|_{r_{\text{D}}} = 0, \qquad (3.25)$$

which leads to

$$\frac{r_{\text{D}}}{r_0^2} - \frac{aN^2v}{r_{\text{D}}^4} = 0. \qquad (3.26)$$

Rearranging Equation 3.26 and substituting the definitions of r_0 and a gives

$$r_{\text{D}}^5 = aN^2vr_0^2 = (1-g)b^2vN^3. \qquad (3.27)$$

Equation 3.27 predicts that $r_{\text{D}} \sim N^{3/5}$. This prediction is in excellent agreement with experiments. **Figure 3.19** shows the results of small-angle neutron scattering experiments on 28 different proteins, which indicate that $r_{\text{D}} \sim N^{0.598}$ [9].

What is the importance of this 3/5-power dependence? Classical polymer theory predicts three different regimes for the radius of a polymer molecule versus chain length: (1) an exponent of $1/3$ ($R \sim N^{1/3}$) implies solvent conditions (called a Flory *poor solvent*) in which a chain is collapsed; (2) an exponent of $1/2$ implies solvent conditions (called a Flory *theta solvent*) in which the chain conformations follow an ideal random flight, where the intrachain attractions exactly balance the excluded-volume repulsion. (3) an exponent of 3/5, as we have here, implies solvent conditions (called a Flory *good solvent*), in which the chain is expanded, and the excluded-volume repulsions are greater
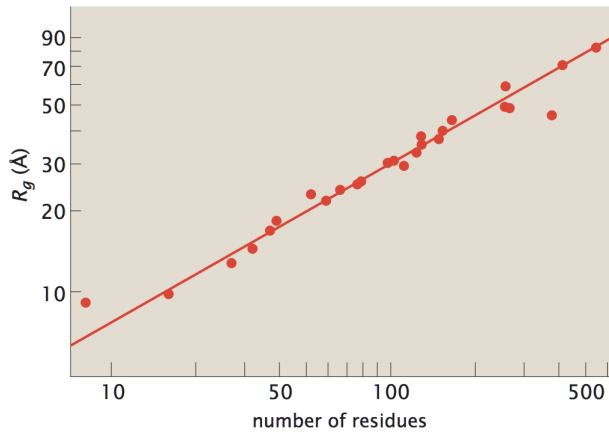
Figure 3.19 Average radii of denatured proteins increase with chain length. The radii of gyration R_g (radius measured from the center of mass) for chemically denatured, cross-link-free, and prosthetic-group-free proteins and peptides were determined by small angle X-ray scattering [9]. The R_g, or average end-to-end distances for denatured proteins, scales with N, the number of amino acids in the chains, as $r_D \sim N^{0.598}$. This agrees with the relation predicted by Flory for polymers in a good solvent, $R_g \sim N^{3/5}$ (From JE Kohn, IS Millett, J Jacob, et al. *Proc. Natl. Acad. Sci USA*, 101:12491–12496, 2004. Copyright (2004) National Academy of Sciences, USA.)

than the intrachain attractions. Therefore, proteins in native conditions resemble collapsed polymers in poor solvents, while proteins in strong denaturants act like expanded polymers in good solvents. Box 3.1 summarizes these scaling relationships.

**Box 3.1 Geometric Scaling Relationships**

	Native	Intermediate	Denatured
Radius	$R \sim N^{1/3}$	$R \sim N^{1/2}$	$R \sim R^{3/5}$
Surface area	$S \sim N^{2/3}$	$S \sim N^{1/3}$	$S \sim N^{6/15}$
Volume	$V \sim N^{1}$	$V \sim N^{3/2}$	$V \sim N^{9/5}$

Individual proteins can vary. They are not perfect spheres [10].

We are now in a position to explain how proteins behave in situations where they are in crowded conditions or in confined spaces. A protein is said to be *confined* when it is contained within a fixed space, for example inside a chaperone or ribosome cavity. A protein is *crowded* when it is in a solution with other inert molecules (such as polysaccharides or sometimes other proteins) that restrict the volume available to it. Proteins are crowded in normal cell environments, where nearly 25% of the volume is occupied by other protein molecules.

Confinement or Crowding Can Increase a Protein's Folding Stability

Putting a protein in a tight space can often increase its folding stability. Consider a protein inside a space that is *inert*, that is, a space that imposes only steric constraints, and does not otherwise interact energetically with the protein. Steric confinement limits the possible conformations that the denatured state of the protein could have. Some of the denatured conformations that would otherwise have been highly expanded when the protein is free of constraints will not be allowed within the confinement volume. This means that confinement will reduce the conformational entropy of the denatured chain, increasing the free energy of the denatured chain. That is, confining the chain causes the difference, $\Delta\Delta G_{\text{fold}} = (\Delta G_{\text{fold}})_{\text{confined}} - (\Delta G_{\text{fold}})_{\text{free}} < 0$. So, confining the chain effectively makes the folded state more stable (Figure 3.20). Therefore, confinement also increases a protein's denaturation temperature (Figure 3.21).
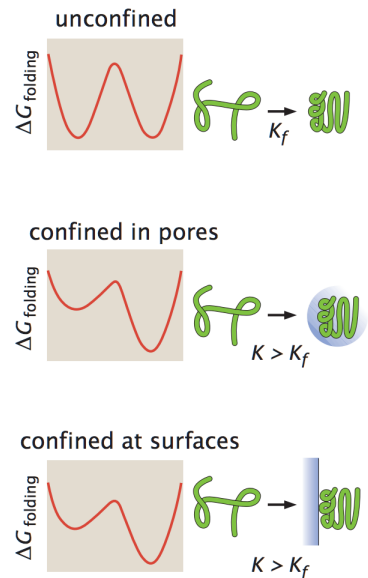
Figure 3.20 A protein in a tight space has greater folding stability, because its denatured state has fewer conformations and thus less conformational entropy. (Adapted from H-X Zhou and KA Dill. *Biochemistry*, 40:11289–11293, 2001.)
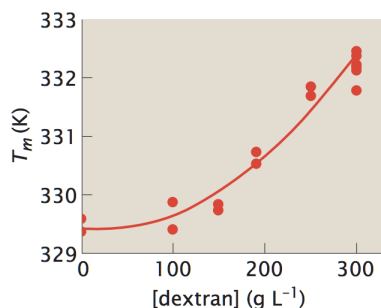
Figure 3.21 Crowding stabilizes lysozyme. The thermal denaturation temperature T_m of hen egg-white lysozyme, determined by circular dichroism, increases with the concentration of dextran, an inert crowding agent. The line indicates a square-law dependence of T_m on the volume excluded by the dextran, consistent with a decrease in the entropy of the unfolded state. (From K Sasahara, P McPhie, and AP Minton. *J Mol Biol*, 326:1227–1237, 2003. With permission from Elsevier.)

SUMMARY

Proteins fold into compact native states because the attractions between amino acids (from hydrophobic and van der Waals interactions, opposite-charge interactions, and hydrogen bonding) are greater in magnitude than the chain conformational entropy that favors the open denatured states. The reason that one particular sequence folds to one particular 3D structure can be understood in terms of a binary hydrophobic (H) and polar (P) code. The HP model is a lattice model that captures basic features of the collapse process and the sequence code. A two-state thermodynamic model explains how the folding free energy, enthalpy, and entropy depend on chain length, temperature, and denaturants. The denatured state has both higher energies (fewer contacts) and higher entropies. Ensembles of denatured conformations are not all the same. Changing solution conditions can expand or contract the denatured state. The scaling powers depend on the state of a protein: $N^{1/3}$ for the native state and $N^{3/5}$ for the denatured state. Confining a protein molecule in an inert container can stabilize it. When a protein is in an acidic or basic solution, its titratable side chains can be charged, and if the net positive or negative charge on the protein is sufficiently high, the protein will denature because of like-charge repulsions.

APPENDIX 3A: A SIMPLE ELECTROSTATIC MODEL OF DENATURATION BY ACIDS AND BASES

How do acids and bases destabilize folded proteins? First, to model the effects of solution *pH* on the side-chain protonation equilibria, let q_a be the net charge on each of the N_a acidic groups. Then the fractional charge per acidic group, f_a, is

$$f_a = \frac{q_a}{N_a} = \frac{[A^-]}{[A^-] + [HA]} = \frac{\alpha}{1 + \alpha},$$ (3.A.1)

where $\alpha = [A^-]/[HA]$. The acid dissociation constant K_a is
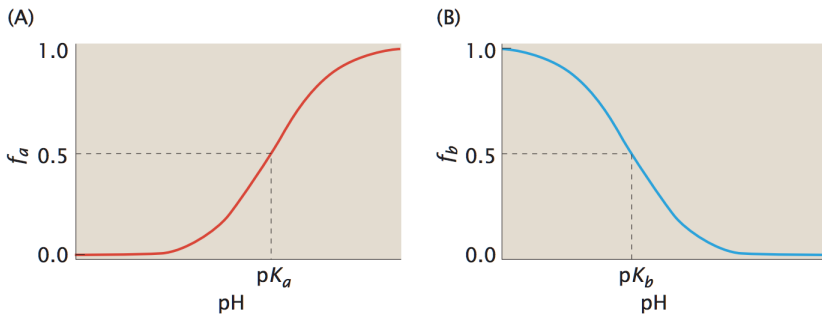
$$K_a = \frac{[A^-][H^+]}{[HA]} = \alpha[H^+].$$ (3.A.2)

Now, we switch to the notation $p = -\log$. That is, $pH = -\log[H^+]$ and $pK_a = -\log K_a$. Side-chain pK_a values are given in Table 3.2. Taking the logarithm (base 10), Equation 3.A.2 becomes

$$\alpha = 10^{\log \alpha} = 10^{pH - pK_a}.$$ (3.A.3)

Substituting Equation 3.A.3 into Equation 3.A.1 gives

$$f_a = \frac{q_a}{N_a} = \frac{10^{pH - pK_a}}{1 + 10^{pH - pK_a}}.$$ (3.A.4)

Equation 3.A.4 gives the *titration curve* for a given type of acidic group. Examples are shown in **Figure 3.A.1**.

Figure 3.A.1 **Titration curves calculated for (A) acids and (B) bases by Equations 3.A.4 and 3.A.6.** When the pH of the solution equals the pK_a or pK_b, half of the ionizable groups carry a charge.

When the pH of the solution is lower than the pK_a of an acidic group, you have $K_a < [H^+]$. In this case, the acidic group will be mostly protonated (uncharged). On the other hand, when pH $> pK_a$, the acid will be mostly deprotonated (charged).

The same type of analysis applies to a basic group. The deprotonation equilibrium for a base is

$$BH^+ \xrightarrow{K_b} B + H^+, \qquad (3.A.5)$$

where the fractional charge per basic group, f_b, is

$$f_b = \frac{[BH^+]}{[BH^+] + [B]} = \frac{q_b}{N_b} = \frac{10^{pK_b - pH}}{1 + 10^{pK_b - pH}}, \qquad (3.A.6)$$

leading to a titration curve of the form shown in Figure 3.A.1B.

Basic groups become deprotonated (that is, they take the form B, rather than BH^+) when pH $> pK_b$. Basic groups that are deprotonated have no net charge; basic groups that are protonated have a net positive charge.

Now let's apply this reasoning to protein denaturation. Suppose a protein has N_a acidic and N_b basic groups. To keep the math simple, suppose all the acids have identical pK_a values and all the bases have identical pK_b values. The protein will then have q_b positive charges and q_a negative charges, where

$$q_a = N_a \left(\frac{10^{pH - pK_a}}{1 + 10^{pH - pK_a}} \right) \qquad (3.A.7)$$

and

$$q_b = N_b \left(\frac{10^{pK_b - pH}}{1 + 10^{pK_b - pH}} \right). \qquad (3.A.8)$$

The net charge on the protein will be

$$q_{net} = q_b - q_a. \qquad (3.A.9)$$

The particular solution condition that causes a protein to have a net charge of $q_{net} = 0$ is called the *isoelectric point* of the protein. To put a protein at its isoelectric point, you adjust the pH of the solution. Different proteins have different isoelectric points because of their different collections of charged side chains.

Here is an approximate model for how a protein's electrostatic charges affect its stability. We compute an electrostatic contribution, ΔG_{es}, to the free energy of folding. We assume that the native protein is a sphere of radius r_N having charge Q_N and that the denatured protein is a sphere of radius r_D and charge Q_D (which is sometimes taken as equal to Q_N). The electrostatic free energy is the free energy of charging up the native sphere in water minus the free energy of charging up the denatured sphere in water.

The total electrostatic free-energy difference between native and denatured states is[4]

$$\frac{\Delta G_{es}(T, pH, c_s)}{RT} = \frac{q_N^2 l_b}{2r_N(1 + \kappa r_N)} - \frac{q_D^2 l_b}{2r_D(1 + \kappa r_D)}, \qquad (3.A.10)$$

where q_N is the total charge on the native protein, q_D is the total charge on the denatured protein, and l_b is a constant (for fixed temperature) called the *Bjerrum length*. κ is the inverse of the *Debye length*. The Debye length describes the distance through space over which interactions between charges diminish due to an intervening salt solution. The Debye length gets shorter with increasing salt concentration c_s according to the following relation:

$$\kappa^2 = 2c_s l_b. \qquad (3.A.11)$$

The justification for using the dielectric constant of water for the native protein is that charged side chains are mainly on the protein's surface, and are largely solvated in both native and denatured states. The value of r_N can be determined by knowing the radius of gyration in the native state. The radius of gyration for the denatured state, r_D, can be obtained from experimental measurements, such as those shown in Figure 3.19.

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[4]The electrostatic free energy for charging a sphere of radius r in a solvent having dielectric constant ϵ from zero charge to a charge of Q is

$$\Delta G_{es} = \frac{1}{2} \int \sigma \psi \, ds,$$

where $\sigma = q$ is the final surface charge. The integral is taken over the surface of the sphere, and ψ is the electrostatic surface potential [2]:.

$$\psi(r') = \frac{Cq}{\epsilon r(1 + \kappa r)} e^{-\kappa(r'-r)},$$

where r' is the distance from the center of a sphere of radius r, $Ce^2 \mathcal{N}$ is an electrostatic constant equal to 1.386×10^{-4} J m mol^{-1}. Integrating gives the free energy of charging the sphere [2]:

$$\frac{\Delta G_{es}(r)}{RT} = \frac{q_{net}^2 l_b}{2r(1 + \kappa r)},$$

where l_b is the Bjerrum length,

$$l_b = \frac{1.386 \times 10^{-4} \text{ J m mol}^{-1}}{\epsilon RT}.$$

R is the gas constant, T is the absolute temperature, and ϵ is the dielectric constant of water. The Bjerrum length is the distance over which the electrostatic energy between two unit charges in a given solution diminishes to RT. At room temperature, $l_b = 7.13$ Å, using the dielectric permittivity of water, $\epsilon = 78$, at room temperature.
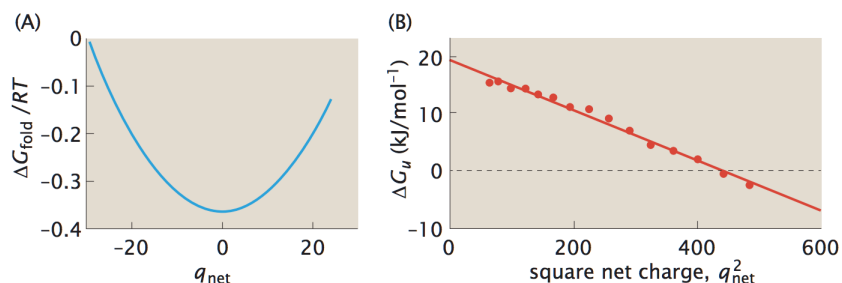
Figure 3.A.2 **Protein stability depends on the square of the net charge.**
(A) The free-energy difference between folded and unfolded states is a function of the protein's net charge. The net charge on metmyoglobin is calculated from the pK_a values of its ionizable groups (B) The free energy of unfolding the ferricytochrome c molten globule depends on the net charge. Free energies of thermal unfolding of the acid-induced molten globule state were determined by scanning calorimetry for various charge states of horse ferricytochrome c. The line is fit to the linearized square of the net charge. (pK_a values in (A) are from E Breslow and FRN Gurd. *J Biol Chem*, 237:371–381, 1962. (B) From Y Hagihara, Y Tan, and Y Goto. *J Mol Biol*, 237:336–348, 1994. With permission from Elsevier.)

The pK_a of an acidic or basic side chain, as given in Table 3.2, is a property of its protonation equilibrium measured in water. But sometimes, in folded structures, protonatable side chains are buried in native hydrophobic cores. In those cases, the pK_a of a side chain can be different than its value in water. Those altered values of pK_a should be used in the given model, if they are known.

Equation 3.A.10 shows how to interpret charge effects on protein stability. It says that if you systematically vary the net charge, $q_{net} = q_b - q_a$ (Equation 3.A.9) by mutating a protein's acidic or basic amino acids, the protein's stability will decrease in proportion to the square of the net charge, q_{net}^2, on the protein. **Figure 3.A.2A** shows this prediction that the more net charge on the protein, the less stable the folded state will be. This is why extremes of pH, which lead to extremes of net charge on the protein, can unfold proteins (**Figure 3.A.3**). Experimental confirmation of this prediction through random acetylation experiments [11] is shown in Figure 3.A.2B.

In summary, at low pH (high concentrations of H^+ ions), both the acidic and basic side chains are protonated—the acidic side chains are mostly in the form of $-COOH$ and the basic side chains are in the form of $-NH_3^+$, so the protein has a net positive charge. At high pH (low concentrations of H^+ ions), both the acidic and basic groups are deprotonated—mostly in the forms of $-COO^-$ and $-NH_2$, giving a net negative charge. In the middle range of pH, both acidic and basic side chains are charged, in the forms of $-COO^-$ and $-NH_3^+$. So, if the numbers of acidic groups is about the same as the number of basic groups, the net charge on
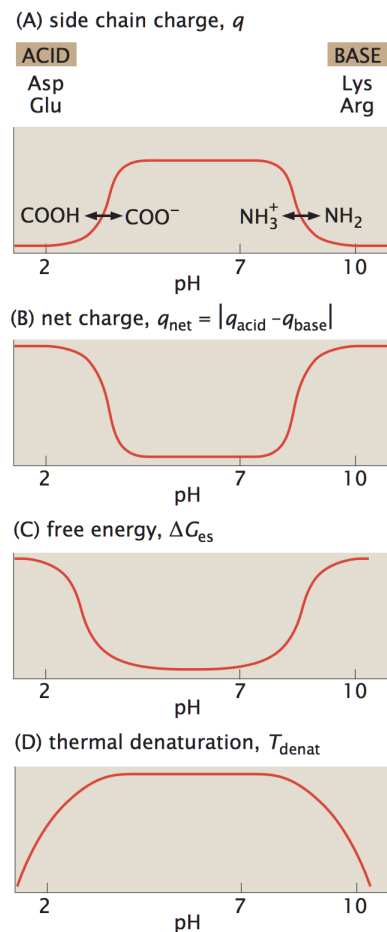
Figure 3.A.3 **The charge and stability of a protein depend on solution pH.** (A) As the solution is titrated from acidic to basic pH, most of the acidic $-COOH$ side-chain groups become deprotonated. At high pH, basic $-NH_3^+$ side-chain groups give up a proton. (B) At very low pH, the protein has both $-COOH$ and $-NH_3^+$ groups, so it is positively charged. At very high pH, the protein has both $-COO^-$ and $-NH_2$ groups, so it is negatively charged (C) The electrostatic free energy follows a pattern similar to the net charge. (D) These charge effects destabilize the protein for thermal denaturation at both extremes of pH.

the protein will be small in the mid-range of pH. Hence, proteins will denature in acids or bases because, at those extremes of pH, the protein becomes highly charged, leading to repulsions that drive the protein toward the denatured state.

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