

13. MULTIMERIZATION

17 May 2021

Although most of the preceding chapter focused on proteins in a generic way, a substantial fraction of proteins in virtually all organisms operate by binding to other proteins, either transiently or in semi-permanent structures. The focus here is on multimers, i.e., assemblages of polypeptide subunits held together at binding interfaces in a noncovalent manner. All cells contain large numbers of beautiful, symmetric complexes of proteins, called dimers when there are two subunits, trimers when there are three, tetramers when there are four, etc. (Figure 13.1). Indeed, virtually all biological pathways and cell structural features involve one or more multimers. Some of these consist of higher-order complexes built out of lower-order multimers, prime examples including flagella, nuclear pore complexes, centrosomes, and ATP synthase, all of which are discussed in separate chapters.

Our focus here is on the evolution of the basal building blocks of such complex structures. Before getting started, a brief overview of nomenclature will be useful. When the subunits of a multimer are encoded by the same genetic locus, the complex is referred to as a homomer. Complexes consisting of subunits from two or more loci are heteromers, the extreme situation being the case in which each subunit is encoded by a unique genetic locus. The interfaces between subunits are critical to multimer assembly, and it is useful to distinguish between isologous and heterologous forms. In the former case, both participants deploy the same surface patch (although generally rotated) in binding, whereas in the latter case, each binding partner utilizes a different surface patch at the interface. By definition, all heteromeric interfaces are heterologous.

Even when homomeric, odd-mers (e.g., trimers, pentamers, etc.) almost exclusively rely on heterologous interfaces, as these are required to create a closed structure (Figure 13.2). Closed structures with just one type of heterologous interface are referred to as cyclical multimers. A special case is the domain-swapping homodimer, which has a geometric configuration that allows two symmetric heterologous interfaces to form a closed loop. In contrast, dihedral multimers have multiple axes of symmetry and require more than one interface type, with each usually being isologous (Figure 13.2).

It is often assumed that the kinds of organized diversity embodied in multimers could only be a product of natural selection, with higher-order multimers sometimes being referred to as the ‘end points’ or ‘pinnacles’ of stepwise evolution. Should this be true, then an obvious question is why only a subset of lineages have been able to achieve such lofty heights. In fact, as will be shown below, there are numerous examples of evolutionary reversions to lower oligomeric states. Eukaryotes may have

a higher incidence of heteromeric than homomeric structures relative to orthologs in bacteria (Figure 13.3; Reid et al. 2010; Nishi et al. 2013; Marsh and Teichmann 2014), but there is no compelling evidence that the former are functionally superior (Chapter 24). Because the extent to which multimeric structures are promoted or maintained by selection remains unclear, it is useful to think of them as ‘biology’s snowflakes,’ to remind us that beauty and diversity can often arise for purely physical reasons.

How and why multimeric structures originate constitute two of the more significant issues in evolutionary cell biology. Sometimes a multimeric complex achieves a new function by virtue of special features associated with the interface, a process that will often require gene duplication and neofunctionalization unless such a modification can be achieved without compromising the original gene function. There are also numerous cases in which higher-order complexes assemble into containers (e.g., some chaperones, and cages for vesicles) or fibrils (actins and tubulins). However, in the case of enzymes, more often than not all subunits retain their original monomeric functions, in which case for example, a dimeric enzyme would simply have two catalytic sites but no new function.

We first consider the incidence of various multimeric types of molecules across the Tree of Life, highlighting the apparent lack of association between molecular and organismal complexity. We then review some of the major biophysical challenges to adopting a multimeric protein form. Following this entrée into the biophysics of aggregation and the general features of known interfaces, the basic theory for understanding how multimeric architectures evolve will be reviewed.

The Incidence and Architectural Features of Multimers

There is astounding diversity in the higher-order structure of proteins. Surveys of proteins with known quaternary structures indicate that multimers are very common, on the order of 60% of all characterized proteins, with homomers being about twice as frequent as heteromers (Levy et al. 2006; Maryianayagam et al. 2004; Lynch 2012a). Indeed, because complex proteins are more difficult to characterize structurally, it is likely that the incidence of multimers is even greater than 60%. The vast majority of multimers are dimers, with a roughly exponential pattern of decline in frequencies with elevated numbers of subunits, and odd-mers tending to be under-represented relative to even-mers (Figure 13.3; Goodsell and Olson 2000; Mei et al. 2004).

Strikingly, these distributions are essentially independent of phylogenetic context, with those for bacteria being quite similar to those for vertebrates and land plants (Figure 13.3). Although these distributions are potentially biased by the nature of proteins that have been studied at the structural level, a comparison of orthologous metabolic proteins across taxa supports the conclusion that there is no gradient of molecular complexity (number of subunits) with organismal complexity, and further demonstrates that within and among most major lineages, there is substantial variation in the multimeric states of the same proteins with the same functions (Reid et al. 2010; Lynch 2013). For example, it is not uncommon to see the same enzyme operating as a monomer in some bacteria, a dimer in others, and a

tetramer in still others. Hemoglobins provide a textbook view of the extraordinary diversity of complexes that can exist (from monomers to structures containing more than 100 subunits, in metazoans alone) (Shionyu et al. 2001). Levy et al. (2006) suggest that at the point of 30-40% amino-acid sequence divergence, the probability of two species sharing quaternary structure drops to $\sim 70\%$.

Numerous studies also reveal that orthologous proteins with the same numbers of subunits in different phylogenetic lineages often utilize different binding interfaces. For example, for glycosyltransferases, Hashimoto et al. (2010) found a dramatic diversity of monomeric, homodimeric, and homotetrameric states across the Tree of Life, with many of the independently evolved dimers utilizing different binding interfaces, sometimes even on opposite sides of the monomeric subunit. Examples are also known in which some species use isologous and others use heterologous interfaces (Vassylyev et al. 2006). For proteins with known multimeric structures in more than one species, $\sim 36\%$ of cases exhibit variants with different interfaces across species, with $\sim 4\%$ of cases exhibiting more than five different binding modes across lineages (Dayhoff et al. 2010).

Ahnert et al. (2015) produced a ‘periodic-table’ classification of types based on the total number of repeating units, the number of subunit types (gene products) per repeat, and the number of ways in which these can assemble into multimers. Just a small set of the possible topologies for heteromers are shown in Figure 13.4, with two to three repeats and two subunit types per repeat. When there are four or more repeats, the final complexes can be cyclical or dihedral in form. For example, a cyclic homomeric tetramer has just one type of face-to-back interface repeated four times in a closed loop, whereas a dihedral homomeric tetramer utilizes two different isologous interfaces twice (Figure 13.2). If there are two subunits per repeat and three repeats, the resultant structure is a hexamer, which can take on two configurations (Figure 13.4). A wide variety of higher-order topologies beyond those outlined in Figures 13.2 and 13.4 have been discovered in biology (Ahnert et al. 2015), and those with larger numbers of repeats and subunits/repeat continue to be identified. Whenever there exists the possibility of either a dihedral or cyclical closed structure (as with tetramers; Figure 13.2), dihedral forms are $\sim 10\times$ more frequent than cyclical forms (Levy et al. 2008), suggesting a greater ease of evolution towards isologous binding interfaces, a point that will be further discussed below.

For heteromeric even-mers beyond dimers (tetramers, hexamers, etc.), there is the possibility of uneven stoichiometry from the contributing proteins (e.g., AAAB as opposed to AABB). Uneven stoichiometry is thought to be nearly twice as common in bacteria as in eukaryotes, and the incidence of unevenness increases with the number of contributing proteins, e.g., $\sim 10\%$ when just two or three proteins participate, but $\sim 30\%$ when there are five or more contributing proteins (Marsh et al. 2015). To achieve unevenness, at least one protein must self-associate to produce a dyad, and Marsh et al. (2015) suggest that a higher propensity to homomerize may explain the excess degree of unevenness in bacteria. With all of these observations, there is reason for caution on quantitative details, as a balanced analysis based on orthologous proteins remains to be done.

Propensity to Aggregate

To achieve multimeric architectures, the cellular concentration of monomeric subunits must be at a sufficiently high level to allow reasonable encounter and aggregation rates between partner molecules. Because proteins come at a biosynthetic cost (Chapter 8), this may then entail an excess investment that would otherwise not be required. If multimers are less subject to degradation, the overall cost of monomer production could be mitigated, but there is also a potentially fine line between the assembly of functional complexes vs. collateral damage, as adhesive interfaces can promote promiscuous interactions with noncognate molecules and/or lead to the runaway production of harmful self-aggregates, including fibrils (Jucker and Walker 2013).

Just how close to the edge of aggregation/disassembly proteins are is demonstrated by experimental work in *E. coli* showing how single amino-acid substitutions to hydrophobic surface residues can shift proteins into supramolecular states (Garcia-Seisdedos et al. 2017). Similarly, in a well-known case in human biology, a single glutamate-to-valine substitution in the hemoglobin molecule induces the fibril formation that causes sickle-cell anemia. From experimental observations in yeast, Zhang et al. (2008) estimate that promiscuous binding leads to $\sim 25\%$ of proteins being at least transiently bound to inappropriate partners.

The clear implication here is that any involvement of natural selection in the evolution of higher-order protein structures must include not just the enforcement of productive self-binding but the elimination of surface feature that promote harmful structures (Zabel et al. 2019). Mechanisms of mitigating the latter problems are not just restricted to the primary amino-acid sequence of a protein, but include the spatial configuration of adhesive residues in ways that influence the relative rates of monomer folding and subunit aggregation. For example, locating interfaces residues on the C-terminal end of a protein (the last to emerge from the ribosome) reduces the likelihood of premature aggregation of localized and/or partially folded proteins (Natan et al. 2018; Gartner et al. 2020). The spatially restricted nature of transcription also reduces the likelihood of off-target binding. For example, when mRNAs are cotranslated by groups of ribosomes (so-called polysomes jointly processing the same mRNA), this creates a situation in which protein monomers are initially colocalized at substantially higher concentrations than expected under a random distribution, increasing the association rate, and sometimes even leading to co-translational assembly (Wells et al. 2015). In the case of bacteria deploying operons, this spatial clustering likely even allows for enhanced assembly of heteromers (Wells et al. 2016).

Theory of association. Acknowledging that there are many underlying molecular factors, we now consider in the simplest and most general terms the principles underlying the rate and stability of molecular aggregation, building on the concept of molecular diffusion introduced in Chapter 7. After emerging from a ribosome, amino-acid chains will initially be monomeric in form, and will retain that state until encountering a binding partner. Even then, the maintenance of an appropriate multimeric form requires sufficient binding energy across the interface. In principle, given steady-state conditions (e.g., a constant concentration of protein per unit cell volume), an equilibrium will be reached within the cytoplasm reflecting equal rates of association into multimers and dissociation of the latter back to monomers. In

this sense, few proteins can be viewed as having a single form, and when one is referred to, this should simply be viewed as the dominant phase.

The equilibrium distribution of multimeric forms within a cell depends on at least three factors: 1) the cellular concentration of proteins, which dictates the encounter rate of monomeric subunits; 2) the rate of particle movement and binding, which dictates the assembly rate; and 3) the stability of the binding interface, which dictates the longevity of a newly formed multimer. The basic principles can be understood by focusing on a system allowing for just monomers and dimers.

Owing to the destabilizing forces of molecular motion, two subunits will have a thermodynamically determined probability of being in complex depending on the rates of association of monomers and dissociation of dimers. Letting $[A]$ and $[AA]$ denote the cellular concentrations of solo A molecules and AA complexes, the fraction of total *particles* contained within complexes is denoted as

$$p_{AA} = \frac{[AA]}{[A] + [AA]}. \quad (13.1a)$$

By multiplying the numerator and denominator by $[A]/[AA]$, the expected fraction of dimers at equilibrium can be expressed as

$$p_{AA} = \frac{[A]}{K_D + [A]}, \quad (13.1b)$$

where

$$K_D = \frac{[A][A]}{[AA]} \quad (13.2a)$$

is the dissociation constant, which depends on the strength of binding (Foundations 13.1).

Written in this way, it can be seen that K_D is equivalent to the concentration of monomers in the cell at which aggregation leads to an equivalent concentration of dimeric complexes, i.e., $p_{AA} = 0.5$ when $K_D = [A]$, which requires $[AA] = [A]$. Note that $[A]$, $[A]$, and K_D are all in molar concentration units, although often converted to μM (where $1 \mu\text{M} = 10^{-6} \text{ M}$) below, as cytoplasmic protein concentrations are typically in the μM range (Chapter 7). In the ideal situation in which there are no interfering molecules, K_D is equivalent to the ratio of dissociation and association rates, where the latter can be expressed in terms of the random encounter rate (Chapter 7) and the probability of proper binding (Foundations 13.1). Because the rate of encounter is a function of the molecular concentration, proteins with lower binding affinities are expected to require higher cellular concentrations to achieve an effective level of complexation.

To put Equation 13.2a into a more biophysical perspective, the dissociation constant can be written in energetic terms as

$$K_D = e^{-\Delta E/RT}. \quad (13.2b)$$

where ΔE denotes the excess energy required for dissociation (in kcal/mol), and RT denotes the standard background energy associated with Brownian molecular motion (where for most biological temperatures, $RT \simeq 0.6 \text{ kcal/mol}$) (Foundations 13.1).

Thus, as the binding energy across the interface between two subunits increases, K_D asymptotically approaches zero and the probability of complexation approaches 1.0.

It should be emphasized that the quantitative value of the dissociation constant is context dependent. As defined in Equation 13.2b, K_D has a rather precise meaning from a pure chemistry perspective, and is typically recorded in an aqueous solution containing only A molecules. Written in the form of Equation 13.1b, however, K_D can be thought of in much more general terms, as a simple (inverse) indicator of the degree of affinity of two A molecules for each other in a system of arbitrary complexity. Within the cellular environment, which can contain thousands of proteins encoded by other loci, a monomeric subunit is confronted not just with the challenge of adhering to its binding partner, but with the additional problem of avoiding inappropriate, promiscuous engagement with noncognate molecules. Assuming that the molecule of interest, A, is sufficiently adhesive that it is essentially always in complex with either a self or foreign molecule, the methods in Foundations 13.1 can be used to derive a modified expression for the fraction of molecules bound up in appropriate homodimeric complexes, maintaining the structure of the preceding formula but using a modified definition of the dissociation constant,

$$K'_D = K_D \cdot \phi e^{\Delta E'/(RT)}. \quad (13.3)$$

Here, ϕ is the effective concentration of foreign proteins with a capacity for promiscuous binding, and $\Delta E'$ is the strength of binding associated with promiscuous liaisons. In effect, the terms to the right of K_D amount to a weighting factor that increases the level of dissociation as a function of background interference. Because ϕ could easily be on the order of 10-fold greater than $[A]$, $\Delta E'$ must be much smaller than RT if a high incidence of engagement in nonproductive (and possibly harmful) complexes is to be avoided.

As will be discussed below, a typical value of ΔE for a binding interface is 10 kcal/mol, which implies $K_D = e^{-10/0.6} \simeq 0.058 \mu\text{M}$. Under these conditions and assuming no promiscuous binding, Equation 13.1b indicates that when equilibrium monomeric concentrations are 0.1, 1.0, and 10.0 μM , the fractions of dimers are 0.633, 0.945, and 0.994, respectively. Supposing, however, that $\phi = 10$ and that $\Delta E'/(RT)$ is 1 kcal/mol, then K_D is modified to $K'_D \simeq 1.6 \mu\text{M}$, and for equilibrium monomeric concentrations of 0.1, 1.0, and 10.0 μM , the fraction of dimers is now reduced to 0.058, 0.385, and 0.862 respectively, owing to the fact that many monomers will be sequestered in inappropriate complexes.

The physical features of interfaces. Geometric considerations dictate that the interface between two globular molecules cannot exceed a moderate fraction of the total surface area, and direct observations from a large number of proteins suggest a range of ~ 5 to 30%, with a weak positive linear scaling between interface size and total monomeric surface area (Chothia and Janin 1975; Jones and Thornton 1996; Bordner and Abagyan 2005; Lynch 2013). Whether the latter is a simple consequence of geometry or a result of larger proteins requiring larger interfaces for stabilization remains unclear.

Although little work has been done on the energetic features of homomeric interfaces, surveys of a diverse set of transient protein-protein interactions (e.g., antigen-

antibodies, enzyme-inhibitors, etc., from a variety of taxa; Jones and Thornton 1996; Bogan and Thorn 1998; Horton and Lewis 1995; Kastiris et al. 2011) provide the basis for two conclusions (Figure 13.5). First, the average interfacial binding strength is $\sim 18RT$ with a standard deviation of $5RT$ (recalling from above that $RT \simeq 0.6$ kcal/mol). We can expect the binding strength of more permanent multimers to be somewhat greater, and Brooijmans et al. (2002) suggest an average of $22RT$ for homodimer interfaces. Second, there is only a weak relationship between binding strength and total interface area, with the minimum observed interface area being $\sim 1000 \text{ \AA}^2$ and the minimum binding energy being ~ 6 kcal/mol, i.e., $10RT$. Drawing from a diverse set of observations, Bahadur et al. (2003) find an average interface area of $\sim 4000 \text{ \AA}^2$ for homodimers and $\sim 2000 \text{ \AA}^2$ for hetero-complexes, although the distributions are quite asymmetric with modes lower than the mean.

An approximate mechanistic argument can be derived for the lower limit to a functional interface area as follows (Day et al. 2012). From Figure 13.5, it can be seen that a rough upper limit to the binding strength per $\text{\AA}^2$ is ~ 0.012 kcal/mol. For two proteins to have any affinity at all, the total strength of the interaction must be at least equal to the energetic cost of simply holding the two interfaces in a particular orientation, which is on the order of 8.0 kcal/mol (Janin 1995; Zhang et al. 2008; Day et al. 2012). The minimum interface size necessary to cover this cost is $\sim 8.0/0.012 = 667 \text{ \AA}^2$, also in accord with Figure 13.5. Taking an average binding strength of a dimeric interface to be $22RT$ (from above), a very approximate average ΔE is $(22 - 8)RT = 16RT$, which from Equation 13.2b implies K_D on the order of 0.1 μM . The latter is of the order of magnitude of molecular concentrations often seen in cells (Chapter 7).

Observed interfaces typically consist of no more than a few dozen amino-acid residues (Chothia and Janin 1975). For example, broad surveys over diverse organisms suggest means of ~ 80 to 100 for homodimer interfaces and 50 to 60 for heterodimers, again with strong asymmetries in the distributions yielding lower modes than means (Bahadur et al. 2003; Zhanhua et al. 2005). The absolute lower bound appears to be ~ 10 residues, with most interfaces being in the range of 10 to 40 with a mode near 20 (Bordner and Abagyan 2005). However, these total sizes need not provide accurate counts of the actual residues involved in adhesion, as the total binding energy is usually concentrated in a small number of hot spots and fewer than ten residues are typically involved (Bogan and Thorn 1998; Hochberg et al. 2020; Pillai et al. 2020).

Many homomers utilize symmetric isologous interfaces rotated 180° with respect to each other, and the commonness of this feature may reflect a simple geometric ‘two for the price of one’ argument made by Monod et al. (1965). Supposing that residue A binds with a complementary residue B on a parallel flat interface, then rotating around this affinity pair by 180° will yield a complementary B-A match, thereby doubling the strength of binding with just a single mutational change. Extensions of this argument lead to the conclusion that proteins have an innate propensity for self-assembly relative to random surfaces (Ispolatov et al. 2005; Lukatsky et al. 2007; André et al. 2008). For a dihedral tetramer (Figure 13.2), the preceding logic leads to a ‘four for the price of one’ argument.

However, the flip-side of this view is that an isologous interface is also expected to have a 50% reduction in the mutation rate to binding residues (because the mu-

tational target sized is reduced by 50%), and that any mutation to a binding residue will have double the effect (because two binding pairs of sites are lost simultaneously). Thus, it remains unclear whether the ‘two for the price of one’ argument is a sufficient explanation for the excess abundance of isologous interfaces. Instead, it appears that symmetrical isologous interfaces are more common simply because mutations more frequently confer stability to such configurations (André et al. 2008; Plaxco and Gross 2009).

Binding can come about in a multiplicity of ways, although utilization of hydrophobic residues and hydrogen bonds are the predominant factors. Interface hydrophobicity is generally intermediate to that of interior cores and exterior surfaces, with a typical enrichment of the more hydrophobic and polar uncharged residues, most notably F, C, L, M, I, Y, W, and V (Bordner and Abagyan 2005; Levy 2010). A summary from diverse proteins and organisms indicates that the average numbers of hydrogen bonds are ~ 11 and 18 for heterodimer and homodimer interfaces, with a scaling of ~ 0.2 per interface residue (Jones and Thornton 1995; Bahadur et al. 2003; Zhanhua et al. 2005).

Notably, the structural evolution of multimeric proteins extends beyond the conjoining interfaces. For example, Marsh and Teichmann (2014) found that monomeric subunits that engage in multimeric assemblies tend to be significantly more flexible than those that operate exclusively as monomers. Their interpretation of this pattern is that flexibility facilitates the conformational changes necessary for successful binding. There are, however, open questions of cause vs. effect here. In particular, selection for self-rigidity may be relaxed in a protein that is secondarily stabilized by a binding partner. The average flexibility of subunits increases with the number of nonhomologous subunits in a heteromeric complex, and as a protein complex acquires new subunits over time, the consecutive additions tend to be increasingly flexible (Marsh and Teichmann 2014).

As in the case of folding stability (Figure 12.6), once a highly refined level of binding efficiency has been obtained, the fitness advantages of further improvement can be expected to become increasingly negligible, rendering a situation in which excess capacity for binding remains unutilized (Figure 13.6). Several studies have been performed in which alanine residues are individually substituted for the amino-acid constituents of interfaces, and the effects on binding strength typically have distributions of effects in which the highest density is near zero, with $\sim 10\%$ having slightly positive effects on binding (Bogan and Thorn 1998). A number of examples also exist in which multimers engineered to have stronger interfaces also have increased thermostabilities (Griffin and Gerrard 2012).

Given that there are typically multiple unutilized residues at most binding interfaces, these observations suggest that there are a large number of alternative and nearly energetically equivalent amino-acid constitutions at individual interfaces (Figure 13.6). This further implies that interface residues can evolve in multiple ways with minimal effects on molecular functionality, with such sequence wandering eventually leading to situation in which orthologous proteins in different lineages have different interface constitutions (as noted above). Consistent with this view, amino-acid sequences at interfaces do not evolve at unusual rates – on average not more than 50% more slowly and sometimes slightly more rapidly than residues on the exposed surface and in the internal core (Grishin and Phillips 1994; Caffrey et

al. 2004; Bordner and Abagyan 2005; Mintseris and Weng 2005).

Over time, this combination of multiple degrees of evolutionary freedom and the diminishing fitness advantages of increased binding, and the resultant effectively neutral interface evolution, is expected to lead to a situation in which the interfaces on molecules derived from different phylogenetic lineages are fine in isolation but nonfunctional in a cross-species construct (e.g., as mixtures of the monomeric subunits from two species). The few attempts to evaluate heterologous compatibilities have led to mixed results, with interpretations made difficult by the fact that most examples focus on constructs where the catalytic function is built from the interface.

Cross-species molecular hybrids of thymidylate synthase from *E. coli* and *Lactobacillus casei*, two very distantly related bacteria, appear to be fully functional (Greene et al. 1993). Functionality has also been demonstrated for hybrid ornithine decarboxylase dimers between the monomeric subunits of *Trypanosoma brucei* and mouse, whereas cross-species dimers between *T. brucei* and *Leishmania donovani* (both trypanosomes) are nonfunctional (Osterman et al. 1994). For triose phosphate isomerase, Sun et al. (1992) find that cross-species hybrid dimers involving mammals, chicken, and yeast have lower levels of catalytic efficiency and enzyme stability than within-species dimers (in this case, the catalytic site is not created by the interface). Careful comparative analyses of this sort involving a gradient of phylogenetic relationships, and extended to situations in which catalysis is independent of interface binding, will be essential to furthering our understanding of how interfaces evolve.

Evolutionary Considerations

Given the penchant for most biologists to assume that virtually every aspect of biology owes its origin to natural selection, it will come as no surprise that most attempts to explain the existence of multimers start with this implicit assumption (e.g., Monod et al. 1965; Goodsell and Olson 2000; Marianayagam et al. 2004; Mei et al. 2004; Hashimoto et al. 2011; Griffen and Gerrard 2012). The proposed advantages are diverse. First, as noted in Chapter 12, it is generally easier to fold multiple small proteins than a single long one, and cases are known in which multimerization can actually enhance the folding rates and stability of the monomeric subunits (Zheng et al. 2012).

Second, the encounter rate of an enzyme and a small substrate is proportional to the effective radius of the enzyme (Chapter 7), and the elimination of extraneous protein surface may further enhance the frequency of productive encounters between catalytic sites and their substrates. However, these considerations need to be tempered by the fact that multimerization reduces the number of enzymatic complexes subject to diffusion. For example, a dimer with double the volume of a monomer would be expected to have a radius (and hence encounter rate) $\simeq 2^{1/3}$ times that of the monomer but half the number of particles, which would reduce the encounter rate to a factor of $2^{-2/3}$, unless there were additional favorable factors involved.

Third, multimerization may have secondary advantages. A smaller surface-area to volume ratio might reduce a protein's vulnerability to denaturation or engagement in promiscuous interactions (Bershtein et al. 2012). Higher-order structures might

also reduce the sensitivity of catalytic sites to internal motions, thereby increasing substrate specificity. In addition, complexation offers increased opportunities for allosteric regulation of protein activity.

Finally, multimerization can sometimes lead to the creation of an entirely new function by inducing secondary structural changes that alter the nature of the catalytic site. For example, some Archaea have a CS₂ anhydrase that converts CS₂ into H₂S by a process similar to carbonic anhydrase's conversion of CO₂ to HCO₃⁻, but whereas the latter is a monomer, the former has a hexadecameric architecture that prevents access of CO₂ to the catalytic site (Smeulders et al. 2013).

Weighted against these potential advantages, one must also consider the negative side effects that can result from proteins with a tendency to multimerize. First, unless a newly emerging dimer has a single isologous interface, concatenations into indefinite fibrils can arise. Human disorders involving the production of amyloid fibrils, such as Alzheimer's and Parkinson's diseases, amyotrophic lateral sclerosis, and sickle-cell anemia, are prominent examples of the consequences of overly adhesive proteins (Chiti and Dobson 2009).

Second, as noted above, to achieve a critical concentration of an active multimer, the expression of monomeric subunits must be raised to a high enough level to ensure an adequate number of encounters for successful complex assembly. Any increase in subunit production will entail an energetic cost, and highly expressed proteins are also vulnerable to promiscuous interactions (Semple et al. 2008; Vavouri et al. 2009). If, however, multimeric proteins are less vulnerable to thermo-instabilities and degradation, multimerization might actually result in a lower energetic demand, as the necessity of protein replacement is reduced.

Third, it bears emphasizing that even if a particular multimeric structure is advantageous, it need not follow that a new mutation to such a form can be easily promoted by selection, as the fixation process critically depends on the background conditions in which the mutation first appears. As will be noted below, in diploid species, some dimerizing mutations may have deleterious effects when combined with ancestral non-dimerizing proteins in heterozygous individuals (where potentially detrimental heterotypic complexation can occur). In diploid species, where new mutations are always present on a heterozygous background, such a process has the potential to greatly reduce the probability of establishment of a dimerizing allele.

In summary, although the large pool of multimeric structures in today's organisms cannot possibly be strongly maladaptive, this need not imply that they have arisen or are currently maintained by adaptive processes. Indeed, despite the plausibility of many of the above hypotheses, empirical evidence for the adaptive value of alternative multimeric structures is quite scant. Moreover, a number of examples can be pointed to in which a more complex structure seemingly operates no more efficiently in its lineage than a simpler structure in others.

For example, the mismatch-repair system, which plays important roles in replication fidelity, DNA repair, and recombination, is comprised of monomeric proteins in eubacteria but dimers in eukaryotes (Kunkel and Erie 2005; Iyer et al. 2006), yet the repair efficiency of eukaryotic systems appears to be lower than that in prokaryotes (Lynch 2011). Freist et al. (1998) point out that the overall fidelity amino-acid loading of class I tRNA synthetase enzymes in translation tends to be

much greater than that of the class II enzymes, despite the fact that the former operate as monomers and the latter as dimers. As still another example, the sliding clamps used in DNA replication are homodimeric in eubacteria but homotrimeric in eukaryotes, with both structures having very similar overall architecture (Kelman and O'Donnell 1995), yet replication-fork progression rates are nearly an order of magnitude faster in prokaryotes (Lynch 2007). A more practical example involves the insulin hormone, which normally operates as a homoheptamer in humans, but has been engineered to become a monomer by incorporating just one or two amino-acid changes in the interface (Brange et al. 1988); in the treatment of diabetes, the monomers are absorbed much more rapidly than the multimers and have equivalent efficacy, although they can sometimes lead to the production of amyloid fibers at the site of injection. Finally, as noted in Chapter 6, although the ribosome has a much more complex protein repertoire in eukaryotes than in prokaryotes, as will be discussed in Chapter 20, there is no evidence that translation fidelity is elevated in the former.

With these considerations in mind, we now consider the more formal evolutionary theory essential to understanding how the evolution of alternative multimeric structures proceeds. Our focus here is primarily on situations in which the gene function is independent of the number of subunits in the complex, and it bears emphasizing that quite different outcomes are expected when the interfaces between subunits are essential to function, as this strongly constrains the acceptability amino-acid substitutions (Abrusán and Marsh 2018). Although the basic issues appear clear, we are still a long way from being able to formally test the theory, which will require a thorough combination of comparative analysis and experimental work on orthologous proteins across diverse phylogenetic taxa.

Transitions from monomeric to higher-order states. In one of the few experimental studies of the causes and consequences of dimerization, Hochberg et al. (2020) compared the features of estrogen and steroid receptors, hormone-activated transcription factors that diverged from a common ancestor at the base of the chordates. The former operate as dimers, and the latter as monomers. Through phylogenetic comparisons of the sequences from a diverse set of species, the authors were able to reconstruct and evaluate the functional properties of key ancestral sequences. The key finding was that the functional features of monomeric and dimeric family members are likely not a consequence of multimerization *per se*, but that following its initial establishment, the dimeric form gradually became more entrenched as mutations to hydrophobic residues accumulated in the interface.

This proposed scenario is very similar to the kind of evolutionary trajectory postulated under the constructive neutral-evolution hypothesis (Chapter 6), which envisions the effectively neutral establishment of higher-order complexes as interfaces become a permissive environment for the accumulation of mutations that are only deleterious if exposed on the surface of a molecule. What remains, however, is an understanding of the evolutionary events involved in the transition from monomers to dimers (and vice versa), which ultimately requires a consideration of the underlying population-genetic processes.

Guided by knowledge on the physical features of interfaces noted above, we start with a relatively simple null model for homomers in which the architectural

features of a protein can be described as a linear series of alternative structural states, with each class having a finite probability (per unit of evolutionary time) of transitioning to an adjacent class by gaining or losing a binding residue (Figure 13.6). The alternative molecular phenotypes range from an extreme case of a pure monomer through a series of states in which the dimeric interface is increasingly stable owing to the additions of adhesive mutations.

As time proceeds, the molecular state will wander over the landscape of alternative phenotypes to a degree depending on the evolutionary transition rates. However, provided the transition possibilities are bidirectional, given enough time and constant rates, a quasi-steady-state distribution is expected to emerge, at which point the long-term average evolutionary rates of movement into and out of each class must be equal. This implies that transitions towards a more dimeric state occur at exactly the same rate as transitions towards a more monomeric state. Such a condition follows from the fact that more abundant states will have lower rates of export to adjacent states (per probability mass), whereas less abundant states will have higher rates of export, such that the products of probability mass and exchange rates per probability mass are equal in both directions between any two adjacent classes. This distribution holds for both the long-term history of a protein in a particular phylogenetic lineage or across independent lineages, given a constant set of conditions. It also applies to the states occupied by different proteins experiencing similar evolutionary forces at any particular point in time.

A useful feature of this model, developed in more general and technical form in Foundations 5.3, is that the equilibrium probability of being in any particular state is proportional to the product of all of the coefficients pointing towards the state in both the upward and downward directions. Equivalent to the product of the number of mutations arising in the population per generation and the product of the probability of fixation, these transition coefficients potentially depend on a multitude of factors jointly determining the relative directional powers of mutation and selection, combined with the stochastic force of random genetic drift.

The logical starting point is a neutral model in which each alternative state has equivalent fitness (i.e., all dimers operate with equal efficiencies as each other and as monomers). Here, we will assume a constant upward mutation rate between classes, i.e., $\mu_{i,i+1} = u$ for all i , as would be closely approximated if the number of potential surface residues that can mutate to adhesive states is large. Letting v be the mutational rate of loss of an adhesive residue, the downward mutation rate must increase linearly with the allelic state, i.e., $\mu_{i,i-1} = (i-1)v$, owing to the increase in the number of adhesive residues at the interface subject to loss with increasing i . As u is an aggregate mutation rate over a large number of sites, and v is a per-site mutation rate, we expect u/v to be generally > 1 , and perhaps greatly so.

The general approach immediately suggests two things. First, because each adjacent pair of states is separated by a pair of upward and downward coefficients, the steady-state distribution depends only on the ratios of pairs of mutation rates independent of their absolute values. Second, because such ratios decrease with increasing numbers of binding sites, the equilibrium frequencies of the higher states must eventually diminish toward zero, ensuring the existence of a quasi-steady-state probability distribution of the array of possible alleles.

Under neutrality, because the number of mutations occurring is a function of

the number of + alleles and the probability of fixation is inversely proportional to the initial frequency of a mutation (which is the inverse of the number of gene copies in the population), the transition rates are entirely independent of population size, and using the expression in Foundations 5.3, the equilibrium probability distribution simplifies to

$$\tilde{P}_i = \frac{e^{-\alpha} \alpha^{i-1}}{(i-1)!}, \quad (13.4)$$

where $\alpha = u/v$, which is equivalent to a Poisson probability distribution with parameter α . The probability of the extreme monomeric state is simply $\tilde{P}_1 = e^{-u/v}$, which for $u/v = 0.01, 0.1, 1.0$, and 10.0 , respectively, becomes $\tilde{P}_1 = 0.99, 0.90, 0.37$, and 0.000045 . Thus, even in the absence of adaptive differences among allelic states, the probability of a dimeric structure ($1 - \tilde{P}_1$) can be substantial. It also follows that for large u/v substantial variation in interface binding strength is expected among lineages (Figure 13.6), consistent with the observations noted above on variation for interfacial areas. Given the independence of these results of population size, assuming the mutation ratio u/v is reasonably constant, this neutral model suggests that the probability distributions of monomers and dimers should be independent of the phylogenetic lineage, i.e., approximately the same in bacteria as in multicellular eukaryotes, which is consistent with the observations in Figure 13.3.

What are the consequences of selection towards one extreme or the other? Because the probability of fixation depends on the effective population size when selection operates, we can anticipate a shift in the form of the distribution from the neutral expectation as the efficiency of selection increases with increasing N_e . The key modification that must be made is the weighting of the mutation pressure in each transition coefficient by the probability of fixation of the mutant allele, which is no longer the initial frequency.

Although there are numerous ways in which fitness might change over the phenotypic array displayed in Figure 13.6, for each pair of upward and downward coefficients, the ratio of fixation probabilities in the two directions is equal to $e^{4N_e s}$ assuming diploidy, where s is the relative fitness difference between the two adjacent states (s being positive from the perspective of the more beneficial allele). If one assumes weak positive selection in the direction of increasing i , such that the difference in selective advantage between adjacent states remains constant, Equation 13.4 still holds but with $\alpha = (u/v)e^{4N_e s}$, i.e., the Poisson distribution is maintained, except that the mutation-pressure ratio is weighted by the selection pressure ratio. If the prevailing selection pressure is in the direction of the monomeric state, then $\alpha = (u/v)e^{-4N_e s}$.

Thus, the Poisson parameter is now a composite function of the ratio of mutation pressure in the upward vs. downward direction (u/v), the selection differential between adjacent alleles (s), and the magnitude of random genetic drift ($1/2N_e$). The ratio of the power of selection to the power of drift, $s/(1/2N_e) = 2N_e s$ (for diploidy, and half that for haploidy), simply enters as an exponential pressure, pushing the system further in the favored direction than would be expected on the basis of mutation pressure alone. If the power of drift is substantially greater than that of selection, $1/N_e \gg s$, which implies $4N_e s < 1$, $e^{4N_e s} \simeq 1$, and the distribution of state frequencies will still closely approximate the neutral expectation.

There are a number of ways in which the mutational model used above might be

modified to endow further biological reality. For example, in the preceding discussion, the entire pool of mutations was treated as homogeneous with respect to their physical nature. However, initial transitions to a dimeric state may frequently be precipitated by a type of mutation (e.g., a major insertion or deletion; Hashimoto and Panchenko 2010) that is fundamentally different than those accruing later. In addition, once the protective environment of an interface has developed, a multimeric condition is likely to encourage the accumulation of secondary mutations that would otherwise be rejected by selection, e.g., substitutions causing hydrophobic or electrostatic interactions that further enhance interfacial binding strength but would be harmful if exposed on a protein's surface. This type of reinforcement scenario is qualitatively consistent with numerous studies showing that lab-induced point mutations that prevent interfacial binding usually retain some catalytic ability while also experiencing a substantial reduction in structural stability (Griffin and Gerrard 2010), presumably a result of exposure of conditionally deleterious interface mutations. It is also entirely consistent with the concept of interface entrenchment noted above.

In principle, all of these complications can be accommodated by modifying the preceding model to allow for mutational pathways to additional alternative states, although the motivation for such modifications will have to be informed by observations on the distribution of mutational effects and their influence on fitness. The consequences of more complex selection scenarios, including a bottleneck in fitness for the low- to mid-level dimerizing states, can be readily evaluated by a simple modification of the transition coefficients in Figure 13.6. One could argue that the selective advantages of additional binding residues will decline, owing to the nonlinear relationship between binding energy and the dissociation constant, but this will not greatly alter the conclusions outlined above, except that the long-term evolutionary distribution would no longer be exactly Poisson.

A final point of emphasis is that the model presented assumes a low mutation-rate regime, such that the waiting times between subsequent mutations are long enough that populations are almost always in a monomorphic state, which restricts transitions to adjacent states. In sufficiently large populations, however, mutant haplotypes may incur secondary mutations while segregating in the population, raising the possibility of vaulting to more distant states in a single haplotype-fixation event, a process that can be particularly significant when intermediate-state alleles are deleterious. The general principles of such stochastic-tunneling events, outlined in Chapter 5, are specifically applied to multimer evolutionary issues in Lynch (2012a).

The basic structure of this model has also been extended to allow for the secondary evolution of homotetramers from homodimers (Lynch 2013). In this regard, it is notable that dimers appear to often constitute the initial step in the evolution of higher-order multimers. For example, Levy et al. (2008) found that whenever a tetramer in some lineage has a shared evolutionary relationship with a dimer in another, the dimer interface is usually conserved in the tetramer. This suggests that tetramers typically evolve via an intermediate evolutionary step involving simple dimerization, with the dominant dimeric interface evolving first (Levy et al. 2008; Dayhoff et al. 2010), followed by the joining of a potentially weaker interface to form a complex of four. Although this argument appears logical for a dihedral tetramer,

it less obviously applies to a cyclical architecture, as in this case, all interfaces are identically heterologous. Moreover, given the steady-state distributional properties noted above, there is further need for care in interpretation here, as the sharing of interfaces between orthologous dimers and tetramers need not always reflect the emergence of the latter from the former, but may result from changes in the opposite direction. In any event, consistent with a stepwise evolutionary link between alternative levels of multimerization, the order of assembly events of higher-order multimers within cells appears to reflect the postulated order of evolutionary emergence of different interfaces, with the stronger interface typically being formed first (Marsh et al. 2013; Marsh and Teichmann 2015).

Regardless of the exact details of the model, two central points emerge from the preceding analyses, relevant not just to the issue of quaternary protein structure but to the distributions of all complex traits. First, substantial phenotypic variation can arise among lineages experiencing identical intensities of selection, mutation, and random genetic drift. Each of the curves in Figure 13.6 represents such a situation, raising the clear caveat of how risky it can be to pursue adaptive explanations for phenotypic divergence among lineages. For example, if the composite parameter $\alpha = 1$, the probability of being in the pure monomeric state is 0.37, and that of being in the remaining dimeric categories is 0.63. That is, a substantial amount of phenotypic diversity would exist among phylogenetic lineages despite being confronted with identical evolutionary pressures, and any attempt to explain these differences in terms of imagined lineage-specific selection pressures would be quite misplaced.

Second, the most common state is not necessarily the optimum. Even with negative selection against multimers, they will still be common provided the mutational bias towards binding affinity is sufficiently large. The mode of the distribution is entirely determined by the composite parameter α , and if $2N_e s \ll 1$, the prevailing molecular phenotype will be essentially defined by the mutation spectrum. Notably, mutation pressure in many phylogenetic lineages is biased toward the production of A/T nucleotides (Hershberg and Petrov 2010; Hildebrand et al. 2010; Lynch 2010), encouraging a bias toward more hydrophobic, and hence more adhesive, surface residues (with A/T-rich codons) (Knight et al. 2001; Bastolla et al. 2004; Hochberg et al. 2020). This highlights the risk of making the usual assumption that the most common phenotype reflects the optimal state.

In summary, the preceding analyses suggest that the substantial phylogenetic variation that exists in the multimeric states of proteins is not necessarily a consequence of idiosyncrasies in modes of selection in different lineages. Rather, it is an expected outcome of the stochastic evolutionary dynamics that arise in finite populations when the combined pressures of mutation and selection are not overwhelmingly large in one direction. If this hypothesis is correct, and one had the capacity to sample a single evolutionary lineage over a very long period of time, orthologous proteins in different phylogenetic lineages would be observed to occupy various multimeric states in frequencies reflecting the underlying transition probabilities.

Although we do not have the luxury of making such observations directly, for reasons discussed above, provided enough evolutionary time has elapsed for the Tree of Life to have reached the steady-state distribution, a corollary can be tested – the number of transitions from a monomeric to a dimeric state on the branches

of a phylogeny should equal that in the opposite direction, and the same symmetry should hold for dimer-tetramer transitions, etc. Unfortunately, owing to the huge imbalance in the taxa and proteins with existing structural data, such a test cannot yet be made, and if such efforts are to be pursued, it must be kept in mind that the phylogenetic depth for sampling must be substantially greater than the expected transition times between alternative states. For example, if the likely transition rate between states is on the order of 10^{-8} per year, a focus on a lineage that diverged more recently 10^8 years ago would be uninformative.

The domain-swapping model. Although the preceding section focused on a fairly general model for evolution along a gradient of adjacent allelic classes, the mechanisms underlying transitions between adjacent states need not always be as gradual as implied above, and the consecutive steps need not all be unidirectional with respect to fitness effects. One specific and frequently invoked model for the origin of homodimers is encapsulated in the domain-swapping model (Bennett et al. 1994; Kuriyan and Eisenberg 2007), whereby a monomeric protein with two interfacing domains (within the same polypeptide chain) is physically altered in such a way that intramolecular binding is no longer possible (e.g., by a major deletion in the linker between the two binding domains that prevents them from meeting). In principle, such a modification can be compensated by reciprocal domain swapping between monomeric subunits, the result being a dimer with two heterologous interfaces (Figure 13.7).

An attractive feature of this model is that the well-endowed binding domains already present in the ancestral protein do not have to go through a phase of incremental improvement. In addition, depending on the configuration of mutant monomers, the process envisioned is not restricted to the origin of dimers, but extends to the establishment of higher-order multimers as well (Ogihara et al. 2001). Finally, it is plausible that the process is bidirectional, with insertion mutations in the linker sequence between domain-swapping dimers sometimes causing reversion to the monomeric condition.

Although there are plenty of convincing examples of domain-swapping proteins (Liu and Eisenberg 2002), the conditions required for such evolution are sensitive to the population-genetic environment. Here, we consider the simplest case in which an allele for the domain-swapping protein arises by a single deletion mutation that denies self-accessibility within the ancestral monomer. If the dimer is beneficial, such a mutant allele can readily proceed to fixation by positive selection in a haploid species. However, in a diploid outcrossing species, the mutant allele will initially be present exclusively in heterozygotes, raising potential challenges for establishment. Most notably, if heterozygote fitness is compromised by the production of malfunctioning composites of the two alternative monomeric subunits, e.g., chimeras between proteins with and without a deletion, there will be a barrier to the spread of the mutant allele unless it can somehow rise to a high enough frequency that beneficial homozygotes become likely. Presumably, the magnitude of any heterozygote disadvantage will depend on the rate of folding of the ancestral monomer and the overall cellular concentration of both allelic products, as slow folding and/or high concentration should magnify the likelihood of chimeric assemblies. Concern with the fitness consequences of the heterozygous state is further motivated by the obser-

vation that even normally well-behaved domain-swapping dimers can concatenate into harmful fibrils in some cellular environments.

The mathematical details relevant to this domain-swapping model in diploid populations have been worked out (Lynch 2012a), but here we simply provide a heuristic guide to the most salient issue – the low likelihood of an evolutionary sojourn through a bottleneck in mean population fitness caused by heterozygote disadvantage unless the latter is overwhelmed by the power of random genetic drift. Of special interest is the critical effective population size (N_e^*) beyond which the efficiency of selection is so strong that there is effectively no possibility of making a transition to a domain-swapping allele.

With heterozygotes having a fractional fitness reduction of δ , homozygotes an advantage of s , and p being the frequency of the domain-swapping allele **a** in the population, under the assumption of random mating, mean population fitness reaches a minimum at $\hat{p} = \delta/(s + 2\delta)$ (Figure 13.8). When $p < \hat{p}$, there is net selection against the domain-swapping allele, but when $p > \hat{p}$, there is net selection in favor of allele **a**. Thus, because the initial frequency of a novel domain-swapping allele is very low (on the order of the reciprocal of twice the absolute population size), the key issue is whether a mutant allele can drift against a gradient of negative selection up to frequency $\hat{p}$. When rare, **a** will be essentially exclusively found in heterozygotes, and will therefore act like a deleterious mutation being removed from the population at rate δ .

The population-size barrier to the establishment of the domain-swapping protein is

$$N_e^* \simeq \frac{s + 2\delta}{\delta^2}. \quad (13.5)$$

which reduces to $N^* \simeq 2/\delta$ if there is no selective advantage of the domain-swapping homozygote. For example, if the deleterious effect in heterozygotes is just 0.002, unless the effective population size is smaller than 1000, there is essentially no chance of establishment of an otherwise neutral domain-swapping allele. Even if the domain-swapping allele had a 1% advantage ($s = 0.01$), the barrier is still a very small $N_e^* = 3500$. Thus, a small heterozygote disadvantage is a very strong impediment to the establishment of an allele that is advantageous when fixed.

To sum up, under the domain-swapping model, a transition from a monomeric to a dimeric state is most plausible under two sets of conditions: 1) a haploid population, in which heterozygote disadvantage is never experienced; or 2) a diploid population in which selection against heterozygotes is inefficient, either because the effective population size is small (which allows selection to be overcome by drift) or because the reduction in heterozygote fitness is negligible. Unfortunately, although a knowledge of the fitness consequences of mixtures of the products of ancestral and derived alleles is essential to resolving how readily domain-swapping can evolve in diploid populations, there appear to be no data on this key issue or even on whether domain-swapping dimers confer greater or lesser fitness than monomers.

Notably, the theory presented above is entirely general in that a simple change in definition of terms is all that is required for considering the reverse transition of homodimer to monomer, a scenario that certainly cannot be ruled out on the basis of existing data. Indeed, for the simplest case in which there is no heterozygote disadvantage, if u is the rate of mutation to dimers and v is the reverse mutation rate,

and s_d is the selective advantage of dimers (negative if dimers are disadvantageous), the steady-state probability of being in the dimeric state is

$$\tilde{P} = \frac{\alpha}{1 + \alpha}, \quad (13.6)$$

where $\alpha = (u/v)e^{4N_e s_d}$, and $1 - \tilde{P}$ is the probability of monomers. Note that this formula follows directly from the theory discussed in the preceding section, being the special case in which there are just two possible states (Foundations 5.3).

Heteromers from homomers. Although heteromers can, in principle, arise from promiscuous interactions among nonorthologous proteins, most seem to originate from interactions between paralogs arising from gene duplication, and this will be our final focus. Duplication might occur first in an ancestral gene with no intrinsic tendency to form dimers, with secondary complementary mutations resulting in complexation of the paralogous products. However, an alternative, and perhaps more likely, scenario involves the situation in which an ancestral gene already engages in homodimerization and therefore has a well-established interface at the outset.

The initial steps in developing a heteromeric interface would then require the accumulation of unique interface mutations in both paralogs that encourage heterodimerization while discouraging homodimer formation. On the one hand, such a process might be facilitated if there were no intrinsic benefit to dimerizing, as this would eliminate any negative consequences of relinquishing homodimerization. On the other hand, without some form of reinforcement by selection, the long-term maintenance of a heterodimer would also be evolutionarily unstable owing to the fact that each locus would be subject to loss by degenerative mutations without the intervention of a preservational mechanism. The population-genetic details associated with such a scenario remain to be worked out.

There is compelling evidence for the origin of heteromeric complexes via this pathway. For example, Mcm1 is a MADS-box transcriptional regulator that operates as a homodimer in many fungal species. Following duplication in *S. cerevisiae*, the paralogous copies acquired complementary mutations that cause heterodimer assembly; ancestral homodimeric constructs are fully functional in *S. cerevisiae*, but the loss of either duplicate is lethal (Baker et al. 2013). Experimental work involving historical reconstructions suggests that hetero-tetrameric hemoglobin, deployed in a number of metazoan species, evolved from a homodimer, with the transition following gene duplication and just two amino-acid substitutions sufficient to confer a new binding interface (Pillai et al. 2020). A somewhat more complex scenario involves the vacuolar ATPase proton pump, which contains a hetero-hexameric ring composed of two components in most species, but three in fungi, where the additional participant is a paralog of one of the pre-existing components (Finnigan et al. 2012). Pereira-Leal et al. (2007) found that following whole-genome duplication in yeast, many homomeric complexes made a transition to heteromeric states of the same order. Presumably, the spatial proximity of the monomeric units at the subcellular level helps pave the way for such evolution, as it helps ensure that the participants will be interacting on a regular basis. Many other examples of homomer-to-heteromer transitions in eukaryotes, covered in individual chapters, are summarized in Chapter 24.

This being said, however, it must be pointed out that duplication of a homomerizing gene product need not always lead a heteromer. For example, by inserting different sequence tags into various gene copies, Billerback et al. (2013) created subunit variants of the normally homomeric barrel-shaped, bacterial chaperone, GroEL (Chapter 14), and found that instead of assembling as heteromeric complexes, the resultant assemblies were individualized homomers. Moreover, Hochberg et al. (2018) found that following gene duplication most homomers actually evolve to avoid the construction of heteromeric complexes, apparently becoming preserved by the evolution of distinct functions, either by subfunctionalization or neofunctionalization of different homomeric complexes. On the other hand, the individualization of duplicated homomers may be slowed by the fact that mutations discouraging the production of complexes between the products of duplicate genes may have parallel negative effects on the capacity for self-assembly (Marchant et al. 2019).

There are established mechanisms by which reinforcement might occur. Supposing, for example, that each monomeric subunit from the ancestral gene had multiple, independently mutable subfunctions, then gene duplication followed by complementary degenerative mutations (the process of subfunctionalization) would open up the possibility of joint preservation of both paralogs. Under this model, the evolved heterodimer would carry out the combined functions of the ancestral gene, one on each subunit. Alternatively, if for other structural reasons the heterodimer outperformed the homodimeric products of each individual locus, this could lead to positive selection for heterodimeric complexation.

Notably, the latter scenario need not always await the arrival of new mutations affecting function (Lynch 2012a). Consider, for example, the situation in which an ancestral locus encoding a homodimer harbors two alleles, such that the cross-product dimer created within heterozygotes elevates fitness beyond that for either of the two pure types produced in homozygotes. Prior to duplication, heterozygous cells would be expected to produce three types of dimers (e.g., **AA**, **Aa**, and **aa**) in a binomial 1:2:1 ratio. Letting the fitnesses of the three genotypes at the ancestral locus be $1 - s_1$, 1, and $1 - s_2$, respectively, with $0 < s_1, s_2 < 1$, the two alleles will have been maintained in the ancestral (pre-duplication) population by balancing selection, with frequencies $s_1/(s_1 + s_2)$ and $s_2/(s_1 + s_2)$. Because the **Aa** individuals with highest fitness always segregate equal numbers of both alleles into the next generation, there is no possibility of a pure hetero-allelic state.

Gene duplication alters this situation by providing the opportunity for each locus to fix the alternative allele, with each descendent locus becoming fixed for a different ancestral allele (Lynch et al. 2001). Once this point has been reached, then every member of the population would have the expression pattern found in the ancestral heterozygote (Figure 13.9) – fitter than the average member of the ancestral population, but in the early stages with every individual still producing the three dimeric types in a 1:2:1 ratio, provided the strength of selection exceeds that of drift. However, following the establishment of this complementing duplication state, subsequent mutational modifications involving the interfaces of one or both loci would be expected to lead to further refinements facilitating heterodimerization, eventually to the point at which homodimer assembly no longer takes place. This particular model is, of course, irrelevant for haploid species, which cannot harbor ancestral heterozygosity.

Summary

- At least 60% of proteins assemble into multimeric higher-order structures, with homomers being about twice as frequent as heteromers. Dimers are more common than tetramers, which are more common than hexamers, and so on, with odd-mers being under-represented relative to even-mers.
- These distributions are very similar across all phylogenetic groups of prokaryotes and eukaryotes, indicating that there is no gradient of molecular complexity (number of subunits) with organismal complexity. Moreover, orthologous proteins often have different numbers of subunits in different phylogenetic groups, and even when the level of multimerization is conserved, it is not uncommon for different taxa to utilize different binding interfaces between monomeric subunits.
- Proteins have a tendency to be naturally self-adhesive, leading to a situation in which monomeric proteins are often just one or two amino-acid substitutions away from switching to a dimeric state or to the production of harmful open-ended fibrils.
- Individual binding interfaces of multimers are relatively simple – typically, < 20% of the total surface area, involving fewer than ten key residues, and having binding strengths generally in the range of 15 to 24× the level of background thermal energy.
- The binding interfaces of multimers typically have excess capacity in the form of a subpopulation of nonadhesive residues. This enables the specific binding sites of any particular lineage to wander in an effectively neutral fashion over evolutionary time, which in turn can lead to incompatibilities between subunits from divergent taxa.
- Although numerous adaptive explanations have been proposed for the widespread use of multimers, other than for the cases in which a new catalytic function is conferred by the interface or in which a functional cage or fiber is produced, there is very little direct evidence that multimeric proteins are selectively advantageous. Moreover, any proposed advantages must be weighed against several costs of relying on multimers, such as the engagement in promiscuous binding and the necessity of producing elevated numbers of monomers.
- Because of mutation bias towards adhesive amino-acid residues, there is an innate tendency for monomeric proteins to move in the direction of becoming homomers, and this can gradually lead to a situation in which such complexes appear to be entrenched by reinforcing binding sites, even though the process need not have

been driven by selection. As a consequence of such directionality and stochastic gains and losses, there can be an expected broad distribution of molecular phenotypes in different lineages exposed to identical processes of selection, mutation, and drift. Moreover, long-term mean phenotypes can deviate significantly from the optimum.

- A celebrated mode of origin of homodimers is domain-swapping, wherein a monomer containing two internal binding domains incurs a deletion in the linker that prevents self-binding and encourages assembly into dimers with two heterologous binding interfaces. The alternative route, in which an insertion in a linker in the latter encourages self-binding, is also possible. A major challenge of such transitions in diploid species is the possibility of production of harmful chimeric complexes between the two allelic products in heterozygous individuals, which imposes a barrier in mean population fitness that can only be overcome by a sufficiently small population size to enable drift across the fitness valley.
- Transitions from homomeric structures to heteromers (as well as reversions) are commonly observed, although more so in eukaryotes, and usually emerge following gene duplication with the sister genes then becoming specialized binding partners. Such transitions may be incited when there is an ancestral polymorphism at the locus, with the heterozygote having superior fitness, but fixation of the hetero-complex being impossible until duplicated loci each adopt a particular allelic type.

Foundations 13.1. Association / dissociation equilibria. To understand a variety of issues with respect to reaction dynamics and equilibria, knowledge of a few basic features of molecular thermodynamics is required. Consider the interaction between two molecules A and B, with the potential to join together to form a noncovalent complex AB, e.g., a dimer. Regardless of the starting concentrations, nearly all such systems will reach an equilibrium containing fixed relative concentrations of A, B, and AB. In this particular chapter, the focus is often on the special case in which $A = B$, i.e., the two molecules are of the same type, but the more general solution is given here. There are two ways of obtaining the equilibrium solution.

The first approach takes a macroscopic view of the problem, using only information on the concentrations of the system components and their rates of interchange. Letting k_{on} be the association rate of A and B to form AB, and k_{off} be the reciprocal dissociation rate of AB to A and B, at equilibrium the rate of formation of AB must equal its rate of dissociation,

$$k_{\text{on}}[A][B] = k_{\text{off}}[AB], \quad (13.1.1)$$

where the quantities in brackets denote equilibrium concentrations. This general relationship is known as the law of mass action. The dissociation constant K_D (not to be confused with the dissociation *rate* k_{off}) is the ratio of the reverse and forward rates, which in turn relates to the ratio of reactant molecules under equilibrium conditions. Rearranging Equation 13.1.1,

$$K_D = \frac{k_{\text{off}}}{k_{\text{on}}} = \frac{[A][B]}{[AB]}, \quad (13.1.2)$$

defined at 1 M total concentrations of A and B. Although the underlying on/off rates, k_{on} and k_{off} , will not be considered further here, it should be emphasized that these are dictated by the structural features of the molecular participants, which determine the rates of encounter and efficiency of binding (critical to k_{on}) and degree of complex stability (critical to k_{off}) (Kastritis and Bonvin 2012).

Letting p_C denote the fraction of A molecules that are in complex, and rearranging and substituting from Equation 13.1.1, at equilibrium,

$$p_C = \frac{[AB]}{[A] + [AB]} = \frac{[B]}{([A] \cdot [B]/[AB]) + [B]} = \frac{[B]}{K_D + [B]}. \quad (13.1.3a)$$

This expression shows that K_D is equivalent to the equilibrium concentration of B at which half of the A molecules are in complex with B. If $A = B$, then

$$p_C = \frac{2[AA]}{[A] + 2[AA]} = \frac{2}{([A]/[AA]) + 2} = \frac{2[A]}{K_D + 2[A]} \quad (13.1.3b)$$

because there are two A molecules within each complex.

The second approach takes a more detailed, thermodynamical view of the alternative states of the system. The key here is that from the perspective of a single A molecule, there are a number of potential local microstates (the set of all possible configurations of the entire population of A and B molecules) involving the states of all B molecules in the system (some of which include a B molecule in complex with A). A classical result from the field of statistical mechanics is that the probability of a particular microstate i of a molecular system is proportional to the function $e^{E_i/(K_B T)}$, where E_i is the energy associated with the state (a more positive number implying

a more energetically favorable state), K_B is the Boltzmann constant, and T is the temperature in degrees Kelvin. Microstates that are more thermodynamically stable have more positive values of E , and at the molecular level, this needs to be expressed relative to the background energy related to thermal motion of the solvent molecules ($k_B T$).

Consider the situation in which there are N molecules of B for each molecule of A, with a B molecule having energy E_{on} when bound to A and E_{off} when free. Focusing on a specific molecule of A, the total energy of the local system will then be NE_{off} if A is unbound, and $E_{\text{on}} + (N-1)E_{\text{off}}$ if a particular A molecule is bound to a single B. The one significant remaining issue is the number of ways in which this one particular A molecule can be bound with various alternative molecules of B, relative to the number of microstates in which none of the local B molecules is bound. This turns out to be simply equal to the concentration of B (see Phillips et al. 2013, p. 237-244, for an explicit derivation). To account for this effect, the probability of an individual AB association must be multiplied by $[B]$, whereas the weighting factor for the situation in which A is unbound is just 1. Thus, an alternative way of expressing the probability that a molecule of A is complexed with B is

$$p_C = \frac{[B]e^{[E_{\text{on}} + (N-1)E_{\text{off}}]/(RT)}}{e^{NE_{\text{off}}/(RT)} + [B]e^{[E_{\text{on}} + (N-1)E_{\text{off}}]/(RT)}} \quad (13.1.4a)$$

$$= \frac{[B]e^{\Delta E/(RT)}}{1 + [B]e^{\Delta E/(RT)}} \quad (13.1.4b)$$

where $\Delta E = E_{\text{on}} - E_{\text{off}}$.

Note that there has been a change in notation here. The usual convention is to express molecular concentrations as well as energies associated with them in terms of molar quantities (mol/liter), and so K_B has been scaled up to its molar equivalent R , which is simply K_B times Avogadro's constant (6.022×10^{23} molecules / mol). With R being equal to $1.987 \text{ cal} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$, at standard temperature 25°C (equivalent to 298 K), $RT \simeq 0.6 \text{ kcal/mol}$ (an approximate constant that we will adhere to throughout, as even a 25°C change in temperature alters RT by $< 10\%$). For Equation 13.1.4b to work properly, ΔE must also have units of kcal/mol, and $[B]$ must be the molar concentration of B.

Comparing Equations 13.1.3 and 13.1.4b shows that

$$K_D = e^{-\Delta E/(RT)} \quad (13.1.5)$$

provides an alternative definition of the dissociation constant in thermodynamic terms. The binding-energy differential ΔE will be positive for a pair of molecules with an energetically favorable interaction, so that with increasing affinity, $K_D \rightarrow 0$ and $p_C \rightarrow 1$. In the absence of affinity between A and B ($\Delta E = 0$), $K_D = 1$, implying that at equilibrium $[A][B] = [AB]$.

The approach leading to Equation 13.1.4b is a general one that can be used to estimate the frequency of alternative states in any localized molecular system at equilibrium. In this particular case, there are only two alternatives for any particular molecule, so the solution is relatively simple, but with multiple reactants, the book-keeping for alternative, combinatorial states becomes increasingly complex. The sum of terms in the denominator of Equation 13.1.4a is known as the partition function, as it insures that the probabilities of all possible microstates sums to 1.0. The overall set of probabilities for alternative states is generally referred to as the Boltzmann distribution. In this particular example, there are just two alternatives, A being bound to B with probability p_C , and A being unbound with probability $(1 - p_C)$, the latter being equivalent to Equation 13.1.4b, but with a 1 for the numerator.

Note that Equation 13.1.4b for the probability of one of two particular molecular states in a population of molecules is identical in form to Equation 13.6, which

expresses the probability of one particular allelic state in a population (fully derived in Foundations 5.3). There is, thus, a remarkable convergence in the form of these and evolutionary-genetic equations, with the prefix terms ($[B]$ and u/v , respectively) being measures of the intrinsic pressure towards the state (owing to molecular concentration and mutation bias, respectively), and the exponential terms denoting the added pressure associated with energetic favorabilities and selective advantages.

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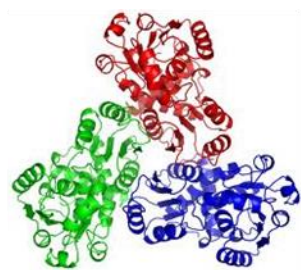
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Figure 13.1. Some examples of the varied forms of multimeric complexes. In each case, the subunits are presented in different colors, although each subunit might be encoded by the same genetic locus (homomers).



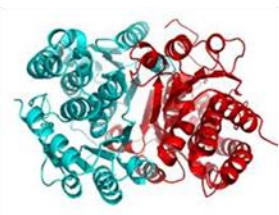
monomer



trimer



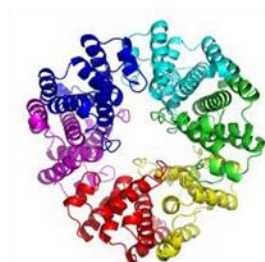
pentamer



dimer



tetramer



hexamer

Figure 13.2. Structures of monomers, dimers, and tetramers for the case of homomeric proteins. Blue-blue and red-red denote isologous interfaces, and blue-red heterologous interfaces. For a closed dimer, with one isologous interface, there is only one possible topology. A dimer with a heterologous interface (top) is subject to the development of open fibrils, unless it is geometrically configured to form a closed loop, in which case an odd-mer would have to be produced (as an even-mer would necessitate one interface being isologous). With two interfaces, a tetramer can take on two possible topologies: cyclical when heterologous interfaces attract, and dihedral when isologous interfaces attract. In the latter case, the tetramer can be viewed as a dimer of dimers.

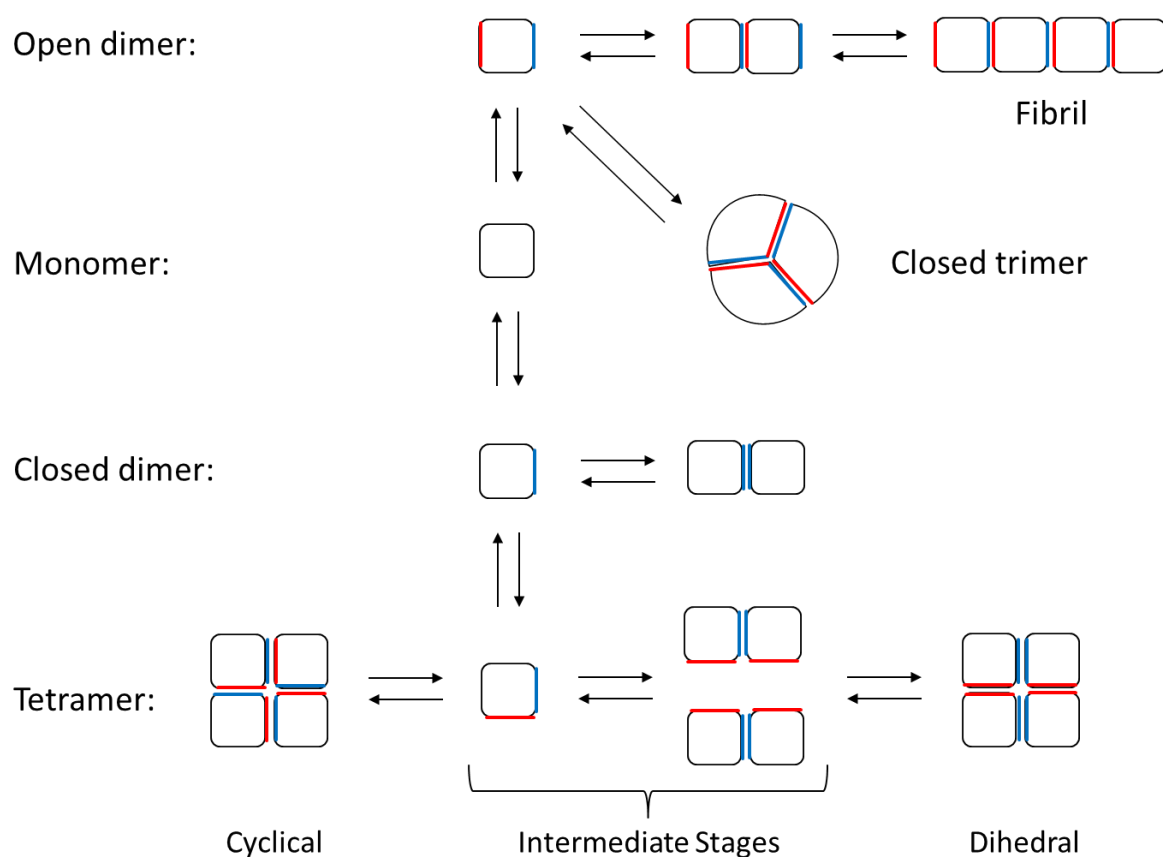


Figure 13.3. **Left)** Relative frequencies of the three main classes of protein structures in three phylogenetically broad groups. **Right)** Frequency distributions of levels of multimeric complexity (including monomers) for homomers for six major taxonomic groups; although the data are more limited, the distributions are similar for heteromers. The data are drawn from a diversity of proteins that have been structurally characterized in a broad range of organisms, but are by no means balanced in coverage (from Lynch 2012a).

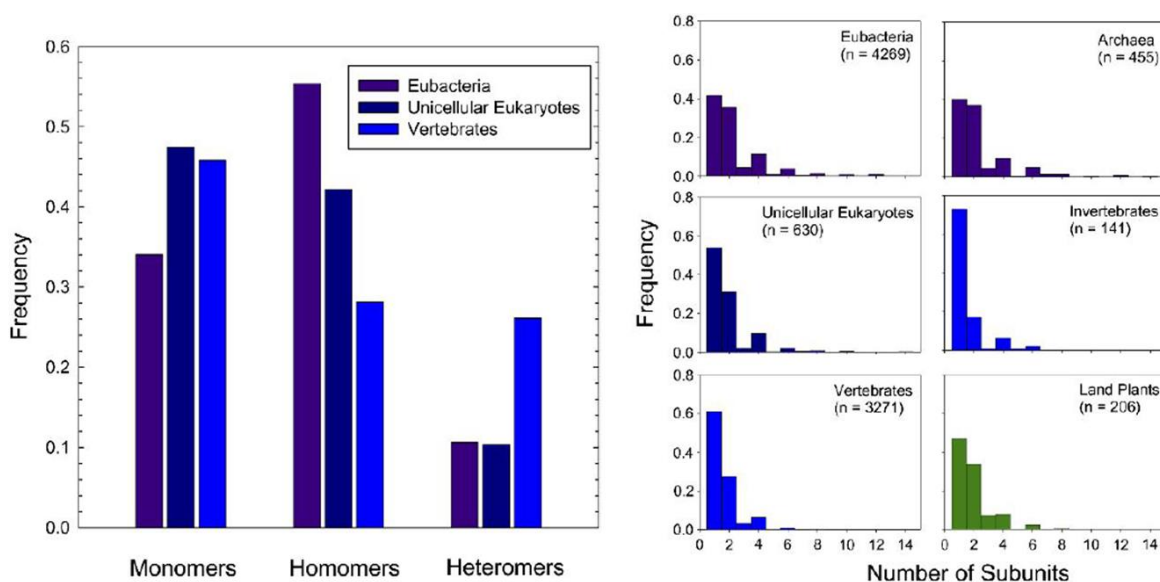


Figure 13.4. Structural topologies of heteromers involving two subunits per repeat (one bounded by black solid lines and the other by dashed lines). All interfaces in the structures shown here are heterologous, except the green-green pair for the tetramer on the left.

Heterodimer:



Tetramer
(dimer of dimers):



Hexamer
(trimer of dimers):

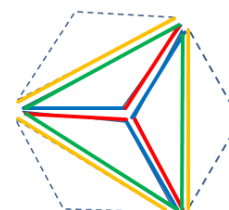
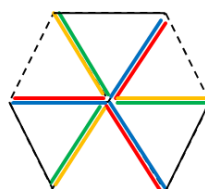


Figure 13.5. Binding strengths associated with interfaces of heterodimeric complexes. **Left)** The weak relationship between binding energy of a complex and the area of the interface. Solid circles (Kastritis et al. 2011); open circles (Horton and Lewis 1992). The dashed line denotes an approximate upper limit to the binding energy of $\sim 0.012 \text{ kcal}\cdot\text{mol}^{-1}\cdot\text{\AA}^{-2}$, where $\text{\AA}$ denotes Angstroms. **Right)** An approximately normal distribution of interface binding strengths, with mean = $18.3RT$ and SD = $4.8RT$. Data are extracted from a wide variety of sources (summarized in Kastritis et al. 2011).

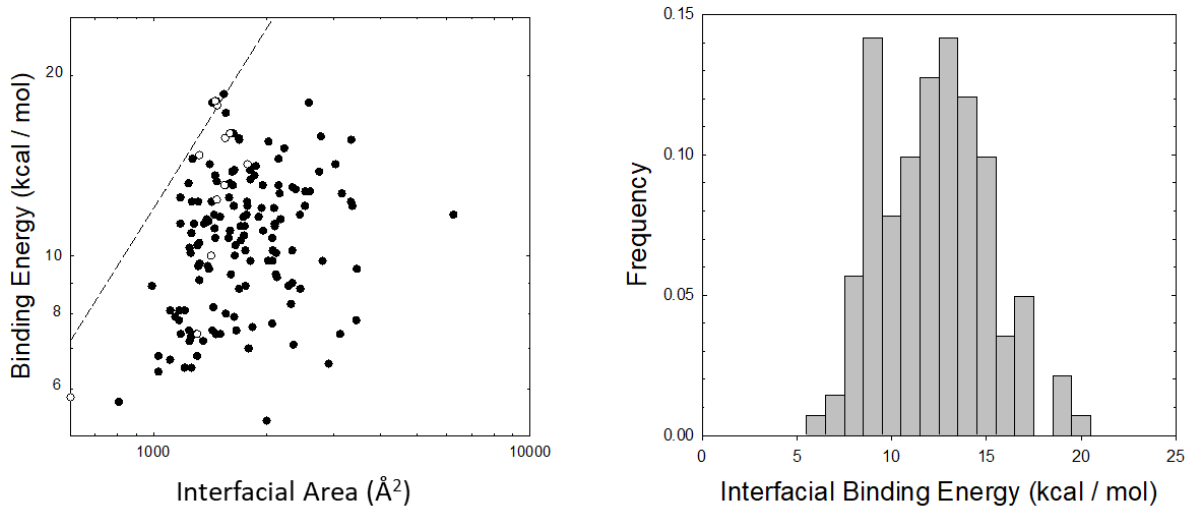


Figure 13.6. Effectively neutral evolution of new binding configurations at an interface patch. There are 25 potential binding residues in the patch, but at any point in time here, there are only six adhesive residues (in red). Over time, the specific residues involved in binding are free to wander over the surface, so long as six remain actively engaged. In principle, the binding number could wander above six by mutation and drift, although here it is assumed that six are sufficient to confer maximum fitness.

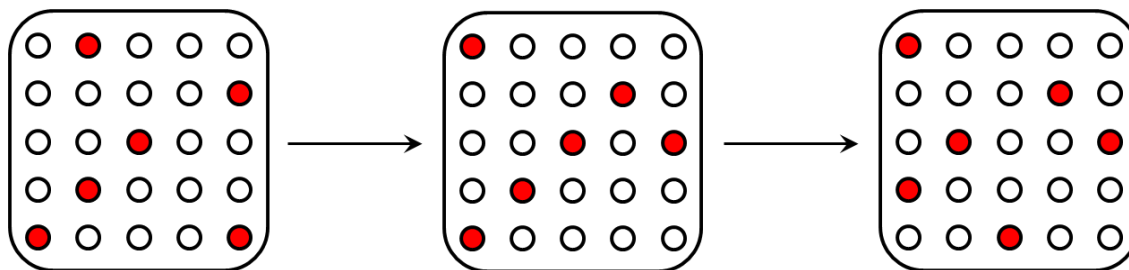


Figure 13.7. An idealized model for evolution along a linear array of alternative quaternary states of a protein. Here, the states describe a series of molecular variants with increasing numbers of binding sites at the interface, so that the state on the left is essentially a pure monomer, and moving to the right, the states have progressively stronger binding sites. The coefficients m_{ij} are evolutionary rates of transition from state i to state j , the values of which will depend on the power of mutation, random genetic drift, and selection, which together govern the composite parameter α in Equation 13.4. In effect, α is a measure of the net pressure from mutation and selection in the upward direction. The lower panel gives the equilibrium probability distributions of i for different values of α for the model described in the text.

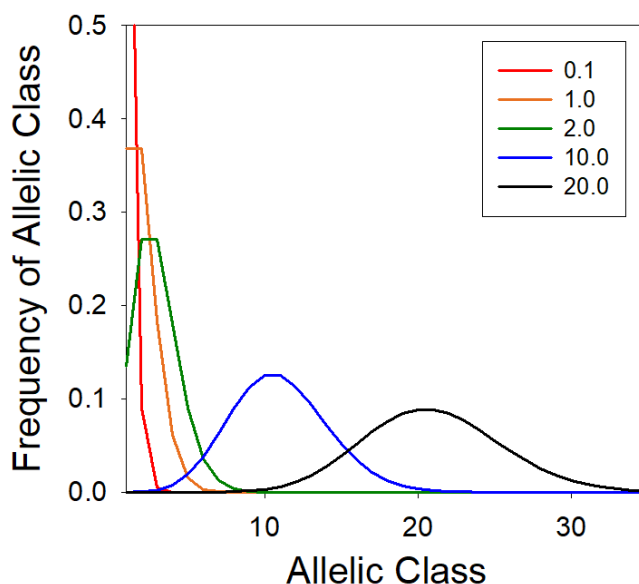
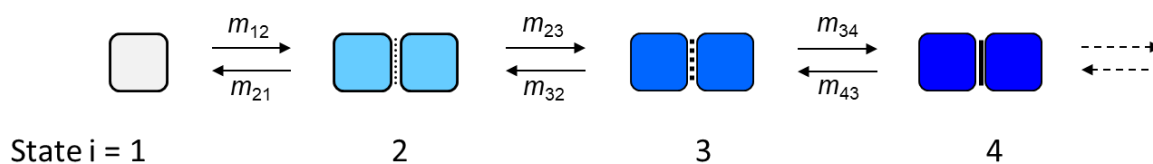


Figure 13.8. The domain-swapping model in a diploid population. **Left)** Relative to the ancestral monomeric type, the domain-swapping allele has a fitness deficit of δ in the heterozygous state, but advantage s in the homozygous state ($s = 0$ meaning that the alternative fixed states are equivalent in fitness). **Right)** Such a scenario results in a fitness surface (as a function of the frequency of the domain-swapping allele) with a valley at an intermediate frequency, owing to the fact that low-frequency alleles are present almost exclusively in deleterious Aa heterozygotes. If the frequency of allele a is to the left of the bottom of the valley, the prevailing pressure of selection is to remove the allele from the population, whereas to the right of the valley, selection promotes allele a, potentially driving it to fixation.

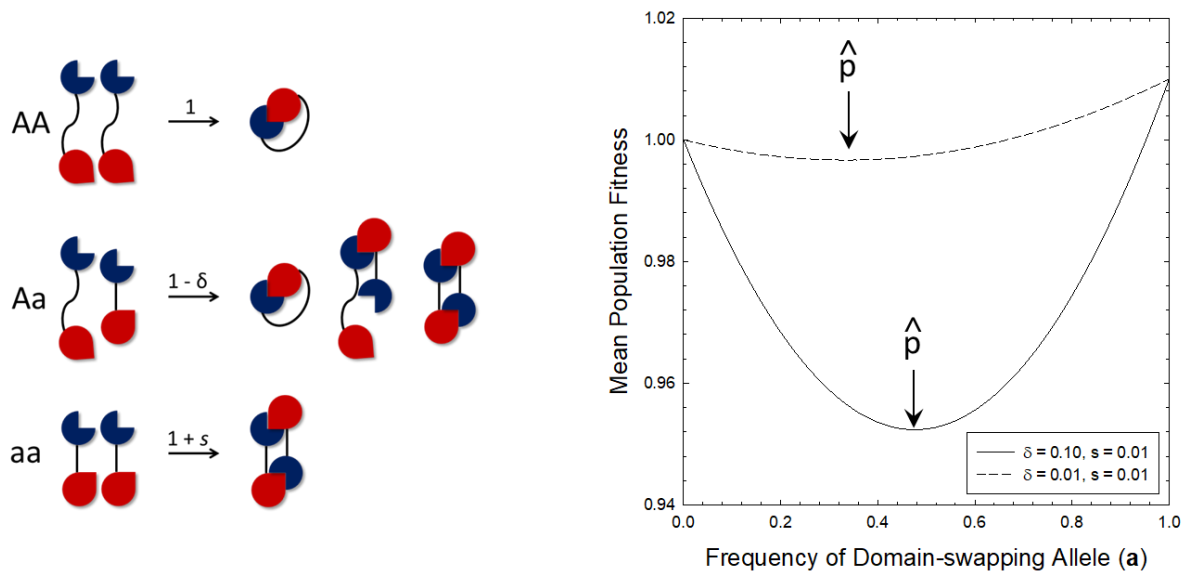


Figure 13.9. A potential path to the evolution of heterodimers from a homodimeric state following gene duplication at a heterozygous locus. Solid circles represent individual proteins derived from alternative alleles, which together make dimers consisting of A (blue) and/or a (red) subunits; ancestral heterozygotes (prior to duplication) produce three types of dimers in a 1:2:1 ratio, assuming random assembly. Following gene duplication, each locus becomes fixed for an alternative allele, and the encoded products at each locus subsequently diverge to the point that self-assembly is avoided, leading to ‘fixed heterozygosity.’

