The spectrum of biomolecular states and motions

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The universe of conformational substates of a protein molecule is huge. The complete energy landscape of proteins is, therefore, complex when studied at low temperature. Many experiments under physiological conditions commonly reveal a simpler spectrum of states. These states are individually ensembles of low temperature substates. That is, room temperature experiments probe the low free energy part of the spectrum of excitations. This paper describes how the complete landscape and the spectrum of these thermally excited motions can be related to each other. On funneled landscapes, partially folded ensembles of states are the most important excited states. Their properties and their free energy spectrum can often be predicted by native topology based models. Frustration, i.e., the conflict between inconsistent stabilizing interactions that have evolved for other purposes than optimizing folding, offers another mechanism for forming low free energy excitations. Frustration can be localized and quantified using energy landscape theory. Symmetry provides an obvious route to low free energy states in oligomeric systems, where simply repositioning parts of the molecule in ways quasi-equivalent to their relation in the native structure gives nearly degenerate energies. [DOI: 10.2976/1.3003931]

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Frauenfelder, whose cryochemical experiments long ago revealed the complexity of protein energy landscapes (Austin *et al.*, 1975), has often emphasized the analogy between the study of biomolecular physics during the last few decades and atomic physics a century earlier. In the atomic physics of the Edwardian era, a range of spectroscopic experiments showed that atoms indeed had moving parts and that patterns existed in the spectra that were characteristic of families of elements. Bohr, Sommerfeld, and others brought some mathematical order to this pattern on the basis of what we now call "old" quantum theory but a complete understanding awaited the birth of quantum mechanics proper with its still heavier mathematical apparatus (Sommerfeld and Bopp, 1951). In biomolecular physics, the fact that proteins and nucleic acids have moving parts is now completely accepted. The facts are indeed becoming clear to everyone, just as in early atomic science. Many feel, in some sense, that molecular dynamics simulations already provide a reliable theory, analogous to mature quantum mechanics, that can answer all conceivable questions about biomolecular motions.

While this assessment of the power of molecular dynamics is true in principle, the oracular nature of direct simulations is, in practice, challenged by the time scales involved. Motions of direct physiological significance often occur on a millisecond time scale or longer. This time scale still is a bit beyond what can be routinely done, but should soon be accessible. Is it therefore reasonable to say we have, or should have, skipped the "old quantum theory" stage for biomolecules? In this paper, I suggest the answer to both forms of the question is "No!" Like the development of the old quantum theory the interplay of theory and experiment has already led to approximate models

and simple but quantitative pictures that allow us to understand the patterns of the slow motions of proteins, what might be called the lower lying parts of the biomolecular free energy spectrum. These patterns reflect the topology, symmetry, and frustration of biomolecules. The understanding of the patterns of biomolecular motion that emerges from present day energy landscape theory is far from rigorous but is sufficiently powerful to guide both new experiments and future detailed molecular dynamics calculations as our computational abilities continue to increase.

THE FREE ENERGY SPECTRUM OF BIOMOLECULAR MOTIONS VERSUS THE ENERGY LANDSCAPE

The key to Frauenfelder's discoveries was low temperature. Unfortunately, today still very few biochemical experiments are done under cryogenic conditions and certainly no physiology takes place there. Yet, the cryochemical experiments established the existence of an energy landscape. This realization provides the basis for understanding how motions occur even at physiological temperatures. Low temperature has two important features that facilitate understanding biomolecular motions. First, low temperature slows down all motions (apart from vibrations). More important, crucially, the unavailability of activation energy at low temperature spreads out the range of times involved. Just as separating light emissions with a spectroscope or purifying an enzyme from a raw biological extract allows the properties of each single component to be ascertained, individual motions (or groups of motions) by occurring on wider ranges of time scales can now be separately studied when studied at low temperatures. Second, the Third Law of Thermodynamics implies that the configurational entropy of a system (i.e., its large multiplicity of states) can be neglected in determining the rates of the elementary events occurring at the lowest temperatures. In fact, glass transitions of the solvent-protein complex always prevent such a severe simplification from being completely accurate but nevertheless the entropic contributions to ensemble properties are minimized by studying motions cryogenically and this helps the purely energetic components of biomolecular transformation to come better into focus. These two features of low temperature phenomena have allowed biocryochemical studies to be pretty well mapped onto an energy landscape (Frauenfelder *et al.*, 1991). The upshot of the cryochemical experiments is that the complete energy landscape of a protein is indeed complex. A simplifying feature, however, emerges: the studies show that while biomolecular motions cover a very wide ranges of time scales in the low temperature regime most but not all these time scales tend to merge at higher physiological temperatures (Frauenfelder and McMahon, 1998). The residual slow motions that we think of as playing a role in physiology at higher temperatures therefore are not generally transitions between specific substates conceived as completely atomically defined configurations as in the caricatures provided by x-ray crystallography but involve ensembles of many substates, diverse in energy and possessing significant entropy. Unlike what happens in the cryogenic case, at high temperature how entropy and energy play off each other therefore becomes a very significant feature defining the spectrum of available states and rates of slower motions. The spectrum of biomolecular states and motions, physiologically, must always be considered a free energy spectrum, combining entropy and energy.

THE FUNNELED NATURE OF THE ENERGY LANDSCAPE DOMINATES THE FREE ENERGY SPECTRUM

The specificity of biological function usually gives a fitness premium to molecules which at least locally have a most dominant structure. By having a reasonably (but not perfectly) well defined structure, evolved biomolecules end up doing one thing and not another in the cell. In this way, rigidity of a biomolecule allows it to harness the information in its sequence to be functionally useful, rather than problematic, to the organism. While rigidity conveys information and therefore is eventually needed, spaghetti-like, flexible chains are easier to synthesize, transport, and assemble into bigger structures. Proteins are therefore often found in an unfolded state *in vivo*. Nevertheless, when biomolecules buckle down to work, their tasks and capabilities must be clear, thus the importance of average structure.

The previous paragraph summarizes the teleological argument for why proteins fold but why most man-made macromolecules do not fold. In any event, although natively disordered proteins are biophysically important, for very many biomolecules we can take the existence of a favored average structure as a fact, in thinking about the biomolecular fee energy spectrum. A large number of the slower motions of protein can be understood as following patterns arising from this fact of a dominant structure along with, crucially, the requirement of locality of the action of forces.

One part of the spectrum of biomolecular states and motion should resemble that of small molecules. As for small molecules, the uniqueness of the average structure of a protein first tempts us to conclude there are low energy (and therefore free energy) excitations that merely distort the structure locally (Go *et al.*, 1983; McCammon *et al.*, 1976). These are quite loosely called "normal modes." Those normal modes that harmonically distort large regions of a protein lead to small local strains and since the energy is quadratic in the strain, ironically, the largest scale motions cost the least energy, in general. The topology of the protein indicates where the springs are placed and so determines these low energy motions. These motions, thus can be predicted from quite crude structural models (Tama and Brooks, 2006). If the native state of the molecule possesses weakly connected regions having few interactions to perturb ("hinges"), displacing these residues specifically may lead to

structures that have especially low energies too. Although such large scale motions involve configurations low in free energy, these motions are generally not particularly slow because they do not need to overcome any activation barrier nor carry out any significant entropic search. In fact, as pure harmonic motions, such modes are expected to lie in the terahertz regime (Leitner *et al.*, 2006). Really slow motions arise in a different way: such motions require free energy degeneracy of states coupled to free energy barriers which slow the interconversion.

The process of folding itself involves an entropic barrier and may be seen as the paradigm of slow motions. In the simplest cases folding involves two structurally distinct ensembles. These two ensembles are nearly degenerate in free energy. Because there is little driving force for change these ensembles slowly interconvert (on the time scales of the elementary motions). Structures in the unfolded ensemble have high energy but this disordered ensemble also has a very high entropy encompassing a cosmological number of individual atomic structures on the energy landscape. The folded ensemble has almost the same free energy as the hugely diverse, unfolded ensemble but it is much more nearly unique in structure and has a compensating stabilization free energy (when the solvent is considered). This stabilizing free energy is made up of many locally stabilizing interactions. This locality of interaction implies partially folded structures corresponding to ensembles in which only parts of the molecule has been organized also can have significant probability of being observed. Usually such partially folded structural ensembles do not have such complete compensation of energy for entropy as do the endpoint, completely folded and unfolded ensembles. The corresponding free energy imbalance means such structures correspond with "excited states" and are rarer than the folded or unfolded ensembles at thermal equilibrium. These partially folded structural ensembles, however, can act as bottlenecks in the folding process, i.e., they may be transition state ensembles for folding. Such excited states determine folding rates and can be probed via mutagenesis and kinetics (Oliveberg and Wolynes, 2005). Sometimes these free energetically excited states may even become populated intermediates in the mechanism of folding and may be directly detected.

When we consider the balance between the energetic dominance of a single structure and the entropic dominance of many high energy structures having no particularly strong contacts, and furthermore take into account the principle that energies are made up of local contributions we see that in overall sense the protein energy landscape resembles a funnel (Leopold *et al.*, 1992; Bryngelson and Wolynes, 1987). The limiting case of this landscape is a "perfect funnel" in which contacts not found in the native structure are completely neglected (Go, 1983). Bryngelson and Wolynes argued that under conditions of "minimal frustration" (see below) the residual effects of non-native interactions, while present, could be summarized as a sort of friction acting on the stochastic flow of structures which is largely guided by the native contacts of a perfect folding funnel (Bryngelson and Wolynes, 1989). The friction would only dominate at low temperatures through a glass transition.

The possible free energy spectra of molecules described by funneled landscapes, in turn, are largely determined by the "topology" of contacts in the native protein structure, since the pattern of contacts encodes both the entropy and energy scales locally. Proteins in the same topological structural class have similar free energy spectra, just as atoms in a column of the periodic table of elements have similar spectra.

The structures of folding transition state ensembles (Onuchic *et al.*, 1996) and folding intermediates should be and are often well predicted by such native topology-only models.

Nearly degenerate folding routes and transition state ensembles are often predicted from such models. The multiplicity of paths often arises because of symmetry. In such cases, while in one member of a protein family, laboratory experiments may see only one of the folding routes, another route to the folded structure may emerge as kinetically dominant for a closely related variant. Such switching of folding "pathways" shows the closeness and near degeneracy of structurally distinct ensembles in overall free energy. Several reviews of the successes of perfect funnel models for folding kinetics already exist (Onuchic and Wolynes, 2004; Wolynes, 2005; Clementi, 2008).

Some of the ensembles involved in the folding process also may be thought of as contributing to the free energy spectrum of the low excited states probed in experiments carried out on largely folded proteins. Transient excursions to the low excited states allow chemical processes to take place such as ligand entry as in Frauenfelder's venerable myoglobin study, or for H/D exchange to occur (Woodward, 1994). These excited states will be related to folding intermediates. On a funneled landscape, the actual excited state must be considered an ensemble, really a set of structures in which a local region of the protein becomes disordered. At low temperature, on the real landscape, one of the disordered structures in the ensemble may win out and be called a "taxonomic substate" when trapped and studied cryogenically. In Fig. 1, the assembly of cytochrome *c* predicted from a perfectly funneled energy landscape is shown (Weinkam *et al.*, 2005).

The order of the folding subunits is predicted from a native topology based funneled landscape model (but one that also includes the dominant role of interactions of the chain with the heme cofactor). This set of partially ordered structural ensembles corresponds quite precisely with the ladder of excitation free energies of substructures inferred from H/D exchange experiments by Englander's group (Bédard *et al.*, 2008). The scale of the energy landscape (a funnel) is

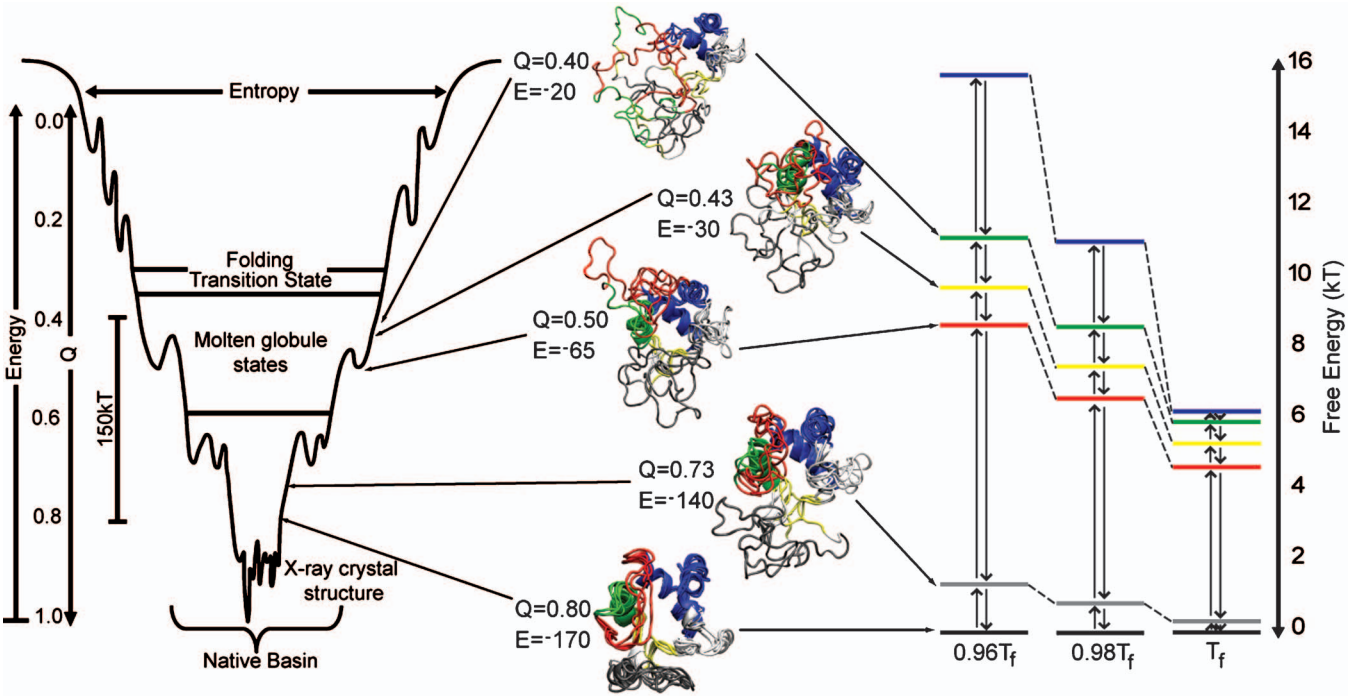

Figure 1. The authors compare a schematic funneled energy landscape (on the left) with the spectrum of free energy of partially folded states of cytochrome *c* relevant to H/D exchange. The calculations were carried out with a non-additive native topology based model (Weinkam *et al.*, 2005). The funnel on the left is indicated both by a *Q* scale (that measures the amount of native structure) and an energy scale *E*. The two scales are nonlinearly related due to nonadditivity. The energy scale of the different states is however quite large, in the hundreds of units of the thermal energy, kT. Representative structures of the ensembles of excited states are pictured in the middle of the diagram, along with their *Q* values and average energies. These ensembles all lie within the native basin, since they possess more order than does the folding transition state ensemble, which has a lower *Q*. The free energy spectrum is shown on the right. At several temperatures near the folding temperature T_F, the free energy cost of each excited state subensemble is shown. Notice the range of free energies (~15 kT) is much smaller than the energies of excitation. The near-degeneracy of free energies arises from the compensating entropy released on partial unfolding. The large *T* dependence of the spectrum reflects this entropic contribution. Increasing temperature or adding denaturant reduces the free energy cost of excitations. Denaturants thus can catalyze rearrangements between the so-called taxonomic substates on the energy landscape.

much larger than the scale of the experimentally probed free energy spectrum which is much sparser. The relatively low free energy of these low lying excited state ensembles comes from the high entropy made available by opening up regions of the protein which compensates for the ensuing lack of stabilizing interactions. On a funneled landscape, entropy and therefore topology determine the pattern of the free energy spectrum.

FRUSTRATION AND THE LOW-LYING FREE ENERGY SPECTRUM

There is another way to achieve nearly degenerate excited state ensembles which by virtue of their possessing low excitation free energies can be seen in experiment. This mechanism involves a violation of the funnel paradigm, called "frustration." Frustration denotes the conflict of interactions (Anderson, 1978)—it is essentially the opposite of funneledness. Instead of local interactions acting in concert to stabilize the same structure the various energetic contributions can be in conflict in any one single structure. This opens up the possibility of there being two or more structures using these interactions in different ways. In each of these structures there are still energetic conflicts so all these individual structures have similar energies. If frustration were very prevalent in natural proteins, proteins would commonly exhibit globally degenerate states and ensembles (Bryngelson and Wolynes, 1987). Although a few examples of such near degeneracy are showing up, it still seems quite rare in natural monomeric proteins (He *et al.*, 2008). An example of a designed monomeric protein that with very few sequence changes exhibits two disparate structures is shown in Fig. 2. While such monomeric structural diversity is rare in nature, dimerization often allows frustrated conflicts to be resolved in new ways. When two proteins with such similar sequences exist it is extremely probable that each "incorrect" structure can be considered a low free energy excited state for one sequence, which becomes a ground state for the other sequence. In this case, we can have near degeneracies in free energy without needing entropic effects.

Frustration can at least crudely be detected and localized

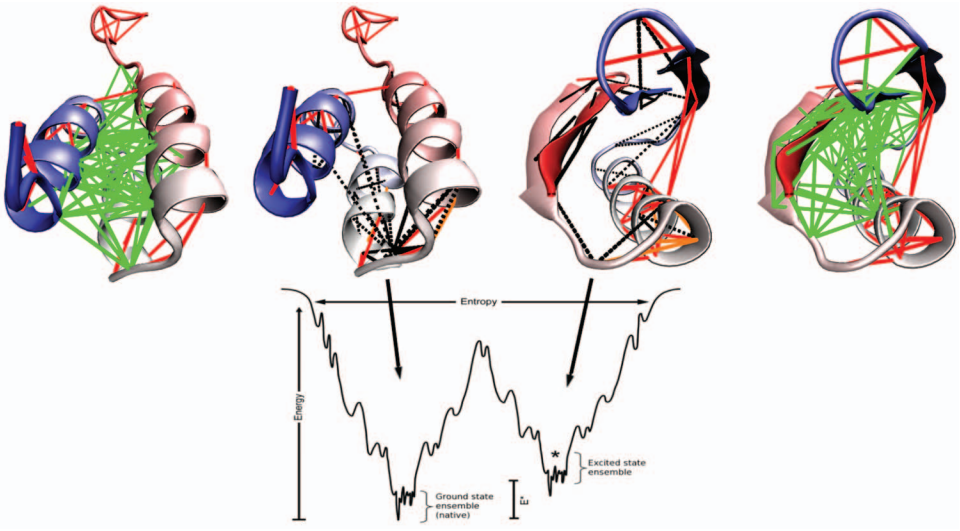

Figure 2. Frustration allows low free energy excited states without entropy compensation. The landscape then has multiple funnels. Configurations of a designed switch protein G_A88 (PDB 2JWS) are shown. Just a few residue changes allows a new structure to become the ground state. The two outer figures show the frustration patterns of the two structures. Green lines indicate a minimally frustrated, very stable interaction. Red lines indicate highly frustrated interactions that are rather unstable. The minimally frustrated interactions in both cases form connected webs. The middle figures omit the minimally frustrated web and show the highly frustrated interactions in the given conformation in red, along with black dotted lines showing pairs of residues that are highly frustrated in the alternative, excited state conformation. The interchange of these highly frustrated interactions (along with more weakly frustrated interactions omitted for visual clarity) between the two conformations allows a near degenerate excited state on the dual funneled landscape pictured below.

in x-ray crystal structures (Ferreiro *et al.*, 2007; Sutto *et al.*, 2007). Ferreiro *et al.* have developed an algorithm to locate frustrated regions of proteins given their native structures. The frustration patterns of the two designed high sequence identity proteins with near degenerate structures are shown also in Fig. 2. While both structures possess many minimally frustrated interactions, there are numerous frustrated interactions as well. These are interchanged in the ground state when the molecule is mutated and would also allow an excitation that simultaneously costs little free energy and little energy.

While there are proteins with an energy landscape having a multifunnel structure, the single funneled nature of the landscape of natural proteins becomes apparent from studies of localized frustration patterns: for most proteins there is a strongly connected web of individually minimally frustration interactions which have few possibilities of alternate structures (see Fig. 3). In most naturally evolved proteins there are only a few remaining frustrated clusters of residues. These frustrated regions often correlate with binding sites: their frustration is usually relieved by interacting with a partner. See Fig. 3 for the example of Im7 interacting with colicin. In some cases, frustrated clusters of residues also can provide a route to low energy excitations of the monomer—structures that preserve much of the funneled interactions but that use near degenerate arrangements of the frustrated residues in order for the molecule to move cooperatively. Such frustrated regions can act as pivots that reconfigure in an allosteric transition. One expects to see frustrated regions especially in motor proteins. Frustration, therefore, can be a main determinant of the pattern of the free energy spectrum, but it is generally subordinate to topological patterns for natural proteins.

SYMMETRY AND THE FREE ENERGY SPECTRUM

As in atomic physics, biomolecular symmetry gives rise to degenerate free energy ensembles and therefore slow motions (Wolynes, 1996). Symmetry always has a chance to arise in oligomeric assemblies, since there is a repetition of sequence and therefore of precisely symmetrical interactions in multiple parts of the assembly. Oligomers therefore quite

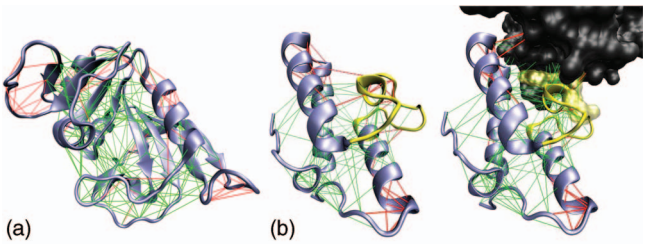

Figure 3. In the left panel the strongly connected minimally frustrated web of the enzyme dihydrofolate reductase (PDB ID code 1RX2) is shown in green, along with some clusters of frustrated sites. The right panel shows Im7, a protein that binds to colicin. The highly frustrated region is involved in the binding process. The folding of Im7 exhibits an intermediate with non-native contacts. This excited state seen in folding kinetics is caused by the residual frustration in this smaller protein.

commonly have very slow interconversions between degenerate ensembles. Domain swapping is a key example of symmetry induced degeneracy of the free energy spectrum (Yang *et al.*, 2004). Symmetry giving rise to near degenerate structures also explains the odd folding kinetics of ROP dimer variants (Levy *et al.*, 2005). Symmetry now seems a specialized mechanism in determining the free energy spectrum but it very likely played a key role in early prebiotic existence when peptides first emerged, just as it does in inorganic chemistry.

COMPOSITE MOTIONS

We have seen that low free energy excited state ensembles for a biomolecule can come about in several ways: harmonic motions of large length scale or special harmonic motions utilizing weakly connected links, local folding/unfolding on a funneled landscape, frustrated local interactions giving energetically degenerate structures and sequence symmetry that gives rise to alternate structures. Various combinations of such low free energy cost motions occurring at the same time will also give low free energy excited states and kinetically dominant reaction paths.

An example of a combination mode is "cracking" in which a biomolecule makes excursions along a low frequency normal mode (not costly in energy), during this excursion the molecule locally unfolds (not too costly in free energy) and then refolds (Miyashita *et al.*, 2003). Obviously if the region that unfolds is frustrated, so much the better: its unfolding will be still cheaper in free energy terms and faster. Cracking seems to be a likely mechanism for many physiologically relevant biomolecular processes (Whitford *et al.*, 2008).

FROM ENERGY LANDSCAPE TO FREE ENERGY SPECTRUM

The richness and complexity of the complete energy landscape of any biomolecule can be reconciled with the relative simplicity often seen in the free energy spectrum of motions at physiological temperatures. This essay has listed some of the mechanisms which have so far been found to explain the surprising sparsity of the low free energy excited states seen in standard biochemical experiments. While no other mechanisms have yet occurred to us, the list is probably not complete. Nevertheless, the authors believe that examining any particular biomolecular dynamic process in light of the theme of how a simple free energy spectrum can emerge from a complex energy landscape will be at least entertaining and will also very likely be quite useful in planning experiments and simulations.

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