Energy landscape along an enzymatic reaction trajectory: hinges or cracks?

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Are the most dynamically flexible regions around the equilibrium structure of an enzyme the same regions involved in the transition state for rate limiting processes involved in the enzymatic reaction? Kern and co-workers (Wolf-Watz *et al.*, 2004; Henzler-Wildman *et al.*, 2007a, 2007b) have shown that insights about functionally relevant motions that determine the overall enzyme turnover rate can be obtained by investigating conformational dynamics around the equilibrium basin of the enzyme adenylate kinase. An allosteric change in protein structure turns out to be the controlling process. In this commentary we compare results of this study with earlier predictions of the route by which the enzyme undergoes its conformational change. These predictions are based on the idea that the energy surface for the protein is determined by the end structures of the conformational change. A key issue is whether the protein moves by specific hinges or whether it “cracks” and accesses partially unfolded states during its structural change. [DOI: 10.2976/1.2894846]

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The very idea of an enzyme as an agent that speeds up reactions connotes understanding molecular motion. In spite of this, the most fruitful early concept in enzymology, due to Haldane and Pauling (Haldane, 1930; Pauling, 1948), takes a rather static view of the enzyme's role. They argued that an enzyme fits the transition states of its substrates as a lock does its key, thermodynamically encouraging the reaction. Certainly we all agree the detailed molecular structure of transition states' ensembles must be crucial to enzyme kinetics. Decades after Pauling, however, we now know protein molecules are dynamic entities (Frauenfelder *et al.*, 1991) “forever kicking and screaming” in the words of Gregorio Weber (Weber, 1975). The question now has become how have these inevitable atomic motions been trained and disciplined by evolution to carry out catalysis. Building on decades of study of protein motions, Dorothee Kern and co-workers have assembled a picture of the role of protein internal motions in the enzymatic function of a paradigm system, adenylate kinase (Wolf-Watz *et al.*, 2004; Henzler-

Wildman *et al.*, 2007a, 2007b). In their work, the vast range of time scales involved has been scanned by a broad panel of approaches: near static x-ray crystallography on homologues, nuclear magnetic resonance (NMR) relaxation spectroscopy, and single molecule fluorescence. These are all brought together with computation and graphical structural analysis.

Many studies have made evident the complexity of catalysis of the seemingly simple disproportionation brought about by adenylate kinase (Adk). This enzyme maintains adenosine monophosphate, adenosine diphosphate, and adenosine triphosphate in a dynamic equilibrium within the cell. The rate limiting catalytic step is not covalent bond transformation, the key focus of the Haldane–Pauling hypothesis. Covalent chemistry has indeed been speeded up by evolutionary design. With such near perfection of covalent chemistry, conformational rearrangement of the protein between an open form and a closed form in which the substrates are cradled and protected from solvent now has become the controlling process.

The first hints of conformational dynamics limited catalysis were seen in experiments by Tsou's group (Fan *et al.*, 1995; Zhang *et al.*, 1997). They made the striking observation that the enzymatic function was speeded up by the addition of small amounts of denaturant. In larger quantities the denaturant would unfold the protein leading to loss of function. The possible involvement of the whole energy landscape in catalysis was also plausibly suggested by the observation that the thermophilic homologues of the enzyme function poorly at room temperature and achieve optimal rates only near to their natural environmental temperature. Kern's earlier NMR studies on the enzyme in the presence of its substrates directly demonstrated the limiting nature of conformational dynamics (Wolf-Watz *et al.*, 2004).

Since conformational dynamics is rate limiting, many questions arise: what is the structural nature of the motion?, what is the origin of the barrier of the motion (if any)?, how did the functional motion arise in evolution? Theoretical studies along with the new experiments give insightful but still incomplete answers to these questions.

Examining (with x-ray crystallography) the structures not only of the end points but also along the range of motion obtained in homologues, shows that the overall conformational change is both of large amplitude and, perhaps of greater significance, long wavelength. At a crude level the main changes can be thought of as being made up of a few of the lowest frequency normal modes of motion of the adenylate kinase structure. It is perhaps a little misleading to characterize these large scale motions, as "low frequency," because their speed is really not their crucial characteristic. Indeed these kinds of motions in reality are likely both to be highly damped (McCammon *et al.*, 1976) and more importantly nonlinear. Rather, these motions, because they involve only modest relative displacements of the residues, have weak restoring forces: they are easily driven by thermal fluctuations providing easy paths for conformational change. These modes, by their global character, average over many of the details of the atomic force, and are largely determined by protein topology. Such an approach is successful due to the protein landscape being, in the main, minimally frustrated with an overall funnel nature; in this regime native interactions are dominant over non-native ones (Bryngelson and Wolynes, 1987; Leopold *et al.*, 1992; Onuchic and Wolynes, 2004).

This nature of the protein landscape allows successful characterization of the relevant collective modes using models of varying levels of detail (see for example: Tiron, 1996; Tama and Sanjouand, 2001; Atilgan *et al.*, 2001; Tama and Brooks, 2002; Miyashita *et al.*, 2003; Whitford *et al.*, 2008).

Completely harmonic descriptions of the allosteric motions however must be inadequate to capture the slow millisecond conformational change. These global motions will be accompanied by more local changes that can pin the molecule, not only in its end point structures but also at various

intermediate locations along the allosteric transformation/s. Here the story becomes more interesting. One limiting possibility is that the anharmonicity can be understood by viewing the molecule as having a limited number of hinge points. At the hinge points, a single (highly anharmonic!) dihedral angle change can be made, allowing the rotation, translation and screw distortion of a neighboring secondary structure element. Comparison of the crystal structures and snapshots of molecular dynamics trajectories leads the Kern collaboration to identify eight such hinges (Henzler-Wildman *et al.*, 2007a). Molecular dynamics shows the molecule makes excursions towards the open form from the closed one in less than 10 nanoseconds, but does not complete the transition. These small amplitude motions utilize the hinge motions.

The overall motion between open and closed states, however, takes much longer than nanoseconds. The opening and closing is actually a rather rare event. The orders of magnitude of extra time available certainly raises the possibility of there being a larger diversity of reaction paths than is revealed by short time molecular dynamics. A few years ago observations of Miyashita, Onuchic and Wolynes suggested the possibility of a different mode of reconfiguration in large protein conformational changes—called "cracking." In cracking, the global motions needed to reach the transition state stress regions of the protein locally. This stress in the transition state might be relieved by specific motions, like those postulated for the hinges. On the other hand, the high local stresses also would allow a diverse set of locally unfolded states to compete, in free energy terms. Even if any one such state were high in energy, the number of possibilities would balance this out (see Fig. 1.)

At the level of the whole molecule, the balance of entropy and energy leads to the well-known marginal thermodynamic stability of protein. Whether cracking occurs or not, certainly will be a function of local stability but it will also be influenced by the large scale topological features of the conformational change, just as normal modes are. While the open and closed forms must be different, perforce giving a multiply funneled landscape, many features of both structures are the same, so most of the structure is kept in place by a funneled landscape, which constrains the possible local motions. Local frustration allowing deviations from a perfect funnel (Ferreiro *et al.*, 2007) is thought to play a role in such allosteric motions (Whitford *et al.*, 2007).

In Fig. 2, we show the regions that were predicted to crack in the transition state for reconfiguring adenylate kinase. These areas were identified in two ways. The first approach due to Miyashita, Onuchic and Wolynes (Miyashita *et al.*, 2003) was to force the system to move along combinations of the low frequency collective modes, making note of where regions of high stress emerge. These regions are then allowed to relax by cracking if their stress exceeds a threshold. Alternately explicit molecular dynamics calculations using multiple funnel models based on end point structures

Figure 1. Schematic diagram illustrating the effects of cracking on the transition state ensemble for conformational rearrangement during enzyme catalysis. The x axis is the reaction coordinate, largely made up of low frequency normal modes. The local excitations shown around the open minimum include the jumps between substates probed by the NMR order parameter. In the cracking scenario, the transition state may contain many partially disordered states of slightly higher energy. The multiplicity of higher energy states reduces the free energy barrier. If the energy variance is large enough only a small number of substates would contribute to the rate. These would likely involve motion only around a few hinges. Since the protein landscape is globally funneled, one expects some overlap between the protein regions involved in the ensemble of substates accessible in the transition region and the local excitations found near the open basin. There is no reason, however, to expect that every dynamically flexible region in the open basin is important to the rate of conformational rearrangement.

can be used to identify potential cracking regions, as Whitford *et al.* have done (Whitford *et al.*, 2007). In Fig. 2(a) the hinges identified by Kern's collaboration are indicated on the same representation. Clearly most of the hinges occur within or just proximate to the high stress regions in the transition state predicted for rearrangement. Some of the hinges do not enter into the predicted cracking regions. It would seem that they are either unused in unlocking the doors to configurational change or are involved in later steps of the rearrangement, as indeed Whitford *et al.* have predicted.

A longstanding question in protein dynamics has been the relation of motions in equilibrium ensembles and those that are important in the transition states for activated processes. Strictly speaking these two kinds of motion need not have any relation to each other. This point has been forcefully made by Fersht in discussing analyses of protein folding routes hypothetically inferred via hydrogen/deuterium exchange (Fersht *et al.*, 1991). Often, though, there is some correlation. Just as low frequency normal modes of motion provide easy distortion paths, local melting of the most labile regions (by balancing favorable energy by large entropy), provides easy places for cracks to occur. These labile regions therefore may be featured prominently in the transition state ensemble. Such local motion should correlate with local order parameters measured by NMR relaxation, as NMR probes fluctuate on the nanosecond time scale and below.

Figure 2(b) shows the order parameters reported by Kern, superimposed on the regions predicted to be likely to crack in

Figure 2. Schematic representations of the apo (ligand free) structure of adenylate kinase (Adk). A comparison between the flexible regions around the equilibrium structure of the enzyme and the regions involved in the transition state is presented. (a) Comparison between strained regions seen in the transition state ensemble predicted by following the nonlinear low frequency modes to the "hinges" suggested by structural analysis by Kern and collaborators. The stress is indicated as increasing from the yellow regions, which are unstrained, to the dark red regions that are predicted to crack. Residues in the hinges are indicated by spheres at the C α carbon position. As expected for a largely funneled landscape, several of the regions where cracking is expected to occur overlap with some of the proposed hinges. Many other regions, however, exhibiting such local motions appear not to be functionally relevant. Potential hinges undergo substantial distortions in dihedral angles, which may lead to accumulation of strain and cracking in regions around, or proximal, to them. (b) Comparison between the strained regions predicted in the transition state ensemble and the experimentally determined regions of high amplitude local excitations as probed by an NMR order parameter by Kern and collaborators. Strain is indicated using the same color code as in (a). Residues with an order parameter less than 0.8 are shown as spheres at the C α carbon position (Henzler-Wildman *et al.*, 2007a). The local excitations found by NMR take place in the context of the unstressed ground state. While not all excitations are necessarily excited in the transition state, local excitations and cracking apparently coincide for some residues.

the transition state. We again see that there is significant overlap. Although the authors of Henzler–Wildman *et al.* suggest there are discrepancies between the earlier work and their observation, this is entirely expected since the predictions referred to transition state properties and are therefore related to which residues should influence rates, not equilibria. The predictions do not refer directly to the amplitudes of motion in equilibrium, the quantity measured by the Kern experiments.

Biased molecular dynamics (MD) simulations give free energy barriers (Arora and Brooks, 2007) which agree with the predictions of elastic network models that allow for cracking. While energetic barriers are accessible through short time scale simulations, longer sampling times will allow one to quantify the extent to which cracking is occurring.

Finally then we must ask does adenylate kinase really crack in its transition state for rearrangement, or has it evolved to smoothly readjust only at hinges. We think the jury is still out on this question. Mutational studies, like the Φ -value analyses of protein folding, would give much more information. The Tsou observations *prima facie* support cracking but one could argue that strictly the denaturant's influence on catalysis only suggests there are additional binding sites for urea in the transition state for rearrangement. Also one should recognize that fluctuations in energy caused by non-native interactions in different cracked substates may have allowed evolution to select a small handful of structures like those seen in the biased MD simulations.

In any event it seems one should bear in mind both of these limiting possibilities for allosteric motions. Cracking may not be limited to Adk as it has also been implicated in many molecular motors (Hyeon and Onuchic, 2007a, 2007b; Wonmuk *et al.*, 2008), where fragile connecting structures are involved.

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