Influenza hemagglutinin: kinetic control of protein function

In response to decreased pH, influenza hemagglutinin changes to a more stable conformation. Such changes, which can be controlled thermodynamically or kinetically, are the method by which many biological 'switches' are thrown.

Many enveloped viruses such as influenza virus enter cells through receptor-mediated endocytosis. Once in the endosome, the low pH triggers the fusion of the viral and endosomal membranes allowing efficient delivery of the viral genome into the cytoplasm [1,2]. In the case of influenza virus, cell surface binding and membrane fusion are mediated by a single protein, called hemagglutinin (HA), whose high-resolution structure was determined by the Wiley lab in 1981 [3]. As a consequence of this early progress, HA has served as a model system for understanding the general processes of viral entry and membrane fusion.

Recent studies, culminating in the stunning paper by Bullough *et al.*, which appeared recently in *Nature* [4], indicate that dramatic and irreversible conformational changes in HA are fundamental to the mechanism of HA-mediated membrane fusion. Here we will try to summarize these findings and to consider the results from the perspective of novel mechanisms for regulating protein function.

Native HA is a 200 kDa trimer of identical subunits; each subunit consists of two chains, HA_1 (328 residues) and HA_2 (221 residues) linked by a disulfide bond. Although normally anchored in the membrane via the carboxyl terminus of the HA_2 chain, soluble trimers can be released by proteolysis. Analysis of the structure of this soluble molecule at neutral pH (HA-N) indicates that HA_1 forms a globular head perched atop the predominantly HA_2 α-helical stem region, which comprises a coiled coil of three α-helices, each 70 Å long (one from each monomer). The receptor binding sites for sialic acid and the major antigenic sites are on HA_1, while residues 1–24 of HA_2 form a hydrophobic helix believed to be crucial in membrane fusion. In HA-N, this 'fusion peptide' is buried in the hydrophobic interface of the trimer. Moreover, the fusion peptide is located ~100 Å away from the top of the molecule, where the target membrane would bind, and ~35 Å away from the viral membrane.

Clearly this geometry would make membrane fusion problematic, as fusion requires these surfaces to be brought into close apposition. A wide variety of biochemical, biophysical, and immunological evidence indicates that the protein undergoes a dramatic conformational change after being exposed to the low pH of the endosome, adopting a low-pH form (HA-L). The fusion peptide becomes accessible, the globular heads dissociate, the molecule becomes very protease sensitive, and significant changes are observed by electron microscopy. The sharp change in properties as a function of pH is suggestive of a highly cooperative structural transition. This conformational change is required for fusion activity. Both small molecules that block the exit of the fusion peptide [5] and engineered disulfide bonds that prevent the globular head domains from dissociating inhibit fusion. Not surprisingly, a major goal has been to understand the structural changes that occur on transition to low pH.

Last year Carr and Kim [6] suggested that a remarkable coil–helix transition may be involved. At the time, they were searching through the sequence data base for proteins containing coiled coils (two or more α-helices wrapped around one another in a characteristic fashion). Because of the large coiled coil (helices C,D in Fig. 1) that comprises much of the trimer interface, it was no surprise that HA was found in this search. What was surprising was that the non-helical loop region B (Fig. 1) was strongly predicted to form a coiled coil. In fact, a similar prediction was initially made in 1980 [7] before the X-ray structure was determined, but this prediction was seemingly disproved by the X-ray structure. Not willing simply to ignore these predictions on the basis of structurally determined 'reality', Carr and Kim went on to show that while synthetic peptides corresponding to this loop and some flanking regions were disordered at pH 7, they would become helical at low pH. From this came the intriguing suggestion that the pH-induced conformational changes could be driven by a coil–helix transition. Such a transition would move the fusion peptide ~100 Å towards the distal tip of the HA molecule and the host cell membrane.

At about the same time, the Wiley group was making significant progress towards determining the structure of a soluble, trimeric fragment of HA-L. While a significant fraction of the molecule (23%) has yet to be modeled due to lack of connected electron density, the rest indicates conformational changes even more dramatic and intriguing than predicted by the Carr and Kim model.
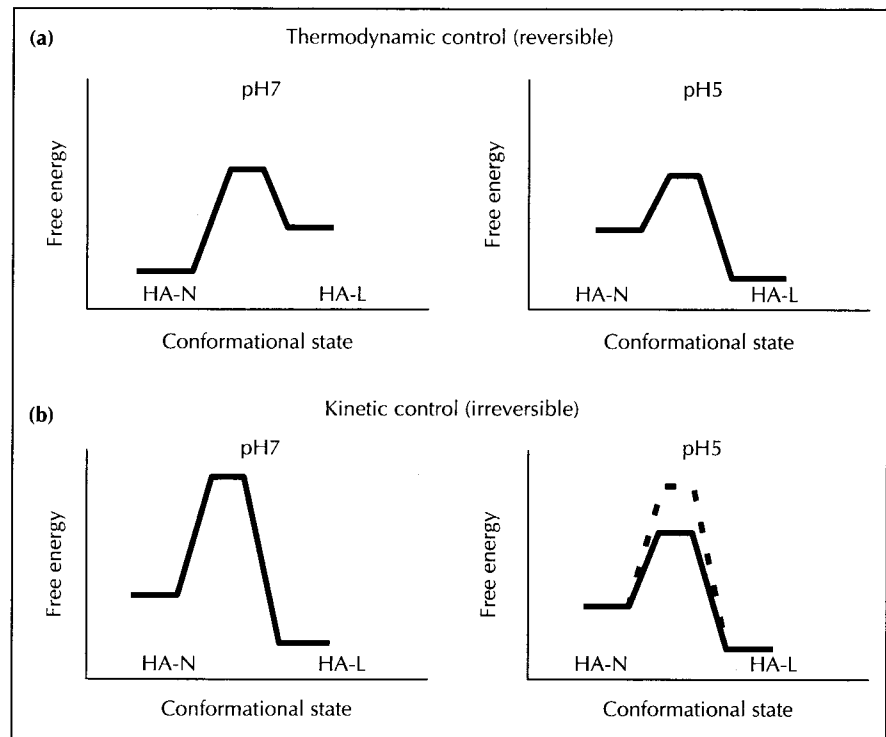
Fig. 1. Structures of the HA_2 part of the HA molecule before (left) and after (right) the conformational change induced by lowering the pH. Helices are labeled A–H. The first β-strand of HA_1 is also shown, labeled 1. Figure reproduced from [4] with permission.

The dominant feature of the structure of the HA-L fragment is an ~100 Å helical coiled coil comprising single, long α-helices from each monomer (helices ABC, Fig. 1). A small loop connects this long helix to a shorter α-helix (helix D) that packs antiparallel to the main α-helix. Remarkably, only 30 residues (helix C, Fig. 1) have the same structure in HA-N and HA-L. In both structures, this segment forms a part of the central triple-stranded α-helical coiled coil. The extended loop in HA-N (B) does indeed convert into an α-helical coiled coil at low pH as predicted by Carr and Kim. However, what was not predicted was a helix–coil transition which breaks the continuous long α-helix (CD) into a loop with a 180° bend followed by the rest of helix D that now packs antiparallel with ABC.

The helix–coil transition appears to be due to the loss of helix-stabilizing interactions with the fusion peptide. In addition, many of the other small structural elements that were packed against the carboxy-terminal end of D, are flipped and pack against C and D in HA-L.

The net result of these structural gymnastics is the movement of the fusion peptide by ~100 Å towards the host membrane and a rearrangement of the portion of the molecule adjacent to the viral membrane.

Kinetic *versus* thermodynamic control

Generally speaking, there are two ways in which a change in an external factor such as the pH could produce a protein conformational change such as the huge changes between HA-N and HA-L (Fig. 2). Firstly, a change in pH could alter the relative stability of two states so that a state disfavored at high pH becomes favored at low pH. This modulation of the stability of the two states is thermodynamic control. Secondly, a pH change could greatly increase the rate of transitions from one state to another. This modulation of the rate of transitions between the states is kinetic control.

Which mechanism holds for the low pH-induced conformational change of HA? Two lines of evidence suggest that the transition is under kinetic control. Firstly, the conformational change induced by low pH is irreversible. HA-L does not convert back to the initial state when the pH is raised, and it is more thermostable than HA-N, even at the higher pH. Secondly, the 52 residue peptide studied by Carr and Kim folds in isolation into a stable coiled coil. These data suggest that HA-L is lower in energy and hence that HA-N is metastable. An attractive model is that folding at neutral pH leads to a structure under strain, and that lowering the pH allows the strain to be relieved with the adoption of coiled coil structure by a portion of the chain which was distorted into a loop in the initial structure (loop B, Fig. 1).

To highlight the differences between thermodynamic and kinetic regulation, it is instructive to compare HA with hemoglobin, another protein subject to pH regulation [8]. In order to deliver oxygen from the lungs to the muscle, hemoglobin must have a higher affinity for oxygen in the former than in the latter. An important component of this regulation — the Bohr effect — is that the lower pH in the muscle reduces the affinity of hemoglobin for oxygen by stabilizing a low affinity state (the T state) relative to a higher affinity state (the R state). This is partly due to an increase in the pK of a histidine in the T state, which comes about because the histidine moves closer to an aspartate residue. This is clearly thermodynamic control: the change in pH changes the relative stabilities of the R and T states.

In the case of HA, a large conformational change results from a rather limited change in pH. This is highly suggestive of a cooperative transition. Cooperativity results from the combination of multiple binding (protonation) sites with two different conformational states in such a way that the binding equilibria are linked to the conformational equilibria. Again the comparison with hemoglobin is instructive. For hemoglobin, the two relevant conformational states are the R and T states, whereas in HA, because of the kinetic control, the two relevant states are HA-N and the transition state. In tetrameric hemoglobin, the two conformational states differ mainly in their quaternary structure. Based on the dramatic conformational changes observed in HA-L, it is quite likely

Fig. 2. Possible free energy diagrams for the pH-induced transition in influenza hemagglutinin. **(a)** The thermodynamic control situation where the most stable state is altered in response to lowering the pH. Such changes could be fully reversible. **(b)** Kinetic control. Here, the initially formed structure at neutral pH (HA-N) is kinetically prevented from obtaining the most stable state, but lowering the pH reduces the energy barrier, allowing the transition to the more stable low-pH state (HA-L). In this case, the process would be irreversible.

that changes in both quaternary organization and the tertiary structures of the individual subunits occur in the transition state.

What is the biological utility of kinetic *versus* thermodynamic regulation? If the goal is to build a conformational switch that is to be triggered by a fixed free energy change, under what circumstances is it more efficient to modulate the energy of a transition state rather than a starting or final state? Efficiency can be taken to be the degree of change in the population of one of the states following the switch. A quick calculation shows that for the simple case of two states separated by a single barrier,

$$a \leftrightarrow b$$

thermodynamic control is always more efficient. However, if a second reaction can occur which rapidly consumes one of the states,

$$a \leftrightarrow b \rightarrow c$$
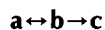
kinetic control becomes much more powerful. In fact, if the rate of the $b \rightarrow c$ transition is much greater than that of the $b \rightarrow a$ transition, thermodynamic effects clearly become irrelevant. Here the relative population of states depends critically on the height of the transition state separating a and b. In the case of HA, the $b \rightarrow c$ step could correspond to premature fusion or intercalation of the fusion peptide into a membrane bilayer.

A second and perhaps more important type of situation in which kinetic control is preferable over thermodynamic control is when the switch must be irreversible. Irreversibility cannot be built into a thermodynamic switch, which must necessarily be 'history independent' — upon return to the starting conditions, the initial

distribution of states must also return. Kinetic control has no such limitation; if the final state is significantly lower in energy than the initial state, even under the initial conditions, the switch will be irreversible. Indeed, as mentioned above, HA-L is more thermostable than HA-N even at high pH, and the transition appears to be irreversible. Zymogen activation can also be viewed as an instance of kinetic control with the added complication of a change in covalent chemistry: in chymotrypsin, for example, following proteolytic activation the new amino terminus turns inward to make a strong salt bridge with a buried aspartate and triggers a number of conformational changes [9]. The activated state is lower in energy than the starting state, but is not kinetically accessible without a protease to catalyze the activating cleavage.

The difference in behavior of different systems is very closely linked to the height of the activation barrier. If the barrier is small, the system will be governed by thermodynamic concerns, if the barrier is moderate in height the system can either spontaneously convert from one state to another or be subject to kinetic regulation, and if the barrier is extremely high, conversion between the two states will require enzymatic catalysis.

Kinetic control thus provides a mechanism for ensuring irreversibility in biological systems. In contrast, allostery or thermodynamic control allow for reversible regulation. Thus, kinetic control may be expected to occur in situations in which irreversibility is advantageous. The plasminogen activator inhibitor (PAI-1), a member of the serpin family of protease inhibitors, provides an interesting case in point [10]. After synthesis *in vivo* or refolding after denaturant treatment *in vitro*, PAI-1 folds first to a state which is an active protease inhibitor. Remarkably,

this active form converts to an inactive, latent form over a period of several hours. This spontaneous inactivation is effectively irreversible unless the protein is denatured again and allowed to renature. The irreversible inactivation of PAI-1 may be important for the proper regulation of the activity of key enzymes such as tissue plasminogen activator and urokinase and part of a more complex role in cell signalling.

Kinetic regulation can also be critical during protein folding. A recent example is the pro region-mediated folding of proteases such as α-lytic protease and subtilisin [11]. The molecular chaperones also kinetically regulate the outcome of folding reactions by circumventing aggregation (reviewed in [12]). A related example of the kinetic control of protein conformation is the conversion of the endogenous prion protein into amyloid plaques, mediated by infectious prion particles which act as plaque nuclei [13].

The conformations of influenza HA and PAI-1 which are synthesized initially appear to be metastable, since in both cases the initial conformation is not the lowest energy state of the chain. In effect, this metastability means that the initial conformation is 'spring-loaded'; designed to switch to a second conformation with the appropriate environmental trigger or after some length of time. How the lower energy states are avoided during folding to the initial metastable states is an intriguing open question.

The recent discoveries summarized here suggest that kinetic regulation may be a new paradigm for control in biology.

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