Phenylalanine hydroxylase catalyzes a critical step in the phenylalanine catabolic pathway, and impairment of the human enzyme is linked to phenylketonuria. Phenylalanine is also a positive allosteric regulator of the enzyme, and the allosteric binding site has been determined by crystallography. However, the allosteric activation mechanism remains unclear. Using large-scale simulations to explore how phenylalanine binds to the regulatory site, Ge et al. discovered gating motions of the protein that suggest a conformational selection mechanism.

Human phenylalanine hydroxylase (PAH) is a tetrameric protein that catalyzes the conversion of phenylalanine (Phe) to tyrosine, and mutations in its gene underlie disorders such as phenylketonuria, the most common heritable disorder in amino acid metabolism. PAH binds Phe not only at its active site, but also at a second site that allosterically regulates PAH. This site is formed by two ACT domains, a widespread regulatory domain used for ligand sensing. Binding of Phe to the regulatory site increases PAH activity, enabling the cell to maintain constant levels of Phe. However, the exact mechanism by which Phe allosterically regulates PAH is unclear. As revealed by the recently solved crystal structure of the Phe-bound ACT domain dimer, the Phe-binding allosteric site is positioned at an inter-subunit interface located between the two ACT domains and is well-defined in activated PAH but absent in the resting state of the enzyme.

Substrate recognition by a protein involves many steps, from the initial encounter complex formation to the final catalytically active form. During this process, both the ligand and receptor may undergo a series of conformational changes to improve their fit. The two extremes of this process are termed induced fit (2) and conformational selection (3). In the first case, ligand binding causes the protein receptor to transition to the substrate-bound state. In the latter case, the protein is in a pre-existing equilibrium between substrate-unbound and substrate-bound conformations, and the ligand selectively stabilizes the bound conformation. As a result of external and internal factors, proteins may shift the balance between these two recognition mechanisms (4). A detailed analysis of binding pathways can provide novel insights into biological recognition processes, and this is the approach Ge et al. (5) pursued.

The authors explore the mechanism of binding of Phe to one of the ACT domains in human PAH by using the crystal structure of the Phe-bound ACT domain dimer as a starting point. A previous model of the unbound ACT domain dimer showed that the allosteric site is obstructed by a helical turn containing four protein residues. This raised the central question in this study: How does the allosteric site open and does the binding of Phe influence this opening? The authors addressed this question by using molecular dynamics (MD) simulations, a computational approach that can greatly complement experimental techniques by revealing protein dynamics at the atomic scale. One major challenge of MD simulations (often at microseconds or shorter) is to reach sufficient duration, where relevant protein dynamics occurs (at milliseconds or even longer). Markov State Models (MSMs) provide a powerful approach to address this challenge by modeling long timescale dynamics from many short MD simulations and have been widely applied to study protein conformational changes.

The authors used MD simulations and constructed MSMs to analyze the PAH ACT domain dimer and monomer, in both the presence and absence of Phe. This work integrates state-of-the-art techniques for the MSM construction, including adaptive sampling, dimensionality reduction through time-lagged independent component analysis (tICA), and an application of transition path theory to elucidate kinetic pathways. By employing the community-based platform Folding@home, the authors produced a massive simulation dataset containing an aggregated simulation time of over 630 μs. This is almost a thousandfold improvement on previous MD simulations of PAH (8) and allows the authors to observe slow protein conformational changes expected to be involved in allosteric activation of PAH. Through tICA, they identified key motions of the ACT dimer in the binding process. Several independent approaches converged on an estimation of rates for Phe binding to a pre-formed ACT domain dimer. Their tICA analysis further revealed that the bending motion of a hairpin loop is as slow as...
binding, but surprisingly independent of it. Several biochemical assays demonstrated that a mutant with a bulky substitution in this loop (L72W) displays no changes in activity compared with the WT PAH, confirming that the loop motion is unlikely to be involved in the allosteric regulation.

The authors make two key observations supporting a conformational selection mechanism of binding. First, their apoprotein simulations indicate that the slowest motion of the protein opens the allosteric site in the absence of ligand. Second, their binding simulations further reveal that this opening of the allosteric site is sufficient for ligand entry. They also show that the allosteric site opening is regulated by a gating loop carrying the WT PAH, confirming that the loop motion is unlikely to be involved in the allosteric regulation.

After the initial binding, the ligand enters the allosteric site and further induces the closure of the gating loop to complete the recognition process (Fig. 1). This observation of structural rearrangement is interesting, as the closure of the gating loop will further stabilize binding between protein and ligand by forming new contacts. The finding indicates that the initial stage of conformational selection and the later stage of structural rearrangement collaboratively enable precise molecular recognition. In fact, this mechanism has been reported in other biological systems, including the binding of arginine to a periplasmic binding protein (9).

The gatekeeping motion of Val-45 and the conformational selection step in effector binding were clearly demonstrated in this study. With enhanced computation power, we anticipate that a similar MSM approach could be applied to investigate conformational changes occurring at longer timescales such as ACT dimerization and the impact of Phe binding on this dynamic process. Coupled with experiments, these studies could provide great new insights into the molecular mechanisms of PAH regulation. Moreover, proteins operating via the conformational selection mechanism often exhibit ligand promiscuity, whereby multiple pre-existing conformations of the apoprotein are available to accommodate different ligands (10). It will be interesting to explore if there are other, yet unknown, effectors, apart from Phe, which could stabilize the active form of PAH by selectively binding to other pre-existing PAH conformations. Undoubtedly, the work by Ge et al. (5) greatly expands our understanding of PAH regulation and also lays a solid foundation for the above-mentioned future studies.

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Figure 1. Conformational selection followed by structural rearrangements for Phe binding to PAH. A, the slowest tic from ticA analysis demonstrates the process of binding: In the absence of Phe, the ACT domain dimer exists in equilibrium of open and closed conformations (1); Phe selects the open conformation to bind (2); The initial binding induces further closure of the ACT domain dimer to reach the bound state (3). This figure was reproduced from Ref. 5. This research was originally published in the Journal of Biological Chemistry. Ge, Y., Borne, E., Stewart, S., Hansen, M. R., Arturo, E. C., Jaffe, E. K., and Voelz, V. A. (2018) Simulations of the regulatory ACT domain of human phenylalanine hydroxylase (PAH) unveil its mechanism of phenylalanine binding. J. Biol. Chem. 2018; 293:19532–19543. © Ge et al. B, cartoon representations of several key conformations described in the binding process in A.

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