Structure and Catalysis: Conformational Flexibility and Protein Motion

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While enzymes can be studied from the viewpoint of (a) the active site environment, (b) kinetic mechanism, and (c) chemical mechanism, the enzyme is a unit, and all these approaches tell valid but partial tales. A complete understanding of enzyme mechanism requires a correlation of these and other information, like enzyme structure and its dynamics. Although chemical mechanisms have been elucidated for many enzymes, how they achieve a catalytically competent state has become approachable only recently through experiments and computation. Synergy between structure and plasticity results in the unique power of enzymes. Structural enzymology aims to address these catalytic motions in detail.

Structural Enzymology This aspect of enzymology is concerned with molecular architecture of enzymes, especially how they acquire their unique catalytically competent structures and how alterations in these structures affect their function. This subject is of great interest to enzymologists because it is only when proteins fold into specific three-dimensional shapes that they are able to perform catalytic function. Amino acids are joined via peptide bonds; this bond has partial double bond characteristics and is almost always in the *trans* conformation. The *primary structure* (amino acids covalently joined in a particular order through peptide bonds in the polypeptide) dictates the higher-order structures – as elegantly demonstrated with RNase A folding by C. Anfinsen (see Table 2.2) (Anfinsen 1973). The polypeptide sequence locally folds into *secondary structure*s like α-helices, β-sheets, or random coils. These in turn fold into the polypeptide *tertiary structure*. The complete tertiary structure of an enzyme may consist of a single domain or a few domains juxtaposed in a suitable arrangement. Many proteins are oligomers consisting of subunits, and this defines their *quaternary structure*. Hemoglobin is a α2β2 tetramer (Fig. 8.1), while aspartate transcarbamoylase has a $3R_2$-$2C_3$ architecture (with 12 subunits where R is regulatory subunit and C is a catalytic subunit; see Chap. 37 for more details).

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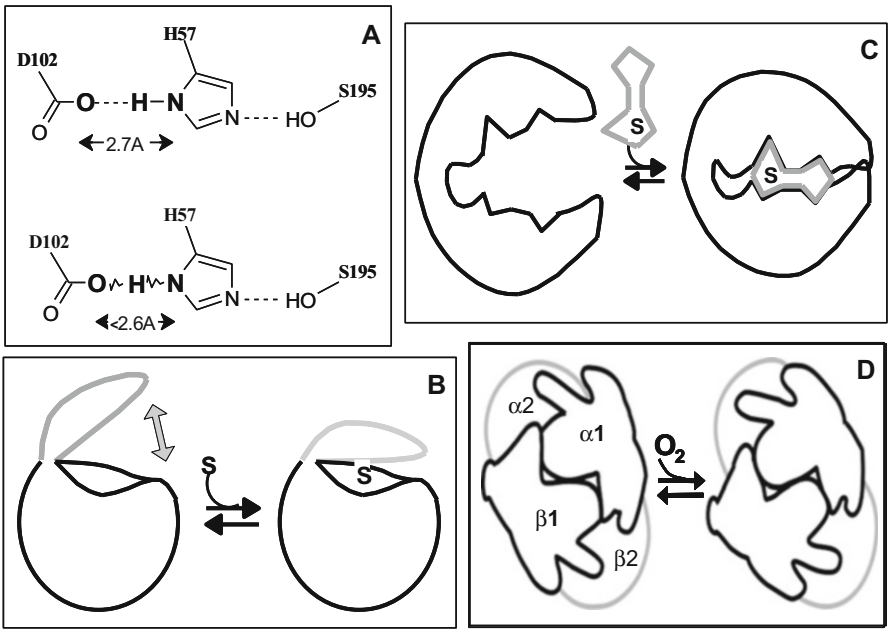

Fig. 8.1 Range of conformational changes relevant to enzyme function. (**A**) A LBHB between Asp102 and His57. (**B**) Flexible loop of triose phosphate isomerase to lock up the substrate. (**C**) Domain movement and substrate-induced fit. (**D**) The R (oxy) and T (deoxy) states of hemoglobin tetramer with two distinct positions of its α1β1 dimer relative to the α2β2 dimer

Structural details of an enzyme, the largely unchanging three-dimensional forms as also the conformational flexibility with associated mechanical motions, provide valuable clues in understanding the basis of catalysis. Toward this end, structure determination methods contribute immensely to the understanding of enzyme mechanisms. The X-ray crystallography has provided many insights to enzyme structure function, and a few historically important ones are listed below.

- Lysozyme (from hen egg white) was the first enzyme whose crystal structure (X-ray diffraction) was solved by D. Phillips in 1965. Co-crystallization with its substrate analog $(GlcNAc)_3$ provided considerable insight into the enzyme mechanism. This was the first example of structure providing clues to the mechanism of enzyme action. The so-called Phillips mechanism proposed the role of Glu-35 and Asp-52 in catalysis. This "Phillips mechanism" was further sorted and revised after almost 50 years using another structural technique (electrospray ionization mass spectrometry, ESI-MS) in combination with a E35Q mutant of lysozyme (Kirby 2001; Vocadlo et al. 2001). It was shown that active site residue Asp52 acts as a nucleophile and forms a covalent bond to the C1 carbon of the substrate glycoside.
- Another model enzyme whose three-dimensional structure was established (in 1967 by David Blow's group) was bovine pancreatic α-chymotrypsin (Matthews et al.

1967). With this structure, the catalytic triad (Asp-His-Ser) typical of so many serine proteases was discovered (Perona and Craik 1995). Whether the N-3 hydrogen on the imidazole of His-57 is actually transferred to Asp-102 during catalysis could not be ascertained by X-ray crystallography – because H atoms are too small to be resolved by this technique. Additional structural tools – like neutron diffraction studies with deuteriated His-57 and ^{15}N NMR analysis – confirmed later that N-3 hydrogen actually remains attached to His-57. It is this H atom which participates in the low-barrier hydrogen bond during chymotrypsin catalysis.

- A super-secondary structure consisting of a parallel sheet formed by three extended parallel β-sheets connected by α-helices (the β-α-β fold) was first discovered in 1970 in M Rossmann's Laboratory. This motif forms the nucleotide binding domain of NAD-dependent lactate dehydrogenase. Similar alternating motif of β-α-β-strand secondary structures (known as the Rossmann fold) is found in most enzyme proteins that bind nucleotide cofactors FAD, NAD^+, and $NADP^+$ (Laurino et al. 2016).

Over the years, many more enzyme structures have been solved and mechanisms better understood. Improvements in X-ray diffraction methodology and the ease of heterologous expression of almost any protein in *E. coli* (with or without an affinity tag for purification!) have accelerated the enzyme structure elucidation since 1980s. Besides X-ray crystallography, other structural methods have also significantly contributed to our understanding of enzymes. Many proteins do not always crystallize easily, especially when parts of the structure are flexible or the complex has structural heterogeneity. In such cases, methods like cryoelectron microscopy (cryo-EM) were developed to obtain high-resolution 3-D images of proteins, and structures of many enzymes like glutamine synthetase, β-galactosidase, isocitrate dehydrogenase, and glutamate dehydrogenase are available now (Vonck and Mills 2017). It is beyond the scope to cover extensively on all the tools available to study the structural aspects of enzymology. However, Table 8.1 lists the more commonly used methods along with comments on their strengths and constraints.

Many of the structural methods listed above provide snapshots of the enzyme protein. Catalysis being a dynamic kinetic process of such information is of limited value. Therefore, time-resolved spectroscopic tools are being developed with many of these methods to capture the dynamics of the enzyme catalysis.

Conformational Flexibility and Enzyme Catalysis The native structure of a protein is simply the most thermodynamically stable conformation accessible to the folding polypeptide chain. As such polypeptide chains have many degrees of freedom, it is likely that the molecule has several other accessible conformations with almost as low ΔG° of formation as the native structure. Consequently a population of rapidly equilibrating stable conformations for the same protein is possible. For instance, a protein could have two conformations – the native structure and another conformation – in equilibrium with each other. Crystallographic evidence is available for two conformational states of hemoglobin, aspartate transcarbamylase, hexokinase, citrate synthase, and triose phosphate isomerase.

Table 8.1 Methods for structural analysis of enzymes

Technique/method	Information/outcome	Constraints
High resolution		
X-ray diffraction	Complete 3-D structure at atomic resolution	Requires high purity crystalline protein; snapshots with limited time resolution
Cryoelectron microscopy	3-D structure with detailed subunit arrangement; works with soluble proteins, without crystallization needs	Resolution of small protein structures; need further improvement
Nuclear magnetic resonance (NMR) spectroscopy	Complete 3-D structure at atomic resolution; structural dynamics, loop flexibility; side-chain movements and ionization of residues; ligand binding	Limited to small-sized proteins; larger sample size; kinetics possible but at longer timescales
Global features		
UV-Visible spectroscopy	Gross structural changes and ligand binding; kinetics possible	Sample purity and composition (availability of intrinsic chromophores)
Fluorescence spectroscopy	Gross structural changes and ligand binding; kinetics possible; excellent sensitivity	Sample purity and composition; availability of fluorophores (intrinsic or extrinsic)
Circular dichroism (CD) spectroscopy	Overall secondary structure details and dynamics; environment change effects	Sample purity and size
Mass spectrometry	High-resolution molecular mass characterization and sequencing of proteins	Sample processing and purity
Dynamic light scattering	Size distribution profile; aggregation behavior; effective particle diameter	Presence of small impurity
Calorimetry	Protein folding (differential scanning calorimetry, DSC); thermodynamic parameters of interactions in solution (isothermal titration calorimetry, ITC)	Only overall interaction data
Hydrodynamic		
Analytical ultracentrifugation	Shape and molecular mass of the enzyme protein	Only gross hydrodynamic information
Gel filtration chromatography	Molecular weight of the native enzyme protein	Only gross hydrodynamic information
Gel electrophoresis	Molecular weight and gross quaternary structure (native, denatured, and cross-linked proteins)	Only gross hydrodynamic information
Chemical		
Chemical modification	Amino acid residues relevant for structure/function	Supporting evidences required
		Supporting evidences required

(continued)

Table 8.1 (continued)

Technique/method	Information/outcome	Constraints
Site-directed mutagenesis	Amino acid residues important in structure, active site binding, etc.	
Hydrogen–deuterium exchange	Solvent accessibility of various parts of the enzyme molecule; protein tertiary structure; folding pathways characterization	Involved experimentation
Computational		
	Protein structure prediction	Tools improve with better database and availability of computational time
	Molecular dynamics simulation; molecular docking	Computational time intensive

Often the two states represent the unliganded conformation (most stable in the absence of a specific ligand) and liganded conformation (most stable with one or more specific ligands bound to it). In any case, if two conformations of a protein exist in a definite equilibrium, then we could also define an equilibrium constant for the same. This was invoked as one of the mechanisms for allostery in regulating enzyme activity (the R and T states of the Monod–Wyman–Changeux model; see Chap. 37).

An enzyme active site can accommodate either the substrate or the product of the reaction it catalyzes. Also, S and P are distinct chemical entities but are structurally related. For instance, glucose and glucose-6-phosphate (in hexokinase reaction) differ by a phosphate group – the rest of the sugar structure is by and large identical. Whatever be their individual affinities, S and P have to interact with (and hence be complementary to) the enzyme pocket. Therefore, an enzyme cannot be a rigid structure ("lock for a key") but exhibit local conformational changes – in the vicinity of the active site at least! In order for the enzyme to participate in catalysis, protein motion (however small!) and conformational plasticity are a must. Most enzymes handle a substrate that is larger than an electron (with the exception of cytochromes!). In all these cases the space-filling needs of reactants and products are obviously different in the enzyme active site. Since the enzyme has to reach the *TS*, starting from either reactant or product, some things have to move. Conformational flexibility and mechanical motion of the enzyme protein are thus a necessity. Enzymes may therefore be also viewed as *dynamic mechanical devices*.

For many enzymes, snapshots of conformations that are sampled during catalysis have been obtained using ligands, substrates, and inhibitors. The protein motions promoted by such ligand binding are most interesting. Intrinsic motions along an enzymatic reaction trajectory could be monitored through X-ray crystallography, NMR, single-molecule FRET (fluorescence resonance energy transfer) and molecular dynamic simulations. A range of conformational changes

Table 8.2 Conformational flexibility and enzyme catalysis

Conformational change	Effect	Example
Orbital steering and small structural changes	Large changes in kinetic property, cooperativity	Chymotrypsin, NADP-isocitrate dehydrogenase, ketosteroid isomerase, most enzymes
Flexible loops	Hold or protect the substrate (ligand)	Triosephosphate isomerase; adenylate kinase; HIV protease
Domain movements	Induced fit, generation of active site	Hexokinase; citrate synthase; transglutaminase; cAMP-dependent protein kinase; calcium/calmodulin-dependent kinase II; adenylate kinase
Subunit communications	Allostery, cooperativity	Aspartate transcarbamylase; hemoglobin

are observed and are relevant to enzyme function (Table 8.2). These differ in the extent to which the changes transmit and extend from the active site.

Orbital Steering and Small Structural Changes Local protein motion at the enzyme active site must occur. Consequences of small conformational changes are profound and are easily detected by the discriminatory power of enzymes. Orbital overlap produced by optimal orientation of reacting orbitals play major quantitative role in the catalytic power of enzymes. In a large measure, the ability of enzyme to maximize orbital steering contributes to catalysis. Often such conformational changes are barely detectable by the best physical tools and structure elucidation methods! An interesting question was posed by Koshland – "How small a conformational change is big enough?" A fraction of an Å shift in an active site group of NADP-isocitrate dehydrogenase is "big enough" to be functional through large catalytic consequences (Koshland 1998; Mesecar et al. 1997). Chymotrypsin active site has a catalytic triad of Asp-His-Ser. The normal hydrogen bond between His57 and Asp102 goes through a low-barrier hydrogen bond (LBHB) during the catalytic cycle (Fig. 8.1). A short contact distance is necessary for an LBHB to form. Thus the two heteroatoms (N of His and O of Asp) are drawn close together. This is again an example of movement, of atoms/groups during catalysis, in sub-Angstrom scale. Ketosteroid isomerase binds to its substrate through hydrogen bonds that tighten up as the *TS* is approached. One of them is a LBHB. Tiny variations of the order of 10 picometer (about 0.1 Å) make a remarkable difference in efficiency of enzymatic catalysis (Kirby and Hollfelder 2008).

Flexible Loops Examples of enzymes in this group display movement of a relatively small loop upon ligand binding. This movement encloses the ligand in a cage-like structure and excludes it from the bulk solvent water. Often such loop regions are ill-defined in the X-ray or 2D NMR data indicating their conformational flexibility. Best example of this type of conformational motion may be found in triose phosphate isomerase (Table 8.1). Upon substrate binding, a short loop (residues 168–177) of this protein closes over the substrate to lock it in

the active site – unstable intermediate formed during the enzymatic reaction is protected from decomposition by solvent water. This lid has to open for the product to leave the active site after each catalytic cycle. In fact, this conformational change (closing and opening motion of the loop) has to be much faster than the overall rate of catalysis. Similar functionally significant motions occur in some proteins containing nuclear localization signals. Here the signal sequence may occur in exposed state or be tucked in to prevent nuclear entry.

Domain Movements Member enzymes of this group show large-scale structural movements. Two large domains of the same polypeptide chain may move in relation to each other about a flexible hinge region. Subsequent substrate binding and rearrangement of various amino acid residues occur to produce a functional active site. This *induced fit* and *productive binding* are an important manifestation of protein flexibility (Koshland 1958). Hexokinase active site is functionally assembled by closing two large domains, only upon binding glucose (Fig. 8.1). This ensures that ATP cleavage and phosphate transfer to glucose are strictly coupled – transfer to water cannot occur (nonproductive binding). Similar domain closure is observed when oxaloacetate binds to citrate synthase.

Adenylate kinase is yet another classic example of "induced fit" and is a representative member of NMP kinases. These enzymes contain a glycine-rich sequence (known as Walker A motif) forming the P-loop. The P-loop typically contains an amino acid sequence of the form Gly-X-X-X-X-Gly-Lys-(S/T) and interacts with the phosphoryl groups of the bound nucleotide. Interaction of the nucleotide substrate with adenylate kinase results in the movement of P-loop – this in turn closes the two domains to engulf the substrate. The P-loop NTPase domains (and Walker motifs) are encountered in a number of enzymes (Laurino et al. 2016) that undergo substantial conformational changes on NTP binding and/or hydrolysis.

Subunit Communications It is possible, but not necessary, that the conformational change observed upon ligand binding may be restricted to in/around the enzyme active site. In multimeric enzyme proteins, conformational changes may be communicated across the subunits. These may lead to profound biochemical consequences such as cooperativity and regulation. Examples of subunit communication of this kind are observed in hemoglobin (an honorary enzyme!) and aspartate transcarbamylase. Apart from conformational changes within a subunit, an alteration in the spatial relationship among the subunits in an oligomer is also possible (Fig. 8.1).

Precise orientation of catalytic groups is required for enzyme action. Substrate binding causes an appreciable change in the three-dimensional relationship of amino acids of the protein – at least at/around the active site. In contrast to the rather rigid key-lock concept of Emil Fischer, *induced fit theory* by Koshland proposes protein flexibility as an essential characteristic of enzymes (Koshland 1958). Such a perspective for an enzyme explains many important phenomena like the ability of enzymes to

exclude omnipresent water, regulation outside the active site, and noncompetitive inhibition. Clearly, conformational changes are at the root of feedback inhibition, enzyme activation, cooperativity, etc. (refer to Chap. 37 for a detailed treatment on these topics). Small conformational changes having large effects explain the process of evolution of proteins and why enzymes are large.

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