

PROTEIN FLEXIBILITY AND ENZYMATIC CATALYSIS

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ABBREVIATIONS

1-CPI	1-(4-chlorophenyl)imidazole
3D	three dimensional
4-BP	4-benzylpyridine
4-CPI	4-(4-chlorophenyl)imidazole
4-NBP	4-(4-nitrobenzyl)pyridine
(<i>S/R</i>)-GOP	(<i>S/R</i>)-glycidol phosphate
TIM	Triosephosphate Isomerase
UDX	UDP- α -D-xylose
NMR	Nuclear Magnetic Resonance
NSE	Neutron Spin Echo
CYP	Cytochrome P450

ABSTRACT

The dynamic nature of protein structures has been recognized, established, and accepted as an intrinsic fundamental property with major consequences to their function. Nowadays, proteins are considered as networks of continuous motions, which reflect local flexibility and a propensity for global structural plasticity. Protein–protein and protein–small ligand interactions, signal transduction and assembly of macromolecular machines, allosteric regulation and thermal enzymatic adaptation are processes which require structural flexibility. In general, enzymes represent an attractive class among proteins in the study of protein flexibility and they can be used as model systems for understanding the implications of protein fluctuations to biological function. Flexibility of the active site is considered as a requirement for reduction of free energy barrier and acceleration of the enzymatic reaction while there is growing evidence which concerns the connection between flexibility and substrate turnover rate. Moreover, the role of conformational flexibility has been well established in connection with the accessibility of the active site, the binding of substrates and ligands, and release of products, stabilization and trapping of intermediates, orientation of the substrate into the binding cleft, adjustment of the reaction environment, etc.

I. INTRODUCTION

Long before the appearance of any experimental evidence supporting the dynamic character of protein structures, their view as static and rigid objects was proving increasingly insufficient to explain a large body of biochemical data, which were gathered mainly in the field of enzymes. The “key-lock” hypothesis, proposed at the end of the nineteenth century by Fisher (1894) and used for the first half of the twentieth century to explain enzymatic activity was based on the concept of fixed structures, that is, the substrate had to fit into a structurally well-defined active site of fixed shape which was also complementary to the substrate’s shape. However, the predictions of the “key-lock” theory were not always sufficient to explain the experimental data. Thus, the need for revision led Koshland in 1958 to propose the “induced fit” theory which introduced the concept of an enzyme active site which undergoes structural changes induced by the binding of the substrate. This theory is based on the idea that protein structures or at least their active sites possess a flexibility which enables them to adopt more than one

conformation. This was proposed even before the determination of the first protein three-dimensional (3D) structure (myoglobin; [Kendrew et al., 1958](#)). One year later, [Linderstrom-Lang and Schellmann \(1959\)](#) proposed a breath-like continuous movement of the protein structures, an idea which was experimentally probed during the following decades. Evidence came from the analysis of crystallographic temperature factors which had lower values for buried/semi-buried areas and systematically higher for solvent-exposed residues. In some cases, loops and side chains exposed to the solvent have very high values indicating high mobility. Moreover, relatively high temperature factors also characterize regions of the active site of enzymes. The same conclusions were drawn by comparing the apo- and holo-states of a protein complexed with different compounds or by comparing homologous proteins from different organisms. Beginning with the analysis of crystallographic structures of lysozyme, the importance of conformational flexibility for catalytic efficiency was widely accepted. In the 1970s, Karplus and coworkers studied the molecular dynamics of folded proteins by solving the equations of motion for the atoms, applying empirical potential energy functions, and the mobile character of protein structures was confirmed for once more. In the 1980s, the development of nuclear magnetic resonance (NMR) provided an additional experimental tool to explore the dynamical aspects of protein structures. Nowadays, the dynamic nature of structures is well established and proteins are considered as a network of continuous motions, which reflect local flexibility and a propensity for global structural plasticity; an implication of the latter is a mechanism for the establishment of new folds as proposed by [Glykos et al. \(1999\)](#). Internal motions have different amplitudes and their frequencies range over different time scales. The motions which affect the protein structure include bond vibrations at the time scale of femto- to pico-seconds, side-chain rotations at the time scale of nanoseconds, and more complicated motions of larger time scales such as motion of flexible termini and loops, large concerted domain motions, and conformational adjustment upon substrate binding.

A growing variety of methods was used and/or developed to study the protein mobility at time scales ranging from femtoseconds to seconds. Next to classical macromolecular crystallography, NMR and molecular dynamics simulations, the protein motions can be studied by a variety of time-resolved techniques and a wide range of spectroscopies such as time-resolved Laue diffraction and intermediate trapping, single-molecule fluorescence resonance energy, neutron spin echo (NSE), etc.

Some types of protein functions can be easily related to structural flexibility. For example, almost any interaction between a protein and another molecule is associated with conformational changes. Ligand recognition and binding, protein–protein interactions, and processes as complicated as signal transduction and assembly of multiprotein machines are based on protein flexibility (Gazi et al., 2009; Teilum et al., 2011). Allosteric regulation is achieved through conformationally flexible regulatory proteins. Even the evolutionary process of thermophilic, mesophilic, or psychrophilic adaptations of protein structures, affect protein sequences such that structural flexibility is directly affected with implications for protein stability and enzymatic catalysis. In general, enzymes represent an attractive class among proteins in the study of protein flexibility and they can be used as model systems for understanding the implications of protein fluctuations to biological function.

Enzymes catalyze a variety of biological processes and may accelerate them by as many as 20 orders of magnitude in comparison with the corresponding uncatalyzed reaction (Wolfenden and Snider, 2001). One of the most intriguing questions in biochemistry is how the enzymes achieve this high rate enhancement. Numerous studies have been reported aiming to elucidate dynamic effects associated with catalysis. These studies demonstrate that among other factors, the ability of enzymes to adopt different conformations is of high importance for the efficiency of the catalytic processes (Kamerlin and Warshel, 2010). Flexibility of the active site is considered as a requirement for reduction of free energy barrier and acceleration of the enzymatic reaction (Hammes-Schiffer and Benkovic, 2006; Henzler-Wildman and Kern, 2007; Nashine et al., 2010). During the catalytic cycle, the enzyme molecule passes through different states and each state is associated with a different active site conformation. Rapid transition between the different conformational states is therefore mandatory for the maximum enzyme activity. Some aspects of the role of this conformational flexibility have been established in connection with the accessibility of the active site, the binding of substrates and ligands and release of products, stabilization and trapping of intermediates, orientation of the substrate into the binding cleft, adjustment of the reaction environment, etc. Furthermore, growing evidence concerns the connection between flexibility and the substrate turnover rate (reviewed in Yon et al., 1998; Hammes, 2002; Agarwal, 2006).

Extreme cases of protein flexibility are the molten globule state and the intrinsically disordered proteins. These exhibit significant conformational flexibility which is frequently necessary for the establishment of intermolecular interaction networks (Ohgushi and Wada, 1983; Gazi et al., 2009; Schlessinger et al., 2011). In the partially folded molten globule state, the polypeptide chain adopts a nearly native-like secondary structure, and a dynamic tertiary structure due to the partial absence of native intramolecular packing interactions.

During the last decade, many proteins have been described that fail to adopt a stable tertiary structure under physiological conditions and yet display biological activity (Dunker et al., 2008; Uversky and Dunker, 2010). This state of the proteins, defined as intrinsic disorder, has been found to be rather widespread; disordered regions lacking stable secondary and tertiary structure are often a prerequisite for biological activity, suggesting that structure–function relationships can be frequently only understood in a dynamic context in which function arises from conformational freedom. Fully or partly nonstructured proteins are described as intrinsically disordered or intrinsically unstructured proteins. The term natively unfolded proteins indicates that protein function is associated with a dynamic ensemble of different conformations (Gazi et al., 2008, 2009).

Here, we will focus on the flexibility of natively folded proteins and, in particular, of enzymes. We will review the different ways in which flexibility is linked to enzymatic catalysis through conformational flexibility of loops surrounding the active site, and of domain and active site residue dynamics. Specific examples from the literature will be reviewed. In addition, an overview of widely used specific methodologies for studying protein flexibility will be presented, as well as some more recently developed ones. The issues of allostericity, thermal adaptation, and ligand specificity in relation to flexibility will be reviewed in detail. The topic is presented from the structural point of view with an emphasis on X-ray crystallography results.

II. SELECTED METHODS USED FOR STUDYING PROTEIN FLEXIBILITY

A summary of selected methods used today to investigate and analyze protein flexibility is presented below. A detailed application of X-ray crystallography, mutagenesis, and molecular dynamics simulations to a specific example is described in a following section.

A. *Crystallography and Time-Resolved X-ray Methods*

X-ray crystallography is the method of choice for obtaining the molecular structure of proteins at atomic resolution. The X-ray diffraction pattern of the crystalline specimen is recorded and the electron density map of the molecule under study is calculated. The molecular model is built into the electron density map and refined and thus the position of the atoms is determined with high accuracy. In fact, crystallography provides a static model of a dynamic molecule; therefore this model represents an average, in space and time, of 3D structure of the molecule. However, even the static crystal structure contains useful information for the dynamic nature of the molecule, as it is explained below.

The refined atomic model contains information for the degree of thermal motion of the atoms. The mean-squared atomic displacement is commonly expressed as the temperature factor (B-factor). Temperature factors express not only the static disorder, which is an ensemble of substates present in solution and trapped in the crystal but also the dynamic disorder, which represents fluctuations in the crystal or otherwise crystal defects. Thus, B-factors cannot be interpreted simply as the amplitude of atomic fluctuations because both true intramolecular motion and lattice disorder contribute to them. Moreover, crystal contacts affect B-factors. In the context of protein structures, the B-factors can be taken as indicating the relative vibrational motion of different parts of the structure and comparing them along the structure may allow one to draw conclusions about which are the most flexible parts. Atoms with low B-factors belong to a part of the structure that is well ordered while atoms with large B-factors generally belong to a part that is very flexible.

It is quite common that some protein segments, that is, amino and carboxyl termini, loops or even other protein segments yield weak or nondetectable electron density. A common reason (apart from crystal lattice defects) for missing electron density is that the unobserved region fails to scatter X-rays coherently due to variation in position of the atoms of this particular segment, that is, the unobserved atoms are disordered or highly flexible.

Sometimes there are residues in a structure which present clear electron density for two positions, or they present what we call multiple distinct conformations. This is a direct evidence that the side chain spend time in more than one conformations or otherwise that present a relative flexibility. In other cases however, the movement from one conformation to the

other occurs so quickly that there is no definite electron density for some of the atoms of the side chain.

Several time-resolved X-ray structural methods (for a review see [Westenhoff et al., 2010](#)) have been developed in the recent years aiming to follow in real time the conformational changes of a protein while performing its action. Time-resolved Laue diffraction, intermediate trapping, time-resolved wide-angle X-ray scattering, and time-resolved X-ray absorption are the most common of them. Time-resolved Laue diffraction ([Schottea et al., 2004](#)) provides conformational snapshots of the whole protein in high resolution and allow us to visualize in real time and with atomic detail the conformational evolution of a protein. Detection of structural changes as small as 0.2–0.3 Å with a time resolution of 100 ps is possible. The time-resolved Laue diffraction experiments are of a pump-probe type. The reaction is triggered within the protein crystal by photolysis caused by ultra-short laser pulses which play the role of the pump for the initiation of the reaction. Diffraction patterns are collected at specific time delays after triggering. This cycle must be repeated many times for each spatial rotation of the crystal and many times for the same time delay. The applicability of the method depends on whether the molecule retains its biological activity in the crystalline state, whether the molecule is inherently photosensitive or if it could be engineered as so, whether the system is reversible, whether the concentration of the produced intermediate is sufficiently high to be detectable because at any given time during reaction several intermediates are likely to be present unless they are well separated in time. The method has been successfully applied to heme-containing proteins ([Srajer and Royer, 2008](#)).

As an alternative, the intermediate trapping method could be used. In this method, the lifetime of intermediates is sufficiently extended so that they can be studied by static crystallography. Freezing, changes in pH, chemical modifications, or solvent modifications are some ways which are used to trap the intermediate. The successful application of an alternative approach of this method has been used to reveal the dynamics of the active site of the small guanosine nucleotide-binding protein H-Ras-p21 ([Klink et al., 2006](#); [Klink and Scheidig, 2010](#)).

In time-resolved wide-angle X-ray scattering diffraction, data are recorded as a function of time from the molecule in solution ([Fischetti et al., 2003](#)). Because the structural information obtained is averaged over all orientations of the randomly oriented molecules in the solution, the atomic detail

is poor. This technique is used to characterize large-scale global conformational changes. On the other hand, the time-resolved X-ray absorption spectroscopy is used to characterize the geometry and the changes of the geometry of the coordination structure in the active sites of metalloproteins.

B. X-ray Free Electron Lasers

The X-ray free electron laser (XFEL) is a novel radiation source, which combines a particle accelerator with laser physics. Bunches of electrons are first brought to high energies in a superconducting accelerator. Then they fly through a special arrangement of magnets, called undulator, in which they emit laser-like flashes of radiation. By this way, X-ray flashes of high energy are generated and they can be directed to the sample whose diffraction pattern is recorded just before its explosion. The special characteristics of the generated flashes, that is, coherent radiation, wavelengths at the level of Angstroms, and short pulses at the level of hundreds of femtoseconds allows for atomic resolution snapshots of the molecule under study. XFEL may allow imaging of single particles/molecules and a large variety of different applications such as investigation of protein folding and dynamics, the actions of catalysts, and the splitting of chemical bonds can be afforded (Neutze *et al.*, 2004). Applicability of the method with tiny crystals of photosystem I has been demonstrated (Chapman *et al.*, 2009).

C. Spectroscopies, NMR, NSE, and Hydrogen–Deuterium Exchange

Spectroscopy is the study of the interaction of electromagnetic radiation with matter. An advantage of the spectroscopic methods is that they can give a dynamic picture of the selected part of the molecule, for example, time-resolved intrinsic fluorescence of Trp reflects internal mobility of this amino acid. Real-time information on structural changes for macromolecules containing a chromophore, such as heme proteins, is provided by time-resolved spectroscopic studies including absorption, resonance Raman, and infrared spectroscopy. In those cases, we observe structural changes limited to the chromophore environment.

The single-molecule fluorescence resonance energy transfer (FRET) reports proximity of moieties within a molecule for distances in the range of 1–10 nm. FRET is measured between two dyes, donor and acceptor. If the dyes are separated by a large distance (larger than 10 nm), then

there is little interaction between them and even if the donor emits photons upon its excitation by laser, acceptor will not interact with this energy. However, if the two dyes are brought closer, the acceptor takes the energy from the donor and emits photons of different colors. So, we can use FRET to measure distance changes in the nanometer scale. A recent application of the single-molecule FRET is described by [Kahra et al. \(2011\)](#) for the investigation of conformational plasticity and dynamics of the peptidyl-prolyl *cis-trans* isomerase SlyD.

NMR spectroscopy is used to obtain detailed structural information for the whole system and, in addition, can yield information on the dynamics of specific parts of the structure. The spin of protons is the property which causes the nucleus to produce NMR signal. Specific nuclei, called NMR active, that is, ^{15}N and ^{13}C , when they are found under a magnetic field absorb radiation at a characteristic frequency and migrate to a higher spin energy level. When spin returns to the basic level, energy is emitted and this signal can be recorded and processed to generate the (NMR) spectrum. Protein NMR spectroscopy is the use of NMR phenomenon to extract structural information. The protein structure determination by this method is a process which results in a convergent ensemble of structures.

Molecular motions generate local fluctuating magnetic fields which have, as a consequence, NMR relaxation. Relaxation times can be measured and used to determine parameters as correlation times and chemical exchange rates which yield information on the dynamics of protein parts as the backbone or side chains. Motions which can be detected occur on the time scale of 10 ps to 10 ns but even slower motions between 10 μs to 100 ms can be studied.

NSE spectroscopy is an inelastic neutron-scattering technique with recent applications to the study of protein dynamics ([Mezei, 1980](#)). It is a technique of high effective energy resolution which determines the time-space correlation function at the temporal scales from nanoseconds to microseconds and at the spatial scale from several Angstroms to some hundreds. NSE is used to determine relaxation processes in a macromolecule, that is, internal dynamic modes and it has the potential to determine the global shape fluctuations and domain motions. NSE has been applied to the NHERF1 multidomain protein to distinguish and characterize couple domain motions which are involved in the dynamic propagation of allosteric signals at the nanoseconds timescale ([Farago et al., 2010](#)). It is shown that NSE can be used to determine the domain mobility tensor

which determines the degree of dynamical coupling between domains. [Bu et al. \(2005\)](#) have used the method to determine internal coupled domain motions within DNA polymerase I from *Thermus aquaticus*.

The hydrogen–deuterium (H/D) exchange is a chemical reaction. A covalently bonded hydrogen atom is replaced by a deuterium atom from the solution. Usually the examined protons are the amides in the backbone of a protein. The method gives information about the solvent accessibility of various parts of the molecule and detects global or local unfolding on time-scales of milliseconds and longer. It is monitored by NMR and/or mass spectrometry. The conformational changes that occur in factor XIII due to monovalent and divalent ion binding has been recently reported by [Woolfer and Maurer \(2011\)](#). The H/D exchange effects observed in the presence of a wide range of ions in different conditions and the deuterium incorporation was analyzed by MALDI-TOF MS. Moreover, [Oyeyemi et al. \(2011\)](#) have applied the technique to elucidate the relationship between flexibility and thermal adaptation in the case of dihydrofolate reductases.

D. Computational Methods

Biomolecular simulations are an important technique for characterizing protein conformational changes ([Klepeis et al., 2009](#)). The advantage of the computational methods is that one can follow the details of the protein dynamics in the pico-second timescale and examine the structural features in the atomic level. The disadvantage is that without experimental validation of the potentials used in the simulations, the predictions are on the risk of questioning. The starting point is a high-resolution structure determined by X-rays or NMR. A prerequisite is also the availability of computational power. Then, an empirical force field, which is a set of parameters describing the potential energy of the system together with the equations of motion, is applied to the system and the successive positions of all the atoms of the system, and thus the progressive motion of the molecule, can be watched. The computational demands prevent the method from reaching timescales greater than milliseconds. To achieve longer timescales, simplified models can be used as the implicit solvent or the coarse-graining models.

Molecular dynamics simulations apply empirical molecular mechanics potential energy functions which are suitable for studying conformational changes and dynamics as the conformational changes during the catalytic cycle, associated with substrate binding and product release as well as

fluctuations around an average structure. However, they cannot be applied to model chemical reactions, so cannot investigate enzyme reaction mechanisms directly. To study the connection between conformational and chemical changes as well as to investigate the hypothesis that protein motions accelerate the reaction rates of catalysis, the quantum mechanics/molecular mechanics (QM/MM) methods are used. An analytic overview of how the computational methods have been used to increase our understanding of the dynamical aspects of enzymatic catalysis has been presented by [McGeagh et al. \(2011\)](#).

III. DETERMINANTS OF ACTIVE SITE FLEXIBILITY

Enzymes are generally characterized by conservation of their functional groups through evolution. On the other hand, the flexibility requirements of many active sites frequently impose a relaxation of strict conservation so that different functional residues are not conserved during evolution while converging toward the same mechanistic role. The resulting plasticity of active sites has been reviewed by [Todd et al. \(2002\)](#). In principle, upon catalysis, residues in and around the active site must undergo conformational changes associated with the binding and release of substrates and cofactors, the protection of the reaction space from the aqueous environment, stabilization of reaction intermediates, and interactions between catalytic residues and binding subsites with various groups of reactants upon completion of catalysis. In some cases, the structural requirements for conformational flexibility are limited to individual amino acids especially to side chains capable of adopting different rotamers. In other cases, the interactions of the macromolecule with substrate and cofactors require conformational changes mainly involving the external loops located in the periphery of the active site. On the other hand, several times the required structural reorganization associated with catalysis involves large global movements and rearrangements of whole protein domains.

A. *Flexible Active Site Loops*

Loop regions belong to the most flexible parts of protein structures. In crystal structures, they are characterized by higher than the average temperature factors and display the highest conformational variability among equivalent regions of homologous protein structures. In some cases, they correspond

to segments of weak or missing density in electron density maps because they fail to scatter X-rays coherently due to their pronounced disorder. The conformational variations of loop regions become evident in NMR structural ensembles, which show a multitude of different conformations for the same loop. The high mobility of loops has been frequently confirmed by trajectories of molecular dynamics simulations.

The extreme flexibility of loops which reflects the absence of conformational constraints is consistent with their low sequence conservation, an indicator of low evolutionary pressure. A pronounced exception to this conservation rule, are the loops participating in the active sites of enzymes. The critical role of loops for the enzymatic function was recognized early and their conformational transitions are now accepted as key events in catalytic processes (Malaban *et al.*, 2010). Such loops, commonly called “lids” or “flaps”, have been the subject of investigation by many research groups. Their location at the entry of active site plays a major role in substrate selectivity and recognition and facilitation of substrate binding into the binding cleft. The structural comparison of apo- and holo-states of various enzymes highlights that the main structural difference between the two states is the different conformations of active/binding site loops. At the unbound state, the flaps adopt an open conformation which leaves the binding cleft and active site accessible to solvent. In the bound state, the flaps adopt a closed conformation which blocks the entry to the active site. A characteristic example is the 60/70 loop of the zinc-dependent endopeptidase BoNT/A LC (Thompson *et al.*, 2011). Binding of three different inhibitors induces a consistent change in the conformation of this loop as a result of the hydrophobic interaction of a loop residue (Pro69) with an aromatic moiety of the inhibitor (Fig. 1A). Compared to the uncomplexed enzyme, ligand binding induces a more compact conformation because the loop is pulled into the active site enclosing more tightly specific subsites of the inhibitor and inducing a more effective inhibition. Thus a disordered, solvent-exposed loop adopts upon substrate binding a more compact and ordered conformation-making interactions with subsites of the ligand and/or other residues of the protein. The closing of the flaps traps the ligand into the cleft and shields fully or partially the ligand molecule from the aqueous environment, thereby stabilizing the bound state. Moreover, the access of the active site to other molecules is prevented and the reaction intermediates are protected and stabilized (Kember, 1993). This mechanism provides an effective way

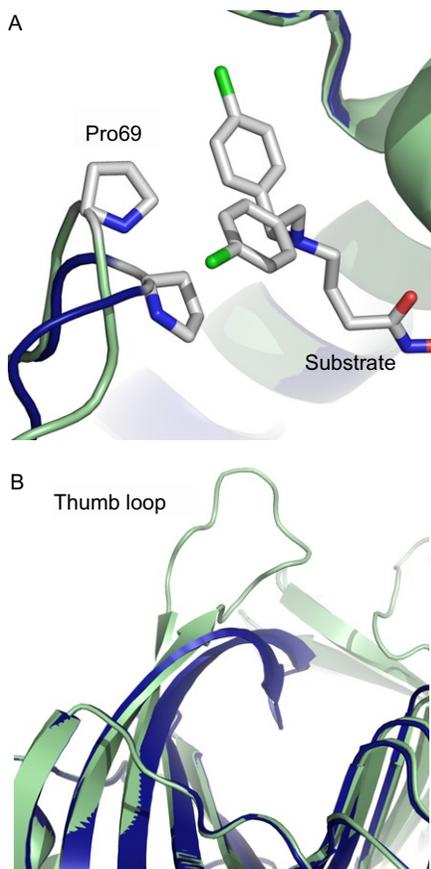


FIG. 1. (A) Structural comparison of the active site, 60/70 loop of the zinc-dependent endopeptidase BoNT/ALC at the apo- (green) and holoenzyme states (blue). Hydrophobic interactions of Pro69 with the substrate, which is also shown by stick representation inside the active site, induce a more compact protein conformation by pulling the 60/70 loop toward the active site (PDB entries 3QIX and 3QIY). All figures have been prepared by PYMOL. (B) The “thumb” loop is the most flexible part of the structure of glycoside hydrolase family 11 xylanases. Here, two conformations of this loop, the open (green) and closed one (blue) are shown (PDB entries 3EXU and 2B46).

to control the accessibility to the active site. After the end of the reaction, the opening of the loops permits release of the product and the beginning of a new catalytic cycle. For example, in the glycoside hydrolase family 11 xylanases, a highly conserved “thumb loop” in the proximity of the active site has been found in three conformational states, a closed, a loose, and an extended open one (Pollet et al., 2009; Fig. 1B). Molecular dynamics simulations suggest that the thumb is the most flexible part of the xylanase structure: a dynamic catalytic cycle has been proposed based on these three different conformations of the thumb. These conformational changes are directly associated with the binding of the substrate and the release of the product. Moreover, a mutation which hinders the thumb movement results in a fourfold decrease of turnover number thus suggesting a direct relation between thumb movement and catalytic properties.

Flaps are not the only flexible structural elements around active sites. Quite often, the rearrangement of the active site geometry is accompanied or facilitated by movements and conformational changes of secondary structural elements directly adjacent to the active site, for example, helices which frequently precede or follow the flaps. Examples are presented at the following paragraphs for the cases of EryK P450 cytochrome and *Bc*ZBP deacetylase.

At least two models are used to describe and mechanistically explain the transition from the open to the closed conformation. The first of them, the well-known “induced fit” model, postulates that large conformational changes at a local level, and the transition to the closed conformation are induced by substrate binding into the active site cleft. In a number of examples, the unbound enzyme state has as a favored conformation the open one and the bound enzyme state, the closed one. The extensively studied loop 6 of triosephosphate isomerase (TIM) is a typical case interpreted through the “induced fit” model (Lolis and Petsko, 1990). TIM is a homodimer in which the subunits catalyze the interconversion of D-glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. Each subunit is a classical ($\beta\alpha$)₈ barrel (Branden and Tooze, 1999) with the active site being formed by the loops located at the C-termini of the eight β -strands (loops 1–8) of the barrel. A detailed structural comparison of the unbound and bound forms revealed that the only significant difference is the movement of the 10-residue loop 6 resulting in the blockage of the active site entry at the bound state. Some residues of the active site loop are moved up to 7 Å from their original position.

However, in some cases, experimental evidence supports as an alternative a “conformational selection” scenario in which the enzyme preexists in two alternative and stable conformations which differ in their ability to bind ligands. This concept has been reported for the monomeric cytochrome P450 (CYP) from *Saccharopolyspora erythraea*, commonly called EryK (Savino et al., 2009). X-ray crystallography studies revealed two different structures, an open and a closed one, which were determined for the unbound state of EryK in different ionic strength conditions. Characterization of binding kinetics of the ErD substrate to EryK could be sufficiently explained by the “conformational selection” model and allowed for the exclusion of the “induced fit” model. Comparison of the two structures revealed conformational shift involving among others a movement of an active site loop of about 11 Å and a shift of the N-terminus of an α -helix of about 10 Å toward this active site loop. This reorganization closes the access channel for the active site.

The rigid body movement observed for the TIM active site loop 6 is the most common type of loop motion in general, and is termed “hinge-bending motion” because the loop is linked to the protein through two regions known as hinge regions and moves around them as a rigid body. For α -helices, a common movement is rotation around one of their ends which moves the other end away from its original position, occasionally up to several Angstroms. The high flexibility of the loops is often associated with the presence of glycine residues while in hinges alanines and prolines are also quite common. Mutagenesis has been frequently used to probe the significance of loop flexibility for catalysis with hinge residues being quite often the targets of such experiments. In the case of glutathione synthetase (Tanaka et al., 1993), replacement of Gly and Pro in the hinge by Val significantly impaired the enzymatic activity. Structural studies show that the mutations caused no structural alterations to the rest of the structure. Absence of electron density indicated that a high degree of flexibility has been retained for the mutant loop as well as for the native one. The observed loss of catalytic activity could reflect problems in conformational adaption of the mutant loops in the closed state which results in disruptive interactions with the bound substrate. A study by Kursula and coworkers (2004) demonstrated by mutagenesis the importance of small residues at position 3 of the C-terminal three-residue hinge (LysThrAla) of TIM loop 6. When the small Ala residue at position 3 was replaced by bulky residues (Gln, Leu, or Lys), the unliganded form of the

mutants resembled the closed conformation. The results suggest that these mutations shift the equilibrium of the oscillation motion of loop in favor of the closed conformation. Kinetic data implies that in these mutants substrate binding is the rate-limiting step.

Except from hinge residues, the importance of residues in the middle of loops for active site flexibility has also been investigated. When Gly76 of the active site loop (residues 71–80) of pepsin was replaced by Ala, Val, and Ser, a lower catalytic efficiency was measured and interpreted as the result of lower flap flexibility. A lower flexibility could alter the pattern of interactions (hydrogen bonds) which are responsible for substrate alignment in the active site (Okoniewska et al., 2000).

In conclusion, mobile loops in the proximity of active sites frequently play various roles in catalysis and it is widely accepted that their pronounced conformational flexibility may contribute to their functions. Mutagenesis experiments support the concept of this flexibility being essential for catalytic efficiency and activation processes.

B. Active Sites Located at Domain Interfaces

A significant portion of oligomeric enzymes form their catalytic and binding sites at the interface between subunits while, in the case of monomeric enzymes, active and binding sites often lie between domains (Ali and Imperiali, 2005). Among the most well-studied examples of enzymes with their active/binding sites formed between domains are the alcohol and glutamate dehydrogenases, citrate synthase and hexokinase, glutathione transferases and ribonuclease A. An obvious consequence from the location of active sites in the interdomain or intersubunit interface is that even subtle local conformational changes between the domains, which are commonly called lobes, could affect catalysis and enzyme interaction with substrate and products. A model consistent with many experiments and with the Koshland's (1958) "induced fit" model implies that the ligand is first bound to one lobe; subsequent movement of the other lobe brings it near to the ligand permitting additional interactions which stabilize the structure (Stillman et al., 1993; Hayward, 2004).

Lesk and Chothia (1984) classified domain motions as hinge or shear domain motions. In the former case, the two domains are connected through a short extended and flexible peptide fragment, the hinge region. Hinge permits rotations while the lobes move as rigid bodies with

their internal structure being preserved and all the deformations restricted to the hinge region. As a result, the binding cleft may exist in many different intermediate states between the open and the closed ones. On the other hand, the shear motion is generated by small rearrangement motions, that is, small changes in torsion angles and side-chain motions causing small shifts of secondary structure elements with respect to the original position. The shear domain closure is thus the cumulative effect of relatively small shifts of loosely packed secondary structures. This type of motion is more likely to occur when there is an extended surface between domains. Domain motions around the active sites of citrate synthase and hexokinase have been grouped with this type of motions.

Large-scale domain motions that can be classified as hinge motions were observed for the homodimeric restriction endonuclease PvuII in connection with the binding of cognate oligonucleotides. The U-shaped dimer exists in an open (Athanasiadis et al., 1994) and a closed, DNA-bound conformation (Cheng et al., 1994). Each PvuII monomer consists of a DNA-binding subdomain which also carries the catalytic site and a helical dimerization subdomain. Although the structures of the individual subdomains are highly conserved in the open and the closed conformation, their relative orientation changes upon DNA binding, with the opening of the DNA-binding cleft being reduced by nearly 28 Å upon transition from the open to the closed conformation. This large conformational change results mainly from small changes ($<10^\circ$) in the φ, ψ backbone conformational angles of two Gly residues located in a loop region at the interface between the dimerization and DNA-binding subdomains.

Alcohol dehydrogenases are present in many different organisms and catalyze the conversion of alcohols to aldehydes functioning as dimers or tetramers (Lesk and Chothia, 1984). Each subunit consists of a catalytic and a coenzyme-binding domain which are linked together through two α -helices (termed α_2 and α_3). Comparing the crystal structures of the apo- and holo-liver alcohol dehydrogenase, Eklund et al. (1981) described the conformational changes between these states as a rigid body rotation of one domain relative to the other one. The two hinge regions were determined at the α_2 , α_3 helices and was found to consist of three and four residues, respectively. Colonna-Cesari and co-workers (1986) applied empirical energy functions to simulate the domain rotation. The analysis showed that most of the hinge residues undergo small changes at their main chain, φ/ψ angles ($<15^\circ$) while motion of the side chains with

variations in χ torsion angles of about 50° was observed. The internal dynamics of the enzyme was investigated by small angle neutron scattering and NSE spectroscopy and confirmed a large-scale correlated domain motion (Biehl et al., 2008). By normal mode analysis, this motion can be understood as the result of the movement of the outside catalytic domain with respect to the rigid core. Moreover, this motion results in a cleft opening which is much larger than the relatively narrow pocket observed in the crystal structures.

RNase A is a model system which has been used in numerous experiments for the study of dynamical properties of proteins and it is represented in PDB with more than a hundred deposited structures (e.g., see Wlodawer et al., 1988; Santoro et al., 1993; Toiron et al., 1996; Leonidas et al., 1997). It is a stable monomeric enzyme which catalyzes the cleavage of the P—O⁵ bond in single-stranded RNA. Its structure consists of two all-beta domains which form a V shape and are linked by a hinge. The binding and active sites are formed at the interface between the domains with the active site located at the bottom of the deep cleft. Petsko and colleagues have shown that the enzyme is unable to bind the inhibitor cytidine 2'-monophosphate (2'-CMP) at temperatures lower than -50°C (Rasmussen et al., 1992). Since no structural differences could be noticed comparing the crystal structures at cryo temperatures and room temperature, they suggested that flexibility is a required property for binding and catalysis (Tilton et al., 1992). Vitagliano and co-workers (2002) could observe in the crystal a significant, reversible, hinge-bending domain motion upon ligand binding which leads to a more compact structure. Upon substrate binding, the hinge region is moved, the angle between the domains decreases, and residues belonging to different domains and involved in substrate binding present a significant reduction of their between distances.

C. Flexibility of Active Site Residues

Active site residues play many different roles. They may be involved in catalysis and substrate binding, stabilize the intermediates of the reaction or the structure of the binding cleft. They provide the suitable for the catalysis microenvironments and enable substrates to form enough contact points for strong binding. Some degree of flexibility is inevitable for the active site residues to achieve their functions and accommodate the

conformational changes which are necessary during the catalytic cycle. This flexibility facilitates active site rearrangements during the numerous intermediate steps of the reaction. The flexibility is achieved at a cost, usually in form of local strain or instability. A systematic mutational analysis of active site residues of barnase protein was one of the first studies to demonstrate that there is an inverse relationship between stability of active site and activity (Meiering et al., 1992).

The side chains of catalytic residues usually adopt more than one conformation although only some of them may be catalytically competent. In addition, their conformational flexibility has been related to specific steps of the catalytic cycle as, for example, for the proton shuttling. A characteristic example comes from the hydrolytic aldehyde dehydrogenases (ALDHs). Analysis of the crystal structures of several different ALDHs from more than nine different organisms determined in the absence or presence of substrates, cofactors, and products showed that although these proteins do not undergo major conformational changes upon binding of cofactors, they exhibit a high degree of flexibility for the catalytic residues Cys302 and Glu268 (González-Segura et al., 2009; Munoz-Clares et al., 2011; Fig. 2). In particular, Cys302 has been found in two conformations called “resting” and “attacking” conformation, which are far and close to the carbonyl carbon of the bound aldehyde, respectively. The catalytic Glu268 adopts three conformations: (i) the “inside” conformation which activates Cys302 for nucleophilic attack, (ii) the “intermediate,” which activates the hydrolytic water molecule, and (iii) the “outside” conformation which releases the proton taken from Cys or water through a proton relay mechanism. Examination of the various residue conformations with respect to the binding cleft architecture in the presence and absence of the cofactors reveals the sterically compatible combinations and helps rationalize the details of the mechanism.

Another recent example comes from the crystal structures of TIM protein complexed with suicide inhibitors (Venkatesan et al., 2011). It is shown that two residues of the active site, the catalytic Glu167 and Glu97, are flexible and can adopt two different conformations when the enzyme exists in the closed liganded state. The differences are mainly associated with changes in the side-chain dihedrals which give rise to two active site geometries. Thus, when the (*S*)-glycidol phosphate ((*S*)-GOP) is bound in the active site, Glu167 adopts its well-known competent conformation and Glu97 is salt bridged. When (*R*)-glycidol phosphate ((*R*)-GOP) is bound

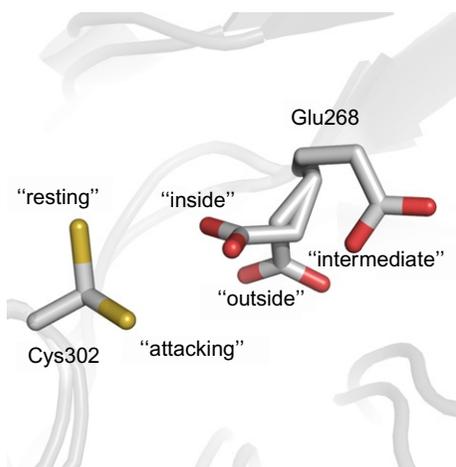


FIG. 2. Local flexibility of the active site residues of aldehyde dehydrogenases is demonstrated by the multiple conformations of Cys302 and Glu268. As it is shown, Cys302 can adopt two conformations called “resting” and “attacking” while Glu268 adopts three, the “inside” “intermediate” and “outside” conformations. Combinations of these conformations determine different stages of the catalytic cycle (PDB entries 1UZB and 1O02).

both residues adopt unusual conformations. The first geometry enables Glu167 to attack the terminal carbon of (S)-GOP in a stereochemically favored (linear) arrangement, but this is not possible for (R)-GOP at the second geometry. These could explain the higher chemical reactivity of (S)-GOP compared with (R)-GOP.

D. A Specific Example: Flexibility of the BcZBP Deacetylase

BcZBP is a zinc-dependent deacetylase from *Bacillus cereus* whose crystal structure has been reported at the resolution of 1.8 Å (Fadouloglou et al., 2006, 2007). The biological pathway and the function of the protein were unknown until recently when the ortholog from *Bacillus anthracis* *BaBshB* (with 97% sequence identity) was identified as a deacetylase involved in the bacillithiol biosynthesis (Newton et al., 2009; Parsonage et al., 2010). *BcZBP* is a hexamer and possesses six structurally equivalent active sites which are formed by the association of two monomers. X-ray crystallography,

mutagenesis, and molecular dynamics simulations have been used to elucidate aspects related with function and flexibility of the protein.

B-factor analysis of the crystal structure and structural superposition of different states of the enzyme demonstrates a high mobility for the three active site loops (42–51, 129–140, and 180–192) and highlights changes of the active site cavity between states. Structural comparison of *BcZBP* with the homologous TT1542, which shares 38% sequence identity and has been used as the search model for the molecular replacement which led to the *BcZBP*'s structure, locates the greater differences between the enzymes in the immediate environment of the active sites. In the case of *BcZBP*, the N-terminus of $\alpha 2$ -helix has been moved 4 Å closer to the active site together with the preceding loop (42–51) which has been shifted toward the top of the active site. Because *BcZBP* represents the structure of apoenzyme and TT1542, the structure without the zinc bound in the active site, we suggest that the observed differences highlight a zinc-induced organization of the active site environment by promoting a more closely packed conformation. On the other hand, the temperature factors of crystal structures demonstrate as the most flexible part of both enzymes the active site loop 180–192, joining helices $\alpha 5$ and $\alpha 6$.

A 50 ns molecular dynamics simulation study ([Fadouloglou et al., 2009](#)) supports the X-ray crystallography findings. The analysis resulted in an agreement between the simulation-derived atomic fluctuation and the crystallographically determined atomic temperature factors. Thus, the three loops which frame the active site, have atomic fluctuations which are significantly greater than the average for the rest of the structure demonstrating their high flexibility. The trajectory also reveals a concerted loop motion, which generates the effect of a breathing-like motion around the active site with successive opening and closing events.

The functional significance of the active site loops flexibility was further investigated by mutagenesis of the hinge residues Arg140 and Ala42 ([Deli et al., 2010](#)). Arginine 140 is located at the rim of the substrate-binding cleft. In the crystal structure, its side chain adopts two distinct conformations. The one conformer blocks the active site's entry while the other one, which is stabilized by electrostatic interactions, keeps the active site accessible. Arg was replaced by the small, hydrophobic Ala residue and by the oppositely charged Glu residue. In both variants, the ability for distinct conformations at position 140 has been disrupted. Both variants showed a decrease in their catalytic efficiency compared with the wild type. Ala42,

on the other hand, which is located in the proximity of the substrate was replaced by a Ser residue. Ser is able to form putative hydrogen bonds either with the substrate or with adjacent amino acids. With either of these ways, the mutation is predicted to increase the rigidity or even to trap the loop to a closed-like conformation. Indeed, the produced variant exhibited a dramatic reduction of efficiency.

IV. FLEXIBILITY AND SPECIAL ASPECTS OF ENZYME PROPERTIES

A. *Flexibility and Thermal Enzymatic Adaptation*

Given the environmental variations at the different areas of earth, many organisms have evolved adaptation mechanisms for very low or very high temperatures. As a consequence, there are enzymes isolated from such organisms whose optimal temperature for function is below 10 °C (psychrophilic) or above 45 °C (thermophilic). Catalytic residues are generally conserved in homologous psychrophilic and thermophilic enzymes which imply that cold adaptation resides on other parts of the structure. In the effort to elucidate the molecular basis of enzymatic temperature, adaptation was observed that the cold adaptation is related with a reduced thermal stability (cold adaptation is reviewed by [Siddiqui and Cavicchioli, 2006](#)). This led to the hypothesis that optimization of the catalytic activity at low temperatures may be associated with an increased structural flexibility ([Feller and Gerday, 1997](#)). Since psychrophilic enzymes must function in low temperatures, the evolutionary pressure to retain structural features responsible for conformational rigidity (e.g., disulfide and salt bridges) is relaxed. In the absence of conformational constraints for stability, they present an increase in flexibility which possibly leads to a reduction of the activation energy ([D' Amico et al., 2002](#)). In other words, the increased active site flexibility results in a higher number of conformational states of the enzyme/substrate complex. The energy of activation is used many times as a criterion for the evaluation of psychrophilicity as it is usually lower in the cold-adapted enzymes than to their mesophilic counterparts. Sequence comparison among homologues from thermophilic, mesophilic, and psychrophilic organisms shows that generally in the cold-adapted enzymes ([Siddiqui and Cavicchioli, 2006](#)): (i) buried residues tend to be smaller and less hydrophobic, (ii) surface

residues tend to be less polar, probably because polar residues confer stability by forming additional intramolecular H-bonds. (iii) Less Ile residues are found, probably due to their good packing properties inside the protein cores, (iv) Lys residues usually replace Arg which facilitate a greater number of electrostatic interactions and H-bonds over Lys, (v) a less number of salt bridges are observed. Radestock and Gohlke (2011) probed by computational means the corresponding states hypothesis which claims that homologues from different thermal-adapted organisms are in corresponding states of similar rigidity and flexibility at their respective optimal temperatures. Comparing a sample of 19 pairs of homologues from meso- and thermophilic enzymes, they showed that adaptive mutations of thermophilic enzymes maintain the balance between overall rigidity important for thermostability and local flexibility important for activity at the respective temperature at which the proteins function.

In several studies, the psychrophilicity has been related to an overall flexibility throughout the protein structure. Studies on the α -amylase from *Pseudoalteromonas haloplanktis* have shown that the cold adaptation strategy for this enzyme leads to a uniformly unstable protein (Feller et al., 1999). Site-directed mutagenesis and comparison with mesophilic and thermostable α -amylases demonstrated a weakening of intramolecular interactions which lead to an overall decrease of the thermostability of the psychrophilic protein. This provides the appropriate plasticity around the active site, necessary to adapt the catalytic efficiency to low temperatures. In the case of Zn-metalloproteases of thermolysin family, an optimization of the overall protein flexibility is achieved via the reduction of the hydrogen bonds stability in the dynamic structure due to a decrease of amino acids which form hydrogen bonds (Xie et al., 2009).

However, the need for a global elevated flexibility was questioned and another cold adaptation model was proposed which implies that the requirement for increased structural flexibility can be limited only to a small, crucial region of the protein structure and, in particular, around and inside the active site (Fields and Somero, 1998). Many studies present evidence which supports this model. Comparing A4-lactase dehydrogenases from Antarctic (optimum temperature of function -2 to 1 °C) and South American (4 – 10 °C) notothenioid species was found that the active site residues are fully conserved. Combination of kinetic, sequence, and structural data suggested that cold adaptation is based on increasing

flexibility in small areas of the molecule, outside the active site, that affect the mobility of adjacent to active site structures (Fields and Somero, 1998). The work of Watanabe and Takada (2004) demonstrates the importance of amino acid substitutions around the active site to the thermal adaptation of isocitrate lyases. Gln207 and Gln217 of the psychrophilic lyase from *Colwellia maris* were replaced by a His and a Lys, respectively, which are the residues occupying the equivalent positions at the lyase from *Escherichia coli* and other organisms and are essential for catalysis. The catalytic activity at low temperatures is decreased or diminished respectively while the enzyme remains active at moderate temperatures. It is believed that each of the two residues affects the thermal adaptation through a different mechanism since the Gln217Lys replacement is associated with structural changes expressed as an increase in the thermostability and changes in the CD spectra (enhancement of α -helical components) while the Gln207His replacement resulted in slight conformational changes. Comparative molecular dynamics studies show that the strategy of members of elastase family for being adapted at cold also concerns increase of localized flexibility (Papaleo et al., 2006; Riccardi and Papaleo, 2010). The most obvious difference between mesophilic and psychrophilic elastases concerns the amino acid composition and flexibility of loops which are clustered around the active site and specificity pocket.

The importance of local flexibility into the active site for cold adaptation has been demonstrated in several cases. The psychrophilic alkaline phosphatase from the Antarctic strain TAB5 has been used as a model system, and the possibility of modifying its psychrophilic properties by introducing—via mutagenesis—predictable flexibility changes to key active site residues (Tsigos et al., 2001) or residues to the direct vicinity of active site (Mavromatis et al., 2002) has explored. Tsigos et al. (2001) have modified the side-chain flexibility of the catalytic residues Trp260 and Ala219 and that of His135 from the Mg^{2+} binding site. Trp260Lys is less active than the wild type at low temperatures, while the double mutant Trp260Lys/Ala219Gln has lost its psychrophilic character, although its activity at elevated temperatures exceeds that of the wild type. Finally, substitution of His135 by Asp resulted in stabilization of the enzyme and in the case of the triple mutant restored a low energy of activation. Thus, the psychrophilic character of an enzyme can be strongly affected by very slight variations of its amino acid sequence which however is expected to drastically change the local flexibility. As it was mentioned above,

psychrophilicity has also been related with increased levels of Gly residues which ensure local flexibility. Psychrophilic alkaline phosphatase positions 261 and 262, in the vicinity of active site are occupied by Gly residues. [Mavromatis et al. \(2002\)](#) have investigated their importance in the establishment of the psychrophilic character of the enzyme. To constrain the conformational flexibility of the main chain with the minimum perturbation of the local structure, it was chosen to mutate Gly to Ala. The Gly262Ala mutant is completely inactive while the Gly261Ala has lost the psychrophilic character although it is active at elevated temperatures. Thus, Gly clusters in the vicinity of active sites in combination with their structural environment are frequently essential determinants of the psychrophilic character.

B. Flexibility as an Essential Component of Enzymatic Allostery

Allostery is the process by which conformational changes at one site of a protein (called regulatory or allosteric site) are coupled with changes to a different and usually distant functional site (active site). The most common way for triggering the allosteric response of an enzyme is the binding of a specific allosteric effector molecule which could be an activator or an inhibitor of the enzymatic function. Studies mainly by X-ray crystallography showed that the functional states of an allosteric enzyme are well represented by two, structurally different forms: (i) the “R” form (relaxed), which has an optimal affinity for the substrate and (ii) the “T” form (tensed), which has a minimal affinity. Attempts to explain the nature of the allosteric transition have led to two models, the so-called concerted and sequential models. Both of them have been developed under the condition that allosteric enzymes are symmetric oligomers with identical protomers. Each subunit in the oligomer can adopt one of the “R” or “T” conformations. According to the concerted model ([Monod et al., 1965](#)), the conformational changes induced by the binding of an allosteric effector to one subunit is transmitted to all other subunits, thus switching them to the same conformation, corresponding to one of the two possible states, either “T” or “R”. On the other hand, the sequential model ([Koshland et al., 1966](#)) permits a number of different global states, that is, subunits can change conformations independently and alterations to one of them are not necessarily transmitted to the others. Thus, the oligomer could be found in a number of hybrid “TR”

combinations. A relatively new idea for rationalizing the allosteric regulation is based on the concept that allosteric enzymes, as all proteins, exist as a dynamic ensemble of conformational states. According to the ensemble model of allostery, ligand binding to the allosteric site leads to redistribution within the ensemble enabling an altered conformation at the region around the active site (Gunasekaran et al., 2004; Wrabl et al., 2011). The shift in the relative populations of the protein may affect protein function, that is, as the distribution substrate affinities are altered within the conformational ensembles (Goodey and Benkovic, 2008). The dependence of protein function on conformational flexibility favors an expanded view of allostery as an intrinsic property of all dynamic proteins which are potentially allosteric. Based on this idea and in contrast to what was believed in the past, allostery is recognized now as a property of monomeric proteins as well (Gunasekaran et al., 2004). The binding of the effector favors the transition from the relatively rigid inactive T-form to a more flexible active ensemble and the increased flexibility facilitates the conformational transitions during enzyme turnover. Hilser and Thompson (2011) have used the ensemble concept of allostery to discuss the behavior of steroid hormone receptors which are much more dynamic systems than is represented by traditional models. Thus, a dynamic ensemble of structures and the presence of intrinsically disordered segments which are stabilized upon ligand binding can explain the broad variety of ligands which drive remote allosteric responses. The importance of this model to the explanation of cooperativity of the bacterial flagellar switch was recently discussed by Bai et al. (2010). Novinec et al. (2010) showed by intrinsic fluorescence spectroscopy that Cathepsin K, a human cysteine peptidase, has a flexible structure which converts slowly among distinct conformational states. Addition in a protein sample of glucosaminoglycans, which are allosteric regulators of Cathepsin K, causes a change in the enzyme conformation resulting in a rapid binding of the inhibitor stefin A. So, it is proposed that glucosaminoglycans affect the distribution of the preexisting conformational equilibrium.

An intuitively very appealing mechanism for the coupling of the effector binding to one site with the conformational changes to another site is the existence of amino acid networks (Goodey and Benkovic, 2008). According to this model, propagation of the signal through a protein structure is based on networks of physically interconnected and thermodynamically linked residues. Amaro et al. (2007) combining data from crystallography,

biochemical kinetic assay, and molecular dynamics simulations have revealed a network of interactions directly correlated to the transmission of the allosteric signal of the imidazole glycerol phosphate synthase. This network is formed by a set of highly conserved amino acids which lead from the allosteric site of the enzyme to its active site, more than 25 Å apart. A correlated motion analysis confirms the involvement of these residues to a stream of coupled motions and their role in the allosteric response.

Two examples which clearly demonstrate that structural flexibility is an essential component of allosteric activation are those of glucosamine-6-phosphate deaminase (Bustos-Jaimes et al., 2002; Rudino-Pinera et al., 2002) and of the human hexameric UDP-glucose dehydrogenase (Kadirvelraj et al., 2011; Sennett et al., 2011). Glucosamine-6-phosphate deaminase catalyzes the reversible isomerization–deamination of glucosamine 6-phosphate and it is allosterically activated by the N-acetyl glucosamine-6-phosphate. Comparing a variety of structures Rudino-Pinera et al. (2002) demonstrated that the main structural differences between the T- and R-states are located at the active site lid. This enzyme possesses a complex active site lid (residues 158–187) formed by a helix–loop segment and a β -strand (Fig. 3A). The lid is directly connected with both, the active and allosteric sites, that is, Arg172 is an active site residue which participates at the substrate binding while Arg158, Lys160, and Thr161 belong to the allosteric site. Experiments support the correlation of the lid flexibility with the allosteric transition and the substrate-binding properties of the active site. It was shown that the allosteric transition from the “T” to “R” state is not associated with defined geometrical changes of the lid but with pronounced changes to its conformational flexibility. Especially, the atoms in the central segment of the lid show a marked decrease in their crystallographic B-factor from a B average of 80 at the T-state to a B average of 40 Å² at the R-state. Moreover, when the enzyme is at the R-state, the active site lid has been found in three distinct conformations (Rudino-Pinera et al., 2002). Substrate binding stabilizes the lid in one of the three conformers and produces a general reduction in the atomic vibration of the whole protein.

The connection of the conformational flexibility of the lid and the function of the deaminase was investigated by mutating Phe174 to Ala. This mutation had, as a result, such an increase of the conformational flexibility of the T-form that no electron density was visible for part of the lid (Bustos-Jaimes et al., 2002). The mutant was inactive in the absence of

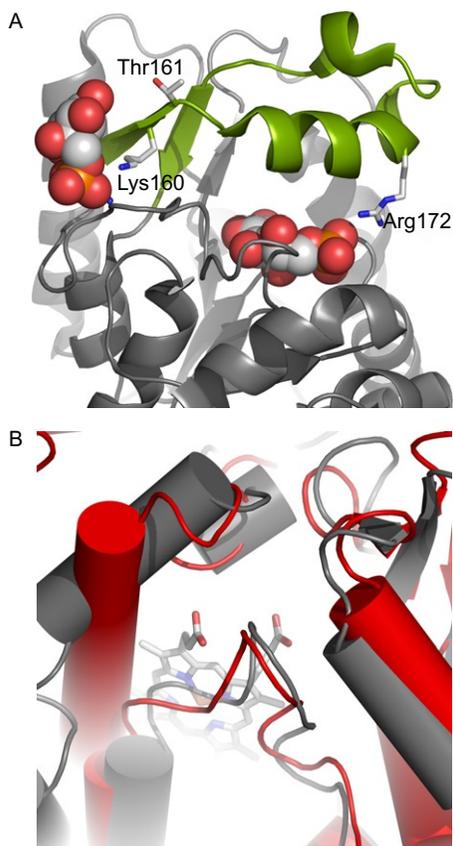


FIG. 3. (A) Cartoon representation of the glucosamine-6-phosphate deaminase. The enzyme possesses a complex active site lid (shown in green) which is directly connected with both, active and allosteric sites. To illustrate the location of these sites, a glucosamine 6-phosphate (space-filling model) is shown bound to each of them (PDB entry 1FS5). (B) Structural comparison of the P450 cytochromes CYP101 (P450cam, PDB entry 2L8M) in gray from *Pseudomonas putida* and CYP108 (P450terp, PDB entry 1CPT) in red, a bacterial enzyme from *Pseudomonas*. The enzymes adopt a conserved fold with a substantial variability around the substrate-binding region. The molecule of heme is shown as stick representation. Small displacements of structural elements which abuts the active/binding toward the heme together with the segmentation of one of the helices (shown on the left of the figure) decreases the size of the active/binding site of CYP101 relative to that of the CYP108.

the allosteric activator, a proof that, conformational flexibility of the active site lid alters the binding properties of the active site.

Except from the active site lid, the 144–154 loop has been shown to be directly associated with the allosteric transition through interactions of Glu148 with residues of the active site lid, which stabilize the R-form or the allosteric site, that is, a salt bridge with Lys 160, which stabilize the T-form. Structural superposition of R-form complexed with either both sites occupied or only the allosteric site shows that there is a correlation between the movement of 144–146 part and the active site lid. When the active site is free, the 144–146 part moves to open the lid and when the active site is occupied moves to close the lid.

The human hexameric UDP-glucose dehydrogenase is an example of an allosteric enzyme whose active site is bifunctional and can bind either substrate or allosteric effector with distinct “induced fit” conformations (Kadirvelraj et al., 2011). In this case, it was shown that packing defects in the protein core in combination with structural flexibility constitute a mechanism for the evolution of allostery. Binding of the UDP- α -D-xylose (UDX) allosteric inhibitor activates a distinct “induced fit” allosteric response: The buried Thr131 loop directly connects the hexamer building interface to the active site and plays the role of an allosteric switch. Upon substrate binding is positioned in a way which supports the formation of a functional binding site. On the other hand, when the allosteric inhibitor binds, this loop moves about 4 Å and rotates about 180°. This movement changes the packing interactions of the protein core and rotates a neighboring helix, resulting in the remarkable repacking of the core and the conversion of the enzyme into an inactive oligomer. Deletion of the buried Val132 traps the enzyme to an open intermediate conformation of the allosteric response (Sennett et al., 2011). Comparison of the closed wild type and the open Δ 132 structure identified a hinge-bending axis between two residues of the dimerization domain. A concerted hinge-bending motion between adjacent subunits was proposed to be the basis of the allosteric transition.

C. Flexibility and Ligand Specificity

The broad substrate specificity which characterizes some enzymes has been explained under the view of a flexible active site cavity capable of accommodating stereochemically diverse substrates. The different substrates may either induce conformational rearrangements to the

broader area of the flexible active site or, if the protein structure is seen as a dynamic ensemble of conformations, each substrate is bound to different conformations with different affinity. The impact of plasticity and flexibility on ligand binding has been illustrated in the literature, among others, with examples from the family of endonucleases V (Feng et al., 2005), glutathione S-transferases (Hou et al., 2007), dehydrogenases (Deng et al., 2009), and CYP (Pochapsky et al., 2010). In some cases, the local flexibility around the active site and the possible reposition and repack of only few individual side chains could cause sufficient space and shape alterations inside the active site to switch from one substrate to another without substantial structural changes. However, in other cases, global flexibility seems to be a requirement for promiscuity.

CYPs are a superfamily of enzymes which catalyze the addition of an oxygen to an C—C or C—H bond, that is, they are oxidoreductases catalyzing the oxidation of structurally dissimilar organic substrates. CYPs adopt a similar global fold which is combined with a remarkable adaptivity for substrate recognition. This is possibly due to the modularity of secondary structure features which surround the active site (Fig. 3B). These features vary even between different states of the same enzyme (Pochapsky et al., 2010). Some CYPs show broad substrate and hydroxylation specificity and it has been proposed that their high flexibility and plasticity around the active site is responsible for the wide substrate selectivity and specificity they present. The typical topology of CYPs contains a four alpha helical bundle which also accommodates the heme group which is ligated by a Cys at the beginning of one of the bundle helices. In total, the structure is composed by 14 helices and 5 beta sheets. In an early spectroscopic study of five individual CYPs (Anzenbacher and Hudecek, 2001), it was demonstrated that their active sites exhibit significant differences in their flexibility and stability although in these enzymes these properties cannot be related by a simple relationship since in some cases, a low stability does not necessarily reflect a high flexibility. Among the five CYPs which were tested, one demonstrates a significantly higher promiscuity than the others by being able to accommodate and modify a variety of structurally different substrates. Comparison with the other four cytochromes of higher specificity showed that what differentiates it from them is a more flexible active site together with a less conformationally restricted heme group.

Comparison of the crystal structures of P450 2B4 cytochromes complexed with the ligands 1-CPI (1-(4-chlorophenyl)imidazole) and 4-CPI (4-(4-chlorophenyl)imidazole) highlights a significant shift of the backbone of two helices (F and I) with consequence the reposition of side chains reshaping of the active site and change of its volume almost 80 \AA^2 (Zhao et al., 2007). Likewise, comparison of the structures of the human P450 2B6 enzyme complexed with the ligands 4-(4-nitrobenzyl)pyridine (4-NBP) and 4-benzylpyridine (4-BP) reveals that small rearrangements of five Phe residues of the active site resulted in an increase of the active site volume sufficient to accommodate the bulkier 4-NBP in comparison to 4-BP (Shah et al., 2011).

The *Plasmodium falciparum* dihydroorotate dehydrogenase (*PfDHODH*) is an example of enzyme which is able to accommodate a wide range of structurally different classes of inhibitors due to a drastic reorganization which creates different binding pockets (Deng et al., 2009). Structural studies demonstrate that the high flexibility of the enzyme allows for two alternative binding sites close to each other for interactions with different structural classes of ligands. In addition, local flexibility inside the active site pocket contributes to its ability to bind ligands of variable size. The different binding modes result as a consequence of a large conformational change in the position of Phe188 between the two structures which is also accompanied by α -helical shifts.

Studies on glutathione S-transferases suggest that in some cases ligand promiscuity may most easily be achieved by distributing flexibility throughout the structure than limiting it to the active site (Hou et al., 2007). The structurally related isoforms of glutathione, A1-1, and A4-4 represent benchmarks for high catalytic promiscuity and selectivity, respectively. H/D exchange and tryptophan fluorescence showed significantly greater solvent accessibility throughout of the A1-1 sequence compared with A4-4. Moreover, a double mutation to the A1-1 which introduces an aromatic interaction present to the A4-4 and the reverse double mutation to A4-4 were sufficient to change local and global flexibility and partially invert their relative substrate specificities.

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