

# Protein Conformational Disorder and Enzyme Catalysis

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**Abstract** Though lacking a well-defined three-dimensional structure, intrinsically unstructured proteins are ubiquitous in nature. These molecules play crucial roles in many cellular processes, especially signaling and regulation. Surprisingly, even enzyme catalysis can tolerate substantial disorder. This observation contravenes conventional wisdom but is relevant to an understanding of how protein dynamics modulates enzyme function. This chapter reviews properties and characteristics of disordered proteins, emphasizing examples of enzymes that lack defined structures, and considers implications of structural disorder for catalytic efficiency and evolution.

**Keywords** Dynamics · Evolution · Intrinsic disorder · Protein function · Protein structure

## Contents

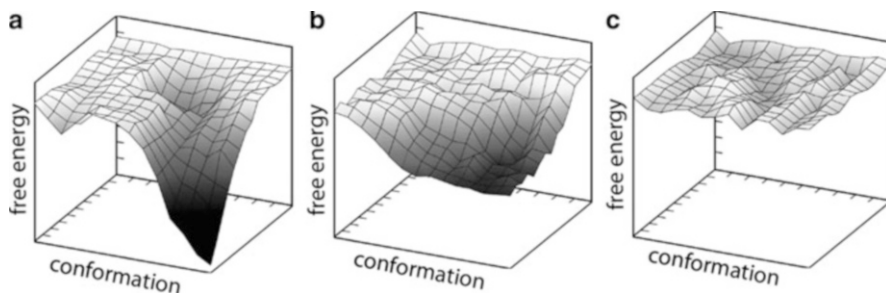
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# 1 Introduction

It is axiomatic in biochemistry that proteins must adopt a stable, well-defined tertiary fold to be wholly functional. Given this, sequence–structure–function analyses have generally concentrated on fully structured proteins. In addition to a stable fold, however, proteins require certain flexibility. Because fluctuating interactions of defined amino acid residues or conformational movements of entire loops or domains are crucial to many biological activities, interest is mounting in proteins that present increased conformational flexibility and even lack stable tertiary structure.

Amino acid sequences dictate protein conformation, but not every amino acid sequence is able to produce a stable tertiary structure and not all proteins within a cell are well structured. Proteins that cannot adopt a stable tertiary fold are called natively unfolded, intrinsically unfolded, or intrinsically disordered. Such molecules are not typically fully unstructured. Local residual secondary structures may be present but insufficient to stabilize long-range tertiary interactions under physiological conditions. High intrinsic flexibility results in dynamic ensembles of rapidly interconverting conformational states of comparable energy [1]. Within these ensembles, individual polypeptide chains may adopt collapsed (molten globule) or extended, random coil-like (pre-molten globule) conformations (Fig. 1). Other proteins may contain partially disordered regions. When positioned at protein termini, flexible segments can serve as signal sequences. When located within multi-domain proteins they may function as dynamic linkers connecting individual domains.

Intrinsically disordered proteins (IDPs) and proteins with large intrinsically disordered regions are found surprisingly frequently in all domains of life. Interestingly, the proportion of IDPs and proteins containing disordered regions increases with organismal complexity [3]. Algorithms based on the biased amino acid compositions and specific biochemical properties of IDPs predict that approximately one-third of



**Fig. 1** Energy landscapes for increasingly disordered proteins. (a) The native conformation of a well-folded protein has a well-defined minimum energy. (b) A molten globular protein exhibits multiple, partially folded conformations. (c) An intrinsically unstructured protein lacks a deep minimum and consists of many different interconverting species of comparable free energy. The figure is partially adapted from [2]

all eukaryotic proteins are partly or completely disordered [4, 5]. In mammals, one quarter is fully disordered and half contains extended disordered regions [6]. Disordered proteins play important roles in many fundamental biochemical processes, ranging from transcription and translation to signal transduction and regulation [7]. Even enzymes, the biological catalysts responsible for accelerating nearly all metabolic reactions in the cell, can exhibit significant structural disorder.

In this review we examine the properties of intrinsically disordered proteins with special emphasis on enzyme catalysis. The implications of intrinsic disorder for enzymatic efficiency and evolution are considered.

## 2 Intrinsically Disordered Proteins

Conformational flexibility is an inherent property of all polypeptide sequences, whether folded or non-folded. The diversity of motions observed in proteins extends from local fluctuations of amino acid side chains and loops on the picosecond to nanosecond timescale to domain movements and complete rearrangements of entire protein folds on the microsecond to second timescale [8]. Such jostling may give rise to altered protein conformations at any time, but most states represent modest structural excursions around the native protein fold [9]. Because motions on different timescales are coupled, local fluctuations in the picosecond to nanosecond time scale can influence larger structural changes [10]. Intrinsic disorder takes these normal dynamic attributes of proteins to extremes.

Both protein folding and protein non-folding are encoded at the level of amino acid sequence [11]. Disordered proteins typically exhibit relatively low sequence complexity compared to well-ordered proteins [12]. They contain few hydrophobic amino acid residues [1], which are usually buried in ordered protein cores and favor formation of compact structures in hydrophilic environments. Many polar and charged amino acid residues lead to high net polypeptide chain charge. Owing to electrostatic repulsion, expanded rather than compact structures result [13–15]. In fact, plotting mean protein hydrophobicity as a function of mean net charge provides a useful means of identifying IDPs at the proteome level [16]. Methionine, which has a flexible aliphatic side chain, and proline, which disrupts secondary structure, are overrepresented in IDPs. Repeated sequences, such as polyalanine or polyglycine stretches, are also prevalent [1].

Given low structural content and expanded structure, disordered proteins have distinctive biophysical properties. For instance, they typically elute from gel filtration columns as broad peaks, unfold non-cooperatively [17–19], and evince greater susceptibility to proteolytic attack than ordered proteins [20]. As hydrophobic residues in unstructured proteins are largely accessible to solvent, protein disorder can often be detected with dyes like 1-anilino-naphthalene-8-sulphonate (ANS) or thioflavin T that bind to exposed hydrophobic patches [21]. Although protein crystallization imparts little useful structural information about unstructured regions, small angle X-ray scattering and NMR spectroscopy can be profitably

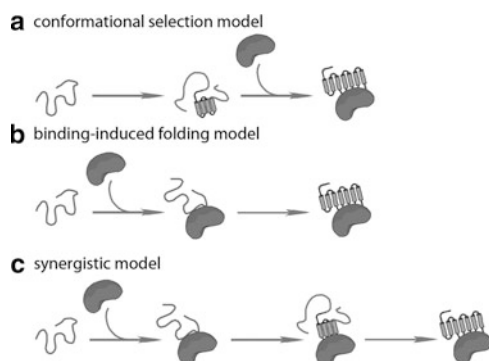
exploited to characterize IDPs [22–26]. Their dynamic properties typically cause peak broadening and/or low signal dispersion in 1D and 2D NMR spectra [18, 27–29]. Fast hydrogen/deuterium exchange, which can be detected by either NMR spectroscopy or mass spectrometry, is another consequence of intrinsic disorder [30].

### 3 Target Recognition and Binding

Although the notion that three-dimensional structure is required for protein function has historically dominated the thinking of chemists and biologists, it is now clear that proteins lacking a defined tertiary structure play a surprising diverse set of biochemical roles. As noted above, many molecules involved in signaling and regulation of the cell cycle are intrinsically disordered [31]. It has been estimated that ~75% of all mammalian proteins involved in cell signaling and regulation contain large unstructured regions or are fully disordered [6]. Transcription and translation are additional key biological processes in which global or partial disorder figures [32, 33]. As a consequence, disorder often figures in disease [34]. Plants and insects utilize such proteins as protection against dehydration and for regulation of intracellular salt concentrations [35]. IDPs can also serve as degradation tags [33] or as chaperones [36, 37].

Typically, IDP activity is expressed through interactions with a specific partner, such as DNA, RNA, or another protein. Upon binding to a target, most IDPs undergo significant conformational change, typically leading to a more ordered state [38]. In the extreme case, these disorder-to-order transitions result in conversion of a random coil into a well-defined three-dimensional structure. Both the binding and folding steps are entropically unfavorable. As unbound IDPs are rarely completely unfolded [39], however, the initial conformational space is not as great as that expected for a fully unstructured polypeptide chain. Moreover, large regions of an IDP may remain highly flexible even when bound. As in more conventional protein folding, the entropic costs of conformational reorganization in these cases are (partially) offset by entropic gains associated with the release of ordered water molecules and by favorable enthalpic interactions within the folded structure. In addition, target-dependent disorder-to-order transitions benefit from intermolecular interactions between the IDP and its binding partner.

The precise mechanism of IDP binding is still debated. A conformational selection model (Fig. 2a) posits rapid interconversion of many different states for an IDP, only one or a few of which are correctly configured for target recognition. Formation of a productive complex shifts the equilibrium toward the folded conformation, which becomes increasingly populated over time [40]. As only two populations – bound and unbound – are kinetically relevant, folding is thought to follow a simple two-state mechanism [41]. A second theory postulates that folding is coupled to target binding (Fig. 2b). In this scenario, binding can take place at any number of initiation sites along the IDP polypeptide chain and induces folding [42]. Parallel folding pathways and multiple structures may result [41].



**Fig. 2** Models for target-dependent folding of intrinsically disordered proteins. (a) In the conformational selection model the target molecule binds to a specific subspecies of the intrinsically disordered protein. (b) In the binding-induced folding model the intrinsically disordered protein folds upon binding to the target molecule. (c) The synergistic model combines aspects of the conformational selection and binding-induced folding models

By analyzing the folding behavior of an IDP in the presence of its binding partner it is sometimes possible to distinguish between the different binding models. Examples of each have been described in the literature [24, 43–46]. In many cases, however, the mechanisms cannot be differentiated. Sometimes, multiple mechanisms may be operative. To account for this observation, a synergistic model for IDP folding (Fig. 2c) was proposed that combines the two pathways [47]. The relative contribution of the individual mechanisms depends on case-specific factors such as binding rates, IDP concentration, protein plasticity, and the specific nature of the disorder-to-order transition. Some IDPs are thought to exploit different binding modes in different environments or in response to different binding partners. For example, the C-terminal segment of tumor suppressor protein p53 adopts four types of secondary structure when complexed with different binding partners [48]. The conformational states and binding modes of this protein are further modulated by posttranslational phosphorylation, methylation, and acylation [49, 50].

Lack of stable tertiary structure and high inherent flexibility confer multiple advantages on IDPs compared to structured proteins with respect to molecular recognition. Almost all biological functions of these proteins depend on an appropriate balance of specificity and affinity for different targets [51]. The increased interaction surface and conformational fluctuations of IDPs can be exploited to maximize the rate of substrate binding and product release. The larger capture radius of an unstructured protein allows it to bind weakly and nonspecifically to its target at large distances – as in “fly-casting” – and fold as it approaches the actual binding site [52]. Consequently, IDPs require fewer encounters than structured proteins to form complexes, accelerating the binding process [53]. Computer simulations suggest that linking binding and folding significantly reduces the free energy barrier for binding [47]. Nonetheless, increased flexibility of the polypeptide

chain in the bound state also yields high  $k_{\text{off}}$  rates relative to fully folded proteins. The resulting rapid turnover rates are likely to be kinetically advantageous for the dynamic biological processes involving IDPs.

When coupled, high binding specificity and moderate binding affinity enable rapid and tunable regulation of the cell cycle and other biological processes in response to changing environmental conditions [54]. The extended conformations of IDPs enhance binding diversity. Indeed, IDPs often co-operate with multiple binding partners, forming hubs, or nodes, of large protein–protein interaction networks [55, 56]. Such hubs are so essential to key biochemical functions that deletion of the IDP can result in organism death. Alternatively, interaction networks can be established by binding of multiple IDPs to a single folded partner [57].

## 4 Intrinsically Disordered Enzymes

Catalysis poses a particularly demanding molecular recognition challenge. Biological enzymes make life possible by accelerating all metabolic reactions in the cell by enormous factors. Pauling postulated that protein catalysts lower the activation barrier of the reaction they promote by binding the transition state more tightly than the substrate(s) [58]. In this view, enzymes utilize a defined structure and preorganized sets of functional groups to provide maximum shape and chemical complementarity to this transient, high-energy species, selectively stabilizing it.

Recognition that proteins are not rigid led Koshland to modify this model [59]. He proposed that substrate not only binds to the preformed enzyme active site but also brings about structural changes that orient the catalytic residues for productive reaction. Subsequent biochemical and crystallographic studies of many enzymes support this induced fit hypothesis [60]. In most textbooks the induced fit model is conventionally depicted as a conversion of one tight conformational ensemble (free enzyme) to another distinct ensemble (bound enzyme) through local substrate-mediated structural rearrangements. It is now evident, however, that enzymes, like other proteins, exhibit an extensive array of motions over many timescales [61]. In extreme cases they even evince significant structural disorder.

Several naturally occurring enzymes are either fully disordered or include large disordered regions (Table 1). RNase E, an endoribonuclease found in the *Escherichia coli* RNA degradosome, consists of an evolutionarily conserved, well-structured N-terminal region [65], yet much of its C-terminal region is intrinsically disordered judging from sequence analysis, CD spectroscopy, and X-ray studies [66]. While its catalytic center is located in an ordered N-terminal region, its disordered C-terminus is essential for mediating interactions with the RNA substrate and other binding partners in the RNA degradosome [67].

The hepatitis C virus NS3 protease (HCVP) is a partially unstructured  $\text{Zn}^{2+}$  dependent serine-protease [19]. In the presence of  $\text{Zn}^{2+}$  ions it cleaves viral precursor proteins in non-structured regions. While not directly involved in catalysis, the  $\text{Zn}^{2+}$  ion induces large conformational changes that yield stable, catalytically active enzyme conformations [68]. UreG, a GTPase that functions as a

**Table 1** Representative examples of intrinsically disordered enzymes

Enzyme	Organism	Metal ion	Structural disorder	Catalytic activity	Reference
RNase E (nuclease)	<i>E. coli</i>	–	Partially disordered	$k_{\text{cat}} = 1.4 \text{ s}^{-1}$	[62]
HCVP (protease)	Hepatitis C virus	$\text{Zn}^{2+}$	Partially disordered	$k_{\text{cat}} = 2.7 \times 10^{-2} \text{ s}^{-1}$	[19, 63]
UreG (GTPase)	<i>Bacillus pasteurii</i>	$\text{Zn}^{2+}$ , $\text{Ni}^{2+}$	Largely disordered	$k_{\text{obs}} = 6.7 \times 10^{-4} \text{ s}^{-1}$ <sup>a</sup>	[27]
UreG (GTPase)	<i>Mycobacterium tuberculosis</i>	$\text{Zn}^{2+}$ , $\text{Ni}^{2+}$	Largely disordered	$k_{\text{obs}} = 1.7 \times 10^{-4} \text{ s}^{-1}$ <sup>a</sup>	[64]
Anhydrin (endonuclease)	<i>A. avenae</i>	$\text{Mg}^{2+}$ , $\text{Mn}^{2+}$	Largely disordered	$k_{\text{obs}} = 5.5 \times 10^{-4} \text{ s}^{-1}$ <sup>a</sup>	[35]
TPPP/p25 (GTPase)	Human	$\text{Mg}^{2+}$	Largely disordered	$k_{\text{obs}} = 2.3 \times 10^{-4} \text{ s}^{-1}$ <sup>a</sup>	[28]

<sup>a</sup>It is unclear whether these enzymes were assayed at saturating or subsaturating substrate concentrations, hence only observed rate constants are available

chaperone in nickel trafficking and urease activation, is another example [64, 69]. It exhibits rapidly interconverting conformational sub-states [17]. Transitions among these disordered ensembles occur non-cooperatively but reversibly. Binding of  $\text{Zn}^{2+}$  ions induces a conformational change and protein stabilization, but has no effect on GTPase activity [27]. Like many other intrinsically disordered proteins [57, 70], UreG appears to function as a scaffold protein, coordinating the binding of multiple partners.

A third catalytically active IDP, anhydrin, is found in the nucleus of the anhydrobiotic nematode *Aphelenchus avenae* [35]. Anhydrin discharges two entirely independent functions. In addition to acting as a chaperone to reduce protein aggregation, it is an endonuclease that acts on supercoiled, linear, and chromatin DNA. Though approximately ten times less active than T7 endonuclease I, its specific activity is sufficiently high for physiological function. While  $\text{Ca}^{2+}$  ions do not influence its catalytic activity,  $\text{Mg}^{2+}$  and  $\text{Mn}^{2+}$  ions enhance it. Even in its DNA bound state, anhydrin remains largely unstructured [35].

The tubulin polymerization promoting protein/p25 (TPPP/p25) is an unstructured brain-specific protein that induces aberrant tubulin assemblies in vitro [71]. Intracellular TPPP/p25 levels influence cell differentiation and proliferation and might be implicated in Parkinson's disease and other nervous system pathologies [72]. The TPPP/p25 amino acid sequence exhibits characteristics typical of IDPs [72]; CD measurements attest to extensive random-coil structure [73], whereas 1D and 2D NMR spectra indicate multiple protein conformations and increased protein flexibility [28, 74]. Extended disordered segments are located primarily at the N- and C-termini, but the mid-region also exhibits high flexibility. Sequence alignments have identified multiple potential GTP binding sites. The micromolar GTP binding affinity increases in the presence of  $\text{Mg}^{2+}$  ions crucial for TPPP/p25 GTPase activity. GTP hydrolysis by TPPP/p25 is comparable in rate to the intrinsic GTPase activity of other small G proteins [28].

Although the number of naturally disordered enzymes is still small, our perspective is strongly biased by the dominance of X-ray crystallography as a structural tool. Others may well come to light in the future through intensified NMR spectroscopic study of proteins in solution.

## 5 Inducibly Disordered Enzymes

Protein disorder is inducible *in vitro* under conditions such as high temperature, extreme pH, and distinct denaturant or salt concentrations [75]. In these circumstances many proteins lose their defined tertiary structure and biochemical function. On occasion, however, a modicum of activity can be retained or recovered by a disorder-to-order transition induced by substrate or another ligand (Table 2).

Disordered states are often transiently populated during protein folding [81]. For example, molten globule conformations are obligatory intermediates in the folding pathways of many proteins [82, 83]. Generally speaking, these highly dynamic protein states do not exhibit catalytic activity. In the case of *Sulfolobus solfataricus* acylphosphatase, however, an enzymatically active non-native folding intermediate has been identified [76]. Although its active site is structurally heterogeneous, the folding intermediate exhibits 80% of the catalytic activity of the fully folded enzyme. Control experiments showed that enzymatic activity does not derive from the fraction of native protein in the sample, but substrate-induced organization of the active site could not be ruled out. NMR studies on a homologous *Bacillus subtilis* acylphosphatase indicated a structurally disordered active site in the absence of substrate that became more ordered upon substrate binding [84]. Conformational heterogeneity was particularly pronounced at acidic pH, where the enzyme exhibits optimal activity, possibly accounting for the relatively broad specificity of the enzyme. Phosphate was found to alter the distribution of states present in solution by selectively binding to the active conformation.

Human topoisomerase I catalyzes topological changes in DNA by a two-step mechanism involving a covalent enzyme–DNA intermediate. The highly conserved C-terminal domain contains the active site nucleophile Tyr723, but lacks enzymatic activity on its own. Catalysis arises only in the presence of the core domain, which contains additional catalytically relevant residues. In solution, the isolated C-terminal domain adopts a largely  $\alpha$ -helical molten-globule-like state whose protein surface is a virtual patchwork of hydrophobic elements [77]. Association with the core domain affords a complex that still exhibits some structural fluctuations. Within a narrow salt-concentration range, and despite 20-fold lower DNA affinity, this reconstituted complex maintains topoisomerase activity [78]. The conformational flexibility of the C-terminal domain may be biologically important for accommodating the large conformational changes that occur during the catalytic reaction [77].

Point mutations sometimes induce disorder. For example, the substrate binding domain of *E. coli* adenylate kinase was destabilized by the introduction of two point mutations [79]. Although locally unfolded, the resulting variant exhibited enhanced affinity for ATP and retained ca. 5% of the wild-type catalytic activity. These results suggest that the conformational switch between an open and closed state that normally



**Table 2** Representative examples of inducibly disordered enzymes

Enzyme	Organism	Inducer	Structural disorder	Catalytic activity	Reference
Acylphosphatase	<i>S. solfataricus</i>	Guanidinium chloride	Partially disordered	79 wt%	[76]
Human topoisomerase I	<i>Homo sapiens</i>	Fragmentation/recombination	Molten globule	~11 wt%	[77, 78]
I116G/L168G adenylate kinase	<i>E. coli</i>	Mutation	Partially disordered	5 wt%	[79]
S54G/P55N RNase T1	<i>Aspergillus oryzae</i>	Mutation	Partially disordered	40 wt%	[80]

limits the rate of catalysis occurs by cooperative unfolding and refolding of large segments of the protein. This novel molecular mechanism for achieving large-scale conformational transitions could conceivably be useful for other proteins as well.

A ribonuclease T1 mutant provides another example of a molten globule with substantial enzymatic activity. During refolding of an enzyme variant containing only a single *cis*-proline, a transient intermediate accumulates prior to proline isomerization that exhibits extensive secondary structure but only partial packing of the hydrophobic core. Despite its low tertiary structure content, this species possesses 40% of the RNase activity of the native protein toward the dinucleotide GpC [80]. Similarly, an intermediate containing a non-native peptidyl–prolyl bond is formed during refolding of RNase A that exhibits RNase activity similar to that of the native folded enzyme. However, subsequent studies showed that its active site region is largely in a native-like conformation [85]. In nature, kinetically trapped folding intermediates, which arise because of slow folding events like disulfide bond formation and proline isomerization, may also exhibit catalytic activity. Though not fully active, these species may contribute, at least to some extent, to biological function. These intrinsically disordered states might also shape the evolutionary potential of polypeptides by facilitating the generation of alternative folds and functions.

## 6 Designed Disordered Enzymes

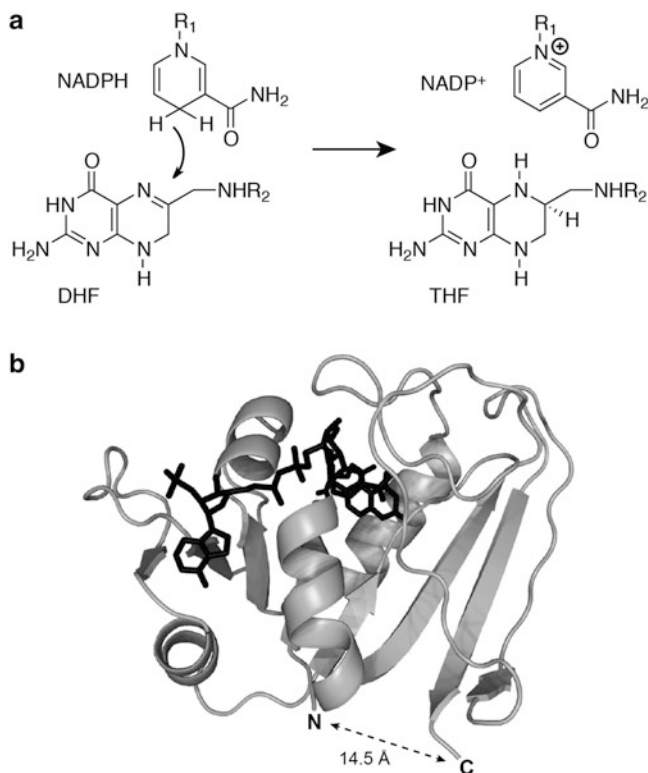
While few instances of enzymatically active IDPs may be known in nature, a number of engineered proteins have been found to possess considerable catalytic power despite being intrinsically disordered (Table 3). In these cases, the sequence hallmarks of natural IDPs are not always evident.

The enzyme dihydrofolate reductase (DHFR) has been an important model system for studying protein folding, enzyme catalysis, and the relevance of protein dynamics for function [95]. It is a monomeric two-domain protein that catalyzes the reduction of 7,8-dihydrofolate (DHF) to 5,6,7,8-tetrahydrofolate (THF) by a

**Table 3** Representative examples of designed enzymes that exhibit significant disorder

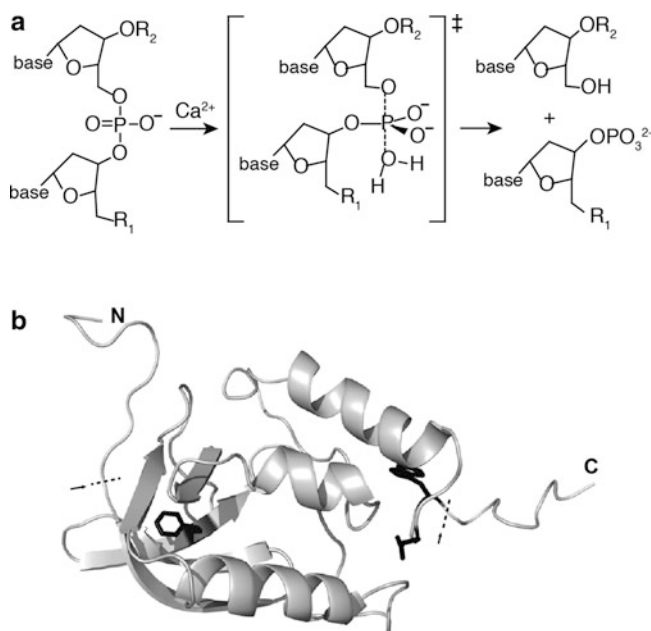
Enzyme	Organism	Modification	Structural disorder	Catalytic activity	Reference
DHFR (reductase)	<i>E. coli</i>	Circular permutation	Molten globule	1–5 wt%	[86]
C85A/C152S DHFR	<i>E. coli</i>	Fragmentation/recombination	Molten globule	15 wt%	[87]
SNase (nuclease)	<i>S. aureus</i>	N-terminal truncation	Molten globule	65 wt%	[88]
F34W/W104F SNase	<i>S. aureus</i>	Mutation	Molten globule	87 wt%	[89]
N138D SNase <sub>141</sub>	<i>S. aureus</i>	C-terminal truncation	Partially disordered	72 wt%	[90]
SNase	<i>S. aureus</i>	C-terminal truncation	Partially disordered	90–110 wt%	[91]
$\Delta$ 131 $\Delta$ SNase	<i>S. aureus</i>	C- and N-terminal truncation	Largely disordered	wt	[92]
TIM (isomerase)	–	Consensus design	Molten globule	0.002 wt%	[93]
CM (mutase)	<i>M. jannaschii</i>	Topological redesign	Molten globule	30 wt%	[94]

stereospecific hydride transfer from the cofactor NADPH to the C6 atom of the pterin ring of DHF (Fig. 3a). Five different ligand-complexed states in the catalytic cycle have been detected [95, 96]. These vary in the orientation and inherent flexibility of the Met20 loop, which governs access to the active site during catalysis [97]. In studies of chain connectivity effects on protein folding, the *E. coli* enzyme was extensively circularly permuted [87, 98–100]. The natural N- and C-termini were connected by a short peptide linker and new termini were sequentially introduced between every pair of protein residues (Fig. 3b). Certain permutations resulted in complete loss of structure and activity, while others had little or no effect. Two of the circularly permuted DHFR variants studied in detail possessed molten globular attributes but retained low catalytic activity, ranging from a 20- to 100-fold decrease in efficiency compared to wild-type DHFR [86]. Although they lack a rigid tertiary structure, addition of ligands like the potent inhibitor methotrexate leads to a gain of native-like structural properties, including cooperative thermal unfolding [101], suggesting that the small molecules may initiate folding. In an even more extreme modification, cleavage of the DHFR backbone after residue 86 produced two poorly structured, catalytically inactive fragments [87]. Nonetheless, when mixed, these formed a high affinity complex having substantially less secondary structure content than the wild-type protein but 15% of its DHFR activity. When exposed to inhibitors, the structural content of the complex increased significantly, indicating the potential of small molecules for stabilizing the active site architecture.



**Fig. 3** Dihydrofolate reductase (DHFR). (a) The enzyme catalyzes stereospecific hydride transfer from NADPH to dihydrofolate (DHF) to give tetrahydrofolate (THF) and NADP<sup>+</sup>. (b) Tertiary structure of DHFR (PDB code: 1RX2). The substrate folate and the cofactor NAD<sup>+</sup> are shown in *black*. The N- and C-termini of the protein are close in space, facilitating circular permutation

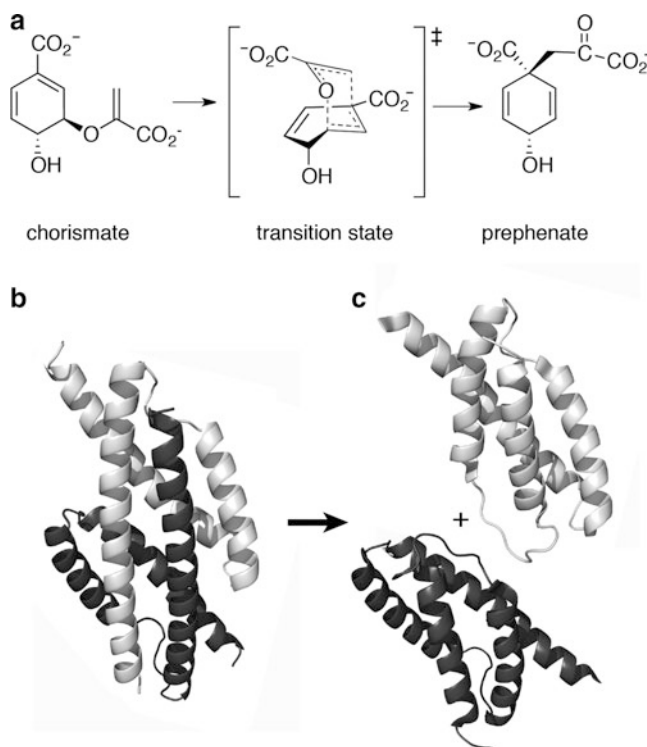
Diverse modifications of staphylococcal nuclease (SNase), a Ca<sup>2+</sup>-dependent enzyme that catalyzes DNA and RNA hydrolysis, similarly produce intrinsically disordered but active enzyme variants (Fig. 4). For example, the double point mutant, F34W/W104F SNase, has molten globular-like characteristics and essentially wild-type catalytic activity [89]; in this case, ligand binding apparently causes the mutant to fold into its functionally active conformation. SNase<sub>141</sub>, a truncated variant lacking the last eight C-terminal amino acids, has a compact, well-folded structure, but introduction of the N138D point mutation disrupts its conformational integrity and stability by eliminating a key hydrogen bond [90]. Nevertheless, this variant retains 72% of the activity of SNase<sub>141</sub>. Another variant, whose last 13 amino acid residues were deleted, partly unfolds and lacks stable secondary structure [91, 102]. This variant is fully active at low salt concentrations. Calcium ions and the inhibitor thymidine 3',5'-bisphosphate induce a conformational transformation to a more folded state [91].



**Fig. 4** Staphylococcal nuclease. (a) The enzyme is a calcium-dependent endonuclease that cleaves both single and double-stranded DNA and RNA. (b) Tertiary structure of wild-type staphylococcal nuclease (PDB code: 2SNS). Both N- and C-terminal truncations (*dashed lines*) produce intrinsically disordered protein. Residues Phe34 and Trp140, whose substitution to F34W/W140F results in disorder, are depicted in *black*. Residue Asn138, whose substitution to N138D combined with an eight amino acid C-terminal truncation results in disorder, is shown in *black*

Truncating the N-terminus of SNase also results in significant structural perturbations. Deleting 11 or 12 amino acids, for instance, produces a molten globular state [88, 103]. Whereas the  $-11$  variant refolds to a distinct structure in the presence of substrate,  $-12$  no longer adopts a native-like conformation. These variants possess 65% and 0.3% wild-type SNase activity, respectively. When residues 4–12 and 141–149 are excised, another variant is obtained –  $\Delta 131\Delta$  SNase – whose native state is composed of different conformational species that rapidly convert between helical and extended conformations. Some secondary structure elements, such as the C-terminal  $\alpha$ -helix or the fourth and fifth  $\beta$ -strands, are disordered in the native state. Nevertheless,  $\Delta 131\Delta$  SNase assumes wild-type-like structures in the presence of the substrate and inhibitor, enabling efficient catalysis at high substrate concentrations [92, 104].

Studies on the capsid protease of Semliki Forest virus (SFVP) further illustrate the catalytic potential of IDPs [18]. Variants of this enzyme lacking one to seven C-terminal residues show biophysical properties characteristic of natively unfolded proteins, including the absence of a defined three-dimensional structure. The truncated proteins nevertheless efficiently catalyze the hydrolysis of activated aromatic amino acid esters with a  $10^4$ -fold rate acceleration over background.



**Fig. 5** Chorismate mutase. (a) The enzyme catalyzes the Claisen rearrangement of chorismate to prephenate via a pericyclic transition state. (b) Tertiary structure of a dimeric AroQ chorismate mutase (PDB code: 1ECM). (c) Insertion of a hinge-loop into the long N-terminal helix of structure of the dimeric AroQ mutase MjCM from *M. jannaschii* affords the highly active monomeric mutase mMjCM (PDB code: 2GTV), which exhibits the properties of a molten globule

Moreover, the reported  $k_{\text{cat}}$  and  $k_{\text{cat}}/K_{\text{m}}$  values of  $15 \text{ s}^{-1}$  and  $5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ , respectively, are comparable to the efficiencies of typical structured proteases.

In other work, a molten globular variant of triosephosphate isomerase (TIM) was inadvertently generated by consensus design [93]. The protein, cTIM, shares only 70% identity with its closest naturally occurring homolog. Biophysical characterization showed that it is monomeric, in contrast to native TIM dimers, and also less well folded. Although its catalytic efficiency ( $133 \text{ M}^{-1} \text{ s}^{-1}$ ) is four orders of magnitude lower than that of the wild-type enzyme, this level of activity is sufficient to complement the TIM deficiency of an engineered *E. coli* strain. Interestingly, a relatively small number of mutations at unconserved positions sufficed to convert this consensus design into a protein with more native-like properties, including a well-folded structure and high catalytic efficiency.

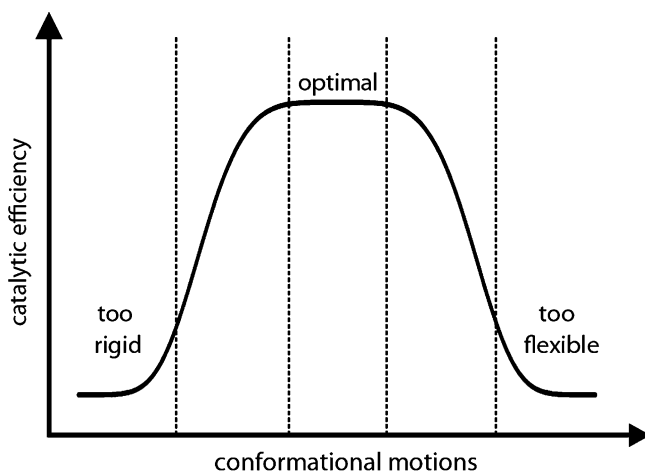
One well-studied disordered enzyme is a designed chorismate mutase (CM) that catalyzes prephenate formation from chorismate, a key step in the biosynthesis of aromatic amino acids (Fig. 5). The homodimeric mutase from *Methanocaldococcus*

*jannaschii* [105] was converted into a monomer by inserting a randomized hinge-loop sequence into the middle of the long, dimer-spanning N-terminal helix and selecting for functional variants in a CM-deficient *E. coli* strain [94]. The resulting protein, mMjCM, has 30% native activity but, unlike its thermostable parent, it possesses a fluctuating tertiary structure as judged by rapid H/D exchange, poor NMR signal dispersion, non-cooperative thermal denaturation, and ANS binding [106]. Upon binding of a transition state analog, this loosely packed and highly dynamic ensemble undergoes substantial structural ordering. This process is characterized by substantial entropy–enthalpy compensation as well as elevated rates of ligand association and dissociation compared to MjCM [107]. The solution structure of the complex, which was determined by NMR spectroscopy [24], confirmed that the protein adopts a bundle fold, as designed, although it still exhibits unprecedented millisecond flexibility across its entire length.

## 7 Protein Dynamics and Catalysis

The observation that structural disorder is compatible with catalysis is intriguing in the context of ongoing discussions regarding the role dynamics plays in the function of more conventional enzymes [108–114]. A variety of techniques, including fast kinetic methods, NMR relaxation data, alone or in combination with ambient-temperature X-ray crystallography, and molecular simulation techniques, have provided detailed information on the conformational dynamics along the reaction trajectories of selected enzymes [61, 96, 115, 116]. Conformational changes in otherwise well-structured proteins are often important for substrate binding and product release. By sequestering substrates from aqueous solution, structural rearrangements can create a protective environment for a reaction and position functional groups for effective transition state stabilization [117]. Sometimes conformational changes occur on a timescale similar to that of catalytic turnover and may even limit the overall rate of transformation [115, 118].

Attempts to link enzyme dynamics directly to catalytic efficiency – and particularly the ability of protein motions to influence the nature of the chemical step itself – have sparked vigorous debate [108–113, 116, 119–122]. Nevertheless, a consensus seems to be emerging that conformational sampling enhances the probability of generating active site environments that are conducive to reaction [109]. Protein fluctuations help to generate landscapes preorganized for molecular recognition of substrate(s) and stabilization of rate limiting transition states [123]. In so doing, they may help to reduce the reorganization energy required to move from the reactant to the transition state relative to the solution reaction. Nuclear quantum tunneling, which depends strongly on the hydrogen donor–acceptor distance, has been exploited to probe protein motions during catalysis of hydride transfer reactions [124]. The temperature dependence of the kinetic isotope effects, for example, has been cited as evidence for this type of conformational sampling [108]. By orienting the substrates for efficient reaction, protein motions influence the hydrogen donor–acceptor distance and create a suitable electrostatic environment

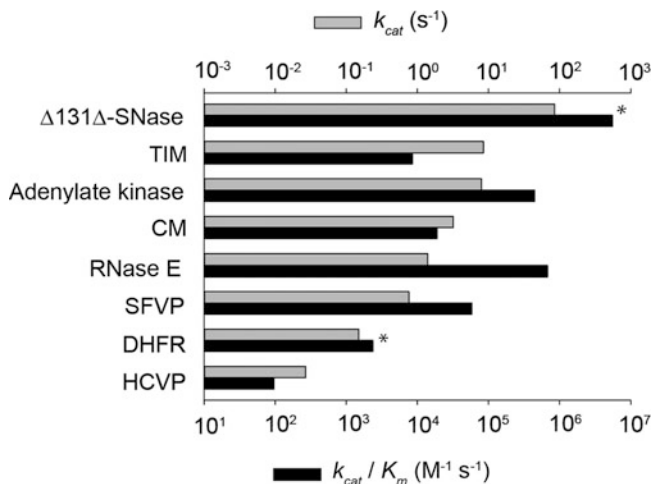


**Fig. 6** Schematic representation of the relationship between protein flexibility and catalytic efficiency. Too high or too low inherent protein flexibility is expected to be disadvantageous for enzyme catalysis

for the reaction [113]. Such fluctuations are likely to be particularly important for multistep reactions that require continual active site reconfiguration to maximize chemical complementarity to the transition states of consecutive chemical steps. By funneling the protein through a series of catalytically competent conformations, a dynamic energy landscape can channel a reaction along a preferred kinetic path.

While some protein flexibility is clearly desirable to accommodate structural changes in the reactants as chemical bonds are made or broken, excessive structural disorder would be expected to diminish catalytic efficiency (Fig. 6). In fact, intrinsically disordered proteins – when enzymatically active – often exhibit modest catalytic proficiency compared to their well-folded counterparts [79, 86–88, 93]. Nevertheless, the turnover numbers for disordered enzymes, which vary from  $10^{-2}$  to  $10^2 \text{ s}^{-1}$  (Fig. 7), are comparable to the  $k_{\text{cat}}$  values reported for many natural enzymes [125]. Because the corresponding  $K_{\text{m}}$  parameters are in the micromolar to millimolar range,  $k_{\text{cat}}/K_{\text{m}}$  values can be as low as  $100 \text{ M}^{-1} \text{ s}^{-1}$  or as high as  $10^7 \text{ M}^{-1} \text{ s}^{-1}$  (Fig. 7). While these data are suggestive, relatively few disordered enzymes have been subject to detailed kinetic characterization, so only tentative conclusions regarding the influence of bound substrates on conformational landscapes can be made.

Structural heterogeneity should diminish the efficiency of conformational sampling. If the time required to generate productive conformations exceeds biologically relevant turnover times, which are typically in the millisecond to second range [126], the search for configurations capable of substrate binding could severely limit overall enzyme efficiency [127]. This may explain why many disordered catalysts found in nature exploit metal ions as organizing elements to nucleate and stabilize catalytically competent conformations.



**Fig. 7** Comparison of  $k_{cat}$  and  $k_{cat}/K_m$  parameters for disordered enzymes that catalyze mechanistically diverse reactions. The *asterisk* denotes unpublished data from Stouffer and Schulenburg

The reduced activity observed for many disordered enzymes compared to their ordered counterparts may also reflect the difficulty of maintaining a productive catalytic environment for the time required for the chemical step to proceed [127]. The large number of charged amino acid residues could disfavor formation of a compact, catalytically competent fold even in the presence of bound ligand. The entropic cost associated with placing active site residues in a catalytically relevant conformation is likely to be high in disordered proteins, raising the free energy barrier for reaction and decreasing the overall rate. Nevertheless, tightening of non-covalent interactions throughout the complex upon ligand binding can give rise to enthalpic gains that (partially) offset such entropic losses [107]. In contrast, substantially less reorganization energy is required for the substrate to access transition state conformations in a well-folded and preorganized enzymatic active site.

As we have seen, some intrinsically disordered enzymes retain near native activity. These tend to be molten globules having largely intact secondary but fluctuating tertiary structures. In such cases, protein fluctuations are likely to represent modest departures from the mean structure observed for their well-folded homologs. As a consequence, locking the catalytically active conformation in place as the reaction proceeds has minimal energetic penalty. One case in point is the molten globular mMjCM chorismate mutase. Computational studies suggest that this enzyme undergoes greater ordering upon ligand binding than MjCM, its conventionally folded counterpart, but it also accesses a broader ensemble of catalytically competent conformations without significant preorganization penalty [128]. Consequently, the catalytic efficiency of the two systems differs by only a



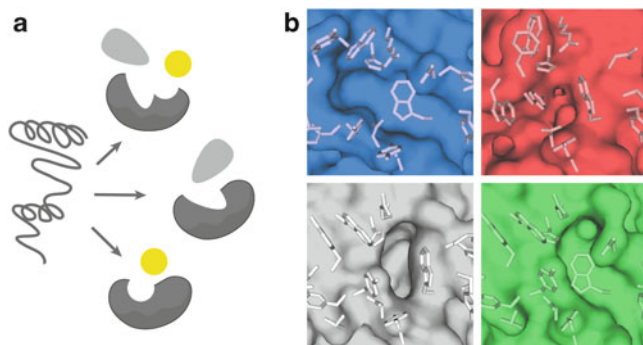
factor of three. Although conformational disorder decreases ligand affinity somewhat, mMjCM binds substrate and releases product significantly faster than MjCM [107]. In this case, partial structural disorder actually accelerates molecular recognition. The greater active site accessibility of the molten globule state probably facilitates formation of weak long-range protein–ligand interactions by a fly-casting mechanism, which strengthen as the enzyme accommodates to its target.

The proposal that chemical steps of an enzymatic reaction can be accelerated by channeling protein motions into vibrations along the reaction coordinate has generated considerable controversy. Experiments with “heavy” enzymes in which all nonexchangeable carbon, nitrogen, and hydrogen atoms were substituted with  $^{13}\text{C}$ ,  $^{15}\text{N}$ , and  $^2\text{H}$  have shown that a dynamic link might exist between mass-dependent bond vibrations of the enzyme and events in the reaction coordinate [129, 130]. However, the relevance of fast femtosecond to picosecond dynamic motions to catalysis is still debated. For example, the similar isotope effects on hydride transfer promoted by DHFR enzymes possessing widely different flexibilities argues against a direct coupling of protein motions to the chemical step [131]. Similarly, the fact that ordered and disordered proteins can both achieve high levels of enzymatic activity suggests that protein dynamics is not the source of the enormous rate acceleration over the reference solution reaction. The chorismate mutase mMjCM monomer exhibits motions on the same millisecond timescale as catalytic turnover [24], but it is difficult to envision how these stochastic fluctuations might be coupled dynamically to the chemical step. Instead, catalytically relevant active site configurations appear to be populated sufficiently frequently that little difference in rate is observed relative to the more ordered protein [128]. A recent computational study of cyclophilin A suggests that, while substrate dynamics associated with the chemical step of a reaction may be coupled to the dynamics of the surrounding medium, such effects do not lower the chemical barrier of an enzymatic reaction [132]. Instead, they alter the pre-exponential factor, effectively impeding the rate acceleration relative to the solution reaction.

Flexibility and cooperative conformational changes are general hallmarks of proteins generally and of enzymes more specifically. Most likely shaped by natural evolution, these dynamic properties vary widely across different scaffolds and even within the same protein fold family. For each specific case, there may be an ideal balance of conformational motion for optimal catalysis. However, establishing whether dynamical effects significantly influence the chemical step of an enzymatic reaction, and hence the reaction rates achieved, will require further research.

## 8 Structural Disorder and Evolution

Conformational dynamism has been proposed to be a cornerstone of protein evolvability [133–135]. Sampling scores of conformational options facilitates exploration of many potentially productive states. It may also favor the emergence of promiscuous binding and catalytic activities [49, 136, 137], which can be amplified in the course of divergent evolution [138]. Optimizing such activities through multiple rounds of mutagenesis and selection provides a route to new function.



**Fig. 8** Promiscuous functions of intrinsically disordered proteins. **(a)** Intrinsic disorder allows a protein to adopt different conformations, facilitating interactions with different binding partners. **(b)** Antibody SPE7 adopts multiple conformations in the absence of ligand (*blue*, PDB: 1O AQ and *red*, PDB: 1O CW), complexed with a low molecular weight hapten (*gray*, PDB: 1O AX), or bound to a protein (*green*, PDB: 1O AZ) [140]. The ligand has been omitted in the latter two panels to facilitate visualization of the respective binding pockets

Mammalian immune defense against diverse pathogens illustrates this point [139]. In response to antigenic challenge, antibody receptors are produced that bind foreign molecules with high affinity and selectivity. An enormously diverse pool of immunoglobulin sequences are initially assembled at the genetic level by combining multiple gene segments. Structural studies on germ-line antibodies show that the proteins in the primary immunological repertoire often exhibit high conformational diversity. The effective scope of the starting immunological repertoire is further increased by the ability of individual binding pockets to adapt to many different antigens, providing countless viable starting points for the evolution of more stable and selective complexes via somatic mutation and affinity selection. Over the course of affinity maturation, mutations that stabilize specific conformations from the ensemble of possibilities are selected, converting a low-affinity, imperfect binding site to a high-affinity complex with tailored binding interactions [135]. In some instances, even mature antibodies have been shown to adopt multiple structures and bind structurally unrelated antigens (Fig. 8) [140].

While all proteins and their functions are subject to evolutionary forces, disordered proteins should be easier to alter and adapt for new tasks than their fully structured counterparts. Individual amino acids in flexible segments take on diverse conformations more freely, and are less constrained by structure, enabling successful accumulation of neutral mutations and emergence of new function. Further facilitating evolution in IDPs is their large fraction of solvent exposed residues; surface residues are generally subject to more frequent substitution than amino acids in the buried protein core [141]. Indeed, sequence variability correlates with protein packing density [142], implying that loosely packed proteins evolve more rapidly than proteins with high tertiary structural content. By comparing genetic distance in protein families, proteins having sizable disordered regions were found to

have evolved significantly faster than ordered proteins in 19 out of 26 instances [143]. In only two cases did disordered proteins evolve more slowly than their ordered counterparts.

In a direct experimental test of connections between active site flexibility and evolvability, mutational tolerance of the intrinsically disordered chorismate mutase mMjCM was compared to that of a homologous thermostable enzyme [144]. It was found that analogous point mutations in ordered and disordered scaffolds have widely divergent and context-dependent effects on catalytic activity. Because variations in activity were not caused by decreases in secondary structure or thermodynamic stability, the results substantiate the idea that enzymes lose their activities more easily than their folds upon mutation. Less stable structures such as molten globules are evidently viable starting points for evolution. In fact, when the molten globular mMjCM was subjected to three rounds of random mutagenesis and high-stringency selection, a robust and more native-like variant was obtained that exhibited catalytic efficiency comparable to that of a natural dimeric chorismate mutase [20]. Biophysical characterization demonstrated that the evolved enzymes were substantially better folded and more stable than their molten globular precursor.

While such findings support the premise that disordered structures are promising starting points for evolution, a pre-existing activity was optimized in the mMjCM experiments. Changing the catalytic mechanism of an enzyme represents a far greater challenge. The catalytic promiscuity that many enzymes exhibit provides a possible handle for redesigning substrate specificity and catalytic mechanism by directed evolution. Several reports describe the optimization of such activities by random mutagenesis and screening [145–151], but it remains to be seen whether high structural plasticity further enhances evolvability. Although it should be easier for a conformationally diverse ensemble of molecules to access new catalytic activity than a conventionally folded protein, small structural changes could further destabilize the molecule, resulting in complete unfolding or faster degradation. Preliminary attempts to convert the molten globular chorismate mutase into an isochorismate pyruvate lyase have proven surprisingly difficult, for example (K. Höland, K.J. Woycechowsky, C. Jäkel, and D. Hilvert, unpublished data). Despite the likelihood that some natural isochorismate pyruvate lyases evolved from AroQ mutases [152], and clear sequence, structural, and chemical homology between the two systems, it has not been possible to achieve the desired functional transformation through mutagenesis and screening. Improved understanding of the interplay between protein disorder, promiscuity, and evolvability will hopefully emerge from additional study.

Many, if not most, modern enzymes are believed to derive from primordial molten globules [153, 154]. Given this, the design of new enzymes in the laboratory may also profit from intrinsic disorder. Protein engineers have created many *de novo* folds that possess the properties of molten globules; these could serve as the starting point for novel biochemical activities.

## 9 Perspectives

Over the past two decades, numerous IDPs have been described in the biochemical literature. Due to high flexibility and enlarged interaction surfaces, these proteins possess significant advantages over fully folded proteins for specific biological tasks. While high specificity and moderate binding affinity enable interaction with many different binding partners, increased cellular turnover also enables rapid response at fundamental points of the cellular signaling network [155]. The ability to perform many biologically important activities outside the native state is evidently more prevalent than previously thought. That enzymes too can also be substantially disordered is, surely, still something of a surprise.

The fact that some disordered proteins achieve substantial levels of catalytic activity challenges the long-held view that efficient catalysis requires a high degree of structural preorganization. Though conformational heterogeneity might be expected to reduce catalytic efficiency, it is now clear that the energetic cost of forming a suitable environment for a chemical reaction need not be terribly high. The disorder-to-order transition observed for intrinsically disordered enzymes upon ligand binding is analogous to the conformational sampling and reorganization processes that also take place in most conventionally folded enzymes, differing perhaps only in degree.

The finding that catalysis is not necessarily coupled to a stable and persistent fold nevertheless opens new perspectives on enzyme action and promises to enhance our understanding of the role of dynamics on normal enzyme function. From a practical viewpoint, intrinsic disorder may provide some enzymes with a novel selectable advantage in much the same way as it extends the properties of protein receptors and interaction partners. Rapid ligand binding and release with only modest losses in affinity can ensure rapid flux through the catalyst and avoidance of product inhibition. The ability to sample multiple catalytically relevant configurations could be especially helpful for multi-step reactions that require continuous reorganization of the binding pocket to accommodate consecutive transition states. Tight regulation of the production, posttranslational modification, and degradation of these catalysts, as for other disordered proteins, should allow rapid adjustment of intracellular concentrations as needed. Promiscuous binding to structurally distinct substrate molecules provides a means of rapid evolutionary adaptation. In the future it may be possible to exploit these properties for the purposes of design.

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