

Enzymology: some paradigm shifts over the years

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It seems that many enzymes have broader specificity than what was originally thought. This very broad specificity exhibited in different contexts has great significance for their biological functions. A practical application of this is that robust affinity ligands, with no biological relationship, are used in industrial enzymology. Moonlighting protein and biological promiscuity are the *in vivo* phenomena exploiting this broad specificity. Similarly, both order and disorder in protein structure are biologically relevant. Intrinsically disordered proteins (IDPs) seem to indicate the same. Enzyme catalysis in nearly anhydrous organic solvents is an example of condition promiscuity. Some IDPs show moonlighting property and promiscuity in molecular recognition. It is time to revisit concepts of native structure, biological specificity and even binding site.

Keywords: Catalytic promiscuity, enzyme catalysis, intrinsically disordered proteins, moonlighting proteins, protein purification.

‘The difficulty lies, not in the new ideas; But in escaping the old ones.’

John Maynard Keynes

THE last few decades have witnessed dramatic developments in biological sciences. It all started with the recombinant DNA technology. Approaches like directed evolution¹ and metagenomics² have become possible. In science, new areas and new approaches bring excitement to a discipline; attract the bright minds who further enrich the science. Some old areas do not fade away. Something happens – rekindles the interest like an occasional gust of breeze would give fresh life to the dying embers. Protein structure and function is one such area. Discovery of involvement of amyloid aggregates in several neurodegenerative diseases³ and formation of inclusion bodies during overexpression in bacterial host systems⁴ renewed the interest in denaturation and aggregation of proteins. Proteomics brought back the interest in methods of protein separation and even chemical modification⁵. In addition to these obvious and better noticed developments, there are several other changes which have happened in our understanding of protein structure and enzymology.

These developments are slowly finding their way into the books written for specialists^{1,3,6}. With time, these are likely to be part of the standard textbooks and courses of study. It may perhaps be useful to list few of these. (It is very likely that another author may come up with a different list.) The present article is an overview of these concepts. The references to more comprehensive and critical reviews are provided.

Relevance of Lindstrøm-Lang’s view of protein structure

Richards⁷ provided an excellent account of the seminal contributions of Lindstrøm-Lang’s laboratory in the area of protein structure. He mentioned that it was during Lane medical lectures at Stanford in 1952 that Kaj Ulrik Lindstrøm-Lang ‘presented and defined in detail the concepts (and names) of the primary, secondary and tertiary structure of a peptide chain’⁷. By that time Sanger had 1-fluoro-2,4-dinitrobenzene (FDNB) end-group analysis in place. It was few years later in 1955 that Sanger completed the primary sequence of insulin. The numerous tools for determination of secondary, tertiary and quaternary structure developed in later years. Interesting enough, Richards made the comment regarding this nomenclature of the hierarchical order of protein structure: ‘The concepts and names survive to this day’⁷. That of course is correct since all textbooks of biochemistry mention these terms in their introduction to protein structure. However, some subtle points of the conceptual nature are worth noting¹.

(1) Our current understanding of protein folding is clear on one issue: During folding the protein chains do not pass through primary structure → secondary structure → tertiary structure stages. As the peptide synthesis proceeds, the folding process takes place simultaneously. The complex details can be found elsewhere⁸.

(2) The concept of post-translational covalent modifications was unknown in early years. These modifications affect structure, stability and function of proteins¹.

(3) The concept of secondary structure has proven relevant and useful even in the light of the subsequent knowledge about the protein structure. Secondary structure contributes significantly to overall stability of the folded protein. It also provides a convenient way of describing various folds³.

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(4) Till about a decade ago, the phrase ‘conformational change’ had slight vagueness about it. It was sort of implied that any change in secondary structure was bound to affect tertiary structure significantly and vice versa. Today, there are many instances that either tertiary structure or secondary structure can change without affecting the other level of structure. In the past, most of the structural changes were measured by far-UV circular dichroism (CD) or steady-state fluorescence. The former focuses just on secondary structure content. The latter tracks changes in microenvironments of tryptophan/tyrosine. The situation is changing with both techniques. Near-UV CD is used more often⁹ and both fluorescence resonance energy transfer (FRET) and time-resolved fluorescence are providing more details about changes in the protein¹.

Many techniques are now able to look at the action of a single enzyme molecule. Apart from the fluorescence techniques such as total internal reflection fluorescence microscopy or fluorescence correlation spectroscopy, optical tweezers and atomic force microscopy have been used for studying *in singulo* enzymology^{1,10}.

Greater understanding of the effect of movement of various parts of the polypeptide chain on protein function has started to emerge in the last decade. Of course ‘different types of motions occur on time scales that may or may not be relevant to the reaction of interest’¹¹. The various important classes of motions occurring in the protein structure are: atomic fluctuations (exemplified by interatomic vibrations), motions of the ‘bonded and non-bonded neighbouring group of atoms’ and induced conformational changes³. The ranges of the time involved are 10^{-15} – 10^{-11} sec, 10^{-12} – 10^{-3} sec and 10^{-9} – 10^3 sec respectively³. Henzler-Wildman *et al.*¹² used multiple tools to show (with adenylate kinase as the system) that ‘the larger scale motions in substrate free adenylate kinase are not random, but preferentially follow the pathways that create the configuration capable of proficient chemistry. Such preferred directionality, encoded in the fold, may contribute to catalysis in many enzymes’. Nagel and Klinman¹³ also emphasized the importance of understanding the role of various internal motions in catalysis.

(5) In the last few decades, it is more common to talk of super secondary structures like helix–loop–helix and give description of local tertiary structures in terms of domains, modules and folds^{1,3}.

(6) The paradigm shift has not been so drastic here as moving from Dalton’s atomic theory to Schrodinger’s H-atom but more subtle in nature, mostly in terms of understanding finer details.

Proteins and organic solvents

The old wisdom was that organic solvents denature proteins. That general impression persists. It is not totally incorrect, but it is not totally correct either! It is a ques-

tion of which organic solvent and in how much percentage of the solution. Let us start with the latter. So, we have the following situations:

(a) About 0–20% (v/v) of water-miscible organic solvents: In many enzyme assays, 0–5% addition of an organic solvent like DMSO or DMF is needed to dissolve the substrate. The frequently used assay of trypsin using BAPNA is a well-known example¹⁴. Generally, it has insignificant effect, although it is advisable to run a control. The organic solvent in 0–20% (v/v) range in fact often leads to higher initial rates¹⁴. The reasons can be complex, but both medium effects and enhanced flexibility of the protein conformation have been implicated. Lower alcohols could be bad, especially if low temperature is not used.

(b) 20–95% (v/v) of water-miscible organic solvent: This is generally the bad range and responsible for the general impression that organic solvents denature the protein. Some organic solvents are better than others and a parameter called ‘denaturation capacity’ has been defined¹⁵.

(c) 95–100% (v/v) of organic solvent: This is the medium which is often referred to as ‘low water media’ or ‘nearly anhydrous media’ or ‘neat organic solvents’^{16–18}. This is of considerable interest to synthetic organic chemists as fairly inexpensive hydrolases can be used for synthesis. With bulk water not available, thermodynamic equilibria shift in the opposite direction. So, proteases can synthesize peptides¹⁹. Esterases can form esters. The ‘medium engineering’ becomes possible and the initial rates, conversions and specificity can be altered by changing the organic solvent. Regiospecificity, substrate specificity and stereospecificity, all can be altered¹⁷. The Unilever group had given some general rules about which organic solvents are good for obtaining higher catalytic efficiency¹⁷. The parameter $\log P$ (the partition coefficient of the organic solvent between octanol and water) is generally considered the best guiding parameter in this respect, although other parameters have also been discussed¹⁴. This is an area of very high research activity, even if general textbooks of biochemistry are silent about it.

It may be pointed out that in such low water media, the enzyme molecules do not dissolve unless modified by PEG²⁰ or are used in the form of surfactant complexes²⁰. The most frequently used form is to generally use lyophilized powders. Lyophilization or freeze-drying proteins is a classical technique and has been used by enzymologists for decades. In classical enzymology, lyophilization is considered a benign technique routinely used by enzymologists, manufacturers of pharma proteins and vaccines. So, it came as a surprise when it was found that lyophilization causes extensive changes in the secondary structure²¹. This fact was missed earlier as these changes are reversible upon dissolution of protein powders in bulk water. However, when these lyophilized powders are used in non-aqueous media, these structural changes con-

tribute to the observed low catalytic activity²². In fact, lyophilization is a complicated process and its understanding has been valuable to people drying proteins for the high-value applications. Knowledge from those areas has generated techniques like use of cryoprotectants and lyoprotectants to minimize the structural changes during lyophilization²¹⁻²³. This has been put to good use by workers in the area of non-aqueous enzymology²⁴. Improving catalytic activity in such media is immensely useful and several ways for this have been suggested in the literature. Biocatalyst preparations like immobilized enzymes²⁵, enzyme precipitated and rinsed with propanol (EPRP)^{26,27}, crosslinked enzyme aggregates (CLEA)^{25,28}, protein-coated microcrystals (PCMC)^{29,30} and crosslinked protein-coated microcrystals (CLPCMC)³¹ are known to give much better catalytic rates as compared to just lyophilized powders or 'straight from the bottle' preparations obtained from the commercial sources. Use of enzymes in reversible micelles³² and ionic liquids^{33,34} also is part of non-aqueous enzymology. Inasmuch as, knowledge from the area provides considerable insight about the interplay among flexibility, specificity and stability²⁷, familiarity with the area is beneficial to the workers in the general areas of protein structure, conventional enzymology and biotechnology.

(d) 0→95% (v/v) of water-immiscible organic solvents: Even water-immiscible organic solvents have limited solubility in water. For example, solubility of some water immiscible organic solvents is given in Table 1 (refs 35 and 36). To provide some quantitative idea after the solubility limit is reached, the added organic solvent separates out as a different phase. Such two-phase systems also form excellent reaction systems in biocatalysis^{37,38}. The usefulness of such systems relates to working with substrates of poor solubility. Such substrates are dissolved in organic solvent phase, the biocatalyst remains in aqueous phase. The reaction takes place at the interface of the two phases. If the product also separates out in the organic solvent-rich phase, equilibrium is disturbed and the reaction continues in the forward direction. Additionally, any substrate or product inhibition is also relieved¹.

This is an area which has become extremely important in synthesis of drug intermediates, agrochemicals and materials. Enzymes per se show stereospecificity; changing reaction media provides a tool to even alter or control it. As chirally pure compounds are highly desirable in many areas (for example, drug industry), this research area would increasingly become difficult to ignore by biochemists/enzymologists.

Enzyme purification

Here, a significant change for the classical protein chemist/enzymologist has been that one requires much smaller amounts of pure proteins for analysis. Primary sequence is increasingly determined via gene sequences (unless one

is concerned with post-translational covalent modification). In the context of proteomics, one does not have to 'obtain' a pure protein. It can be done by putting together an appropriate battery of instruments in the right sequence. The protein purification is increasingly done via construction of an appropriate fusion protein. Consequently, a whole generation of biochemists thinks enzymes can be easily purified by purchasing a kit from the commercial vendor.

Enzymes/proteins in large scale (for pharma proteins this could be in gram quantities, for industrial enzymes it is in kilograms) have to be obtained for practical applications and it is neither practical nor economical to follow the current practice prevailing in the academic sector.

There are considerable developments in the area of protein purification (at preparative scale) which most of the textbooks have ignored. Few of the separation techniques for protein purification are listed in Table 2 (refs 39–58). The two paradigm shifts which have occurred over the last few decades are the following:

(1) Affinity-based separations have assumed greater importance. This has largely become possible because we have begun to understand that this concept goes beyond *in vivo* relationship among molecules^{59,60}. Hence, robust affinity ligands (for example, textile dyes) can be used. Scaffolds designed to produce libraries of compounds have made it possible to search out a potential affinity ligand with almost any combination of functional groups⁵⁹.

(2) Chromatography has been an important component of any purification protocol wherein very high purity is desirable. Such end applications include pharma proteins. These proteins already constitute a multibillion dollar market. Monoclonal antibodies, as a single sub-class of pharma proteins are projected to grow by a huge amount as therapeutics in the coming decade. Chromatography

Table 1. Solubility of some water immiscible solvents^{35,36}

Solvent	Formula	Solubility in water (g/100g)	Relative polarity
Cyclohexane	C ₆ H ₁₂	0.005	0.006
Pentane	C ₅ H ₁₂	0.0039	0.009
Hexane	C ₆ H ₁₄	0.0014	0.009
Heptane	C ₇ H ₁₆	0.0003	0.012
Carbon tetrachloride	CCl ₄	0.08	0.052
Carbon disulfide	CS ₂	0.2	0.065
<i>p</i> -Xylene	C ₈ H ₁₀	0.02	0.074
Toluene	C ₇ H ₈	0.05	0.099
Benzene	C ₆ H ₆	0.18	0.111
<i>N,N</i> -dimethylaniline	C ₈ H ₁₁ N	0.14	0.179
Chlorobenzene	C ₆ H ₅ Cl	0.05	0.188
Anisole	C ₇ H ₈ O	0.10	0.198
Ethyl benzoate	C ₉ H ₁₀ O ₂	0.07	0.228
di- <i>n</i> -Butyl phthalate	C ₁₆ H ₂₂ O ₄	0.0011	0.272
1-Octanol	C ₈ H ₁₈ O	0.096	0.537
1-Heptanol	C ₇ H ₁₆ O	0.17	0.549
1-Hexanol	C ₆ H ₁₄ O	0.59	0.559

Table 2. Separation techniques seldom used for protein purification by enzymologists

Technique	Reference
Aqueous two-phase separation	39
Expanded bed chromatography	40–44
Perfusion chromatography	45, 46
Three-phase partitioning	47–49
Precipitation with smart polymers	50, 51
Macro affinity ligand-facilitated three-phase partitioning	52–54
Micellar extractions	55, 56
Separation based upon magnetism	57, 58

results in a very high resolution and can be a part of the robotic platform for a purification protocol. Hence, efforts to replace it completely by a more economical and more easily scalable non-chromatographic step are unlikely to succeed⁶¹.

Nevertheless, most of the industrial enzymes for application in detergents, food processing, leather processing, oil and fat processing, and textile industries⁶² are required at fairly low purity level. Chromatography is hardly used. One constraint with chromatography has been the need for the feed to be clean and free of suspended impurities (like cell debris). Some versions of chromatography like expanded bed adsorption (EBA) have been available now for some time⁴¹. Non-chromatographic techniques like aqueous two-phase separations (ATPS)³⁹ and macro-affinity ligand-facilitated three-phase partitioning (MLFTPP)^{52–54} are the only two other techniques which can deal with a feed with suspended impurities. The use of smart polymers in protein purification and protein refolding has also been reported^{4,50}. The awareness of classical enzymologists about non-chromatographic strategies unfortunately has not changed since the era of Cohn or even Kunitz.

Catalytic promiscuity

This phenomenon refers to an enzyme showing a different activity from what is expected from its assigned enzyme commission (EC) number. Many generations of biochemists grew up with the notion that specificity is the hallmark of biology. The belief in tight correlation between structure and function of biological molecules like proteins and nucleic acids has spurred so many developments in biology. Who can forget the iconic sentence with which Watson and Crick concluded their classical paper announcing the discovery of double-helix structure of DNA: ‘It has not escaped our notice that the specific pairing that we have postulated immediately suggests a possible copying mechanism for the genetic material’⁶³. So, it is not surprising that discoveries of catalytic promiscuity and moonlighting proteins seem to have resulted in classical enzymologists going to a state of denial! Both the concepts were discussed in this journal recently⁶⁴, so only an update should be sufficient.

The IUPAC classification created well-defined classes of enzymes⁶⁵. For example, one of the six classes is constituted by hydrolases, the enzymes which hydrolyse various types of bonds. As discussed elsewhere in this article, there are now several hundreds of papers showing that hydrolases and proteases can be generally employed for the synthesis as well using nearly anhydrous organic solvents. This is one example of condition promiscuity⁶⁶. Now, it transpires that lipases can carry out several condensation reactions even in predominantly aqueous media^{67,68}. This has led to enhancing or tweaking of such activities by directed evolution⁶⁹. This is a revolutionary change in the concept of biocatalysis and requires a wider awareness and appreciation.

Khersonsky and Tawfik⁶⁹ have suggested a way by which enzyme promiscuity could be fitted in with the existing EC Numbers. According to these authors, one should grade promiscuity in terms of problems with first, second or third digit of the EC numbers. Non-enzyme proteins like BSA showing enzyme catalysis are cited as examples of highest degree of promiscuity. Further refinement of these suggestions is necessary in the context of Hult’s classification of promiscuity in terms of condition promiscuity, substrate promiscuity and catalytic promiscuity⁶⁶.

Babtie *et al.*⁷⁰ have analysed kinetic data of promiscuous enzymes and observed ‘... that binding and catalysis can be highly efficient for more than one reaction; challenging the notion that proficient catalysis requires specificity’. Mutti *et al.*⁷¹ suggest that alkene cleavage activity of peroxidase which requires heme group only should be called ‘Ostensible enzyme promiscuity’.

Humble and Berglund⁷² have provided an overview on biocatalytic promiscuity recently. Busto *et al.*⁷³ have written a comprehensive review on promiscuous activity on hydrolases. A more focused review just on lipases is also available⁷⁴. Clinton *et al.*⁷⁵ believe that the promiscuous activity of subtilisin proteases may have a role in bacterial degradation of strobilurin fungicides.

In the cases of catalytic and condition promiscuity, it has been clearly established that the same active site is involved in both normal and promiscuous activities⁶⁸.

Some examples of promiscuity are given in Table 3 (refs 67, 73, 76–80). It looks as if we have just seen the proverbial tip of an iceberg. More examples and various other aspects of promiscuity are sure to emerge in the coming years.

Moonlighting proteins

It turns out that many proteins have multiple binding sites for very different kinds of ligands and each ‘molecular recognition’ results in a different biological activity. The term was coined by Constance Jeffery and in her words these are the ‘proteins in which the two functions are found in a single polypeptide chain. They do not include

proteins that are multifunctional due to gene fusions, families of homologous proteins, splice variants or promiscuous enzyme activities⁸¹. Such different biological functions may be observed when: (a) such a protein occurs in different parts of the cell; (b) an intracellular protein becomes extracellular or vice versa; (c) such a protein is expressed in a different cell type (*in vivo*); (d) state of aggregation and (e) different concentrations of a substrate/ligand^{64,82}.

For a long time, scientists have asked why enzymes have to be macromolecular in nature when very few residues actively participate in the actual catalysis? The answer to the question, we think, has grown along with our understanding of the biology. Organic chemists or those who work with supramolecular systems especially seem to think that higher catalytic efficiencies of the enzymes could have been achieved with a much smaller structure. Way back in 1960, Koshland Jr⁸³ talked of ‘junk’ and ‘non-junk’ amino acids in the context of this debate. The first component of this answer was that to fashion appropriate specificity, one needed a large scaffold. The second insight came when signal hypothesis focused attention on the need of a targeting structural component so that the protein lands up at the right place within or outside the cell. Even earlier, zymogen existence and discovery of allosteric phenomenon pointed to the need for bigger structure for necessary regulation of the biological activity. An extension of this is that glutamine synthetase, an enzyme which regulates entry of nitrogen in the metabolism is among the larger molecular weight enzymes with multi-subunits. It has to be responsive to concentrations of multiple ligands which collectively dictate the need for entry of inorganic nitrogen in the metabolism. The enzyme has to be big enough to interact with those multiple ligands. The existence of moonlighting protein is yet another part of that answer. Often the different biological activities require different binding sites on the moonlighting protein⁸². As Huberts and van der Klei⁸⁴ point out ‘... important criteria for a moonlighting protein is the

independency of both functions, meaning that inactivation of one of the functions (for example by mutation) should not affect the second function and vice versa’. The classical example of a moonlighting protein is the eye lens protein crystallins; duck ϵ -crystallin also shows lactate dehydrogenase activity; turtle τ -crystallin is also α -enolase. An updated list of examples have been given by Jeffery⁸¹ and Huberts and van der Klei⁸⁴ and includes thioredoxin/T7 DNA polymerase subunit, aldolase/host cell invasion and murl which is glutamate racemase/DNA gyrase inhibitor. Apparently, very few changes in amino acid sequence are required for acquiring a new moonlighting function⁸⁴. Further details on moonlighting proteins are available in the literature^{64,85–89} (other than the references already cited). Recently, Jeffery⁹⁰ has talked of ‘neomorphic’ moonlighting proteins. These are the proteins ‘... in which a mutation or conformational change imparts a second function on a protein that is not a “normal” function of the protein’. She reported that in some cases, these ‘neomorphic moonlighting functions’ have been found to be associated with some cancer and neurological disorders. Examples of such proteins include β 2-microglobulin, glyceraldehyde-3-phosphate dehydrogenase and triose phosphate isomerase.

Intrinsically disordered proteins

While the intrinsically disordered proteins (IDPs) may not yet have made to the general textbooks of biochemistry, they are very likely to figure in their next editions. IDPs are already well known to a fairly wide section of biochemists in the context of protein aggregation and neurodegenerative diseases⁹¹. They are being mentioned here since just like the concepts of promiscuity and moonlighting, their discovery questions a well-established paradigm about proteins. The biological function of a protein is considered to be heavily dependent upon the native structure. The denaturation – the departure from the native structure – was generally associated with transition to a less ordered structure. Old timers would recall the excitement associated with the discovery of RNAase S^{92,93}. This reassociated complex of S-peptide and S-protein obtained from proteolytic digestion of RNAase A is completely active. X-ray structure of RNase S (compared with that of RNAase A) shows a disorder in the region of association of the S-peptide with S-protein⁹³. Denaturation was associated with unfolding of the polypeptide chain and a more disordered tertiary structure. In IDPs, as the name implies, the native structure is highly disordered. How do you define denaturation here? In the classical picture, unfolding of the chain led to exposure of buried hydrophobic residues and hence aggregation¹. In IDPs, aggregation is associated with the formation of the ordered structures⁹¹. In alpha synuclein, for example, aggregates are reported to have higher β -sheet content⁹⁴.

Table 3. Some recent examples of catalytic promiscuity useful for biotransformations

Reaction	Enzyme used	Reference
Aldol reaction	Nuclease p1 Lipase Protease	73, 76
Aldol condensation	Lipase	67
Knoevenagel condensation	Lipase	67
Knoevenagel–Doebner condensation	Lipase	67
Markovnikov addition	Penicillin G acylase	77
Baeyer–Villiger oxidation	Lipase	73
aza-Michael addition	Acylase	78
Henry reaction	Lipase Protease	79
Mannich reaction	Transglutaminase Lipase	80

It may be interesting to point out that ‘The so called ‘hub’ proteins, which bind to many partners and are thus central to protein interaction networks, use conformational disorder to provide the required plasticity to interact with large number of different proteins’⁹⁵. So, size of the protein again is an issue. In the classical picture, size was believed to be required for creating an ordered structure. Now we find that size is equally important to have diverse binding sites in the disordered region.

Thinking rationally, we did realize long ago that lock-and-key hypothesis with a rigid active site was not the correct picture. That was the message of induced fit hypothesis by Koshland⁹⁶. Flexibility of the conformation is absolutely necessary for catalysis. The rigid structure of the proteins in low water media, which makes them stable at 100°C, is also largely responsible for their dismally low catalytic efficiency¹⁷. Similarly, relatively rigid conformation of thermostable enzymes from thermophilic organisms shows much lower K_{cat}/K_m than corresponding enzymes from mesophiles¹. The disorder of IDPs just extends that a little further. One does not require a rigid structure to create a specific binding site; it is the ultimate ‘induced fit’ which is essential. ‘To permit specific recognition, disordered targets usually undergo coupled or concomitant folding/ordering and binding’⁹⁵. As Mittag *et al.*⁹⁵ also point out, this plasticity allows a protein with the same primary sequence to moonlight.

Some excellent reviews on IDPs are available. A good introduction is by Uversky⁹¹, who is a leading worker in the area. In 2010, there was a Gordon Research Conference on IDPs with a subtitle of ‘Introducing unfoldome and unfoldomics’⁹⁷. Uversky⁹¹ has listed more than a dozen names which have been used earlier for IDPs. These include ‘floppy’, ‘natively unfolded’, ‘dancing proteins’ and ‘protein clouds’. Interesting enough, the term ‘partially folded’ was used by Lindstrom-Lang and Shellman wayback in 1959 and is included in this long list⁹¹.

IDPs are characterized by absence of any significant level of secondary and tertiary structure, have low mean hydrophobicity and high net charge. Given their pliability, many IDPs show promiscuity⁹¹.

IDPs are attracting considerable attention for various reasons. They are found to have a role in a wide variety of diseases: ‘cancer, cardiovascular disease, neurodegenerative diseases, amyloidoses,’⁹¹. Some IDPs are known to moonlight extensively; an example is that of some proteins of 50S subunit of *Escherichia coli* ribosome which can facilitate folding of both RNA and protein molecules. These are called ‘Janus chaperones’⁹⁸.

Other examples of IDPs include clustrin, calsequestrin, neuromodulin, kinesin and titin⁹⁹. Some recent articles^{100,101} suggest that we need to consider disorder as an extreme case of flexibility. So, in IDPs ‘much higher flexibility’ is the key to molecular recognition.

What is a binding site in a protein?

In classical enzymology as it is described in any textbook of biochemistry, enzymes can have two sites at which molecules bind specifically – the active site at which the substrate and some classes of inhibitors bind and the allosteric site at which effectors bind in regulatory enzymes. For transport proteins and other biologically active proteins, similar sites are well defined. Ringe¹⁰² wrote an article entitled ‘What makes a binding site a binding site?’, which needs wider attention. The article started with an illustrative example of thrombin (a protease involved in blood-clot formation) with which hirudin (a protease inhibitor from leech) was found to interact at two different sites; only one of these was the known active site of thrombin. It is pointed out that computational approaches for mapping binding sites ignore two facts: proteins are flexible and water at 55M concentration is a competing ligand¹⁰². Using elastase (another protease), Ringe tested the hypothesis: ‘Is it possible that the characteristics of a binding site include the presence of not only suitable electrostatic partners and van der Waals partners for the ligand, but also a water structure that allows the ligand to compete successfully for such a site in the face of 55M water?’. The author concluded ‘experimental studies of protein binding surfaces are beginning to suggest a set of general guidelines for what makes a binding site a binding site. Such regions are usually depressions in the protein surface in which there is a greater than average degree of exposure of hydrophobic groups. They contain bound water molecules that are not tightly held but which do make specific interactions with the polar groups that are in the site. Such water molecules are easily displaced, allowing the polar functional groups on the ligand to make the same sort of interactions with the target protein. Although shape and charge complementarity are obviously important, they are not the whole story. Proteins are flexible molecules and many regions on their surfaces can adapt their shapes to those of incoming ligands’.

Another article which takes this discussion further is in the question and answer format and is about pharmacological chaperones¹⁰³. ‘... pharmacological chaperones are small molecules, and instead of assisting in folding, they usually stabilize an already folded macromolecule (usually a protein) by binding to it and stabilizing it against thermal denaturation and proteolytic degradation’¹⁰³. This links to the binding site issue since pharmacological chaperones bind specifically. These are not to be confused with glycerol and trehalose-type molecules which stabilize proteins in a non-specific fashion¹⁰⁴⁻¹⁰⁶. A good review on pharmacological chaperones is given by Arakawa *et al.*¹⁰⁷. The pharmacological chaperones are important in therapeutics and have shown potential as drugs for protein misfolding diseases (for example Parkinson’s and Lou Gehrig’s)^{108,109}. While all pharmacological

chaperones currently under clinical trials are reversible inhibitors which bind to the active site^{110–114}, in principle, pharmacological chaperones binding to other sites should also work with certain advantages. Hence, there is a need for further understanding about what is a binding site and developing improved methods to identify binding sites.

Conclusion

(1) Biological specificity is not limited to *in vivo* context. Almost any class of an organic compound can be used to tailor a 'specific ligand' for a given protein. A lectin can recognize a peptide 'specifically'. Robust and economical protein purification methods have emerged out of this basic departure from the classical picture of biological activity.

(2) Assigning of EC numbers has been based upon the activity of the enzymes in aqueous media. With hydrolases showing synthetic activity in nearly anhydrous organic solvents and ionic liquids, there is a need for a serious rethink about the system. Similarly, both moonlighting and promiscuity show that classifying the enzyme in six classes may no longer be a valid system.

(3) Both short-range and long-range interactions spanning the entire protein chain may be crucial for catalysis/biological activity.

(4) One protein structure need not have just one biological activity. Promiscuity and moonlighting properties of proteins/enzymes have established this beyond doubt. It raises interesting questions about the old paradigm of structure and function of proteins.

(5) Flexibility and order in protein structure is tailored for a given protein, keeping in mind both the organism, its milieu and function. Denaturation could mean obtaining a more ordered structure.

Just as there is a phrase 'method in madness', the design of IDPs shows that biological function is rooted in disorder in the protein structure. To quote Uversky⁹¹, 'Modern protein science is at the turning point'. An old blessing is 'May you live in interesting times.' These are interesting times for enzymologists or whatever this tribe prefers to be known as today.

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ACKNOWLEDGEMENTS. We thank the Department of Biotechnology [Grant No. BT/PR14103/BRB/10/808/2010] and Department of Science and Technology, New Delhi [Grant No. SR/SO/BB-68/2010] for financial support. J.M. thanks the Council of Scientific and Industrial Research, New Delhi for award of Senior Research Fellowship.

Received 1 January 2013; revised accepted 22 March 2013