

# Contents

|                                   |             |
|-----------------------------------|-------------|
| <b>List of contributors .....</b> | <b>xv</b>   |
| <b>Preface.....</b>               | <b>xvii</b> |

|   |          |
|---|----------|
| <b>Chapter 1: Enzymology: early insights.....</b> | <b>1</b> |
|---|----------|

*Munishwar Nath Gupta and Vladimir N. Uversky*

|   |    |
|---|----|
| 1.1 Introduction .....                              | 1  |
| 1.2 Isolation and purification of proteins .....    | 3  |
| 1.3 The dawn of structural biology .....            | 4  |
| 1.4 The early work on enzyme kinetics .....         | 7  |
| 1.5 Assaying enzymes.....                           | 8  |
| 1.6 Enzyme immobilization .....                     | 11 |
| 1.7 Applied enzymology and white biotechnology..... | 13 |
| 1.8 Enzymes in neat solvents .....                  | 18 |
| 1.9 Some myths about applications of enzymes.....   | 21 |
| 1.10 Conclusions .....                              | 22 |
| References .....                                    | 23 |

|   |           |
|---|-----------|
| <b>Chapter 2: Deep mutational scanning to probe specificity determinants in proteins.....</b> | <b>31</b> |
|---|-----------|

*Jayantika Bhowmick, Soumyanetra Chandra and Raghavan Varadarajan*

|  |    |
|--|----|
| 2.1 Proteins, enzymes, and disorder .....  | 31 |
| 2.2 Deep mutational scanning—a high-throughput method to explore protein sequence–function landscapes..... | 33 |
| 2.3 Deep mutational scanning of globular proteins .....  | 38 |
| 2.3.1 Deep mutational scanning of enzymes .....  | 38 |
| 2.3.2 Deep mutational scanning of nonenzymatic globular proteins.....                                      | 47 |
| 2.4 Study of sequence–disorder–function relationships in Intrinsically Disordered Proteins (IDPs) .....    | 49 |

---

|   |    |
|---|----|
| 2.4.1 Deep mutational scanning of intrinsically disordered regions in transcription factors .....   | 50 |
| 2.4.2 Deep mutational scanning to probe aggregation of intrinsically disordered proteins.....   | 54 |
| 2.4.3 Nonproteinogenic deep mutational scanning in intrinsically disordered proteins.....   | 57 |
| 2.4.4 Deep mutational scanning to investigate residue-specific contributions to partner binding in intrinsically disordered protein ..... | 58 |
| 2.5 Discussion.....   | 59 |
| Acknowledgments.....  | 59 |
| Author contributions .....  | 60 |
| References .....  | 60 |

**Chapter 3: Protein flexibility and cryoenzymology: the trade-off between stability and catalytic rates.....** 73

*Munishwar Nath Gupta and Vladimir N. Uversky*

|   |    |
|---|----|
| Abbreviations.....  | 73 |
| 3.1 Origin of cryoenzymology .....  | 73 |
| 3.2 Search for the solvents suitable for the low-temperature studies .....            | 77 |
| 3.3 Looking into the protein folding at subzero temperatures .....                    | 79 |
| 3.4 Cryoenzymology: analysis of the enzymatic reactions at subzero temperatures ..... | 81 |
| 3.5 X-ray cryoenzymology .....  | 84 |
| 3.6 Cryo-electron microscopy enzymology .....   | 85 |
| 3.7 Cryoenzymology and psychrophiles.....   | 86 |
| 3.8 Concluding remarks .....  | 90 |
| References .....  | 91 |

**Chapter 4: Thermodynamic perspective of protein disorder and phase separation: model systems.....** 97

*Riley J. Workman, Justin A. Drake and B. Montgomery Pettitt*

|  |     |
|--|-----|
| 4.1 Introduction .....                             | 97  |
| 4.1.1 Thermodynamic uses of protein disorder ..... | 99  |
| 4.1.2 Role of modeling and simulation .....        | 100 |
| 4.2 Conformational landscapes .....                | 100 |
| 4.2.1 Populations and free energy .....            | 100 |
| 4.2.2 Enthalpy of order.....                       | 102 |
| 4.2.3 Entropy of disorder .....                    | 103 |

|  |     |
|--|-----|
| 4.3 Determinants of the disordered ensemble.....   | 105 |
| 4.3.1 Sequence composition: collapsed and extended disorder.....                                       | 105 |
| 4.3.2 Polymer physics and the quality of water as a disordered region solvent .....                    | 106 |
| 4.4 Lessons from a protein backbone model.....   | 107 |
| 4.4.1 Polyglycine: a protein backbone and disordered region model.....                                 | 107 |
| 4.4.2 Protein backbone collapse: a solubility-driven event.....  | 108 |
| 4.5 Aggregation of many peptides—liquid–liquid phase separation is a type of order.....                | 112 |
| 4.5.1 Solubility limits measure more than hydrophobicity .....   | 112 |
| 4.5.2 Backbone conformational entropy and liquid–liquid phase separation.....                          | 113 |
| 4.5.3 Towards tuning the properties of liquid–liquid phase separation.....                             | 116 |
| 4.6 Future perspectives .....  | 117 |
| 4.6.1 Simple systems and modeling approaches.....  | 117 |
| 4.6.2 Conformational entropy reservoir: thermodynamic coupling through order–disorder transitions..... | 118 |
| Acknowledgments .....  | 119 |
| References .....   | 120 |

***Chapter 5: Structure and disorder: protein functions depend on this new binary transforming lock-and-key into structure-function continuum .....*** 127

*Munishwar Nath Gupta and Vladimir N. Uversky*

|  |     |
|--|-----|
| 5.1 Introduction .....   | 127 |
| 5.2 Keys in locks and hands in gloves: classical representations of protein functionality.....   | 128 |
| 5.3 Structure-function paradigm cannot be stretched far enough to include protein “moonlighting,” multifunctionality, binding promiscuity, and scaffolding ..... | 130 |
| 5.4 A new player in the block: functional proteins without unique structures .....   | 133 |
| 5.5 Structural heterogeneity of IDPs and IDRs .....  | 134 |
| 5.6 Proteoforms as a solution for the “one gene—many proteins” challenge .....   | 136 |
| 5.7 Intrinsic disorder, proteoforms, and protein-structure continuum .....   | 137 |
| References .....   | 139 |

***Chapter 6: Methods for measuring structural disorder in proteins .....*** 149

*Frank Gondelaud, Antoine Schramm, Stefania Brocca, Antonino Natalello, Rita Grandori, Carlo Santambrogio and Sonia Longhi*

|                        |     |
|------------------------|-----|
| 6.1 Introduction ..... | 149 |
|------------------------|-----|

---

|       |   |     |
|-------|---|-----|
| 6.2   | Obtaining hints of intrinsic disorder.....                                    | 150 |
| 6.2.1 | Anticipating intrinsic disorder from the amino acid sequence.....             | 150 |
| 6.2.2 | SDS-PAGE and limited proteolysis.....   | 151 |
| 6.2.3 | Resistance to denaturing conditions .....                                     | 151 |
| 6.3   | Assessing protein hydrodynamic properties .....                               | 152 |
| 6.3.1 | Size-exclusion chromatography.....  | 152 |
| 6.3.2 | Dynamic light scattering .....  | 153 |
| 6.3.3 | Analytical ultracentrifugation.....   | 154 |
| 6.3.4 | Small-angle X-ray scattering.....   | 156 |
| 6.4   | Assessing protein secondary structure content .....                           | 160 |
| 6.4.1 | Circular dichroism spectroscopy .....   | 160 |
| 6.4.2 | Fourier transform infrared spectroscopy.....                                  | 162 |
| 6.4.3 | Nuclear magnetic resonance spectroscopy .....                                 | 163 |
| 6.5   | Assessing protein tertiary structure .....                                    | 164 |
| 6.5.1 | Near-ultraviolet circular dichroism spectroscopy .....                        | 164 |
| 6.5.2 | Differential scanning calorimetry .....                                       | 165 |
| 6.5.3 | Fluorescence spectroscopy .....   | 165 |
| 6.5.4 | Electrospray ionization mass spectrometry.....                                | 168 |
| 6.5.5 | Ion mobility-mass spectrometry .....  | 169 |
| 6.5.6 | Hydrogen–deuterium exchange approaches.....                                   | 171 |
| 6.6   | High-speed atomic force microscopy .....                                      | 172 |
| 6.7   | Approaches relying on protein labeling and/or site-directed mutagenesis ..... | 172 |
| 6.7.1 | Fluorescence resonance energy transfer .....                                  | 172 |
| 6.7.2 | Vibrational spectroscopy of cyanlated cysteines.....                          | 175 |
| 6.7.3 | Electron paramagnetic resonance spectroscopy .....                            | 178 |
| 6.7.4 | Tryptophan triplet cysteine quenching .....                                   | 178 |
| 6.8   | In vivo assessment of disorder.....   | 180 |
| 6.9   | Modeling intrinsically disordered proteins as conformational ensembles .....  | 182 |
| 6.10  | Assessing binding events .....  | 186 |
|       | Acknowledgments.....  | 187 |
|       | References .....  | 187 |

---

**Chapter 7: Prediction of protein structure and intrinsic disorder in the era of deep learning .....** 199

*Gábor Erdős and Zsuzsanna Dosztányi*

|     |  |     |
|-----|--|-----|
| 7.1 | Introduction .....   | 199 |
| 7.2 | A brief overview of protein structure prediction approaches.....                 | 202 |
| 7.3 | Critical assessment of structure prediction—structure prediction evaluation..... | 204 |

|   |     |
|---|-----|
| 7.4 Machine learning revolution through deep learning ..... | 206 |
| 7.5 Deep learning methods in structure prediction .....     | 209 |
| 7.6 Predicting protein disorder.....                        | 213 |
| 7.7 Predicting the functions of disordered regions .....    | 217 |
| 7.8 Conclusions .....                                       | 218 |
| References .....  | 219 |

**Chapter 8: Roles of intrinsically disordered regions in phosphoinositide 3-kinase biocatalysis .....225**

Vrushank Dave' and Vladimir N. Uversky

|   |     |
|---|-----|
| 8.1 Biochemistry of PI3K enzymes.....   | 225 |
| 8.2 Class I PI3K enzymes.....   | 227 |
| 8.3 Class II PI3K enzymes.....  | 230 |
| 8.4 Class III PI3K enzyme.....  | 230 |
| 8.5 Normal and aberrant cellular functions of PI3K enzymes .....                | 231 |
| 8.6 Normal functions of class I PI3K in cellular signaling and physiology ..... | 232 |
| 8.7 Aberrant hyperactivation of PI3K as a major driver of diseases.....         | 232 |
| 8.8 Structural biology and biocatalysis of class I PI3K family .....            | 233 |
| 8.9 Role of intrinsically disordered regions in the PI3K functions .....        | 234 |
| References .....  | 238 |

**Chapter 9: The various facets of protein promiscuity: not just broad specificity of proteins .....241**

Munishwar Nath Gupta and Vladimir N. Uversky

|  |     |
|--|-----|
| 9.1 Introduction .....   | 241 |
| 9.2 Protein specificity .....  | 242 |
| 9.3 Protein promiscuity as a driver of protein evolution .....                       | 245 |
| 9.4 Types of promiscuity .....   | 249 |
| 9.5 Promiscuity of alkaline phosphatase superfamily .....                            | 253 |
| 9.6 Quantifying enzyme promiscuity .....   | 255 |
| 9.7 Engineering enzyme promiscuity .....   | 255 |
| 9.8 Promiscuity in protein–protein interactions.....                                 | 257 |
| 9.9 Calmodulin promiscuity.....  | 259 |
| 9.10 $\alpha$ -Synuclein promiscuity, multifunctionality, and polypathogenicity..... | 262 |
| 9.11 Promiscuity in drug design .....  | 267 |
| 9.12 Conclusions .....   | 267 |
| References .....   | 268 |

---

|  |            |
|--|------------|
| <b>Chapter 10: Role of plasticity and disorder in protein moonlighting: blurring of lines between biocatalysts and other biologically active proteins.....</b> | <b>279</b> |
| <i>Munishwar Nath Gupta and Vladimir N. Uversky</i>  |            |
| 10.1 Introduction .....  | 279        |
| 10.2 Description of “moonlighting” as a phenomenon.....  | 280        |
| 10.3 What is a binding site? .....   | 283        |
| 10.4 Moonlighting proteins in health and diseases.....   | 284        |
| 10.5 Protein conformational plasticity .....   | 286        |
| 10.6 Disordered moonlighting regions .....   | 288        |
| 10.7 Intrinsic disorder roots of moonlighting: multifunctionality<br>as a consequence of the disorder-based structural heterogeneity .....                     | 293        |
| 10.8 Moonlighting in virulence activity of pathogens .....   | 294        |
| 10.9 Conclusions and future perspectives .....   | 296        |
| References .....   | 297        |
| <b>Chapter 11: Molten globular enzymes.....</b>  | <b>303</b> |
| <i>Vladimir N. Uversky</i>   |            |
| Abbreviations .....  | 303        |
| 11.1 Enzymes as a cornerstone of the “lock-and-key” model of protein<br>functionality.....   | 303        |
| 11.2 Molten globular enzymes: machines at the edge of stability .....  | 307        |
| 11.2.1 Dihydrofolate reductase mutants with amino acid<br>replacements in the active center .....  | 310        |
| 11.2.2 Circularly permuted dihydrofolate reductase .....   | 311        |
| 11.2.3 Truncated $\Delta 131\Delta$ mutant of staphylococcal nuclease .....  | 311        |
| 11.2.4 Double-point mutant F34W/W140F of staphylococcal nuclease.....  | 312        |
| 11.2.5 C-terminally truncated mutants of the capsid protease from<br>Semliki Forest virus .....  | 313        |
| 11.2.6 Monomeric chorismate mutase from <i>Methanococcus jannaschii</i> .....  | 313        |
| 11.2.7 Simplified chorismate mutase with 9-amino acid alphabet.....  | 314        |
| 11.2.8 Molten globular active sites of the detoxication enzyme<br>glutathione transferase A1–1 .....   | 314        |
| 11.2.9 Urzyme: an ancestral tryptophanyl-tRNA synthetase precursor.....  | 315        |
| 11.2.10 De-evolved dephospho-CoA kinase.....   | 315        |
| 11.2.11 Catalytically active alkaline molten globular enzyme:<br>5-aminolevulinate synthase .....  | 316        |
| 11.2.12 Nucleolytic activity of folding intermediates of RNase T <sub>1</sub> .....  | 317        |
| 11.2.13 Nonnative intermediate transiently populated during folding<br>of the acylphosphatase from <i>Sulfolobus solfataricus</i> .....                        | 317        |

---

|         |  |     |
|---------|--|-----|
| 11.2.14 | Oxaldies: artificial, rationally designed catalytic polypeptides ..... | 318 |
| 11.2.15 | Biologically active de novo proteins .....                             | 318 |
| 11.3    | Concluding remarks .....   | 319 |
|         | Acknowledgments .....  | 319 |
|         | References .....   | 319 |

***Chapter 12: Intrinsic disorder and allosteric regulation ..... 327***

*Qiaojing Huang, Limin Chen, Luhua Lai and Zhirong Liu*

|      |   |     |
|------|---|-----|
| 12.1 | The ensemble view of allostery and its applications .....   | 327 |
| 12.2 | The role of intrinsic disorder in protein allosteric regulation:<br>representative cases.....             | 331 |
| 12.3 | Small allosteric molecules targeting intrinsically disordered proteins .....                              | 335 |
| 12.4 | Allostery of multidomain proteins with disordered linkers.....  | 338 |
| 12.5 | Phase separation of intrinsically disordered proteins .....   | 341 |
| 12.6 | Computational methods to study allostery in disordered proteins and<br>mechanism of phase separation..... | 344 |
| 12.7 | Outlook.....  | 346 |
|      | References .....  | 346 |

***Chapter 13: Macromolecular crowding: how it affects protein structure,  
disorder, and catalysis..... 353***

*Munishwar Nath Gupta and Vladimir N. Uversky*

|      |   |     |
|------|---|-----|
| 13.1 | Introduction .....  | 353 |
| 13.2 | What do we know about crowding inside cells? .....                                  | 353 |
| 13.3 | Crowding agents employed to simulate intracellular environments .....               | 354 |
| 13.4 | How crowding affects catalytic activity of enzymes .....                            | 355 |
| 13.5 | Effect of crowding on proteins with intrinsic disorder .....                        | 361 |
| 13.6 | Effect of crowding on protein assembly, aggregation, and amyloid<br>formation ..... | 363 |
| 13.7 | Miscellaneous recent observations .....   | 365 |
| 13.8 | Conclusions and future perspectives .....   | 368 |
|      | References .....  | 369 |

***Chapter 14: Intrinsic disorder and posttranslational modification:  
an evolutionary perspective..... 377***

*Paul M. Harrison*

|      |   |     |
|------|---|-----|
| 14.1 | Introduction .....  | 377 |
| 14.2 | PTMs prevail in intrinsically disordered regions and intrinsically<br>disordered regions are enriched in PTMs ..... | 380 |

---

|        |   |     |
|--------|---|-----|
| 14.3   | Links between posttranslational modification and disorder-to-order transitions of IDRs.....                                 | 381 |
| 14.4   | An evolutionary perspective.....  | 381 |
| 14.4.1 | Intrinsically disordered regions and the evolution of phosphorylation sites.....  | 382 |
| 14.4.2 | Phosphorylation sites in intrinsically disordered regions and pathogenic mutations in human proteins .....                  | 384 |
| 14.4.3 | Intrinsically disordered regions and modifications on lysine and arginine residues .....                                    | 384 |
| 14.5   | Intrinsically disordered regions as fertile substrates for the evolution of posttranslational modification cross talk ..... | 385 |
| 14.6   | Conclusions .....   | 387 |
|        | References .....  | 388 |

**Chapter 15: *The roles of prion-like domains in amyloid formation, phase separation, and solubility* .....**397

*Eric D. Ross and Sean M. Cascarina*

|       |  |     |
|-------|--|-----|
| 15.1  | Discovery of yeast prion proteins.....                                 | 397 |
| 15.2  | Prion domains .....  | 399 |
| 15.3  | Amyloid fibrils.....   | 399 |
| 15.4  | Kinetics and thermodynamics of amyloid formation .....                 | 401 |
| 15.5  | Prions as protein-based genetic elements .....                         | 404 |
| 15.6  | Stable, nontransmissible assemblies as a form of cellular memory ..... | 405 |
| 15.7  | PrLDs in the formation of biomolecular condensates .....               | 408 |
| 15.8  | FUS as a model for LLPS by PrLDs .....                                 | 409 |
| 15.9  | Misregulation of phase behavior .....                                  | 412 |
| 15.10 | PrLDs as regulators of phase behavior .....                            | 413 |
| 15.11 | Conclusion .....   | 414 |
|       | Acknowledgments.....   | 414 |
|       | References .....   | 414 |

**Chapter 16: *Disordered protein networks as mechanistic drivers of membrane remodeling and endocytosis* .....**427

*Wade F. Zeno, Feng Yuan, Kristin D. Graham and Jeanne C. Stachowiak*

|      |  |     |
|------|--|-----|
| 16.1 | Introduction .....   | 427 |
| 16.2 | Disordered proteins as sensors of membrane curvature .....                     | 429 |
| 16.3 | Disordered protein networks as catalysts of trafficking vesicle assembly ..... | 433 |
| 16.4 | Disordered proteins as drivers of membrane curvature.....                      | 438 |

---

|   |     |
|---|-----|
| 16.4.1 Repulsive interactions drive convex curvature .....          | 440 |
| 16.4.2 Attractive interactions drive concave curvature.....         | 441 |
| 16.5 Disordered protein networks as drivers of vesicle coating..... | 444 |
| 16.6 Disordered proteins as drivers of vesicle uncoating .....      | 447 |
| 16.7 Conclusion and outlook .....                                   | 448 |
| Acknowledgments.....  | 448 |
| References .....  | 448 |

***Chapter 17: How binding to surfaces affects disorder?.....455****Ary Lautaro Di Bartolo and Diego Masone*

|   |     |
|---|-----|
| 17.1 Introduction.....  | 455 |
| 17.2 Lipid bilayers.....  | 456 |
| 17.3 Membrane fusion .....  | 458 |
| 17.4 Membrane curvature .....   | 459 |
| 17.5 Hemifusion stalk .....   | 460 |
| 17.6 The fusion pore.....   | 462 |
| 17.7 Membrane proteins .....  | 463 |
| 17.8 Binding proteins to surfaces.....  | 463 |
| 17.9 Synaptotagmin-1 C2A and C2B domains.....                                     | 464 |
| 17.10 The Bin/Amphiphysin/Rvs domain with an N-terminal<br>amphipathic helix..... | 464 |
| 17.11 The dynamin family.....   | 464 |
| 17.12 Intrinsic disorder .....  | 465 |
| 17.13 The acrosome reaction .....   | 466 |
| 17.14 Proteins within the acrosome reaction.....                                  | 468 |
| 17.15 $\alpha$ -Synuclein .....   | 469 |
| 17.16 Computational methods.....  | 470 |
| 17.17 Collective variables.....   | 472 |
| 17.18 Coordination and radial distribution function .....                         | 473 |
| 17.19 Radius of gyration.....   | 474 |
| 17.20 Root mean square fluctuations .....   | 475 |
| 17.21 Lindemann disorder index.....   | 475 |
| 17.22 Conclusions.....  | 476 |
| References .....  | 477 |