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VOLUME 62

Unfolded Proteins

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CONTENTS

The Expanded Denatured State: An Ensemble of Conformations Trapped in a Locally Encoded Topological Space

DAVID SHORTLE

Identification and Functions of Usefully Disordered Proteins

A. KEITH DUNKER, CELESTE J. BROWN, AND ZORAN OBRADOVIC

Unfolded Proteins Studied by Raman Optical Activity

L. D. Barron, E. W. Blanch, and L. Hecht

What Fluorescence Correlation Spectroscopy Can Tell Us about Unfolded Proteins

Carl Frieden, Krishnananda Chattopadhyay, and Elliot L. Elson

Unfolded Peptides and Proteins Studied with Infrared Absorption and Vibrational Circular Dichroism Spectra

Timothy A. Keiderling and Qi Xu

Is Polyproline II a Major Backbone Conformation in Unfolded Proteins?

ZHENGSHUANG SHI, ROBERT W. WOODY, AND NEVILLE R. KALLENBACH

Toward a Taxonomy of the Denatured State: Small Angle Scattering Studies of Unfolded Proteins

IAN S. MILLETT, SEBASTIAN DONIACH, AND KEVIN W. PLAXCO

Determinants of the Polyproline II Helix from Modeling Studies

TREVOR P. CREAMER AND MARGARET N. CAMPBELL

Hydration Theory for Molecular Biophysics

MICHAEL E. PAULAITIS AND LAWRENCE R. PRATT

Insights into the Structure and Dynamics of Unfolded Proteins from Nuclear Magnetic Resonance

H. JANE DYSON AND PETER E. WRIGHT

Unfolded State of Peptides

XAVIER DAURA, ALICE GLÄTTLI, PETER GEE, CHRISTINE PETER, AND WILFRED F. VAN GUNSTEREN

A New Perspective on Unfolded Proteins

ROBERT L. BALDWIN

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JOHN T. EDSALL AND ADVANCES IN PROTEIN CHEMISTRY

Today the advances in almost any aspect of science are proceeding at an ever increasing rate. There is a strong tendency for each individual to feel that his/her area is indeed the most exciting. However, these same individuals do not read and think any faster now than their forebears did, with the result that science on a daily basis is breaking into smaller and smaller segments of the overall pie. Each small segment usually develops its own vocabulary which then inhibits easy communication between segments, even in very similar areas. This can easily be seen in *Nature Magazine* which, in just a few years, has fragmented itself into seven or so different journals for research articles (and five separate volumes for reviews). The number seems likely to increase markedly in future years.

Because of this behavior at the level of the primary literature, the so-called secondary literature (i.e., reviews) has become increasingly important. There are two limiting major types of reviews: (1) A list of what has been published in several different related segments over some usually short time interval with minimal attention to tying it all together. Such reviews can be useful, but will still be uninterpretable to those at any distance from the general field. (2) Reviews, usually triggered by some recent publication, but based on fundamental points, drawing on work from diverse areas, selecting literature from studies that may go far back in time, and resulting in a synthesis that may change the direction and attitude of an entire field. Both types of reviews serve useful functions, but the most sought after, and the hardest to find, are the latter.

The rising importance of the whole area of reviews is seen in the output from commercial publishers and many scientific societies. This has generated a competition for the best formats for conveying the desired

information. Developments in electronic communication have not yet solved the basic problems or eliminated the need for paper, but they certainly have altered the playing field. The younger members of the scientific community, say those under 70, tend to think of the current explosion in reviewing as a recent phenomenon, but this seems not to be so.

The first volume of *Advances in Protein Chemistry* appeared in 1944, near the end of the Second World War. The papers it contained were written during the height of the battles, a *tour de force* considering the difficulty of communications at that time. The following quotations are taken from the Preface of Volume 1:

Paragraph 1: "In the last generation, protein chemistry, which was once a relatively narrow branch of organic and biological chemistry, has spread out into the most varied fields of physics, chemistry, and biology. Enzymes, viruses, and many substances of immunological importance are now known to be proteins. The techniques now used for the study of proteins range from the most elaborate form of X-ray analysis to quantitative measurements of antibodies. Workers in the most diverse fields of science have not only contributed to the development of techniques, but have become interested themselves in applying the techniques they helped develop in the study of the problems of protein chemistry . . . "

Paragraph 2: "The rapid pace of the advances in protein chemistry, the varied character of the work being done, and its practical applications to industry and medicine have given rise to an increasing need for thoughtful and critical evaluation of the results achieved, and of their implications. We hope that this series of volumes will give the opportunity to workers in special subjects to present their views in more organized form than is possible in the regular journals, and also to express their personal judgment on problems which are still unsettled. We hope too that, as the reviews accumulate, they will provide a useful and comprehensive picture of the changing and growing field of protein chemistry, and a stimulus to its further development."

This statement accurately describes today the feelings and intents of the current editors. The lack of any nonnegotiable length of a manuscript we feel has contributed to the construction of some important and first class reviews.

This review series and all of its attitudes, approaches, and coverage was started by John T. Edsall and M. L. Anson (known to everyone as "Tim"). Interestingly enough, much of the pressure on, and help for, these two laboratory scientists was provided by Kurt Jacoby, who was then Vice President of the new version of Academic Press set up in

America by German immigrants displaced by the war. Later members of the senior staff, most recently Shirley Light, have maintained their strong support for this series for at least 60 years. The Series Editors have been a self-reproducing group. In chronological order, by addition or replacement, they have been John T. Edsall, Vols. 1–48 (emeritus); Tim Anson Vols. 1–23; Kenneth Bailey Vols. 5–17; Christian B. Anfinsen Vols. 12–47; Frederic M. Richards Vols. 19– ; David S. Eisenberg Vols. 39– ; Peter Kim Vols. 48–56; John Kuriyan Vols. 59– .

As these numbers show, John Edsall's tenure as an Editor of *Advances in Protein Chemistry* dwarfs the work of the rest of us. For most of this period, his eminence as one of the pioneering researchers of protein chemistry was crucial to attracting high-quality manuscripts to the series. Edsall, always a stickler for proper notation and clear writing, edited every submitted review in detail, often in successive drafts. He also worked diligently to encourage authors to finish important reviews that they had begun, but somehow had difficulty in finishing. One author of a review long past the deadline reported that he was surprised to see Dr. Edsall, then late in his ninth decade, at the door of his office, and in a city far from his home university. Edsall greeted the author by saying that the long-delayed review was so important that he had come in person to request that it be completed so that he, Edsall, could live to read it in print. The astonished author promised to finish the review quickly.

Advances in Protein Chemistry and the references contained therein provide a documented history of the field of protein chemistry. Not all applications of the theories and techniques are covered in this series, but the foundations are laid. There are occasional articles in which a single publication changes the thinking and approaches of an entire field. For example: Fred Sanger in Volume 7 in 1952 described the very start of the sequencing era, giving both methods and results on a pure protein, insulin. This work solved a long-standing argument on the covalent structure and biological significance of linear polypeptide chains. (The following year, the famous paper of Watson and Crick on DNA set Sanger off on what would be his next great sequencing success.) W. Kauzmann in Volume 14 pointed out the importance of nonpolar interactions, the hydrophobic effect, in both structure and function, drawing data from many fields in chemistry. J. Porath in Volume 17 introduced Sephadex as a resin for column separations based solely on the Stokes radius of the particle and not specific binding properties. Gel filtration is now a very widely used procedure for all manner of macromolecular materials. G. N. Ramachandran and V. Sasisekharan in Volume 23, with the primitive computers available at that time, produced the "Ramachandran Map," the starting point to this day for the analysis of the structure

of polypeptide chains. Jane Richardson in Volume 34 provided a classification of the structures of the then known proteins, and developed by hand (no computers) the basic drawing procedures for macromolecular structures which have been further extended in the currently available computer-produced modeling programs. Charles Tanford produced the enormous review on denaturation (a total of 255 pages in three parts appearing in Volumes 23 and 24) which now serves as a starting summary for all the later reviews on disordered structures, a current area of great interest in many biological systems, and particularly as background for this current volume.

While protein chemistry could easily be divided into a variety of subfields, each with its own review journal, the editors decided a few years ago on a different approach. They now pick a subfield and, perhaps more importantly, an individual to serve as editor for that particular volume. This editor is free to choose the authors asked to write the chapters for the whole volume focused on the selected subject area. So far this has worked quite well. The volumes are small enough so that we hope that all members of the field will read all of the volumes both in and outside of their particular area. Such an action would be a small step in trying to keep a broad overview alive. The mix of techniques, biological systems, and results will, of course, vary in each volume. There may be some overlap, but in our view that would be good. George Rose has done an excellent job of collecting a very interesting series of papers for this volume, and "by good luck" (a euphemism for "careful planning"), they all happen to be in a part of protein chemistry that has long been one of John Edsall's favorites.

David S. Eisenberg
John Kuriyan
Frederic M. Richards

GETTING TO KNOW U

Review articles fall somewhere between two extremes: the he said this, she did that category and the what it all means category—summaries vs. syntheses. The reviews in this volume tend toward the latter type. Indeed, the whole volume can be regarded as one long synthesis describing the unfolded state of proteins as depicted in multiple, complementary perspectives.

For years, the reigning paradigm for the unfolded state has been the random coil, whose properties are given by statistical descriptors appropriate to a freely jointed chain. Is this the most useful description of the unfolded population for polypeptide length scales of biological interest? The answer given by this volume is clear: there is more to learn. But first a word about the occasion that prompted this volume.

John T. Edsall will be 100 in November 2002, and this volume is our birthday present to him. Fred Richards has written the dedicatory Preface on behalf of the three Series Editors: David Eisenberg, John Kuriyan, and himself. John Edsall was a founding editor of *Advances,* an editor of legendary prowess as described by Richards. I cannot resist recounting a personal story about his effectiveness in this role. About 20 years ago, Lila Gierasch, John Smith, and I agreed to write a review on peptide and protein chain turns for this series (Rose *et al.,* 1985). Several years came and went, with the promised review still "in aspiration." I confess to being the rate-limiting author. As it happens, I was under consideration for promotion about this time, and the dean solicited John's opinion. Apparently, John neglected to mention my failings, and promotion was forthcoming. Soon after, the phone rang. The voice was unmistakable:

"George, John Edsall here. How are you?"
"Very well," I stammered.

"I want to congratulate you on your promotion. I trust my letter was helpful."
More stammering: "Yes, it was, John, thank you very much."
"Very good", he responded. And then, without noticeable pause, "turning now to the matter of your overdue review . . ."

It was ready for the next issue.

In contrast to my own delinquency, contributors to this volume have been quite punctual. The occasion of John's 100th birthday was such an effective forcing function for all of us that no editorial nagging was needed, and perhaps it would have been unnecessary in any case. But, there are no transferable hints for future editors. An event like this occurs only once!

Background

Arguably, the discipline called *protein folding* was established early in the last century in publications of Wu (Wu, 1931; Edsall, 1995) and Mirsky and Pauling (1936). Both historic papers sought to provide a theory of protein denaturation. At a time when proteins were widely regarded as colloids, these prescient articles had already recognized that many disparate properties of proteins are abolished coordinately on heating. Was this mere coincidence? To Wu and Mirsky and Pauling it seemed more plausible that such properties are all a consequence of some root-level cause—hypothesized to be the protein's structure. When that structure is melted out, the properties are abolished. Yesterday's far-sighted hypothesis is today's fact, as confirmed by Kauzmann (Simpson and Kauzmann, 1953).

These early investigations of protein denaturation set the stage for the contemporary era. Less than a decade after Mirsky and Pauling (1936), Anson summarized earlier evidence that denaturation is a reversible process (Anson, 1945). Any lingering doubt was dispelled by the work of Anfinsen and co-workers showing that urea-denatured ribonuclease could be renatured spontaneously and reversibly (Anfinsen and Scheraga, 1975). This demonstration placed the folding problem squarely in the realm of equilibrium thermodynamics. Instructions for folding are encoded in the amino acid sequence and, therefore, in the genetic code, and no cellular components are needed, i.e., the problem can be studied *in vitro*. These instructions establish the link between the one-dimensional world of replication, transcription, and translation and the three-dimensional world of folded, functioning proteins. What process could be more elegant or more profound. All of this is implicit in

Anfinsen's thermodynamic hypothesis, which states that the native state for a protein in its physiological milieu is the one of lowest Gibbs free energy (Anfinsen, 1973).

The three overall questions arising from this understanding of folding are how to characterize the folded state, the unfolded state, and the transition between these two populations. The characterization of folded proteins is on familiar ground, with more than 15,000 structures currently in the Protein Data Bank (Berman *et al.*, 2000). Given that folding is spontaneous and reversible, the transition can be well characterized by equilibrium thermodynamics as described by Tanford (Tanford, 1968; Tanford, 1970). There is no better introduction to this topic. For small, biophysical proteins, the transition is effectively two-state under experimental conditions of interest, meaning it can be described by only two rate constants, k_{forward} and k_{reverse}, with a single activated state that can be obtained from these rates with respect to temperature, denaturant concentration, etc. As shown by Ginsburg and Carroll (1965) for temperature-denatured ribonuclease, diverse probes yield identical folding curves on normalization. Consequently, we study the unfolding transition for a protein, not a family of such curves, each corresponding to a different probe.

The apparent two-state folding process is disappointing news for the chemist seeking intermediates, but welcome news for the protein thermodynamicist, who can now treat the transition as $\text{U} \rightleftharpoons \text{N}$, i.e., the native state population, N, is in equilibrium with the unfolded population, U. Almost all contemporary work has been along these lines (Schellman, 2002). On closer inspection, the definition of the denaturation process has been somewhat fuzzy from the beginning. Experimentalists have come to accept an operational definition such as a change in observable properties, e.g., Ginsburg and Carroll (1965), while theoreticians tend to opt for an abstract definition, such as the random chain model. In either case, the actual situation is more complex than simple two-state behavior: partially folded intermediates are present within the equilibrium population (Mayne and Englander, 2000).

We turn now to the remaining question—how to characterize the unfolded population, the topic of this volume.

The equilibrium population is said to have a *structure* when a substantial fraction of the molecules adopts similar conformations. But the phrase *lacking structure* does not imply that individual molecules comprising the ensemble lack a conformation; rather, the population is too heterogeneous to be readily characterized using a coherent, structure-based descriptor. The unfolded state resists ready characterization because it is so diverse. Typical biophysical methods report ensemble-

averaged properties in which important components of the population may be concealed beneath the background or the intrinsic distributions may be lumped into a misleading average. It is here that our thinking depends most critically on underlying models.

The current model, which originated with Flory (Flory, 1953; Flory, 1969) and Tanford (Tanford, 1968; Tanford, 1970), treats unfolded proteins as random chains as summarized in Baldwin's concluding chapter. Specifically, under conditions that favor unfolding, the chain is free to adopt all sterically allowed (Ramachandran and Sasisekharan, 1968) values of ϕ,ψ-angles, and when it does so, the population is found to be Gaussian-distributed around the radius of gyration expected for random, freely jointed chains of the same length in good solvent, but with excluded volume constraints.

It is important to realize that the random-chain model need not imply an absence of residual structure in the unfolded population. Formative articles—many of them appearing on the pages of *Advances in Protein Chemistry*—recognized this fact. Kauzmann's famous review raised the central question about structure in the unfolded state (Kauzmann, 1959):

> "For instance, one would like to know the types of structures actually present in the native and denatured proteins. ... The denatured protein in a good solvent such as urea is probably somewhat like a randomly coiled polymer, though the large optical rotation of denatured proteins in urea indicates that much local rigidity must be present in the chain" (pg. 4).

Tanford cautioned that a random chain and an α-helix are expected to have similar values of the radius of gyration at chain lengths approximating ribonuclease and lysozyme (Tanford, 1968, pg. 133). Indeed, the problem is one of scale. As Al Holtzer once remarked, "even a steel I-beam behaves like a random chain if it's long enough," but this realization does not figure heavily in designs on the length scale of my office at Hopkins.

The energy landscape for authentic random chains is maximally disordered and therefore temperature-independent, and this model prompts key questions about the unfolded population. Subjected to folding conditions, how do chains search out the native state in biological real-time while avoiding metastable traps along the way (Levinthal, 1969)? Such questions have engendered the 3Fs: frustration, funnels, and foldability, e.g., Sali *et al.* (1994); Bryngelson *et al.* (1995); Dill and Chan (1997); Tiana *et al.* (2000); Brooks *et al.* (2001).

Meanwhile, evidence continues to mount that the unfolded state is far from a random chain at length scales of interest, even under strongly

denaturing conditions. Critical examination of that evidence is the main purpose of this volume. In particular, the left-handed polyproline II helix (P_{II}) is found to play a central role in the unfolded population, as discussed in many chapters. It should be noted that the name of this helix is something of a misnomer in that the conformation often occurs in a proline-free sequence (Stapley and Creamer, 1999).

In P_{II} conformation, the backbone dihedral angles are near $\phi = -76^\circ$, $\psi = 149^\circ$, a favored location on the ϕ,ψ-map (Han *et al.*, 1998; Poon and Samulski, 2000; Pappu and Rose, 2002), resulting in a left-handed helix with a perfect threefold repeat. Unlike the α-helix, a P_{II} helix lacks intrasegment hydrogen bonds (Fig. 1, see color insert); moreover, residues are free to fluctuate independently in this part of the ϕ,ψ-map (Pappu *et al.*, 2000). Consequently, a high degree of fraying across the entire P_{II} segment is expected.

Although the P_{II} conformation is found in folded proteins (Adzhubei and Sternberg, 1993; Stapley and Creamer, 1999), it is not abundant. Approximately 10% of all residues have P_{II} ϕ,ψ-values. This too is expected because in P_{II}, the backbone cannot make either intrasegment H-bonds (like an α-helix) or intersegment H-bonds (like a β-sheet), only H-bonds to water. In sum, the P_{II} helix is a highly plausible unfolded conformation because hydrogen bonding promotes protein:solvent interactions at the expense of protein:protein interactions, and the structure is compatible with both preferred ϕ,ψ-angles and dynamic disorder (i.e., fraying).

The preceding issues underscore the crucial importance of models. Although $\sim$10% of all residues in proteins of known structure do have ϕ,ψ-values in the P_{II} region, the prevalence of P_{II} in the unfolded population could not have been deduced from this inventory.

Chapters in This Volume

Turning now to the chapters in this volume, a variety of complementary techniques and approaches have been used to characterize peptide and protein unfolding induced by temperature, pressure, and solvent. Our goal has been to assemble these complementary views within a single volume in order to develop a more complete picture of denatured peptides and proteins. The unifying observation in common to all chapters is the detection of preferred backbone conformations in experimentally accessible unfolded states.

Shortle has focused on the unfolded state for more than a decade, leading up to his recent demonstration using residual dipolar couplings that staphylococcal nuclease retains global structure in 8 *M* urea. His chapter on "The Expanded Denatured State" sets the stage. Dunker *et al.* then explore the complementary world of disordered regions within

folded proteins, asking how disorder, rather than order, can be specifically encoded by the sequence in these cases.

Most of the experimental information about P_{II} in the unfolded state comes from optical spectroscopy. The chapter on "Unfolded Proteins Studied by Raman Optical Activity" by Barron *et al.* provides a definitive exposition of this technique that emphasizes structural signatures present in unfolded proteins, with particular attention to P_{II}. "What Fluorescence Correlation Spectroscopy Can Tell Us About Unfolded Proteins" by Chattopadhyay *et al.* describes how fluorescence can be used to follow changes in conformation by measuring changes in the diffusion coefficient over a broad range of time scales. "Unfolded Peptides and Proteins Studied with Infrared Absorption and Vibrational Circular Dichroism Spectra" by Keiderling and Xu emphasizes identification of residual structure in the unfolded state, including P_{II}. "Is Polyproline II a Major Backbone Conformation in Unfolded Proteins" by Shi *et al.* consolidates existing CD and other data on unfolded peptides and proteins, again emphasizing P_{II}.

"Toward a Taxonomy of the Denatured State: Small Angle Scattering Studies of Unfolded Proteins" by Millett *et al.* assesses denatured states induced by heat, cold, and solvent for evidence of residual structure, while "Insights into the Structure and Dynamics of Unfolded Proteins from NMR" by Dyson and Wright describes their extensive investigations of residual structure in the unfolded state.

Three theory papers are also included. "Determinants of the Polyproline II Helix from Modeling Studies" by Creamer and Campbell reexamines and extends an earlier hypothesis about P_{II} and its determinants. "Hydration Theory for Molecular Biophysics" by Paulaitis and Pratt discusses the crucial role of water in both folded and unfolded proteins. "Unfolded State of Peptides" by Daura *et al.* focuses on the unfolded state of peptides studied primarily by molecular dynamics.

A final summary is provided by Baldwin, who articulates the new direction implied by these chapters and points the way toward the future.

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George D. Rose

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JOHN T. EDSALL
(November 3, 1902–June 12, 2002)

John Edsall passed away just as this volume was headed to press. It was hoped by the editors that this volume would be one of the 100th birthday presents that John would receive on November 3. This was not to be.

No brief biography can convey the enormous impact that John Edsall had on the students, scientists, and scholars who crossed his path. He seemed larger than life: someone who virtually personified the unselfish search for truth that science is supposed to be. As a researcher, he spent decades applying physical chemistry to proteins, showing, among other things, that proteins bristle with charges. As a person of action during World War II, he and his co-workers learned how to fractionate blood into proteins that can be used in medicine and surgery. As a community leader during the McCarthy era, he stood bravely in defense of the right of Linus Pauling and other scientists to take unpopular political stands [this episode is described in my brief biographical sketch on Edsall in *Protein Science,* **1,** 1399–1401 (1992)]. As a teacher, he inspired generations of undergraduate and graduate students to become biochemists, bolstered by his example as a gentle, humane scientist and his example that biochemists can contribute to society as a whole as well as to our own discipline. As a scholar, he worked to understand the history of ideas in protein science. As a writer of texts and monographs, he conveyed the most difficult concepts of energetics and function to new generations of scientists. More on all of this will be available in the November issue of the *Journal of Biophysical Chemistry* dedicated to John Edsall.

John Edsall's mind and body remained vigorous in years when most humans have surrendered to old age. When the Protein Society met in Boston in the hot summer days of 1997, John, nearing 95, went on foot and subway from his home in Cambridge to the Boston Convention