

# The hydrodynamic and conformational properties of denatured proteins in dilute solutions

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**Abstract:** Published data on the characterization of unfolded proteins in dilute solutions in aqueous guanidine hydrochloride are analyzed to show that the data are not fit by either the random flight or wormlike chain models for linear chains. The analysis includes data on the intrinsic viscosity, root-mean-square radius of gyration, from small-angle X-ray scattering, and hydrodynamic radius, from the translational diffusion coefficient. It is concluded that residual structure consistent with that deduced from nuclear magnetic resonance on these solutions can explain the dilute solution results in a consistent manner through the presence of ring structures, which otherwise have an essentially flexible coil conformation. The ring structures could be in a state of continual flux and rearrangement. Calculation of the radius of gyration for the random-flight model gives a similar reduction of this measure for chains joined at their endpoints, or those containing loop with two dangling ends, each one-fourth the total length of the chain. This relative insensitivity to the details of the ring structure is taken to support the behavior observed across a range of proteins.

**Keywords:** protein; radius of gyration; intrinsic viscosity; loop formation

## Introduction

In a 2005 article devoted to the discussion of “the case for (and against) residual structure in chemically denatured proteins” the authors cited a number of articles to that date suggesting that “nuclear magnetic resonance (NMR) studies suggest that significant secondary structure and long-range hydrophobic clusters persist in unfolded proteins even at high concentrations of urea or guanidine hydrochloride (GuHCl)”, whereas “intrinsic viscosity, hydrodynamic radii, and small-angle scattering experiments, some of which date back almost four decades, have been taken as evidence that the unfolded state is an effectively random coil ensemble.”<sup>1</sup> Since that publication, studies have continued to reveal secondary structure in strongly denatured proteins and polypeptides in dilute solution, mostly based on NMR

analysis, with such studies often motivated by interest in the folding of the denatured chain. The aforementioned use of the random flight model, which has dominated most of the interpretations of hydrodynamic behavior of proteins and polypeptides in dilute solution, starting with the seminal studies of Tanford *et al.*,<sup>2</sup> led to the conclusion that the chains may be modeled by an ensemble of random flight chains with excluded volume effects.<sup>1,3</sup> One study treated the same data to conclude that the data on the intrinsic viscosity could be represented by a wormlike chain model with the chain having an appreciable persistence length, but negligible excluded volume.<sup>4</sup> In this study, we suggest that taken together the experimental data on intrinsic viscosity, hydrodynamic radii, and the radius of gyration suggest a somewhat compact structure of the denatured proteins and polypeptides studied above, possibly owing to intramolecular association to form transient loops among chain elements widely separated along the chain backbone.

*Abbreviations:* NMR, nuclear magnetic resonance

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## Results and Discussion

### The published data

The addition of data on the root-mean-square radius of gyration  $R_G$  for a number of denatured proteins and polypeptides in aqueous (2–6) $M$  GuHCl or (4–8) urea,<sup>5</sup> motivates this reconsideration of the previously available data on the intrinsic viscosity  $[\eta]$ , second virial coefficient  $A_2$ , and hydrodynamic radius  $R_H$  as functions of the molecular weight  $M$  in (5–6) $M$  GuHCl. The data on  $R_H = \Xi/6\pi\eta_{\text{solv}}$ , with the molecular friction coefficient  $\Xi$  determined from the sedimentation coefficient in the original study,<sup>2</sup> have been augmented in recent study by estimates based on NMR studies on denatured proteins in a gradient field and by dynamic light scattering, discussed in detail in Ref. 6; here,  $\eta_{\text{solv}}$  is the solvent viscosity. The experimental data on these parameters are reproduced in Figure 1, where  $M$  is replaced by the number  $N$  of residues in the backbone, to more nearly represent the behavior in terms of the chain contour length  $L$ .

As noted above, prior treatments based on the behavior of  $[\eta]$ ,  $A_2$ , and  $R_H$  as functions of  $M$  have utilized a random flight flexible model for linear chains or a wormlike chain model in an attempt to elucidate the dependence of  $R_G$  on  $M$ , and the value  $(R_G/\sqrt{M})_\Theta$  of  $R_G/\sqrt{M}$  under Flory theta conditions, for which  $A_2 = 0$ . As the GuHCl solvent system is found to be a thermodynamically good solvent for the proteins studied, with  $MA_2 \approx [\eta]$ , as often observed for solutions of flexible or wormlike chains,<sup>7</sup> the evaluation of  $(R_G/\sqrt{M})_\Theta$  from the dependence of  $[\eta]$  on  $M$  requires the use of models and extrapolations to zero  $M$ , both of which introduce opportunities for error, especially in view of the relatively narrow range of  $M$  for which data are avail-

able. For example, the analyses with the random flight and wormlike chain models, though quite different in conformational interpretations, provide reasonable fits to the data on  $[\eta]$  as a function of  $M$  in the aqueous GuHCl solutions.<sup>2,4</sup> However, both treatments provide estimates of  $(R_G/\sqrt{M})_\Theta$  that are too large in comparison with the recent experimental data on  $R_G/\sqrt{M}$ ,<sup>5</sup> unavailable to those earlier studies.

As may be seen in Figure 1, although the data are somewhat scattered, it appears that  $\partial \ln [\eta] / \partial \ln M = 0.64_0$ ,  $\partial \ln R_G / \partial \ln M = 0.59_7$ , and  $\partial \ln R_H / \partial \ln M = 0.51_6$ . If interpreted alone, the value of  $\partial \ln R_G / \partial \ln M$  would indicate a very strong intramolecular excluded volume effect, and that has been the usual interpretation.<sup>1,5</sup> Similarly, if taken alone the values of  $\partial \ln [\eta] / \partial \ln M$  and  $\partial \ln R_H / \partial \ln M$  would each represent a more modest excluded volume effect. As mentioned above the data in the intermolecular thermodynamics given by the dependence of  $A_2$  versus  $M$  would suggest a so-called good solvent, consistent with a substantial intramolecular excluded volume for a flexible-coil chain, but irrelevant to such behavior for rodlike or even wormlike chains with a large persistence length  $\hat{a}$ .<sup>7</sup> The significance of these possibly disparate tendencies is considered next.

### An analysis involving $[\eta]$

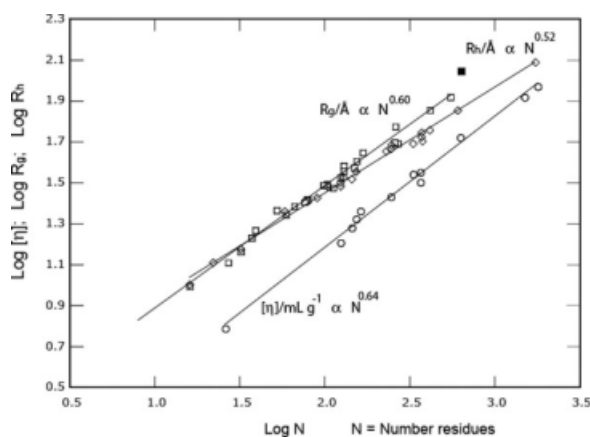
In a previous study, theoretical expressions for the parameter  $K$  were presented for a number of models, including linear and branched random flight chains, linear rodlike, and wormlike chains and ellipsoids of revolution (including spheres), where<sup>8</sup>

$$K = \frac{M[\eta]}{\pi N_{\text{AV}} R_G^2 R_H}, \quad (1)$$

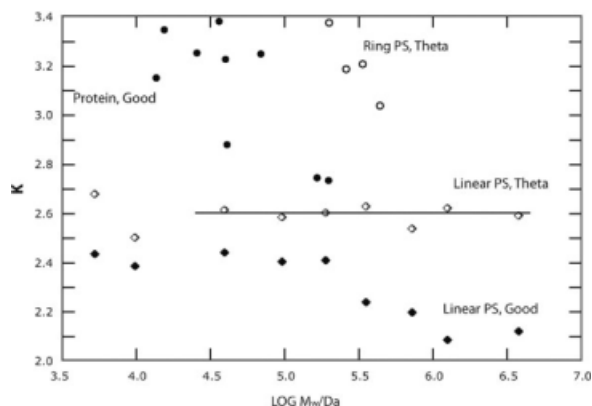
with  $N_{\text{AV}}$  the Avogadro number. Of course,  $K$  is trivially related to the more familiar Flory-Fox parameter  $\Phi'$  given by<sup>9</sup>

$$\Phi' = M[\eta]/R_G^3, \quad (2)$$

so that  $\Phi' = \pi N_{\text{AV}}(R_H/R_G)K$  includes the dependence of  $R_H$  on  $M$  encoded into the definition for  $K$  such that for linear flexible or wormlike chain models  $K$  spans a much smaller range of values and is much more weakly dependent on  $M$  than is  $\Phi'$ . For example, calculations with the random flight or wormlike chain models without intramolecular excluded volume show that  $K_0 \leq K \leq K_\infty$ , with  $K_0$  and  $K_\infty$  the limiting values of  $K$  as  $M$  tends to zero and infinity, respectively; for example,  $K_0 = 1$  and  $K_\infty \approx 3$  for linear chains, depending on the details of the model used.<sup>8</sup> Values of  $K_\infty$  for branched chains tend to exceed that for linear molecules, as does  $K_\infty$  for the random flight or wormlike chain models for ring-shaped molecules without intramolecular excluded



**Figure 1.** Experimental data reported in the literature on various proteins in strongly denaturing solvents giving the dependence on the number of residues  $N$  for the intrinsic viscosity,<sup>2</sup>  $[\eta]$  (circles), the root-mean-square radius of gyration,<sup>5</sup>  $R_G$  (squares) and hydrodynamic radius,<sup>6</sup>  $R_H$  (diamonds).



**Figure 2.** The function  $K = [\eta]M / \{\pi N_A V R_G^2 R_H\}$  for several systems: unfolded proteins based on the data in Figure 1, filled circles; linear polystyrene under Flory theta conditions and good solvent conditions, unfilled and filled diamonds, respectively,<sup>14–16</sup> and ring-shaped polystyrene, unfilled circles.<sup>11–13</sup>

volume, for example, for a ring-shaped molecule,  $K_{\infty, \text{RING}} = 1.519K_{\infty, \text{LIN}}$ , with  $K_{\infty, \text{LIN}}$  the value for the same model for a linear chain.<sup>10</sup>

Values of  $K$  calculated as a function of  $M_w$  for the data on proteins and polypeptides for the data in Figure 1 are shown in Figure 2, along with data on synthetically prepared well-defined model linear and ring-shaped macromolecules. In fact, there are not many such studies, owing principally to the difficulty of preparing well-defined models. Two studies on ring-shaped polymers prepared by the anionic polymerization of styrene, followed by ring closure give comparable results.<sup>11–13</sup> Thus, for linear polystyrene, the experimental  $K \approx K_{\infty}$  is constant for  $10^4 < M < 10^6$  ( $K \approx 2.6$ ) under Flory theta conditions, and in a good solvent (toluene) it decreases from this value as  $K \propto M^{-0.04}$  with increasing  $M$ , reflecting the differing effects of intramolecular excluded volume on  $[\eta]$  and  $R_G^2 R_H$ .<sup>14–16</sup> Neither of these studies provided the behavior in good solvents needed to compute  $K$ . The data on the ring-shaped polystyrene determined under Flory Theta conditions depart from expectations in two ways: (i)  $K_{\text{RING}}$  is not expected to depend on  $M$  under these conditions and (ii)  $K_{\text{RING}}$  is smaller than the value 3.95 expected by comparison with  $K_{\text{LIN}}$  for the same polystyrene. The unexpected behavior may reflect the presence of linear chains remaining from the difficult synthesis of the ring-shaped polymers, as observed in some chromatography studies.<sup>17</sup> The data on proteins and polypeptides are seen to be scattered, but with a tendency to decrease from about 3.4 to 2.7 with increasing  $M$  over the range studied, with  $K \propto 10^{-0.07}$ . As the protein and polypeptide samples used in the various studies differ, resort is made to the smoothed correlations shown in Figure 1 to compute  $K$ . It is seen that the resulting  $K$  for the large  $M$

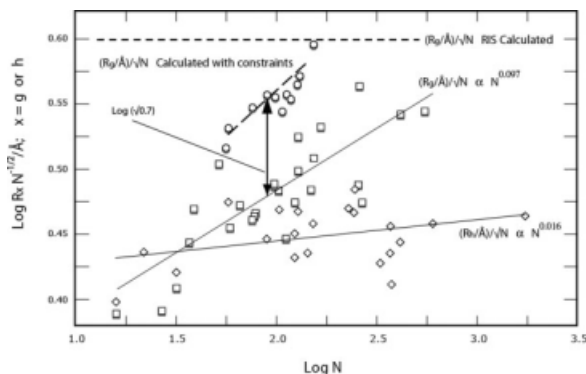
limit is comparable to  $K_{\infty}$  for a linear random-flight or wormlike chain for large  $M$  under Flory theta conditions, but increases to larger values with decreasing  $M$ , so that the overall pattern is similar to that seen in Figure 1 for ring-shaped polystyrenes. The effects on  $K$  or related to those note on the Flory-Fox parameter  $\Phi'$  in recent considerations of size exclusion chromatography on denatured proteins.<sup>18,19</sup> It may also be noted in Figure 1 that  $R_H \approx R_G$  for the lower values of  $M$ . Although one can conclude that the protein conformation appears to be more compact than a linear random flight or wormlike chain at the lower range of  $N$ , and that such behavior is not an indication of free draining hydrodynamics, which would decrease  $K$ , it is not possible to interpret these data in terms of a definitive model. Possibilities are discussed in the following.

### The effects of association among chain residues

Intermolecular association would lead to branch-shaped aggregates, and as mentioned above,  $K$  will tend to be increased above that for the linear counterpart, offering one possible interpretation. However, this possibility is discounted as the existence of such intermolecular association would be expected to have characteristic features in the dependence of the viscosity and light (or X-ray) scattering behavior, not reported for the studies cited.

Studies by NMR and MCD over the past decade have revealed that the so-called denatured state of proteins and polypeptides contain locally ordered sections of a helical nature, even in solvents that strongly suppress hydrogen bonding.<sup>1,20–22</sup> This includes evidence that these sections may be involved in intramolecular association among units far removed along the chain backbone, forming loops that flicker in and out of existence. As observed above, the result that  $K$  for the lower molecular weight proteins in aqueous GuHCl solutions is comparable to the behavior seen for the ring-shaped polystyrene, suggesting the presence of ring-shaped molecules or chains with containing loops, at least in that range of  $M$ . As with the data on polystyrene, the modestly decreasing value of  $K$  noted with the data on the protein solutions in aqueous GuHCl solution may reflect a modest intramolecular excluded volume effect, with the higher value obtained at low  $M$  closer to the limit that would be observed under Flory theta solvent conditions.

The data on  $R_G/N^{1/2}$  and  $R_H/N^{1/2}$  for the data in Figure 1 are shown in Figure 3, along with data on  $(R_G/N^{1/2})_{\text{STAT}}$  computed via statistical coil model for the unfolded state of a range of proteins as a function of  $N$ , including excluded volume effects, and taking account of conformational constraints consistent with NMR analysis revealing (non-H-bond stabilized) helical sequences in aqueous GuHCl



**Figure 3.** Experimental data on  $R_G/N^{1/2}$  and  $R_G/N^{1/2}$  versus  $\log N$  using the data in Figure 1, along data on  $(R_G/N^{1/2})_{\text{STAT}}$  calculated via a statistical model with conformational constraints (aside from intramolecular association)<sup>23</sup> and  $(R_G/N^{1/2})_{\text{STAT}}$  versus calculated with rotational-isomeric-state model.<sup>24</sup>

solution,<sup>23</sup> and a constant value of  $(R_G/N^{1/2})_{\text{RIS}}$  calculated for the rotational-isomeric-state model in the limit of large  $N$ .<sup>24</sup> It may be seen that the experimental values of  $R_G/N^{1/2}$  are about equal to  $\sqrt{0.7(R_G/N^{1/2})_{\text{STAT}}}$ . Thus, if  $(R_G/N^{1/2})_{\text{STAT}}$  is presumed to be an accurate measure of  $R_G/N^{1/2}$  for the chain without any intramolecular association producing ring structures,  $g = R_G^2/(R_G^2)_{\text{LIN}} \approx 0.7$ , larger than the value  $g = 1/2$  expected for a pure ring-shaped polymer. Although not impossible and perhaps possible for some unfolded proteins,<sup>25</sup> it would perhaps be unlikely for transient intramolecular hydrophobic clusters to form rings by association of their endgroups, so it is of interest to consider values of  $g$  for a model with a loop formed by association of more generally placed repeating units.

A simplified model with the results shown in Figure 4 comprises a chain with an association of residues each a distance  $xL$  from the ends of a chain of length  $L$ , resulting in a loop with  $(1 - 2x)L$  residues, or length  $(1 - 2x)L$ , and two “branches,” each with  $xL$  residues, emanating from the crosslink locus. Use of the random flight model to compute  $g$  including the constraint imposed by the effective intrachain crosslink site according to the usual expression for a chain with  $n$  statistical segments of length  $b = L/n$ .<sup>26</sup>

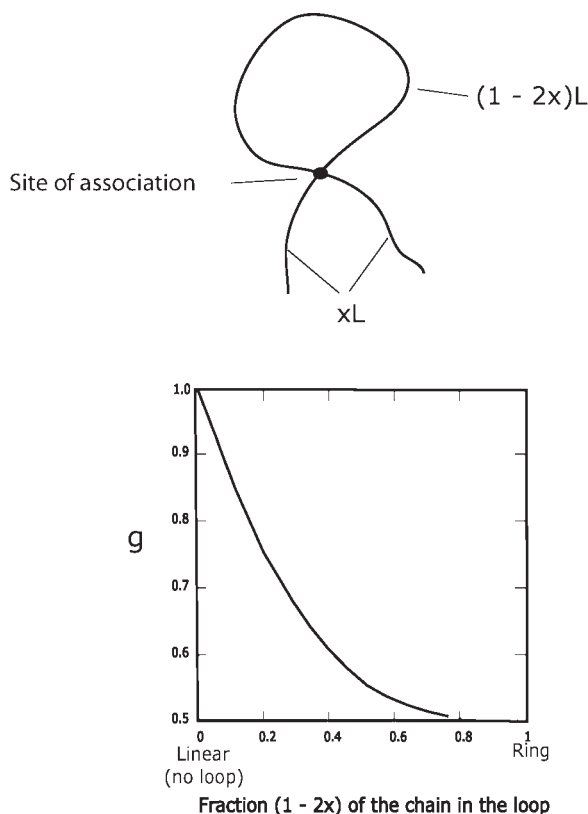
$$R_G^2 = \frac{b^2}{2n} \sum \sum (i - j) \quad (3)$$

where the sums are over all possible segments  $i$  and  $j$ , consistent with the crosslink constraint. The calculation may be conveniently accomplished by summing the contributions segment pairs confined to several components of the total structure: (1) a loop with  $n - 2xn$  segments, (2) a linear chain with  $2xn$  segments made from the two branches, (3) four lin-

ear chains, each with  $n/2$  segments, comprising a  $1/2$  loop + one branch, and (4) less the over-count from four linear chains, each with  $(n - 2xn)/2$  segments from four  $1/2$  loops and four linear chains, each with  $xn$  segments from four branches. The calculation gives  $g$  as a function of the fraction  $1 - 2x$  of the chain in the loop as shown in Figure 4, with

$$g(x) = 4x^3 + 1/2. \quad (4)$$

As required,  $g$  is unity or one-half for fractions of residues in the loop equal to zero ( $x = 1/2$ ) or unity ( $x = 0$ ), respectively. Although this model is certainly too simplified to permit a definitive quantitative conclusion, it may be noted that the observed  $g \approx 0.7$  would correspond to about a fraction 0.25–0.3 of the residues in the loop. However, even if the conclusion cannot be considered to be quantitatively accurate, the existence of such transient structures with a life-time long enough to be seen in the parameters of interest here would seem to be reasonable in view of the properties found in the NMR studies and could be relevant to the refolding process of the denatured protein.



**Figure 4.** Upper: schematic diagram of a loop of length  $(1 - 2x)L$ , and two “branches,” each of length  $xL$ , emanating from the site of the association creating the structure. Lower: a plot of  $g$  versus the fraction  $1 - 2x$  of the residues in the loop.



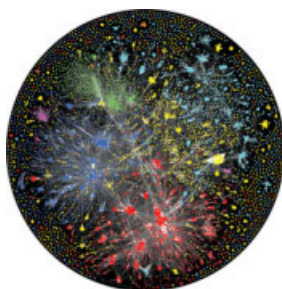
## Conclusions

Based on the preceding analysis of published data on the characterization of proteins in dilute solutions in aqueous GuHCl, it is concluded that the data are not fit by either the random flight or worm-like chain models for linear chains, but can be rationalized if it is assumed that transient intramolecular association form temporary ring conformers among the chain units. The analysis includes data on the intrinsic viscosity, root-mean-square radius of gyration, from small-angle X-ray scattering, and hydrodynamic radius, from the translational diffusion coefficient. Such residual ring structures in a state of continual flux and rearrangement in otherwise unfolded proteins are consistent with behavior deduced from NMR on these solutions. Calculation of the radius of gyration for the random flight model gives a similar reduction of this measure for chains joined at their endpoints, or those containing loop with two dangling ends, each one-fourth the total length of the chain. This relative insensitivity to the details of the ring structure is taken to support the behavior observed across a range of proteins.

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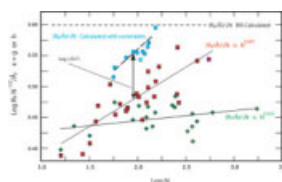
# IN THIS ISSUE



## 124 **A galaxy of folds**

Vikram Alva, Michael Remmert, Andreas Biegert, Andrei N. Lupas, and Johannes Söding

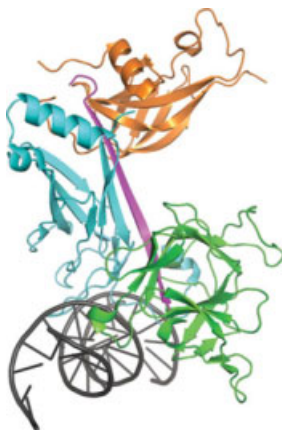
Despite their enormous sequence diversity, proteins are built of recurrent domains belonging to only a few thousand fold types. Did these folds originate independently multiple times, converging on similar structural solutions, or did they evolve divergently from ancestral prototypes? In this study, Söding and co-workers clustered domains of known structure by their sequence similarity, a property that reflects common descent. They report that, while some highly populated folds indeed appear to have evolved convergently, most domains possessing the same fold arose from an ancestral prototype. They also report that occasionally, domains belonging to different folds cluster together, connected by recurrent fragments which may be descendants of an ancestral pool of peptide modules from which the first folded proteins arose.



## 94 **The hydrodynamic and conformational properties of denatured proteins in dilute solutions**

Guy C. Berry

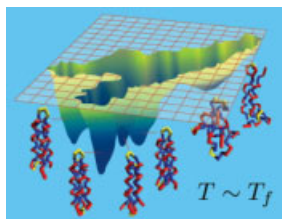
Estimates of the root-mean-square radius of gyration  $RG$  of denatured proteins versus the number  $N$  of residues based on the intrinsic viscosity versus  $N$  using expressions appropriate for linear flexible-chain polymers give estimates of  $RG/\sqrt{N}$  consistent with the rotational-isomeric state RIS model. Recent direct measurements of  $RG$  give smaller values of  $RG/\sqrt{N}$  than deduced from those estimates, or  $RG/\sqrt{N}$  computed using a model with conformational constraints. It is suggested that consistent with NMR observations, transient loops created by intrachain association in the denatured state, resulting in an effective nonlinearity of the denatured chain in dilute solution can reconcile these apparently disparate observations.



## 34 **Thermodynamic and structural insights into CSL-DNA complexes**

David R. Friedmann and Rhett A. Kovall

The Notch pathway is a highly conserved signaling mechanism, in which extracellular signals are ultimately transduced into changes in gene expression via the DNA binding protein CSL. Despite being identified over twenty years ago, very little is understood at the quantitative level regarding the affinity of CSL for DNA. Here Friedmann and Kovall report a comprehensive thermodynamic and structural analysis of CSL binding to the two DNA sites that comprise the HES-1 response element - a known Notch target gene. Previously, it was thought that CSL binds DNA with high affinity; however, our findings demonstrate that CSL only has moderate affinity for DNA, which suggests that DNA binding by CSL is likely a more regulated process than previously appreciated.



## 141 **Sequence periodicity and secondary structure propensity in model proteins**

Giovanni Bellesia, Andrew Iain Jewett, and Joan-Emma Shea

In this report, Bellesia, et al. use a simplified computational model to study the relative importance of local effects - those originating from the amino acids intrinsic secondary structure propensity - and non-local effects - those reflecting the sequence of amino acids as a whole - in determining a protein's fold. Their simulations show that the fold of small, globular proteins is mainly determined by the pattern of polar and non-polar amino acids regardless of their intrinsic secondary structure propensities. These results quantitatively agree with, and extend, previous experimental observations.

# OBITUARY

## In Memoriam: Reflections on Charles Tanford (1921–2009)

### C. Nick Pace

Biochemistry and Biophysics Department, Texas A&M University, and Department of Molecular and Cellular Medicine, Texas A&M Health Science Center, College Station, Texas

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On October 1st, Jackie Reynolds notified me that Charles Tanford had died. I forwarded her message to the people on my email list who study proteins. The replies below were received the same day along with many others expressing similar thoughts.

“Sorry to hear this news. He was one of my scientific heroes. I named the position of the transition state on the reaction pathway from denaturant dependence as betaT in his honor, a name that has stuck. Farewell to a great protein scientist. His name will live on.”

—Alan Fersht

“Thank you for sharing the passing of one of the giants of our field, it is truly remarkable how much insight his classic experiments provided to our understanding of protein folding, experiments that still are carefully considered in the way we think about protein folding today.”

—Jeff Kelly

“It’s a very sad day. Charlie was truly a legendary biophysicist. I, for one, learned a huge amount from his work and writings, particularly his lucid books.”

—Ken Dill

“Very sorry to hear of Charles Tanford’s passing. A great scientist, an original thinker and a major impetus for the way that I and many others think about the folding problem.”

—Bob Matthews

“Thanks so much Nick for sharing this notice of the passing of a great historical figure in our field...his intellectual influence on me and others was enormous.”

—Paul Schimmel



“I’m really sad to hear that Nick. He was one of the giants of biophysical chemistry in the last century, and his work influenced me enormously.”

—Wayne Bolen

“Sad News. He was truly a great scientist.”

—Neville Kallenbach

“Thanks Nick. I guess we saw this coming. Fred, then Walter, now Charles ... a year of loss.”

—George Rose

“Oh my. Sad day it is. I was just thinking of him last night when I was with van Moudrianakis and Bertrand. They were reminiscing days of old.”

—Carolyn Fitch

Carolyn Fitch received her Ph.D. from Johns Hopkins in 2002. I was the Outside Examiner on her dissertation committee. I was surprised to see that her thesis was dedicated to Charles Tanford. Carolyn said she never met him, but he had a great influence on her Ph.D. studies.

I was fortunate to be a graduate student in Charles Tanford's laboratory from 1962 to 1966. My dissertation was titled “The Reversible Denaturation of  $\beta$ -lactoglobulin A” and in the Acknowledgments I wrote: “I am indebted to Dr. Charles Tanford for providing advice, encouragement, criticism, a lab full of equipment and interesting people, and, perhaps most important, a very good example of how a very good scientist proceeds. For all of this, I am grateful.”

All of these sentiments reflect the enormous impact that Charles Tanford had on those of us who study proteins.

Charles Tanford was born on 29 December 1921 in Halle, Germany. After the Nazi party did well in the elections in 1930, his parents, Max and Charlotte Tannenbaum, moved to England and changed their name to Tanford. Many of his relatives stayed behind in Germany and perished in the holocaust. In 1939, at the outbreak of the war in Europe, Charles moved to New York and lived with relatives. He earned a B.A. from NYU in 1943, then worked on the Manhattan Project at Oak Ridge for a year, and then earned a Ph.D. in chemistry from Princeton in 1947. He did postdoctoral work in protein chemistry in the laboratory of Edwin Cohn and John Edsall at Harvard University. Charles began his academic career at the University of Iowa in 1950 and moved to Duke University in 1960. In 1970, he was named the James B. Duke distinguished professor. Charles retired in 1988, moved to Easingwold, England, and began a second career writing about the history of science.

Charles married Lucia Brown while at Harvard and they had three children, Vicki, Alex, and Sarah. They were divorced in 1968, and soon thereafter Charles began a professional and personal relationship with Dr. Jacqueline (Jackie) Reynolds, a fellow biochemist, that lasted until his death.

Charles published over 200 articles during his scientific career. His first was an experimental study: “The Mercury-Sensitized Reaction between Hydrogen and Nitric Oxide” and it was published while he was an undergraduate at NYU.<sup>1</sup> At Princeton, Charles had planned to work with Henry Eyring, but he was required to work with R.N. Pease for his Ph.D. (Eyring was Tanford's favorite teacher at Princeton.) His Ph.D. work led to three theoretic-

cal papers on the combustion of gases.<sup>2</sup> Walter Kauzmann returned to Princeton in 1946 during Tanford's final year, and he had decided to become a protein physical chemist after reading the Cohn and Edsall treatise, *Proteins, Amino Acids, and Peptides*<sup>3,4</sup> and other books on proteins in a cabin in the Colorado mountains. Kauzmann recalled<sup>4</sup>: “Tanford attended my informal lectures on proteins and we talked a lot about the subject. He decided that his future lay with proteins rather than with flames, and he went on to a postdoctoral position in the Cohn group at Harvard, and, of course, to a very distinguished career in protein chemistry. So, perhaps I can claim him as one of my most important discoveries.”

In the laboratory of Cohn and Edsall at Harvard Medical School, Tanford began his career as a protein chemist. His research led to his first paper on proteins,<sup>5</sup> a careful experimental study of the hydrogen ion titration of human serum albumin, and a theoretical analysis of the results based on the model of Linderstrom-Lang.<sup>6</sup> In the Acknowledgments, Tanford “... expresses his gratitude to Dr. E.J. Cohn for suggesting this problem, and to Drs. J.T. Edsall, J.L. Oncley, George Scatchard, and W.L. Hughes, Jr., for many invaluable discussions.” This experience and the lectures by Kauzmann left Tanford well equipped to begin his work on proteins.

At Iowa, Tanford continued his studies of the hydrogen ion equilibria of proteins, and related topics. Titration curves were determined for bovine serum albumin, insulin, lysozyme, and ribonuclease. The study of bovine serum albumin in 1955 was cited over 500 times,<sup>7</sup> and the figures used in several textbooks. In 1956/1957 he did a sabbatical with J. G. Kirkwood at Yale to improve the theoretical treatment of the acid-base properties of proteins. The older Linderstrom-Lang model represented the protein molecule as a sphere with a continuous and uniform distribution of charge on its surface.<sup>6</sup> In the new model, discrete charges were placed at fixed positions on the surface of the protein.<sup>8</sup> The Abstract ends with: “General equations are obtained which express the titration curve as a function of the locations of ionizable sites and of their intrinsic properties. It is concluded that the intrinsic properties may themselves be quite sensitive to the location of the dissociable site with respect to the surface of the protein molecule.” This paper triggered an interest in the factors that determine pK values of the ionizable groups of proteins that continues to the present day. The work from this period is summarized in a review published in 1962.<sup>9</sup>

Tanford taught a course on the physical chemistry of polymers at Iowa and decided to write a textbook. His 10 years of work led to the publication in 1961 of *Physical Chemistry of Macromolecules*.<sup>10</sup> He has written an interesting “Recollections” article



about the book.<sup>11</sup> He noted: "There were two reviewers and their criticism was scathing; I had got it all wrong, they said, and the book was declared unpublishable." When he met with the publishers he told them "... that I had every confidence in what I had written and would not change a word." The book was a great success and has now sold over 25,000 copies, and has been republished, unaltered. For many of us, it was an essential reference book for our teaching and research.

Tanford moved to Duke in 1960 and he expanded his research into new areas. When I arrived in 1962, he had a group studying antibody structure, and another group studying various aspects of protein folding. Laboratory meetings were held at 3 pm on Friday afternoons and generally went to at least 6 pm. This was when we learned from Tanford. (Tanford and Bob Hill taught an excellent course on proteins and enzymes that was also a great learning experience.) The laboratory was crowded and my desk also served as my lab bench. We had two Beckman Model E ultracentrifuges (one inherited from Hans Neurath) and, shortly after I arrived, Cary 60 spectropolarimeter #3. (The first two had gone to Elkan Blout and Henry Eyring.) It was an exciting time to be in the laboratory. The experiments that led to the characterization of the denatured state were underway, and Yas Nozaki was overseeing solubility measurements on amino acids and peptides that led to the  $\Delta G_{tr}$  values for urea and GdnHCl used to understand how these compounds unfold proteins. The studies of protein folding were reviewed by Tanford in a 1968 article<sup>12</sup> that has been cited over 2200 times, his most cited paper, and in a continuation article.<sup>13</sup> These articles paved the way for the explosion of research in protein folding that occurred when site directed mutagenesis became available.

In the late 1960s, Tanford began his long, productive collaboration with Jackie Reynolds. With Tanford's interest in the hydrophobic effect and Reynold's background in protein-lipid interactions, the laboratory moved into the area of membranes and membrane proteins with great success. The two papers they published together in 1970 were cited over 1500 times, and revolutionized the study of membrane proteins.<sup>14,15</sup> The following year, Nozaki and Tanford published the first hydrophobicity scale.<sup>16</sup> In 1973, Tanford published his second book *The Hydrophobic Effect: Formation of Micelles and Biological Membranes* and it was also a great success.<sup>17</sup> Tanford had a long and very successful academic career. He published 14 articles that were each cited over 500 times. Tanford remained at Duke until 1988 when he retired, and began writing about the history of science and related topics.

In Tanford's second career, his first book was titled: *Ben Franklin Stilled the Waves: an Informal*

*History of Pouring Oil on Water with Reflections on the Ups and Downs of Scientific Life in General*.<sup>18</sup> Next Tanford and Reynolds wrote two travel guides for scientists. *The Scientific Traveler*<sup>19</sup> is a great resource for anyone who wants to visit the sites in Europe where important scientific discoveries were made. A related book, *A Travel Guide to Scientific Sites of the British Isles*,<sup>20</sup> was published 3 years later. Their last book, *Nature's Robots: A History of Proteins* was published in 2001.<sup>21</sup> The last line of Henryk Eisenberg's review in *Nature*<sup>22</sup> was "... anyone interested in proteins will find Nature's Robots an absorbing and often exciting story, as well as a major contribution to scholarship." In addition to these books, Tanford and Reynolds wrote many always interesting book reviews for *Nature*.

I will conclude with a few personal observations on Charles Tanford during my time in his laboratory. Tanford suggested three possible projects for my Ph.D. research. One caught my interest. Kauzmann's seminal review showed convincingly that hydrophobic bonds stabilize proteins, and the model compound data showed that they become stronger as the temperature goes up.<sup>23</sup> Tanford pointed out that this did not make sense because everyone knows that proteins unfold at higher temperatures. My project was to figure this out. He suggested that I work on  $\beta$ -lactoglobulin because we had 12+ g in the freezer that had been given to him by Serge Timasheff and Bob Townend. This was a fortunate choice. It turned out that  $\beta$ -lactoglobulin is most stable at 35°C and unfolds at both higher and lower temperatures.<sup>24</sup> Consequently, we were the first to observe the cold denaturation of a protein and show that proteins can be unfolded by either lowering or raising the temperature.  $\Delta H$  for unfolding was strongly temperature dependent varying from -40 to +40 kcal/mol between 10°C and 50°C. This was a reflection of the large change in heat capacity,  $\Delta C_p$ , that accompanies protein unfolding,<sup>24</sup> as shown earlier by Brandts.<sup>25</sup> This research benefitted others in ways we had not suspected. Efraim Racker wrote me a nice note to thank us because they began purifying their protein at room temperature rather than in the cold room and got a better yield.

Tanford and Kazuo Kawahara published a paper in 1966 showing that aldolase is a tetramer with a molecular weight of 158,000.<sup>26</sup> (Kazuo took the nice picture of Tanford that accompanies this article.) Three earlier papers in *Biochemistry* had suggested that that aldolase was a trimer with a lower molecular weight. Later that year at Federation Meetings in Atlantic City, a talk by a graduate student presented overwhelming evidence that aldolase was in fact a trimer. I wondered how Tanford could possibly respond. Tanford had broken his leg earlier and was on crutches. He hobbled to the microphone and said something like: we have measured the molecular

weights of proteins A, B, C, etc. and got them all exactly right and I am absolutely certain that aldolase is a tetramer. Tanford was right, as usual.

Robinson and Jencks had used peptide models to measure the  $\Delta G_{tr}$  of a peptide group from water to urea and GdnHCl solutions.<sup>27</sup> Tanford was interested in this because they had used different peptide models for the same purpose and the results did not agree. (This question was only recently resolved by Auton and Bolen.<sup>28</sup>) Consequently, Tanford invited Bill Jencks to Duke to give a seminar. In the introduction, Tanford said that despite the fact that Jencks did not understand thermodynamics at all, he had some interesting experimental results to present.

I was fortunate to have two long car rides with Tanford; they were a chance to learn about things other than proteins. In one, we were riding from Atlantic City to Durham and he explained to me why he enjoyed bird watching and classical music, two things I knew little about. Later, he would loan me records to take home and play with the hope that I would develop an appreciation of classical music. Tanford did not succeed in all of his ventures.

Tanford called me into his office to set me straight on two occasions. The first was when I suggested that Philip Handler did not deserve to be elected to membership in the National Academy of Science. (Handler later served two terms as President.) Tanford explained to me that Handler had done more than anyone to gain support for scientific research in Congress, and he certainly deserved to be a member. (Like many of us, Tanford was mightily impressed by Handler, as he has described.<sup>29</sup>) The second was when I had a disagreement with a faculty member and used some inappropriate language. In this case, he began the conversation with "I hope you are still in graduate school."

Finally, near the end of my post doc with Gordon Hammes at Cornell, I began looking for a job in industry. I interviewed at four companies and at each they asked me who this fellow Tanford was. It turns out that in my letter of reference he said I was good, much too good for industry. I guess it was a good strategy, they all offered me a job.

We celebrated Tanford's retirement on Cape Cod in 1988. At the time, Tanford suggested that his success resulted from all of the good experimental data gathered by his students. He was just being nice to us. More important was Tanford's ability to take the experimental results and write the great papers that helped so many of us gain a better understanding of proteins. Few if any made a greater contribution to protein science than Charles Tanford. (For more information on Tanford's origins and his thoughts on his research and his career, see his article "Fifty Years in the World of Proteins" published in 2003.<sup>29</sup>)

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