The State of Aggregation of α-Helical Poly(L-glutamic acid) in Aqueous Salt Solutions

Alfred Holtzer* and Roland B. Hawkins

Department of Chemistry, Washington University St. Louis, Missouri 63130

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Highly protonated poly(L-glutamic acid) is predominantly α-helical in aqueous salt solutions, whereas the fully charged poly(L-glutamate) polyion is randomly coiled. Potentiometric titration of poly(L-glutamic acid) with base therefore allows one to sweep through the entire helix−coil transition at a given temperature.[1−4] Such titration curves reveal four distinguishable regions as the solution is brought from highly acidic to more basic pH. These have been interpreted as a region of aggregation (A), a region of helical, but dispersed, polymer (H), a transition region (HC), and a region of random coil (C).[1,3,4]

These titration curves have been used to determine the standard free energy, entropy, and enthalpy changes for transition of the *uncharged* poly(L-glutamic acid) from helix to coil.[3,4] Recently, these long-extant thermodynamic data were used to raise certain questions concerning older and newer literature values for helix propensity, a highly significant quantity in protein-folding studies.[5] However, the possibility exists that poly(L-glutamic acid) is not molecularly dispersed, even in the pH region earlier identified as comprising only single-chain helices.[6] If, in fact, aggregates persist in that region, then dissociation free energies would contribute an unknown amount to the observed free energies earlier ascribed solely to the helix−coil transition.

In the original titration study of interest, the molecular dispersal of poly(L-glutamic acid) in the H region is accepted on the basis of two types of experimental evidence: (1) certain characteristics of the titration experiments themselves[4] and (2) direct measurements of molecular weight by light scattering.[7] The latter demonstrate that the polymer in the H region consists of single chains. These light scattering data were explicitly cited in ref 4, but, although a few for neutral pH have been published,[8] the relevant data at acid pH have not hitherto appeared except in dissertation form.[7]

At the time that the potentiometric titration data of ref 4 were being collected, we were concerned that aggregation might persist beyond A and into the H region, perhaps even leading to metastable states and hysteresis.[9] The latter possibility was eliminated by preparing several solutions (each in 100 mM NaCl unless otherwise noted) in *five* different ways.[4] Ordinarily, one starts with a water solution of sodium poly(L-glutamate), which is first protonated and its Na^+ counterions removed, then mixed with NaCl solution to bring the concentration of added salt to the desired level; titration with NaOH then follows. The different protocols are as follows: (i) protonating by mixing with excess HCl, then dialyzing to remove Na^+ counterions and excess HCl, yielding a flocculent white precipitate, which redissolves and clarifies before reaching the H region in the titration with NaOH that follows addition of salt; (ii) protonating and deionizing the polymer with an ion-exchange resin, then adding salt only after titrating to a neutralization fraction (α) of 0.1, a procedure that yields no precipitate or marked turbidity; (iii) adding salt to resin-protonated and deionized solution, causing opacity and partial precipitation that disappears before the H region is reached; (iv) titrating to beyond the end point with NaOH, then back-titrating potentiometrically with HCl; and (v) adding salt (to 50 mM) to the resin-protonated and deionized sample, then completely titrating one aliquot immediately and titrating a second aliquot to $\alpha = 0.345$, then completing the second titration only after allowing to stand overnight. All these protocols yield the same potentiometric titration curve, powerful evidence that no metastable states or hysteresis are involved outside the A region. Moreover, it was also shown that using the ion-exchange resin column- or batch-wise yields indistinguishable results.[4]

Titration evidence also exists that these equilibrium states in the H region in fact comprise single chains. Most obviously, the titration curves are essentially independent of polymer concentration,[3,4] which could not be true in a system that dissociates when it unfolds. Moreover, the small observed concentration dependence implies only a negligible difference in the measured Gibbs energies.[4] Finally, such concentration dependence as is observable appears only at low concentrations of added salt,[3,4] probably because of contributions of the polymer to the ionic strength. This is opposite to expectations from the aggregation hypothesis, since salt fosters aggregation of poly(L-glutamic acid).

To provide more direct evidence, light scattering studies were performed at 25 °C for various ionic strengths throughout the H, HC, and C ranges on two of the four samples used in the titration experiments.[7] Although available in dissertation form since 1967, these data have been published previously only for the C region, in connection with a detailed study of the intrinsic viscosity and molecular dimensions of the randomly coiled form.[8] The weight average molecular weights and second virial coefficients for all conformational regions are now presented in Table 1, along with the pH, fraction neutralized, and titration-categorized region (H, HC, or C) in which they reside.

Each row in Table 1 contains the results of a Zimm plot, the scattering having been measured at 15 angles (30−135°) to the incident beam for each of at least four solutions differing in concentration. The molecular weights follow from the usual double extrapolation to zero angle and zero concentration. Complete experimental details and a sample Zimm plot have been given previously.[7,8] Since only the rows marked "C" in the last column of Table 1 have appeared earlier,[8] the newly reported information stems from 12 Zimm plots. The weight average molecular weights for a given polymer in the H and HC regions are seen to agree, within error, with those for the completely charged, fully unfolded (C) region, no matter what the ionic strength.

Finally, the second virial coefficients in Table 1 provide a strong argument against the notion that the polymer is aggregated in the H region. Second virial coefficients are not determinable with great precision, and one certainly does not expect them to be independent of molecular weight, ionic strength, charge, or polydispersity. However, all the values determined are strongly positive, indicating a marked preference for solvent−polymer over polymer−polymer interactions. Such behavior is not characteristic of systems of aggregates. Indeed,

(1) Wada, A. *J. Mol. Phys.* **1960**, *3*, 409−416.
(2) Zimm, B. H.; Rice, S. A. *J. Mol. Phys.* **1960**, *3*, 391−407.
(3) Nagasawa, M.; Holtzer, A. *J. Am. Chem. Soc.* **1964**, *86*, 538−543. Appendix I gives a brief derivation of how one can obtain the standard free energy of the helix−coil transition in the uncharged molecule from titration data.
(4) Olander, D. S.; Holtzer, A. *J. Am. Chem. Soc.* **1968**, *90*, 4549−4560. Line 7 on the right column of page 4551 should read 62%, not 26%. Line 15 of the right column of page 4559 should say "to" not "of"; and, same page and column, 13 lines from the bottom, "glutamic" should be replaced by "aspartic".
(5) Holtzer, A. *J. Am. Chem. Soc.* **1994**, *116*, 10837−10838.
(6) Spek, E. J.; Gong, Y.; Kallenbach, N. R. *J. Am. Chem. Soc.* **1995**, *117*, 10773−10774.
(7) Hawkins, R. B. Ph.D. Dissertation, Washington University, 1967. Appendix A explains in detail why molecular weights provided with commercial sources of poly(L-glutamic acid) must be considered nominal. For those interested in this dissertation, A. H. will provide errata on request.
(8) Hawkins, R. B.; Holtzer, A. *Macromolecules* **1972**, *5*, 294−301. In eq 2, replace "0.15" by "0.51". The middle expression in eq 4 should read $\Phi_\theta[\langle L_\theta^2\rangle/M]^{3/2}$.
(9) Schuster, T. M. *Biopolymers* **1965**, *3*, 681−686.

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Table 1. Light Scattering Results for Poly(L-glutamic acid)

[NaCl]/ mM	pH	α/%	M_w/kDa	A_2/ (mmol cm^3 g^{-2})	region
			Sample G-61		
10	4.945	22.4	158	4.58	H
50	4.845	28.3	153	1.76	H
50	4.890	29.3	143	2.09	H
50	5.029	33.1	145	2.20	H/HC
50	5.552	60.1	155	4.67	HC
100	4.896	33.9	148	1.26	H/HC
100[a]	7.05	97.3	160	3.13	C
400	4.796	45.4	151	0.73	HC
400[a]	6.798	99.0	158	1.85	C
1000[a]	7.10	100.	152	1.45	C
			av 152 ± 6 (SD)		
			Sample G-72		
50	4.750	26.2	110	0.87	H
50	4.793	27.0	110	1.56	H
100	4.670	27.6	123	0.98	H
100	4.671	27.6	117	0.90	H
400	4.532	34.0	111	0.70	H
400[a]	6.798	99.1	122	1.52	C
1000[a]	7.10	100.	111	1.37	C
			av 115 ± 6 (SD)		

[a] Also contained 10 mM sodium phosphate.

strong aggregation, which leads to increase of molecular weight with concentration, gives apparent second virial coefficients that are strongly *negative*. These data prove that the poly(L-glutamic acid) used in the titration experiments at issue was not aggregated in the H region.

In dealing with acidic solutions of poly(L-glutamic acid), one nevertheless must be rather careful to identify the range of pH that yields the unaggregated H region. Examination of the published titration curves from this laboratory[3,4] shows plainly that the H region is not reached until a pH > 4.6 at ordinary conditions of ionic strength and temperature. By this criterion, for example, a long-extant demonstration of hysteresis in certain properties of solutions of poly(L-glutamic acid) is seen to involve the A region.[9]

Another example is the recent finding that glutamic acid peptides of mean degree of polymerization near 5 residues are significantly helical by CD, which certainly suggests aggregation.[6] However, these studies were also performed at pH values in the A region, where aggregation is expected. Moreover, these samples are polydisperse, so the helix content may be due to the presence of material of much higher chain molecular weight than average. Two prior studies of L-glutamic acid oligomers found very low helix content for 5-mers; these earlier studies employed samples fractionated by ion-exchange chromatography.[10] In any event, such small helices, if they exist, may very well prefer the 3_{10}-helix conformation,[11] a structure not relevant to the present discussion.

In addition to exercising care in choosing pH, one should test samples for chemical purity, where aggregation is suspected. Poly(L-glutamic acid) is usually made by anhydride polymerization of poly(γ-benzyl-L-glutamate), followed by removal of the benzyl groups. Residual benzyl is known to cause aggregation in aqueous solution. This was tested for and found absent in the samples used in our experiments.[4]

In the case of the polymers studied by potentiometric titration, then, light scattering experiments demonstrate that there is no significant aggregation in the helical region, properly defined, or in any other region from which titration data were accepted as usable. It follows that the substantial body of data from a variety of laboratories on poly(L-glutamic acid) need not be re-evaluated, provided the above-described precautions have been taken. In particular, the principal conclusion of ref 5 remains intact: the *intra*molecular side chain interactions in poly(L-glutamic acid) are mimicked, perhaps fortuitously, in both entropic and enthalpic components, by the host polymers employed in obtaining the older values of the helix propensity for a glutamic acid guest residue.

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