Protein-Polyelectrolyte Complexes. How to Suppress Thermoaggregation without Noticeable Denaturing of the Protein?

Vladimir A. Izumrudov

Summary: Homotetrameric enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a model protein for elucidation a capability of the oppositely charged polyelectrolyte to suppress the protein thermoaggregation. Charge density and degree of polymerization of the chains, relative content of GAPDH in the mixture, as well as pH and ionic strength proved to be the factors of the thermoaggregation control. However the electrostatic binding of the most active suppressors, e.g. sodium poly(styrenesulfonate) was attended with marked changes of secondary, tertiary and quaternary structure of GAPDH and clearly defined denaturing. The performed comprehensive study of GAPDH interaction with different tightly bound polysulfoanions revealed the key role of a degree of polymerization and hydrophobicity of the chains in the denaturing, specifically the pronounced adverse effect on the enzyme of relatively hydrophobic short-chained polyanions. Accordingly, the required criteria have been stated as follows: relatively hydrophilic highly polymerized polyelectrolytes that are able to form with a protein of fairly stable water-soluble complexes are best suited to prevent thermoaggregation without drastic change of the protein structure.

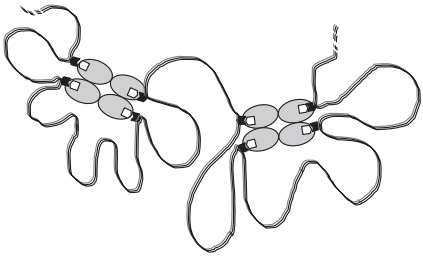
Keywords: chains length; enzymes; hydrophobicity; thermoaggregation; thermodenaturing

Introduction

The searching for ways of stabilization of globular proteins (enzymes) solutions, specifically by complexing with oppositely charged polyelectrolyte [1–4] has assumed a new significance with the revealed key role that plays the proteins aggregation in formation of insoluble amyloid structures responsible for the development of neurodegenerative diseases like Alzheimer disease, bovine spongiform encephalopathy and Huntington disease.[5–11] Recently we used oligomeric enzymes, mainly glyceraldehyde-3-phosphate dehydrogenase (GAPDH) that are prone to form the amyloid structures *in vivo* as a model proteins for elucidation a capability of polyelectrolytes to suppress thermoaggregation of the electrostatically bound protein *in vitro*.[12,13] Charge density and degree of polymerization of anionic [12] or cationic [13] polyelectrolyte, relative content of the protein in mixtures, as well as pH and ionic strength proved to be the factors of thermoaggregation control. The important role of hydrophobic interactions in stabilization of GAPDH against thermoaggregation was evidenced on studying the influence of different polyamines, specifically ionene bromides or hydrophobic derivatives of poly(N-alkyl-4-vinylpyridinium) cations on the enzyme. The suppression of thermoaggregation was greatly enhanced with increase in a number of methylene groups in either the ionenes or N-alkyl moieties, i.e. with the growth in hydrophobicity of the polycation repeat units.[13] The revealed beneficial effect was attributable to ability of

Department of Chemistry, M.V. Lomonosov Moscow State University, Leninskiye Gory, 119991 Moscow, Russia
Fax: +74959390174;
E-mail: izumrud@genebee.msu.ru

the hydrophobic groups to interact with hydrophobic "spots" that appeared on the surface of the protein globule while heating as the result of the protein unfolding. Blocking of the "spots" (depicted as white squares) with the hydrophobic groups (black squares) protected the contacts between hydrophobic "spots" of different globules and hence, prevented the protein thermoaggregation.

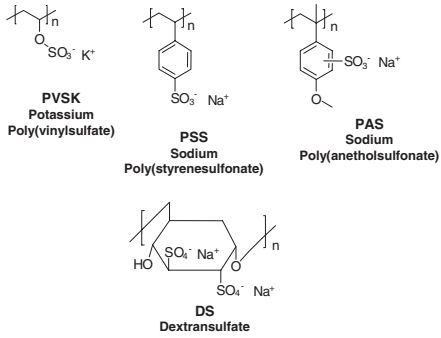


Complexing with polyelectrolytes suppressed thermoaggregation, but not thermodenaturation that was, on occasion, even facilitated. Thus if rather active thermoaggregation suppressors were used, then the denaturing of GAPDH by such hydrophobic polyelectrolytes was profound and virtually irreversible.[13] This finding implies that hydrophobic groups of the highly charged chains not only blocked the "spots" but exert an adverse effect on the protein globules. Argument in favor of this assumption was the finding that relatively hydrophobic sodium poly(styrenesulfonate) (PSS) exhibited much higher efficacy in both preventing of thermoaggregation and enhancing thermodenaturation as compared with the activities of hydrophilic poly(carboxylic) anions.[12]

To verify this suggestion, we conducted a comprehensive study of influence of the polyelectrolyte binding on secondary, tertiary, and quaternary structure of GAPDH. Of special interest was to check whether tightly bound poly(sulfoanions) (i.e. the polymers containing SO_3^- or SO_4^- groups in the chain) of different hydrophobicity which readily suppressed GAPDH thermoaggregation [12] are able to form soluble complexes without noticeable harmful effect on the protein. The reported data of the model study can serve as a basis for development of the approaches capable of suppressing thermoaggregation of the proteins without marked changes in the enzyme functionality.

Results and Discussion

GAPDH (pI 8.5) was extracted from rabbit muscle as described elsewhere.[12] Samples of sodium poly(styrenesulfonate) of different degree of polymerization (DP) and potassium poly(vinylsulfate) (DP 1100) (Serva, Germany), as well as sodium poly(anetholsulfonate) (DP 800) and samples of sodium dextransulfate of different molecular mass (Fluka, Switzerland) were used as poly(sulfoanions).



Judging from the calculated values of Hansch parameter log*P*,[15] the poly(sulfoanions) varied widely in hydrophobicity and could be arranged in the order DS < PVSK << PSS < PAS according to the growth in hydrophobicity of the repeat units. The Hansch parameter for the units of poly(carboxylic) anions, e.g. salts of poly(acrylic acid) or poly(methacrylic acid) differed only slightly from that of PVSK repeat units. Nevertheless, the efficacy of suppression of GAPDH thermoaggregation by all studied poly(sulfoanions) including PVSK was pronounced being two orders of magnitude greater as compared with the poly(carboxylic anions).[14] In other words, the stabilization of the soluble

protein complex by poly(sulfoanions) is determined by the presence of SO_3^- or SO_4^- groups in the chains rather than by hydrophobicity of the macromolecules. Note that it is precisely these groups which form particularly stable ion pairs with amino groups of polycations. Thus, the dissociation of poly(sulfoanion)-containing polyelectrolyte complexes occurred at approximately one order of magnitude higher concentration of the added low-molecular-weight salt as compared with the complexes of poly(carboxylic anions).[16] For the poly(sulfoanion) chains tightly bound with the protein, a contribution of hydrophobic interactions in the complexing seems to be relatively insignificant. Moreover, an increase in hydrophobicity of the poly(sulfoanion) chains even slightly impaired their ability to prevent thermoaggregation. A different situation arises with systems wherein the protein-polyelectrolyte interactions are not so pronounced. Thus the affinity of weakly bound quaternized polyamines for the protein was apparently reinforced by hydrophobic interactions that stabilized the soluble complex and hence, hindered thermoaggregation.[13]

The revealed clearly defined capacity of different poly(sufoanions) to prevent thermoaggregation makes it of primary importance to elucidate the extent of thermodenaturing caused by the polyanions binding. So, we aimed to study the influence of the tightly bound polyanions on GAPDH molecular structure with emphasis on the chains hydrophobicity in order to clarify the chains capable to form soluble complexes without noticeable deleterious effect on the protein.

Effect of Polysulfoanion Binding on GAPDH Secondary Structure

Each subunit of the homotetrameric enzyme contains an active center that comprises catalytic domain with α-helix as the first element of the secondary structure. The circular dichroism spectra make it possible to follow changes in the secondary structure of protein during complexation. The corresponding spectrum shows a minimum at 222 nm, which is associated with $n \rightarrow \pi^*$ transition of the α-helix. Accordingly, an increase in $[\Theta]_{222}$ indicates a decrease in the fraction of α-helices and evidences disorganization of the protein structure as a whole.

The kinetic curves that illustrate changes in above parameter during incubation of the mixtures at 45 °C (Figure 1) imply that the binding of hydrophobic PSS anions results in more serious distortions in GAPDH secondary structure (curves *5*, *6*) as compared with the binding of relatively hydrophilic PVSK anions (curve *4*). Interaction of GAPDH with the most hydrophilic DS anions, both oligomer (curve *2*) and highly polymerized sample (curve *3*), exerts practically no effect on the secondary structure during heating (curve *1*).

Of special interest is rather high activity of short PSS chains containing eight repeat units (curve *6*). The binding resulted in most pronounced breakdown of α-helices, which was completed even before the onset of measurements, cf. the starting values of $[\Theta]_{222}$. For PSS chain consisted of 430 repeat units, the denaturing action weakened to some extent, although the adverse effect remained significant and strengthened with time (curve *5*).

So, the disturbing effect on GAPDH secondary structure is enhanced with hydrophobicity of the polyanions, being most pronounced for the short hydrophobic chains.

Effect of Polysulfoanion Binding on GAPDH Tertiary Structure

Data of differential scanning calorimetry (DSC) that was conducted over a temperature range of 20–90 °C with scan rate of $1\ ^\circ C \cdot min^{-1}$ [14] evidenced marked changes in thermodynamic parameters of the protein melting, i.e. distortion of tertiary structure of GAPDH molecules caused by binding of the poly(sulfoanion). The analysis leaned upon the relative height of the peak h/h_{GAPD}, the temperature corresponding to maximum heat absorption T_m, the half-width of the peak ΔT_m, and its area

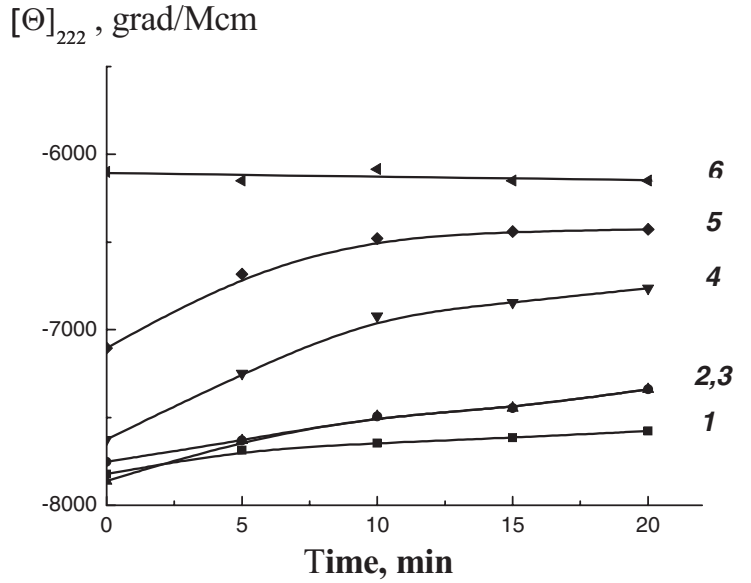


Figure 1.
Time-depended changes in the ellipticity measured at 45 °C for GAPDH (*1*) and GAPDH in mixtures with different poly(sulfoanions) (*2*–*6*): DS, 5 kDa (*2*) DS, 10^3 kDa (*3*), PVSK(*4*), PSS, DP 430 (*5*), and PSS, DP 8 (*6*). 10 mM phosphate buffer, pH 7.5.

reflected the calorimetric enthalpy ΔH_{cal}. Above parameters correlated with values of Hansch parameter log*P* indicating the polyanions hydrophobicity. The peaks of heat absorption of the enzyme in mixtures with hydrophilic poly(sulfoanions), e.g. DS were characterized by both comparatively large height and small value of the half-width (Table 1). In other words, melting of the bound GAPDH occurred cooperatively with retention of a large part of structural elements, although this process proceeded at lower temperature as compared with free protein. In contrast, GAPDH binding with relatively hydrophobic PSS and especially the most hydrophobic PAS resulted in lower and wider peaks that indicated disorder of the tertiary structure caused by disruption of a great part of GAPDH structural elements. Intermediate situation was realized on binding with PVSK.

Degree of polymerization of the polyanion proved to be another factor controlling tertiary structure of the protein. For instance, when highly polymerized DS, 10^3 kDa was replaced with the sample of much lower molecular mass, 10^2 kDa and, in particular, with oligomeric DS, 5 kDa, the successive destabilization of GAPDH occurred. The parameters T_m and ΔT_m therewith remained virtually the same, but the height of the peak, i.e. ΔH_{cal}

Table 1.
Thermodynamic parameters of melting of GAPDH and the bound GAPDH

Sample	Log *P*	h/h_{GAPDH}	T_m °C	ΔT_m °C	ΔH_{cal}, kJ/mol
GAPDH	–	1	62.1	4.9	2250
GAPDH + DS	−5.96	0.78	49.1	5.2	2050
GAPDH + PVSK	−5.86	0.39	51.0	5.8	1010
GAPDH + PSS	−4.48	0.24	50.1	6.9	675
GAPDH + PAS	−4.21	0.17	45.5	14.6	620

value decreased noticeably. A similar effect was revealed on binding of negatively charged GAPDH with positively charged poly(*N*-ethyl-4-vinylpyridinium) bromides of different DP. Thus the decrease in DP from 1600 to 10 halved the value of ΔHcal, but T_m remained practically unchanged.[14]

GAPDH binding with relatively hydrophobic PSS anion revealed particularly high capacity of the short chains to destabilize the enzyme (Figure 2). Even highly polymerized PSS chains consisted of 430 repeat units exerted profound disturbing effect on tertiary structure of the protein (curve *2*), whereas the shortening of the chains resulted in further subsequent decrease in ΔH_{cal} and T_m values (curves *3*, *4*). Notice that in the case of oligomeric PSS containing only eight repeat units in the chain, our attempts to detect a maximum on the heat absorption curve were not successful. The short chains interacted with GAPDH so effectively that the protein tertiary structure destroyed before heating, most probably at room temperature. Accordingly, the inactivation of GAPDH bound with the PSS oligomer accomplished completely for 10 min at room temperature.[14]

It is apparent that the revealed distinct denaturing action of relatively hydrophobic short chains should be taken into account on design of protein-polyelectrolyte systems. To maintain the protein in active state, one should avoid the use of such oligomeric chains or non-fractionated polyelectrolyte samples. The latter is exemplified most clearly by melting of GAPDH in solution of non-fractionated PSS sample (Figure 2, dotted line). The peak is arranged in extremely left position despite the highest value of the average degree of polymerization, DP 950. So, the presence of even small amounts of short chains in the sample could exert primary control over stability of the bound protein.

Most likely, the dramatic denaturing action of the short chains is conditioned by less pronounced steric hindrances. The charged oligomers are able to penetrate deeply inside the protein globule and form more contacts with GAPDH molecule, especially due to hydrophobic interactions that assisted the enzyme destabilization. Note that PSS and poly(methacrylate) anions behaved in a similar manner on binding with rigid cationic dendrimers.[17] The negatively charged oligomers were

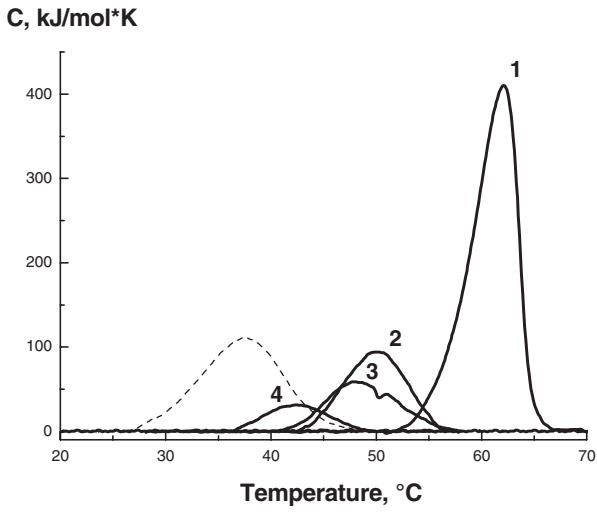


Figure 2.
DSC melting curves of free enzyme (*1*) and GAPDH mixtures with PSS of different DP: 430 (*2*), 77 (*3*), and 30 (*4*). The dotted line corresponds to GAPDH mixture with non-fractionated sample of PSS, DP 970.

able to form ion pairs with cationic groups located in the internal sphere of the dendrimer, whereas the electrostatic binding of relatively long chains occurred only on the surface of the dendrimer molecule.

Effect of Polysulfoanion Binding on GAPDH Quaternary Structure

Native GAPDH molecule comprises four identical subunits. The quaternary structure of the enzyme can dissociate into dimers and even monomers, depending on the concentration of the protein and the level of denaturation. The interaction between dimers that form the tetramer is much weaker than that between the subunits of the dimer.

The results of high-speed sedimentation assay performed under heating made it possible to study the effect of a polyanion binding on quaternary structure of the protein (Figure 3). The sedimentation patterns of GAPDH mixed solutions with relatively hydrophobic PSS anions containing 430 and 360 repeat units clearly display two peaks which move faster than corresponding free PSS anions. On the patterns of shorter PSS chains, only one peak was elucidated. The components in these mixtures moved together as a united front, and the values of sedimentation coefficient S noticeably exceeded those of the corresponding polyanions. GAPDH mixture with oligomeric anions composed of 31 repeat units was characterized by one peak, which moved much faster than the short chains that did not even detach from the meniscus during the experimental run. In other words, in all mixtures the bound enzyme formed soluble complexes of higher molecular mass than the corresponding polyanions.

A comparison of the determined S values with $S = 8.90$ svedberg of native GAPDH does not allow arriving at the analogous unequivocal conclusion about the protein. Binding of the tetrameric form which should result in sedimentation coefficient exceed $S = 8.90$ svedberg, is likely to take place only for highly polymerized PSS chains containing 430 and 360 units (Figure 3). However, even in these mixtures, the fast sedimenting particles coexist with other particles that move slower than free native protein. The patterns of the protein mixtures with shorter chains reveal a single peak with S close to the values of 4.7 and 3.0 svedberg corresponding to GAPDH dimer and monomer, respectively. This finding evidences the disturbing action of PSS anion on GAPDH quaternary structure which is successively strengthened on a decrease in DP of the polyanion. It is not inconceivable that relatively long PSS chains form with the homotetrameric enzyme of soluble complexes which coexist

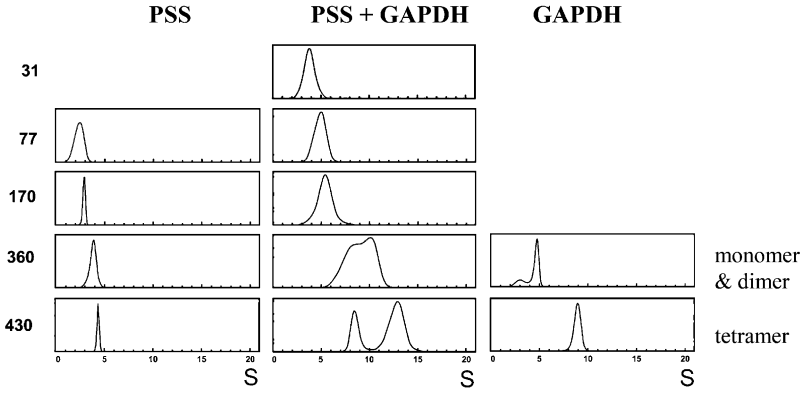
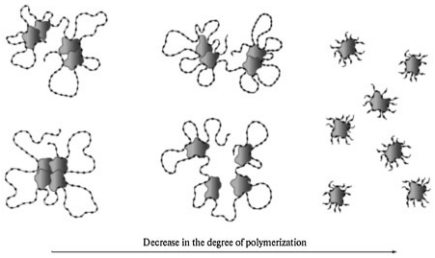


Figure 3.
Sedimentation patterns of different GAPDH forms (right column), PSS of different DP (left column), and GAPDH mixtures with the PSS samples. 10 mM phosphate buffer, pH 7.5, 60 °C.

with the complexes of the protein dimers. However, relatively short chains destroy quaternary structure of GAPDH and bind mainly with the monomeric form of GAPDH.



Noteworthy that contrary to the binding of hydrophobic PSS, GAPDH interaction with hydrophilic DS anions did not result in noticeable changing of the quaternary structure. The sedimentation patterns revealed one peak with the sedimentation coefficient that exceeded *S* of the native tetrameric enzyme even for oligomeric DS, 5 kDa. This proves conclusively much weaker disturbing effect of the bound hydrophilic macromolecules on GAPDH quaternary structure.

Conclusion

The data reported here and published in [12–14] allow one to propose the following guide lines for the monitoring of polyelectrolytes that are able to prevent thermoaggregation with a minimum adverse action on structure and function of the protein. For the effective suppression of thermoaggregation, the polyelectrolyte should have a protein affinity high enough to provide formation of a stable soluble complex. Poly(sulfoanions) and polyamines with primary amino groups in the chain form the most stable polyelectrolyte complexes with oppositely charged partners.[18] Accordingly, the using of these polymers for binding with enzymes seems to be the most promising. Additional non-electrostatic interactions, e.g. hydrogen bonds and hydrophobic interactions make it possible to enhance the affinity of the polyelectrolyte for the protein. However in this case, some undesirable consequences like the drop in catalytic activity as a result of disturbing effect on GAPDH structure may emerge. Therefore, hydrophilic and highly polymerized charged macromolecules that form stable soluble complexes with the enzyme provide the best protection against thermoaggregation without marked loss in catalytic activity. These requirements are fulfilled by sulfated polysaccharides, specifically DS anions, which in addition are biocompatible and biodegradable compounds. The preliminary experiments with heparin [14] demonstrated high antiaggregative activity of this natural sulfated polysaccharide which was not attended with noticeable denaturing action on GAPDH.

Acknowledgements: We are grateful to Dr. S. Stogov for measurements of the protein denaturing and Prof. V. Muronetz for helpful discussion.

[1] K. W. Mattison, I. G. Brittain, P. L. Dubin, *Biotechnol.Prog.* **1995**, *11*, 632.
[2] J. L. Doublier, C. Garnier, D. Renard, C. Sanchez, *Curr. Opin. Colloid. Interface*, **2000**, *5*, 202.
[3] S. L. Turgeon, M. Beaulieu, C. Schmitt, C. Sanchez, *Curr. Opin. Colloid. Interface*, **2003**, *8*, 401.
[4] C. L. Cooper, P. L. Dubin, A. B. Kayitmazer, S. Turksen, *Curr. Opin. Colloid. Interface.* **2005**, *10*, 52.
[5] C. Nerelius, J. Johansson, A. Sandegren, *Front. Biosci.* **2009**, *14*, 1716.
[6] B. Bulic, M. Pickhardt, B. Schmidt, E. M. Mandelkow, H. Waldmann, Mandelkow E., *Angew. Chem. Int. Ed. Engl.* **2009**, *48*, 1740.
[7] T. Wisniewski, M. Sadowski, *BMC Neurosci.* **2008**, *9* (Suppl 2), S5.
[8] D. Drago, S. Bolognin, P. Zatta, *Curr. Alzheimer Res.* **2008**, *5*, 500.
[9] Y. Yan, C. Wang, *Curr. Alzheimer Res.* **2008**, *5*, 548.
[10] N. Sahara, S. Maeda, A. Takashima, *Curr. Alzheimer Res.* **2008**, *5*, 591.
[11] K. Beyer, A. Ariza, *Curr. Med. Chem.* **2008**, *15*, 2748.
[12] I. N. Shalova, R. A. Asryants, M. V. Sholukh, L. Saso, B. I. Kurganov, V. I. Muronetz, V. A. Izumrudov, *Macromol. Biosci.* **2005**, *5*, 1184.
[13] I. N. Shalova, I. N. Naletova, L. Saso, V. I. Muronetz, V. A. Izumrudov, *Macromol. Biosci.* **2007**, *7*, 929.

[14] S. V. Stogov, V. A. Izumrudov, V. I. Muronetz, *Biochemistry (Moscow)* **2010**, *75*, 437.
[15] C. Hansch, T. Klein, *Acc. Chem. Res.* **1986**, *19*, 392.
[16] V. A. Izumrudov, V. V. Parashchuk, A. V. Sybachin, *J. Drug Del. Sci. Tech.* **2006**, *16*, 267.
[17] Z. B. Shifrina, N. V. Kuchkina, P. N. Rutkevich, T. N. Vlasik, A. D. Sushko, V. A. Izumrudov, *Macromolecules* **2009**, *42*, 9548.
[18] V. A. Izumrudov, *Russian Chemical Reviews* **2008**, *77*, 381.

