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Structural Changes of a Protein Bound to a Polyelectrolyte Depend on the Hydrophobicity and Polymerization Degree of the Polyelectrolyte

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Abstract—Influence of polyelectrolytes of different chemical structure and degree of polymerization on aggregation and denaturation of the oligomeric enzyme glyceraldehyde-3-phosphate dehydrogenase has been studied to ascertain molecular characteristics of the polymer chains providing the efficient prevention of aggregation of the enzyme without drastic changes in its structure and catalytic activity. The best polymers meeting these requirements were found to be hydrophilic high-molecular-weight polyelectrolytes forming stable complexes with the enzyme. The revealed pronounced negative effect of short polymer chains on the enzyme must be taken into account in the design of protein–polyelectrolyte systems by using thoroughly fractionated polymer samples containing no admixture of charged oligomers.

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Key words: glyceraldehyde-3-phosphate dehydrogenase, polysulfoanions, protein–polyelectrolyte complexes, thermoaggregation suppression, differential scanning calorimetry

Synthetic and natural polyelectrolytes are widely used in biochemistry and medicine for different purposes from protein immobilization to targeted transport of drugs. An appropriate polyelectrolyte is usually selected for an experiment based of one or two parameters: chain length and/or charge. Other parameters (hydrophobicity, charge distribution along the polymer chain, and presence of fractions that significantly differ in molecular weight) are not always considered; this yields results that are hard to interpret and reproduce (for example, because of differences in the fractionation of polyelectrolyte preparations from different manufacturers). For these reasons, investigation of the influence of different parameters of polyelectrolytes on proteins as simple biological systems is of importance. Such experiments make it possible to predict the effect of polyelectrolytes (including natural ones) on proteins, to select polyelectrolytes that assist proteins in either stabilization or denaturation, or to influence the process of protein aggregation. Investigation of approaches for prevention of protein aggregation is of special importance considering that this phenomenon plays a crucial role in the formation of amyloid structures that are responsible for the development of a number of neurodegenerative diseases (Alzheimer's disease, bovine spongiform encephalopathy, and Huntington disease) [1-7].

Previously, oligomeric enzymes (mainly glyceraldehyde-3-phosphate dehydrogenase (GAPDH) that is capable of forming amyloid structures *in vitro* and also involved in their formation *in vivo*) were used as models for the comparative investigation of the ability of synthetic polyelectrolytes to suppress protein aggregation [8, 9]. The efficiency of the suppression of protein thermoaggregation was found to be dependent on such factors as charge density on the polymer chain, polymerization degree of polyelectrolytes, polyelectrolyte concentration, and pH and ionic strength of the solution. It should be noted that the formation of protein–polyelectrolyte com-

Abbreviations: DP, degree of polymerization; DS, dextran sulfate; GAPDH, D-glyceraldehyde-3-phosphate dehydrogenase; PAA, polyacrylic acid; PAMS, potassium poly-2-acrylamido-2-methyl-1-propane sulfonate; PAS, sodium polyanethol sulfonate; PMAA, polymethacrylic acid; PSS, sodium polystyrene sulfonate; PVS, potassium polyvinyl sulfate.

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plexes protected the enzyme from thermoaggregation, but not from thermodenaturation, which even increased under certain conditions. However, inactivation of GAPDH under the action of polyanions was reversible, since the addition of a synthetic polycation that released the enzyme into the solution resulted in significant (40%) reactivation of the enzyme [8]. These results were an important step on the way to creation of artificial chaperones since they allowed creation of a system imitating the main step of chaperone functioning. However, the compounds that suppressed GAPDH thermoaggregation with the highest efficiency caused virtually complete and irreversible denaturation of the enzyme. For example, the ability of relatively hydrophobic sodium polystyrene sulfonate (PSS) to prevent thermoaggregation and to increase thermodenaturation was much more pronounced compared to hydrophilic polycarboxylic anions [8]. An important role of hydrophobic interactions in complexing with GAPDH was demonstrated during the investigation of the influence of poly-N-alkyl-4-vinylpyridine cation on the enzyme: the effect significantly increased with elongation of the alkyl group, i.e. with the growth in the hydrophobicity of the monomeric unit of the polycation [9].

In the present work we elaborated criteria that a polyelectrolyte should meet so as to not only efficiently prevent aggregation, but to little affect the structure and functional state of proteins. It was of importance to reveal a correlation between the degree of polymerization and hydrophobicity of a polyelectrolyte and the stability of the bound protein. The results can serve as a basis for the creation of compounds possessing the ability to prevent the formation of protein aggregates and to disaggregate them without serious disturbance of the protein structure.

MATERIALS AND METHODS

The following chemicals were used: sodium chloride, potassium phosphate, EDTA, glycine, and glyceraldehyde-3-phosphate diethylacetal (Sigma, USA), NAD (Fluka, Sweden).

Polycarboxylic anions. Samples of high-molecular-weight polyacrylic acid (PAA) with degree of polymerization (DP) of 1200 and polymethacrylic acid (PMAA) with DP of 950 were from Fluka.

Polysulfonate anions. We used strongly charged polymers bearing SO_3^- or SO_4^- groups (the structural formulas of their units are presented in Fig. 1); fractionated samples of sodium polystyrene sulfonate (PSS) with DP of 8, 30, 77, 430, and 1710, and a non-fractionated sample (average DP of 970); potassium polyvinyl sulfate (PVS) with average DP of 1100 (Serva, Germany); sodium polyanethol sulfonate (PAS) with DP of 800, as well as samples of dextran sulfate (DS) with the molecular weights of 1000, 100, and 5 kDa (Fluka); sodium salt of heparin and poly-2-acrylamido-2-methyl-1-propanesulfonic acid (PAMS) with average DP of 9700 (Sigma-Aldrich, USA).

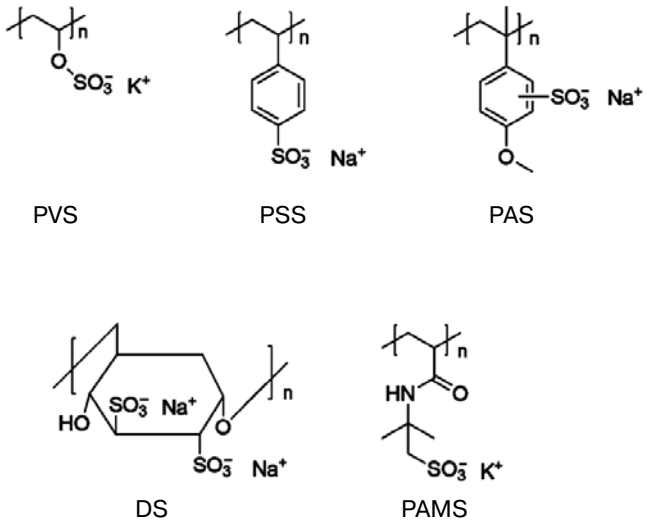

Fig. 1. Structural formulas of monomers of polysulfoanions.

Homotetrameric enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was isolated from rabbit muscles according to the method described by Scopes [10]. The experiments with the enzyme were performed in 10 mM potassium-phosphate buffer, pH 7.5, containing 0.5 mM EDTA.

Kinetics of thermoaggregation was investigated by the turbidimetric assay, monitoring the increase in the turbidity of the solution by absorption at 320 nm. At this wavelength, the light scattering of protein aggregates makes the main contribution into the light absorption. The measurements were made at 60°C in 10 mM phosphate buffer, pH 7.5, using an SLM Aminco DW-2000 spectrophotometer (USA). Protein concentration in the sample was 0.7 μM. The absorption of the sample stopped growing 2 min after the initiation of the reaction, so the values of the absorption for the mixture of GAPDH with polyanions (A_{320}) and for the free enzyme ($A_{320,GAPDH}$) were determined 5 min after the initiation of the reaction. The ratio $A_{320}/A_{320,GAPDH}$ indicates the degree of the suppression of thermoaggregation: 0 corresponds to complete suppression, while 1 is the absence of the effect of the polyelectrolyte.

Differential scanning calorimetry (DSC). The effect of binding of polyanions on the thermodynamic parameters of the protein melting was studied by differential scanning calorimetry assay using a DASM-4 adiabatic microcalorimeter (Biopribor, Russia) with the cell volume of 0.47 ml. During the experiments, the temperature was varied from 20 to 90°C at the heating rate of 1°C/min. The data were processed using the computer program Arina developed in the Belozersky Institute of Physico-

Chemical Biology and OriginPro 7.0 (MicroCal Inc., USA).

Enzymatic activity of GAPDH (both free enzyme and GAPDH in the complex with polyanions) was measured at 25°C in 100 mM glycine buffer, pH 8.9, containing 1 mM EDTA, 100 mM potassium phosphate, 1 mM glyceraldehyde-3-phosphate, and 1 mM NAD^+. The reaction was monitored by the increase in the absorption at 340 nm due to the formation of NADH. Neither GAPDH nor polyanions absorb at this wavelength. The measurements were made using a Shimadzu UV 1601 spectrophotometer (Japan). The original activity of GAPDH constituted 80 μmol NADH/min per mg protein. GAPDH was incubated with polysulfoanions at 45°C. During the incubation, aliquots were taken from the reaction mixture for measuring the enzymatic activity. Concentration of polyanions was calculated and expressed as the molar concentration of the charged groups.

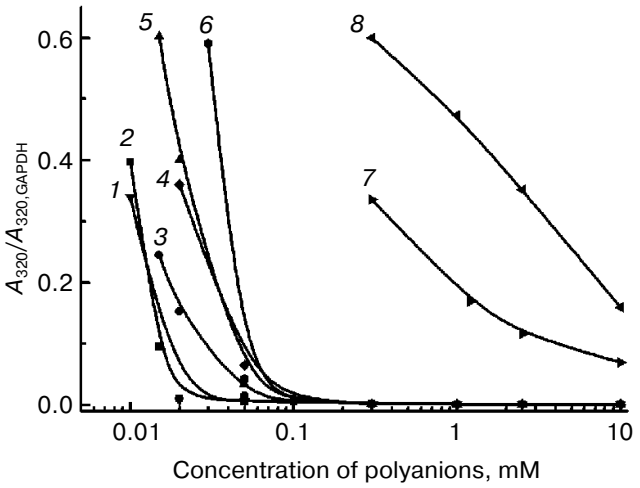

Fig. 2. Thermoaggregation of GAPDH in the presence of different polysulfoanions (*1-6*) and polycarboxylic anions (*7*, *8*): PVS (*1*), DS of 1000 kDa (*2*), PSS with DP of 430 (*3*), PAMS (*4*), PAS (*5*), heparin (*6*), PAA (*7*), and PMAA (*8*). The reaction mixture contained 0.7 μM GAPDH in 10 mM phosphate buffer, pH 7.5, at 60°C.

RESULTS AND DISCUSSION

We investigated earlier the effect of some polyanions and polycations on denaturation and aggregation of GAPDH. GAPDH is a convenient subject of research since the mechanisms of its spontaneous and chaperone-assisted folding are well known, as well as the influence of heat shock proteins on these processes [11]. The choice of GAPDH is also explained by the fact that this enzyme is capable of forming not only random protein aggregates, but also amyloid structures that are responsible for the development of neurodegenerative diseases [12-14]. Earlier, we observed an extremely high anti-aggregative activity of sodium polystyrene sulfonate. The reason for this phenomenon remained unclear, but it could be related to the presence of the sulfo groups or to the high hydrophobicity of this polyanion. Thus, the goal of the present work was to reveal the crucial parameters of polyelectrolytes determining the character of their interaction with proteins and characteristic features of the formed protein–polyelectrolyte complexes.

Thermoaggregation of GAPDH. Influence of hydrophobicity of the charged chains. Interaction between GAPDH and polysulfoanions was investigated at pH 7.5. Under these conditions, molecules of the enzyme are charged positively (p*I* for GAPDH is 8.5 [15]), while molecules of polysulfoanions are charged negatively.

The dependence of aggregation level on polyanion concentration is shown in Fig. 2. To analyze the effect of hydrophobicity, the Hansch coefficient of hydrophobicity (log P) [16] was computed using the ACD Labs program. According to the results of these computations, the investigated polysulfoanions can be ranged in the order of increasing hydrophobicity of their structural units as following: heparin < DS < PAMS < PVS < PSS < PAS. The hydrophobicity of polycarboxylic anions is virtually the same as the hydrophobicity of PVS. However, polycarboxylic anions (curves *7* and *8*) suppress aggregation of GAPDH at much higher concentrations than polysulfoanions (curves *1-6*) including PVS.

The left position of curves *1* and *2* in the figure indicates that relatively hydrophilic PVS and DS exhibit the highest ability to suppress the aggregation, while much more hydrophobic PSS (curve *3*) and PAS (curve *5*) suppress the thermoaggregation of GAPDH with less efficiency. These results suggest that a crucial role in the stabilization of soluble complexes belongs to SO_3^- and SO_4^- groups, but not to hydrophobic interactions.

The clearly pronounced anti-aggregative activity of polysulfonates is presumably explained by their strong electrostatic binding to GAPDH. It was demonstrated that polyanions containing SO_3^- or SO_4^- groups form extremely stable bonds with the amino groups of positively charged polymers [17].

Thermoaggregation of GAPDH. Influence of length of polyelectrolyte chain. Figure 3 shows the difference in the ability of polysulfoanions of various chain lengths to suppress thermoaggregation, which was estimated by the polymer concentration in the point where $A_{320}/A_{320,GAPDH} = 0$. According to these values, it can be concluded that the ability to suppress thermoaggregation of high-molecular-weight 1000-kDa dextran sulfate (curve *1*) is more than 3-fold higher than that of dextran sulfate of 100 kDa (curve *2*) and approximately 10-fold higher than that of oligomeric sample of 5 kDa (curve *3*).

A similar dependence was revealed for another polysulfoanion, PSS: the value $A_{320}/A_{320,GAPDH}$ (0.614, 0.4, and

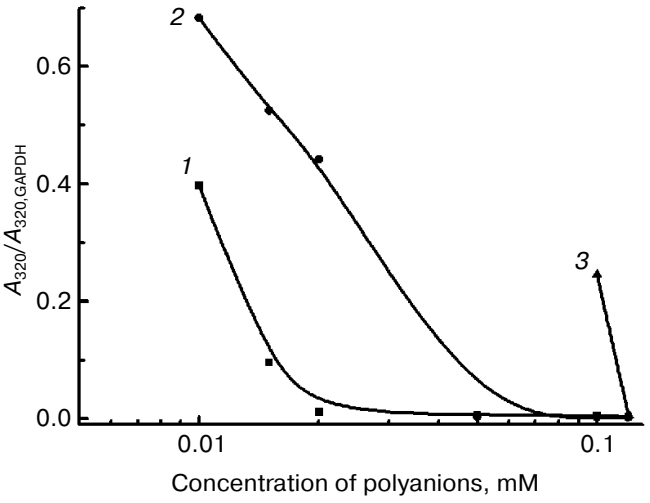

Fig. 3. Thermoaggregation of GAPDH in the presence of dextran sulfate samples of different molecular weights (in kDa) 5 min after starting the reaction: 10^3 (*1*), 10^2 (*2*), 5 (*3*). The conditions are the same as in Fig. 2.

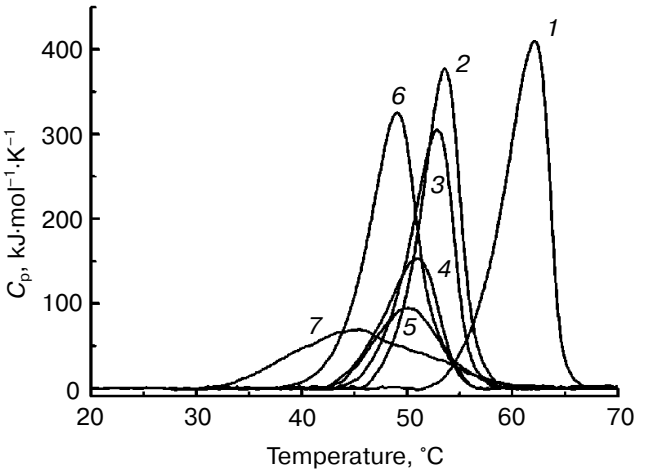

Fig. 4. Heat absorption curves of free GAPDH (*1*) and its complexes with different polysulfoanions: PAMS (*2*), heparin (*3*), PVS (*4*), PSS (*5*), DS (*6*), and PAS (*7*) in 10 mM phosphate buffer, pH 7.5. Concentration of the charged groups of polysulfoanions was 1 mM. GAPDH concentration was 7 μM.

0.245) decreased with the increase in the DP value of the sample (77, 430, and 970, respectively). These results agree with the previously published data on the efficiency of high-molecular-weight polycarboxylic anions and polycations in suppressing of protein aggregation [8, 9]. It should be noted that even the shortest dextran sulfate with the molecular weight of 5 kDa suppresses thermoaggregation of GAPDH much more efficiently than high-molecular-weight polycarboxylic anions (compare curve *3* in Fig. 3 and curves *7* and *8* in Fig. 2).

Thus, there are two factors negatively affecting the anti-aggregative activity of polysulfoanions: shortening of the polymer chain and increase in the hydrophobicity.

Thermodenaturation of GAPDH. Influence of the hydrophobicity of a polyelectrolyte. As demonstrated above, polysulfoanions prevent thermoaggregation, which is likely explained by the prevention of the interaction of the denatured protein molecules with each other. However, at the same time polysulfoanions destabilize the protein structure. The data obtained by DSC (Fig. 4) demonstrate significant changes in thermodynamic parameters of GAPDH melting and, consequently, in the structure of GAPDH molecule in the presence of polysulfoanions.

The heat absorption curves can be divided into three groups. The first group comprises the curves obtained in the presence of hydrophilic polymers PAMS, DS, and heparin (curves *2*, *3*, *6*). The peaks are relatively high with low values of the peak width at half height. Consequently,

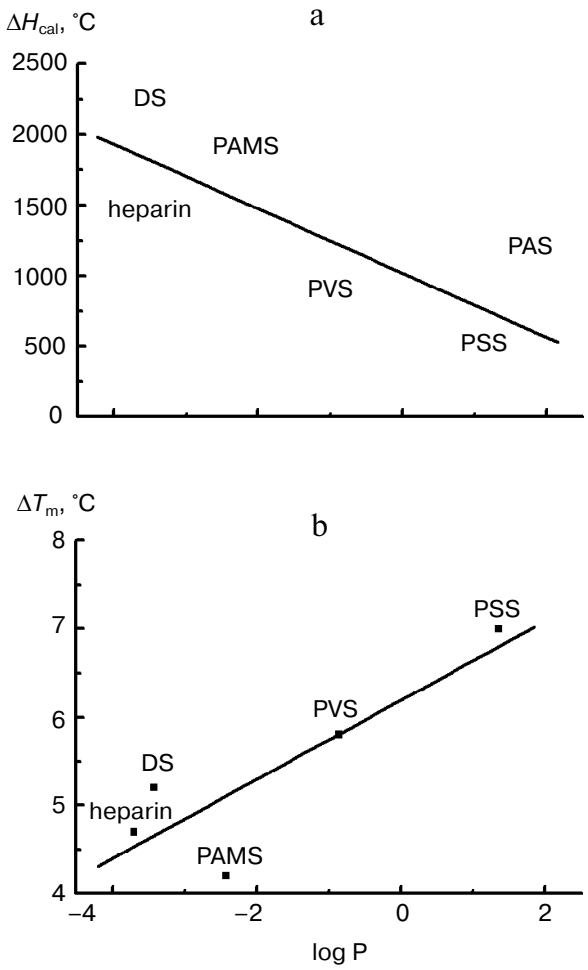

Fig. 5. Dependence of parameters of GAPDH melting on hydrophobicity coefficient of bound polysulfoanion: a) calorimetric enthalpy; b) peak width at half height.

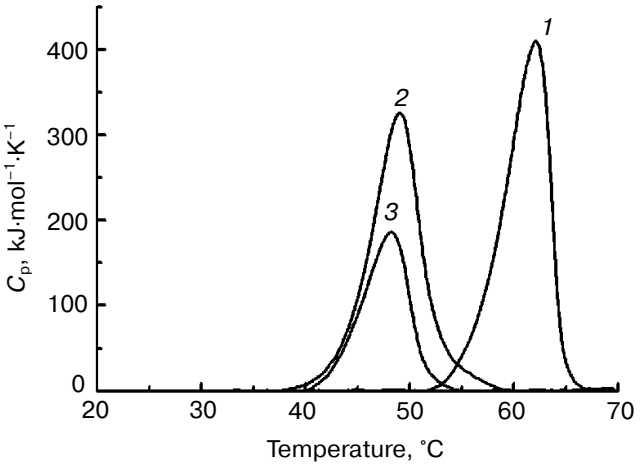

Fig. 6. Heat absorption curves of free GAPDH (*1*) and GAPDH in the presence of DS of 10^2 (*2*) and 5 kDa (*3*). Other conditions are the same as in the legend to Fig. 5.

the bound GAPDH melts cooperatively, retaining the most part of structural elements, although at much lower temperature than the free enzyme. The heat capacity curves of the third group obtained in the presence of PSS and PAS (curves *5* and *7*) significantly differ from those described above. The peaks are low and wide, this indicating the reduction of the cooperativity of melting and the decrease in the ratio of the ordered structures in the enzyme molecule. The curve obtained in the presence of PVS takes an intermediate position between the first and third groups (curve *4*).

To analyze data obtained by DSC assay, the following parameters were used: maximum of the heat absorption curve (T_m), the peak width at half height (ΔT_m), and calorimetric enthalpy (ΔH_{cal}). These parameters were analyzed in terms of Hansch hydrophobicity coefficient (log P). The dependences of ΔH_{cal} and ΔT_m on the hydrophobicity coefficient are adequately fit to straight lines (Fig. 5, a and b, respectively). The presented data indicate that the destabilizing action of polysulfoanions significantly grows with the increase in the hydrophobicity of their structural units.

Measuring the catalytic activity also showed that the denaturing action of a polysulfoanion grows with the increase in its hydrophobicity.

Thermodenaturation of GAPDH. Influence of the length of the polyelectrolyte chain. In the present work, we first demonstrated that the most pronounced effect on the protein is observed in the presence of short polyelectrolyte chains composed of several tens of structural units. For example, reduction of the molecular weight of dextran sulfate from 1000 (curve *2* in Fig. 6) to 5 kDa (curve *3*) resulted in destabilization of the GAPDH molecule: the values of T_m and ΔT_m did not change, but the peak height and, as a consequence, the ΔH_{cal} value significantly decreased.

The described behavior is characteristic not only for polysulfoanions. We demonstrated a similar effect investigating the interaction of GAPDH with poly-N-ethyl-4-vinyl pyridinium cation. Reducing DP from 1600 to 30 did not lead to any changes, but further reduction of DP to 10 units significantly decreased the ΔH_{cal} value, not affecting the maximum of the heat absorption curve.

Destabilizing action of short charged chains was much more pronounced in the presence of relatively hydrophobic PSS (Fig. 7). Binding GAPDH to PSS with DP of 430 strongly destabilized the enzyme (curve *2*). Reduction of DP decreased the ΔH_{cal} and T_m values. When DP of PSS reached eight units, the absorption peak of the enzyme disappeared, this indicating significant disturbances in the protein structure. Such a strong effect of PSS on the structure of GAPDH agrees well with the report on the strong denaturing action of PSS on lysozyme [18] and suggests an important role of the hydrophobic interactions in the stabilization of protein structure.

There is no doubt that the revealed denaturing action of short charged chains should be taken into consideration while creating protein–polyelectrolyte systems. To maintain the active state of the enzyme, the use of oligomeric and non-fractioned samples should be avoided. The effect of the non-fractioned PSS sample can be illustrated by its effect on the heat absorption curve of GAPDH: the peak significantly shifted to the left (dotted curve in Fig. 7) in spite of the fact that the maximal value of average DP of the used sample corresponds to 970. Thus, the presence of even a little admixture of short chains can strongly affect the stability of a protein bound to a polyelectrolyte.

The data obtained in the experiments on investigation of the enzymatic activity of GAPDH agree well with

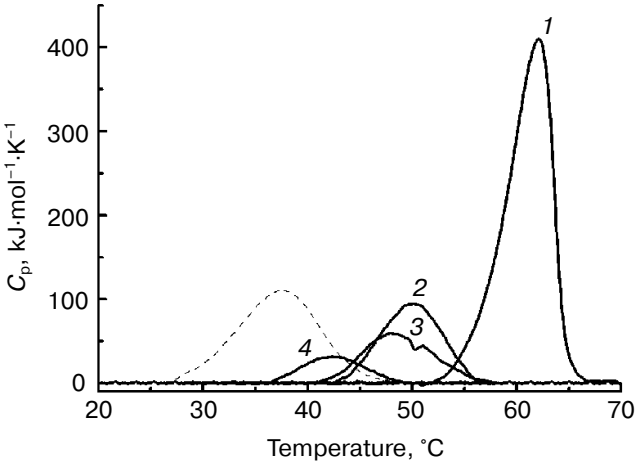

Fig. 7. Heat absorption curves of free GAPDH (*1*) and GAPDH in the presence of PSS with various DP values: 430 (*2*), 77 (*3*), and 30 (*4*). The dotted curve corresponds to GAPDH in the presence of non-fractioned PSS with average DP of 970. Other conditions are the same as in Fig. 4.

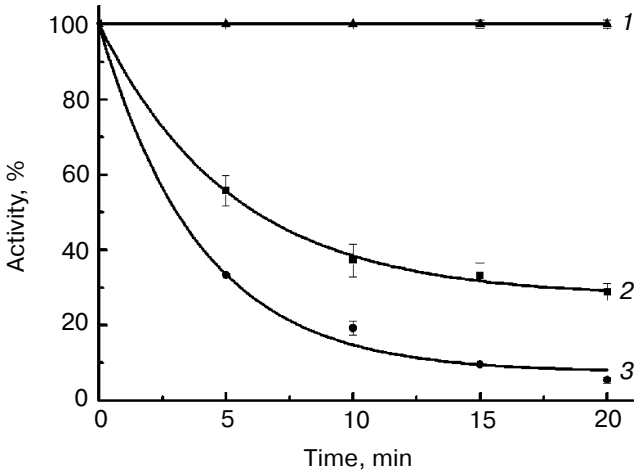

Fig. 8. Specific activity of free GAPDH (*1*) and GAPDH in the presence of DS of 10^3 (*2*) and 5 kDa (*3*). Incubation medium contained 0.7 μM GAPDH in 10 mM phosphate buffer, pH 7.5, 45°C. Concentration of negatively charged groups of polysulfoanions was 100 μM.

the results of DSC assay. The activity of the enzyme decreases with shortening of the chain length of DS (Fig. 8), the oligomeric sample exhibiting the strongest denaturing action (curve *3*).

At first sight, the described ability of the short chains to affect significantly the protein structure is in contradiction with the low stability of the oligomer-containing complexes [19]. This contradiction can be explained as following: shorter chains have less steric hindrance and are capable of penetrating deep into the protein globule. Consequently, the short chains are able to form more contacts with the protein, this allowing them to affect more strongly the protein structure. It should be noted that similar processes are known to proceed during the interaction of rigid cationic dendrimer with polyanions: oligomeric polyanions are able to reach the internal regions of the dendrimer, while high-molecular-weight polyanions are not [20].

The data presented here and previously [8, 9] indicate that to suppress aggregation efficiently, polyelectrolyte must possess a rather high affinity to the protein, this providing the formation of a stable soluble complex. Polysulfoanions and probably polycations with primary amino groups [21] exhibit a high affinity to proteins due to electrostatic interactions only. There is another possibility to increase the stability of protein–polyelectrolyte complexes: to introduce additional non-electrostatic interactions. However, this can result in negative consequences. For example, increase in the hydrophobicity of polycations decreases not only the level of protein aggregation, but also the enzymatic activity. Thus, sulfated polysaccharides appeared to be the best anti-aggregants, being at the same time biocompatible and biodegrading compounds.

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