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Antibodies to Inactive Conformations of Glyceraldehyde-3-phosphate Dehydrogenase Inactivate the Apo- and Holoforms of the Enzyme

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Abstract—Polyclonal antibodies produced after the immunization of a rabbit with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from *Bacillus stearothermophilus* were used to isolate two types of antibodies interacting with different non-native forms of the antigen. Type I antibodies were purified using Sepharose-bound apo-GAPDH that was treated with glutaraldehyde to stabilize the enzyme in the tetrameric form. Type II antibodies were isolated using immobilized denatured monomers of the enzyme. It was shown that the type I antibodies bound to the native holo- and apoforms of the enzyme with the ratio of one antibody molecule per GAPDH tetramer. While interacting with the native holoenzyme, the type I antibodies induce a time-dependent decrease in its activity by 80-90%. In the case of the apoenzyme, the decrease in the activity constitutes only 25%, this indicating that only one subunit of the tetramer is inactivated. Differential scanning calorimetry experiments showed that the formation of the complex between both forms of the enzyme and the type I antibodies resulted in a shift of the maximum of the thermal capacity curves (T_m value) to lower temperatures. The extremely stable holoenzyme was affected to the greatest extent, the shift of the T_m value constituting approximately 20°C. We assume that the formation of the complex between the holo- or apo-GAPDH and the type I antibody results in time-dependent conformational changes in the enzyme molecule. Thus, the antibodies induce the structural rearrangements yielding the conformation that is identical to the structure of the antigen used for the selection of the antibodies (i.e., inactive). The interaction of the antibodies with the apo-GAPDH results in the inactivation of the subunit directly bound to the antibody. Virtually complete inactivation of the holoenzyme by the antibodies is likely due to the transmission of the conformational changes through the intersubunit contacts. The type II antibodies, which were selected using the immunosorbent with unfolded enzyme form, do not affect the activity of native holo- and apo-GAPDH, but prevent the reactivation of the denatured GAPDH, binding the denatured forms of the enzyme.

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Among the numerous experimental approaches used in protein chemistry, methods based on the ability of antibodies to recognize different conformations of antigen proteins are of special interest [1, 2]. Antibodies are useful in the investigation of the mechanisms of protein folding. Binding to specific epitopes formed during different steps of folding of a protein, they can stabilize the protein in the corresponding conformations, thus allowing identification of these conformational states. The use of monoclonal antibodies revealed an intermediate form of β-lactoglobulin that was virtually identical to the completely folded protein in terms of its physical and chemical properties, differing only in the capability of interacting with the antibodies [3]. Using the monoclonal antibodies against the β-subunit of bacterial tryptophan synthase that were sensitive to a certain conformation of the enzyme, different intermediates were detected during the renaturation of the enzyme [4].

The ability of antibodies to affect the conformation of proteins involved in the formation of the complex has been little studied. This property of antibodies suggests

Abbreviations: GAPDH) glyceraldehyde-3-phosphate dehydrogenase; PBS) 20 mM potassium phosphate, pH 7.5, containing 0.15 M NaCl.

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that if an antibody is produced against a non-native conformation of an antigen, the binding of the antibody to the native protein induces the transformation of the protein into the corresponding non-native structure. One of the goals of the present study was to test such a possibility using antibodies to non-native forms of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The second goal was to compare the effect of two types of antibodies produced against different non-native conformations of GAPDH on the activity of the enzyme and on the processes of its denaturation and renaturation.

We chose GAPDH for our investigation for the following reasons. First, to date two conformational states of the tetrameric molecule of GAPDH have been characterized: apo-conformation and holo-conformation. Due to a high affinity to NAD^+, the enzyme is usually isolated as the holoenzyme (the ratio A_{280}/A_{260} is 1.1-1.15). The removal of NAD^+ during the preparation of the apoenzyme results in a significant destabilization of the protein structure. The formation of the GAPDH · NAD^+ complex results in conformational rearrangements leading to an increase in the maximum of the partial capacity curves (T_m) by 15°C during thermal unfolding of the protein [5]. The role of NAD^+ in interdomain and intersubunit interactions that are necessary for the formation of the active conformation was studied in detail [6].

Second, interest in the non-glycolytic functions of GAPDH has been significantly increased in recent years [7-12]. There are some reasons to assume that some of these functions can be performed by non-native (conformationally changed) forms of the protein [9, 12]. Consequently, the elaboration of methods allowing the detection of such forms of GAPDH in the cell seems to be of importance. We suppose that one of the most promising approaches to solve this problem is the use of specific antibodies that are capable of recognizing different protein conformations. The goal of the present work was to obtain and investigate the properties of two types of antibodies: type I binding to the inactive tetrameric GAPDH treated with glutaraldehyde and type II interacting with the denatured unfolded form of GAPDH.

MATERIALS AND METHODS

In this work we used the following chemicals: NAD^+, glycine, Tris, D-glyceraldehyde-3-phosphate, EDTA, urea, Affi-Blue Sepharose, Sepharose 4B, Sephadex G-50 (fine), Sephadex G-200, ion-exchange resin AcA-34, Q-Sepharose, and phenyl-Sepharose from Sigma (USA); dithiothreitol from Serva (Germany); glutaraldehyde from Merck (Germany). Protein immobilization was performed with the use of CNBr freshly synthesized from KCN and Br_2. Other chemicals were of domestic production (Reakhim). All solutions were prepared using bidistilled water. Urea was recrystallized from ethanol. Standard phosphate buffer (PBS) contained 20 mM potassium phosphate and 0.15 M NaCl, pH 7.5.

Purification of the enzyme. Glyceraldehyde-3-phosphate dehydrogenase from *Bacillus stearothermophilus* was isolated from the cells of *E. coli* strain GM-109 transformed with pBluescript II plasmid containing the *gap* gene under the control of the lac promoter according to the method described by Roitel et al. [13]. The plasmid was kindly provided by Prof. Guy Branlant (Henri Poincare University, France). The enzyme was purified using gel filtration on AcA-34 and chromatography on Q-Sepharose and phenyl-Sepharose. The specific activity of the purified enzyme constituted 150 U/mg.

Determination of protein concentration. Concentration of soluble proteins was determined by absorption at 280 nm considering $A_{280}^{0.1\%}$ 1.0 and 1.45 for the holoform of GAPDH and the antibodies, respectively, or by the Bradford method [14]. Concentration of the immobilized proteins was determined using a modification of the Bradford method [15].

Determination of enzymatic activity. The activity of GAPDH was determined spectrophotometrically at 340 nm by the rate of NADH accumulation. The reaction mixture contained 50 mM glycine buffer, pH 10.0, 50 mM potassium phosphate, and 5 mM EDTA. The samples also contained 1 mM glyceraldehyde-3-phosphate and 1 mM NAD^+ in the case of the soluble enzyme and 2 mM concentration of both substrates in the case of the immobilized enzyme. The reaction was started by the addition of the enzyme.

Preparation of the apoform of GAPDH. The apoenzyme was prepared using Affi-Blue Sepharose [16]. A solution of the protein in 50 mM Tris-HCl, pH 8.0, containing 2 mM EDTA was applied on an Affi-Blue Sepharose column. The protein was eluted with the same buffer containing 1 M KCl. Then the protein solution was desalted on a Sephadex G-50 column equilibrated with PBS. The resulting preparation exhibited A_{280}/A_{260} ratio of 1.85.

Protein immobilization. Immobilization of GAPDH on CNBr-activated Sepharose was performed as described earlier [17], using 5 mg of CNBr per ml of the settled gel for the activation of Sepharose. The activity of the immobilized enzyme constituted 85-90% of the activity of the soluble enzyme.

Preparation of immunosorbents. In the present work, we used two types of immunosorbents. The first was used for isolation of the polyclonal antibodies specific to the apoenzyme stabilized in the tetrameric form by cross-linking with glutaraldehyde. The procedure was the following: the apoform of GAPDH immobilized on Sepharose was treated with glutaraldehyde (4 μl of 25% solution per ml of the settled Sepharose gel with the immobilized protein). The mixture was incubated for 18 h at 25°C, and then glycine was added (final concentration, 50 mM) to block the non-reacted groups. After 3 h of incubation, the sorbent was washed with PBS. The cross-

linking of the enzyme with glutaraldehyde resulted in the loss of catalytic activity.

The immunosorbent of the second type was used for isolation of the antibodies specific to the unfolded monomeric species of GAPDH. To prepare the sorbent, the native immobilized GAPDH was washed with glycine buffer (50 mM glycine, 0.15 M NaCl, pH 2.3) at 25°C until no absorption was detected in the eluate at 280 nm. The washing with the glycine buffer, pH 2.3, decreased the total content of the immobilized protein 4-fold, indicating that the tetrameric enzyme dissociated into monomers. The treatment also resulted in the loss of catalytic activity. The obtained sorbent was washed with PBS, but this did not lead to restoration of the activity.

We assume that after the denaturation of the enzyme at pH 2.3 and the subsequent washing with PBS only inactive denatured monomers were retained covalently bound to the matrix. This was supported by CD experiments. Since it was impossible to take the CD spectra of the immobilized protein, we compared the spectra of the native soluble protein with the spectra of the soluble protein treated under the same conditions that were used for the treatment of the immobilized protein. It was shown that the CD spectrum of the soluble GAPDH after the incubation in 50 mM glycine containing 0.15 M NaCl, pH 2.3, exhibited characteristic features of a denatured protein and differed from the CD spectrum of the native soluble GAPDH in PBS (data not shown). Incubation of the denatured enzyme in PBS resulted in neither restoration of its activity, nor change in the CD spectrum (data not shown).

Purification of polyclonal antibodies specific to different GAPDH forms. A rabbit was immunized with native GAPDH using Freund's adjuvant according to the standard procedure [18]. Then the IgG fraction was precipitated with ammonium sulfate to 33% saturation. Two types of antibodies to GAPDH were separated from the same antiserum using the standard procedure of affinity chromatography [19]. Low-affinity antibodies were eluted with buffer containing 0.05 M acetate, pH 4.3, 0.15 M NaCl, and discarded. High-affinity antibodies were eluted with 50 mM glycine-HCl buffer, pH 2.3, containing 0.15 M NaCl.

In the beginning, the antibodies to the modified tetrameric GAPDH (type I antibodies) were purified on a column with the immobilized apo-GAPDH tetramers cross-linked by glutaraldehyde (immunosorbent of the first type). The protein fraction that passed through the first column was applied on the type II affinity column containing immobilized GAPDH denatured by pH 2.3, and the type II antibodies were collected.

Immunoprecipitation of antigen–antibody complexes with protein G. The type I and II antibodies were incubated with soluble native GAPDH in PBS for 30 min at 25°C. The Sepharose gel with immobilized protein G was also washed with PBS. The mixture of GAPDH and the antibodies (150-200 μl) was added to the settled gel (20 μl) of the Sepharose with protein G and was incubated for 40 min with gentle stirring at 25°C. After the incubation, the gel was washed with PBS to remove unbound proteins, and the proteins bound to the protein G were analyzed by SDS-PAGE. The PSBAS 2.08 program was used to process the data.

Analytical ultracentrifugation was performed using a Spinco model E analytical ultracentrifuge (Beckman, USA) equipped with a photoelectric scanner, a multiplexer, and a monochromator. A An-F-Ti titanium rotor and double-sector cells were used. Scanning was carried out at 280 nm and 261,600*g* in PBS. The sedimentation coefficients were normalized to standard conditions.

Differential scanning calorimetry measurements were made using a DASM-4 adiabatic microcalorimeter (Biopribor, Pushchino, Russia) as described earlier at a constant scan-rate of 1°/min [5, 6]. MicroCal Origin software was used to process the data.

RESULTS

Isolation of antibodies interacting with different non-native forms of GAPDH. To isolate antibodies recognizing different conformational states of GAPDH, we used affinity immunosorbents with different immobilized forms of the enzyme. We assumed that such sorbents could be used for the selective extraction of the corresponding antibodies directly from the mixture of the polyclonal antibodies obtained after immunization of a rabbit with a preparation of native GAPDH. The adequacy of this approach is based on a conventional concept, according to which proteins in a solution exist in the equilibrium of numerous conformational states varying in their functional characteristics. It can be assumed that among different populations of polyclonal antibodies produced against the immunization with a certain protein, there may be some clones capable of interacting not only with native GAPDH, but also with conformationally altered forms of the protein. The results obtained in the present work support this assumption.

Using the corresponding affinity sorbents, we prepared two types of antibodies. The type I antibodies recognizing the non-native form of GAPDH were obtained using the inactive GAPDH forms immobilized on CNBr-activated Sepharose and stabilized in the tetrameric state by treatment with glutaraldehyde. The type II antibodies capable of binding the denatured unfolded enzyme forms were obtained using the immobilized denatured GAPDH forms. The type I antibodies exhibited the same affinity to the apo- and holoform of GAPDH. The extent of cross-reactivity of the antibodies of types I and II did not exceed 10%.

Influence of the antibodies on the activity of GAPDH. Figure 1 presents the effect of the two types of antibodies

on the activity of GAPDH. In each case, the reaction mixture contained 1 mol antibody per mol tetrameric enzyme. It was shown that the activity of apo- and holo-GAPDH in the control samples did not change during 300 min incubation in PBS at 25°C. The type I antibodies specific to the tetrameric apoenzyme cross-linked with glutaraldehyde inactivated both the apo- and holoform of the enzyme. In the latter case (curve *1*), the activity decreased by 50% after 20 min and by 80% after 180 min of incubation. Figure 1 also shows that the effect of the type I antibodies on the apoenzyme was less pronounced (curve *2*), but nevertheless 20 min of incubation resulted in 25% inactivation. Considering that the ratio antibody/GAPDH in the complex constituted 1.0, and the enzyme is a tetramer, it can be assumed that the binding of the antibody to the apoform of GAPDH results in the inactivation of only one subunit of the tetramer, while the binding of the antibody to the holoenzyme inactivates several subunits. The data presented in Fig. 1 also show that the type II antibodies specific to the unfolded conformation of GAPDH slightly affected the activity of apo- and holoforms of the native enzyme (curves *3* and *4*).

To clarify the mechanism of the effect of the antibodies on the enzyme activity, it was necessary to compare the rate of the formation of the complex [holo-GAPDH · type I antibody] with the rate of the enzyme inactivation, and to determine the stoichiometry of the formed complex. For this purpose, the immobilized tetrameric GAPDH was incubated in PBS in the presence of 3 mM NAD^+ and type I antibodies (the molar ratio type I antibody/GAPDH constituted 1 : 1, 2 : 1, and 4 : 1). After different time intervals, the gel of Sepharose bearing the immobilized enzyme was removed by centrifugation and the content of the unbound antibodies was determined in the supernatant. The results presented in Fig. 2 demonstrate that in the sample containing the antibodies and the tetrameric enzyme in the ratio 1 : 1, the binding of one antibody molecule was completed in 5 min. Similar results were obtained with the immobilized apoform of GAPDH. Thus, we can conclude that the formation of the [GAPDH · antibody] complex occurs faster than the inactivation of the enzyme. Since the increase in the concentration of the antibodies did not affect the stoichiometry of the binding, we can conclude that the maximal antibody/GAPDH ratio in the complex does not exceed 1.0.

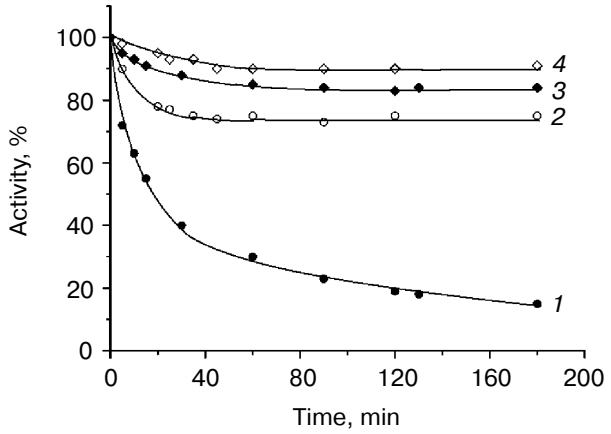

Fig. 1. Effect of antibodies on the activity of GAPDH. GAPDH (final concentration, $1.5 \cdot 10^{-6}$ M calculated per tetramer) was incubated in PBS in the presence of the antibodies (final concentration, $1.5 \cdot 10^{-6}$ M) at 25°C. *1*) Holo-GAPDH (with 3 mM NAD^+) in the presence of the type I antibodies; *2*) apo-GAPDH in the presence of the type I antibodies; *3*) holo-GAPDH (with 3 mM NAD^+) in the presence of the type II antibodies; *4*) apo-GAPDH in the presence of the type II antibodies.

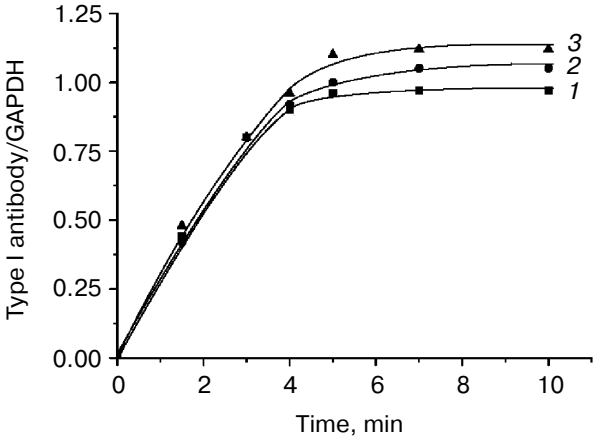

Fig. 2. Time-dependence of the formation of the [GAPDH · type I antibody] complex. The preparation of the tetramers of holo-GAPDH immobilized on Sepharose through one subunit was incubated in PBS in the presence of 3 mM NAD^+ and the type I antibodies with gentle stirring. The molar ratio type I antibody/tetrameric GAPDH constituted 1 : 1 (*1*), 2 : 1 (*2*), and 4 : 1 (*3*). After different time intervals, the Sepharose containing the immobilized enzyme was settled by centrifugation, and concentration of the unbound antibodies in the supernatant was determined.

Taken together, the data led us to conclude that the inactivation of the native enzyme presented in Fig. 1 occurs within the binary [GAPDH · antibody] complex and must be a consequence of time-dependent conformational changes induced by the antibody. To provide experimental support for the existence of such changes, we isolated the binary complexes of apo- and holo-GAPDH with the antibodies and analyzed them using differential scanning calorimetry.

Separation of [GAPDH · type I antibody] complexes and their analysis by differential scanning calorimetry. To obtain the complexes, GAPDH (both holo- and apoenzyme) were incubated in the presence of the type I antibodies in the ratio 1 : 1 (the concentration of each protein being $1.5 \cdot 10^{-6}$ M) in PBS for 300 min with the subsequent

gel filtration. The data shown in Fig. 1 suggest that the holoenzyme isolated after 300 min of incubation with the type I antibodies was completely inactivated, whereas the apoenzyme retained about 75% activity. The molecular weights and stoichiometry of the complexes were evaluated by ultracentrifugation. The values of the Svedberg coefficient (s_{w20}) constituted 12.8 ± 0.39 S, 6.4 ± 0.35 S, and 6.5 ± 0.42 S for the complex (both with apo- and holoenzyme), the free antibodies, and free GAPDH, respectively. These data supported our conclusion according to which the formed complex consisted of the tetrameric GAPDH molecule bound to one antibody molecule.

Figure 3 presents the excess heat capacity profiles of these complexes and their components. Curves *1* and *2* represent the heat capacity profiles of the native apo- and holoenzyme, respectively. The data show that the binding of NAD^+ significantly increases the protein thermostability. Incubation of the holoenzyme with the type I antibodies (curve *6*) sharply decreases the enzyme stability, approaching that of the free apoenzyme (curve *1*) or the apo-GAPDH in the complex with the antibodies (curve *5*). According to these data, the inactivation of the holoenzyme induced by the antibodies is accompanied by a decrease in the T_{max} value by 20°C. In the case of the apoenzyme, the effect of the antibodies is less pronounced (the decrease in the T_{max} value does not exceed 3-4°C).

Influence of antibodies on the refolding of GAPDH. To confirm the conclusion that the type I antibodies selected on the immunosorbent with the folded but nonnative conformation of the tetrameric enzyme recognize mainly conformationally-dependent epitopes, but do not bind the unfolded structures of the denatured protein, we

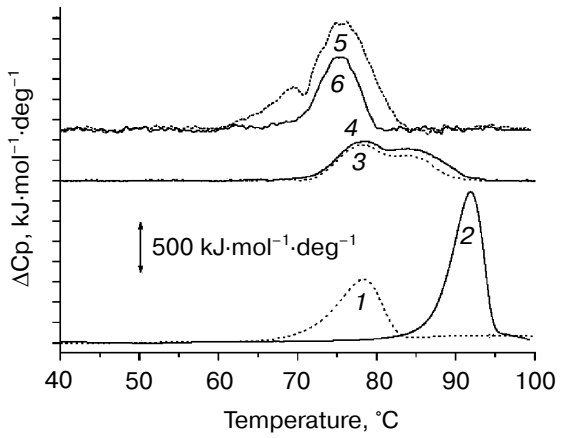

Fig. 3. Partial heat capacity curves for apo-GAPDH (*1*), holo-GAPDH (in the presence of 3 mM NAD^+) (*2*), type I antibodies (*3*), type I antibodies in the presence of 3 mM NAD^+ (*4*), [apo-GAPDH · type I antibody] complex (*5*), and [holo-GAPDH · type I antibody] complex in the presence of 3 mM NAD^+ (*6*). All samples were prepared in PBS.

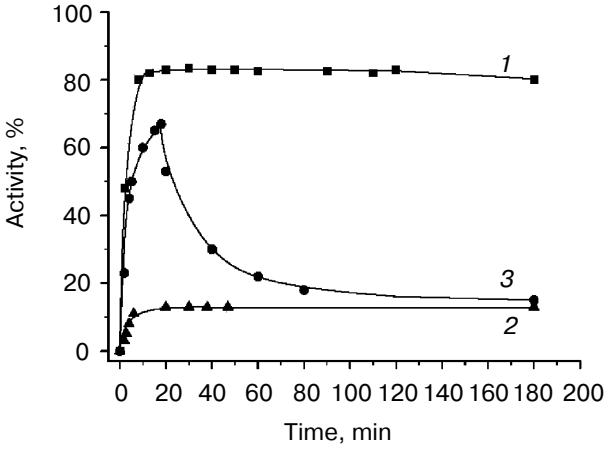

Fig. 4. Effect of antibodies on the spontaneous reactivation of GAPDH at 25°C. The enzyme was denatured by incubation in PBS in the presence of 8 M urea for 24 h and 25°C. The reactivation was started by a 70-fold dilution of the denatured enzyme with PBS containing 3 mM NAD^+. The resulting mixture contained $3 \cdot 10^{-7}$ M GAPDH (*1*), $3 \cdot 10^{-7}$ M GAPDH and $3 \cdot 10^{-7}$ M type II antibodies (*2*), or $3 \cdot 10^{-7}$ M GAPDH and $3 \cdot 10^{-7}$ M type I antibodies (*3*).

investigated the effect of the antibodies on the kinetics of spontaneous reactivation of the enzyme, which must reflect its refolding. In parallel, we investigated the effect of the type II antibodies selected on the immunosorbent with the denatured forms of the enzyme. It could be expected that the type II antibodies would stabilize the unfolded state of the protein, binding the unfolded molecules to yield a firm complex, thus decelerating the process of the renaturation. As seen in Fig. 4, this was really observed. The extent of GAPDH spontaneous reactivation in the control experiment reached 80% in the first 5 min after initiating the process, this level remaining constant during all the time of the experiment (Fig. 4, curve *1*). The addition of the type II antibodies into the incubation medium significantly decreased the efficiency of the reactivation, which did not exceed 15% (curve *2*).

Another situation was observed in the case of GAPDH refolding in the presence of type I antibodies (Fig. 4, curve *3*). The extent of reactivation reached approximately 68% in the first 13 min, and then a gradual decrease in the activity was observed. Apparently, the type I antibodies, being incapable of binding unfolded protein molecules, do not affect the initial stages of refolding. But as the number of folded molecules increases, the antibodies start to form complexes, this resulting in the enzyme inactivation.

DISCUSSION

Thus, using affinity chromatography, two types of antibodies (type I and type II interacting with the

tetramers and denatured polypeptide chains, respectively) were isolated from antiserum containing different antibodies against GAPDH. Since we used polyclonal antibodies, it remains unclear whether antibodies of type I or type II are individual protein molecules interacting with a certain epitope. We cannot exclude that each of these types can contain proteins interacting with different antigen determinant of GAPDH. More strict analysis could be performed using monoclonal antibodies, but as demonstrated previously, in such a case the selection of the necessary clones would be more complex [20]. The use of immunosorbents containing different conformational states of the same antigen allows selection of the required types of antibodies from the serum, decreasing the content of various clones in the resulting preparation of the antibodies.

The most important result of the present investigation is the finding that the type I antibodies purified on the immobilized inactive tetrameric apo-GAPDH cross-linked with glutaraldehyde are capable of interacting with the native enzyme, leading to its time-dependent inactivation. To explain this effect, two possibilities were considered. First, the binding of the type I antibodies results in the immediate inactivation of the enzyme. In this case, the dependence of the process on time reflects the shift in the equilibrium between different conformational states of GAPDH in a solution exhibiting different affinity to the antibodies.

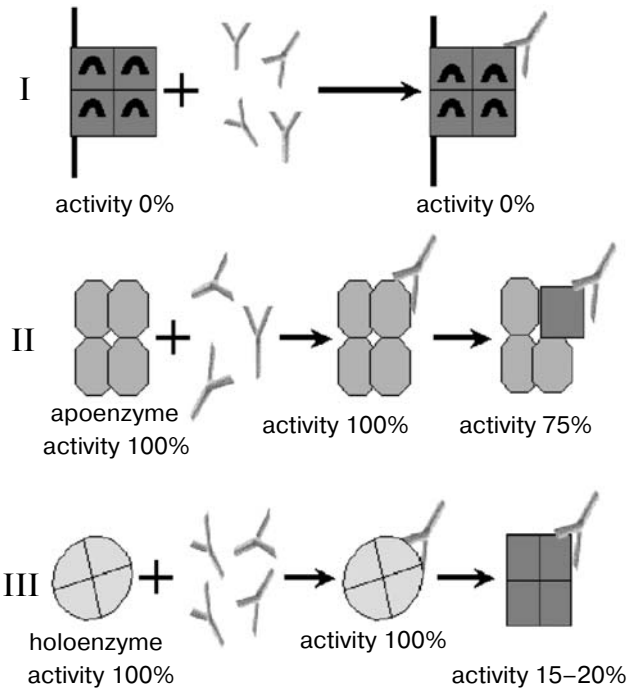

Fig. 5. Scheme of the interaction of the type I antibodies with different GAPDH forms and their effect on the enzyme activity. I) Binding of the type I antibodies to the inactive GAPDH immobilized on CNBr-activated Sepharose and cross-linked with glutaraldehyde. II) Binding of the type I antibodies to the native apo-GAPDH resulting in the inactivation of only one subunit. III) Binding of the type I antibodies to the native holo-GAPDH resulting in the inactivation of several subunits. Symbols of different shape designate different conformational states of GAPDH.

The second possibility suggests that the process of inactivation takes place within the formed binary complex [GAPDH · antibody] and is a consequence of time-dependent conformational changes induced by the binding of the antibody. In this case, the time course of the complex formation should be considerably shorter than the time course of the enzyme inactivation. To discriminate between these possibilities, we determined the time required for complex formation between GAPDH and the type I antibodies. It was shown that the binding of one antibody molecule to apo- and holo-GAPDH tetramers is accomplished in 5 min, i.e., it proceeds much faster than the inactivation (Figs. 1 and 2). Since the experiments were performed with GAPDH immobilized on Sepharose, where diffusion limitations could slow down the reaction, it seems possible that in a solution, the [GAPDH · antibody] complex is formed faster.

These data suggest that the binding of the type I antibodies is able to induce time-dependent enzyme inactivation according to the scheme presented in Fig. 5. The type I antibodies isolated with the use of the immobilized inactive tetramers of GAPDH (Fig. 5, I) bind not only the antigen determinants of the inactive form, but also the antigen determinants that are present in holo- and apo-GAPDH. Presumably, the binding of the type I antibodies to the holo- and apoforms yields a conformation of GAPDH that is close to that of the inactive enzyme cross-linked with glutaraldehyde, i.e., the conformation used for the selection of these types of antibodies (Fig. 5, II and III).

If this assumption is true, the behavior of the type I antibodies can be considered in the frame of the concept according to which an antibody is capable of inducing some deformation, or strain, of the structure of a partner involved in the formation of the complex, adjusting its conformation according to that of the antigen-binding site. The emergence of such a strain has been recently shown for the interaction between Fab-fragments of the antibodies produced against the intermediate of the ferrochelatase reaction and the substrate (mesoporphyrin) [21]. The formation of the complex with the antibody resulted in changes in the substrate structure yielding the structure of the transition state against which the antibody was produced. We assume that the effects observed in our work can have a similar nature, with the difference that another protein (GAPDH) serves as the substrate, and the effect of the antibodies is directed to the change in its conformation.

The suggested interpretation completely explains the experimental data on the effect of the type I antibodies on the activity of the apoform of GAPDH. Binding of one

molecule of the antibody to one of the subunits of the tetrameric apo-GAPDH results in a gradual inactivation of this subunit without inactivation of the other subunits (the loss of the activity constituted 25%) (Fig. 5, II). Another situation is observed in the case of the binding of the same antibodies to the holo-GAPDH. In spite of the same stoichiometry of the formed complexes (one antibody molecule per tetramer GAPDH), the extent of inactivation of the holo-GAPDH is significantly higher and reaches 80% (Fig. 1, curve *1*). Consequently, the inactivation involves not only the subunit bound to the antibody, but also the subunits that do not contact directly with the antibody. Thus, in the case of the holoenzyme, the antibody alters the conformation of one subunit, and then the conformational changes are transmitted through the intersubunit contacts, leading to the inactivation of the neighboring monomers (Fig. 5, III). The different effects of the antibodies on the apo- and holo-GAPDH are likely due to the different strength of the intersubunit interactions in these forms. It is well known that the interdimer and intermonomer contacts in holo-GAPDH from different species are significantly stronger than those in the apoforms of GAPDH [22]. The described effect of the antibodies is analogous to the characteristic for GAPDH effect of "half-of-the-sites reactivity" that is also transmitted through the intersubunit contacts [23, 24]. The latter effect was observed during the association of the native subunits and the subunits modified at the active sites [25]. It should be noted that the investigation of the mechanisms of the formation of an "incorrect" conformation of a protein upon its interaction with an altered partner acquires a special significance considering that the formation of the infectious prions is based on similar processes [26].

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REFERENCES

1. Huse, K., Bohme, H. J., and Scholz, G. H. (2002) *J. Biochem. Biophys. Meth.*, **51**, 217-231.
2. Muronetz, V. I., and Korpela, T. (2003) *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.*, **790**, 53-66.
3. Hattori, M., Ametani, A., Katakura, Y., Shimizu, M., and Kaminogava, S. (1993) *J. Biol. Chem.*, **268**, 22414-22419.
4. Blond, S., and Goldberg, M. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 1147-1151.
5. Levashov, P., Orlov, V., Boschi-Muller, S., Talfournier, F., Asryants, R., Bulatnikov, I., Muronetz, V., Branlant, G., and Nagradova, N. (1999) *Biochim. Biophys. Acta*, **1433**, 294-306.
6. Roitel, O., Ivinova, O., Muronetz, V., Nagradova, N., and Branlant, G. (2002) *Biochemistry*, **41**, 7556-7564.
7. Nagy, E., and Rigby, W. F. (1995) *J. Biol. Chem.*, **270**, 2755-2763.
8. Sirover, M. A. (1999) *Biochim. Biophys. Acta*, **1432**, 159-184.
9. Saunders, P. A., Chen, R. W., and Chuang, D. M. (1999) *J. Neurochem.*, **72**, 925-932.
10. Modun, B., Morrissey, J., and Williams, P. (2000) *Trends Microbiol.*, **8**, 231-237.
11. Mazzola, J. L., and Sirover, M. A. (2002) *Neurotoxicology*, **23**, 603-609.
12. Arutyunova, E. I., Danshina, P. V., Domnina, L. V., Pleten', A. P., and Muronetz, V. I. (2003) *Biochem. Biophys. Res. Commun.*, **307**, 547-552.
13. Roitel, O., Sergienko, E., and Branlant, G. (1999) *Biochemistry*, **38**, 16080-16091.
14. Bradford, M. (1976) *Analyt. Biochem.*, **72**, 248-254.
15. Asryants, R., Duszenkova, I., and Nagradova, N. (1985) *Analyt. Biochem.*, **151**, 571-574.
16. Talfournier, F., Colloc'h, N., Mornon, J. P., and Branlant, G. (1998) *Eur. J. Biochem.*, **252**, 447-457.
17. Cherednikova, T., Muronetz, V., and Nagradova, N. (1980) *Biochim. Biophys. Acta*, **613**, 292-308.
18. Green, J., and Manson, M. (1998) *Methods in Molecular Biology*, Vol. 80, Humana Press, Totowa, New Jersey, p. 1.
19. Cherednikova, T. V., Muronetz, V. I., and Nagradova, N. K. (1981) *Mol. Immunol.*, **18**, 1055-1064.
20. Grigorieva, J. A., Dainiak, M. B., Katrukha, A. G., and Muronetz, V. I. (1999) *Arch. Biochem. Biophys.*, **369**, 252-260.
21. Yin, J., Andryski, S. E., Beuscher, A. E., Stevens, R. S., and Schultz, P. G. (2003) *Proc. Natl. Acad. Sci. USA*, **100**, 856-861.
22. Bell, J. E., and Dalziel, K. (1975) *Biochim. Biophys. Acta*, **410**, 243-251.
23. Levitzki, A. (1974) *J. Mol. Biol.*, **90**, 451-468.
24. Golovina, T. O., Muronetz, V. I., and Nagradova, N. K. (1978) *Biochim. Biophys. Acta*, **524**, 15-25.
25. Muronetz, V. I., Ashmarina, L. I., and Nagradova, N. K. (1983) *Biochem. Int.*, **6**, 443-450.
26. Sparrer, H. E., Santoso, A., Szoka, F. C., Jr., and Weissman, J. S. (2000) *Science*, **289**, 595-599.