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Novel Inhibitors of Glyceraldehyde-3-phosphate Dehydrogenase: Covalent Modification of NAD-Binding Site by Aromatic Thiols

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Abstract—Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12) is a glycolytic enzyme catalyzing the formation of 1,3-diphosphoglycerate from glyceraldehyde-3-phosphate and inorganic phosphate. In cooperation with E3 ubiquitin-kinase Siah1, GAPDH directly participates in the apoptotic death of neurons in Parkinson's disease. Potential GAPDH inhibitors were screened *in silico*, and three compounds with high affinity to the NAD-binding site and theoretically capable of forming a disulfide bond with amino acid residue Cys149 were found among cysteine and glutathione derivatives. The inhibitory effect of these compounds was tested on GAPDH from rabbit muscles using isothermal calorimetry and kinetic methods. As a result of experimental screening, we selected two compounds that inhibit GAPDH by forming disulfide bonds with the Cys149 residue in the enzyme active site. Since Cys149 is the key residue not only for the catalyzed reaction, but also for interaction with Siah1, the compounds can be assumed to inhibit the formation of the proapoptotic complex GAPDH–Siah1 and therefore have potential effect against Parkinson's disease.

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Key words: glyceraldehyde-3-phosphate dehydrogenase, covalent inhibition, cysteine and glutathione derivatives, NAD-binding domain, apoptosis, Parkinson's disease

It is well known that the inhibition of catalytic activity of the central glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12) leads to blockage of glycolysis. Due to uniquely high reactivity of a sulfhydryl group of the GAPDH active center [1], oxidants, sulfhydryl poisons, and many other compounds effectively inhibit the activity of this enzyme. Some of them react at very low concentrations that are close to the concentration of the enzyme active centers [2, 3]. For example, adding iodoacetate in stoichiometric amounts (four molecules per tetramer, or one molecule per monomer) completely inactivates the enzyme [4]. Moreover, manifestation of the "half-of-the-sites reactivity" effect, or more precisely reactivity of half of GAPDH active centers, leads to the fact that in some cases one modifier molecule results in a loss of activity of two enzyme active centers at once [5].

Inhibition of GAPDH activity can be achieved not only in experiments *in vitro*, but also by addition of various sulfhydryl group modifiers in a cell culture, and a 10-20-fold decrease in the enzyme activity can be achieved without significant changes in the activity of other enzymes [6]. These observations indicate that the selection of effective GAPDH inhibitors for the inhibition of its glycolytic function is not a difficult problem. However, recently it has been clearly shown that GAPDH participates in other cellular processes that are not directly related to its glycolytic function [7]. Many of these processes are due to pathological disturbances of the cells functioning and are directly involved in the development of neurodegenerative diseases, apoptosis, and malignant transformations [8]. Most clearly the participation of GAPDH in such processes is demonstrated in apoptosis, when translocation of certain forms of the enzyme into the nucleus and their binding to nucleic acids is observed [9, 10]. Since these noncanonical functions of GAPDH are not directly linked to its catalytic dehydrogenase activity, inhibition of the enzyme cannot lead to immediate changes in its participation in these processes. For regu-

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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lation of such noncatalytic functions of GAPDH, we need to search for some other specific ligands interacting with the enzyme and altering its properties. There are several classes of compounds that specifically interact with GAPDH, not just modifying the sulfhydryl groups of the enzyme. They include furoxan derivatives that interact with the enzyme and inhibit its activity, as well as derivatives of deprenyl, which served as a lead compound for development a drug for treatment of Parkinson's disease (CGP 3466) [11]. The latter compound does not modify GAPDH in a covalent manner, but binds to its active centers (possibly in a state free of NAD) and possesses antiapoptotic and pronounced therapeutic effect on Parkinson's disease [12].

GAPDH also participates in other ways in the induction of apoptosis. For example, NO-S-nitrosylation of catalytic Cys149 of the GAPDH active site leads to the binding of the enzyme to E3 ubiquitin-kinase Siah1, which has nuclear localization signal (NLS). The resulting complex enters the nucleus, where Siah1 performs proapoptotic functions through proteolytic cleavage of its substrates. It is suggested that the proapoptotic role of GAPDH is in stabilization of the normally short-living Siah1 [13, 14]. Therefore, inhibition of the interaction of GAPDH with Siah1 should lead to prevention of neuronal apoptosis in case of Parkinson's disease. Thus, we speculate that addition of such thiol compounds might be an effective way to prevent apoptosis by forming a specific covalent bond with the SH-group of Cys149 of the GAPDH catalytic site, as such a modification should result in the failure of the dehydrogenase to form a complex with Siah1.

In this work we have studied a new class of ligands – aromatic thiols that interact with the NAD-binding domain of the GAPDH active center and modify the sulfhydryl group of its active center. The ligands should primarily interact with those active centers that do not contain a cofactor or bind it weakly. Such an approach would lead only to partial inhibition of GAPDH, while protecting some of the active centers from the modification of sulfhydryl groups and subsequent changes in the enzyme structure. At the first stage cysteine and glutathione derivatives were screened *in silico* using molecular docking to find potential GAPDH inhibitors. In the second stage the most promising compounds were experimentally studied as GAPDH inhibitors using isothermal calorimetry and kinetic techniques.

MATERIALS AND METHODS

We used the following reagents: dithiothreitol and Tris (Fluka, Germany); NAD, glyceraldehyde-3-phosphate, glycine, Sephadex G-100 and G-50, ammonium sulfate, and EDTA (Sigma, USA), and KH_2PO_4 (Merck, Germany). Glyceraldehyde-3-phosphate dehydrogenase from rabbit skeletal muscles was isolated using the method of Scopes [15] followed by gel filtration on Sephadex G-100. The protein concentration was determined spectrophotometrically by absorption at 280 nm assuming $A_{280}^{1\%} = 1.0$ for GAPDH. The molecular weight of native GAPDH (tetramer) is 144 kDa. GAPDH activity was detected spectrophotometrically in a standard system from the increase in optical density at 340 nm during the enzymatic oxidation of 3-phosphoglyceric aldehyde in the presence of NAD.

Molecular docking. We used ChemSketch (http://www.acdlabs.com) for building structures of low molecular weight compounds. Molecular docking was carried out using Lead-Finder [16]. During covalent docking the length of S–S bonds was taken to be 1.8 Å. The center of the cell was specified by the coordinates of the Cys149 sulfur atom, and the size of the cell was taken to be 20 × 20 × 20 Å. In cases of *in silico* screening and noncovalent docking, the cell was set by coordinates of the reference structures. VMD 1.8.6 was used for visualization of molecular structures (http://www.ks.uiuc.edu/ Research/ vmd/vmd-1.8.6/).

Isothermal titration calorimetry. GAPDH from rabbit muscle, stored as a suspension in ammonium sulfate, was desalted by dialysis against 50 mM phosphate buffer (pH 7.5) overnight in the cold. After dialysis, for the experiment we used 2 ml protein preparation with concentration 1 mg/ml. The enzyme had a catalytic activity of about 100 units per mg protein, and the ratio of absorption at 280 nm to absorption at 260 nm was 1.1, indicating the presence at an average of three molecules of NAD cofactor for the enzyme tetramer. Aqueous solutions of analyzed compounds were thawed and diluted in 50 mM phosphate buffer (pH 7.5) to a final concentration of 0.4 mM and volume of 0.5 ml, after which they were used for titration.

A VP-ITC isothermal titration calorimeter (Microcal, USA) was used for titration at 25°C. The data were processed using MicroCal Origin 7.0 software.

Measurement of GAPDH activity in the presence of inhibitors. GAPDH was desalted on a column with Sephadex G-50 equilibrated with 50 mM phosphate buffer (pH 7.5) and diluted to a final concentration of about 2 mg/ml (14 μM per tetramer). Then, at room temperature, the protein was incubated with inhibitors under stirring in 50 mM phosphate buffer (pH 7.5) in the noted concentration. The concentration of inhibitors was taken in 10-fold excess in relation to the SH-groups of the protein active center (i.e. at a final concentration of 0.56 mM). The samples for activity measurement were taken at regular intervals. Activity was measured at pH 8.9 (0.1 M glycine, 0.1 M sodium phosphate, 5 mM EDTA) with 1 mM NAD and 1 mM 3-PGA. Similar experiments were performed to study the effect DTT, adding 5 mM DTT to the enzyme incubation medium.

RESULTS AND DISCUSSION

Docking of thiols into the active site of GAPDH. In this work, we studied N-acyl derivatives of natural SH-compounds (cysteine and glutathione) for which biocatalytic synthesis methods have been recently developed [17] as potential inhibitors of GAPDH. For computer screening we used a library of thiols theoretically capable of forming a disulfide bond with the SH-group of the catalytic residue Cys149. A GAPDH structure with resolution of 1.75 Å was used as the target (1U8F in the Protein Data Bank) [13]. In the primary evaluation of effectiveness of binding of the SH-compounds in the region of Cys149, we tested the possibility of docking potential inhibitors into the free and NAD-bound active site of GAPDH. Based on the *in silico* screening and evaluation of the free energy of binding, three compounds were selected for further study (Fig. 1): GSH (N-(phenylacetyl)-glutathione), NAC (N-(phenoxyacetyl)-L-cysteine), and NMC (N-((R)-mandelyl)-(S)-cysteine). To study the interaction of these compounds with GAPDH, we modeled covalent modifications of the Cys149 SH-group by covalent docking, determined positions of the investigated inhibitors in the target active site while a cysteine bridge is formed, and studied the stability of the covalent complexes.

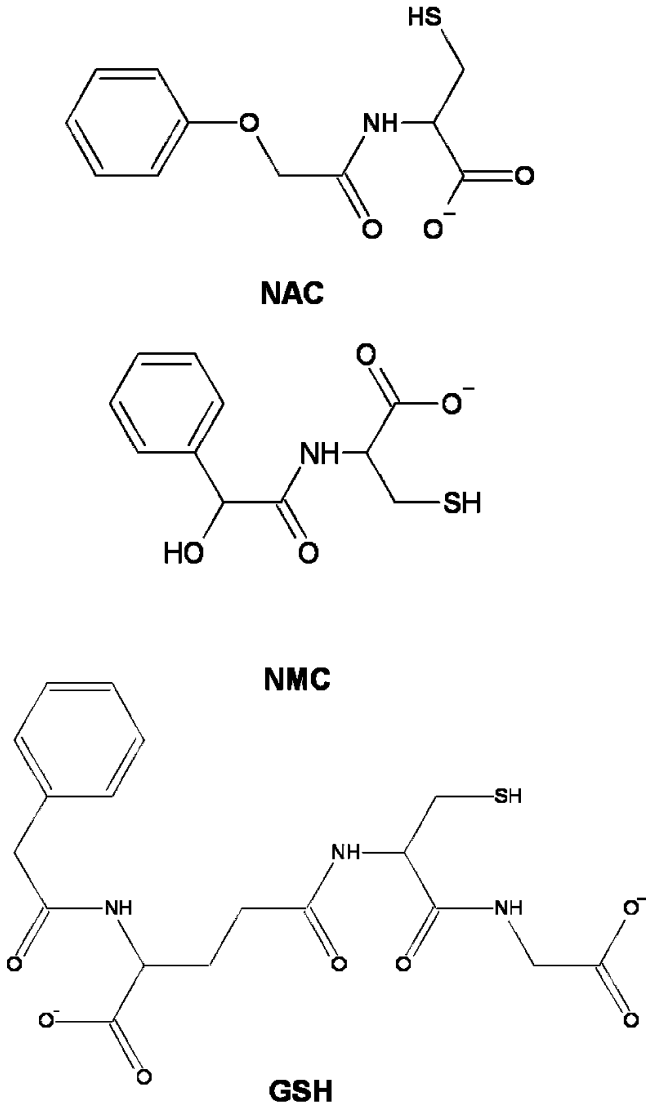

Fig. 1. Structural formulas of compounds NAC, NMC, and GSH.

Noncovalent docking of GSH into the free active site of GAPDH showed that its SH-group is located near the catalytic Cys149, which indicates a high probability of disulfide bridge formation. Molecular modeling of the disulfide bond through the GSH covalent docking also showed that the link is stable, and covalent binding of the inhibitor does not cause steric constraints in the active site. However, it was found that the covalent binding of GSH and the disulfide bond formation are not possible if the active center contains bound coenzyme. Thus, molecular modeling showed that the compound is able to interact only with the free active site of GAPDH.

A more complex pattern is observed when GAPDH interacts with the significantly smaller molecule of NAC. On noncovalent docking of the compound into the GAPDH free active site, it was found that the orientation of SH-groups of the inhibitor and Cys149 did not provide the possibility of disulfide bridge formation. However, binding of the inhibitor in the active site with already bound cofactor is possible in the case of NAC, and the SH-group of NAC can occupy the position reactive for the formation of disulfide bridge (Fig. 2).

Noncovalent docking of NMC into the GAPDH free active site showed the possibility of formation of a disulfide bond between SH-groups of the inhibitor and Cys149 (distance between sulfur atoms was about 4 Å). The molecular modeling established that the covalent binding of NMC is possible only when the GAPDH active site is unoccupied, i.e. binding of NAD prevents it.

To test the adequacy of the findings obtained by the computer screening of potential inhibitors, it was decided to conduct experimental studies of the inhibitory properties of the selected compounds *in vitro*.

Study of interaction of GAPDH with compounds capable of modifying the SH-group in the enzyme active site by isothermal titration calorimetry (ITC). To quantitatively characterize interactions of the three selected compounds (GSH, NMC, and NAC) with GAPDH from rabbit muscles, we used isothermal titration calorimetry, which allows determination of the thermodynamic parameters of the processes occurring while adding ligands to the analyzed protein. As seen in Fig. 3, practically no absorption or emission of heat occurs during the titration GAPDH by the NMC solution. Another situation was observed during the titration of GAPDH by the two other SH-compounds. The experimental data show (see titration diagram in Fig. 3) that the addition of GSH

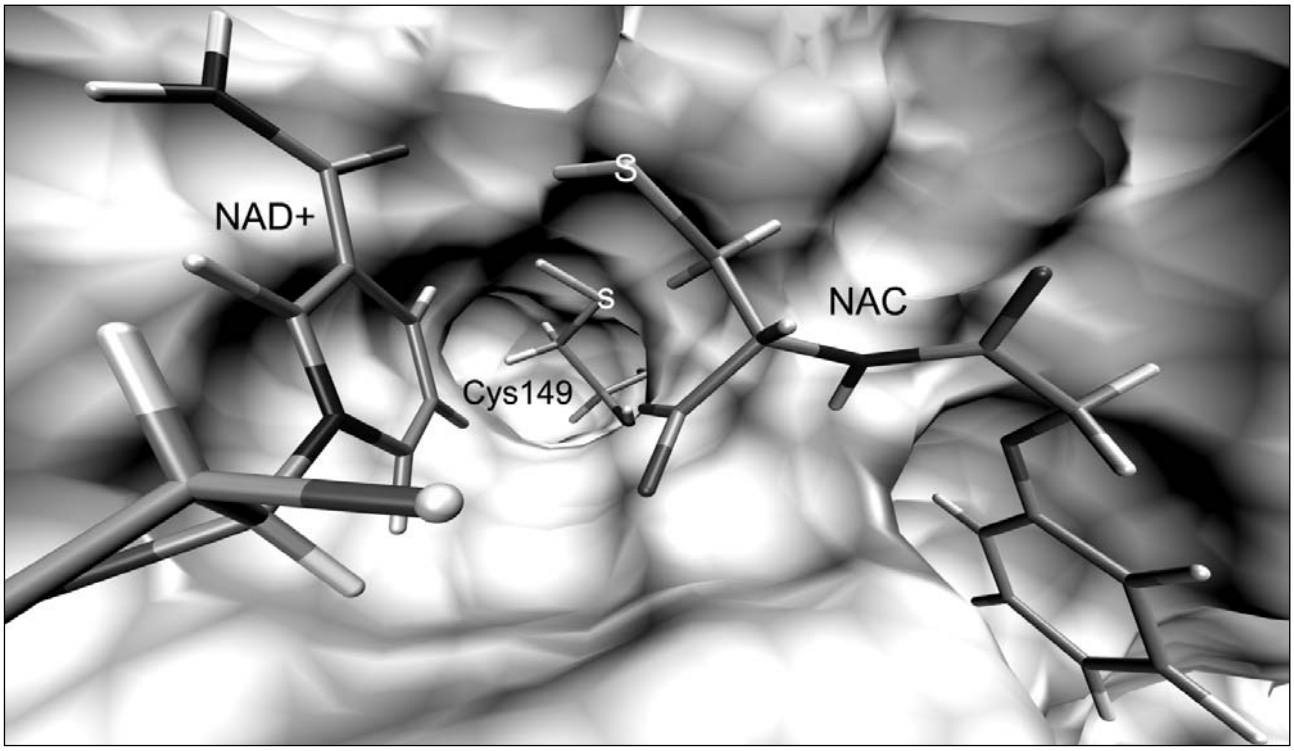

Fig. 2. NAC binding in GAPDH active site.

and NAC evoke absorption of heat, which indicates that the enzyme interacts with the inhibitors. However, in the course of titration we did not observe any isothermal plateau, i.e. under the experimental conditions saturation indicative of complete transformation of the free enzyme into the enzyme–inhibitor complex has not been reached. The reason for this may be the complexity of the processes occurring during the interaction of the ligands with the enzyme. One should note that in this case there are at least two stages of interaction – the formation of a noncovalent complex of GAPDH with the SH-compound and subsequent covalent modification of the Cys149 in the enzyme active site. In addition, the formation of a disulfide bond may be accompanied by the displacement of the cofactor from the active center, which can cause a very complex pattern of overall heat absorption.

Thus, from these data we conclude that an interaction between GAPDH and NMC is missing or cannot be detected by the method we use. GSH and NAC, of course, interact with GAPDH, but since these interactions are very complex, we could not determine the association constant and enthalpy of the noncovalent stages. We presume that these parameters could be evaluated during the titration of GAPDH apoenzyme completely free of cofactor. Unfortunately, it is practically impossible to get native preparations of GAPDH apoenzyme, since removal of the last two (per tetramer) cofactor molecules

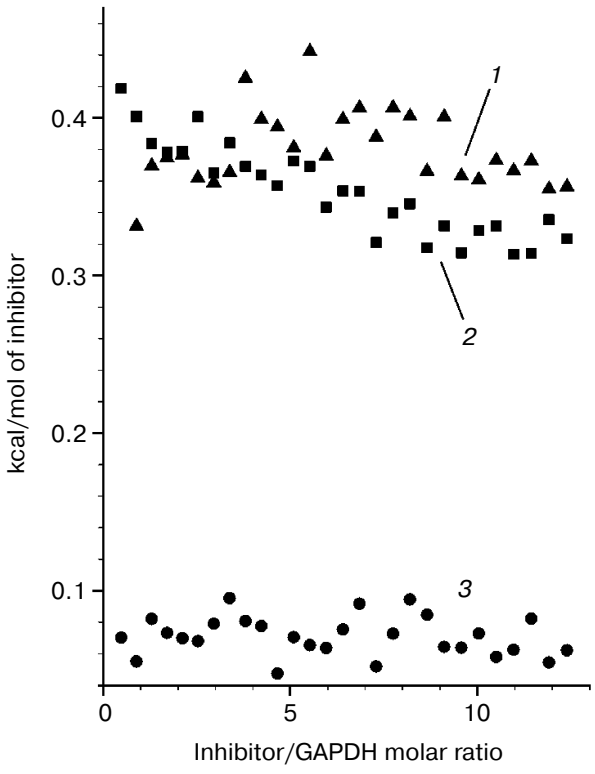

Fig. 3. Isothermal calorimetric titration of native somatic GAPDH by the three sulfhydryl compounds: triangles correspond to GSH (*1*), rectangles to NAC (*2*), and circles to NMC (*3*).

leads to disruption of the enzyme spatial structure. In future we plan to obtain mutant forms of GAPDH with point substitutions in the NAD-binding domain, which might provide apoenzyme, although it would somewhat complicate the interpretation of data on the binding of the ligands.

Inhibiting effect of GSH and NAC on catalytic activity of GAPDH. The inhibiting action of the SH-compounds was studied experimentally on somatic GAPDH preparation that was isolated from rabbit skeletal muscle by the standard method described in "Materials and Methods". The specific activity of GAPDH was 101 ± 5 U per mg of the protein. Absorption ratio A_{280}/A_{260} was close to one, indicating the binding of 3-4 molecules of NAD per enzyme tetramer. The preparation was homogeneous according to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

The experimental results are shown in Fig. 4. As can be seen from the curves, during the incubation of somatic GAPDH in the presence of GSH and NAC time-dependent growth in inhibition of the enzyme activity was observed, reaching in the case of NAC 65%, in the case of GSH 45%. However, the third of the studied compounds (NMC) had no effect on GAPDH activity up to the NMC concentration of 55 mM. The results indicate that only the active centers of the enzyme are modified that contain relatively weakly bound cofactor (K_d 10^{-4}-10^{-5} [18]). Binding of the investigated ligands with active centers containing strongly bound cofactor (K_d 10^{-9}-10^{-11} [18]) under these conditions does not occur.

Inhibition of GAPDH by NAC and GSH is due to interaction between inhibitor SH-groups and a cysteine residue of the enzyme. Since interaction with NAC and GSH could lead to covalent modification of the enzyme sulfhydryl groups, it was necessary to evaluate the contribution of this process to the inhibition of GAPDH. The inhibitory effect of the two selected compounds was studied in the presence of a low molecular weight dithiol – dithiothreitol (DTT). As follows from the data shown in Fig. 5, the addition of DTT completely prevents the inhibitory effect of NAC and GSH on the enzyme. This observation indicates that inhibition of GAPDH activity does not occur without disulfide bond formation during the modification of the enzyme by the studied ligands. We also studied the restoration of GAPDH activity by DTT after GAPDH modification by the tested compounds. It was shown that the addition of DTT did not lead to full restoration of activity, probably due to partial irreversibility of the enzyme inactivation. In addition, for the restoration of activity it is necessary to use rather high concentrations of DTT (10 mM and more), which led to partial aggregation of the protein and, consequently, to a decrease in activity (data not shown). A combination of several factors affecting the activity of the enzyme in these experiments does not allow us to uniquely interpret the results of the experiments on the restoration of activity. Nevertheless, on the basis of the protective effect of DTT described above, we assume that for the inhibitory effect of the two studied compounds interaction between their SH-groups and the SH-groups of a cysteine residue of the enzyme is required.

Thus, derivatives of cysteine and glutathione were found that can covalently bind to the free active site of GAPDH. For selecting the compounds, we used two main criteria. On one hand, these substances should covalently modify the sulfhydryl group of the active site of GAPDH, and on the other they should interact with the NAD-binding site. The latter property would solve two

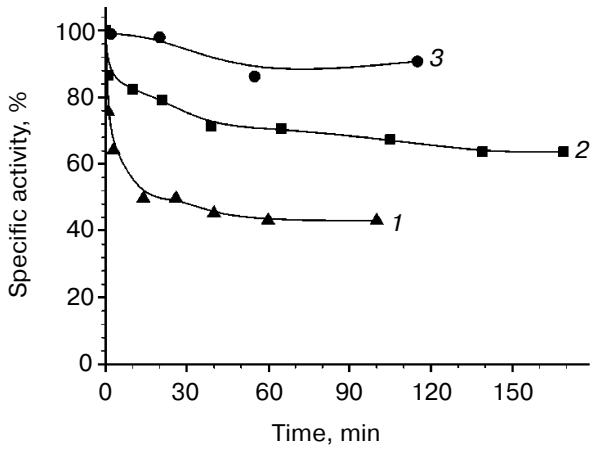

Fig. 4. Change in activity of somatic GAPDH from rabbit muscles during incubation with the proposed inhibitors: *1*) interaction of GAPDH with GSH; *2*) GAPDH with NAC; *3*) GAPDH with NMC.

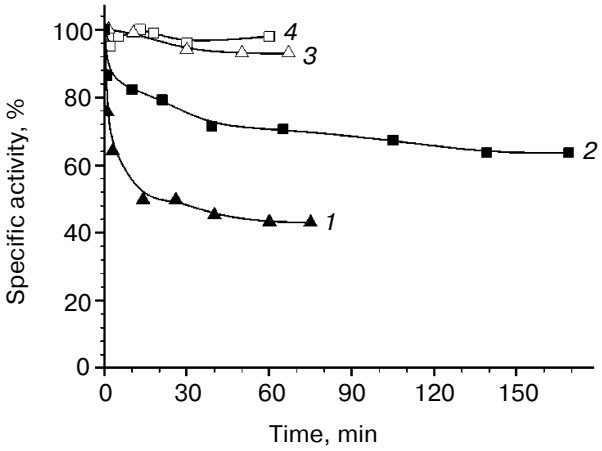

Fig. 5. Effect of dithiothreitol (DTT) on inhibition of somatic GAPDH from rabbit muscle. Filled symbols represent specific activity of GAPDH in the presence of inhibitor, and unfilled in the presence of DTT and inhibitor: *1*) interaction of GSH and GAPDH; *2*) NAC and GAPDH; *3*) GSH, DTT, and GAPDH; *4*) NAC, DTT, and GAPDH.

problems at once – highly specific modification of sulfhydryl group directly in the active site and modification of only those active sites that are free of cofactor or that bind it relatively weakly. Indeed, it was shown experimentally that the binding of the inhibitors and subsequent modification of sulfhydryl groups occurs in two monomers of the tetramer, i.e. it does not affect the active centers containing strongly bound cofactor. It is this circumstance that allows obtaining hybrid forms of the tetrameric enzyme possessing catalytic activity (about 50% of the original), but containing the covalently bound inhibitors in two active centers of the four. Such a hybrid form of GAPDH may differ from the native enzyme by its ability to interact with both nucleic acids and other proteins, which provides a noncanonical role of GAPDH in the regulation of cellular processes. In future, to clarify the possible role of hybrid forms of GAPDH in pathological processes, we intend to explore the antiapoptotic effect of the found covalent inhibitors on cellular models of Parkinson's disease.

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