ISSN 0006-2979, Biochemistry (Moscow), 2008, Vol. 73, No. 2, pp. 185-191. © Pleiades Publishing, Ltd., 2008.
Published in Russian in Biokhimiya, 2008, Vol. 73, No. 2, pp. 228-236.
Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM07-252, November 11, 2007.

Investigation of Glyceraldehyde-3-phosphate Dehydrogenase from Human Sperms

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Received July 16, 2007
Revision received September 3, 2007

Abstract—Glyceraldehyde-3-phosphate dehydrogenase (GAPDs) was purified from human sperms and properties of the enzyme were investigated. After sonication of sperms, the most part of GAPDs is associated with the insoluble cell fraction. Trypsin treatment results in the cleavage of part of the N-terminal domain of the enzyme yielding a soluble fragment that was purified by hydrophobic chromatography on Phenyl-Sepharose. The isolated fragment was shown to be a tetramer with molecular weight of approximately 150 kD (according to Blue Native PAGE) and composed of subunits of 40 kD (according to SDS-PAGE). The specific activity of the isolated fragment reached 374 U/mg. It is supposed that GAPDs exists in sperms as the tetrameric molecule bound to the fibrous sheath of the flagellum through the N-terminus of one or two subunits. Comparative study of the amino acid sequences of mammalian GAPDs revealed conservative cysteine residues (C21, C94, and C150) that are specific for the sperm isoenzyme and absent in the somatic isoenzyme. Residue C21 can be involved in the formation of the disulfide bond between the N-terminal domain of GAPDs and fibrous sheath proteins.

DOI: 10.1134/S0006297908020107

Key words: glyceraldehyde-3-phosphate dehydrogenase, spermatozoa, fibrous sheath, trypsin

D-Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12) is a glycolytic enzyme catalyzing oxidative phosphorylation of glyceraldehyde-3-phosphate yielding 1,3-diphosphoglycerate. In the organism of mammals, there are two isoenzymes: somatic enzyme (GAPDH) and sperm enzyme (GAPDs). GAPDH is present in all tissues of the organism and located in the cell cytoplasm. After breaking of the cells, GAPDH is easily extracted with aqueous solutions. The enzyme consists of 4 noncovalently connected subunits of 36 kD. Each subunit of GAPDH from human muscle consists of 333 amino acid residues and contains NAD-binding (1-148) and catalytic (149-333) domains [1]. GAPDs is a specific isoenzyme that is expressed only in sperms at definite stages of spermiogenesis. In mammals, the somatic and sperm isoenzymes are encoded by the genes located in chromosomes 12 and 19, respectively. Somatic GAPDH has not been found in spermatozoa. GAPDs of mammals possess an additional N-terminal domain containing from 72 (human) to 105 (mouse) amino acid residues with a high content of hydrophobic proline residues [2, 3]. Immunochemical staining of mammalian spermatozoa demonstrated that GAPDs is bound to the fibrous sheath (a structure surrounding the axoneme in the principal piece of the flagellum) [2-4]. The fibrous sheath consists of two longitudinal columns connected with transversal ribs. The columns and the ribs are formed at the early and the late stages of spermiogenesis, respectively [5]. According to recent studies, GAPDs is incorporated into the ribs of the fibrous sheath at the last stage of its formation [6].

Previously, the fibrous sheath was considered as a structural component of the flagellum [7]. However, investigation of fibrous sheath proteins showed that this structure plays a significant role in regulation of sperm motility. The main components of the fibrous sheath are the proteins AKAP4 (A-kinase anchoring protein) [8], TAKAP-80 [9], and AKAP3 [10]. It is still unknown which of the proteins is responsible for binding GAPDs.

It was demonstrated that the binding of GAPDs to the fibrous sheath is provided by the interaction of the N-terminal domain of GAPDs with fibrous sheath proteins

Abbreviations: GAPDH) somatic glyceraldehyde-3-phosphate dehydrogenase; GAPDs) glyceraldehyde-3-phosphate dehydrogenase from spermatozoa; G-3-P) glyceraldehyde-3-phosphate.

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[3]. Considering the strength of these interactions, it was suggested that GAPDs is covalently bound to the fibrous sheath [4]. The presence of the N-terminal domain results in abnormal motility of GAPDs in polyacrylamide gel. Analysis of mouse sperm lysates by SDS-PAGE reveals the band of GAPDs with the apparent molecular weight of 69.2 kD (the molecular weight determined from the amino acid sequence is 47.5 kD) [3]. Similarly, lysates of human sperms give a band corresponding to 56 kD, although the calculated molecular weight of human GAPDs is 44.5 kD [2].

The strong connection with the fibrous sheath of the flagellum may indicate an important role of GAPDs in sperm motility. Actually, it was demonstrated that the energy for the motility of mammalian sperms is provided mainly by glycolysis [11, 12]. Disturbances in the expression of GAPDs block the progressive motility of the sperm [13]. It is noteworthy that GAPDs is expressed in a certain period of spermiogenesis. In mouse cells, mRNA of the gene *GAPDs* was found at stages 4-7 of spermiogenesis. However, GAPDs was not detected in the cells until stages 12-13, which was supported by immunohistochemical methods [3]. Such a long existence of *GAPDs* mRNA without appearance of the protein indicates that the translation is controlled by some mechanism. Such a mechanism is provided by the protein transline, or TB-RBP (testis brain RNA-binding protein), which is a DNA- and RNA-binding protein with numerous functions. *In vitro*, mouse transline selectively forms a complex with *GAPDs* mRNA. Presumably, such a regulation is necessary to provide the incorporation of GAPDs into the growing fibrous sheath [14]. Unusual structure of GAPDs is of special interest for enzymologists. Unlike somatic GAPDH, properties of GAPDs are virtually unexplored. This is explained by the fact that GAPDs, like most of the fibrous sheath proteins, cannot be extracted with aqueous solutions or detergents, which hampers the isolation of the enzyme. The recombinant enzyme does not possess enzymatic activity. The only method that allowed isolation of GAPDs from boar sperm included destruction of the interactions between the protein and the fibrous sheath using trypsin treatment with subsequent purification of the active GAPDs fragment on Phenyl-Sepharose [4].

The goal of the present work was to investigate the properties of GAPDs in human sperm extracts, isolate the active GAPDs fragment after trypsin treatment of human sperms, and investigate the resulting preparation.

MATERIALS AND METHODS

In the present work we used ε-aminocaproic acid, Bis-tris, and glycine from ICN Biochemicals (USA); diaminobenzidine hydrochloride, dithiothreitol, Coomassie G-250, Coomassie R-250, NAD^+, ammonium sulfate, and glyceraldehyde-3-phosphate from Sigma (USA); trypsin from SPOFA (Czechoslovakia); Tricine from Serva (USA); Phenyl-Sepharose 6 Fast Flow from Pharmacia Biotech (Sweden); Tween-20 from Ferak (Germany); mouse monoclonal antibodies (clones 2E3-2E10) against GAPDs from Abnova (Taiwan). Samples of human sperms were provided by Pasteur Diagnostic Center (Moscow).

Mass-spectrometric analysis of the GAPDs fragment was performed in ZAO "PINNI" ("Postgenomic and Nanotechnological Innovation") on the basis of Innovation Center of Medical Nanobiotechnologies (Institute of Physico-Chemical Medicine of the Ministry of Health of the Russian Federation).

Isolation of active GAPDs fragment from human sperms. To isolate the active fragment of GAPDs, we used the procedure described for isolation of GAPDs from boar sperms [4] with some modifications. Human sperms were centrifuged (2500*g*, 10 min), washed with buffer (10 mM KH_2PO_4, 150 mM NaCl, pH 7.4), centrifuged (2500*g*, 10 min), and then frozen. Before isolation of the enzyme, the cells were thawed, suspended in an equal volume of 10 mM KH_2PO_4, pH 7.5, and broken by sonication (3 × 20 sec). The suspension was centrifuged (13,000*g*, 15 min), and the supernatant was discarded. The pellet was suspended in an equal volume of the same buffer and supplemented with trypsin (0.2 mg per ml of the pellet). The suspension was incubated at 30°C for 15 min, and then a solution of phenylmethylsulfonyl fluoride was added (final concentration, 2 mM). The suspension was centrifuged (13,000*g*, 10 min, 4°C), and the resulting supernatant was used for isolation of GAPDs using hydrophobic chromatography on Phenyl-Sepharose as described in [4]. GAPDs was eluted while washing the column with 50 mM potassium-phosphate buffer without ammonium sulfate. All fractions eluted from the column were supplemented with dithiothreitol (final concentration, 2 mM). The fractions containing the dehydrogenase activity were pooled and concentrated using 50,000 MWCO Amicon Ultra centrifugal filters (Millipore, USA).

Blue Native PAGE. The molecular weight of native GAPDs was determined using Blue Native PAGE in 5.5-12% gradient of polyacrylamide gel [15]. In the presence of Coomassie G-250, proteins acquire negative charge at neutral pH and migrate in the electric field, the rate being inversely proportional to their molecular weight. Samples of the purified enzyme or a sperm extract in the sample buffer (20 mM Tris-HCl, pH 7.4, 500 mM ε-aminocaproic acid, 10% glycerol) were applied to the gel. Electrophoresis was run at 4°C (200-300 V).

Immunoblotting. After electrophoresis, the proteins were transferred from the gel to an Immobilon P membrane (Millipore) (100 V, 45 min). Transfer buffer contained 25 mM Tris, 192 mM glycine, 20% ethanol, pH 8.3. Monoclonal antibodies against GAPDs (1 : 1000)

(Abnova) were used as the primary antibodies. These antibodies interact with GAPDs and do not bind to the somatic isoenzyme. The secondary antibodies were anti-mouse antibodies conjugated with peroxidase. The membrane was stained with a solution containing 3 mg of diaminobenzidine hydrochloride, 30 mg of $NiSO_4 \cdot 7H_2O$, and 10 μl of 30% H_2O_2 in 10 ml of 0.1 M Tris-HCl, pH 7.6.

Assay of the activity of GAPDs. Activity of GAPDs was determined by measuring the increase in the absorption at 340 nm during oxidation of glyceraldehyde-3-phosphate (G-3-P). The reaction mixture (1 ml) contained 50 mM glycine, 50 mM potassium phosphate, pH 9.0, 5 mM EDTA, 0.5 mM NAD^+, 1 mM G-3-P, and 1 μg of the purified enzyme (or 20 μl of the investigated extract).

Modeling of three-dimensional structure of human GAPDs tetramer. Three-dimensional structure of the tetrameric GAPDs molecule without consideration of the N-terminal domains was modeled by means of Swiss-Model [16] using the known structure of human somatic GAPDH (PDB ID 1ZNQ). The percentage of overlapping sequences for human GAPDs and GAPDH constitutes approximately 68%.

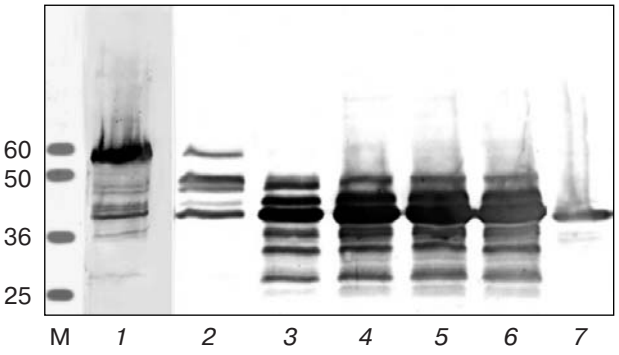

Fig. 1. Immunoblotting of different fractions of human sperms. The sperms were washed, suspended in 10 mM potassium-phosphate buffer, pH 7.5, and broken by sonication. The cell pellet (*1*) was separated by centrifugation, and the supernatant (*2*) was removed. The cell pellet was suspended in an equal volume of the buffer and supplemented with trypsin (0.5 mg per ml of the pellet). The suspension was incubated at 30°C for 15 min. After 3, 7, 11, and 15 min of incubation, the suspension was centrifuged and the supernatant (lanes *3-6*, respectively) and the pellet after 15 min of incubation (lane *7*) were analyzed by SDS PAGE with subsequent transfer to a nitrocellulose membrane. The membrane was stained with specific antibodies against GAPDs. The molecular weights of protein standards (M) are given on the left (kD).

RESULTS AND DISCUSSION

Extraction of GAPDs with trypsin. Analysis of sperm extracts by SDS-PAGE with subsequent immunoblotting showed that after sonication of sperms, most of the GAPDs remained bound to insoluble components of the cell. As seen from Fig. 1 (lane *1*), the cell pellet contains a protein band of 58 kD corresponding to the full-length subunit of human GAPDs, and a shorter fragment of 40 kD. In the extract, there are several protein bands (58, 48, 47, and 40 kD) interacting with the antibodies against GAPDs (Fig. 1, lane *2*): the band of 58 kD corresponds to the full-length subunit of human GAPDs, and the others are likely to be products of its proteolysis. As seen from the data presented in the figure, only a minor part of the full-length GAPDs is released into the solution after sonication: the extract contains mainly light fragments of GAPDs, the main part of the full-length GAPDs remaining in the pellet (Fig. 1, lane *1*). After treatment of the pellet with trypsin, the content of the 40-kD fragment in the extract significantly increases (Fig. 1, lanes *3-6*), and the 58-kD band in the pellet disappears (Fig. 1, lane *7*). Since the trypsin treatment releases GAPDs into the solution, it can be assumed that trypsin splits off the N-terminal domain that is involved in the binding of GAPDs to fibrous sheath proteins.

Isolation of the fragment of GAPDs trypsinolysis. The extract obtained after trypsin treatment of the sperms under mild conditions (0.2 mg of trypsin per ml of the cell pellet) was purified by hydrophobic chromatography on a Phenyl-Sepharose column. As a result, an active fragment of GAPDs was isolated with the molecular weight of 40 kD according to SDS-PAGE (Fig. 2). The fact that the fragment obtained by GAPDs trypsinolysis interacts with Phenyl-Sepharose indicates that the isolated fragment retains a part of the N-terminal hydrophobic sequence.

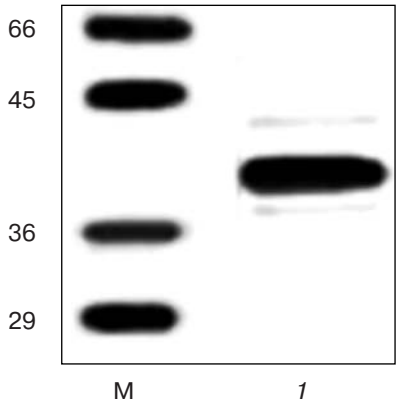

Fig. 2. Immunoblotting of the GAPDs preparation purified from the extract after the trypsin treatment of the sperms (*1*). After SDS-PAGE, the proteins were transferred to a nitrocellulose membrane and stained with specific antibodies against GAPDs. The molecular weights of protein standards (M) are given on the left (kD).

Mass-spectrometry (MALDI) analysis of the isolated GAPDs fragment. The isolated fragment was analyzed by the peptide mass fingerprint method, suggesting complete cleavage of the analyzed protein with trypsin with subse-

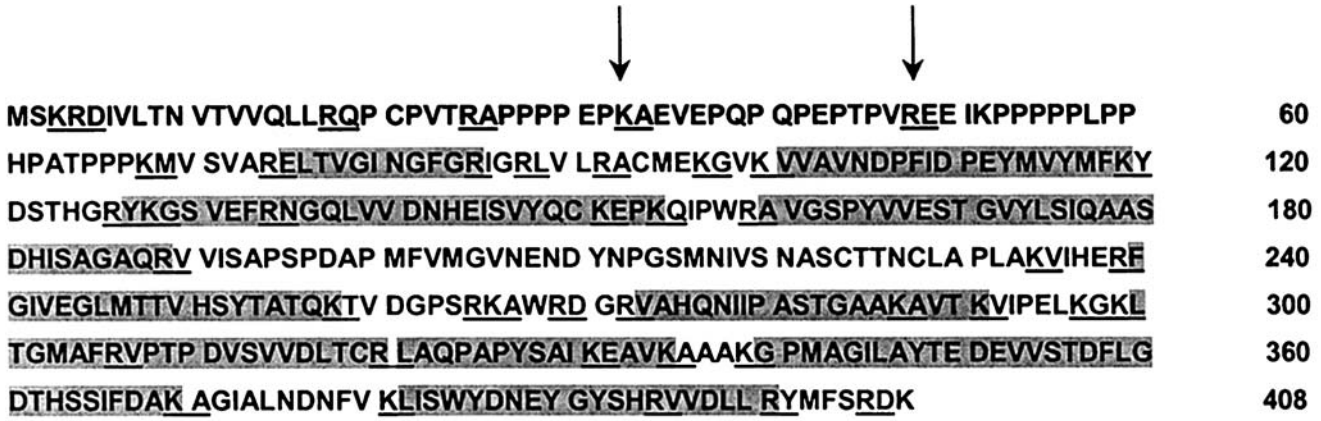

Fig. 3. Amino acid sequence of human GAPDs (Swiss Prot, O14556) (www.expasy.org). The first 73 amino acid residues correspond to the additional N-terminal domain that is characteristic for GAPDs. The peptides identified in the 40-kD fragment of GAPDs are highlighted gray. All possible sites of trypsinolysis are underlined. The sites of trypsin cleavage calculated from the molecular weight of the resulting GAPDs fragment (40 kD) are shown by arrows.

quent analysis of the resulting peptides by MALDI assay. The results of the analysis are presented in Fig. 3. The comparison of the set of peptides with the database showed that the analyzed 40-kD protein is a fragment of human GAPDs (Probability Based Mowse Score, 208). As seen from Fig. 3, the identified peptides overlap virtually all amino acid sequence of GAPDs. Two relatively long sequences are absent: 189-239 (50 amino acid residues) and the N-terminal sequence of 73 amino acid residues. Since the molecular weight of the full-length GAPDs calculated from the amino acid sequence is 44.5 kD, the fragment of 40 kD can result from the removal of a 4.5-kD polypeptide from one of the termini of the GAPDs molecule. As seen from Fig. 3, the peptides of the C-terminus are found in the sequence of the investigated protein, while the peptides of the N-terminal domain are absent. The peptide 189-234 (45 amino acid residues) has no sites of trypsinolysis. It should be noted that long peptides (more than 3.5 kD) are not analyzed, since they can be products of the incomplete cleavage of an analyzed protein. Presumably, the peptide 189-234 is not identified for this reason.

The presented data suggest that the investigated protein of 40 kD corresponds to the GAPDs lacking in a part of the N-terminal domain (complete cleavage of the N-terminal domain at the R74-E75 position must yield a fragment of 36 kD). Considering the molecular weight of the resulting fragment, the N-terminal domain must split at positions K33-A34 or R48-E49 (indicated by arrows in Fig. 3) yielding the products of 40.8 or 39.4 kD, respectively. As seen from the figure, in these cases the GAPDs molecule retains a small fragment of the N-terminal domain (25-40 amino acid residues) enriched with proline residues that interacts with Phenyl-Sepharose during hydrophobic chromatography. The fact that the trypsin treatment results in the liberation of GAPDs into the solution supports the assumption concerning the removal of the N-terminal domain.

Presumably, during the trypsin treatment the cleavage occurs predominantly in the area of the stem connecting the globular part of the enzyme with the fibrous sheath proteins, this explaining the increased accessibility of this site to trypsin. Other sites of trypsinolysis (underlined in Fig. 3) are screened by the fibrous sheath or hidden inside the protein globule, becoming accessible during denaturation of the protein.

Thus, it can be concluded that the mild treatment of the sperm pellet with trypsin (0.2-0.5 mg of trypsin per ml of the pellet, 30°C, 15 min) results in the elimination of the N-terminal sequence of the GAPDs molecule (33-48 residues) and liberation of the active GAPDs fragment into the solution.

Investigation of properties of the isolated GAPDs fragment. Incubation of 0.5 ml of the cell pellet with 0.1 mg of trypsin resulted in isolation of 30 μg of GAPDs. The specific activity of the isolated preparation constituted 50-374 U/mg in different fractions (20°C, pH 9.0). Increasing trypsin concentration decreased the activity of the purified preparation. Within the pH range of 7.0-9.5, the pH dependence of the enzymatic activity of GAPDs preparation was studied. The maximal activity was observed in the range of pH 8.0-9.0. The K_m values for NAD^+ and G-3-P constituted 22 and 770 μM, respectively. The parameters of somatic GAPDH from different human tissues [17] and human GAPDs are presented in the table.

As seen from the table, the activity of GAPDs in the most active fraction exceeds 6-fold the activity of GAPDH from human liver. pH-Dependence of GAPDs activity is characterized by a broader maximum (8.0-9.0) compare to that of somatic isoform. The K_m value of GAPDs for NAD^+ is close to the corresponding values

Catalytic characteristics of human GAPDH from different tissues and human GAPDs

Isoenzyme	Specific activity	pH optimum for the reaction of G-3-P oxidation	K_m for NAD^+	K_m for G-3-P
GAPDH	62 U/mg (liver)	8.0-8.3* (for different tissues)	13-98 μM** (for different tissues)	10-32 μM** (for different tissues)
GAPDs	50-374 U/mg	8.0-9.0****	22 μM***	770 μM***

* In the presence of arsenate.
** At pH 8.2.
*** 50 mM glycine, 50 mM potassium phosphate, 5 mM EDTA, pH 9.0.
**** The buffer contained 50 mM potassium phosphate, 50 mM Taps, 50 mM glycine, 5 mM EDTA, pH 7.0-9.5.

determined for GAPDH from different tissues. The K_m value of GAPDs for G-3-P exceeds more than 20-fold the corresponding parameter for somatic GAPDH.

Investigation of the oligomeric composition of GAPDs. By analogy with the somatic isoenzyme GAPDH, GAPDs is supposed to be a tetramer. However, it has been known that GAPDs is bound to the fibrous sheath through the N-terminal domain. Figure 4 presents a model of three-dimensional structure of GAPDs showing the position of the N-terminal domains. As seen from the model, the N-terminal domains are located at different sides of the molecule. Thus, the tetrameric molecule can be attached to the fibrous sheath through all four N-terminal domains only in the case if the surface of the fibrous sheath forms outgrowths. If the surface is flat, such an attachment is impossible, since it would result in the distortion of the spatial structure and, as a consequence, in the loss of the activity of the enzyme. In the latter case, it can be assumed that the enzyme is not a tetramer, or only one or two subunits of the tetramer are involved in the interaction with the fibrous sheath. Since there is no information concerning the fibrous sheath structure, it is necessary to consider all possibilities. To investigate the oligomeric composition of GAPDs, sperm extracts were analyzed using Blue Native PAGE.

Blue Native PAGE of the sperm extract obtained by trypsin treatment of the sonicated cells gives a band (Fig. 5a, lane *1*) that migrates at the same rate as rabbit muscle GAPDH (144 kD; Fig. 5a, lane *2*) and is stained with the antibodies against GAPDs (Fig. 5b, lane *1*). The R_f value of the rabbit muscle GAPDH does not fit the plot $\log M_r(R_f)$, created using protein standards. Obviously, this is connected with characteristic features of GAPDH. However, considering that GAPDs exhibits a significant similarity with GAPDH (68% of homology), the molecular weight of GAPDs was evaluated from the position of the band of rabbit muscle GAPDH. Thus, the molecular weight of the GAPDs fragment obtained after trypsin treatment of the sperm pellet constitutes approximately 150 kD. The data of SDS-PAGE show that this extract contains mainly the subunits of 40 kD (Fig. 1, lanes *4-6*). Consequently, the band of GAPDs (lanes *1* in Figs. 5a and 5b) corresponds to the tetrameric form of the enzyme. As seen from the presented data, no monomeric or dimeric species of GAPDs were observed in the extracts. Consequently, trypsin treatment of GAPDs bound to the fibrous sheath results in the formation of the soluble tetrameric GAPDs formed by the subunits lacking the N-terminal domain.

Blue Native PAGE of the extract obtained after sonication of the sperms revealed a band that migrated with a

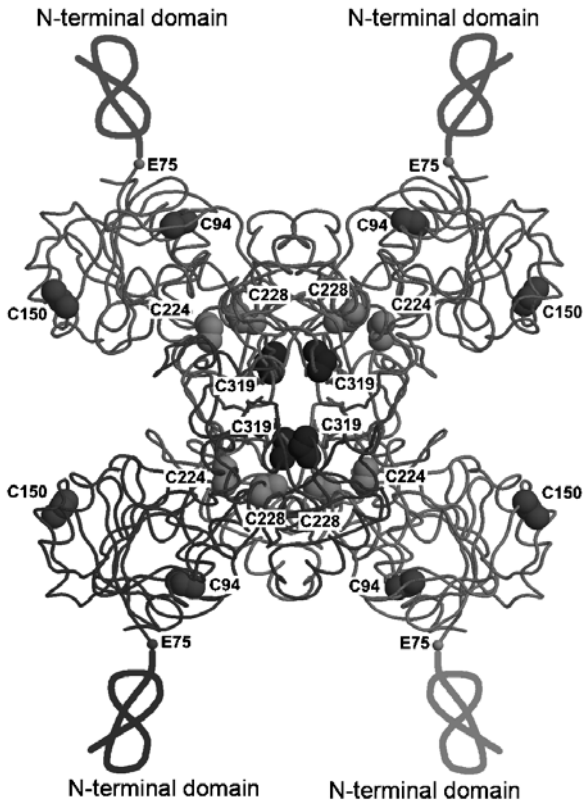

Fig. 4. Model of three-dimensional structure of human GAPDs: C94 and C150 are the cysteine residues specific for GAPDs; C224, C228 (the active site cysteines), and C319 are the cysteine residues that are present in both isoenzymes. Situation of the N-terminal domain is shown.

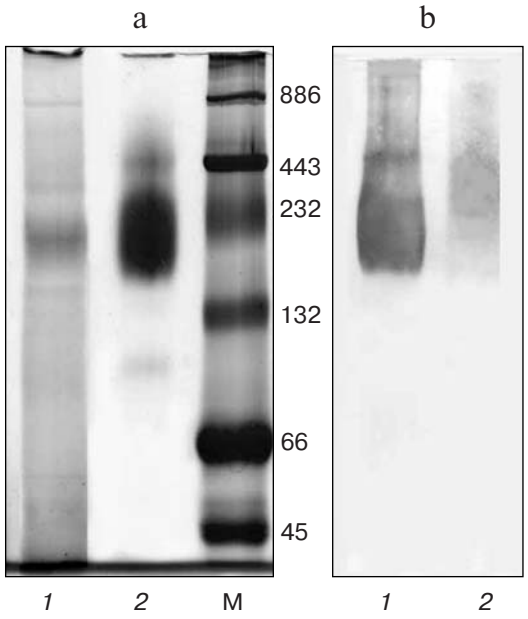

Fig. 5. Blue Native PAGE of human sperm extracts. a) Polyacrylamide gel after staining for protein: *1*) extract after the incubation of the cell pellet with trypsin; *2*) rabbit muscle GAPDH (144 kD); M) protein standards—ferritin (886 and 443 kD), catalase (232 kD), BSA (132 and 66 kD), and ovalbumin (45 kD). b) The membrane after staining with the antibodies against GAPDs: *1*) extract after the incubation of the cell pellet with trypsin; *2*) extract after sonication of the cells.

lower rate than the tetramer formed by the 40-kD subunits. Since the content of GAPDs in the extract after sonication is much lower than after trypsin treatment, the GAPDs band can be observed only after immunoblotting (Fig. 5b, lane *2*). According to the results of SDS PAGE, the extracts after sonication contain subunits of 58, 48, 47, and 40 kD (Fig. 1, lane *2*). Consequently, the GAPDs band (Fig. 5b, lane *2*) corresponds to the tetrameric form of GAPDs formed by the subunits of different length (58, 48, 47, and 40 kD) and therefore is characterized by the higher molecular weight.

The results suggest that the full-length GAPDs bound to the fibrous sheath of the flagellum is also a tetramer. However, the way of the attachment of the tetrameric molecule to the fibrous sheath remains unclear. The fact that a part of the subunits of the enzyme observed in the extracts is lacking in the N-terminal domain (Fig. 1, lanes *1* and *2*) suggests that the N-terminus of some subunits is free and can be easily removed by endogenous proteinases.

Proteolysis of GAPDs by endogenous proteinases. To understand what form of GAPDs exists in native sperms and when the N-terminal domain is removed (*in vivo*, in storage, or while preparing the samples), we investigated sperm extracts obtained under different conditions. To exclude the possibility of proteolysis of GAPDs during storage, sonication, and extraction, the pellet of fresh cells was suspended in the sample buffer, and the samples were immediately heated at 90°C for 5 min. As seen from Fig. 6, the proteolytic cleavage can be avoided when the fresh cells are suspended in the buffer preheated to 90°C (Fig. 6, lane *1*). If the sample is heated gradually, the 40-kD band emerges (Fig. 6, lane *2*). A prolonged incubation of the broken sperms results in the accumulation of fragments of proteolysis (Fig. 6, lanes *3* and *4*).

The data presented in Fig. 6 indicate that proteolytic decomposition of GAPDs yielding short fragments occurs during the preparation of the samples. Thus, sperms contain the full-length chains of GAPDs (58 kD) that are cleft by endogenous proteinases after the cell death. During the incubation of the broken cells, products of proteolysis of 48, 47, and 40 kD (Fig. 6, lanes *3* and *4*) are accumulated. At the same time, a significant part of the GAPDs remains as the full-length chain. These data suggest that some of the subunits of the tetrameric GAPDs are less accessible to proteinases. Different accessibility of the subunits of the tetramer to proteinases suggests that the tetrameric molecule of GAPDs is bound to the fibrous sheath through one or two subunits, the N-terminal domains of the other subunits being free. The subunits that are bound directly to the fibrous sheath are less susceptible to proteolysis, since their N-terminal domains are screened by the fibrous sheath proteins. The free N-terminal domains are easily removed by proteinases.

Role of the additional cysteine residues in the GAPDs molecule. The comparative analysis of the primary structure of mammalian GAPDs showed that, compared to somatic GAPDH (Swiss Prot, PO4406), GAPDs contains three additional cysteine residues. In human GAPDs these are the residues C21, C94, and C150 (Swiss

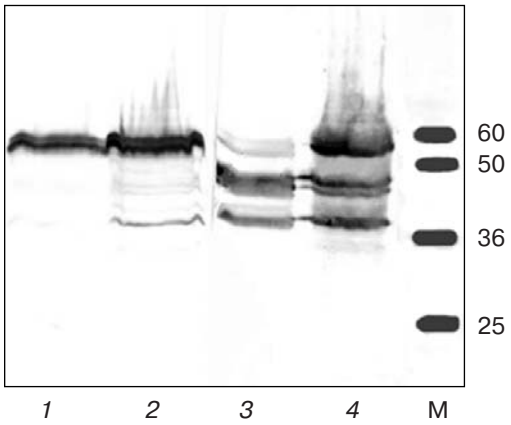

Fig. 6. Proteolysis of GAPDs by endogenous proteinases. Fresh sperms were separated by centrifugation and placed into preheated to 90°C sample buffer (lane *1*) or heated gradually (lane *2*); the sonicated cells were incubated at 4°C for 24 h and then centrifuged. The supernatant (*3*) and the pellet (*4*) were analyzed. M, protein standards.

Prot, O14556). One of them (C21) is located in the N-terminal domain (Fig. 3) and presumably forms the disulfide bond with the cysteines of the fibrous sheath proteins. Two other cysteine residues (C94 and C150) are situated in the NAD-binding domain (Fig. 4). The residue that is homologous to C94 of human GAPDs is found in all known sequences of mammalian GAPDs (11 species). The residues C21 of the N-terminal domain and the residue corresponding to C150 of human GAPDs is found only in GAPDs of placental mammals. These results suggest that the indicated residues are necessary for some specific functions of the sperm isoenzyme. So, it was supposed that these residues could be involved in the interactions with fibrous sheath proteins. As seen from the model of three-dimensional structure of human GAPDs (Fig. 4), the residue C150 is situated on the surface of the protein globule, this allowing the possibility of its interaction with other proteins. Besides, cysteines of the N-terminal domain, namely C21, can be involved in the formation of disulfide bonds with fibrous sheath proteins. To test the possibility of the participation of these cysteine residues in the formation of disulfide bonds with fibrous sheath proteins, we investigated the effect of β-mercaptoethanol on the extraction of GAPDs. The pellet of broken sperms was incubated in the presence of 5 mM β-mercaptoethanol for 2 h and the resulting extract was investigated. It appeared that the presence of β-mercaptoethanol did not affect significantly the extraction of GAPDs (data not shown). The full-length GAPDs is poorly extracted independently of the presence of β-mercaptoethanol, and the short fragments of 40-48 kD are easily released into the extract both in the presence and in the absence of β-mercaptoethanol. The results indicate that C150 does not form covalent bonds with fibrous sheath proteins. The residue C21 can be involved in the formation of disulfide bonds, but interactions of other nature also can exist between the N-terminal domain and the fibrous sheath, so the reduction of the disulfide bonds does not release GAPDs into the solution. Thus, the role of residues C94 and C150 remains unclear. However, the presence of the additional cysteine residues makes the enzyme more sensitive to oxidants and sulfhydryl poisons.

Thus, it was shown that human GAPDs is a tetramer composed of similar subunits of 58 kD. Based on the three-dimensional structure of GAPDs together with the analysis of the products of proteolytic degradation of GAPDs by endogenous proteinases, it was assumed that the tetrameric molecule of the enzyme is bound to the fibrous sheath through the N-terminal domain of one or two subunits. During storage and preparation of the samples, the free N-terminal domains are split off by endogenous proteinases. As a result, SDS-PAGE of the samples gives a set of characteristic bands with apparent molecular weights of 58, 48, 47, and 40 kD. Trypsin treatment of the sperms results in the cleavage of the N-terminal domain yielding the soluble fragment of GAPDs that can be purified using hydrophobic chromatography. The purified fragment of GAPDs is a tetramer of approximately 150 kD (according to Blue Native PAGE) and composed of the subunits of 40 kD (according to SDS-PAGE). The specific activity of the isolated fragment of GAPDs reaches 374 U/mg, while the specific activity of GAPDH from different sources constitutes 60-120 U/mg. A high activity of GAPDs is likely connected with the peculiarities of the association of the active sites and cooperative interactions between the subunits. Comparative analysis of the primary structure of mammalian GAPDs revealed conservative cysteine residues that are specific for GAPDs (C21, C94, and C150). The residue C21 is likely to be involved in the formation of disulfide bonds with fibrous sheath proteins. Further investigations are needed to reveal the role of residues C94 and C150.

This work was supported by the Russian Foundation for Basic Research (06-04-48240).

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