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# Molecular Characterization of Tumor Associated Glyceraldehyde-3-phosphate Dehydrogenase

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**Abstract**—Here we describe the purification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from normal leukocytes of healthy subjects and leukocytes of chronic myeloid leukemia (CML) patients and from normal mouse muscle and sarcoma tissue. The data indicate that some properties of GAPDH of leukocytes of CML patients and sarcoma tissues are similar and also similar to those of EAC (Ehrlich ascites carcinoma) cellular GAPDH but distinctly different from those of the normal cellular GAPDH. Polyclonal antiserum raised against the 54 kDa subunit of EAC cell GAPDH strongly reacted with GAPDH of leukocytes of CML patients and sarcoma tissue GAPDH only and weakly reacted with GAPDH of normal leukocyte and normal muscle and a variety of other tissues of normal rats. Both the subunits of GAPDH of sarcoma tissues were partially sequenced from the N-terminus and compared with the known sequences of GAPDH. The altered properties of GAPDH of three different malignant sources might be common feature of all malignant cells, which is discussed in relation to glycolysis and malignant aberrations.

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*Key words*: glyceraldehyde-3-phosphate dehydrogenase, sarcoma, leukemia, cancer marker

An important characteristic of rapidly growing malignant cells is their high capacity for aerobic glycolysis [1-3]. Many explanations have been put forward backed by experimental and theoretical studies, but none of these has been able to identify precisely a causal relationship between a specific change and the observed lactate flux [1-8]. In textbooks, it is generally assumed that glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12) although an important enzyme of the glycolytic pathway is not a control point for glycolysis and hence is unrelated to malignancy.

However, in the last couple of years investigations from several laboratories have indicated that GAPDH might play a primary role in the high aerobic glycolysis of malignant cells. These investigations indicated a strong enhancement of expression of a protein in different types of malignant cells that is apparently identical with the subunits of GAPDH. Enhanced expression of mRNA of GAPDH has also been found in different malignant cells. This overexpression has been found to occur in a wide variety of human and animal malignant cells such as lung cancers [9], prostatic adenocarcinoma [10], renal cell carcinoma [11], glioma cells [12], and breast carcinoma cells [13]. Surprisingly, increased level of mRNA of GAPDH was reported to be present in the plasma of patients with hepatocellular carcinoma [14]. In fact, these and other related studies have raised doubt about GAPDH being a housekeeping protein [15].

Despite this new interest in relating GAPDH with malignant aberration, the enzyme had been purified from only two different malignant cells: by Nakano et al. from HeLa cells [16] and in our laboratory by Bagui et al. from Ehrlich ascites carcinoma (EAC) cells [17]. Previous studies from our laboratory had however indicated that methylglyoxal, a normal metabolite, inactivates GAPDH of a wide variety of malignant cells and tissues but it has no effect on this enzyme of normal tissues and benign tumors, suggesting that malignant cellular GAPDH [18, 19] may be critically altered. Immunological studies with HeLa cells [20] and normal and malignant human prostate cancer cells [21] indicate some difference in GAPDH of normal and malignant cells.

Preliminary studies on the properties of GAPDH purified from EAC cells had indicated that the structural and catalytic properties of this enzyme might be different from those of other (normal) sources. The most striking difference is that the EAC cell GAPDH is a heterodimer

*Abbreviations*: CML, chronic myeloid leukemia; EAC, Ehrlich ascites carcinoma; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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containing two subunits of 33 and 54 kDa, whereas, the enzyme purified from other (normal) sources is a homotetramer of 35 kDa subunit. Some other differences in the properties are pH optimum, inhibition by ATP, and also the nature of the active site [17, 22].

However, there was one important limitation in the previous study of our laboratory. The properties of EAC cell enzyme were compared with that of the enzyme of normal rabbit muscle. The differences in the properties that were observed might be due to differences in tissue and species variation. Moreover, HeLa cells have no normal counterpart. So, the present study was initiated to determine whether the properties of the EAC cell enzyme are common features of all malignant cells. For this, we purified GAPDH from leukocytes of CML (chronic myeloid leukemia) patients and also of sarcoma of mice, which was induced by the chemical carcinogen 3-methylcholanthrene. This enzyme was also purified from leukocytes of normal donors' blood and muscle tissue of normal mice. The properties of the enzyme of normal and malignant cells were compared. The results presented here do indicate that malignant cell GAPDH might be altered in malignant cells.

## MATERIALS AND METHODS

**Chemicals and proteins.** Glyceraldehyde-3-phosphate, NAD, NADH, ATP, rabbit muscle GAPDH, Freund's complete adjuvant, Freund's incomplete adjuvant, anti-rabbit IgG peroxidase conjugated antibody, and anti-mouse IgG (whole molecule) peroxidase conjugated were purchased from Sigma (USA). Anti-α-tubulin and anti-GAPDH antibody and luminol reagents were obtained from Santa Cruz Biotech (USA). Tubulin, purified from goat brain, was a kind gift from Dr. Bhabatarak Bhattacharya, Bose Institute, Kolkata, India. All other chemicals were of analytical grade and obtained from local manufacturers.

**Blood.** Blood samples of leukemic patients suffering from chronic myeloid leukemia, acute myeloid leukemia, or acute lymphocytic leukemia were collected from hematology unit of hospitals. Normal blood was collected from healthy donors. For purification of the enzyme from malignant sources, blood from CML patients was used. Some properties of the enzyme were also tested in partially purified enzyme of leukocytes from acute myeloid leukemia and acute lymphocytic leukemia patients and were found to be similar in nature. However, we present here results that were obtained from CML leukocytes. For collection of blood, informed consent was obtained from the donors. Blood was brought to the laboratory at ambient temperature.

**Development of tumors.** Sarcoma tissue was developed in left hind leg of mice by intramuscular injection of 3-methylcholanthrene. The carcinogen was dissolved in olive oil by placing it in a warm water bath, and 0.1 ml of olive oil containing 0.2 mg of the carcinogen was injected into each mouse thrice with one-week interval. After 12-14 weeks full-grown tumor was developed. The malignant nature of the tissue was confirmed by histology (figure not shown).

Animal experiments were done according to the guidelines of the ethics committee on animal experiments. Appropriate measures were taken to minimize pain or discomfort towards animals.

**Preparation of leukocyte homogenate.** Leukocytes were isolated from human blood by gelatin treatment [23]. About 1 g of packed leukocytes (either leukemic or normal) was suspended in 1.2 ml of 25 mM triethanolamine-HCl buffer, pH 7.4, containing 5 mM EDTA and 5 mM β-mercaptoethanol (buffer A) and kept at room temperature for 30 min. The mixture was then cooled to 4°C and homogenized in a tightly fitting glass-Teflon Potter–Elvehjem homogenizer with 20 up and down strokes. Buffer A (1.2 ml) was added to the homogenate, and the sample was processed for GAPDH purification as described below.

**Preparation of muscle homogenate.** Fifteen grams of either normal mouse muscle or sarcoma tissue was homogenized in an Omni GLH International (USA) homogenizer with four volumes of 50 mM triethanolamine-HCl buffer, pH 7.4, containing 10 mM EDTA and 10 mM β-mercaptoethanol (buffer B). The tissue and the buffer were pre-cooled, and all operations were carried out at 0-4°C.

The homogenization was done four times with 1 min homogenization and 1 min intervals. GAPDH was purified from the homogenate as described below.

**Purification of GAPDH from normal muscle and sarcoma tissue and from normal and leukemic leukocytes.** After preparation of the homogenates, the procedures for purification of GAPDH from the four different sources were essentially similar. However, the leukocyte and muscle enzymes were processed in buffer A and buffer B, respectively, and both these buffers are mentioned as buffer. We describe here a composite purification procedure.

The homogenate was centrifuged at 18,000*g* for 20 min and the supernatant was retained. The pellet was resuspended in the buffer and homogenized and centrifuged as above. The pellet was rejected. This supernatant was combined with the previous supernatant and subjected to $(NH_4)_2SO_4$ fractionation. In this and in all other $(NH_4)_2SO_4$ fractionation steps, the pH of the suspension was maintained at 7.4 by the addition of water diluted $NH_4OH$ (1 : 1). The protein that precipitated at 55-90% $(NH_4)_2SO_4$ saturation was dissolved in a minimum volume of the buffer containing 1 mM NAD and 10% glycerol for CML leukocytes and sarcoma tissue and 10% glycerol for normal leukocytes and muscle. Since we had observed that NAD is required for stability of

GAPDH of malignant cells (see above), for all subsequent purification steps, when the enzyme fraction was suspended in the buffer, 1 mM NAD and 10% glycerol were added to the fraction of malignant cell enzyme, whereas 10% glycerol was added to the fraction of normal cell enzymes. The enzyme was purified by gel filtration and ion-exchange chromatography.

**Preparation of polyclonal antiserum against 54 kDa subunit of EAC cell GAPDH.** Antibody titer was raised in a rabbit against 54 kDa subunit of EAC GAPDH by injecting 100 μg protein of this subunit per week four times. The first injection was 100 μg of protein of 54 kDa subunit of EAC GAPDH, which was eluted from gel after SDS-PAGE, plus Freund's complete adjuvant. The next three injections were the same protein but with Freund's incomplete adjuvant. One week after the fourth injection, blood was collected from the immunized rabbit. We prepared serum from immunized rabbit blood; this serum was used as primary antibody against 54 kDa subunit of EAC GAPDH. The purification of EAC cell GAPDH was described previously elsewhere [17].

**Immunoblotting experiment.** Homogenates of different organs of normal rat or EAC or sarcoma 180 cells were prepared in buffer B using the Omni GLH International homogenizer. The homogenates were centrifuged at 15,000*g* for 15 min. The supernatants (crude homogenates) and purified proteins of normal and CML leukocyte GAPDH and normal muscle and sarcoma tissue GAPDH were subjected to SDS-PAGE (7.5% polyacrylamide) and transferred to a 0.45 μm pore size nitrocellulose membrane.

For immunoblot experiment with the polyclonal antiserum of 54 kDa subunit of EAC GAPDH, the membrane was incubated overnight at 4°C in blocking solution of PBS (50 mM phosphate buffer, pH 7.4, containing 0.9% saline) containing 5% skimmed milk. After washing the membrane with PBS containing 2% Tween 20 (PBST), it was then incubated with the primary antibody (1 : 3000 diluted in PBS) for 1 h at room temperature. The membranes were again washed with PBST and then incubated for 1 h with peroxidase-conjugated anti-rabbit IgG (1 : 30,000 diluted in PBS). After washing the membrane in PBST, the immunoreactive bands were developed by 3,3′-diaminobenzidine tetrahydrochloride.

After transferring the proteins in nitrocellulose membrane as above, for α-tubulin and commercially available GAPDH antibody, we incubated the membranes for 2 h in blocking solution of PBS containing 5% skimmed milk. After washing with PBST, membranes were incubated overnight with primary antibody of α-tubulin (1 : 1000 diluted in PBS) and commercially available antibody of GAPDH (1 : 1000 diluted in PBS). The membranes were washed with PBST and then incubated with secondary antibody (1 : 1000 diluted in PBS) for 1 h and washed again with PBST. Immunoreactive bands were developed on X-ray film by luminol reagent.

**Molecular weight determination.** For determination of molecular weight of GAPDH of normal muscle and sarcoma tissue, the samples were gel filtered on a Sephacryl S-200 column using cytochrome *c*, ovalbumin, BSA, and alcohol dehydrogenase as reference marker proteins. For GAPDH of normal and CML leukocytes, the samples were gel filtered on a Sephadex G-100 column using cytochrome *c*, chymotrypsinogen, ovalbumin, BSA, normal rabbit muscle GAPDH, and aldolase as reference marker proteins.

**Test for purity of enzymes and subunit molecular weight determination.** The purity of the enzymes was tested by PAGE using 7.5% polyacrylamide gels under nondenaturing conditions according to the method of Davis [24]. Subunit composition and molecular weight of GAPDH were determined by SDS-PAGE using 7.5% polyacrylamide according to the method of Laemmli [25]. The reference marker proteins were BSA, ovalbumin, carbonic anhydrase, and normal rabbit muscle GAPDH. The protein bands were identified by Coomassie blue (0.1%) staining.

**N-Terminal amino acid sequencing.** Electrophoretically separated subunits of GAPDH from sarcoma tissue were transferred onto a PVDF membrane using a Bio-Rad Mini Trans blot unit (BioRad, USA) and stained with Ponceau S solution. The 54- and 33-kDa subunit bands were analyzed by N-terminal sequencing using a Procise Model 491 protein/peptide sequencer from Applied Biosystems (USA).

**Stability of GAPDH in presence and in absence of NAD.** A 1-ml portion of GAPDH partially purified after the first Sephadex G-100 column step from four different sources (i.e. normal and CML leukocytes and normal muscle and sarcoma tissue) containing 0.5 mg protein and 40 units of the enzymatic activity was passed through a Sephadex G-50 column to remove previously added NAD. Each of the eluted enzymes was kept in two different tubes frozen at −20°C, one with 1 mM NAD and the other with the same amount of working buffer (buffer B) without NAD. After the indicated period of time, aliquots were removed from these tubes and assayed for the enzyme activity. The activity of the enzyme after passing through the Sephadex G-50 column was taken as 100%.

**Enzyme assay and protein estimation.** Unless indicated otherwise, GAPDH was routinely assayed in triethanolamine-HCl buffer, pH 8.5, as described previously [17]. Assays to determine the optimum pH for the enzymatic activity were performed in three different buffers: 50 mM imidazole-HCl (pH 6.4-8.0), 50 mM triethanolamine-HCl (pH 7.4-8.8), and 50 mM glycine-NaOH (pH 8.2-9.6). All the substrates in the assay mixtures were at saturating concentrations.

Using BSA as a standard, protein was estimated using the method of either Lowry et al. or Warburg and Christian as outlined by Layne [26]. Appropriate control was maintained with triethanolamine buffer to correct for

the interference of this compound, particularly with the Lowry method.

## RESULTS

**Purification of GAPDH.** The purification procedure for GAPDH as described in "Materials and Methods" could be conveniently reproduced using different batches of normal and CML leukocytes and also normal muscle and sarcoma tissues. The enzyme fractions after the DEAE-Sephacel column step were purified 138- and 200-fold for normal and CML leukocytes, respectively, and 28- and 96-fold for normal muscle and sarcoma tissues, respectively. The specific activities of GAPDH at this stage were 105 for normal leukocytes, 357 for CML leukocytes (Table 1), 112 for normal muscle, and 313 for sarcoma tissue (Table 2).

The purified GAPDH from these sources generally showed a single band in non-denaturing PAGE. Occasionally one or two minor band(s) appeared (Fig. 1).

**Molecular weight.** The molecular weight of GAPDH purified from various normal mammalian sources is 140 kDa. This enzyme contains four identical subunits of 35 kDa [8]. However, previous work from our laboratory indicated that the EAC cell enzyme is a heterodimer of molecular mass 87 ± 3 kDa and contains two subunits of 54 ± 2 and 33 ± 1 kDa [17]. So, we investigated whether GAPDH of CML leukocytes and sarcoma tissues is similar to the EAC cell enzyme or to GAPDH of other (normal) sources with respect to molecular weight and subunit composition.

We determined and compared the molecular weight of GAPDH of these four sources by gel filtration under non-denaturing condition. By SDS-PAGE, we also determined the number of subunits and Mr of the subunits of these GAPDHs. The details are described in section "Materials and Methods" under "Molecular weight determination". The molecular masses (in kDa) of GAPDHs in non-denaturing condition were found to be: normal leukocyte, 140 ± 2; CML leukocytes, 87 ± 2; normal muscle, 140 ± 2; sarcoma tissue, 89 ± 2. Under identical conditions, rabbit muscle GAPDH showed a molecular mass of 143 ± 2 kDa, eliminating the possibility of any artifact during determination of Mr of malignant cell GAPDHs.

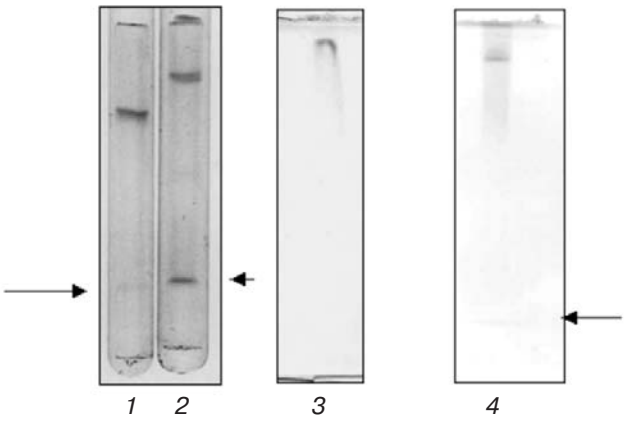


**Fig. 1.** Non-denaturing PAGE of GAPDH purified from normal (*1*) and CML leukocytes (*2*) and normal muscle (*3*) and sarcoma tissue (*4*) (protein load is 5, 5, 6 and 4 μg, respectively). The arrow indicates the dye front.

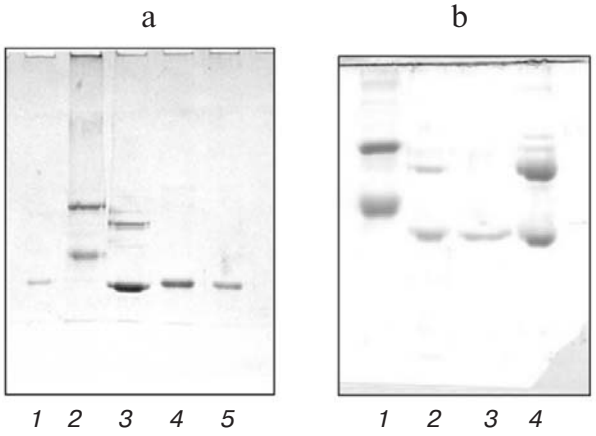


**Fig. 2.** SDS-PAGE of GAPDH purified from normal and CML leukocyte (a) and normal muscle and sarcoma tissue (b). a) Lanes: *1*) normal leukocyte GAPDH (3 μg protein); *2*) BSA (66 kDa) plus ovalbumin (45 kDa); *3*) CML leukocyte GAPDH (10 μg protein); *4*, *5*) rabbit muscle GAPDH (36 kDa), 8 and 5 μg protein, respectively. b) Lanes: *1*) BSA (66 kDa) plus ovalbumin (45 kDa); *2*) sarcoma tissue GAPDH (8 μg protein); *3*) normal muscle GAPDH (6 μg protein); *4*) EAC GAPDH (9 μg protein).

Figure 2 shows that in SDS-PAGE experiments both normal muscle and normal leukocyte enzyme showed a single band corresponding to molecular mass 35 ± 1 kDa, which is similar to that of normal mammalian muscle enzyme such as rabbit muscle GAPDH [8]. In contrast, GAPDH of both CML leukocytes and sarcoma tissues showed two unequal subunits of 54 ± 2 and 33 ± 1 kDa, which is similar to that of EAC cell enzyme as previously reported from our laboratory [17]. These results suggest that the molecular mass and subunits of malignant cell GAPDH might be different from that of the nonmalignant sources.

**Partial sequence of subunits of sarcoma GAPDH.** Because of the unusual nature of the GAPDH of both sarcoma and CML leukocytes, we partially sequenced both the subunits of GAPDH of sarcoma tissue. The partial sequence from the N-terminus for 33- and 54-kDa subunits is presented in Table 3. NCBI Blast Search of the sequences of the two subunits of sarcoma tissue GAPDH had indicated that both 33- and 54-kDa subunits have significant homology with the N-terminus of known GAPDH subunit. For comparison, we present in Table 3 the sequence of the 54 kDa subunit, along with sequence

**Table 1.** Purification of GAPDH from normal (NL) and CML leukocytes (LL)

| Step | Total activity, units | | Total protein, mg | | Specific activity, units/mg | | Purification, fold | | Yield, % | |
|---|---|---|---|---|---|---|---|---|---|---|
| | NL | LL | NL | LL | NL | LL | NL | LL | NL | LL |
| Crude | 56 | 128 | 74 | 72 | 0.76 | 1.78 | 1 | 1 | 100 | 100 |
| 1st $(NH_4)_2SO_4$ | 46 | 90 | 23 | 15.6 | 2.0 | 5.8 | 2.6 | 3.3 | 82 | 70 |
| 2nd $(NH_4)_2SO_4$ | 44 | 84 | 15 | 9 | 2.93 | 9.3 | 4 | 5.2 | 79 | 66 |
| Sephacryl S-200 | 35 | 72 | 1.6 | 1.84 | 21.9 | 39 | 29 | 22 | 62 | 56 |
| 1st Sephadex G-100 | 17 | 38 | 0.56 | 0.47 | 30.4 | 81 | 40 | 45 | 30 | 30 |
| 2nd Sephadex G-100 | 14 | 30 | 0.37 | 0.22 | 37.8 | 136 | 50 | 76 | 25 | 23 |
| 1st DEAE Sephacel | 8 | 17 | 0.087 | 0.05 | 92 | 340 | 121 | 191 | 14 | 13 |
| 2nd DEAE Sephacel | 6.3 | 15 | 0.060 | 0.042 | 105 | 357 | 138 | 200 | 11 | 12 |

**Table 2.** Purification of GAPDH from normal mouse muscle (NM) and sarcoma tissue (ST)

| Step | Total activity, units | | Total protein, mg | | Specific activity, units/mg | | Purification, fold | | Yield, % | |
|---|---|---|---|---|---|---|---|---|---|---|
| | NM | ST | NM | ST | NM | ST | NM | ST | NM | ST |
| Crude | 1820 | 1430 | 460 | 440 | 4 | 3.25 | 1 | 1 | 100 | 100 |
| 1st $(NH_4)_2SO_4$ | 1558 | 1640 | 58 | 54 | 27 | 30 | 7 | 9 | 86 | 114 |
| 2nd $(NH_4)_2SO_4$ | 1516 | 1420 | 37 | 42 | 41 | 34 | 10 | 10 | 83 | 99 |
| Sephacryl S-200 | 860 | 1115 | 16 | 15 | 54 | 74 | 13 | 23 | 47 | 78 |
| 1st Sephadex G-100 | 452 | 625 | 7 | 5.2 | 65 | 120 | 16 | 37 | 25 | 44 |
| 2nd Sephadex G-100 | 232 | 480 | 3 | 3.1 | 78 | 155 | 19 | 48 | 13 | 34 |
| DEAE Sephacel | 133 | 116 | 1.19 | 0.37 | 112 | 313 | 28 | 96 | 7 | 8 |

**Table 3.** Partial sequence of subunits of sarcoma GAPDH

| Protein | Partial N-terminal sequence |
|---|---|
| 33 kDa sarcoma GAPDH (this paper) | **V**N**V**GVNGFGR**IGG**LV VAA |
| 54 kDa sarcoma GAPDH (this paper) | SK**V**GVNGFGRIKRLVTVAA |
| GAPDH (mouse), NCBI Protein Database Acc. No. AAH85315 | **MV**KVGVNGFGRI**G**RLVT**R**AA |
| GAPDH (rabbit), NCBI Protein Database Acc. No. P46406 | **MV**KVGVNGFGRI**G**RLVT**R**AA |
| Tumor protein, transcriptionally controlled, NCBI Protein Database Acc. No. XP_00148705 | **MV**KV**N** GFGRI**R**RLVT**R**AA |

Note: We compared the partial sequence of 54 kDa subunit of sarcoma GAPDH with the partial sequence of other four-subunit/proteins. The differences with the 54 kDa subunit have been marked.

**Table 4.** Kinetic characterization of GAPDH from normal muscle (NM) and sarcoma tissue (ST)

| Substrate | pH | $K_m$, mM | |
|---|---|---|---|
| | | NM | ST |
| Glyceraldehyde-3-phosphate | 8.8 | 0.160 ± 0.005 | 0.110 ± 0.006 |
| | 8.0 | 0.159 ± 0.006 | 0.089 ± 0.005 |
| | 7.3 | 0.149 ± 0.003 | 0.087 ± 0.006 |
| NAD | 8.8 | 0.047 ± 0.006 | 0.040 ± 0.003 |
| | 8.0 | 0.062 ± 0.005 | 0.036 ± 0.002 |
| | 7.3 | 0.071 ± 0.003 | 0.035 ± 0.003 |
| $P_i$ | 8.8 | 4.0 ± 0.2 | 9.9 ± 0.03 |

of the 33 kDa subunit of mouse muscle GAPDH, rabbit muscle GAPDH, and another tumor protein with which it showed similarity.

**Determination of pH optimum and $K_m$.** We investigated the activity of GAPDH of normal and CML leukocytes and normal muscle and sarcoma tissue as a function of pH. For the normal muscle enzyme there was a sharp pH optimum at pH 8.6. But for sarcoma tissue GAPDH there was no sharp pH optimum, and the activities were almost same between pH 7.3 and 8.8. Our previous study on the pH optimum of EAC cell GAPDH showed a similar result with that of the sarcoma tissue enzyme [17].

For both normal and CML leukocyte GAPDH, the increase in activity with increase in pH was similar up to pH 8.8. But when the pH was increased above pH 8.8, for the leukemic leukocyte enzyme the activity showed a sharp fall. However, for normal leukocyte GAPDH there was no similar sharp decline.

The apparent $K_m$ values were determined for normal muscle and sarcoma tissue GAPDH with three substrates (glyceraldehyde-3-phosphate, NAD, and $P_i$) at three pH values (8.8, 8.0, and 7.3) (Table 4).

**Dependence of added NAD on the stability of GAPDH of both leukemic leukocyte and sarcoma tissue.** During previous work from our laboratory on the characterization of EAC cell GAPDH, a distinguishing feature was observed. Unlike GAPDH from other normal sources [8], the stability of the EAC cell enzyme was strongly dependent on externally added NAD [17]. Similar dependence on externally added NAD for the stability of GAPDH of both CML leukocytes and sarcoma tissue had been observed as reported in the present study.

During the course of purification, after dialysis following the first $(NH_4)_2SO_4$ fractionation step, both CML leukocytes and sarcoma tissue GAPDH lost about 80% of its activity when kept frozen overnight at –20°C. For

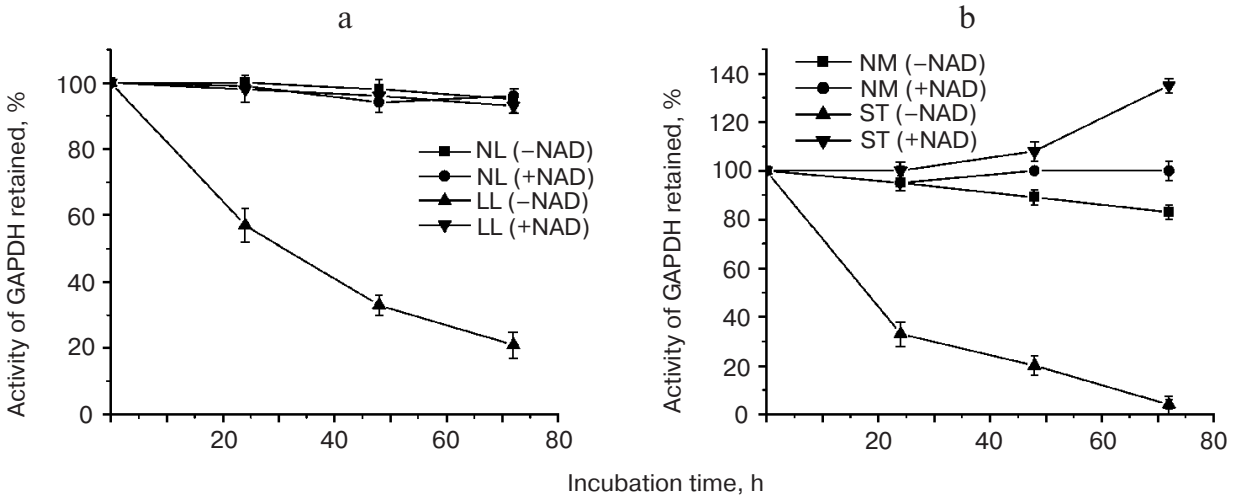


**Fig. 3.** Stability of GAPDH of normal and CML leukocytes (a) and normal muscle and sarcoma tissue (b) in the presence (1 mM) and absence of NAD. The activity of GAPDH at 0 h was taken as 100%. NL, normal leukocyte; LL, CML leukocyte; NM, normal muscle; ST, sarcoma tissue.

GAPDH of both normal muscle and leukocyte there was no appreciable loss of activity. Because we had previously observed that NAD could significantly protect the GAPDH of EAC cells, in the present study we also investigated the role of externally added NAD in protecting the activity of malignant cell GAPDH. The experimental conditions are described in "Materials and Methods".

Figure 3a shows that in the absence of NAD, the CML leukocyte GAPDH lost around 40 and 80% of its activity respectively within 24 and 72 h at −20°C. This GAPDH in the presence of 1 mM NAD, and normal leukocyte GAPDH both in presence and absence of added NAD, could retain more than 90% of their original activity.

A more or less similar pattern was observed when we compared the activity of GAPDH of normal muscle and sarcoma tissue kept frozen in the presence or absence of NAD (Fig. 3b). However, we had observed an increase in the activity from its original activity of sarcoma GAPDH after 72 h kept in frozen condition.

**Inhibition of GAPDH by ATP as function of pH.** Inhibition by ATP is a mechanism for the control of the activity of GAPDH. This inhibition is dependent to some extent on pH. It had been reported previously that ATP is an efficient inhibitor of normal rabbit muscle GAPDH at physiological pH of 7.4 and particularly at a lower pH of 6.8 [27]. The pH of resting muscle is about 7.0, and in fatigued muscle the pH is decreased to below 6.5 due to accumulation of lactic acid [27]. It had also been suggested that this inhibition by ATP is an important mechanism for the control of glycolysis in normal cells. Malignant cells are also highly glycolyzing cells generating high levels of lactic acid and may cause a decrease in the intracellular pH. We therefore undertook a comparative study on the effect of ATP at different pH values on GAPDH of normal muscle and sarcoma tissue and also of normal and CML leukocytes (Table 5).

It appears from Table 5 that this inhibition is less pronounced for sarcoma GAPDH as compared to that of the normal muscle enzyme, particularly at lower pH val-

**Table 5.** Inhibition of GAPDH by ATP at different pH values

| pH | nmol NADH/min | | Inhibition, % |
|---|---|---|---|
| | −ATP | + ATP | |
| **Normal muscle tissue** | | | |
| 6.4 | 2.90 | 1.61 | 44 |
| 6.8 | 3.71 | 2.74 | 26 |
| 7.6 | 6.62 | 5.48 | 17 |
| 8.3 | 7.1 | 6.29 | 11 |
| 8.8 | 5.65 | 5.0 | 12 |
| **Sarcoma tissue** | | | |
| 6.4 | 1.29 | 1.21 | 6 |
| 6.8 | 2.42 | 2.26 | 7 |
| 7.6 | 3.79 | 3.39 | 11 |
| 8.3 | 5.32 | 4.68 | 13 |
| 8.8 | 5.65 | 5.0 | 12 |
| **Normal leucocytes** | | | |
| 6.4 | 25 | 20 | 20 |
| 6.8 | 35 | 32 | 9 |
| 7.4 | 52 | 50 | 4 |
| 8.0 | 71 | 66 | 8 |
| 8.4 | 78 | 75 | 4 |
| 8.7 | 85 | 81 | 4 |
| **CML leukocytes** | | | |
| 6.4 | 34 | 30 | 11 |
| 6.8 | 37 | 33 | 11 |
| 7.4 | 58 | 50 | 12 |
| 8.0 | 67 | 63 | 8 |
| 8.4 | 70 | 66 | 6 |
| 8.7 | 81 | 73 | 13 |

Note: The data are averaged from five individual experiments.

ues. For normal muscle GAPDH, the inhibition progressively increased with the decrease in pH, in contrast to sarcoma GAPDH where the inhibition did not change much with pH.

Table 5 also presents the effect of ATP on both normal and CML leukocyte GAPDH at various pH values. Here we observe that the inhibition of CML leukocyte GAPDH does not change with the change in pH. In contrast, the inhibition of normal leukocyte GAPDH increases with pH decrease.

**Inhibition of normal muscle and sarcoma tissue GAPDH by ATP at various substrate concentrations.** *ATP inhibition with NAD.* ATP was found to be a mixed type inhibitor for rabbit muscle GAPDH with NAD at physiological pH of 7.4 [27]. At pH 9.0, ATP acts as a competitive inhibitor for yeast enzyme with NAD [28]. We studied the nature of inhibition of normal muscle GAPDH and sarcoma tissue GAPDH by ATP with respect to NAD at pH 8.8. As already mentioned, at physiological pH of 7.6, ATP has moderate inhibitory effect (Table 5). The double reciprocal plot of the data indicated a mixed type (nearly competitive) of inhibition by ATP with respect to NAD. By using the Dixon plot, $K_i$ was determined to be 2.1 ± 0.5 mM for normal muscle and 6.0 ± 0.4 mM for sarcoma tissue. By plotting the slope of the reciprocal against inhibitor concentration, the $K_i$ was also calculated and found to be 2.9 ± 0.6 mM for normal muscle and 6.5 ± 0.8 mM for sarcoma tissue.

*ATP inhibition with D-glyceraldehyde-3-phosphate.* The nature of inhibition of ATP with respect to D-glyceraldehyde-3-phosphate at pH 8.8 was found to be noncompetitive for both normal muscle and sarcoma tissue. As previously reported, the same nature of inhibition was obtained with the HeLa cell enzyme [16]. The $K_i$ was calculated from the intersect of the extrapolated lines to the abscissa and found to be 4.4 ± 0.3 mM for normal muscle and 9.6 ± 0.7 mM for sarcoma tissue. A similar $K_i$ value was obtained for D-glyceraldehyde-3-phosphate using the Dixon plot. The noncompetitive nature of the binding suggests that ATP also does not bind to the D-glyceraldehyde-3-phosphate binding site.

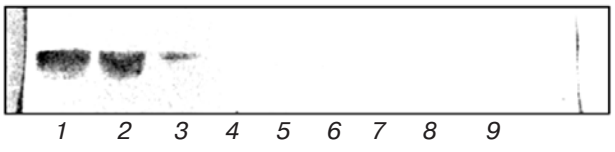


**Fig. 4.** Immunoblots using polyclonal antiserum raised against 54 kDa subunit of EAC cell GAPDH of different malignant cells and different tissues of normal rat. Lanes: *1*) crude homogenates of EAC cell (25 μg protein); *2*) sarcoma 180 (25 μg protein); *3*) purified CML leukocytes (5 μg protein); *4*) normal leukocytes (5 μg protein); *5-9*) crude homogenates (25 μg protein) of skeletal muscle, kidney, intestine, pancreas, and liver of a normal rat, respectively.

**Effect of the reaction product NADH.** *NADH inhibition with NAD.* We also investigated the possible modulating effect of NADH on the enzyme activity of GAPDH at pH 8.8 and it was found that NADH is a strong competitive inhibitor of the enzyme with respect to NAD at pH 8.8. The $K_i$ for NADH was determined by the Dixon plot to be 15 ± 1.2 μM for normal muscle GAPDH and 18 ± 0.9 μM for sarcoma GAPDH at pH 8.8. The nature of inhibition by NADH for both normal and sarcoma GAPDH was found to be competitive at pH 8.8. All these results suggest that NADH binds directly to the NAD binding site.

*NADH inhibition with D-glyceraldehyde-3-phosphate.* We also investigated the inhibition of NADH with various concentrations of D-glyceraldehyde-3-phosphate. The nature of inhibition was found to be noncompetitive for both normal muscle and sarcoma GAPDH. The $K_i$ for NADH determined by the Dixon plot were found to be 19.2 ± 1.0 μM for normal muscle GAPDH and 14.5 ± 1.6 μM for sarcoma tissue GAPDH at pH 8.8. So NADH does not bind to the D-glyceraldehyde-3-phosphate binding site.

**Immunoblot.** As mentioned above and in a previous publication from our laboratory, malignant cell GAPDH can contain an unusual subunit of 54 kDa [17]. It had also been reported that antibody of HeLa cell GAPDH did not cross react with this enzyme from several normal sources [20] suggesting some possible differences of this enzyme of normal and malignant cells. The presence of multiple forms of GAPDH in prostate cells has been reported. Moreover, there was difference in the properties of GAPDH of normal and malignant prostate tissue [21]. So we raised polyclonal antiserum in a rabbit against the 54 kDa subunit of EAC cell GAPDH and tested for the possible cross-reactivity of this polyclonal antiserum with GAPDH of normal and CML leukocytes and normal muscle and sarcoma tissue and several tissue homogenates of normal rat. The results are presented in Fig. 4, which show that the polyclonal antiserum raised against 54 kDa subunit preferentially cross-react with the malignant cell GAPDH, providing further evidence for the presence of 54 kDa subunit in GAPDH of CML leukocyte and sarcoma tissue, which might be a common feature of all malignant cells. We investigated the reactivity of the polyclonal antiserum raised against 54 kDa subunit with the purified GAPDH of sarcoma tissue, normal mouse muscle, and EAC cells. The polyclonal antiserum of the 54 kDa subunit strongly cross-reacted with the 54 kDa subunit and also weakly cross-reacted with 33 kDa subunit. But the intensity of the band is significantly less than that of the 54 kDa subunit (figure not presented). This indicates that there might be some common amino acid sequence in both 33 and 54 kDa subunits, which is corroborated by the results presented in Table 3.

To confirm that GAPDH purified from both normal mouse muscle and mouse sarcoma tissue is a true

GAPDH, we cross-reacted this enzyme from these two sources with commercially available antibody against GAPDH. The results show that GAPDH of both these sources react with this antibody. In both the cases an immunoreactive band appeared at around 33 kDa and no band was visible around 54 kDa even in the sarcoma tissue sample (figure not presented).

Tubulin [29-31] and several other proteins such as SET [32] and Siah1 [31] are known to bind with GAPDH. Of these proteins, α-tubulin is a monomer of 50 kDa, which is similar in molecular mass to the 54 kDa subunit of malignant cell GAPDH as described in this work. So we tested whether commercially available antibody of α-tubulin cross-reacted with the GAPDH of normal muscle and sarcoma tissue of mouse and also of purified tubulin. The crude homogenates of normal mouse muscle and sarcoma tissue and also purified tubulin cross-reacted with the antibody of α-tubulin. In contrast, both purified normal and malignant cell GAPDH did not react. The result indicates that the 54 kDa subunit of malignant cellular GAPDH is not α-tubulin (figure not presented).

## DISCUSSION

The present paper describes the purification of GAPDH from two normal and two corresponding malignant sources. In a limited way, GAPDH of these four sources had also been characterized. Previously, our laboratory had purified this enzyme from rapidly growing, highly dedifferentiated malignant EAC cells. It is remarkable that GAPDH purified in our laboratory from three different malignant sources show some similar properties that are different from the properties of GAPDH purified from other (normal) sources.

The most striking difference in this regard is the molecular weight and subunit composition. While GAPDH of different normal sources including normal human leukocyte and normal mouse muscle is a homotetramer with molecular mass 140 kDa, this enzyme of sarcoma tissue and leukemic leukocyte as presented in this paper and of EAC cells as described previously [17] is a heterodimer of 87 ± 3 kDa containing two unequal subunits of 54 ± 2 and 33 ± 1 kDa. The molecular mass of GAPDH of HeLa cell in non-denaturing condition was not determined [16].

It may be that binding of 33 kDa with the 54 kDa subunit hampers the oligomerization of GAPDH in malignant samples. On the other hand, the association of 54 kDa subunit with the 33 kDa subunit might render the catalytic activity in GAPDH of malignant cells. Although GAPDH is usually catalytically active in its tetrameric form, the presence of catalytically active dimer and monomer has been reported [8, 33]. We attempted to resolve the malignant cell dimeric GAPDH into two subunits by either *p*-hydroxymercuribenzoate or using urea under non-denaturing conditions to assess whether the monomers are catalytically active. However, our attempts to resolve the GAPDH in monomeric form were unsuccessful.

Studies so far reported had identified a single mRNA for the 37 kDa subunit of GAPDH, a homotetrameric protein. However, a unique GAPDH was found to be present in human, mouse, and rat spermatozoa [34]. The cDNA from the human homolog of GAPDH has been cloned and sequenced. The Western blot technique has indicated that the molecular mass of this protein is approximately 56 kDa, whereas the calculated molecular mass from genetic data is 44.5 kDa. Interestingly, the human spermatozoa GAPDH contain 408 amino acids and are 68% identical with somatic cell GAPDH. It has a 72 amino acid segment at the amino terminus that is not present in somatic cell GAPDH [34]. There are reports of appearance of isozymes of GAPDH resulting from amide loss [8]. Moreover, the presence of non-phosphorylating GAPDH of subunit 54 kDa has also been reported [35]. The close association of 54 kDa subunit with 33 kDa of sarcoma tissue and CML leucocytes during different steps of purification suggests that these two subunits are identical components of malignant cell GAPDH. The significant homology of 54 kDa with the known GAPDH suggests that the 54 kDa subunit is a member of isozyme family of GAPDH.

Immunological experiments with the polyclonal antiserum raised against the 54 kDa subunit of EAC cellular GAPDH showed that different malignant tissue samples showed cross-reactivity against this polyclonal antiserum, whereas no cross-reactivity was observed with the nonmalignant tissue samples at the position of the 54 kDa band. However, a weak cross-reactivity was observed at the position of the 33 kDa band.

The apparent association of a 54 kDa subunit polypeptide with the GAPDH of EAC, CML leukocytes, and sarcoma tissue needs further intense investigation for its identification and to ascertain whether this association of 54 kDa polypeptide is a common feature of all malignant cell GAPDHs. There are several reports in the literature that GAPDH can be associated with other proteins, some of which are glycolytic enzymes also. This association can significantly alter the glycolytic flux [36-41]. The presence of a high molecular mass GAPDH has been reported in Huntington's [42] and Alzheimer's diseases [43]. Moreover, GAPDH could be converted to a high molecular mass protein on interaction with a carcinogen-modified protein [44].

Another notable characteristic of the 54 kDa subunit of all these malignant sources is that it is significantly more labile than the 33 kDa subunit. Similar to the present work, the 54 kDa of EAC cell GAPDH has a lower intensity on SDS-PAGE under denaturing conditions. On dialysis or storage, the EAC 54 kDa subunit became

less intense. The preparation buffer also had some influence on band intensity of this subunit [17].

Besides the difference between molecular weight and subunit composition of normal and malignant cell GAPDH, another important characteristic of all the three malignant cell GAPDHs studied is its dependence of added NAD for the stability of the enzymatic activity, suggesting that NAD is not tightly bound at the active site of malignant cell GAPDHs ([17] and this paper). It can be recalled that NAD is tightly bound at the active site of GAPDH of other (normal) cells [8].

It is generally assumed that GAPDH can exert very little control over glycolysis. This idea possibly stems from the fact that *in vitro* the activity of GAPDH of normal cells is very high and this enzyme constitutes a significant portion of the cell protein [8]. However, it has also been suggested that the effective activity of GAPDH in cells is controlled by various factors such as intracellular pH and ATP concentration. Moreover, the change in pH has less influence on the activity of the malignant cell enzyme as compared to the normal counterpart.

Recently several investigations from different laboratories have implicated that GAPDH is not simply a cytosolic enzyme involved in energy production. These investigations have revealed that it is a multifunctional protein in mammalian cells and displays a distinct plasma membrane and nuclear localization also. Besides its already established role in apoptosis [45], GAPDH also functions in endocytosis and membrane fusion, nuclear tRNA transport, DNA replication and repair, translational controls (for a review see [46]). Interestingly despite its multifunctional role, it has been reported that GAPDH could not perform at least some of its functions simultaneously [47]. So it can be assumed that despite being a significant portion of total cellular protein its glycolytic activity is a fraction of its total functions, thereby providing further evidence of this enzyme's role in the control of glycolysis and hence in malignant aberration. This enzyme has recently been identified as a target of antiproliferative agents [48].

So our future research should investigate with a wide variety of malignant cells whether the 54 kDa subunit in GAPDH as reported are common characteristics of all malignant cells and to find the role of this putatively altered subunit on glycolysis and hence on malignant aberrations.

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