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Identification of a critical lysine residue at the active site in glyceraldehyde-3-phosphate dehydrogenase of Ehrlich ascites carcinoma cell

Comparison with the rabbit muscle enzyme

Swapna Ghosh[1], Kasturi Mukherjee[1], Manju Ray[1] and Subhankar Ray[2]

[1]*Department of Biological Chemistry, Indian Association for the Cultivation of Science, Calcutta, India;* [2]*Department of Biochemistry, University College of Science, University of Calcutta, India*

The involvement of the lysine residue present at the active site of Ehrlich ascites carcinoma (EAC) cell glyceraldehyde-3-phosphate dehydrogenase (Gra3*P* DH) was investigated by using the lysine specific reagents trinitrobenzenesulfonic acid (TNBS) and pyridoxal phosphate (PP). Both TNBS and PP inactivated EAC cell Gra3*P* DH with pseudo-first-order kinetics with the rate dependent on modifier concentration. Kinetic analysis, including a Tsou plot, indicated that both TNBS and PP apparently react with one lysine residue per enzyme molecule. Two of the substrates, D-glyceraldehyde-3-phosphate and NAD, and also NADH, the product and competitive inhibitor, almost completely protected the enzyme from inactivation by TNBS. A comparative study of Gra3*P* DH of EAC cell and rabbit muscle indicates that the nature of active site of the enzyme is significantly different in these two cells. A double inhibition study using 5,5′-dithiobis(2-nitrobenzoic acid) and TNBS and subsequent reactivation of only the rabbit muscle enzyme by dithiothreitol suggested that a cysteine residue of this enzyme possibly reacts with TNBS. These studies on the other hand, confirm that an essential lysine residue is involved in the catalytic activity of the EAC cell enzyme. This difference in the nature of the active site of EAC cell Gra3*P* DH that may be related to the high glycolysis of malignant cells has been discussed.

Keywords: glyceraldehyde-3-phosphate dehydrogenase; active site; lysine; cancer.

It has been known for a long time that rapidly growing malignant cells have a high rate of aerobic glycolysis (reviewed in [1]). This phenomenon has been considered by many biochemists to be a fundamental feature of malignancy. Although many explanations have been put forward to explain this high rate of aerobic glycolysis, none of them have fitted properly with the observed lactate flux. However, in the last couple of years, investigations from several laboratories have indicated that glyceraldehyde-3-phosphate dehydrogenase (Gra3*P* DH; EC 1.2.1.12), an important enzyme of the glycolytic pathway, may play a primary role in the high aerobic glycolysis of malignant cells [2–11].

These investigations have indicated a strong enhancement of expression of a protein in different types of malignant cells that is apparently identical with the subunits of Gra3*P* DH [2–4,11]. On the other hand, we have studied the effect of methylglyoxal, a normal metabolite and a potent anticancer agent on Gra3*P* DH, on different normal and malignant cells. These studies have indicated that methylglyoxal inactivates the Gra3*P* DH of a wide variety of malignant cells, but it has no inhibitory effect on this enzyme from cells of several normal tissues and benign tumors [7,12]. These observations suggest that Gra3*P* DH of malignant cells may be modified and that methylglyoxal may act at this modified site. To investigate this, we purified Gra3*P* DH from Ehrlich ascites carcinoma (EAC) cell, a highly dedifferentiated and rapidly growing malignant cell and partially characterized the enzyme [10]. Preliminary results have indicated that structural and catalytic properties of this enzyme may be different from that of other normal sources, suggesting a difference in the primary structure and hence in the active site of the enzyme. In the present paper, we describe our studies with specific amino-acid modifying reagents to explore the nature of active site of Gra3*P* DH of EAC cells and to understand the differences in the active site of this enzyme of normal and malignant cells. Moreover we have partially sequenced the amino-acid residues of the subunits of this enzyme.

Correspondence to M. Ray, Department of Biological Chemistry, Indian Association for the Cultivation of Science, Jadavpur, Calcutta 700 032, India. Fax: + 91 33 473 2805, Tel.: + 91 33 473 4971, E-mail: bcmr@mahendra.iacs.res.in

Abbreviations: EAC, Ehrlich ascites carcinoma; TNBS, trinitrobenzenesulfonic acid; PP, pyrioxal-5′-phosphate; Gra*P*, D-glyceraldehyde-3-phosphate; Gra3*P* DH, glyceraldehyde-3-phosphate dehydrogenase; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

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EXPERIMENTAL PROCEDURES

Materials

All the biochemicals, rabbit muscle Gra3*P* DH and TNBS were purchased from Sigma Chemical Co., St Louis, MO, USA. Pyridoxal 5′-phosphate (PP) and Sephadex G-50 were obtained from Calbiochem and Pharmacia Fine Chemicals, Sweden, respectively. Ehrlich ascites carcinoma cell Gra3*P* DH was purified as described previously [10]. All

other reagents were of analytical grade and obtained from local manufacturers.

The specific activity of Gra3*P* DH of EAC cell [10] and rabbit muscle were approximately 1000 and 100 U, respectively, and the latter is similar with the activity of Gra3*P* DH of other normal sources [12]. The rabbit muscle enzyme showed a single band when PAGE was performed under nondenaturing conditions. In SDS/PAGE, the same enzyme showed a single band of 36 000 Da. As reported previously, in contrast to the property of the enzyme purified from other normal sources, the EAC cell enzyme in its purified form showed a single band of 87 000 ± 3000 Da. In SDS/PAGE, the same enzyme, showed two subunits of 54 000 ± 2000 Da and 33 000 ± 1000 Da [10].

Assay of enzyme and estimation of protein

Unless mentioned otherwise, Gra3*P* DH was routinely assayed in triethanolamine/HCl buffer, pH 8.5 [10]. To monitor the reaction, the increase in absorbance at 340 nm due to the formation of NADH from NAD was noted at 30-s intervals; the rate remained almost linear for 3 min (ΔA: 0.025–0.060 min^{-1}). The assay mixture contained, in a total volume of 1 mL, 50 μmol triethanolamine buffer, 50 μmol Na_2HPO_4, 0.2 μmol EDTA, 1 μmol NAD and 0.2 μmol of D-glyceraldehyde-3-phosphate (Gra*P*). The reaction was started by the addition of an appropriate amount of a solution of Gra*P* containing the requisite amount (0.5 μmol) of Gra*P*. The aqueous solution of Gra*P* was prepared from the water insuluble barium salt of D,L-glyceraldehyde-3-phosphate diethylacetal and the amount of Gra*P* present was measured enzymatically [10]. The enzyme was also assayed by the reverse reaction. The ATP-dependent phosphorylation of 3-phosphoglycerate was catalyzed by phosphoglycerate kinase and the 1,3-bisphosphoglycerate formed was reduced by NADH to Gra*P* by Gra3*P* DH; the oxidation of NADH was monitored at 340 nm [10].

One unit of activity of Gra3*P* DH is defined as the amount of the enzyme required to convert 1 μmol of NAD to NADH per min under standard assay conditions. The specific activity is defined as the units of activity per mg of protein.

Using BSA as a standard, protein was estimated by the method either of Lowry *et al*. or Warburg and Christian as outlined by Layne [14]. Appropriate control was maintained with triethanolamine buffer (where necessary) to correct for the interference of this compound with the Lowry *et al*. method.

Chemical modification experiments

For chemical modification experiments, rabbit muscle Gra3*P* DH (approximately 0.13–0.18 mg of protein containing 12–18 U of activity) or purified EAC cell Gra3*P* DH (0.1–0.15 mg protein containing approximately 90–150 U of activity) was passed through a Sephadex G-50 column (1.1 × 18 cm) previously equilibrated with 50 mM phosphate buffer, pH 8.0 (unless otherwise stated) to remove free NAD and 2-mercaptoethanol. Then the enzymes were reacted with TNBS or PP as described below.

TNBS inactivation

TNBS was dissolved in water and was reacted with sodium glycine buffer, pH 9.0, and analyzed for the adduct at 345 nm assuming an extinction coefficient of 1.45×10^4 M^{-1} [15].

The EAC cell Gra3*P* DH (after the second DEAE–Sephacel step [10]) or the rabbit muscle enzyme was incubated with different concentrations of TNBS. After the indicated time period, aliquots were withdrawn and assayed for the residual enzyme activity. A control tube was maintained with the same amount of the enzyme, but without any TNBS.

Inactivation by PP

Rabbit muscle EAC cell Gra3*P* DH was incubated at 30 °C in 50 mM sodium-phosphate buffer, pH 8.0 containing 1 mM Na/EDTA and in the presence of various concentrations of PP. The incubation mixture was protected from light. At specific time interval, requisite amount of aliquot was withdrawn and assayed for Gra3*P* DH activity. Control tubes were maintained without PP.

In some cases, e.g. in double inhibition and reactivation experiments the incubated reaction mixture after indicated time was passed through a Sephadex G-50 column previously equilibrated with 50 mM phosphate buffer of described pH to stop the enzyme modification or to remove excess modifying reagents. The residual enzyme activity was determined after described addition and experiments were performed.

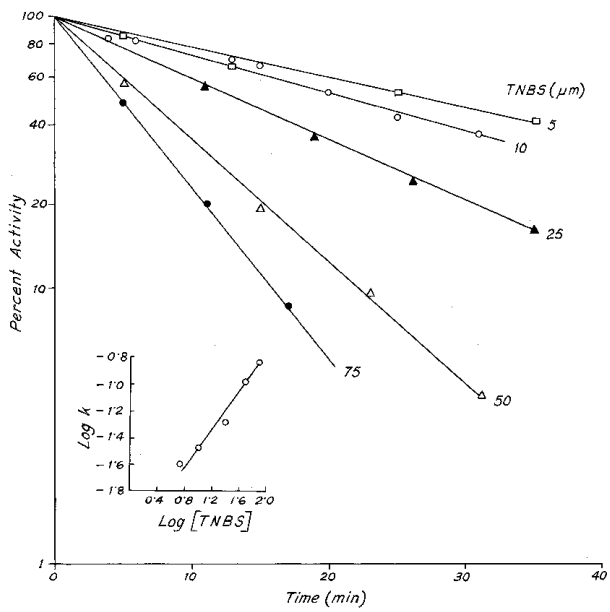

Fig. 1. Kinetics of inactivation of EAC cell Gra3*P* DH by TNBS. The enzyme (35 μg protein·mL^{-1}) was incubated with different concentrations of TNBS in 50 mM phosphate buffer, pH 8.2 at 30 °C. At indicated time intervals, aliquots were removed for the residual enzyme activity. In the control tube the enzymatic rate remained unchanged. Inset shows the plot of log of pseudo-first-order rate constants for inactivation (K_{obs}) obtained at various concentrations of TNBS against log of concentration of the reagent.

The N-terminal amino-acid sequence of the enzyme was determined. Approximately 500 pmol of pure Gra3*P* DH of EAC cells was subjected to SDS/PAGE, transferred to a poly(vinylidene difluoride) membrane and visualized by Ponceau S staining. The region was cut out and partial amino-acid sequence of the subunits was determined using a PPSQ-10 Shimdzu protein sequencer system at the facility provided by Indian Institute of Technology, Mumbai, India.

RESULTS

Two of the substrates of Gra3*P* DH are negatively charged Gra*P* and P_i, which are likely to react with positively charged amino-acid residue(s), e.g. arginine and/or lysine which may be present at the active site of the enzyme. It has already been reported that Gra3*P* DH of muscle of normal rabbit is inactivated by PP, a lysine-specific reagent [13,16,17]. However there has been no systematic study to ascertain whether this inactivation is due to the presence of a lysine residue specifically at the active site of the enzyme of normal sources. Moreover, preliminary evidence has indicated that the catalytic properties of Gra3*P* DH of normal cells and a malignant cell, i.e. EAC cell are significantly different [10,12]. These findings prompted us to investigate whether there is a difference between Gra3*P* DH of EAC cell and rabbit muscle in relation to the presence of critically involved amino-acid residue at the active site of the enzyme.

The α-dicarbonyls such as phenylglyoxal, 1,2-cyclohexanedione,2,3-butanedione known to react with arginine residues in proteins [18,19] when tested could not inactivate the enzyme indicting that this amino acid is not critically involved in the catalytic activity of EAC cell Gra3*P* DH. Therefore, we tested lysine-specific reagents on the catalytic activity of this enzyme

Inactivation of EAC cell Gra3*P*DH by TNBS

Figure 1 shows that TNBS inactivated EAC cell Gra3*P* DH following a pseudo-first-order kinetics. Further kinetic analysis with a plot of log *K* (pseudo-first order rate constant) vs log [TNBS] resulted in a straight line with slope of 1 indicating that at least 1 mol of TNBS per mol of the enzyme was required to produce this inactivation (Fig. 1, inset).

Dependence of TNBS inactivation on pH

The pH-dependence of TNBS inactivation was studied between pH 6.8–9.0 using sodium-phosphate and tricine buffers. We could not use buffers above a pH value of 9.0 due to rapid loss of the enzyme activity of the control sample. It was observed that for the EAC cell enzyme, the rate of inactivation was increased with the increase in pH of the incubation medium (Fig. 2A). With 50 μM TNBS at

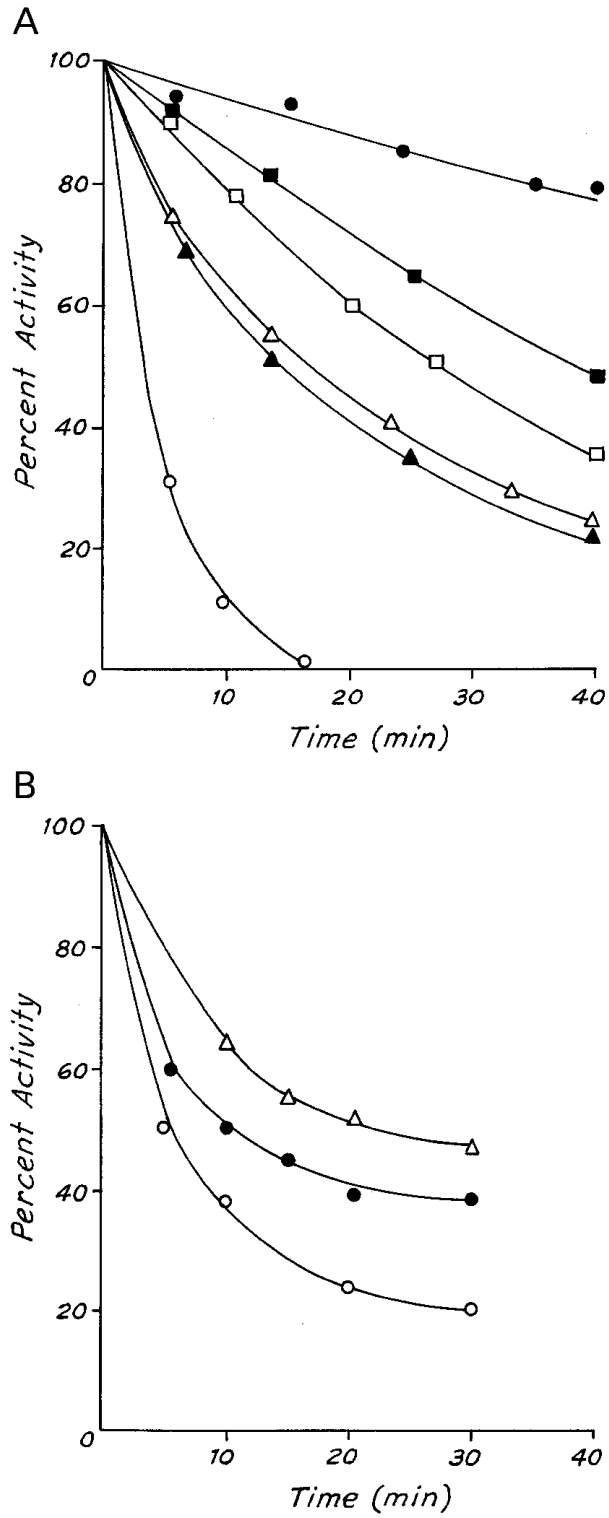

Fig. 2. Inactivation of EAC cell and rabbit muscle Gra3*P* DH **at different pH values.** Each experimental tube contained either 0.3 U of EAC cell Gra3*P* DH or 0.06 U of rabbit muscle Gra3*P* DH in 100 μL of 50 mM sodium phosphate or tricine buffers of different pH values and incubated at 30 °C in different tubes in presence of TNBS (10 μM for the EAC enzyme, 30 μM for the rabbit muscle enzyme). After indicated time intervals, aliquots were removed for measuring the enzyme activity. For both the enzymes, control tubes were maintained at the respective pH values. Percentage activity was calculated by assuming the activity of the enzyme of the control tube as 100%. EAC enzyme (A) buffers and pH were: tricine 9.0 (○), 8.2 (▲); phosphate 8.2 (△), 8.0 (□), 7.4 (■), 6.8 (●). Rabbit muscle enzyme (B) buffer and pH were: phosphate 8.2 (△), 7.4 (●), 6.8 (○).

pH 9.0 and pH 8.2, this enzyme was inactivated by about 90% and 50%, respectively, within 10 min of incubation. However, only about 10% inactivation was observed in the same period of time at pH 6.8.

In contrast, the rabbit muscle enzyme was inactivated by about 60% with 50 μM TNBS at pH 6.8 after 10 min of incubation. The rate of inactivation was further decreased to 35% with the increase in pH to 8.2 of the incubation medium (Fig. 2B). Because TNBS reacts rapidly with thiols [20] and it has also been observed that this reagent reacts more readily with cysteine residues at lower pH values and with lysine residues at higher pH values [21], these results suggest that modification of lysine residues by TNBS results in the inactivation of the EAC cell enzyme, whereas, the inactivation of the rabbit muscle enzyme might be due to the reaction of TNBS with a cysteine residue.

Stoichiometry of modification of the EAC cell Gra3*P*DH by TNBS

As the kinetic order of inactivation was close to 1, i.e. 0.93 (Fig. 1, inset) the minimal number of lysine residue(s) that are involved in the inactivation process can be taken to be one. However, the limitation of the kinetic method for determination of the number of amino-acid residue(s) and also of the stoichiometry of the reaction was indicated by Levy *et al*. [22]. Therefore the stoichiometry of lysine modification was studied by spectral quantitation of the trinitrophenylated protein, using the published molar extinction coefficient of 1.4×10^4 at 345 nm [15]. The EAC cell Gra3*P*DH after treatment with TNBS showed a rapid development of spectrum with an absorption maximum at 345 nm. Figure 3 shows the relationship between the loss of enzymatic activity and the number of lysine residue(s) modified. Extrapolation of the linear plot to zero enzyme activity shows that four residues are modified during complete inactivation. As this method does not usually give the precise number of residue(s) essential for activity, the statistical method of Tsou [23] was used to calculate the number of essential lysine residues for inactivation.

If we assume that all the n modifiable residues including essential residue(s) are approximately equally reactive towards the reagent and modification of any of the essential residue(s) results in complete inactivation, the relationship between the residual activity against lysine modification will be as follows:

$$(A/A_o)^{1/i} = (n - m)/n$$

The number of the essential lysine residue is that value of i which gives a straight line when the residual activity (A/A_o) is plotted against m, i.e. the number of lysine residue(s) modified. From Fig. 3 it appears that Gra3*P*DH activity is dependent upon modification of one critical lysine residue.

Inactivation of EAC cell Gra3*P*DH by PP

The specific reactivity of PP with the lysine residue at the active center of various enzymes, and also our findings that TNBS inactivates EAC cell Gra3*P*DH, prompted us to use PP also for the identification of the essential amino acid at the active site of EAC cell Gra3*P*DH. Treatment of EAC cell Gra3*P*DH with PP resulted in a strong and rapid inactivation of the enzyme (Fig. 4). At a concentration of 1.2 mM, PP inactivated the EAC cell enzyme to the extent of about 90% within 15 min; whereas the rabbit muscle enzyme retains almost 90% activity with the same concentration of PP. The rabbit muscle enzyme could be inactivated to the extent of about 60% with 5 mM PP in 15 min.

The rate of inactivation of the EAC enzyme was a function of the reagent concentration although at any

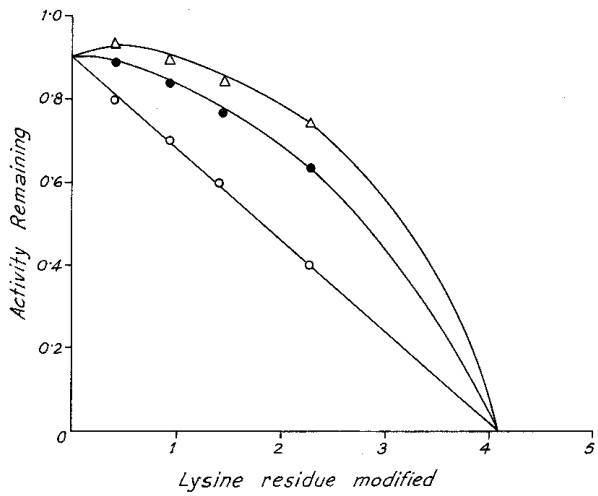

Fig. 3. Correlation between the number of lysine residue(s) modified by TNBS and the residual enzyme activity of EAC cell Gra3*P*DH. The enzyme (2.2 μM) was incubated at 30 °C in 50 mM phosphate buffer, pH 8.0 in presence of 100 μM TNBS. The residual activity and the number of lysine residue(s) modified were measured as described in Experimental procedures. The data are presented as a Tsou plot; for $i = 1$ (○), $i = 2$ (▲), $i = 3$ (△).

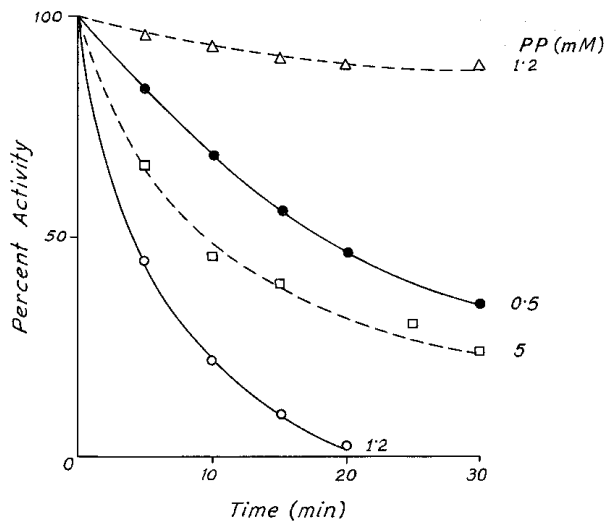

Fig. 4. Inactivation of Gra3*P*DH by PP. The EAC enzyme (36 μg·mL^{-1}) or the rabbit muscle enzyme (43 μg·mL^{-1}) were incubated in different tubes with different concentrations of PP as indicated in the figure. At indicated time, aliquots were removed and assayed for the enzyme activity: Solid lines, EAC; dotted lines, rabbit muscle.

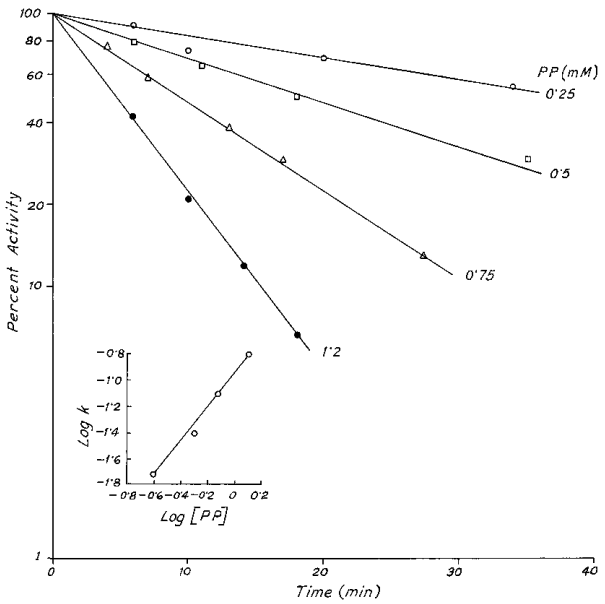

Fig. 5. Kinetics of inactivation of EAC cell Gra3*P*DH by PP. The enzyme (42 μg protein·mL^{-1}) was incubated with various concentrations of PP at 30 °C. At the indicated time intervals aliquots were removed for measurement of the residual enzyme activity. The residual enzyme activity (percentage) was plotted assuming the activity of the enzyme of the control tube as 100. Inset, plot of log of pseudo-first-order rate constant for inactivation (K_{obs}) obtained at various concentrations of PP against log of concentration of the reagent.

particular concentration, the reaction followed pseudo-first-order kinetics. Plot of the log of pseudo-first-order rate constant against the log of the corresponding PP concentration resulted in a straight line with a slope of 1 (Fig. 5) indicating that the inhibition of Gra3*P*DH activity by PP is due to the modification of at least one essential lysine residue on every active unit of the enzyme.

Test for the reactivation by thiol containing reagents of the TNBS- and PP-inactivated Gra3*P*DH

As mentioned before, TNBS is known to react with cysteine residue at lower pH values [21]. To test the whether TNBS or PP reacted with SH-group(s) of EAC cell Gra3*P*DH, we performed the reactivation experiment with thiol containing reagents 2-mercaptoethanol and dithiothreitol.

The results of the above experiment are presented in Table 1, which shows that the EAC cell enzyme inactivated by TNBS or PP could not be reactivated on incubation with either dithiothreitol (10 mM) or 2-mercaptoethanol (10 mM). Increasing the concentration of dithiothreitol or 2-mercaptoethanol and/or increasing the incubation time did not result in any reactivation of the enzyme.

In contrast, when the reactivation experiment was performed in a similar manner with TNBS (100 μM)-inactivated rabbit muscle Gra3*P*DH, the enzyme was reactivated on incubation with dithiothreitol and 2-mercaptoethanol (Table 1). The activity of the TNBS inactivated enzyme was found to be restored to about 80% in presence of dithiothreitol. Similarly, 2-mercaptoethanol can reactivate the TNBS-inactivated enzyme. The PP-inactivated rabbit muscle Gra3*P*DH could also be reactivated to some extent by both dithiothreitol and 2-mercaptoethanol (Table 1).

These results strongly suggest that TNBS and PP react with lysyl residue of EAC cell Gra3*P*DH; whereas TNBS reacts with SH-group in the case of rabbit muscle enzyme. Inactivation of rabbit muscle Gra3*P*DH with high concentration of PP [16,17] might also be due to the reaction of PP with SH-group or with a lysine residue that may be present, but not at the active site of the enzyme.

Table 1. Inactivation of Gra3*P*DH of EAC cells and rabbit muscle by TNBS or PP and reactivation of the enzymes by thiol-containing compounds. Approximately 5 U of the EAC enzyme or 1 U of the rabbit muscle enzyme was incubated in 0.5 mL of 50 mM phosphate buffer, pH 8.0 with indicated concentrations of TNBS or PP. After 30 min of incubation, the enzyme activity in an aliquot of the incubation mixture was measured, which indicated that the enzyme activity was inactivated to the extent of 80–90%. From the residual part of the incubation mixture, after removing excess TNBS or PP, 0.43 U of the EAC enzyme or 0.11 U of the rabbit muscle enzyme was incubated in a total volume of 200 μL 50 mM phosphate buffer, pH 8.0 with different concentrations of 2-mercaptoethanol or dithiothreitol. A tube each containing either the inactivated rabbit muscle or EAC cell enzyme in buffer but with no thiol compounds served as the control.

	Addition to the complete system	Activity retained (%)
EAC	None	100
	TNBS (50 μM)	11
	TNBS (50 μM) + dithiothreitol (10 mM)	9
	TNBS (50 μM) + 2-mercaptoethanol (10 mM)	7
	PP (1.2 mM)	7
	PP (1.2 mM) + dithiothreitol (10 mM)	4
	PP (1 mM) + 2 mercaptoethanol (10 mM)	3
Rabbit muscle	None	100
	TNBS (100 μM)	11
	TNBS (100 μM) + dithiothreitol (10 mM)	87
	TNBS (100 μM) + 2-mercaptoethanol(10 mM)	76
	PP (5 mM)	24
	PP (5 mM) + dithiothreitol (10 mM)	41
	PP (5 mM) + 2-mercaptoethanol (10 mM)	36

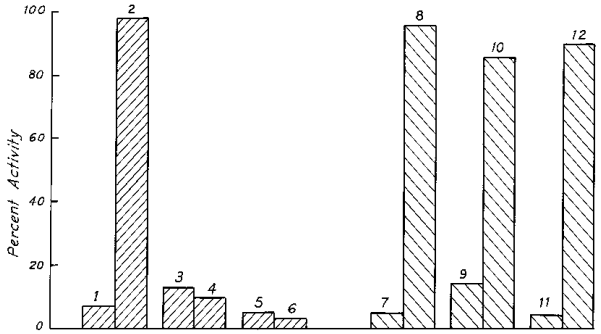

Fig. 6. Reversal of the activity by dithiothreitol of EAC cell and rabbit muscle Gra3*P* DH inactivated by DTNB and/or TNBS. The rabbit muscle (0.13 mg protein, 14 U of activity) or EAC cell (0.1 mg protein, 100 U of activity) Gra3*P* DH was incubated for 15 min in presence of 100 μM and 50 mM TNBS, respectively, and/or DTNB (50 μM for 5 min). After indicated period of time, the residual enzymatic activity was measured by taking an aliquot. The remaining inactivated enzyme after removing excess reagent was then allowed to react with 10 mM dithiothreitol for 20 min and assayed for the enzymatic activity. EAC cell enzyme: bar 1, DTNB; bar 2, DTNB + dithiothreitol; bar 3, TNBS; bar 4, TNBS + dithiothreitol; bar 5, DTNB + TNBS; bar 6, DTNB + TNBS + dithiothreitol. Rabbit muscle enzyme: bar 7, DTNB; bar 8, DTNB + dithiothreitol; bar 9, TNBS; bar 10, TNBS + dithiothreitol; bar 11, DTNB + TNBS; bar 12, DTNB + TNBS + dithiothreitol. For bars 5, 6, 11 and 12 the order of addition of DTNB and TNBS had been reversed but same results were obtained.

Double inhibition studies with DTNB and TNBS

Gra3*P* DH from various sources contains a very reactive cysteine residue at the active site of the enzyme [13]. Involvement of reactive SH-group at the active site of EAC cell Gra3*P* DH was also observed. We have found that this enzyme is strongly inactivated by the thiol reagent DTNB (Fig. 6). Moreover, the inactivated enzyme could be almost completely reactivated by dithiothreitol. By taking advantage of this inactivation–reactivation, we performed a double inhibition experiment by TNBS and DTNB in order to ascertain whether TNBS binds to the lysine residue or to the SH-group of EAC cell Gra3*P* DH.

In one of these experiments, the enzyme was first inactivated by DTNB and then further treated with TNBS. If both the reagents could react with the thiol group, then modification with DTNB would protect the thiol against subsequent reaction with TNBS and hence the activity would be at least partially reversed after final incubation with dithiothreitol. If on the other hand, the loss of activity by TNBS was due to modification of a lysine residue, then the initial modification with DTNB would fail to provide protection against subsequent irreversible reaction by TNBS. In that case, final incubation with dithiothreitol would be unable to regenerate any activity. As shown in Fig. 6 (bars 5 and 6), the EAC cell Gra3*P* DH was first inactivated by DTNB and then treated with TNBS. This inactivated enzyme could not be reactivated by dithiothreitol indicating that TNBS reacted with an essential lysine residue of the EAC cell enzyme. Reversing the order of addition of DTNB and TNBS also resulted in a similar effect. Moreover, dithiothreitol had no reactivating effect on the enzyme inactivated by TNBS alone (bars 3 and 4.).

In contrast, Gra3*P* DH from rabbit muscle inactivated either by DTNB or TNBS could be reactivated by dithiothreitol (Fig. 6, bars 7–10). In the double inhibition experiment, the muscle enzyme was first inactivated by DTNB and then treated with TNBS. In this case, almost complete reactivation of the enzyme activity was obtained on treatment with dithiothreitol (Fig. 6, bars 11 and 12). Changing the order of addition of DTNB and TNBS also yielded the same results (data not shown). These results clearly show that when DTNB is added first, it blocks the reaction of TNBS with the essential thiol group, indicating that both TNBS and DTNB bind to the same thiol group present in the active site of rabbit muscle Gra3*P* DH.

All these studies convincingly demonstrate that the loss of the enzymatic activity on treatment with TNBS was due to the modification of a unique lysine residue of EAC cell Gra3*P* DH and of a cysteine residue of rabbit muscle Gra3*P* DH.

Protection of the activity of EAC cell Gra3*P*DH by the substrates against TNBS- and PP-inactivation

The substrates Gra*P* and NAD were found to protect the enzyme activity against the inactivation by TNBS or PP. NADH, which is a powerful competitive inhibitor with respect to NAD, also afforded almost complete protection against this inactivation (Table 2).

At a concentration of 0.1 mM, which is 2.5 times its K_m value of 0.04 mM, Gra*P* afforded almost complete

Table 2. Protection of the enzymatic activity by substrates Gra*P* and NAD and other nicotinamide nucleotides against TNBS- or PP-inactivation of EAC cell Gra3*P*DH. Each experimental tube containing 0.26 U of the enzyme in 200 μL of 50 mM phosphate buffer, pH 8.0 was incubated with indicated compounds but without TNBS or PP. Three tubes were maintained that contained the same unit of the enzyme in the buffer but no potential protective compounds. After 1 min, indicated concentrations of TNBS or PP was added in the respective tubes and incubated for 30 min. Then equal aliquots were taken from each tube and assayed for the enzymatic activity. The enzymatic activity of the control tube was considered as 100%.

Addition	Activity retained (%)
Control	100
+ TNBS (25 μM)	18
+ Gra*P* (0.05 mM) + TNBS (25 μM)	88
+ Gra*P* (0.1 mM) + TNBS (25 μM)	96
+ NAD (0.1 mM) + TNBS (25 μM)	79
+ NAD (0.2 mM) + TNBS (25 μM)	92
+ NADH (0.05 mM) + TNBS (25 μM)	82
+ NADH (0.1 mM) + TNBS (25 μM)	97
+ NADP (0.4 mM) + TNBS (25 μM)	20
+ NADPH (0.2 mM) + TNBS (25 μM)	17
+ PP (0.75 mM)	10
+ Gra*P* (0.1 mM) + PP (0.75 mM)	82
+ NAD (0.2 mM) + PP (0.75 mM)	87
+ NADH (0.1 mM) + PP (0.75 mM)	79
+ NADP (0.4 mM) + PP (0.75 mM)	8
+ NADPH (0.2 mM) + PP (0.75 mM)	12

protection. Similarly, NAD could provide complete protection at a concentration of 0.2 mM, which is five times its K_m value of 0.04 mM.

At a concentration of 0.1 mM, NADH (K_i 10 μM) could also protect the enzyme from TNBS or PP inactivation. Moreover, NADP and NADPH, which are not substrates or competitive inhibitors for Gra3*P* DH and are supposed to have no binding interaction with the substrate binding site of the enzyme, even at higher concentrations failed to protect the enzyme activity against TNBS or PP inactivation. The small amount of the substrates Gra*P* or NAD or the competitive inhibitor NADH transferred along with the aliquot from the incubation medium to the enzyme assay mixture had no additional effect on the enzymatic rate.

These results confirm that the lysine residue(s) that reacted with TNBS or PP could be completely protected in presence of the substrates Gra*P* or NAD or the competitive inhibitor NADH are therefore located at the substrate binding region of the EAC cell enzyme.

Partial sequence of the subunits of EAC cell Gra3*P*DH

As reported in our previous paper [10] and mentioned above, in contrast to other normal cellular enzyme, Gra3*P* DH of EAC cells is a heterodimer containing two subunits of M_r 54 000 ± 2000 and 33 000 ± 1000.

Therefore we partially sequenced both the subunits of this enzyme. The sequences for M_r 33 000 and 54 000 were found out to be VIVGVNGKGRIGSLVSDDLI and KDLQQWATWTDETWTL, respectively.

DISCUSSION

In the recent past, work from various laboratories has indicated the involvement of Gra3*P* DH in the high glycolytic ability of malignant cells [2–11]. However, this enzyme has been purified only from two malignant cells, HeLa [5] and EAC [10] and partially characterized. Although limited, these studies on the characteristics of the malignant cell enzyme strongly suggest that this enzyme may be significantly different from that of other normal sources in respect to catalytic activity [10] and immunological [6] and structural properties. Therefore, in this paper we investigated the amino-acid residue(s) that are critically involved at the active site of the EAC cell enzyme and whether there is any difference between the malignant and normal cell form by taking rabbit muscle Gra3*P* DH as a representative of the normal cell enzyme.

Studies with the specific lysine modifying reagents TNBS and PP under different reaction conditions provide strong evidence for the presence of a lysine residue at the active site of EAC cell Gra3*P* DH and also suggest a significant difference between the active sites of the malignant cell enzyme and the rabbit muscle enzyme.

The primary structure of Gra3*P* DH had been established from several normal sources [12]. Comparison of these sequences shows that 60% of the amino-acid residues occur in identical sequences indicating that the sequence of Gra3*P* DH has been conserved to a much greater extent than the sequences of other similar enzymes.

In the present work, we have partially sequenced the two subunits of the EAC enzyme: 16 amino acids for the M_r 54 000 ± 2000 subunit and 20 amino acids for the M_r 33 000 ± 1000 subunit. It appears that the smaller subunit has significant homology but it is not identical to the subunit of the enzyme of other normal sources. The presence of the 54 000 subunit in the EAC enzyme appears to be very peculiar. We are unable to assign any function or provide any explanation for the presence of this subunit and also find any similarity with any known protein/subunit. Although conserved in structure, there are reports in the literature that Gra3*P* DH is present in isozymic forms [8], it can remain associated with actin in tumor cells [24], and it can form complexes with other enzymes [25,26]. The presence of a nonphosphorylating Gra3*P* DH of subunit M_r 54 000 had also been reported [27].

Reaction of rabbit muscle holo-Gra3*P* DH with PP resulted in total inactivation, and this inactivation is specific for Lys191 and Lys212 [16]. With the apo-enzyme on the other hand, PP reacted with Lys212 only indicating a conformational change involving Lys191 took place when NAD was removed [17]. It is also possible that at high concentration, PP may remove NAD from the rabbit muscle holoenzyme resulting in conformational change. Another lysine residue, Lys183, present in the rabbit muscle holoenzyme had been shown to have no role in the catalytic activity of this enzyme [13]. It is of interest to note that Lys212 and Lys191 are conserved in all the sequenced species of Gra3*P* DH, but Lys183 is not conserved [13]. Moreover, because NAD is possibly not bound in Gra3*P* DH of EAC cells [10], the conformation of this enzyme may be different, which may impart the catalytic role to an amino-acid residue that has no catalytic role in other normal cellular enzymes.

As mentioned above, this enzyme has been purified from only two malignant cells and the complete primary structure is yet to be determined. The recent experimental evidence from several laboratories has clearly raised the possibility that this enzyme may be altered in malignant cells.

One important limitation of the present study is that we have compared the properties of Gra3*P* DH that originated from two different tissues as well as from two different species. A study of the enzyme from similar sources, e.g. liver and hepatoma or normal and leukemic leukocytes is necessary to understand whether the difference as suggested in this and other papers [10,12] is a fundamental feature of malignancy.

Although it is generally assumed that the major glycolytic control is exerted by the hexokinase-phosphofructokinase system, there is ample evidence that Gra3*P* DH could act as a regulatory enzyme in response to the NAD : NADH and ATP : ADP × P_i ratios in the cells [13]. Moreover we had previously shown that the catalytic potential of EAC cell Gra3*P* DH is much higher than that for other normal sources. In contrast to the rabbit muscle enzyme, this enzyme is not significantly inhibited by a physiological concentration of ATP at physiological pH [10]. Experiments with cell-free extracts of EAC cells have also shown that Gra3*P* DH may significantly contribute in the glucose-dependent L-lactic acid formation in these cells [7].

All these studies point to the difference between Gra3*P* DH of normal and malignant cells that can be truly resolved by determining the full length sequence of this enzyme from a malignant cell.

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