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Evidence for the presence of a critical histidine residue at the active site in glyceraldehyde-3-phosphate dehydrogenase of Ehrlich ascites carcinoma cells

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Ehrlich ascites carcinoma (EAC) cell glyceraldehyde-3-phosphate dehydrogenase (GA3PD) (EC. 1.2.1.12) was completely inactivated by diethyl pyrocarbonate (DEPC), a fairly specific reagent for histidine residues in the *p*H range of 6.0-7.5. The rate of inactivation was dependent on *p*H and followed pseudo-first order reaction kinetics. The difference spectrum of the inactivated and native enzymes showed an increase in the absorption maximum at 242 nm, indicating the modification of histidine residues. Statistical analysis of the residual enzyme activity and the extent of modification indicated modification of one essential histidine residue to be responsible for loss of the catalytic activity of EAC cell GA3PD. DEPC inactivation was protected by substrates, D-glyceraldehyde-3-phosphate and NAD, indicating the presence of essential histidine residue at the substrate-binding region of the active site. Double inhibition studies also provide evidence for the presence of histidine residue at the active site.

Keywords: Glyceraldehyde-3-phosphate dehydrogenase, Ehrlich ascites carcinoma cell, active site histidine, diethyl pyrocarbonate (DEPC), inactivation

An important characteristic of rapidly growing malignant cells is their capacity of high aerobic glycolysis[1,2]. Glyceraldehyde-3-phosphate dehydrogenase (GA3PD) (EC 1.2.1.12), an important enzyme of the glycolytic pathway, catalyzes the phosphorylation of D-glyceraldehyde-3-phosphate (GAP) to 1,3-bisphospho-glycerate. Several studies have suggested the involvement of this enzyme in malignant aberrations[3-7]. Investigation from our laboratory also indicated that methylglyoxal, a normal metabolite (ref. 1 and refs. cited therein) inhibited glycolysis and mitochondrial respiration of Ehrlich ascites carcinoma cell (EAC), a rapidly growing, highly dedifferentiated malignant cell[8]. Moreover, GA3PD of EAC cells was strongly inactivated by methylglyoxal, whereas this enzyme from liver and skeletal muscle of normal mice was very little inactivated[8]. Methylglyoxal also inactivated GA3PD obtained from human post-operative malignant tissues, but not from normal (non-malignant) human tissues and benign tumours[9]. These observations strongly suggest that in malignant cells this enzyme may be critically altered.

Unlike GA3PD from normal cells, which is a homotetramer containing subunits of Mr 36,000, GA3PD from EAC cells was found to be a heterodimer of Mr 33,000 and 54,000[10]. The subunits of GA3PDH from EAC cells had been partially sequenced from N-terminal ends. The smaller subunit appears to be similar with the mammalian enzyme, whereas, the larger subunit appears to be unique[10]. The specific activity of the malignant cell enzyme was also found to be significantly higher, compared to the normal cellular enzyme. The EAC cell GA3PD was found to be active over a wide range of *p*H and not inhibited by physiological concentration of ATP at physiological *p*H. Another distinguishing characteristics of EAC cell GA3PD is that NAD is comparatively loosely bound, than in the normal cell. All these properties raise the possibility that enhanced glycolysis of malignant cells, at least in part may be due to the alteration(s) of this enzyme in these cells. Moreover, since the structural and catalytic properties are apparently different, it is quite likely that architecture of the active site of EAC cell enzyme may also be different. A recent study from our laboratory has indicated a difference in the active site of this enzyme of normal and

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Abbreviations: EAC, Ehrlich ascites carcinoma; GA3PD, glyceraldehyde-3 phosphate dehydrogenase; GAP, D-glyceraldehyde-3-phosphate; DEPC, diethyl pyrocarbonate; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); DTT, dithiothreitol.

malignant cells in relation to the involvement of a lysine residue[11]. The present report describes studies with specific histidine modifying reagent to further explore the nature of the active site of GA3PD of EAC cells and to understand other differences in the active site of this enzyme of normal and malignant cells.

Materials and Methods

Materials

Diethylpyrocarbonate was a product of Aldrich Chemical Co. Sephadex G-50, Sephadex G-100, Sephacryl S-200 and Resource Q column materials were products of Pharmacia Fine Chemicals. D-Glyceraldehyde-3-phosphate (GAP), NAD, NADP, NADPH, dithiothreitol (DTT), and 5,5′-dithiobis-(2-nitrobenzoic acid) [DTNB] were obtained from local manufacturers.

GA3PD was purified to homogeneity from EAC cells, as described previously[10]. This enzyme in non-denaturing PAGE showed a single band. In SDS-PAGE, it showed two subunits of Mr 54,000 ± 2,000 and 33,000 ± 1,000[10]. The specific activity of the enzyme was about 900 Units/mg protein, where 1 unit of the enzyme could convert 1 μmol of GAP to 1,3-bisphosphoglycerate per min under standard assay conditions (see below). The specific activity is defined as the units of activity per mg of protein.

Enzyme assay and protein estimation

Unless indicated otherwise, GA3PD was routinely assayed in triethanolamine/HCl buffer, *p*H 8.5[12]. To monitor the reaction, the increase in absorbance at 340 nm due to the formation of NADH from NAD was noted at 30 sec intervals, the values remained almost linear for 3 min (ΔA: 0.025 to 0.06 min^{-1}). The assay mixture in a total volume of 1 ml contained 50 μmol triethanolamine buffer, 50 μmol Na_2HPO_4, 0.2 μmol EDTA, 1 μmol NAD and 0.5 μmol of GAP. The reaction was started by the addition of an appropriate amount of a solution of D, L-glyceraldehyde-3-phosphate, which contained the requisite amount (0.5 μmol) of GAP. The aqueous solution of GAP was prepared from the water-insoluble barium salt of D, L-glyceraldehyde-3-phosphate diethylacetal and the amount of GAP present was measured enzymatically[12].

The enzyme was also assayed by the reverse reaction[13]. ATP-dependent phosphorylation of 3-phosphoglycerate was catalyzed by phosphoglycerate kinase and the 1, 3-bisphosphoglycerate formed was reduced by NADH to GAP by GA3PD; the oxidation of NADH was monitored at 340 nm. The rate of the reaction was ~ 1.2 times higher than that of the forward reaction with GAP as the substrate.

Protein was estimated using the method of either Lowry *et al*. or Warburg and Christian as outlined by Layne[14], using BSA as a standard. Appropriate control was maintained with triethanolamine buffer to correct for the interference of triethanolamine, particularly with Lowry *et al*. method.

Enzyme modification experiments

Stock DEPC was freshly diluted in absolute ethanol to prepare a working stock solution of required concentration. Since the concentrations of commercial products were variable owing to hydrolysis, the concentration of DEPC was determined spectrophotometrically by reaction with L-histidine[15]. An aliquot of the diluted solution was added to 20 m*M* L-histidine in 20 m*M* potassium phosphate buffer (*p*H 7.0). The increase in absorbance due to the formation of N-carbethoxy-histidine was measured at 230 nm, assuming a molar extinction coefficient of 3.0×10^3 cm^{-1} M^{-1} (refs 15, 16).

Carbethoxylation was carried out by incubating the enzyme with an appropriately diluted DEPC solution in 20 m*M* potassium phosphate buffer (*p*H 7.0). The final concentration of ethanol in the reaction mixture ranged from 1 to 10% by volume and was found to have no significant effect on the activity and stability of the enzyme during the incubation period. The extent of the inactivation was determined by measuring the residual enzyme activity at 25°C in an aliquot removed from the reaction mixture, as described below. The enzyme activity was determined in both the forward and reverse reactions. The extent of inactivation was almost the same in both the assays. Unless otherwise indicated, the activity measurements were usually monitored by the formation of NADH from NAD. The amount of N-carbethoxy histidine in GA3PD in different stages of inactivation was calculated by difference spectra using a molar absorptivity of 3.2×10^3 M^{-1} cm^{-1} at 240 nm[15]. The value of k' (first order rate constant for hydrolysis of DEPC) was obtained by measuring the amount of DEPC remaining at various times on treatment with 20 m*M* histidine in 20 m*M* potassium phosphate buffer (*p*H 7.0), in the absence of the enzyme as described above.

Results

Inactivation of GA3PD by DEPC

When GA3PD was incubated with DEPC in 20 m*M* potassium phosphate buffer (*p*H 7.0), a time-dependent loss of activity was observed. DEPC is readily hydrolysed in aqueous solution, the half-life being

dependent on temperature, *p*H, concentration and composition of the buffer[16-18]. Correction for decomposition of DEPC in buffered solution and the remaining activity at different time after DEPC inactivation is described by the following equation:

$$\ln (A/A_o) = (- k/k') I_o (1 - e^{-k't}) \quad \ldots (1)$$

where A/A_o denotes per cent of activity remaining at time t, the initial concentration of DEPC is I_o and k is the second order rate constant for reaction of the enzyme with DEPC, and k' is the pseudo-first order rate constant for degradation of DEPC[15,18]. The value of k' for DEPC in 1% and 10% ethanol was calculated to be 63×10^{-3} and 28×10^{-3} per min respectively. The kinetics of inactivation was monitored in the presence of both 1% and 10% ethanol because ethanol present in the incubation mixture was in the range of 1% to 10%. The log of remaining activity (A/A_o) when plotted against time $(1\text{-}e^{-k't})/k'$ for DEPC inactivation at different concentrations of the reagents resulted in straight lines in both the cases.

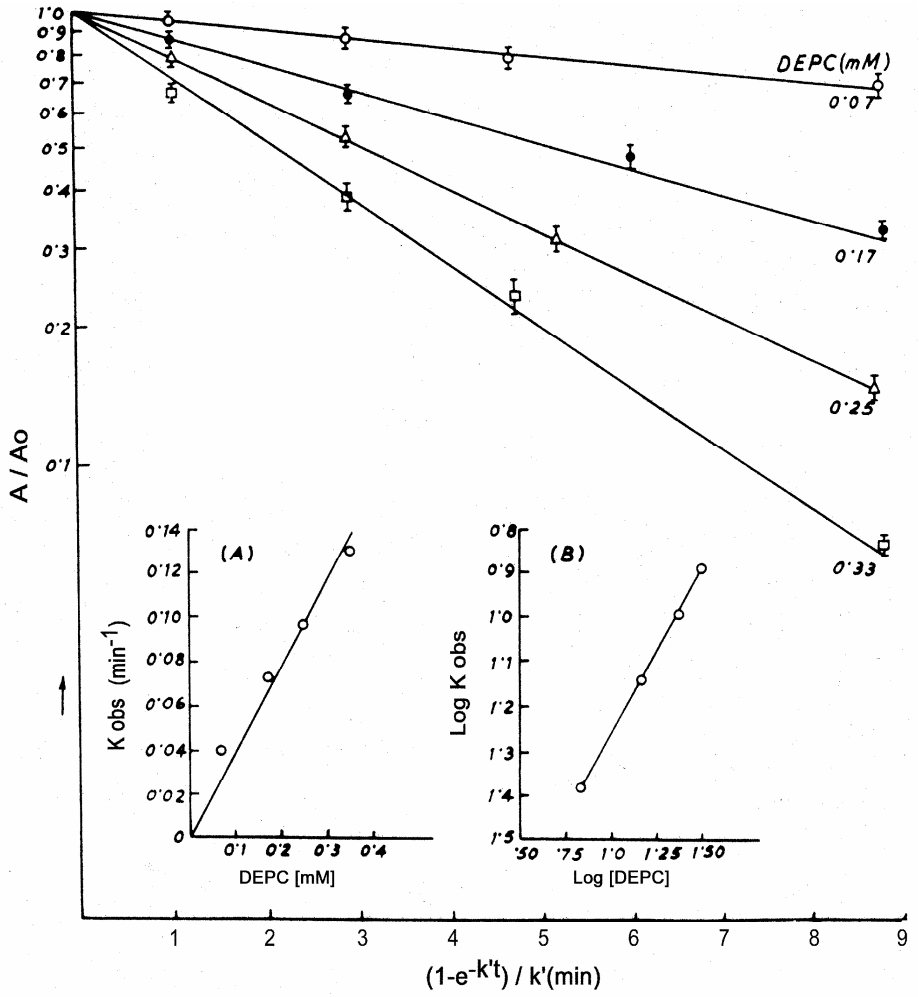

Fig. 1—Kinetics of inactivation of EAC cell GA3PD by DEPC [The enzyme was passed through a Sephadex G-50 column previously equilibrated with 50 m*M* phosphate buffer, *p*H 7.0. The eluted enzyme (38 µg protein/ml) was incubated in different tubes with 0.07 m*M* (○), 0.17 m*M* (●), 0.25 m*M* (Δ), and 0.33 m*M* (□) DEPC in presence of 10% ethanol in 50 m*M* potassium phosphate buffer (*p*H 7.0) at 27°C. At indicated time intervals, aliquots were removed for measurement of the residual enzyme activity. A control tube containing the enzyme, but without DEPC was maintained. The residual enzyme activity (percentage) was plotted assuming the activity of the enzyme of the control tube as 100. The first order rate constant for decomposition of DEPC (k') was determined separately. *Inset A*: the apparent plot of first order rate constant for inactivation (K_{obs}) obtained at various concentrations of DEPC against concentration of this reagent. *Inset B*: shows the plot of log pseudo-first order rate constant for inactivation (K_{obs}) obtained at various concentrations of DEPC against log of concentration of this inactivating reagent]

The results of inactivation kinetics obtained with DEPC in 10 % ethanol are shown in Fig. 1. This figure plots ln (A/A_o) *vs* ($1-e^{-k't}$)/ k'. Therefore, the plot should be linear with a gradient = - KI_o. The plot of pseudo-first order rate constant values for inactivation (K_{obs}) against concentrations of DEPC was linear and the slope yielded a second order rate constant (k) of 400 $M^{-1}min^{-1}$ (Fig. 1, inset A). The reaction order for inactivation with different concentrations of DEPC was calculated from a plot of log (K_{obs}) *vs* log of reagent concentration[15,19]. A value of approx. 1 was obtained from the slope of the plot (Fig. 1, inset B), suggesting that approx. 1 mol of the enzyme was inactivated by 1 mol of DEPC. A similar result was obtained when inactivation experiment with DEPC was carried out in the presence of 1% ethanol.

Effect of *p*H on DEPC-inactivation

The inactivation of GA3PD by DEPC was examined as a function of *p*H using potassium phosphate buffer at the *p*H range of 6.4-8.0, where specifically histidyl residues react[16]. The plot of apparent first order rate constants obtained at different *p*H against various *p*H values showed a typical titration curve with a p_K value around 6.9 ± 0.02 (Fig. 2), which is consistent with the p_K of the histidyl imidazolium group in proteins. Since DEPC reacts only with the unprotonated form of imidazole in a model system and in proteins[20], and p_K value found was within the range expected for a histidine residue, the result was consistent with the proposal that the inactivation results from the modification of histidine residue(s).

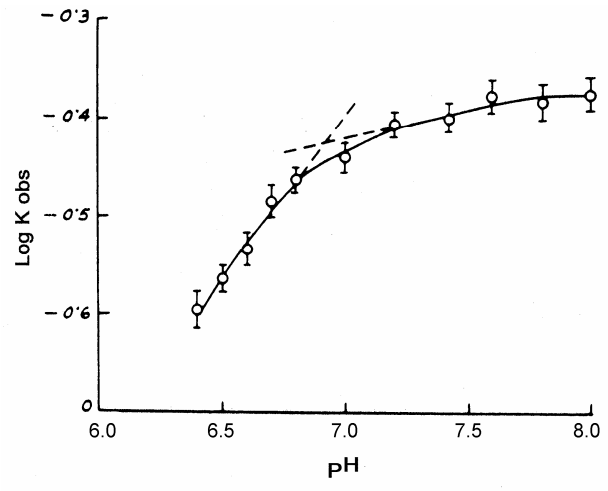

Fig. 2—Effect of *p*H on inactivation [The enzyme (46 μg protein/ml) was incubated at 0°C with 0.1 m*M* DEPC in 0.1 *M* potassium phosphate buffer at various *p*H values as indicated. The value of k' was determined at each *p*H and the apparent first order rate constant for inactivation was obtained as in Fig. 1. The values of log of pseudo-first order rate constant for inactivation (K_{obs}) obtained at different *p*H are plotted against *p*H]

Determination of the number of essential histidine residue(s)

Histidine residues on modification with DEPC give a characteristic band with an absorption maximum between 230 nm to 250 nm depending upon the protein[15,16]. The difference of spectrum of DEPC-treated enzyme and the native enzyme revealed a rapid increase in absorption with maximum at 242 nm, characteristic of an N-carboxyhistidine residue in protein (Fig. 3). The number of histidine residues modified was calculated from the amount of N-carboxyhistidine formed in different stages of inactivation (by difference spectra) assuming a molar absorptivity of 3.2 × 103 $M^{-1}cm^{-1}$ at 240 nm.

The relationship between the loss of enzyme activity and the number of essential histidine residues modified is shown in Fig. 4. Extrapolation of the linear plot to zero activity indicates that almost 17 residues are modified on complete inactivation, which corresponded nicely with the number of histidyl residues modified calculated from difference of increase in absorption spectrum (Fig. 3). As this method does not give the precise number of residue(s) essential for activity, we used the statistical method of Tsou[21] to determine the number of essential residue(s) for inactivation.

If it is assumed that all the modifiable residues (n) including the essential residues (i) are equally reactive towards the reagent and modification of any of the residue will result in a complete loss of the enzyme

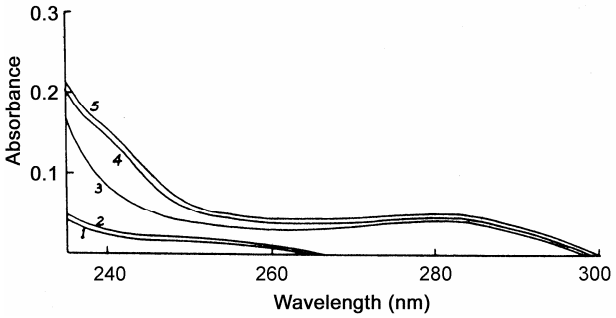

Fig. 3—The difference spectra of DEPC-treated and the native enzyme [The enzyme (2.2 μ*M*) was incubated with 50 μ*M* DEPC in 50 m*M* sodium phosphate buffer, *p*H 7.0 in presence of 10% ethanol. A control tube was maintained in identical condition, but containing no enzyme. Spectra numbered 1 and 2 are of the control tubes on 0 min and 10 min incubation time, respectively while spectra numbered 3, 4 and 5 are of the enzyme at incubation time of 0 min, 10 min and 20 min, respectively]

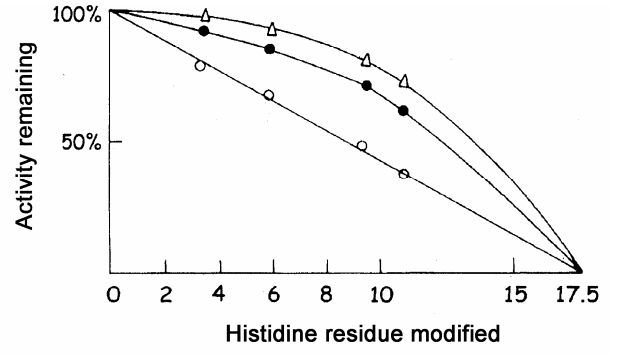

Fig. 4—Correlation between the number of histidine residues modified by DEPC and the residual activity of EAC cell GA3PD [The enzyme (2.2 μ*M*) was incubated at 27°C with 50 μ*M* DEPC in 50 m*M* phosphate buffer (*p*H 7.0) in presence of 10% alcohol, and the residual activity and the number of histidine residue(s) modified were measured as described in 'Materials and Methods'. Data are presented as Tsou plot; for i = 1 (○), i = 2 (●) and i = 3 (Δ)]

activity, the relationship between the residual activity against histidine modification will be as follows:

$$(A/A_o)^{1/i} = (n\text{-}m)/n \quad \ldots (2)$$

where (A/A_o) is the residual activity and m is the number of histidine residue modified. The number of the essential histidine residue is that value of i (essential residues) which gives a straight line when (A/A_o) is plotted against m (Fig. 4). This figure suggests that GA3PD activity is dependent upon modification of one critical histidine residue.

Attempts to reactive the DEPC-inactivated GA3PD by hydroxylamine

DEPC has been shown to react in some proteins with residues other than histidine that includes tryptophan, tyrosine, cysteine etc[16]. Hydroxylamine removes the carbethoxy group from DEPC-modified histidyl residues, but not from other amino acid residues such as lysyl or cysteinyl residues[16]. The EAC cell GA3PD was inactivated by hydroxylamine at different *p*H of the incubation media (data not presented). So, GA3PD inactivated by DEPC could not be reactivated by hydroxylamine. Thus, we could not perform the enzyme inactivation reactivation experiment.

Double inhibition studies with DTNB and DEPC

DEPC is a fairly specific reagent for histidine residues in the *p*H range of 6.0-7.5. GA3PD from various sources contains a very reactive cystine residue at the active site of the enzyme[23]. Involvement of reactive SH groups at the active site of EAC cell GA3PD has also been observed[11]. Table 1 shows that this enzyme is strongly inactivated by the thiol reagent DTNB. Moreover, the inactivated enzyme could be almost completely reactivated by DTT. By taking advantage of this inactivation reactivation, we performed double inhibition experiment by DEPC and DTNB in order to ascertain whether DEPC binds to the histidine residue or to SH group of a cystein residue of EAC cell GA3PD.

Table 1—Test for the reversal of the activity by DTT of DTNB- and/or DEPC-inactivated EAC cell GA3PD

[EAC cell GA3PD (0.12 mg protein, 110 units of activity) was incubated in presence of DEPC (150 μ*M* for 15 min) and/or DTNB (50 μ*M* for 5 min). A control tube in each case was maintained without any inhibitor. After indicated period of time, the residual enzymatic activity was measured by taking an aliquot. The remaining incubation mixture was freed from the excess reagents by passing through a Sepadex G-50 column. The inactivated enzyme was then allowed to react with 10 m*M* DTT for 20 min and assayed for the enzymatic activity. The activity of the control was taken as 100%]

Addition	Percent activity
Nil (control)	100
DTNB	10
DTNB + DTT	98
DTNB + DEPC	7
DTNB + DEPC + DTT	4
DEPC	18
DEPC + DTT	14

For this experiment, the enzyme was first inactivated by DTNB and then further treated with DEPC. If both the reagents could react with the thiol group, then modification with DTNB would protect the thiol against subsequent reaction with DEPC and hence the activity would be at least partially reversed after final incubation with DTT. If on the other hand, the loss of activity by DEPC was due to modification of a histidine residue then modification with DTNB would fail to provide protection against subsequent reaction by DEPC. In that case, final incubation with DTT would be unable to regenerate any activity. As shown in Table 1, the EAC cell GA3PD was first inactivated by DTNB and then treated with DEPC. This inactivated enzyme could not be reactivated by DTT, indicating that DEPC reacted with an essential histidine residue of the EAC cell enzyme. Reversing the order of addition of DTNB and DEPC also resulted in similar effect. Moreover, DTT had no reactivating effect on the enzyme inactivated by DEPC alone (Table 1).

All these studies provide further evidence that the loss of the enzymatic activity on treatment with DEPC was

due to the modification of a unique histidine residue of EAC cell GA3PD.

Evidence for the possible absence of some other amino acid residues at the active site of EAC cell GA3PD

The difference spectrum of DEPC-treated enzyme and native enzyme revealed a peak with absorption maximum at 242 nm, characteristic of N-carbethoxy-histidine residue in proteins (Fig. 3)[18]. However, *o*-carbethoxylation of tyrosine residue usually shows a negative difference spectrum at 278 nm[24]. Since no trough at around 280 nm due to *o*-carbethoxytyrosine was observed, the possibility of modification of tyrosine residue by DEPC was ruled out. In a previous paper[11], we had shown that EAC cell GA3PD is not inactivated by the known arginine specific reagents phenylglyoxal, 1, 2-cyclohexanedione and 2, 3 butanidione[25], indicating that arginine is not involved in the catalytic activity of the enzyme.

Protection of the activity of EAC cell GA3PD by the substrates against DEPC inactivation

The substrates GAP and NAD were found to protect the enzyme activity against the inactivation by DEPC. At a concentration of 0.05 m*M*, which is 1.25 time its K_m value of 0.04 m*M*, GAP could afford about 90% protection (Table 2). Similarly, NAD could provide about 70% protection at a concentration of 0.1 m*M*, which is 2.5 times its K_m value of 0.04 m*M*. At a concentration of 0.05 m*M*, NADH (a competitive inhibitor, K_i 10 μ*M*) could also protect the enzyme from DEPC inactivation. Moreover, NADP and NADPH, which are not substrates or competitive inhibitors for GA3PD could not substantially protect the enzyme from DEPC inactivation (Table 2). All these results indicate that the histidine residue that reacts with DEPC is located at the substrate-binding region of the enzyme.

Table 2—Protection of the activity of EAC cell GA3PD by substrate GAP and NAD and other nucleotides against DEPC inactivation

[Each experimental tube containing about 0.35 units of the enzyme in 200 μl of 50 m*M* phosphate buffer (*p*H 7.0) containing 10% ethanol was incubated with potential protective compounds as indicated, but without DEPC. Two tubes were maintained which contained the same amount of the enzyme in the same buffer, but no potential protective compounds. After 1 min, indicated concentration of DEPC was added in each tube except in one of the above-mentioned two tubes and incubated for 30 min. Aliquots were then taken from each tube and assayed for enzymatic activity. The activity of the enzyme in the tube in which neither DEPC nor any potential protective compound was added served as the control and was considered as 100%]

Addition	Activity retained (%)
Nil (Control)	100
DEPC (0.15 m*M*)	24
GAP (0.05 m*M*) + DEPC (0.15 m*M*)	89
NAD (0.1 m*M*) + DEPC (0.15 m*M*)	67
NAD (0.1 m*M*)+GAP (0.05 m*M*)+ DEPC (0.15 m*M*)	106
NADH (0.05 m*M*) + DEPC (0.15 m*M*)	91
NADP (0.1 m*M*) + DEPC (0.15 m*M*)	21
NADPH (0.2 m*M*) + DEPC (0.15 m*M*)	25

Discussion

GA3PD is a key enzyme of the glycolytic conversion of glucose to pyruvate, which represents an important pathway of carbohydrate metabolism in most organisms. Pure crystalline GA3PD had been isolated from a number of different sources[23] and full amino acid sequence of some of these enzymes had been done. Moreover, many investigations had been carried out on the mechanism of action of this enzyme[23-31]. However, studies on this enzyme of malignant cells are lacking. It had been purified from only two types of malignant cells, HeLa[5] and EAC[10]. Preliminary studies with EAC cell enzyme[10,11] indicated that the structural and catalytic properties of this enzyme are significantly different from that of other normal cells[23]. The specific activity of EAC cell GA3PD is significantly higher, compared to that obtained from other normal sources. In contrast to other normal cellular enzymes, the catalytic activity of EAC enzyme is not much reduced, by lowering *p*H of the assay media; the inhibitory activity of ATP is also less pronounced. Moreover, EAC enzyme requires exogenous NAD for stability. Experimental evidences from several other laboratories have also clearly raised the possibility that this enzyme may be altered and/or over-expressed in malignant cells[3-11]. However, full amino acid sequence and the nature of the active site of the malignant cell enzyme have yet to be determined.

Histidine is implicated in the catalytic action of GA3PD of normal cells[23]. Photo-oxidation studies first showed that inactivation of the rabbit muscle enzyme was associated with the specific destruction of his-38[26]. However, convincing chemical evidence for the direct involvement of a specific histidine in the active site was lacking. Several investigators were unable to label an essential histidine in the enzyme with either 3-bromoacetylpyridyine or bromopyruvate known to react selectively with histidine (ref. 23 for a review). Moreover, the active site cys-149 could not be linked to any neighbouring histidine residue in the rabbit muscle

enzyme with the bifunctional agent dibromoacetone[27]. This cys-149 is the most reactive amino acid present in the active site of normal cellular GA3PD.

However, in the crystallographic model, the highly conserved his-176 occurs within hydrogen bonding distance of cys-149[28]. Subsequently, extensive studies had been made on the role of histidine in the catalysis of this enzyme by using chemical modification experiments and site-directed mutagenesis[29-31]. These studies had clearly shown that his-176 enhances the activity of cys-149 by lowering its p_{Ka} as well as acting a hydrogen donor facilitating the formation of the tetrahedral intermediates. The his-176 also plays a role as a base catalyst involved in hydride transfer and also stabilizes the different intermediates as an acid catalyst[29,30].

In the present study, using histidine-specific amino acid modifying reagent DEPC, we have shown that a histidine residue is critically present at the active site of GA3PD of EAC cells. Recently, we have shown that a critical lysine residue is also present at the active site of the EAC cell GA3PD[11]. A study of the normal cellular GA3PD with DEPC and comparing data with that of EAC cells may actually bring in some conclusion. In order to understand the difference between normal and malignant cellular GA3PD at the precise molecular level, it is necessary to determine the full-length amino acid sequence as well as precise architecture of active site of GA3PD of a malignant cell. Then it will be easy to design a drug, which will inhibit/inactivate this enzyme specifically of malignant cells and thereby, inhibit glycolysis in these cells. Since high glycolysis is a hallmark of malignancy, such a drug may prove to be a truly effective anticancer drug.

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