Biochimica et Biophysica Acta, 569 (1979) 63—69

BBA 68766

CHEMICAL MODIFICATION OF HISTIDYL AND LYSYL RESIDUES IN YEAST ENOLASE

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(Received December 18th, 1978)

Key words: Enolase; Histidine; Lysine; Diethyl pyrocarbonate; Chemical modification; (Yeast)

Summary

Modification of yeast enolase (2-phospho-D-glycerate hydro-lyase, EC 4.2.1.11) by diethyl pyrocarbonate at either pH 6.1 or 6.6 caused a biphasic inactivation of the enzyme. In the presence of excess Mg^{2+}, either an equilibrium mixture of substrates or 3-phosphoglycerate, a competitive inhibitor, prevented the second slower phase of inactivation, but had no effect on the first rapid phase. Complete inactivation by diethyl pyrocarbonate correlates with the modification of six histidyl residues/subunit, while 3-phosphoglycerate protects two histidyl residues/subunit from modification.

Modification of enolase by two lysine-specific reagents, 2,4,6-trinitrobenzenesulfonate and pyridoxal 5′-phosphate, at pH 8.3 caused a slow loss of enzyme activity. However, substrates did not significantly protect against inactivation by either reagent, and inactivation with 2,4,6-trinitrobenzenesulfonate correlates with the modification of 18 lysyl residues/enzyme subunit.

Introduction

The structural features and mechanism of action of yeast enolase (2-phospho-D-glycerate hydro-lyase, EC 4.2.1.11) have been thoroughly studied [1]. However, relatively little is known about specific amino acid residues which are involved in the catalytic mechanism. Recent reports have shown that the enzyme contains a single essential arginyl residue/subunit [2—4], which is probably involved in the binding of the anionic phosphate moiety of substrates

Abbreviations: Bicine, *N,N*-bis(2-hydroxyethyl)glycine; ADA, *N*-(2-acetamido)iminodiacetic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

to the active site [3]. Inactivation of yeast enolase has been correlated with the modification of methionyl [5] and cysteinyl residues under denaturing conditions [6], but in neither instance are the modified residues thought to be present at the active site or involved in catalysis.

It has been reported that enolase is inactivated by photooxidation in the presence of Rose Bengal, and that inactivation is due to modification of histidine which may be involved in Mg^{2+} binding [7]. A recent reinvestigation of photooxidation in the presence of Rose Bengal suggests that inactivation is accompanied by modification of up to four histidyl residues/enolase subunit (Brewer, J.M., personal communication).

We report here further characterization of the role of histidine in yeast enolase, determined by chemical modification with diethyl pyrocarbonate, a reagent highly selective for the modification of histidine [8]. We also report chemical modification studies with two lysine-specific reagents, pyridoxal 5'-phosphate [9,10] and 2,4,6-trinitrobenzenesulfonate [11], and suggest that lysyl residues are probably not involved in substrate binding or catalysis in yeast enolase.

Materials and Methods

Yeast enolase, from Sigma (St. Louis, MO, U.S.A.), was characterized and assayed as previously described [3]. 2-Phosphoglycerate, 3-phosphoglycerate, pyridoxal 5'-phosphate, diethyl pyrocarbonate, and all buffer salts were from Sigma, while hydroxylamine hydrochloride and $MgCl_2 \cdot 6H_2O$ were products of J.T. Baker Chemical Co. (Phillipsburg, NJ, U.S.A.). 2,4,6,-Trinitrobenzenesulfonate was purchased from Nutritional Biochemicals (Cleveland, OH, U.S.A.).

Enolase concentrations were determined from absorbance at 280 nm, using $A_{280}^{0.1\%} = 0.89$ [12] and a molecular weight of 88 000 for the dimeric enzyme [13].

Chemical modification with diethyl pyrocarbonate was performed as described in the figure legends. Stock solutions of diethyl pyrocarbonate were prepared by five-fold dilution into ice-cold ethanol, followed by suitable dilution into the appropriate buffer at 0°C. The concentration of diethyl pyrocarbonate in these stock solutions was determined by the method of Holbrook and Ingram [14] immediately before the initiation of modification. The reagent was determined to have a half-life of 6 h at 0°C under these conditions. Modification was initiated by adding an aliquot of a freshly prepared stock solution of diethyl pyrocarbonate to a solution of enolase, in some cases together with substrates or competitive inhibitor, in the appropriate buffer. Aliquots were periodically withdrawn and assayed for enzyme activity.

Correlation of inactivation of enolase by diethyl pyrocarbonate with the number of histidyl residues modified was made by modifying the enzyme, at 12—24 μM, with diethyl pyrocarbonate (1.8 mM at 0°C, 0.71 mM at 25°C) in 50 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes) (pH 6.1), 1 mM $MgCl_2$, 0.01 mM EDTA, in a Cary 14 spectrophotometer. The number of histidyl residues modified was calculated from the increase in absorbance at 240 nm, using a molar absorption coefficient of $3.6 \cdot 10^3\ M^{-1} \cdot cm^{-1}$ for *N*-ethoxyformyl-

histidine [14]. Determination of the number of histidyl residues modified in the presence of 3-phosphoglycerate was done similarly at 25°C, after preincubation of enolase with 3-phosphoglycerate for 1 h to equilibrate any substrate impurities present in the 3-phosphoglycerate sample.

Chemical modifications with pyridoxal 5'-phosphate and 2,4,6-trinitrobenzenesulfonate were carried out at 25°C in 50 mM *N,N*-bis(2-hydroxethyl)-glycine (Bicine) (pH 8.3), 1 mM $MgCl_2$, 0.01 mM EDTA.

Correlation of the inactivation of 10 μM enolase with the number of lysyl residues/subunit modified by 5 mM 2,4,6-trinitrobenzensulfonate was carried out at 25°C in the same buffer in a Cary 14 spectrophotometer. Matched cuvettes, each having two compartments 0.438 cm in pathlength, were used [15]. Aliquots of enolase were withdrawn and assayed to determine specific activity, while the number of lysyl residues modified was determined by following the increase in absorbance at 367 nm, using a molar absorption coefficient of $1.10 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ for ε-2,4,6-trinitrophenyllysine [16]. This wavelength was chosen because it represents an isosbestic point between the modified lysine and its sulfite complex, and negates interference of the liberated sulfite with the spectrum of ε-2,4,6-trinitrophenyllysine [16].

Results

Inactivation by diethyl pyrocarbonate

When enolase was modified by diethyl pyrocarbonate at 0°C in 50 mM *N*-(2-acetamido)iminodiacetic acid (ADA) (pH 6.6), 1 mM $MgCl_2$, 0.01 mM EDTA, activity was lost in a biphasic manner, decreasing rapidly until 60–65% activity remained, and then in a second slower phase until the enzyme was completely inactive (Fig. 1). The initial rapid rate of inactivation, most obvious

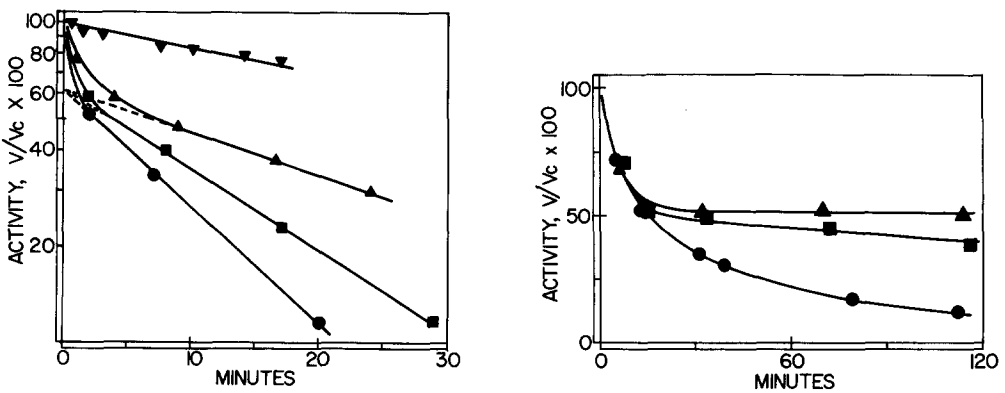

Fig. 1. Semilog plot of changes in the activity of 1 μM yeast enolase in 50 mM ADA (pH 6.6), 1 mM $MgCl_2$, 0.01 mM EDTA, 0°C, on reaction with 0.27 mM (▼), 2.85 mM (▲), 5.70 mM (■), and 8.55 mM (●) diethyl pyrocarbonate. The control retains full activity over this period of time.

Fig. 2. Inactivation of 1 μM yeast enolase by 1.39 mM diethyl pyrocarbonate in 50 mM Mes (pH 6.1), 1 mM $MgCl_2$, 0.01 mM EDTA, at 0°C. Modification was carried out in the absence (●) of substrates or inhibitor, or in the presence of (■) 20 mM 3-phosphoglycerate or (▲) 20 mM 2-hosphoglycerate. The control retains full activity over this period of time.

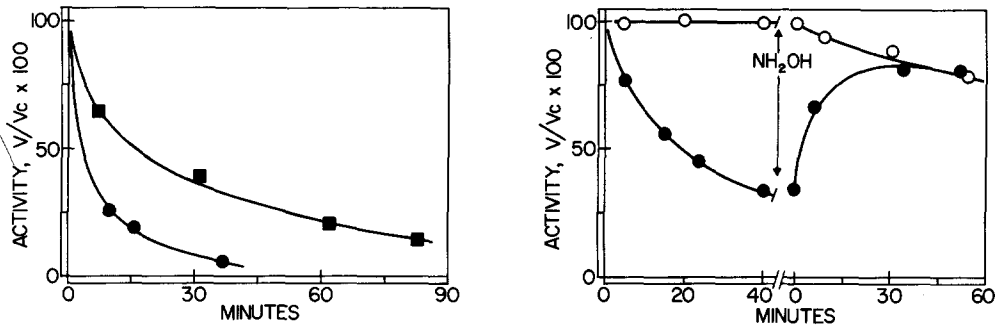

Fig. 3. Effect of Mg^{2+} on the rate of inactivation of yeast enolase by diethyl pyrocarbonate. The enzyme was first made metal free by dialysis overnight at 4°C against three changes of 0.1 mM EDTA, 50 mM Mes (pH 6.1). The enzyme, at 1 μM, was then modified at 0°C by 1.24 mM diethyl pyrocarbonate, either in the absence (•) or presence (▪) of 1 mM $MgCl_2$.

Fig. 4. Use of hydroxylamine hydrochloride to reverse the inactivation of enolase by diethyl pyrocarbonate. Enolase, at 1 μM, was modified at 0°C by 1.36 mM diethyl pyrocarbonate in 50 mM Mes (pH 6.1), 1 mM $MgCl_2$, 0.01 mM EDTA (•), and the change in activity with time was compared to a control (○). At the time indicated by the arrows both control and modified samples were diluted with an equal volume of hydroxylamine hydrochloride at 25°C to a final concentration of 0.25 M NH_2OH-HCl (pH 7.0). A value of 100 for the control in hydroxylamine was determined by assaying immediately after dilution.

when low concentrations of reagent (less than 0.6 mM) were used, has a second-order rate constant of $55 \pm 5\ M^{-1} \cdot min^{-1}$, while the second slower phase occurred with a second-order rate constant of $10.7 \pm 0.6\ M^{-1} \cdot min^{-1}$ at 0°C.

The same biphasic inactivation was observed when modification by diethyl pyrocarbonate was carried out at 0°C in 50 mM Mes (pH 6.1), 1 mM $MgCl_2$, 0.01 mM EDTA (Fig. 2). The second slower phase of inactivation proceeded with a second-order rate constant of $8.1 \pm 0.6\ M^{-1} \cdot min^{-1}$ under these conditions. When modification was carried out in the presence of an equilibrium mixture of substrates (generated by adding 2-phosphoglycerate plus $MgCl_2$ before initiation of modification), the first phase of inactivation remained unaltered, but the second phase was prevented (Fig. 2). Similar protection was provided by 3-phosphoglycerate [17], and by phosphate [3] (data not shown), both competitive inhibitors of enolase.

When enolase was made metal free [7,18,19] by dialysis overnight at 4°C against three changes of 0.1 mM EDTA, 50 mM Mes (pH 6.1), before modification by diethyl pyrocarbonate, the rate of inactivation was greatly increased over that carried out in the presence of 1 mM Mg^{2+} (Fig. 3).

The modification of histidyl residues by diethyl pyrocarbonate is rapidly reversed by neutral hydroxylamine [20]. Reversal of enolase inactivation was complicated by our observation that hydroxylamine hydrochloride caused a time-dependent irreversible inactivation of enolase (unpublished data). Nevertheless, convincing evidence that inactivation of enolase by diethyl pyrocarbonate is due to modification of histidine is shown in Fig. 4. When the enzyme was modified at 0°C to 30% of the initial activity, then diluted to a final concentration of 0.25 M hydroxylamine hydrochloride (pH 7.0) and

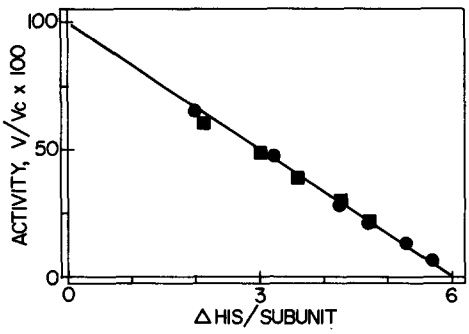

Fig. 5. Correlation of the inactivation of yeast enolase with the number of histidyl residues modified by diethyl pyrocarbonate. Data were collected from modification at (■) 0°C, and at (●) 25°C.

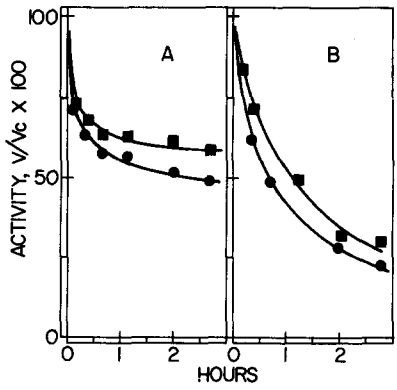

Fig. 6. Inactivation of 1 μM yeast enolase by lysine-specific reagents in 50 mM Bicine (pH 8.3), 1 mM $MgCl_2$, 0.01 mM EDTA, 25°C. (A) Modification by 3 mM pyridoxal 5'-phosphate in either (●) the absence of substrates or (■) the presence of 20 mM 2-phosphoglycerate. (B) Modification by 3 mM 2,4,6-trinitrobenzenesulfonate in either (●) the absence of substrates or (■) the presence of 20 mM 2-phosphoglycerate. The control retains full activity over the duration of these experiments.

incubated at 25°C, activity was rapidly regained, until after 45 min it was identical to the control diluted into hydroxylamine for the same length of time. This rules out the possibility that the inactivation of enolase by diethyl pyrocarbonate is caused by modification of amino groups [20].

At either 0 or 25°C, complete inactivation of enolase by diethyl pyrocarbonate correlates with the modification of six histidyl residues/subunit (Fig. 5). The ultraviolet difference spectrum between native enolase and an enolase sample which had been treated with diethyl pyrocarbonate, and which had 5.3 histidyl residues/subunit modified and 12% activity, showed no change in absorbance of the modified enzyme at 280 nm. This eliminates the possibility that tyrosyl residues of enolase were modified by diethyl pyrocarbonate [20]. Interestingly, when modification was carried out in the presence of 3-phosphoglycerate, a maximum of four histidyl residues/subunit were modified and activity leveled off at 45% of the control (data not shown). This suggests that the combined presence of a competitive inhibitor and Mg^{2+} not only greatly diminishes the slow phase of inactivation by diethyl pyrocarbonate (Fig. 2) but also protects two histidyl residues/subunit from modification.

Modification by pyridoxal 5'-phosphate and 2,4,6-trinitrobenzenesulfonate

When yeast enolase was incubated at 25°C in 50 mM Bicine (pH 8.3), 1 mM $MgCl_2$, 0.01 mM EDTA, with either 3 mM pyridoxal 5'-phosphate (Fig. 6A) or 3 mM 2,4,6-trinitrobenzenesulfonate (Fig. 6B), the activity slowly decreased. However, even after 3 h of modification, 50% activity still remained when pyridoxal 5'-phosphate was used, whereas 25% activity remained in the presence of trinitrobenzenesulfonate. The presence of substrates during modification (generated by adding 20 mM 2-phosphoglycerate before initiation of modification) had very little effect on the rate of inactivation by either

pyridoxal 5′-phosphate (Fig. 6A) or trinitrobenzenesulfonate (Fig. 6B).

Correlation of inactivation by 2,4,6-trinitrobenzenesulfonate with the number of lysyl residues modified suggests that complete inactivation of enolase is achieved only after modification of 18 lysyl residues/subunit (data not shown). The results were essentially identical whether modification was carried out in the absence or presence of substrates.

Discussion

Histidyl residues have been implicated in the catalytic mechanism of yeast enolase by kinetic [17], metal binding [21,22], and chemical modification [7,23,24] studies. Our data suggest that yeast enolase is inactivated by diethyl pyrocarbonate (Figs. 1 and 2). Complete inactivation of enolase correlates with the modificatson of six histidyl residues/subunit (Fig. 5). Neither substrates nor 3-phosphoglycerate, a competitive inhibitor, protect against the initial rapid loss of 35—40% activity on modification by diethyl pyrocarbonate, but both dramatically reduce the second slower phase of inactivation (Fig. 2). Interestingly, the presence of 3-phosphoglycerate plus excess Mg^{2+} protects two histidyl residues/subunit from modification. Although such protection could arise from a number of causes, the likelihood exists that two histidyl residues are present at the active center of enolase and may be involved in catalysis and/or binding of the third and fourth 'catalytic' Mg^{2+} necessary for activity [25].

The data for the modification of enolase by diethyl pyrocarbonate shown in Fig. 1, and Fig. 2 in the absence of substrates or inhibitor, involves modification of the dimeric enzyme to which the first two 'structural' Mg^{2+} are bound [18,25,26]. It is thus interesting to note that when enolase is dialyzed against 0.1 mM EDTA before modification by diethyl pyrocarbonate, a treatment which provides metal-free enzyme [7,18,19], the rate of inactivation is dramatically increased (Fig. 3). These data strongly suggest that histidine(s) may also be involved in binding either or both of the first two tightly bound 'structural' Mg^{2+}. Further experiments to ascertain the validity of this last statement are in progress.

A previous report on the use of low concentrations of pyridoxal 5′-phosphate to modify fifteen enzymes associated with carbohydrate metabolism suggested that enolase is one of only two enzymes not inactivated by this reagent [27]. The results reported herein show that some inactivation of enolase is achieved by using high concentrations of both pyridoxal 5′-phosphate and trinitrobenzenesulfonate. However, concentrations of pyridoxal 5′-phosphate required for inactivation of enolase are much higher than those normally used to modify essential lysines at enzume active sites [9,10,27]. The assumption that modification of essential lysine(s) at the active site of enolase is not involved is greatly strengthened by our observation that substrates afford insignificant protection against inactivation by either reagent (Fig. 6).

The observed inactivation of enolase by both lysine-specific reagents is thus likely to be due to gross structural changes in the enzyme, for not only are large aromatic residues attached when lysyl residues are modified by either reagent, but charge properties of the enzyme are also altered. Lysyl residues normally exist as positively charged moieties under conditions where enolase is

stable, but this changes to a neutral species when ϵ-2,4,6-trinitrophenyllysine is formed and a negative charge when pyridoxal 5'-phosphate is incorporated. The likelihood that gross structural changes are involved is strengthened by our observation that complete inactivation of enolase is achieved only after modification of 18 lysines/subunit by trinitrobenzenesulfonate. This number represents approximately half of the reported number of lysines/enolase subunit [1,28].

The data reported in this paper thus offer additional evidence that histidyl residues play an integral role in mechanism of action of yeast enolase. It is also apparent that lysysl residues are most likely not present at the active site of enolase and thus not involved in substrate binding or catalysis.

Acknowledgements

Acknowledgement is made to the donors of the Petroleum Research Fund, administered by the American Chemical Society, for support of this research. This work was taken in part from the senior Independent Study thesis of A.L.G., the College of Wooster, 1978. Parts of the work were carried out during the summer of 1978 in an undergraduate research participation program made possible by Grant SPI77-25616 from the National Science Foundation.

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