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ESSENTIAL ARGINYL RESIDUES IN YEAST ENOLASE[1]

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Yeast enolase is rapidly inactivated by butanedione in borate buffer, complete inactivation correlating with the modification of 1.8 arginyl residues per subunit. Protection against inactivation is provided by either an equilibrium mixture of substrates or inorganic phosphate, a competitive inhibitor of the enzyme. Complete protection by substrates correlates with the shielding of 1.3 arginyl residues per subunit, while phosphate protects 1.0 arginyl residue per subunit from modification.

Arginyl residues play a very general role in the functional binding of anionic cofactors and substrates to enzyme active sites (1-3). This fact is emphasized by a recent report which suggests that all but one of fourteen enzymes associated with the glycolytic pathway have essential arginyl residues (3). To date the role of arginyl residues has been thoroughly characterized in hexokinase (4), fructose-1,6-bisphosphatase (3,5), aldolase (6), glyceraldehyde-3-phosphate dehydrogenase (7), phosphoglycerate kinase (8,9), phosphoglycerate mutase (10), alcohol dehydrogenase (1), and lactate dehydrogenase (11). However, till now only preliminary data have been available on enolase (3). The work reported herein is a further characterization of the role of the essential arginyl residues in yeast enolase.

MATERIALS AND METHODS

2-Phosphoglycerate was obtained from Sigma and phosphoenolpyruvate was

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from Calbiochem. 2,3-Butanedione was purchased from Aldrich. All other chemicals were reagent grade. Yeast enolase, from Sigma, was determined to be >95% pure by gel electrophoresis (12). It gave an amino acid analysis consistent with published values (13,14) and exhibited a specific activity of 70-140 μmole PEP[4]·min^{-1}·mg^{-1} under the conditions of assay. The samples used for amino acid analysis had an activity of 125. Enolase concentration was determined from absorbance at 280 nm, using A(0.1%) = 0.89 (15) and a molecular weight of 88,000 for the dimeric enzyme (16). Enzymatic activity was determined at 25° by a slight modification of published procedures (17). A standard assay mixture (1.0 ml) contained the following: 50 mM Tris chloride, pH 7.8, 2.0 mM 2-PGA, 1 mM $MgCl_2$, 0.01 mM EDTA. Assays were initiated by the addition of enolase (ca. 0.5 μg) and activity determined from the increase in absorbance at 240 nm as a function of time, using a molar absorption coefficient of 1,400 for PEP in 1 mM Mg^{2+}, pH 7.8 (18).

Modification with butanedione was carried out at 25° under the conditions given in the figure and table legends. Modification of arginine was determined by analysis on a Beckman 120C amino acid analyzer after workup analogous to published procedures (2).

RESULTS

The time course for the inactivation of yeast enolase by 4 mM butanedione in 50 mM borate, 1 mM $MgCl_2$, 0.01 mM EDTA, pH 8.3, is shown in Figure 1. Activity is reduced to 50% of the control after 17 minutes, and only 11% activity remains after two hours modification. If $MgCl_2$ is omitted from the modification mixture, the rate of inactivation is identical to that shown in Figure 1. However, the rate of inactivation is significantly reduced if borate is omitted. Under conditions where the activity is reduced to 17% in the presence of 50 mM borate, 80% activity remains if the borate is replaced by BICINE buffer. Inactivation by butanedione-borate is also reversible. If the enzyme is modified to 8% of the control activity by butanedione-borate and then gel filtered through a Sephadex G-25 column equilibrated with 50 mM Tris chloride, pH 8.3, 62% of the native activity is restored after 4 hr and 90 % activity is observed after 15 hr. If gel filtration is performed in borate, however, no reactivation is observed. This data strongly suggests that inactivation is due to the modification of essential arginyl residues.

Protection against inactivation is provided by either an equilibrium mixture of substrates or by phosphate, a competitive inhibitor of enolase (vide infra) (Figure 1). After 2 hr, 75% of the control activity remains in the presence of an equilibrium mixture of substrates plus Mg^{2+} (generated by adding

[4]Abbreviations used are: PEP, phosphoenolpyruvate; 2-PGA, 2-phosphoglycerate; P_i, inorganic phosphate; Tris, tris-(hydroxymethyl)aminomethane.

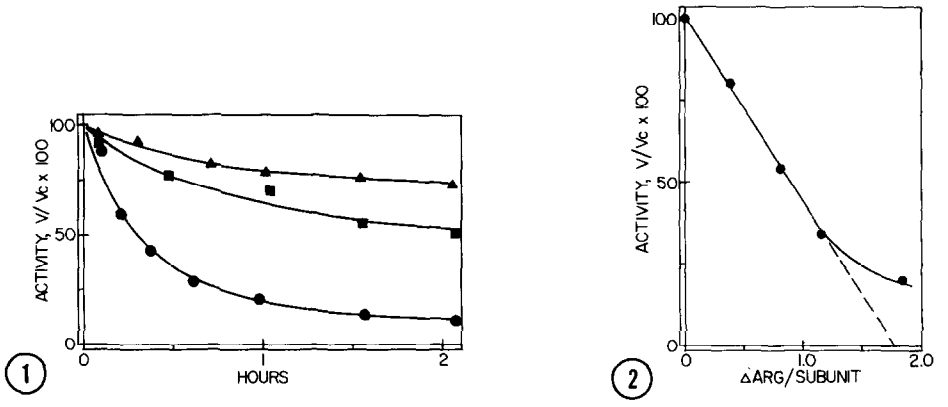

FIGURE 1: Yeast enolase inactivation by butanedione. The enzyme was modified by 4 mM butanedione in 50 mM borate, 1 mM $MgCl_2$, 0.01 mM EDTA, pH 8.3, either in the absence (●) of substrates or inhibitor, or in the presence of (▲)2-PGA, 20 mM, or (■) P_i, 100 mM. The control enzyme retains full activity over this period of time.

FIGURE 2: Correlation of yeast enolase inactivation with arginine modification by butanedione. The enzyme, 30 μM, was incubated with 2 mM butanedione in 50 mM borate, 1 mM $MgCl_2$, 0.01 mM EDTA, pH 8.3, and aliquots were withdrawn periodically and subjected to gel filtration and subsequent analyses as described in Table I. The number of arginines modified is given per subunit of enzyme.

2-PGA, 20 mM, plus $MgCl_2$, 1 mM, before the initiation of modification) compared to only 11% activity in their absence. In the presence of P_i, 100 mM, plus $MgCl_2$, 1 mM, 55% of the control activity remains after two hr. It is significant that either 2-PGA or PEP provide significant protection against inactivation in the absence of added $MgCl_2$, but the extent of protection is increased by the presence of magnesium. When enolase is modified by 4 mM butanedione in 50 mM borate, 0.01 mM EDTA, pH 8.3, 16% activity remains after one hr (data not shown). In the presence of 2-PGA, 2 mM, or PEP, 4 mM, 55% activity remains after one hr, while 70% activity remains after one hr if $MgCl_2$, 0.4 mM, is included with either substrate.

Extrapolation to complete inhibition indicates that inactivation by butanedione correlates with the modification of 1.8 arginyl residues per subunit (Figure 2). When either 2-PGA, 20 mM, or P_i, 100 mM, is present and the modifications are characterized by both inactivation and amino acid analyses, the results shown in Table I are obtained. After 110 min of modification by 2 mM butanedione,

TABLE I: Correlation of Yeast Enolase Inactivation by Butanedione with Loss of Arginine, and Protection by Substrates and Inhibitor[a]

Enzyme	V/Vc x 100	Arg per Subunit	Arg Modified per Subunit
Control	100	13.2	-
+ Butanedione	20	11.4	1.8
+ Butanedione + 2-PGA	80	12.2	1.0
+ Butanedione + P_i	80	12.0	1.2

[a]Modification of yeast enolase, 30 μM, was carried out with 2 mM butanedione in 50 mM borate, 1 mM $MgCl_2$, 0.01 mM EDTA, pH 8.3, in the absence of substrate or inhibitor or in the presence of 2-PGA, 20 mM, or P_i, 100 mM. After 110 min aliquots were subjected to gel filtration on a column (0.9 x 20 cm) of Sephadex G-25 equilibrated with 50 mM borate, 1 mM $MgCl_2$, 0.01 mM EDTA, pH 8.3, assayed for enzymatic activity, and then hydrolyzed with 6 N HCl for 18 hr at 110° and subjected to amino acid analysis as described in the text.

only 20% activity remains when neither 2-PGA or P_i is present and 1.8 arginyl residues per subunit are found to be modified. When 2-PGA is present during modification, 80% activity remains and 1.0 arginyl residues are modified. When P_i is present during modification 80% activity remains and 1.2 arginyl residues are modified.

DISCUSSION

Despite the wealth of information available on various aspects of yeast enolase structure and function, only a few reports have defined the roles of individual amino acid residues by chemical modification techniques. Inactivation of yeast enolase has been correlated with the modification of methionyl residues (19) and cysteinyl residues under denaturing conditions (20). However, in neither case are the modified residues thought to lie at the active site. Photooxidation in the presence of Rose Bengal shows that inactivation correlates with the modification of approximately one histidine per subunit which is suggested to be involved in metal binding (21).

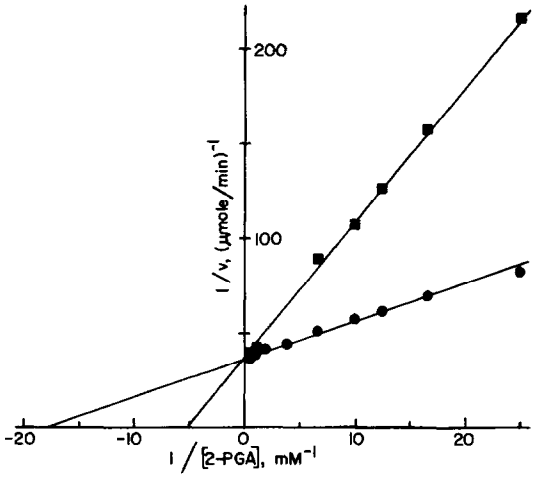

FIGURE 3: Kinetic analysis of inhibition of yeast enolase by phosphate. Assays were carried out at 25° in 50 mM borate, 1 mM $MgCl_2$, 0.01 mM EDTA, pH 8.3, either in the absence (●) or presence (■) of 10 mM P_i. Under these conditions 2-PGA exhibits a K_M of 5.5×10^{-5} M while P_i is a competitive inhibitor with K_I = 3.9 mM.

The data presented here indicate that arginyl residues are critical to the mechanism of action of yeast enolase. Complete inactivation by butanedione-borate correlates with the modification of approximately 1.8 arginyl residues per subunit (Figure 2). The rate of inactivation is greatly reduced when modification is carried out in the presence of an equilibrium mixture of substrates (Figure 1), and complete protection against inactivation correlates with the shielding of 1.3 arginyl residues per subunit (i.e., when 60% activity is protected by substrates 0.8 less arginyl residues per subunit are modified, Table I).

It is widely known that arginyl residues play a very general role in the binding of anionic substrates and cofactors to enzyme active sites (1-11). Thus, it is likely that the single arginyl residue per subunit protected against modification by substrates is involved in binding either the phosphate or carboxylate moiety of 2-PGA or PEP when these are bound to the active site. When modification of yeast enolase is carried out in the presence of inorganic phosphate, complete protection against inactivation correlates with the shielding of 1.0 arginine per subunit (Table I). The previously available data on the mode of inhibition of yeast enolase by P_i suggests that it is a non-competitive inhibitor (22). However, kinetic analysis of the inhibition of yeast enolase by P_i under the conditions of

butanedione modification (50 mM borate, 1 mM $MgCl_2$, 0.01 mM EDTA, pH 8.3) conclusively demonstrates that it is a competitive inhibitor under these conditions (Figure 3), with K_I = 3.9 mM. Thus, a likely interpretation of protection by P_i is that the arginyl residue is involved in binding P_i to the active site, and it is quite conceivable that the same arginine is involved in binding the phosphate moiety of substrates in the functional enzyme-substrate complex of yeast enolase.

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