RABBIT MUSCLE ENOLASE ALSO HAS ESSENTIAL ARGINYL RESIDUES

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1. Introduction

Recent studies have attempted to clarify the roles of various amino acid residues in the catalytic activity of yeast enolase. The yeast enzyme has a single essential arginyl residue per subunit [1–3] which is likely involved in binding the phosphate moiety of substrates to the active site [2]. In addition, the enzyme can be inactivated by the modification of carboxyl [4,5] and histidyl [6–8] residues at the active site, but the roles played by these amino acid residues is less well defined. Inactivation of yeast enolase has also been correlated with the modification of methionyl residues [9,10] and cysteinyl residues under denaturing conditions [11], but in neither case are the modified residues thought to be involved in the catalytic mechanism. Similarly, lysyl residues are not involved in substrate binding or catalysis [8].

Although there have been comparative studies on the structural and catalytic properties of yeast and rabbit muscle enolases [10,12], little information is available on catalytically functional amino acid residues in the muscle enzyme. It has been shown that glycidol phosphate, a substrate analogue, inactivates rabbit muscle enolase by modification of a carboxyl group at the active site [13,14], while cysteinyl residues have been shown to be non-essential [15,16].

Here we report that rabbit muscle enolase, like the yeast enzyme [1–3], has a single essential arginyl residue per subunit which likely serves to bind the anionic substrates to the active site. This conclusion is based on chemical modification with butanedione in borate buffer, a system known to be highly selective for the modification of arginyl residues in proteins [1,2,17–20].

2. Materials and methods

2-Phosphoglycerate, 2,3-butanedione, Sephadex gels, and buffer salts were obtained from Sigma (St. Louis, MO). Commercial preparations of rabbit muscle enolase were obtained from Sigma, Calbiochem, and Boehringer-Mannheim. The preparation from Boehringer-Mannheim (lot 1078414) gave the highest specific activity (53 μmol phosphoenolpyruvate . min^{-1} . mg^{-1}) under the conditions of assay, and a single band on polyacrylamide gel electrophoresis [21], and was used for all further studies. Enzyme concentration was determined from the A_{280}, using A (0.1%) = 0.89 [16] and mol. wt 82 000 [10,15] for the dimeric [22] enzyme. Enzyme activity was determined at 25°C as in [23–25], with the exception that 2-phosphoglycerate was 2 mM. Activity was determined from the increase in A_{240} as a function of time, using a molar absorption coefficient of 1310 M^{-1} cm^{-1} for phosphoenolpyruvate under the assay conditions [26].

Modification with butanedione was carried out at 25°C 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 the procedures in [18,20].

3. Results

The time course for the inactivation of rabbit muscle enolase by butanedione in 50 mM borate (pH 8.3) depends on the concentration of reagent used (fig.1). Butanedione at 2.5 mM reduces the

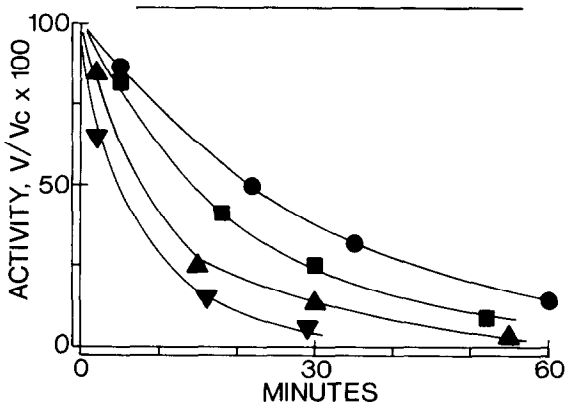

Fig.1. Rabbit muscle enolase inactivation by butanedione. The enzyme (2 μM) in 50 mM borate, 1 mM magnesium acetate, 0.01 mM EDTA, pH 8.3, was modified by: (●) 2.5 mM; (■) 5 mM; (▲) 10 mM; or (▼) 20 mM butanedione. The control retains full activity over this period of time.

Table 1
Correlation of rabbit muscle enolase inactivation by butanedione with arginine modification, and protection by substrates[a]

Enzyme	V/V_c x 100	Arg per subunit	Arg modified per subunit
Control	100	16.3	–
+ Butanedione			
5 min	88	14.1	2.2
20 min	57	14.0	2.3
40 min	33	13.8	2.5
75 min	15	13.3	3.0
+ Butanedione + 2-Phosphoglycerate	95	14.3	2.0

[a] Modification of rabbit muscle enolase (18 μM) was carried out with 4 mM butanedione in 50 mM borate, 1 mM magnesium acetate, 0.01 mM EDTA (pH 8.3). Aliquots were withdrawn at the indicated times, gel filtered to remove excess butanedione, assayed for enzymatic activity, and subjected to amino acid analysis by the procedures in [18,20]. For the sample modified in the presence of 2-phosphoglycerate, 10 mM, an aliquot was subjected to gel filtration after 40 min modification and subsequently analyzed. The values of Arg/subunit listed represent the average of duplicate analyses of single samples for each of the butanedione-treated samples, and an average of duplicate analyses of each of two separate control samples

activity to 50% of the control after 22 min, while only 14% activity remains after 1 h of modification. One feature of the modification of essential arginyl residues in enzymes by butanedione is that the inactivation is enhanced by borate [1,2,17–20]. This is the case with muscle enolase, for under conditions where activity is reduced to 20% in the presence of 50 mM borate, 80% activity remains if the borate is replaced by 50 mM *N,N*-bis-(2-hydroxyethyl)-glycine. This suggests that inactivation of muscle enolase by butanedione is due to the modification of essential arginyl residues.

Protection against inactivation is provided by substrates of muscle enolase (data not shown). After 1 h of modification by 2.5 mM butanedione in 50 mM borate (pH 8.3), 75% of the control activity remains when an equilibrium mixture of substrates

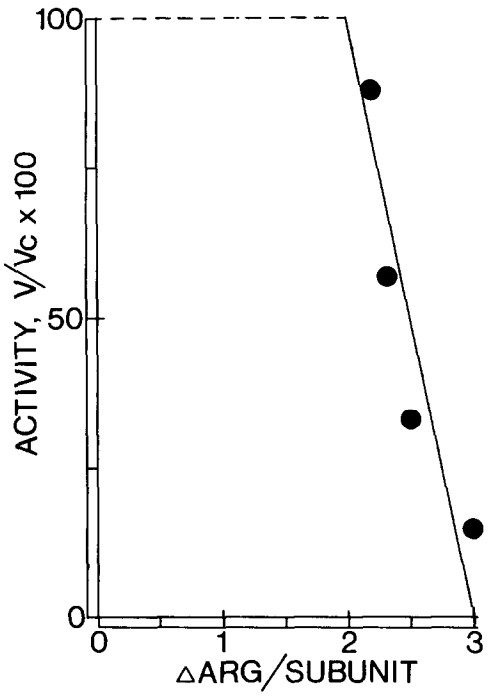

Fig.2. Correlation of the inactivation of rabbit muscle enolase with arginine modification by butanedione. Details of the experiment are given in table 1. The solid line is theoretical, calculated on the assumption that complete inactivation correlates with the modification of the third arginyl residue per subunit.

plus Mg^{2+} (generated by adding 20 mM 2-phosphoglycerate plus 1 mM magnesium acetate before initiation of modification) is also present, while only 14% activity remains when modification is carried out in the absence of substrates.

When inactivation by butanedione is correlated with the modification of arginyl residues, the data shown in table 1 are obtained. Muscle enolase has two very reactive arginyl residues per subunit which can be modified by butanedione without loss of activity. However, as shown in fig.2, activity is completely lost concomitant with the modification of a third arginyl residue per subunit. When modification is carried out in the presence of substrates, only two arginyl residues per subunit are modified and very little loss of enzymatic activity observed (table 1).

4. Discussion

Arginyl residues play a very general role in the binding of anionic substrates and cofactors to enzyme active sites [1]. These data based on chemical modification with butanedione in borate buffer, suggest that rabbit muscle enolase has a single essential arginyl residue per subunit (fig.2). Since substrates protect against the inactivation of muscle enolase and against modification of the essential arginine (table 1), it is very likely that arginine plays a similar role in this enzyme.

An unexpected feature of this study was that muscle enolase has two extremely reactive arginyl residues per subunit which can be modified by butanedione, either in the presence or absence of substrates, without loss of activity (table 1). It has been pointed out that quite often functional arginyl residues at enzyme active sites can be selectively modified by arginine-specific reagents [18,27]. However, these data represent the first example of an arginyl enzyme which has hyper-reactive arginyl residues which can be modified more rapidly than the essential arginyl residue.

In view of the very general role of arginine in enzymes, it is not surprising that enolases from such widely divergent sources as rabbit muscle and yeast [1–3] have essential arginyl residues. It has been suggested that for yeast enolase this arginyl residue is involved in binding the phosphate moiety of substrates to the active site [2]. It is quite likely that the essential arginyl residue of the muscle enzyme plays a similar role, and experiments to ascertain this are in progress.

Cardenas and Wold, comparing structural and catalytic properties of enolases from widely different sources, concluded that the chemical compositions of the active sites of different enolases are identical or very similar throughout the biological world [12]. The yeast and muscle enzymes are now known to contain essential carboxyl [5,13,14] and arginyl residues. Further studies will reveal whether or not other types of amino acid residues which are known to be essential for the activity of the yeast enzyme also play a crucial role in the catalytic mechanism of rabbit muscle enolase.

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