SELECTIVE CHEMICAL MODIFICATION OF ARGININE RESIDUES IN MITOCHONDRIAL MALATE DEHYDROGENASE

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SUMMARY: Mitochondrial malate dehydrogenase (L-malate: NAD^+ oxidoreductase, EC 1.1.1.37) from porcine heart exhibits a time dependent loss in enzymatic activity in the presence of the reagent butanedione. The inhibition occurs concomitant with the modification of 2.4 residues of arginine per molecular weight of 70,000. The presence of the reduced coenzyme, NADH, protects the enzyme from inhibition by butanedione and from modification of arginine residues, suggesting that the residues modified are located near the coenzyme binding site and hence at or near the enzymatic active center of this enzyme.

INTRODUCTION: Butanedione in the presence of borate buffer has been recently reported to be a highly selective reagent for modification of arginine residues in lactate dehydrogenase (1) and in carboxypeptidase A (2). Riordan (2) has suggested that the reversible complex formed between the guanido group of arginine and butanedione is stabilized by borate ion. Riordan also suggested that acidification of the reaction mixture can prevent regeneration of arginine, and, under the conditions employed for protein hydrolysis, a stable hydroxymethylimidazoline compound is formed. The formation of this derivative makes it possible to measure the degree of arginine modification in protein by the loss of arginine on routine amino acid analysis. Treatment of mitochondrial malate dehydrogenase (m-MDH) with this reagent in the presence of borate buffers leads to total inhibition of this enzyme with the course of the reaction following pseudo first order kinetics. Removal of the butanedione and the borate by gel filtration leads to approximately 80% reversa of the inhibition, an observation similar to that reported in the case of butanedione inhibited lactate dehydrogenase and carboxypeptidase.

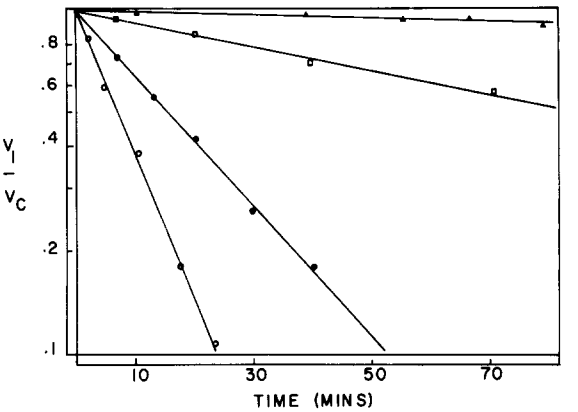

Figure 1

Effect of butanedione on the activity of MDH in 50 mM sodium borate, pH 7.5 at 30°. (O) 250 fold molar excess of butanedione (●) 150 fold molar excess of butanedione; (Δ) 150 fold molar excess of butanedione plus 75 mM NADH; (□) 150 fold molar excess of butanedione plus 150 mM ADP.

MATERIALS AND METHODS: Mitochondrial malate dehydrogenase was purified from acetone powders of fresh pig hearts and enzymatic assays were performed as previously described by Gregory, et. al. (3).

Butanedione obtained from Sigma was redistilled prior to use. A 10% solution of butanedione was prepared by dissolving distilled butanedione in 50 mM sodium borate buffer, pH 7.5, and adjusting the pH to 7.5. The time dependent inhibition of mitochondrial malate dehydrogenase was followed by assaying for dehydrogenase activity as a function of time. Samples were removed at intervals during the course of the inhibition and the reaction was terminated by the addition of 0.2 ml of 6 N HCl to 1.0 ml aliquots of the inhibition mixture. The precipitated protein was then subjected to acid hydrolysis in vacuo for 20 hours in 6 N HCl at 110°. Following removal of HCl, the sample was dissolved in 0.2 M sodium citrate buffer for analysis on a Glenco 100 AS Amino Acid Analyzer by the method of Spackman et. al. (4).

RESULTS AND DISCUSSION: Samples of malate dehydrogenase (3×10^{-5}M) were incubated with a 250 and a 150 fold molar excess of butanedione at 30° in 50 mM sodium borate buffer, pH 7.5. Control samples were incubated under

identical conditions without the addition of butanedione. The effect of this reagent on the enzymatic activity of m-MDH is shown in Figure 1. The inactivation is represented as the log of the ratio $V_I:V_C$ (velocity of inhibited sample to velocity of control sample), as a function of time. The concentration of butanedione in both cases was high with respect to the enzyme, therefore, pseudo first order kinetics were observed. The second order rate constant for the butanedione inhibition was calculated to be 10.0 $min^{-1}moles^{-1}$ liter. The addition of the reduced coenzyme, 75 mM NADH, to the incubation mixture was observed to prevent the inhibition of m-MDH by butanedione (Figure 1). Protection from inhibition by butanedione was also observed with 150 mM adenosine-5'-diphosphate (ADP); however 150 mM nicotinamide, an excess over the K_I of 67 mM for nicotinamide (5), had essentially no effect on the inhibition of m-MDH.

The inhibition by butanedione has been reported to be reversible in the case of lactate dehydrogenase (1) and carboxypeptidase A (2) upon removal of borate and reagent. This was also found to be true for the modification of malate dehydrogenase. The incubation mixture was allowed to react until only 15% of the initial enzymatic activity remained and was then passed over a 1.5 x 25 cm column of BioGel P-4 equilibrated with 50 mM phosphate, pH 7.5 at 4°. The eluting protein was assayed for enzymatic activity and was found to have regained 80% of the initial activity.

To quantitate the reaction of butanedione with m-MDH aliquots of the reaction mixture were removed at intervals during the time course of the inhibition and, according to the method of Riordan (2), the reaction was terminated by the addition of 6 N HCl. After acid hydrolysis of the precipitated protein, the samples were subjected to amino acid analysis. This enzyme contains approximately 16 arginine residues per molecular weight of 70,000. The complete inhibition of m-MDH with butanedione is coincident with the loss of approximately 2.4 moles of arginine per molecular weight 70,000 or approximately 1.2 residue per enzymatic active center. The modification was selective in that modification of residues other than arginine was not detected.

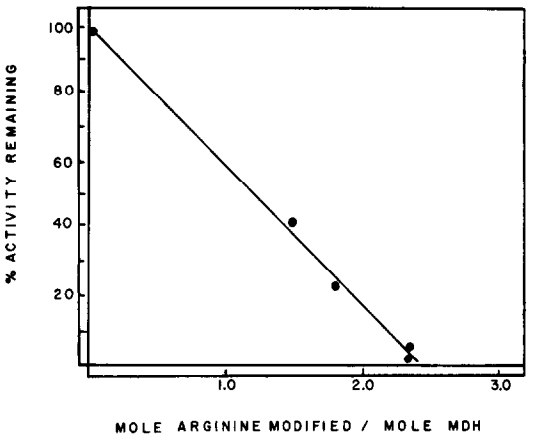

Figure 2

Correlation of the change of enzymatic activity with modification of arginine residues of malate dehydrogenase (3 x 10^{-5}M) with butanedione (7.5 mM) in 50 mM sodium borate, pH 7.5, at 30°. Aliquots of the inhibition mixture were treated as described in the text, and subjected to amino acid analysis to determine the residues of arginine present.

Figure 2 represents the loss of enzymatic activity as a function of the moles of arginine lost per mole of m-MDH. As can be seen in this figure there exists a direct relationship between the loss of enzymatic activity and the loss of approximately 2 residues of arginine. The addition of 75 mM NADH to the incubation mixture prevented the loss of arginine residues. Thus, NADH apparently renders specific residues at or near the coenzyme binding center, and thus, at or near the enzymatic active center, inaccessible for chemical modification by this reagent.

Thus, the inhibition of mitochondrial malate dehydrogenase by butanedione can be correlated with the modification of approximately 2 residues of arginine per molecular weight 70,000. These arginine residues are apparently near the binding site of the coenzyme NADH, or more specifically, near the binding site of the ADP portion of the NADH molecule. Arginine residues have also been implicated at or near the active center in lactate dehydrogenase, as determined from crystallographic techniques (6) and from butanedione modification (1). Additional similarities in active site structures of these dehydrogenases

may be seen in the presence of histidine (7, 8, 9) and cysteine (10) determined from studies on mitochondrial malate dehydrogenase as compared with lactate dehydrogenase (6) as well as glyceraldehyde-3-phosphate dehydrogenase, yeast alcohol dehydrogenase and liver alcohol dehydrogenase (11). The similarities of active center residues, including the implication of arginine by this study, are evidence for the conservation of the nature of these centers.

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