STRUCTURAL STUDIES OF PORCINE MALATE DEHYDROGENASE

SELECTIVE CHEMICAL MODIFICATION

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SUMMARY: Evidence is presented indicating that residues at or near the active center of mitochondrial malate dehydrogenase (L-malate-NAD^+ oxidoreductase, E.C.1.1.1.37) of porcine heart can be selectively carboxamidomethylated with the use of iodoacetamide. Protection against inactivation by addition of the coenzyme NADH and quantitation of incorporation of modifying reagent are discussed.

INTRODUCTION: Selective chemical modification has been utilized in the past as an important technique in the elucidation of the nature of the active centers of numerous enzymes. Recently the active centers of several dehydrogenases, including horse liver alcohol and rabbit muscle glyceraldehyde-3-PO_4 dehydrogenases, have been investigated by the use of various alkylating and sulfhydryl reagents (1,2). Selective modification of active center residues utilizing chromophoric or radioactive labeled reagents, allows one to not only study the residues modified, but these labels can act as markers for active center sequencing studies after limited proteolysis.

Recent reports on the inactivation of malate dehydrogenase (MDH) with various alkylating and sulfhydryl reagents have exemplified the difficulty in inhibiting the enzyme with various of these reagents. Silverstein and Sulebele (3) reported the use of a 28,000 fold molar excess of iodoacetate for 18 hours (pH 8.0) to yield an enzyme 78% inhibited. The use of a 2800 fold molar excess of fluorodinitrobenzene, a 940 fold molar excess of N-ethyl maleimide (NEM), and a 116 fold molar excess of 5,5' dithiobis (2-nitroben-

zoic acid) (DTNB), for 18 hours at pH 8.0 have led to an enzyme 94-100% inhibited. Fondy, Kitto and Driscoll (4) have used a 2 x 10^6 fold molar excess of iodoacetate at pH 7.0 in freeze-thaw studies to yield an enzyme 50-70% inhibited.

The following reagents were investigated as possible site specific modifying agents for MDH; iodoacetate, iodoacetamide, n-ethylmaleimide, and 5,5' dithiobis-2 nitrobenzoic acid. The neutral analogue of iodoacetate, iodoacetamide, was found to be the most effective inhibiting reagent of those listed with respect to required molar excess and time of incubation for complete inhibition. In this respect, iodoacetamide will totally inactivate this mitochondrial enzyme at a level of a 200 fold molar excess in a period of 2 hours. Inactivation of this enzyme with iodoacetamide occurs coincident with the incorporation of approximately two moles of iodoacetamide per mole of enzyme.

MATERIAL AND METHODS: Porcine heart mitochondrial malate dehydrogenase was purified from acetone powders of fresh pig hearts (Harrison; to be published). The resultant preparation exhibited a specific activity of 1600 units/ml/A_{280}, a value some 2 fold higher than previously reported purified enzyme (5). The standard assay consisted of 0.09 M sodium pyrophosphate buffer (pH 10.6), 1.5 mM NAD^+, 33 mM sodium L-malate; the enzyme was added at zero time, and the absorbancy increase at 340 nm was measured. Units of enzymatic activity are expressed as units of enzyme per ml, divided by the "A_{280} value", the absorption of the solution at 280 nm according to previous methods (5). One unit of enzyme is defined as the quantity which catalyzes the reduction of 1 μmole NAD^+/min. Protein concentrations were determined spectrophotometrically at 280 nm utilizing a molar extinction coeffecient of 17,800. Iodoacetate and iodoacetamide purchased from Sigma were recrystallized prior to use. Iodoacetamide-1-C^{14} obtained from Amersham-Searle was used without further purification. Aquaflor scintillation fluid was purchased from New England Nuclear.

Porcine malate dehydrogenase was incubated in 1 mM tris HCl (pH 7.5) at 25°C with the various inactivating reagents, with C^{14} labeled iodoacetamide, or with buffer as a control, and the time dependent inhibition was monitored as a decrease in catalysis of NAD to NADH in the standard assay. Reactions for quantitative C^{14} incorporation were quenched with β-mercapto ethanol and dialyzed prior to scintillation counting using standard radioisotope techniques.

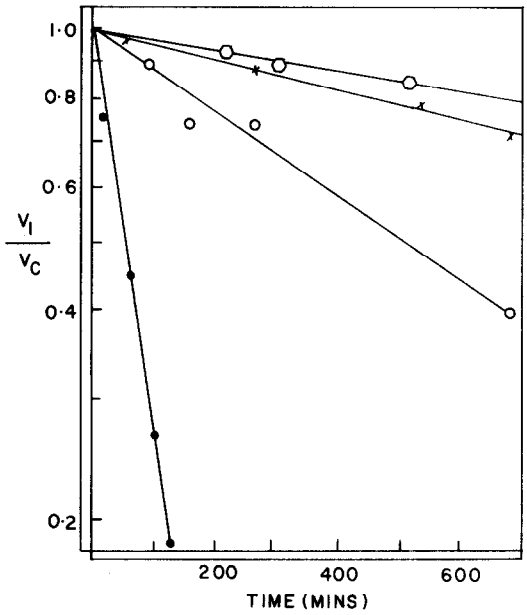

Figure 1

Effect of alkylating and sulfhydryl reagents on mitochondrial MDH activity as a function to time. Incubation was carried out in the dark at 25°C (pH 7.5) in 0.01 M tris HCl buffer as discussed in the text. Concentrations of inhibitors used: ○ 1000 molar excess iodoacetate, 0 1000 molar excess N-ethyl maleimide, X 1000 molar excess 5,5' DTNB, ● 200 molar excess iodoacetamide.

RESULTS AND DISCUSSION: Samples of malate dehydrogenase (8×10^{-6} - 3×10^{-5}M, based on 70,000 molecular weight) were incubated with 1000 fold molar excesses of iodoacetate, NEM and DTNB or a 200 fold molar excess of iodoacetamide at 25°C in 1 mM tris HCl (pH 7.5) for periods up to 8 hours. A control sample with equivalent amount of buffer but no alkylating or sulfhydryl reagent was

also incubated under the same conditions. The incubation was carried out in the dark to prevent formation of iodide ion from iodoacetate and iodoacetamide. Figure 1 represents the effect of these reagents on enzymatic activity expressed as log (velocity inhibited sample/velocity control sample) versus time in minutes. Concentrations of inactivating reagent in each case are high with respect to the enzyme, therefore, pseudo first order kinetics were observed. As can be seen, the 1000 molar excess of iodoacetate yields only 15% inhibition after 8 hours with a second order rate constant value of 0.22 min^{-1} $moles^{-1}$ liters. A 1000 molar excess of DTNB was only slightly more effective yielding a value of 20% inhibition in 8 hours, and exhibiting a second order rate constant of 0.24 min^{-1} $moles^{-1}$ liters. NEM in a 1000 molar excess yielded a value of 50% inhibition and the second order rate constant is 0.44 min^{-1} $moles^{-1}$ liters. The most effective inactivating reagent examined was found to be the neutral analogue of iodoacetate, iodoacetamide, which completely inhibited the enzyme in 2 hours at a concentration one fifth (200 molar excess) that of the other reagents used. The second order rate constant for iodoacetamide inhibition is 3.5 min^{-1} $moles^{-1}$ liter, a value of 8-12 fold that of the rate constants for the other reagents tested.

The rate of inactivation as a function of iodoacetamide concentration is shown in figure 2. The pseudo first order plots of a 100, 200 and 300 fold molar excess is shown. Inhibition was complete in 4.5 hours, 2.0 hours and 1.3 hours respectively. For subsequent work, a 200 fold molar excess was chosen, because this value gave a reasonable molar excess as well as a convenient inactivation time.

The addition of 0.15 m Molar reduced coenzyme, NADH, to the inactivating mixture (200 M excess iodoacetamide) yielded an enzyme 90% protected from inactivation. The addition of 0.2 m Molar NADH (an NADH/Enz ratio of 13/1) to the inactivation mixture caused complete abolition of inactivation as shown in figure 3. Under the same conditions a 200 molar excess of iodo-

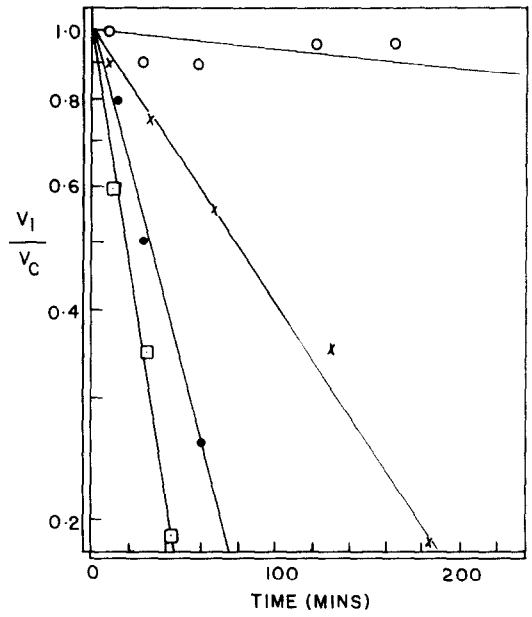

Figure 2

Effect of varying concentrations of iodoacetamide on MDH activity as a function of time. Incubation at 25°C (pH 7.5) in 0.01 M tris HCl was carried out in the dark as described in the text. Concentrations of inhibitors used: ◊ control sample of MDH, no iodoacetamide, X 100 molar excess iodoacetamide, ● 200 molar excess iodoacetamide, □ 300 molar excess iodoacetamide.

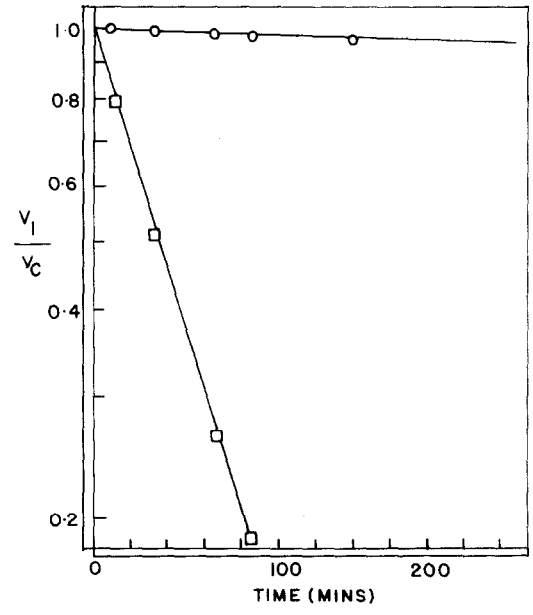

Figure 3

Effect of the reduced coenzyme, NADH, on iodoacetamide inhibition of MDH, Incubation carried out as described in the text. Concentrations of inhibitors used: 0 200 molar excess iodoacetamide + 0.2 mM NADH, □ 200 molar excess iodoacetamide. Control curve and NADH protection curve are identical.

acetamide yielded complete inhibition in 2 hours. NADH thus appears to render 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.

To quantitate (6) the reaction of iodoacetamide with MDH, an aliquot of enzyme was incubated both with and without NADH in the presence of a 200 fold molar excess of 1-C^{14} iodoacetamide. At the end of a 2 hour period, the NADH protected sample exhibited an activity 95% of a control sample, and the unprotected sample showed complete inhibition. The reaction was quenched by the addition of excess β-mercapto ethanol and the samples were subsequently dialyzed exhaustively against neutral phosphate buffer. Aliquots of the protected and unprotected samples were placed into aquaflor scintillation media and C^{14} content was determined by use of a Nuclear Chicago Scintillation counter. By the method of isotopic dilution it was determined that the inhibited sample contained an average of 2.2 moles C^{14} bound covalently and the coenzyme protected sample had an average of 0.2-o.3 moles C^{14} bound per 70,000 MW, thus indicating that the carboxamidomethylation of 2 residues in mitochondrial MDH, presumably at or near the enzymatic active center is responsible for the observed loss of catalytic activity. Pfleiderer (7) and Kaplan (8) have previously reported evidence for 2 catalytically active centers in porcine mitochondrial malate dehydrogenase per molecular weight of 70,000.

Under the conditions here employed, it appears that iodoacetamide can be utilized to selectively carboxamidomethylate a limited number of residues (two) leading to complete inactivation of mitochondrial MDH. The coenzyme, NADH, prevents both the enzymatic change and the modification of these two residues, an average of 1.0 per active, enzymatic site.

Work is currently in progress in our laboratory to determine the nature of the chemically modified residues as a first step in elucidation of the nature of the active center of this enzyme.

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