Role of Tyrosine in the Substrate Binding Site of Mitochondrial L-Malate Dehydrogenase from Bovine Heart Muscle*

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ABSTRACT: The role of functional groups in catalysis was investigated in mitochondrial malate dehydrogenase (MDH) isolated from bovine heart muscle. Of several groups examined, at least one tyrosyl residue was shown to be essential for substrate binding. MDH was strongly inhibited by acetylimidazole. Preincubation of the enzyme with substrates, coenzymes, or certain dicarboxylic acids, protected the enzyme against this reagent. The same dicarboxylic acids were competitive inhibitors of MDH. Spectral examination and chemical analysis of the acetylated enzyme showed that the reaction of tyrosyl residues, and not sulfhydryl or amino groups, caused loss of enzymatic activity. The acetylated enzyme was partially converted into its native form with hydroxylamine, accompanied by a regeneration of enzymatic activity. The acetylated enzyme was also reactivated at pH 10.0. The apparent Michaelis constants for L-malate and NAD were measured with acetylated MDH and did not differ from those values obtained with the native enzyme. MDH was also strongly inhibited by tetranitromethane. This inhibition was prevented by substrates, coenzymes, and the same dicarboxylic acids which protected the enzyme against acetylimidazole. Spectral and chemical analysis proved that tetranitromethane reacted with several tyrosyl residues. After nitration, the enzyme could not incorporate radioactive acetylimidazole. From these studies it was concluded that at least one tyrosyl residue is part of the substrate or coenzyme binding site of the enzyme. Additional experiments ruled out a direct participation of sulfhydryl groups in the catalytic mechanism of MDH.

The participation of a tyrosyl residue in the active site of an enzyme has been investigated in many laboratories. The presence of an "essential" tyrosyl residue in dogfish lactic dehydrogenase was suggested by Shen and Wassarman (1970). DiSabato (1965) proposed that such a residue was part of the pyridine nucleotide binding site in lactic dehydrogenase of chicken heart. The possible role of tyrosine in the mechanism of action of other enzymes such as α-amylase (Connellan and Shaw, 1970), and alkaline phosphatase (Reynolds and Schlesinger, 1969) has also been reported.

We will show that at least one tyrosyl residue is present at the "active site" of MDH.[1] Although we cannot distinguish with certainty between the coenzyme and substrate binding sites, the evidence suggests that the tyrosyl residue is a point of attachment for the substrates.

Experimental Section

Materials. Bio-Gel P-2 was purchased from Bio-Rad Laboratories. PMB, obtained from the Sigma Chemical Co., was recrystallized by the method of Boyer (1954). NAD and NADH were also purchased from Sigma Chemical Co. Recrystallized bovine serum albumin was acquired from Pentex Corp. L-3-Nitrotyrosine, L-3-aminotyrosine, and glutathione were obtained from the Nutritional Biochemical Corp. *N*-Acetylimidazole, purchased from Eastman Kodak Corp., was recrystallized from benzene, dried under vacuum, and stored in a desiccator over P_2O_5 at 5°. Acetyl-*1*-^{14}C-imidazole, obtained from Calatomic Corp. had a specific radioactivity of 1 mCi/mmole. Sodium propionate, maleic acid, and L-(+)-glutamic acid were purchased from Matheson Coleman & Bell. We bought the following compounds from Mann Research Laboratories: *N*-acetyl-L-tryptophan, *N*-acetyl-DL-tyrosine, *N*-acetyl-DL-leucine, *N,O*-diacetyl-DL-tyrosine, L-aspartic acid, D-aspartic acid, DL-β-hydroxybutyric acid, malonic acid, and fumaric acid. From Calbiochem Corp., we bought: L-leucine, L-tryptophan, L-histidine, *N*-acetyl-L-histidine, L-malic acid, D-malic acid, and oxalacetic acid. Tetranitromethane and sodium trinitrobenzenesulfonate were obtained from the Aldrich Chemical Co. Ninhydrin was purchased from the Pierce Chemical Co.

Methods. MDH was purified from bovine heart muscle as previously described (Siegel, 1967). Unless otherwise stated, the enzyme was thoroughly dialyzed against 0.001 M potassium phosphate (pH 7.4, 5°) prior to use; 100% enzymatic activity corresponded to the oxidation of 340–350 μmoles/min per mg of protein of L-malate by NAD in 0.1 M glycine (pH 10.0) at 25° (Siegel and Englard, 1961, 1962; Siegel, 1967). Protein determinations were done by the method of Lowry *et al.* (1951), using bovine serum albumin as a standard.

The sulfhydryl groups of MDH were analyzed by the spectrophotometric method of Boyer (1954). All spectrophotometric measurements were made on a Hitachi Perkin-Elmer Model 139 instrument.

All gel filtration experiments with Bio-Gel P-2 were done at room temperature with a column 100 × 1.3 cm, thoroughly equilibrated with the respective buffer.

MDH was acetylated with acetylimidazole in an automatic titrator (Radiometer) at a constant pH of 7.4 at 25°. The desired pH was maintained during the reaction by the dropwise addition of 0.1 M NaOH. Suitable aliquots were removed after incubation and assayed for enzymatic activity.

The reactions of MDH with sodium trinitrobenzenesulfonate were run in a similar manner at a constant pH of 7.4.

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[1] Abbreviations used are: MDH, mitochondrial malate dehydrogenase; PMB, *p*-hydroxymercuribenzoate; NAD, nicotinamide-adenine dinucleotide; NADH, reduced NAD; TNBS, sodium trinitrobenzenesulfonate.

Amino groups were analyzed by the method of Rosen (1957). Amino acid analysis was kindly done by Dr. Jules Shafer on an automatic analyzer, by the method of Spackman *et al.* (1958). The apparent Michaelis constants for L-malate and NAD were measured as described previously (Siegel and Englard, 1961).

Results

Role of Cysteine in the Mechanism of Action of MDH. MDH contains 12 residues of cysteine/molecule and no disulfide linkages (Siegel and Englard, 1962). The enzyme is inactivated by PMB, although partial protection against this inhibitor can be achieved at high phosphate concentration (Siegel, 1967).

In order to determine whether one or more sulfhydryl groups participated directly in the catalytic function of the enzyme, the following experiment was conducted: MDH (0.5 mg) was incubated with a 30-fold molar excess of PMB at 0° in 3.0 ml of 0.001 M potassium phosphate (pH 7.4). After 30 min, no enzymatic activity remained. In the presence of 0.01 M NAD, NADH, malate,[2] or oxalacetate, only NADH protected the enzyme against PMB. (72% of the initial activity remained after 30 min. The other compounds had no effect. MDH was incubated with PMB in an identical manner (without substrates or coenzymes present), and mercaptide formation was measured (Boyer, 1954). The increase in absorbancy at 250 mμ indicated a complete conversion of all 12 sulfhydryl groups of MDH to mercaptides, with no enzymatic activity remaining. When the reaction was done in 0.02 M phosphate instead of 0.001 M, all 12 sulfhydryl groups reacted but 25% of the enzymatic activity remained. To be sure that the extinction coefficient of the mercaptide did not increase at the higher phosphate concentration, we titrated a sample of glutathione with PMB at both phosphate concentrations, and observed no difference in mercaptide absorbance. Since MDH retains 25% of its optimal activity with all the sulfhydryl groups "immobilized," these groups probably do not participate directly in catalysis, but may stabilize the structure of the protein.

Role of Tyrosine in MDH. REACTION OF MDH WITH ACETYLIMIDAZOLE. In order to study the possible role of tyrosine at the substrate binding site of MDH (which contains nine such residues), we selected acetylimidazole as a potential inhibitor. The enzyme (0.7 mg) was incubated with 4.0 mg of acetylimidazole at 25° for 10 min at pH 7.4 in the presence of each coenzyme, substrate, and compounds related structurally to the substrates. The results are presented in Table I. L-Malate, oxalacetate, NAD, and NADH all protect the enzyme against this inhibitor. D-Malate affords some protection but is less effective than the L isomer. Of the other compounds tested, only dicarboxylic acids ranging from three to six carbon atoms prevented loss of activity. It was previously shown that a substrate of MDH must also be a dicarboxylic acid of three to five carbon atoms (Davies and Kun, 1957; Siegel and Englard, 1961). Monocarboxylic acids were ineffective against acetylimidazole as were L-aspartic and L-glutamic acids. An amino group in the latter two compounds might prevent interaction with MDH. High salt concentration prevented loss of MDH activity, but had no effect at the same concentration as the other compounds.

It seemed likely that the mode of protection involved a direct interaction of the dicarboxylic acids at the substrate binding site of the enzyme. We tested these compounds as possible competetive inhibitors of MDH. The same dicarboxylic acids which protected against acetylimidazole were also competitive inhibitors. The enzyme was assayed by measuring the reduction of oxalacetate by NADH at pH 7.4. A typical double-reciprical plot is shown for fumarate in Figure 1 where competitive inhibition kinetics are observed. Table II summarizes these results using a single concentration of various compounds. All dicarboxylic acids tested were competitive inhibitors of MDH with the exception of L-aspartic acid. Monocarboxylic acids had little effect on enzymatic activity.

Further indication that the dicarboxylic acids interacted at the substrate binding site of the enzyme is seen in Table I. When MDH reacted with acetylimidazole in the presence of 2.0×10^{-5} M NADH, 39% of the enzymatic activity was re-

TABLE I: Protection of MDH against Acetylimidazole.[a]

Compound (K Salt)	% Enzymatic Act. Rel to Control
None	10
NADH	100
Mesoxalate	90
Malonate	77
Glutarate	75
L-Malate	74
α-Ketoglutarate	73
NAD	61
Oxalacetate	60
L-Tartrate	58
Fumarate	57
Maleate	52
Succinate	50
D-Malate	36
D-Tartrate	35
Adipate	28
L-Glutamate	17
L-Aspartate	17
α-Ketobutyrate	14
Pyruvate	14
D-Aspartate	13
β-Hydroxybutyrate	11
Lactate	10
Propionate	8
Acetate	8
Chloride (0.2 M)	100
Sulfate (0.2 M)	100
Tris (Cl^-) (0.2 M)	92
Phosphate (0.2 M)	82
Phosphate (0.01 M)	14
Tris (Cl^-) (0.01 M)	12
Succinate + NADH (0.00002 M)	89
Succinate + malate (0.0004 M)	50
NADH (0.00002 M)	39
L-Malate (0.0004 M)	27

[a] Reaction mixtures of 2.5 ml contained 0.7 mg of MDH, 4.0 mg of acetylimidazole, 25 μmoles of the compound tested, and 0.5 μmole of potassium phosphate. The reaction took place at a constant pH of 7.4, at 25°. The controls contained MDH and the respective compound tested. After 10 min, aliquots were removed for assay.

[2] Refers to the L isomer unless otherwise specified.

TABLE II: Competitive Inhibitors of MDH.[a]

Compound (K Salt)	% Enzymatic Act.
Control	100
L-Aspartate	100
D-Aspartate	99
Pyruvate	94
Lactate	93
Acetate	92
β-Hydroxybutyrate	92
D-Tartrate	69
Adipate	63
Glutarate	52
Succinate	42
Fumarate	28
Mesoxalate	13
Malonate	12
L-Tartrate	9

[a] Reaction vessels of 3.0 ml were prepared at 25° and contained 1.8 μmoles of oxalacetate, 0.5 μmole of NADH, 30 μmoles of potassium phosphate, and 660 μmoles of the compound to be tested, at pH 7.4. The enzyme was added last and initial rates were measured at 340 mμ. Enzymatic activity of 100% corresponds to the oxidation of 1050 μmoles of NADH/(min mg) of protein.

tained. Acetylation in the presence of 0.01 M succinate resulted in 50% retention of enzymatic activity. In the presence of both NADH (2.0×10^{-5} M) and succinate (0.01 M), 89% of enzymatic activity remained. This equals the protection by the two compounds separately. The same experiment with 0.01 M succinate and 4.0×10^{-4} M malate, gave only 50% protection with the combination, compared to a sum of 77% by the compounds separately. These data suggest that succinate and NADH interact at different loci on the enzyme surface, while succinate and malate compete for a site on the enzyme.

SPECTRAL ANALYSIS OF ACETYLATED MDH. In order to identify the site of acetylation on the enzyme we reacted MDH with acetylimidazole until 85% of the activity disappeared. The reaction was terminated by lowering the pH to 3.5 in citrate buffer, followed by exhaustive dialysis against 0.001 M potassium phosphate at pH 7.4. Figure 2 shows the difference spectra of acetylated MDH *vs.* MDH, and *N,O*-diacetyltyrosine *vs.* tyrosine. We inferred from these data that tyrosyl residues in MDH were acetylated.

We attempted to protect MDH against acetylimidazole with several phenolic, imidazole, and indole compounds (Table III). Only *N*-acetyltyrosine was significantly effective.

REACTIVATION OF ACETYLATED MDH. (1) The spectrum of acetylated MDH was partially converted into that of the native enzyme by hydroxylamine. This was accompanied by a regeneration of enzymatic activity (Figure 3). In each case the degree of reactivation was complete in about 15 min and remained constant for at least 17 hr. (2) MDH was inhibited 80% with acetylimidazole at pH 7.4 and allowed to incubate at pH 10.0 in a pH-Stat. Appreciable reactivation occurred, with 65% of the original activity regenerated after 15 min and 75% after 30 min. Such reactivation might be expected in view of the lability of the phenolic ester linkage of acetyltyrosine in mild alkali.

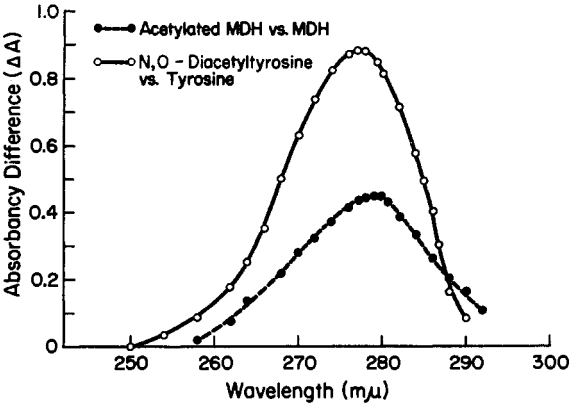

FIGURE 2: Difference spectrum of native *vs.* acetylated MDH. MDH (8.6 mg) reacted with 112 mg of acetylimidazole at a constant pH of 7.4, at 25°. When only 15% of the enzymatic activity remained, the pH was lowered to 3.5 with citrate buffer, and dialyzed thoroughly against 0.001 M potassium phosphate (pH 7.4) at 5°. No enzymatic activity remained by the time the spectra were examined. For comparison, the difference spectrum of *N,O*-diacetyltyrosine (0.0001 M) *vs.* tyrosine (0.0001 M) is also shown in 0.001 M potassium phosphate (pH 7.4).

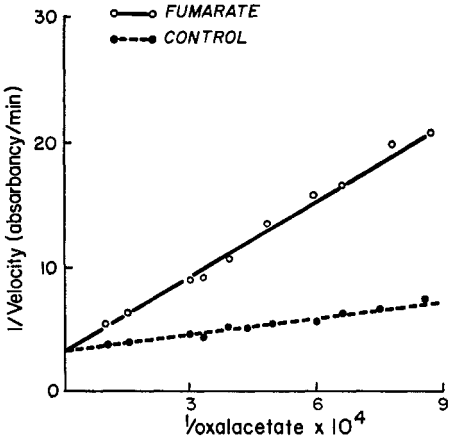

FIGURE 1: A double-reciprocal plot showing competitive inhibition of MDH by fumarate with respect to oxalacetate. See Table II for conditions.

TABLE III: Protection of MDH against Acetylimidazole.[a]

Compound (K salt)	% Enzymatic Act. Rel to Control
None	15
N-Acetyltyrosine	72
N-Acetyltryptophan	33
N,O-Diacetyltyrosine	32
Histidine	31
N-Acetylhistidine	20
Imidazole	18
Leucine	18

[a] The conditions were identical with those in Table I. Each reaction mixture contained 20 μmoles of the respective compound tested in a total volume of 2.0 ml.

TABLE IV: Protection of MDH against Trinitrobenzenesulfonate.[a]

Compound (K Salt)	% Enzymatic Act. Rel to Control
None	15
NADH	85
Tris (Cl^-)	40
Phosphate	32
Oxalacetate	28
Fumarate	27
Propionate	27
Chloride	27
NAD	26
Acetate	25
L-Malate	24
Glutarate	23
Sulfate	18

[a] Reaction mixtures of 2.5 ml contained 0.14 mg of MDH, 7.5 μmoles of TNBS, 0.5 μmole of potassium phosphate, and 25 μmoles of each compound tested. The experiment was conducted at a constant pH of 7.4 and suitable aliquots were removed for assay after 30-min incubation.

POSSIBLE ACETYLATION OF AMINO GROUPS AND SULFHYDRYL GROUPS BY ACETYLIMIDAZOLE. Data thus far did not rule out the possibility that tyrosyl residues were acetylated as a side reaction, with loss of enzymatic activity attributable to a reaction with some other functional group. Acetylation of a sulfhydryl group was ruled out on two grounds. Firstly, malate, oxalacetate, and NAD, all protected MDH against acetylimidazole but not against the sulfhydryl reagent, PMB. Secondly; when 85% of the enzymatic activity was destroyed by acetylation, no decrease in titratable sulfhydryl groups by PMB was observed.

Loss of activity due to acetylation of an amino group was ruled out by two lines of evidence. (a) When 85% of the MDH activity was destroyed by acetylation, ninhydrin analysis indicated no change in the number of free amino groups (35 amino groups in MDH), although further loss of activity was accompanied by acetylation of some amino groups. (b) Additional evidence was obtained by reacting MDH with trinitrobenzenefulfonic acid, a reagent which reacts readily with amino groups. MDH (0.14 mg) was incubated with 3.0 μmoles of TNBS at pH 7.4, 25°, for 30 min (Table IV). TNBS caused extensive inhibition of the enzyme which was prevented only by NADH. NAD, malate, and oxalacetate were not effective. When 85% of the enzymatic activity was destroyed by TNBS, the ninhydrin equivalent of the enzyme decreased 70%. In a separate experiment, MDH was inhibited 50% with TNBS with a 40% decrease in ninhydrin equivalent of the enzyme. No specific protection by NAD, malate, or oxalacetate was observed. These data strongly suggest that amino groups are not part of the substrate binding site of the enzyme, although free amino groups are required for a fully active enzyme.

DETERMINATION OF THE MICHAELIS CONSTANTS FOR L-MALATE AND NAD WITH ACETYLATED MDH. The possibility existed that acetylimidazole did not react at the substrate binding site of MDH but caused a change in protein conformation. To test this hypothesis, we determined the Michaelis constants for the substrates with the acetylated enzyme.

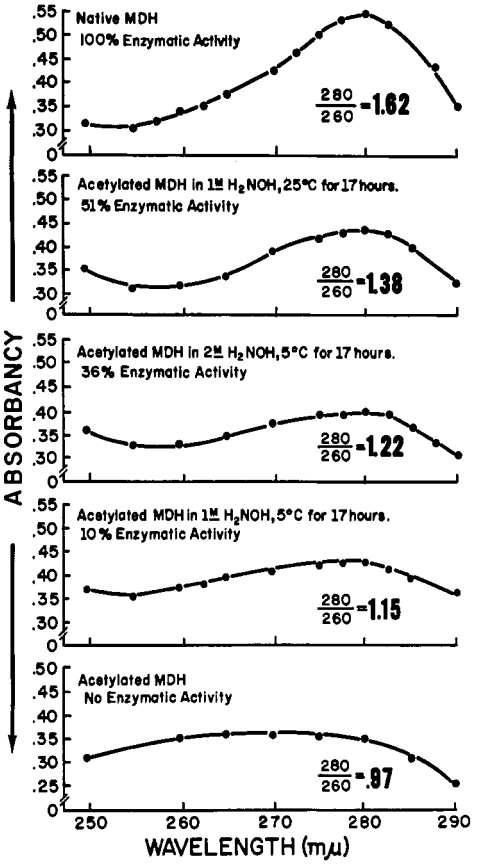

FIGURE 3: Acetylated MDH and reactivation by hydroxylamine. Acetylation was accomplished as described in Figure 2. Samples of acetylated enzyme were incubated with hydroxylamine at pH 7.4 as shown in the figure and redialyzed against 0.001 M potassium phosphate (pH 7.4) prior to examining the spectra.

MDH reacted with acetylimidazole (as described earlier) until 60% of the enzymatic activity still remained. The mixture was dialyzed against 0.001 M potassium phosphate (pH 7.4) at 5° for 1 hr. During dialysis, the enzymatic activity decreased to 35% of its original activity, indicating that further acetylation occurred. The apparent Michaelis constants measured at pH 10.0 were: 3.2×10^{-4} M for L-malate and 1.5×10^{-4} M for NAD. The corresponding values measured with the native enzyme were: 3.7×10^{-4} M for L-malate, and 9.9×10^{-5} M for NAD (Siegel and Englard, 1961). Thus the apparent Michaelis constants for malate and NAD with the partially denatured enzyme (35% active) did not differ significantly from those measured with the native enzyme.

REACTION OF MDH WITH TETRANITROMETHANE. Independent proof that one or more tyrosyl residues were part of the active site of MDH was obtained by a reaction with tetranitromethane.

The enzyme (0.22 mg) was incubated with 8.0 μmoles of tetranitromethane at pH 7.4 for 60 min at 35°. MDH was protected against this reagent with coenzymes, substrates, and related compounds. These results are shown in Table V. As with acetylimidazole, only dicarboxylic acids protected the enzyme, with the exception of L-aspartic acid. Monocarboxylic acids had no significant effect. These results are virtually identical with those obtained with acetylimidazole.

IDENTIFICATION OF 3-NITROTYROSINE IN MDH. To prove that tyrosine residues were nitrated, MDH (6.3 mg) was incubated with 56 μmoles of tetranitromethane in 0.01 M Tris

TABLE V: Protection of MDH against Tetranitromethane.[a]

Compound (K Salt)	% Enzymatic Act. Rel to Control
None	11
NAD	76
L-Malate	73
NADH	60
Oxalacetate	54
Glutarate	40
Fumarate	35
L-Aspartate	16
Acetate	15
Propionate	14
β-Hydroxybutyrate	13
α-Ketobutyrate	12
Oxalacetate + NAD	100
L-Malate + NADH	80

[a] Each reaction mixture contained 2.0 ml of: 0.22 mg of MDH, 0.01 ml of ethanol (used for dilution of TNM), 8.0 μmoles of TNM, 4.0 μmoles of potassium phosphate, and 20 μmoles of the compound tested. After 60 min at pH 7.4, 35°, aliquots were removed for assay. The control tubes contained MDH and the respective compound.

buffer at pH 8.0 for 60 min at 35°. Over 90% of the original enzymatic activity was destroyed. The mixture was separated on a Bio-Gel P-2 column equilibrated with 0.01 M Tris buffer at pH 8.0 (25°). The enzyme fractions were pooled, concentrated by ultrafiltration at 5°, and dialyzed at this temperature for several hours against 0.01 M Tris buffer at pH 8.0. Spectra of the nitrated enzyme at different pH values are shown in Figure 4. Above pH 7.0, an absorbancy maximum of 428 mμ was observed. The isosbestic point was 382 mμ compared to 381 mμ for 3-nitrotyrosine (Sokolovsky *et al.*, 1966). Nitrated tyrosyl residues of MDH, reduced with sodium hydrosulfite, were converted into 3-aminotyrosyl residues, evidenced by a spectral shift shown in Figure 4. We calculated that six of the nine tyrosyl residues of MDH were nitrated.

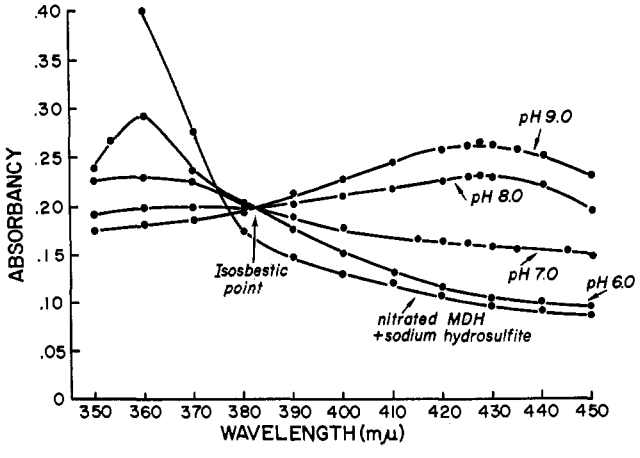

FIGURE 4: Spectra of nitrated MDH before and after reduction with sodium hydrosulfite. MDH was nitrated with tetranitromethane as described in the text. A sample of the nitrated enzyme was reduced to the amino derivative with 40 μmoles of sodium hydrosulfite in 0.01 M Tris (pH 8.0) for 5 min at room temperature. The spectra of nitrated and reduced forms are shown.

COMPLETE AMINO ACID ANALYSIS OF NITRATED MDH. MDH (3.6) mg) was reacted with tetranitromethane (30 μmoles) as previously described. The nitrated enzyme was hydrolyzed for 24 hr in 6 N HCl and subjected to a total amino acid analysis. The results showed a decrease in the tyrosine content of the protein as well as the emergence of a new peak identified as 3-nitrotyrosine. From these data we calculated 4.5 residues of 3-nitrotyrosine/molecule. This reinforces the spectral data, although the values from the complete analysis were somewhat lower.

COMPETITION BETWEEN ACETYLIMIDAZOLE AND TETRANITROMETHANE FOR MDH. Additional proof that both acetylimidazole and tetranitromethane reacted with the same tyrosyl residues of MDH was obtained by successive incubation of each reagent with the enzyme. MDH (3.0 mg) reacted with tetranitromethane (30 μmoles) until more than 95% of the enzymatic activity disappeared. The nitrated enzyme was separated from the excess reagent on a Bio-Gel P-2 column equilibrated with 0.001 M potassium phosphate at pH 7.4 (25°). After dialysis overnight at 5°, it was incubated with 5 μCi of acetyl-*1*-^{14}C-imidazole for 20 min at 25°. The mixture was separated on a Bio-Gel P-2 column and the radioactive enzyme concentrated and analyzed in a liquid scintillation counter (Bray, 1960). A control sample run through this entire procedure without prior exposure to tetranitromethane contained 10,400 cpm (0.8 μmole of acetylimidazole/mole of MDH) compared to 600 cpm for the nitrated enzyme. The low incorporation of radioactive acetylimidazole into native MDH is probably due to the instability of this compound in aqueous media.

TRYPTOPHAN ANALYSIS OF MDH. Analysis for tryptophan in MDH by the method of Harrison and Hoffmann (1961), revealed the presence of two residues of tryptophan per molecule instead of one which was reported earlier (Siegel and Englard, 1962).

Discussion

We investigated the possible role of four functional groups in the substrate binding site of MDH: the sulfhydryl, amino, imidazole, and phenolic groups. Other functional groups such as the guanido, carboxyl, indole, etc., have not yet been considered.

It is unlikely that a sulfhydryl group is involved in either substrate binding or the catalytic mechanism for the following reasons. Firstly, NAD, malate, and oxalacetate did not protect the enzyme against the sulfhydryl reagent, PMB. Secondly, it was possible to react all 12 sulfhydryl groups with PMB in 0.02 M potassium phosphate (pH 7.4) and still retain 25% enzymatic activity. These results resemble a report by Reynolds and McKinley-McKee (1970) who found that liver alcohol dehydrogenase still contained 2–2.5% enzymatic activity after carboxymethylation of an important sulfhydryl group, which may not be essential for catalysis as previously thought.

The presence of some activity at the higher phosphate concentration with the sulfhydryl groups all modified, could be due to a stabilizing effect of inorganic phosphate on the structure of MDH. High phosphate concentration is known to protect MDH against most inhibitors, as well as promote re ombination of inactive subunits into an active enzyme (Siegel, 1967).

We studied the possible role of tyrosine in MDH by reacting

the enzyme with acetylimidazole. Substrates and coenzymes protected the enzyme against this inhibitor. Notably, only compounds similar in structure to malate or oxalacetate (dicarboxylic acids containing three to six carbon atoms) protected MDH against acetylimidazole. This led to believe that the dicarboxylic acids protected the enzyme by interacting at the substrate combining site. As one might expect, these compounds were competitive inhibitors of MDH, although high concentrations were required to demonstrate competitive inhibition. Further support for the interaction of these compounds at the substrate binding site was obtained by demonstrating that succinate and malate compete for a site on the enzyme. This was evident when these compounds were combined to protect the enzyme against acetylimidazole. Less protection was observed than when the two compounds were used separately.

The likelihood that acetylimidazole attacked the active site of MDH was reinforced, but not proven, by demonstrating that the apparent Michaelis constants for the substrate and coenzyme were not altered in the partially denatured enzyme. Acetylimidazole presumably inhibits MDH by (1) attacking a substrate or coenzyme binding site directly, or a point in the active-site region to interfere with the hydrogen-transfer mechanism; (2) attacking some other site, causing a conformational change unfavorable to catalytic function.

In the first case, one would expect a complete loss of activity with the acetylated molecules and no change in K_m with the unreacted molecules. In the second case, one would expect a significant change in K_m if all molecules were acetylated but partially active, and a composite of two or more K_m values (depending on the number and degree of unfavorable conformational states) if some molecules were acetylated (partially active) and some unreacted. A point of ambiguity arises in the second case if a fraction of the molecules were acetylated and completely inactive due to conformation changes. No change in K_m would be expected with the unreacted molecules. This seems to be the least likely possibility since acetylated MDH is rapidly reactivated at pH 10.0 and low ionic strength, a condition which is very unfavorable for restoration of configurationally altered MDH.

Spectral examination of acetylated MDH (completely inactive) revealed that one or more tyrosyl residues reacted. The relationship between enzymatic activity and the presence of free phenolic groups was illustrated by reactivation of acetylated MDH with dilute alkali and with hydroxylamine at neutral conditions.

In order to prove conclusively that the acetylation of tyrosyl residues was not a side reaction, we examined the reactivity of other functional groups in MDH with acetylimidazole. The possibility that sulfhydryl groups were acetylated simultaneously with the phenolic groups, was ruled out. After 85% loss of enzymatic activity by acetylation, all 12 sulfhydryl groups were still titratable with PMB. Furthermore, loss of activity due to modification of the sulfhydryl groups with PMB was not prevented by malate or oxalacetate, whereas these substrates did protect the enzyme aganist acetylation. Regarding possible acetylation of amino groups in MDH, 85% of the enzymatic activity was destroyed by acetylation, with no decrease in the number of free amino groups. When MDH reacted with TNBS, extensive modification of the amino groups occurred with considerable loss of activity. Malate, oxalacetate, and NAD did not protect the enzyme specifically against this reagent, suggesting that an amino group is not part of the substrate binding site of MDH. Although some free amino groups are important for maximal activity, their role at the present time is unclear. The possibility that an imidazole residue was acetylated has not been completely ruled out. However, we have not been able to demonstrate that such a group is acetylated under the conditions of our experiments. Also, compounds such as imidazole, histidine, and *N*-acetylhistidine, did not protect MDH against acetylimidazole.

Independent proof that a tyrosyl residue was part of the active site of MDH was obtained by reacting the enzyme with tetranitromethane. Nitration caused considerable loss of enzymatic activity which was prevented by substrates, coenzymes, and specific dicarboxylic acids. The striking similarity between these results and those obtained with acetylimidazole strongly suggests that both reagents reacted with the same functional group. Thus, the possibility that acetylation of a histidyl residue caused loss of activity seems even more remote. This does not mean that histidine is not part of the substrate binding site of the enzyme, but that the inhibitors used did not react with histidyl residues in the enzyme. The fact that incorporation of radioactive acetylimidazole into nitrated MDH was insignificant compared to the native enzyme, supports the view that both reagents reacted with the same tyrosyl residues.

Final proof that the tyrosyl residues of MDH reacted with tetranitromethane came from spectral and total amino acid analysis, which indicated that five to six of the nine tyrosyl residues were nitrated.

From the data presented, we propose that at least one tyrosyl residue is present at the active site of MDH. It most likely serves as a point of attachment to the substrates, L-malate, and oxalacetate rather than to NAD and NADH for the following resaons. (1) Malate and oxalacetate protected the enzyme only against phenolic reagents, whereas NADH protected MDH against most inhibitors (urea, guanidine, sulfhydryl, and amino reagents, etc.). (2) Only nonsubstrates which bear a structural resemblance to malate or oxalacetate protected the enzyme against tyrosyl reagents. These compounds did not shield the enzyme specifically against other inhibitors. (3) The ratio of protection by malate:oxalacetate against both tyrosyl reagents was almost identical (1.23 for acetylimidazole and 1.35 for tetranitromethane). On the other hand, NADH protected the enzyme much more effectively than NAD against acetylimidazole (1.41), but less effectively against tetranitromethane (0.79). Thus, the substrates showed a greater specificity for protection against phenolic reagents than did the coenzymes.

These observations do not completely rule out an "essential" tyrosyl residue as a point of attachment for the pyridine nucleotides for two reasons. (1) The pyridine nucleotides do protect MDH against tyrosyl reagents. (2) The substrates and related dicarboxylic acids, bind to the enzyme at the substrate combining site which may be adjacent to the pyridine nucleotide site containing an essential tyrosyl residue. The binding of these compounds might sterically block this adjacent site from phenolic reagents. Although this does not seem likely, we have not yet ruled it out.

At the present time, we prefer to suggest that a tyrosyl residue is present at either the substrate or coenzyme binding site, or perhaps both.

Finally, the requirement that a substrate of MDH must have two carboxyl groups, increases the probability that two amino acids in the enzyme may be involved in the binding of the substrates, with additional amino acids required to bind the coenzymes. We are presently investigating the possible role of other functional groups in this process, as well

as attempting to distinguish between the substrate and coenzyme sites, and the essential and nonessential tyrosyl residues.

Acknowledgment

The authors thank Professor Minor J. Coon for his helpful suggestions.

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Interactions of Fructose 1,6-Diphosphate, Substrates, and Monovalent Cations with Yeast Pyruvate Kinase Monitored by Changes in Enzyme Fluorescence*

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ABSTRACT: The tryptophyl fluorescence of yeast pyruvate kinase [like the rabbit muscle enzyme (Suelter, C. H. (1967), *Biochemistry* 6, 418)] is quenched by the addition separately or together of the activating cations, K^+ and Mg^{2+}. The quenching is minimal, however, even in the presence of the substrate, phosphoenolpyruvate (PEP), when compared to the quenching observed in the presence of the activator, fructose 1,6-diphosphate (FDP), either in the presence or absence of the cations or PEP. Titration of the enzyme with FDP in the presence of Mg^{2+} monitored by the fluorescence change reveals a marked dependence of the FDP binding constant on the nature of the effectors present. Addition of 0.23 M K^+ increases the apparent K_D for FDP from 0.48 to 3.1 mM. $(CH_3)_4N^+$ has a similar though smaller effect. On the other hand, the addition of PEP markedly reduces the apparent K_D for FDP to 0.069 mM. K^+ is required to obtain the reduced K_D; $(CH_3)_4N^+$ will not function. Adenosine 5′-diphosphate also promotes a decrease in the apparent K_D for FDP, but no monovalent cation requirement is observed. Changing the temperature from 30 to 0° in the presence or absence of K^+ or PEP decreases the apparent K_D for FDP by an order of magnitude. The data suggest a conformational transition favored by FDP or lowered temperature, which differs from the cation-promoted transition.

Yeast pyruvate kinase (EC 2.7.1.40), like the rabbit muscle enzyme, requires both monovalent and divalent cations for catalytic activity (Boyer *et al.*, 1942; Washio and Mano, 1960; Hunsley and Suelter, 1960b). Aside from this similarity, the two enzymes appear to differ in molecular weight (Kuczenski and Suelter, 1970b; Bischofberger *et al.*, 1970), temperature stability (Kuczenski and Suelter, 1970a), and kinetic properties (Reynard *et al.*, 1961; Hess *et al.*, 1966; Hunsley and Suelter, 1969b). Fructose 1,6-diphosphate, which activates the yeast enzyme, functions by decreasing the substrate K_m's; the same V_{max} is obtained in the absence of fructose 1,6-diphosphate by increasing K^+ from 0.1 to 0.23 M (Hunsley and Suelter, 1969b). The enzyme from *Streptococcus carlsbergenesis* required FDP[1] to achieve optimum V_{max} (Hess and Haeckel, 1967).

Suelter (1967) has shown that the interaction of cations with rabbit muscle pyruvate kinase results in a quenching of the tryptophyl fluorescence of the protein. Since the yeast enzyme exhibits the same cation requirements, we reasoned that a study of the fluorescence properties of the yeast pyruvate

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‡ Research Career Development Awardee 1-K3-GM-9725 of the National Institutes of Health.

[1] Abbreviations used are: FDP, fructose 1,6-diphosphate; PEP, phosphenolpyruvate; PK, pyruvate kinase.