Inactivation of porcine heart mitochondrial malate dehydrogenase (l-malate: NAD⁺ oxidoreductase, EC 1.1.1.37) by selective modification of an active center histidine residue with the reagent iodoacetamide has been further investigated to examine the existence of and the enzymatic activity of a hybrid (half)-modified dimer. The loss of enzymatic activity during iodo(1,14C)acetamide modification is linear with 14C incorporation. Enzyme was modified to various extents and the reaction was quenched. Microzonal electrophoresis was performed to separate native dimeric enzyme, hybrid-modified enzyme, and doubly modified enzyme. The distribution of each species was quantitated by scanning densitometry. The distribution generated preferential association of a modified subunit with an- and the enzymatic activity of a hybrid-modified enzyme indicates that there is no apparent change in kinetic parameters between a subunit of the native dimer and the active subunit in the hybrid-modified dimer. Dissociation and reassociation of a mixture of native enzyme and doubly-iodoacetylamine-modified enzyme indicates that there is no preferential association of a modified subunit with another modified subunit, or of a native subunit with another native subunit, but rather, association is random with respect to native and iodoacetamide-modified subunits.

Incorporation of [14C]iodoacetamide into mitochondrial malate dehydrogenase—The incorporation of iodo(1,14C)acetamide into malate dehydrogenase was assayed by standard isotopic dilution methods (4-10). High purity of a Nuclear Chicago-Searle Isocap/300 liquid scintillation counter. Enzyme solution in 50 mM sodium phosphate buffer, pH 7.0, was diluted into buffer containing 14C-labeled iodoacetamide and the enzymatic activity was determined as a function of time. Aliquots were also removed from the incubation mixture at specific time intervals and the reaction quenched by the addition of β-mercaptoethanol. Following dialysis to remove excess reagent, the incorporation of 14C was determined. Aliquots (400 or 500 µl) were placed in 10 ml of aquasol scintillation mixture (New England Nuclear) and monitored for radioactivity. A blank sample of an equal volume of the final dialysis buffer and an external 14C source were counted with each group of samples.

Enzymatic Assays—Porcine heart mitochondrial malate dehydrogenase was purified from acetone powders of fresh pig hearts as described by Gregory et al. (15). Assays in the direction of oxidation of NADH were performed using a standard assay of 50 mM potassium phosphate buffer, 0.25 mM oxalacetate, and 0.20 mM NADH, pH 7.5. The change in absorbance at 340 nm was measured on a Gilford 250 recording spectrophotometer. Protein concentration was determined spectrophotometrically using the previously reported extinction coefficient of E₁₀₀% = 2.53 (15).

EXPERIMENTAL PROCEDURES

Materials—Porcine heart mitochondrial malate dehydrogenase was purified from acetone powders of fresh pig hearts as described by Gregory et al. (15). NADH, NAD⁺, l-malic acid, oxalacetate, and iodoacetamide were obtained from Sigma. Iodo(1,14C)acetamide was obtained from Amersham-Searle.

Enzymatic Assays—Porcine heart mitochondrial malate dehydrogenase was purified from acetone powders of fresh pig hearts as described by Gregory et al. (15). Assays in the direction of oxidation of NADH were performed using a standard assay of 50 mM potassium phosphate buffer, 0.25 mM oxalacetate, and 0.20 mM NADH, pH 7.5. The change in absorbance at 340 nm was measured on a Gilford 250 recording spectrophotometer. Protein concentration was determined spectrophotometrically using the previously reported extinction coefficient of E₁₀₀% = 2.53 (15).

Incorporation of [14C]iodoacetamide into Mitochondrial Malate Dehydrogenase—The incorporation of iodo(1,14C)acetamide into malate dehydrogenase was assayed by standard isotopic dilution methods (4-10). High purity of a Nuclear Chicago-Searle Isocap/300 liquid scintillation counter. Enzyme solution in 50 mM sodium phosphate buffer, pH 7.0, was diluted into buffer containing 14C-labeled iodoacetamide and the enzymatic activity was determined as a function of time. Aliquots were also removed from the incubation mixture at specific time intervals and the reaction quenched by the addition of β-mercaptoethanol. Following dialysis to remove excess reagent, the incorporation of 14C was determined. Aliquots (400 or 500 µl) were placed in 10 ml of aquasol scintillation mixture (New England Nuclear) and monitored for radioactivity. A blank sample of an equal volume of the final dialysis buffer and an external 14C source were counted with each group of samples.

Microzeoal Electrophoresis of Native and Modified Enzyme—Separation of native, hybrid, and doubly IAM- modified enzyme was achieved by microzonal electrophoresis using a Beckman R-101 electrophoresis cell with cellulose acetate membranes. An LKB 3571 D constant voltage power supply was used with the initial amperage set at 2.5 m amps, approximately 250 volts. The cellulose acetate membranes were equilibrated in a running buffer of 1.4 mM sodium phosphate, 8.4 mM sodium citrate, pH 7.0. The time for electrophoresis was approximately 45 min and membranes were stained for enzymatic activity. Studies have been performed on the kinetics and mechanism of subunit reassociation (14). Recently, investigation of the enzyme which has been immobilized on Sepharose beads has indicated that the mitochondrial malate dehydrogenase monomer can indeed exhibit significant catalytic activity (13), an observation also not fully compatible with a reciprocating-type mechanism. However, both the activity and kinetic parameters for the matrix-bound dimer and matrix-bound monomer were altered somewhat compared to those of native enzyme in solution. In those experiments, it is therefore not clear whether these observed changes might be due to a loss of cooperative subunit interactions which might occur after enzyme immobilization. As an alternative approach to this question, the previously reported inactivation of mitochondrial malate dehydrogenase by selective chemical modification of an active center histidine residue by iodoacetamide (7-10) has been further investigated to examine the possible existence of and the enzymatic activity of a hybrid (half)-modified dimer.

Early kinetic studies of mitochondrial malate dehydrogenase using the substrate analogs hydroxy malonate and keto malonate have led to the proposal of a reciprocating mechanism in which the subunits do not carry out catalysis independently but rather function in concert using a "flip flopping" catalytic sequence (1, 2). More recently, investigations utilizing thenoyltrifluoroacetone have been interpreted as also being consistent with such a mechanism (3). However, the results of numerous studies of the inactivation of mitochondrial malate dehydrogenase by chemical modification have not been supportive of such a reciprocating type mechanism since half-of-the-sites effects have not been apparent (4-10). It has also been observed that the dimeric enzyme, mitochondrial malate dehydrogenase, dissociates into its constituent identical subunits at pH values below neutrality (11, 12). The monomeric species which exist at pH 5.0 has undergone a slight conformational change (13, 14), resulting in loss of enzymatic activity.
protein using a Ponceau S stain as described by Beckman or by using a Coomassie blue stain (0.25% Coomassie blue, 45% methanol, and 9.2% acetic acid). Staining in Coomassie blue was found to be more sensitive and was performed for about 3 min followed by destaining in methanol/acetic acid/water (50:10:50 v/v) followed by 5% acetic acid. For densitometer scans, the stained cellulose acetate membranes were cleared as described by Beckman and scanned using a Gilford 250 scanning densitometer attachment. For activity stains, a 0.11 M sodium L-malate, 0.10 Tris-HCl buffer, pH 8.4 was used containing 7 mg of NADH, 0.23 mg of phenazine methosulfate, and 4 mg of nitroblue tetrazolium/10 ml of buffer. Strips were stained for 2 min in the dark and destained in methanol/acetic acid/water (50:10:50) followed by water.

**RESULTS AND DISCUSSION**

Chemical modification studies of oligomeric enzymes can be used to assess the role of subunit interactions in catalysis. Recent studies by Degani et al. (17-19) with creatine kinase have indicated that the identical subunits of the creatine kinase dimer are associated asymmetrically in solution. In addition, these studies have indicated that the subunits do not function independently but rather interact during catalysis. An important question in the study of mitochondrial malate dehydrogenase, as well as for other oligomeric enzymes composed of identical subunits, is whether or not the subunits can function independently in catalysis.

The data presented in Fig. 1 is a composite from three experiments and demonstrates that the loss of enzymatic activity is linear with the extent of IAM modification. Complete loss of enzymatic activity occurs concomitant with alkylation by 2 moles of IAM/mole of mitochondrial malate dehydrogenase dimer. Previous investigations have shown that the modification occurs at a specific histidine residue producing the 3-carboxyamidomethyl histidyl derivative (8, 10). The solid line in this figure is that predicted if each of the identical subunits of the mitochondrial malate dehydrogenase dimer exhibits identical reactivity toward IAM and the hybrid-modified dimer still possesses one-half of the enzymatic activity. The dashed curve is the theoretical curve predicted if the hybrid-modified dimer is inactive at both subunits. The data points (O, □, △) are a composite of three separate experiments.

Separation of native, hybrid-modified, and doubly modified enzyme was achieved using microzonal electrophoresis. Fig. 2 represents the electrophoresis of native enzyme and enzyme inactivated with IAM to various extents. Bands migrating more slowly in the direction of the cathode are produced during the IAM modification and can be attributed to the hybrid-modified mitochondrial malate dehydrogenase dimer and to the doubly modified dimer. With increasing time of modification, the bands shift from predominantly unmodified plus some hybrid-modified dimer to increasing levels of doubly modified enzyme. A small amount of "isoenzyme" is present in the native mitochondrial malate dehydrogenase which is slower moving on electrophoresis and moves in the position of the hybrid-modified main mitochondrial malate dehydrogenase component. The isoenzyme also undergoes the same shifts in mobility during IAM modification. Previous studies have suggested that minor mitochondrial malate dehydrogenase isoenzymes are generated from deamination of glutamine and asparagine and are slower moving in electrophoresis but are otherwise identical to the main component (20). Our purification procedure yields one major mitochondrial malate dehydrogenase component with varying amounts of one slower migrating isoenzyme. The upper portion of Fig. 2 demonstrates the electrophoresis strip stained for protein. Toward the end of the inactivation time period, as seen in lane 8, most of the protein migrates in a position corresponding to the doubly modified main mitochondrial malate dehydrogenase component. A faint, faster moving band is present due to residual hybrid-modified enzyme and a faint, slower moving band is present due to the doubly modified isoenzyme. The lower portion of Fig. 2 shows an identical electrophoresis strip stained for enzymatic activity. Toward the end of the inactivation time period, most of the activity resides in the position of the hybrid-modified main mitochondrial malate dehydrogenase component. A fainter activity band is present in the position of the doubly modified main enzyme component where most of the protein exists, and corresponds to the
Active Subunits in Malate Dehydrogenase

| LANE | SAMPLE     | %ACTIVITY |
|------|------------|-----------|
| 1    | control    | 100       |
| 2    | quenched   | 55        |
| 3    | quenched   | 100       |
| 4    | " 15 min  | 57.5      |
| 5    | " 30 min  | 57.3      |
| 6    | " 45 min  | 24        |
| 7    | " 60 min  | 10.3      |
| 8    | " 75 min  | 7.0       |

![Image of Microzonal electrophoresis of native mitochondrial malate dehydrogenase and enzyme inactivated with IAM to various extents.](image)

**Fig. 2.** Microzonal electrophoresis of native mitochondrial malate dehydrogenase and enzyme inactivated with IAM to various extents. Top, the cellulose acetate membrane was stained for protein, as described under “Experimental Procedures.” Bottom, the electrophoresis strip was stained for enzymatic activity, as described under “Experimental Procedures.”

![Image of Densitometer scans performed on electrophoresis strips stained for protein and “cleared” for scanning.](image)

**Fig. 3.** The percentage of protein migrating in the position of native, hybrid, or doubly-IAM-modified mitochondrial malate dehydrogenase with increasing extent of IAM modification. The solid curves (—) represent the theoretical curves calculated for the condition where the identical subunits of mitochondrial hybrid-modified isoenzyme.

Densitometer scans were performed on electrophoresis strips stained for protein and “cleared” for scanning as described under “Experimental Procedures.” The results of these scans of protein modified to various extents and then subjected to electrophoresis is presented in Fig. 3, a–c. The solid curves represent the theoretical distribution of unmodified, hybrid-modified, or doubly IAM-modified enzyme with increasing time of IAM modification and inactivation. The theoretical curves are calculated for the condition where the identical subunits of mitochondrial malate dehydrogenase show identical reactivity toward IAM. In the calculation for the theoretical curves, the presence of the minor isoenzyme was considered and accounted for. The data points represent
the observed distribution calculated from the densitometer scans. The reasonably good fit between the observed and the theoretical distributions indicates that the two identical subunits of the mitochondrial malate dehydrogenase dimer do exhibit similar reactivity toward IAM.

Mitochondrial malate dehydrogenase has previously been observed to dissociate from a dimer at higher pH values to monomers at pH 5.0 (11). Equal amounts of native dimeric enzyme and doubly IAM-modified enzyme were mixed and dissociated to monomers by dialysis versus buffer at pH 5.0, and reassociated by further dialysis versus buffer at pH 7.5. The results indicate that reassociation is random with respect to the histidine modification and there is no selection of a modified subunit for another modified subunit or of an unmodified subunit for another unmodified subunit.

Initial attempts to preparatively isolate the hybrid-modified enzyme were unsuccessful. Therefore, the steady state kinetics of native enzyme and of three quarters-IAM-modified and inactivated enzyme were examined and compared. Table I shows the expected distribution for three-quarters-IAM-inactivated enzyme. Of the activity that remains, 75% is contributed by the hybrid-modified enzyme and 25% is contributed by residual native enzyme. Thus, if there are changes in the steady state kinetic parameters of the hybrid-modified enzyme, these changes should be apparent in comparison to this three-quarters-IAM-modified enzyme with native enzyme. Double reciprocal plots are presented in Fig. 5, a and b. The $K_m$ values for all four substrates remain unchanged with the partially IAM-modified enzyme and the $V_{max}$ is reduced directly according to the fraction of subunits that are IAM-modified. This data is tabulated in Table II. The results indicate that there is not a significant change in the kinetic parameters for the hybrid-modified mitochondrial malate dehydrogenase molecule and that the hybrid-modified dimer has one active subunit and one fully active subunit.

Recently, similar studies have been performed by Seelig and Folk on human plasma blood coagulation factor XIIIa (21). The active form of this enzyme consists of two apparently identical catalytic subunits. The enzyme has been shown to react with half-of-the-sites reactivity under some conditions and all-of-the-sites reactivity under other conditions. The all-of-the-sites reactivity has been interpreted to be the result of a positive cooperative reactivity in which modification of the first subunit causes the second subunit to be modified at a much faster rate. In the case of mitochondrial malate dehydrogenase, the IAM modification is shown to exhibit noncooperative reactivity.

Chemical modification studies of creatine kinase have indicated that the identical subunits of the dimer are asymmetrically associated in solution and that subunit interactions are required for catalysis (17-19). However, some reagents have shown apparent all-of-the-sites reactivity. A model was proposed accounting for this result in which each subunit possesses two "communication lines" involved in subunit interaction during catalysis. Only a single, different communication line is functional in each subunit as a result of the asymmetric association of the subunits. Thus, modification of a residue along a functional communication line in one subunit could result in loss of activity on both subunits, while modification of the same residue on the other subunit along a nonfunctional communication line has little or no effect on the activity of either subunit. This type of model is unlikely for mitochondrial malate dehydrogenase, since the histidine residue modified in the IAM reaction has previously been identified as an active center residue suggested to be involved in the catalytic mechanism, as in other "histidine dehydrogenases" as proposed by Lodola et al. (22).
Active Subunits in Malate Dehydrogenase

Fig. 5. The steady state kinetics of native and IAM-modified mitochondrial malate dehydrogenase. A, native enzyme (○) and 76% IAM-modified enzyme (□). The enzyme was assayed in the direction of NADH oxidation, as described under "Experimental Procedures." B, native enzyme (○) and 79% IAM-modified enzyme (□). Assays in the direction of NAD⁺ reduction were performed, as described under "Experimental Procedures."

Table II
Summary of steady state kinetics of native and three-quarters IAM-inactivated mitochondrial malate dehydrogenase

The percentage Vₘₐₓ values in parenthesis are the values expected based on the fraction of subunits that are modified. The observed % Vₘₐₓ values are presented, normalized to a value of 100% for the native enzyme.

In studies of glutamine-aspartate transaminase, a similar approach has been used to investigate site-site relationships in that enzyme (23). With this enzyme, also a dimer of identical subunits, the hybrid-modified enzyme also possessed one fully active and one inactive subunit. Steady state kinetic studies as well as measurement of binary complex formation with several substrates, analogs, and inhibitors indicated no differences between the two active sites of the dimeric native enzyme and the one active site in the hybrid-modified enzyme. Thus, the subunits of this enzyme were also demonstrated to function independently.

Results of this present study with mitochondrial malate dehydrogenase demonstrate that a single subunit can exhibit full enzymatic activity independent of the presence or absence of catalysis on the other subunit. This result directly contradicts the proposed reciprocating mechanism for this enzyme which was based on anomalous inhibition patterns in kinetic studies using inhibitors and substrate analogs (3). Interpretation of the results of equilibrium isotopic substrate exchange experiments performed by Silverstein et al. (24) suggested that the subunits functioned independently, a finding also inconsistent with a reciprocating mechanism. Although recent work has shown that the dimeric structure is important in maintaining the active conformation of the subunits (14), further investigation is required to identify the mechanistic or structural reasons for the dimeric structure of this enzyme.

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