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Affinity Labeling of a Lysine Residue in the Coenzyme Binding Site of Pig Heart Mitochondrial Malate Dehydrogenase†

Siddhartha Roy and Roberta F. Colman*

ABSTRACT: Porcine heart mitochondrial malate dehydrogenase is inactivated with a pseudo-first-order rate constant of 0.00941 min^{-1} by 2.1 mM 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine in 0.02 M sodium barbital buffer, pH 8.0, containing 15% dimethylformamide (DMF). The pseudo-first-order rate constant for inactivation is linearly dependent on the reagent concentration. The adenosine moiety is crucial, as indicated by the observation that *p*-(fluorosulfonyl)benzoic acid (which lacks the adenosine group) inactivates the enzyme 37-fold more slowly. Marked protection against this inactivation is provided by NADH and NAD, but not by malate. The dissociation constants for NADH and NAD, calculated from the decrease in the inactivation rate, are 4.6 and 385 μM, respectively, values close to those obtained by previous direct binding measurements. This dimeric enzyme is known to be dissociated to its monomeric form at low protein concentration and low pH. However, sedimentation equilibrium and light-scattering studies reveal that the native enzyme retains its dimeric structure in the presence of 15% DMF under the conditions of reaction with 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine, and gel filtration experiments demonstrate that the state of aggregation of the enzyme is not altered upon modification by this reagent. A plot of the incorporation of [^{3}H]-5′-(*p*-sulfonylbenzoyl)adenosine vs. loss of enzyme activity is linear throughout the range tested, and extrapolation leads to 0.97 mol of radioactive reagent incorporated per mol of enzyme subunit at 100% inactivation. Fractionation of the acid hydrolysate of the modified enzyme by amino acid analysis or by two-dimensional thin-layer chromatography and electrophoresis leads to the identification of lysine as the modified amino acid. These results indicate the presence of a lysine residue at or near the coenzyme binding site of porcine heart mitochondrial malate dehydrogenase.

Pig heart mitochondrial malate dehydrogenase (L-malate: NAD^+ oxidoreductase, EC 1.1.1.37) is a dimeric enzyme which uses NAD as a cofactor. Although histidine, arginine, and lysine residues have been implicated as being in the catalytic site on the basis of chemical modification with general protein reagents (Anderton, 1970; Anderton & Rabin, 1970; Gregory et al., 1971; Foster & Harrison, 1974; Wimmer & Harrison, 1975), the coenzyme binding site has yet to be fully mapped. Affinity labeling has not thus far been applied in the case of malate dehydrogenase to limit the extent of chemical modification to the region of the active site. We have previously described the synthesis of 5′-[*p*-(fluorosulfonyl)-benzoyl]adenosine, which has been used to label stoichiometrically the NADH regulatory site of glutamate dehydrogenase (Pal et al., 1975) and the adenosine nucleotide sites of rabbit muscle pyruvate kinase (Wyatt & Colman, 1977), RNA polymerase (Kumar et al., 1977), phosphofructokinase (Mansour & Colman, 1978; Pettigrew & Frieden, 1978), mitochondrial F_1 ATPase (Esch & Allison, 1978), chloroplast ATPase (DeBenedetti & Jagendorf, 1979), and human platelet membranes (Bennett et al., 1978). 5′-[*p*-

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(Fluorosulfonyl)benzoyl]adenosine may reasonably be considered as a structural analogue of ATP, ADP, or NAD and, although it has not heretofore been found to react within a catalytically functional NAD site, such a reaction with the relatively simple malate dehydrogenase seemed plausible. This paper presents evidence demonstrating that 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine acts as an affinity label of a single lysine residue per subunit in the coenzyme binding site of malate dehydrogenase.

The interaction between the two subunits of malate dehydrogenase has been the focus of considerable speculation, being variously proposed as being responsible for negative cooperativity in coenzyme binding (Bleile et al., 1977; Frieden et al., 1978; Hodges et al., 1978) and for a reciprocating mechanism ("flip–flop") of enzyme catalysis (Harada & Wolfe, 1968). The linear relationship shown here between the stoichiometry of modification of malate dehydrogenase by 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine and loss of catalytic activity suggests that the subunits may not be obligatorily coupled in the catalytic cycle.

Experimental Procedure

Materials. Pig heart mitochondrial malate dehydrogenase was purchased from Boehringer-Mannheim dissolved in 50% glycerol. The enzyme was dialyzed at 4 °C against 0.1 M potassium phosphate buffer, pH 7.5, for 18 h. The small amount of denatured enzyme present at the end of the dialysis period was removed by centrifugation at 10000 rpm for 10 min, and the supernatant was stored in aliquots at −85 °C. This preparation exhibited only one band when subjected to electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate, and the enzyme was used without further purification. A molecular weight of 35000 (Banazak & Bradshaw, 1975; Murphy et al., 1967) per subunit was used in all calculations involving malate dehydrogenase. For native enzyme the protein was determined by using $E_{280nm}^{1\%} = 3.05$ (Wolfe & Raval, 1969). For modified enzyme, the protein concentration was determined by means of the Bio-Rad protein assay, with native malate dehydrogenase used to establish the standard curve.

5′-[*p*-(Fluorosulfonyl)benzoyl]adenosine was synthesized by reaction of *p*-(fluorosulfonyl)benzoyl chloride with adenosine, as described by Wyatt & Colman (1977). The radioactive [*p*-(fluorosulfonyl)benzoyl]adenosine was prepared in the same way by using [2-^{3}H]adenosine.

The standard modified amino acids N^{ϵ}-(4-carboxybenzenesulfonyl)lysine (CBS-Lys)[1] and *O*-(4-carboxybenzenesulfonyl)tyrosine (CBS-Tyr) were synthesized by reaction of the N^{α}-acetyl amino acids with *p*-(fluorosulfonyl)benzoic acid in accordance with Likos and Colman (unpublished experiments). These standard modified amino acids show very similar properties to those described by Esch & Allison (1978). Oxaloacetic acid and NADH were purchased from Sigma Chemical Co. and were used without any purification.

Determination of Enzymatic Activity. Malate dehydrogenase activity was assayed by measuring continuously the disappearance of NADH at 340 nm by using a Gilford spectrophotometer, Model 240, equipped with an expanded-scale recorder (0–0.1 absorbance full scale). Measurements were made at 25 °C in 0.1 M potassium phosphate buffer, pH 7.5, containing 200 μM NADH and 0.5 mM oxaloacetate in a total volume of 1.0 mL, in a cuvette of total path length of 1.0 cm.

Kinetics of Reaction of 5′-[p-(Fluorosulfonyl)benzoyl]-adenosine with Malate Dehydrogenase. Malate dehydrogenase (0.058 mg/mL) was incubated at 25 °C in 0.02 M sodium barbital buffer, pH 8.0, containing 15% dimethylformamide for 2 h, after which 5′-FSO_2BzAdo was added to initiate the reaction. When other ligands were present, they were added after the 2-h preincubation period but before adding 5′-FSO_2BzAdo. The presence of 15% dimethylformamide was required to maintain the solubility of 5′-FSO_2BzAdo, and 2-h preincubation was necessary to stabilize the activity of the enzyme. During the course of the reaction of malate dehydrogenase with 5′-FSO_2BzAdo, aliquots were withdrawn at given time intervals, diluted with 0.1 M potassium phosphate buffer, pH 7.5, at 0 °C, and assayed by the procedure described above. Since the reagent was diluted approximately 2500-fold in the assay solution, and since high concentrations of NADH (which protects against inactivation) were present in the assay mixture, it was considered that no appreciable additional reaction between malate dehydrogenase and 5′-FSO_2BzAdo took place during the 1–2 min required to conduct the assay. Indeed, the individual assays were linear, and the measured rates were assumed to reflect the residual enzymatic activity at the time of withdrawal of aliquots from the reaction mixture.

Incorporation of 5′-SO_2BzAdo by Malate Dehydrogenase. Enzyme (0.46 mg/mL) was incubated with 2.1 mM [2-^{3}H]-5′-FSO_2BzAdo at 25 °C under conditions similar to those given above. When the enzymatic activity reached a specified point, the entire reaction mixture (1.0 mL) was rapidly centrifuged in a clinical centrifuge to remove any precipitated reagent, and the supernatant was applied to a Sephadex G-25 column (1 × 28 cm), equilibrated with 0.05 M potassium phosphate buffer, pH 7.5, and eluted with the same buffer. Alternatively, when measuring the incorporation at less than 50% residual activity, we made a second addition of 5′-FSO_2BzAdo when the residual activity reached 50%. (The rate of inactivation decreases in time as a result of decomposition and precipitation of the reagent.) At the specified residual activity, 0.025 M dithiothreitol was added to stop the reaction, and solid urea was added to give a final concentration of 5.0 M. The sample was applied to a Sephadex G-25 column (1 × 45 cm) equilibrated with 5.0 M urea in 0.05 M phosphate buffer, pH 7.5, and eluted with the same buffer. The urea was included in the more extensively inactivated samples in order to ensure the solubility of any inactive protein that may have been precipitated. The protein concentration in fractions eluted from the column were determined by the Bio-Rad protein assay using native malate dehydrogenase as the standard. The radioactivity was measured in a Packard Tri-Carb liquid scintillation counter, Model 3300.

Measurements of Molecular Properties of Malate Dehydrogenase under Conditions of Reactions with 5′-FSO_2BzAdo. Malate dehydrogenase was dissolved in 0.02 M sodium barbital buffer, pH 8.0, containing 15% DMF and was preincubated for 2 h. Alternatively, prior to measuring the sedimentation coefficient at protein concentrations above 1 mg/mL, we mixed malate dehydrogenase with the appropriate volume of 0.02 M sodium barbital buffer, pH 8.0, containing 15% DMF and dialyzed it against the same buffer for 3 h.

Sedimentation coefficients were determined in a Beckman Model E analytical ultracentrifuge, at 60000 rpm and 20 °C,

[1] Abbreviations used: 5′-FSO_2BzAdo, 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine; 5′-SO_2BzAdo, 5′-(sulfonylbenzoyl)adenosine; DMF, dimethylformamide; CBS-Lys, N^{ϵ}-(4-carboxybenzenesulfonyl)lysine; CBS-Tyr, *O*-(4-carboxybenzenesulfonyl)tyrosine.

using Schlieren optics. The sedimentation coefficient at zero protein concentration was obtained by linear extrapolation of a plot of $s_{20,DMF}$ vs. malate dehydrogenase concentration. The sedimentation constant thus obtained was corrected for solvent viscosity and density to yield $s^0_{20,w}$. The density for 0.02 M sodium barbital buffer containing 15% DMF was determined experimentally as 0.99706 g/mL, while the viscosity of 15% DMF–85% water was estimated as 0.9915 cP from a linear interpolation of the known viscosities of water and DMF. (The contribution of 0.02 M barbital to the viscosity was neglected.)

For determination of molecular weight by the meniscus depletion sedimentation equilibrium technique (Yphantis, 1964), malate dehydrogenase, dissolved in the appropriate buffers, was dialyzed against the same buffer at room temperature for 4 h. Measurements were made at 24000 and/or 34000 rpm in a Beckman Model E analytical ultracentrifuge using interference optics. Photographs were taken at several time intervals after 14 h. An indication that equilibrium had been reached was assessed by the flatness of the interference fringes in the first half of the cell and a constant slope at different times of a plot of ln C vs. r^2. (C is the fringe displacement and r is the distance from the rotor center.)

For determination of molecular weight by the light-scattering method, malate dehydrogenase was dissolved in 0.02 M sodium barbital buffer containing 15% DMF. This enzyme was preincubated for 2 h and filtered through a superfine sintered-glass funnel to eliminate the dust. The filtrate, which contained malate dehydrogenase in the concentration range 0.7–1.1 mg/mL, was used for weight-average molecular weight measurements at 436 nm by means of a Brice-Phoenix light-scattering photometer.

Identification of the Reactive Amino Acid Residue. Amino acid analyses were performed on both Beckman Model 119C and Beckman Model 120C amino acid analyzers. Amino acid analyses on the Model 119C Beckman amino acid analyzer were performed by using the following automatic buffer change schedule: 0.2 M sodium citrate buffer, pH 3.25, initially until 50 min when it is changed to 0.2 M sodium citrate buffer, pH 4.12; the final buffer, 0.2 M sodium citrate, pH 6.40, is used to elute the column from 67 to 155 min. Under these conditions, CBS-Lys appears at 88 min and CBS-Tyr appears at 97 min. For comparison, the elution times of representative amino acids were as follows: aspartic acid, 24 min; valine, 68 min; tyrosine, 89 min.

Amino acid analyses on the Model 120C amino acid analyzer were performed on the long column by using 110 min of 0.2 M sodium citrate buffer, pH 3.25, followed by 50 min of 0.2 M sodium citrate buffer, pH 4.25. The analyzer was then stopped manually, the pH 4.25 buffer on top of the column was replaced by 0.35 M sodium citrate buffer, pH 5.25, and the automatic analyzer was restarted. Quantification of the CBS-Lys was performed on a Beckman Model 120C amino acid analyzer since a sharper peak, more clearly separated from tyrosine and leucine, was obtained by using this procedure. The elution times for some amino acids are as follows: leucine, 172 min; CBS-Lys, 180 min; Tyr, 184 min. The color constant for CBS-Lys was calculated from a standard run by comparison of the areas exhibited by known amounts of CBS-Lys with those of standard amino acids. The color constant for the red channel thus determined is 1.15 times that of phenylalanine.

Thin-Layer Electrophoresis and Chromatography of Acid Hydrolysate of Malate Dehydrogenase. These procedures were performed by using Eastman cellulose thin-layer plates (plastic backed). Electrophoresis was conducted by using 1.2 M pyridine–acetate buffer, pH 6.4 [pyridine–acetic acid–water (25:1:250)], for 2 h. Electrophoresis was conducted in an apparatus with circulating cold water at 400 V and 10 mA. Butanol–acetic acid–water (25:4:10) was the solvent for thin-layer chromatography. The plates were developed with 0.2% ninhydrin spray in ethanol.

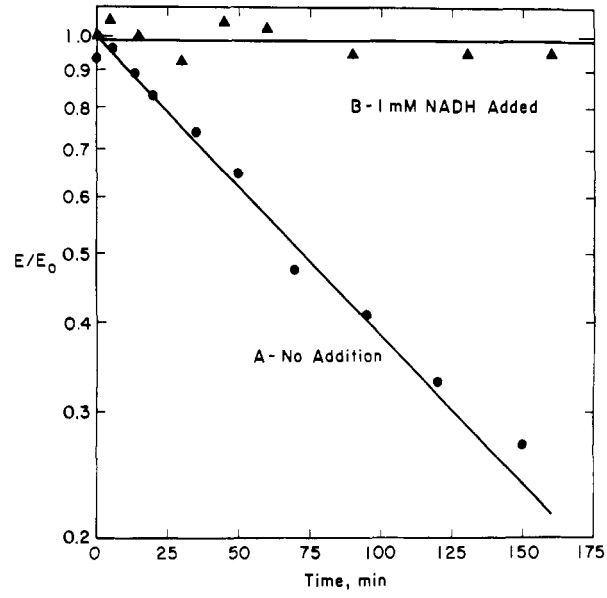

FIGURE 1: Reaction of 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine with malate dehydrogenase in the absence and presence of NADH. Malate dehydrogenase (0.058 mg/mL) was incubated with 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine (2.1 mM) at 25 °C in 0.02 M sodium barbital buffer, pH 8.0, containing 15% DMF. At each indicated time, a 20-μL aliquot was withdrawn and assayed as described under Experimental Procedure. (A) No ligands added to reaction mixture. The pseudo-first-order rate constant is 0.00941 min^{-1}. (B) NADH (1 mM) present in the reaction mixture.

Results

Inactivation of Malate Dehydrogenase by 5′-[p-(Fluorosulfonyl)benzoyl]adenosine. Pig heart mitochondrial malate dehydrogenase is inactivated upon incubation with 2.1 mM 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine at 25 °C in 0.02 M sodium barbital buffer, pH 8.0, containing 15% DMF. In contrast, the enzyme incubated under similar conditions in the absence of reagent loses very little activity. The reaction follows pseudo-first-order kinetics as indicated in Figure 1, line A, by the linear plot of log E/E_0 vs. time, where E and E_0 are the measured enzymatic velocities at the given time and at zero time, respectively. Complete protection against inactivation is observed when 1 mM NADH is included in the incubation mixture (Figure 1, line B), suggesting that reaction of 5′-FSO_2BzAdo with the enzyme takes place in the region of the active site.

The pseudo-first-order rate constant observed in the absence of coenzyme is linearly dependent on the concentration of 5′-FSO_2BzAdo (Figure 2), indicating either that there is no rapid reversible binding of the reagent to the site of reaction on the enzyme or that the dissociation constant of an enzyme–reagent complex is sufficiently high that the plot appears linear over the concentration range tested. A second-order rate constant of 4.0 M^{-1} min^{-1} may be calculated.

In order to evaluate the contribution of the adenosine moiety to the effectiveness of 5′-FSO_2BzAdo in inactivating malate dehydrogenase, we tested the effect of *p*-(fluorosulfonyl)-benzoic acid on the enzyme. Malate dehydrogenase, upon incubation with 10 mM *p*-(fluorosulfonyl)benzoic acid under the same conditions as those of Figure 1, was very slowly

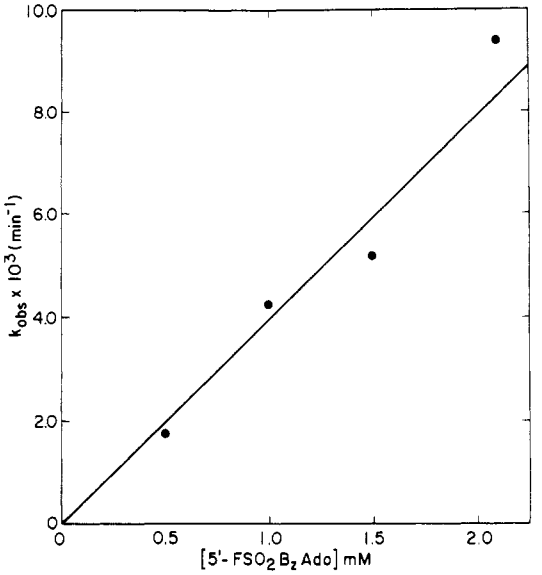

FIGURE 2: Dependence of the pseudo-first-order rate constant on the concentration of 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine. Malate dehydrogenase (0.058 mg/mL) was incubated with the indicated concentrations of 5′-FSO_2BzAdo at 25 °C in 0.02 M sodium barbital buffer, pH 8.0, containing 15% DMF. The rate constant at each concentration was determined as indicated in Figure 1, line A.

Table I: Effect of Ligands on the Inactivation of Malate Dehydrogenase by 2.1 mM 5′-[*p*-(Fluorosulfonyl)-benzoyl]adenosine

additions to reaction mixture	rate constant (min^{-1} × 10^3)	K_d calcd (μM)
none	9.41	
malate (5.0 mM)	4.95	
NADH (2.5 μM)	5.57	3.62
NADH (5.0 μM)	4.26	4.13
NADH (7.5 μM)	3.90	5.30
NADH (15.0 μM)	2.50	5.46
NADH (200 μM)	0.0	
NAD (125 μM)	7.0	363
NAD (250 μM)	5.2	308
NAD (500 μM)	3.9	353
NAD (1000 μM)	3.2	515

inactivated. The reaction obeyed pseudo-first-order kinetics, with an observed rate constant of 0.00108 min^{-1}. The calculated second-order rate constant for *p*-(fluorosulfonyl)-benzoic acid is thus 0.108 M^{-1} min^{-1}, a value 37 times less than that for 5′-FSO_2BzAdo. These data indicate the importance of the adenosine moiety in directing the reaction of 5′-FSO_2BzAdo with malate dehydrogenase.

Table I records the effect of adding various ligands on the pseudo-first-order rate constant. Both the oxidized and reduced coenzyme cause a striking decrease in the rate constant for inactivation, the extent of which depends on the coenzyme concentration. NADH provides essentially total protection above a concentration of 200 μM. In contrast, the substrate malate provides less than a twofold decrease in the rate constant. If it is assumed that coenzymes bind reversibly at the same site attacked by 5′-FSO_2BzAdo, eq 1 can be derived,

$$k_{obsd} = \frac{k_i}{1 + [C]/K_d} \quad (1)$$

which describes the relationship between k_{obsd}, the pseudo-first-order rate constant for inactivation by 5′-FSO_2BzAdo in the presence of coenzymes, k_i, the inactivation rate constant in the absence of added ligands, [C], the coenzyme concentration, and K_d, the dissociation constant for the enzyme–coenzyme complex. Table I records the K_d values calculated from the k_{obsd} obtained at several coenzyme concentrations. Average K_d values of 4.6 and 385 μM can be calculated for NADH and NAD, respectively.

Table II: Molecular Weight of Malate Dehydrogenase Determined by the Sedimentation Equilibrium Method

buffer[a]	coenzyme	speed of centrifn (rpm)	protein concn (mg/mL)	M_r
barbital–15% DMF		24 000	0.45	68 800
barbital–15% DMF		24 000	0.76	63 400
barbital–15% DMF		34 000	0.60	56 400
barbital–15% DMF		34 000	0.47	60 200
barbital–15% DMF	100 μM NADH	34 000	0.50	63 200
barbital–15% DMF	200 μM NADH	34 000	0.50	65 400
barbital–15% DMF	1 mM NAD	34 000	0.60	68 200
phosphate		24 000	0.60	65 800
phosphate		34 000	0.47	56 200

[a] The buffers used were either 0.02 M sodium barbital, pH 8.0, containing 15% dimethylformamide or 0.1 M potassium phosphate, pH 7.5.

Table III: Sedimentation Coefficient in the Presence of Different Buffers

protein	buffer[a]	protein concn (mg/mL)	$s_{20,w}$ (S)
malate dehydrogenase	barbital–15% DMF	extrapolated to 0	3.21
malate dehydrogenase	barbital–15% DMF + equilibrium mixture[b]	1.03	3.20
malate dehydrogenase	phosphate	extrapolated to 0	4.30
bovine serum albumin	barbital–15% DMF	4.84	2.99

[a] Buffers used were either 0.02 M sodium barbital, pH 8.0, containing 15% dimethylformamide or 0.1 M potassium phosphate, pH 7.5. [b] Equilibrium mixture consists of initial concentrations of 0.5 mM oxaloacetate and 200 μM NADH.

Properties of Malate Dehydrogenase in 15% DMF. Since the porcine heart mitochondrial malate dehydrogenase is known to dissociate at low protein concentration and low pH (Bleile et al., 1977) and coenzyme has been reported to favor the associated form of the enzyme (Bleile et al., 1977), it was important to ascertain the state of aggregation of the enzyme in 15% DMF under the conditions of incubation with 5′-FSO_2BzAdo. The molecular weight of malate dehydrogenase in 0.02 M sodium barbital buffer, pH 8.0, containing 15% DMF was measured by the sedimentation equilibrium technique (Yphantis, 1964). Over the protein concentration range 0.45–0.76 mg/mL, the measured molecular weight is not significantly different from that obtained in 0.1 M potassium phosphate buffer, pH 7.5, as shown in Table II. An average molecular weight of 62 000 is found in both buffers. Furthermore, addition of the coenzymes NADH and NAD at concentrations of 200 μM and 1 mM, respectively, does not significantly alter the molecular weight as measured in the presence of 15% DMF.

The weight-average molecular weight of malate dehydrogenase in 0.2 M sodium barbital buffer, pH 8.0, containing 15% DMF was also determined by light-scattering measurements over a protein concentration range from 0.71 to 1.1 mg/mL. An average molecular weight of 73 200 was obtained, indicating that malate dehydrogenase exists predominantly in its dimeric form under the conditions used for incubation with 5′-FSO_2BzAdo.

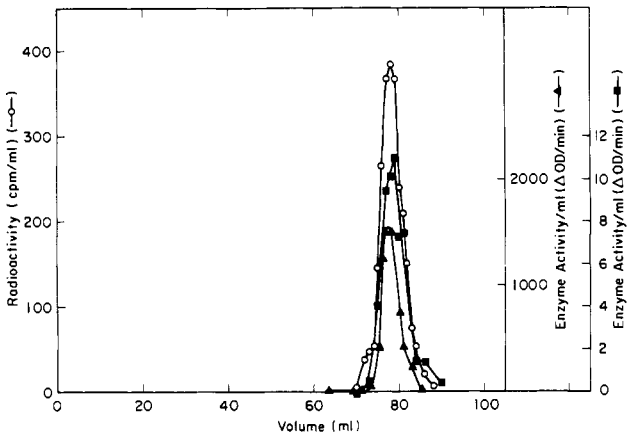

FIGURE 3: Gel filtration of [2-^{3}H]-5′-SO_2BzAdo-modified malate dehydrogenase. Malate dehydrogenase was incubated with [2-^{3}H]-5′-FSO_2BzAdo in 0.02 M sodium barbital buffer, pH 8.0, containing 15% DMF. At 50% inactivation the reaction mixture was centrifuged, and the supernatant was applied to a Sephacryl S-200 column (75 × 1.5 cm) previously equilibrated with 0.02 M sodium barbital buffer, pH 8.0, containing 15% DMF. The total column volume was 133 mL. Radioactivity (○) and enzyme activity (■) in the fractions were determined as described in the text. Native enzyme was separately applied to the same column, and the elution position was measured by enzyme activity (▲).

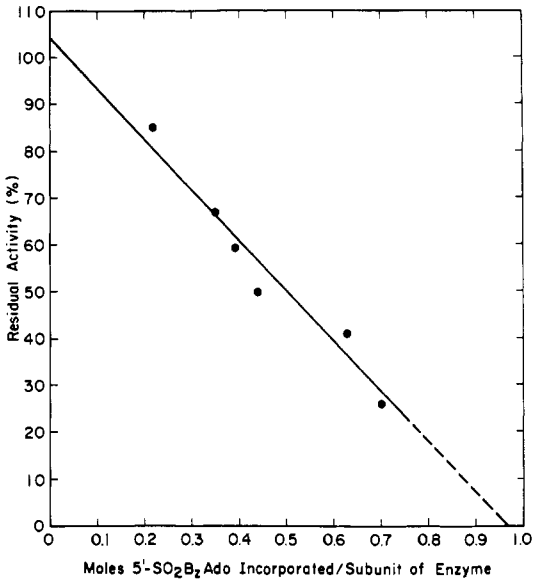

FIGURE 4: Incorporation of radioactive 5′-[*p*-(fluorosulfonyl)-benzoyl]adenosine per subunit of malate dehydrogenase as a function of loss of activity. Malate dehydrogenase (0.464 mg/mL) was incubated with [2-^{3}H]-5′-FSO_2BzAdo (2.1 mM) at 25 °C in 0.02 M sodium barbital buffer, pH 8.0, containing 15% DMF. At each indicated residual activity, the extent of incorporation was determined as described under Experimental Procedure.

Using an analytical ultracentrifuge, we also measured the sedimentation constant of malate dehydrogenase in 0.02 M sodium barbital buffer, pH 8.0, containing 15% DMF, as recorded in Table III. Sedimentation coefficients were measured as a function of protein concentration and, when extrapolated to zero protein concentration, $s^0_{20,w}$ of malate dehydrogenase was found to be 3.21 S in 0.02 M sodium barbital buffer, pH 8.0, containing 15% DMF. It was also found that 200 μM NADH does not significantly alter the sedimentation constant of malate dehydrogenase observed in the buffer containing 15% DMF. The constant obtained is somewhat lower than the value of 4.3 S measured for malate dehydrogenase in 0.1 M potassium phosphate buffer, pH 7.5. However, bovine serum albumin, which does not dissociate below 67 000, was found to have a sedimentation coefficient of 2.99 S in 0.02 M sodium barbital buffer, pH 8.0, containing 15% DMF; this value is also low as compared to that of 4.73 S obtained for bovine serum albumin in the absence of DMF.

In order to ascertain whether there is any change in the state of aggregation after reaction of malate dehydrogenase with 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine, we incubated enzyme (0.46 mg/mL) with [2-^{3}H]-5′-FSO_2BzAdo and allowed the reaction to proceed to 50% inactivation. Gel filtration of this modified enzyme on Sephacryl S-200, equilibrated with 0.02 M sodium barbital buffer, pH 8.0, containing 15% DMF, yielded a peak (determined both by radioactivity and by protein concentration) which coincided with that of native enzyme, determined separately on the same column (Figure 3). This result indicates that there is no change in molecular size of the enzyme upon modification by 5′-FSO_2BzAdo. Furthermore, gel filtration of native mitochondrial malate dehydrogenase over the protein concentration range 0.46–6.0 mg/mL, both in the absence and in the presence of 2 mM NAD, revealed the same elution volume by using a column equilibrated with 0.02 M sodium barbital buffer, pH 8.0, containing 15% DMF. Consequently, it can be concluded that native as well as modified malate dehydrogenase retains its native dimeric structure under the conditions of reaction with 5′-FSO_2BzAdo.

A question might be raised as to whether the enzyme is catalytically functional in 15% DMF under the incubation conditions with 5′-FSO_2BzAdo. Thus, kinetic measurements were made in 0.02 M sodium barbital buffer, pH 8.0, containing 15% DMF of the enzyme-catalyzed NADH-dependent reduction of oxaloacetate. Double-reciprocal plots of 1/[NADH] vs. 1/(initial velocity) at an oxaloacetate concentration of 0.5 mM yields an apparent V_{max} for enzyme in the barbital buffer containing 15% DMF that is approximately 40% of the V_{max} measured in the same buffer except for the absence of DMF. The apparent K_m for NADH is decreased 1.45-fold in 15% DMF as compared to the value obtained without DMF. The enzyme thus appears to be catalytically active, although its kinetic properties are somewhat altered by the presence of the solvent.

Stoichiometry of Reaction of 5′-[p-(Fluorosulfonyl)-benzoyl]adenosine with Malate Dehydrogenase. 5′-[*p*-(Fluorosulfonyl)benzoyl]adenosine reacts irreversibly and in a limited fashion with porcine heart mitochondrial malate dehydrogenase. The stoichiometry of the reaction was determined as described under Experimental Procedure by measurement of the incorporation of [2-^{3}H]-5′-SO_2BzAdo as a function of time of incubation with 2.1 mM reagent. A plot of residual enzymatic activity vs. incorporation of 5′-SO_2BzAdo measured as far as 78% inactivation gives a straight line, as shown in Figure 4. Extrapolation to 100% inactivation yields a value of 0.97 mol of 5′-SO_2BzAdo incorporated per mol of enzyme subunit.

Identification of Modified Amino Acid. In model reactions, both tyrosine and lysine have been found to react with 5′-FSO_2BzAdo to give stable derivatives. Upon treatment with 6 M HCl, the ester linkage between the benzoyl and nucleoside moieties is hydrolyzed to yield the acid-stable products N^ϵ-(4-carboxybenzenesulfonyl)lysine and *O*-(4-carboxybenzenesulfonyl)tyrosine. Both of these compounds are relatively hydrophobic as compared to most amino acids yet have a net negative charge at neutral pH. Because of this unusual combination of properties, these two amino acid derivatives may readily be separated from the normal complement of amino acids present in protein hydrolysates by thin-layer electrophoresis at pH 6.4, followed by thin-layer chroma-

tography in butanol–acetic acid–water as the solvent. Under these conditions, only glutamate, aspartate, CBS-Lys, and CBS-Tyr are negatively charged, and the latter two can readily be distinguished from aspartate and glutamate by their greater hydrophobicity, as reflected in higher R_f values. Indeed, CBS-Lys and CBS-Tyr can also be separated from each other chromatographically since the latter has a higher R_f (CBS-Lys, 0.7; CBS-Tyr, 0.81; in a mixture of standard amino acids). The two-dimensional separation of the acid hydrolysate of 75% modified malate dehydrogenase revealed a new negatively charged amino acid which corresponded in electrophoretic mobility and R_f to CBS-Lys. (A figure showing this chromatogram was provided for the reviewers but is not reproduced here.)

Amino acid analysis on a Beckman 119C amino acid analyzer was used as another way of qualitative detection of CBS-Lys and CBS-Tyr. In this analyzer, with the buffer-change schedule described under Experimental Procedure, both CBS-Lys and CBS-Tyr elute at positions distinct from natural amino acids. In this analysis, by use of 50% modified enzyme, it was shown that CBS-Tyr was not present in significant amounts. In addition, the acid hydrolysate of the modified enzyme was subjected to thin-layer electrophoresis, and the material located between the neutral amino acids and aspartate and glutamate was eluted. This material, when applied to a Model 119C amino acid analyzer, yielded a distinct peak of CBS-Lys as the only amino acid present in appreciable amount.

Quantification of CBS-Lys was conducted with a Beckman 120C amino acid analyzer using the altered buffer-change schedule described under Experimental Procedure. The number of moles of enzyme subunit was calculated from measurements of the amount of proline, valine, and phenylalanine present, together with their known composition in mitochondrial malate dehydrogenase (Banazak & Bradshaw, 1975). The number of moles of CBS-Lys was obtained by using its measured color constant as described under Experimental Procedure. A correction was made for approximately 20% hydrolysis of CBS-Lys under the conditions of acid hydrolysis of proteins, an average value determined in our laboratory and also in that of Esch & Allison (1978). On the basis of these calculations, an average value of 0.59 mol of CBS-Lys per mol of subunit was obtained for 63% inactivated enzyme.

Discussion

The reaction of mitochondrial malate dehydrogenase with 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine exhibits many of the characteristics of affinity labeling. The extent of reaction is limited; i.e., under the conditions used only 1 mol of 5′-FSO_2BzAdo was incorporated per mol of enzyme subunit. The adenosine portion of the structure is essential for reaction of the compound with malate dehydrogenase since *p*-(fluorosulfonyl)benzoic acid, which lacks the adenosine moiety, inactivates the enzyme with a rate constant 37-fold slower than that exhibited by 5′-FSO_2BzAdo. Finally, inclusion of NADH in the reaction mixture completely protects the enzyme from inactivation, indicating that 5′-FSO_2BzAdo and the coenzyme compete for a single site on the enzyme in the region of the catalytic site.

On the other hand, the dependence of the rate constant on the reagent concentration does not indicate saturation kinetics. This result may be interpreted in two ways: either the reagent does not bind to the enzyme prior to reaction or the binding is weak and the highest concentration of reagent used is still low relative to the enzyme–reagent dissociation constant. In view of the other evidence favoring the action of 5′-FSO_2BzAdo as an affinity label and the known poor binding of adenosine and 5′-AMP (K = 2 mM) (Oza & Shore, 1973), the second explanation seems to be more likely.

Both NADH and NAD decrease markedly the inactivation rate constant, and the enzyme–coenzyme dissociation constants calculated from the coenzyme concentration dependence of the inactivation rates are comparable to those reported previously on the basis of direct binding measurements (Hodges et al., 1978; Holbrook & Wolfe, 1972). In contrast to the coenzymes, L-malate does not offer marked protection against the inactivation. One interpretation of this result is that the region where 5′-FSO_2BzAdo reacts does not overlap with the malate binding site. However, the kinetics of the malate dehydrogenase reaction suggests that it is a sequential reaction with coenzyme binding initially to the enzyme (Banazak & Bradshaw, 1975); thus, it is possible that malate fails to protect against inactivation by 5′-FSO_2BzAdo because it cannot bind properly to the enzyme in the absence of coenzyme. Nevertheless, both the structural similarity between NAD and 5′-FSO_2BzAdo and the pattern of protection by ligands are consistent with and most easily explained by postulating that reaction takes places within the coenzyme binding site.

The reaction of 5′-FSO_2BzAdo with malate dehydrogenase was conducted in 15% dimethylformamide, as described under Experimental Procedure. Of prime concern in carrying out this reaction in a mixed solvent is the question of whether 5′-FSO_2BzAdo is reacting with the enzyme while it is in its native state, particularly in view of the known tendency of this enzyme to undergo dissociation from its dimeric to monomeric form at low pH and low protein concentrations (Bleile et al., 1977). If the enzyme is present to an appreciable extent in its monomeric state during the reaction, it might be argued that reaction occurred preferentially with the monomeric form and that protection by coenzyme represented merely a stabilization of the dimeric state of the enzyme rather than a specific blockage of the coenzyme site from attack by 5′-FSO_2BzAdo.

Several different approaches were taken to ascertain the molecular weight of the enzyme under the conditions of its reaction with 5′-FSO_2BzAdo. As determined by the sedimentation equilibrium (Yphantis) method, the molecular weight of mitochondrial malate dehydrogenase in 15% DMF does not indicate any significant difference from that measured in 0.1 M potassium phosphate buffer, pH 7.5; a dimeric structure of approximately 62 000 is found for enzyme samples in both buffers. The molecular weight obtained from the sedimentation equilibrium method is slightly lower than that expected for the dimeric enzyme. However, it is notable that Frieden et al. (1978) obtained a similar molecular weight for dimeric mitochondrial malate dehydrogenase on the basis of sedimentation equilibrium measurements. In the present study confirmation of the existence of malate dehydrogenase in dimeric form was obtained by light-scattering studies using enzyme dissolved in barbital buffer containing 15% DMF.

If malate dehydrogenase were to be dissociated only partially under the conditions used and 5′-FSO_2BzAdo were to react preferentially with the monomer according to the model

$$E_2 \rightleftarrows 2E \xrightarrow{5'\text{-}FSO_2BzAdo} 2E_{modified}$$

two clear predictions could be made: (1) the reaction rate constant should be dependent on protein concentration; (2) the reacted enzyme should exist as a monomer. In contrast to these predictions, the rate constant for reaction of malate dehydrogenase with 5′-FSO_2BzAdo does not exhibit any

systematic dependence on protein concentration. The molecular weight of the enzyme also does not change upon reaction with 5′-FSO_2BzAdo as determined by gel filtration (Figure 3). Therefore, the postulate that malate dehydrogenase partially dissociates in 15% DMF and that the monomer reacts preferentially with 5′-FSO_2BzAdo seems to be excluded.

On the other hand, the sedimentation coefficient of malate dehydrogenase does decrease considerably in 15% DMF, indicating a change in the molecular structure. In view of the observation that the molecular weight of malate dehydrogenase is not altered in 15% DMF and the result that an unrelated protein (bovine serum albumin) exhibits a reduced sedimentation constant although its molecular weight does not decrease in 15% DMF, the observed decrease in the sedimentation constant of malate dehydrogenase in 15% DMF may well be attributed either to a change in the shape of the protein or to an alteration in its state of hydration. In addition, the sedimentation constant of malate dehydrogenase in 15% DMF is not significantly different when NADH is present or absent. If the enzyme were present in its monomeric form, its molecular weight (and, hence, its sedimentation constant) would have been expected to increase in response to added NADH (Bleile et al., 1977; Shore & Chakrabarti, 1976). When enzymatic activity measurements were conducted in the presence of 15% DMF by using malate dehydrogenase which had been preincubated in 15% DMF, it was found that the apparent V_{max} and K_m for NADH at a fixed oxaloacetate concentration were not very different from values obtained in all aqueous solutions. These results suggest that the structure of malate dehydrogenase in 15% DMF is similar to, although not identical with, that obtained in totally aqueous buffers.

The importance of the subunit structure of malate dehydrogenase is not yet understood, but one of the most debated postulates is that the two identical subunits function in alternate manner, with the binding energy of one subunit being transferred to the other subunit in order to facilitate catalysis (Harada & Wolfe, 1968). If this model were correct for malate dehydrogenase, it might be expected that incorporation of 5′-FSO_2BzAdo into one subunit would severely hamper the function of the other subunit. The expected result might then be that the modification of one of the two subunits would lead to total inactivation. (This would be reflected in a nonlinear plot of residual activity vs. incorporation; extrapolation of the initial slope of the plot would lead to 0.5 mol of 5′-SO_2BzAdo per mol of subunit concomitant with 100% inactivation.) Rather, the analysis of the incorporation of radioactive 5′-FSO_2BzAdo as a function of the inactivation of malate dehydrogenase demonstrates a linear relationship over the entire course of the reaction (Figure 4), with total loss of activity occurring at about 2 mol of reagent incorporated per mol of enzyme or 1 mol of reagent per peptide chain. On the basis of these results, we might conclude that in mitochondrial malate dehydrogenase cooperation between the two subunits is not essential to the function of this enzyme but, instead, that the two subunits act independently. However, there is another possible, although less likely, explanation of the results: the initial relatively slow reaction of 5′-FSO_2BzAdo with one subunit may cause a much more rapid reaction with the second subunit with the result that the distribution of the enzyme forms consists predominantly of unmodified enzyme and enzyme with both subunits modified but with a minimal concentration of the species with one of the two subunits reacted. We consider this possibility unlikely because malate dehydrogenase is not known to show positive cooperativity in interactions with its coenzymes and it appears to be at the coenzyme binding sites that 5′-FSO_2BzAdo reacts.

The single modified amino acid per subunit of malate dehydrogenase has been identified as CBS-Lys on the basis of its electrophoretic and chromatographic mobility on thin-layer plates, as well as by its elution position on two amino acid analyzers using different elution schemes. Wimmer & Harrison (1975) using pyridoxal phosphate as a reagent also suggested that a lysine residue was present in the coenzyme binding site, although the conclusion was not so clear-cut in that case since it depended on the difference between 2.85 mol of pyridoxal phosphate incorporated per subunit in the absence of ligands when the enzyme was 54% inactivated, as compared with 2.3 mol of reagent incorporated in the presence of NADH when the enzyme was only 6% inactivated. For inactivation by 5′-FSO_2BzAdo, the conclusion that a lysine residue is at or near the coenzyme binding site of malate dehydrogenase is less ambiguous, since only a single amino acid residue per peptide chain is modified concomitant with inactivation. It is possible that the lysine residue attacked by 5′-FSO_2BzAdo is the same as one of those which react with pyridoxal phosphate.

The reagent [*p*-(fluorosulfonyl)benzoyl]adenosine might reasonably be considered to be structurally analogous to NAD or ADP. In addition to the adenine and the ribose moieties, 5′-FSO_2BzAdo has a carbonyl group adjacent to the 5′ position of ribose, which occupies a similar position to the first phosphoryl group of the naturally occurring purine nucleotides. If the molecule is arranged in an extended conformation, the sulfonyl fluoride moiety may be located in a position analogous to the 5′ position of the ribose adjacent to the nicotinamide group of NAD. Since ADP as well as ADP–ribose are known to act toward malate dehydrogenase as competitive inhibitors with respect to NAD (Oza & Shore, 1973), it seems likely on structural grounds that 5′-[*p*-(fluorosulfonyl)benzoyl]-adenosine would react within the coenzyme binding site whether the reagent is viewed as an analogue of ADP or NAD. The critical lysine which is modified may be located close to the nicotinamide ribose binding region of the NAD site.

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Suicide Inactivation of Bacterial Cystathionine γ-Synthase and Methionine γ-Lyase during Processing of L-Propargylglycine†

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ABSTRACT: L-Propargylglycine, a naturally occurring γ,δ-acetylenic α-amino acid, induces mechanism-based inactivation of two pyridoxal phosphate dependent enzymes of methionine metabolism: (1) cystathionine γ-synthase, which catalyzes a γ-replacement reaction in methionine biosynthesis, and (2) methionine γ-lyase, which catalyzes a γ-elimination reaction in methionine breakdown. Biphasic pseudo-first-order inactivation kinetics were observed for both enzymes. Complete inactivation is achieved with a minimum molar ratio ([propargylglycine]/[enzyme monomer]) of 4:1 for cystathionine γ-synthase and of 8:1 for methionine γ-lyase, consistent with a small number of turnovers per inactivation event. Partitioning ratios were determined directly from observed primary kinetic isotope effects. [α-^{2}H]Propargylglycine displays k_H/k_D values of about 3 on inactivation half-times. [α-^{3}H]-Propargylglycine gives release of tritium to solvent nominally stoichiometric with inactivation but, on correction for the calculated tritium isotope discrimination, partition ratios of four and six turnovers per monomer inactivated are indicated for cystathionine γ-synthase and methionine γ-lyase, respectively. The inactivation stoichiometry, using [α-^{14}C]-propargylglycine, is four labels per tetramer of cystathionine γ-synthase but usually only two labels per tetramer of methionine γ-lyase (half-of-the-sites reactivity). Two-dimensional urea isoelectrofocusing/NaDodSO$_4$ electrophoresis suggests (1) that both native enzymes are $\alpha_2\beta_2$ tetramers where the subunits are distinguishable by charge but not by size and (2) that, while each subunit of a cystathionine γ-synthase tetramer becomes modified by propargylglycine, only one α and one β subunit may be labeled in an inactive $\alpha_2\beta_2$ tetramer of methionine γ-lyase. Steady-state spectroscopic analyses during inactivation indicated that modified cystathionine γ-synthase may reprotonate C_2 of the enzyme–inactivator adduct, so that the cofactor is still in the pyridoxaldimine oxidation state. Fully inactivated methionine γ-lyase has λ_{max} values at 460 and 495 nm, which may represent conjugated pyridoximine paraquinoid that does not reprotonate at C_2 of the bound adduct. Either species could arise from Michael-type addition of an enzymic nucleophile to an electrophilic 3,4-allenic paraquinoid intermediate, generated initially by propargylic rearrangement upon a 4,5-acetylenic pyridoximine structure, as originally proposed for propargylglycine inactivation of γ-cystathionase [Abeles, R., & Walsh, C. (1973) *J. Am. Chem. Soc. 95*, 6124]. It is reasonable that cystathionine γ-synthase is the major in vivo target for this natural acetylenic toxin, the growth-inhibitory effects of which are reversed by methionine.

The acetylenic amino acid propargylglycine (2-amino-4-pentynoate, **1**) is a natural product elaborated by *Streptomycetes* (Scannel et al., 1971). Prior to its isolation as a metabolite from molds, the DL compound had been synthesized and found to be a growth inhibitor of certain strains of *Escherichia coli* and of yeast *Saccharomyces cerevisiae* (Gershon et al., 1949). Scannel et al. (1971) reported the reversal of propargylglycine-induced growth inhibition of *Bacillus subtilis* upon addition of L-methionine to the culture; thus, it seems probable that the major effect of this antibiotic in vivo is the inhibition of microbial methionine biosynthesis.

We have reasoned that pyridoxal phosphate dependent enzymes which generate β-carbanion (C_3) equivalents during catalysis might effect a propargylic rearrangement of the 4,5-acetylene to the electrophilic conjugated allenic PLP quinoid (**2**), which could function, in turn, as an active-site alkylating agent. This expectation has been borne out with studies on γ-cystathionase from rat liver (Abeles & Walsh,

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