Modification of a Thiol at the Active Site of the *Ascaris suum* NAD-Malic Enzyme Results in Changes in the Rate-Determining Steps for Oxidative Decarboxylation of L-Malate†

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ABSTRACT: A thiol group at the malate-binding site of the NAD-malic enzyme from *Ascaris suum* has been modified to thiocyanate. The modified enzyme generally exhibits slight increases in K_{NAD} and $K_{i\,metal}$ and decreases in V_{max} as the metal size increases from Mg^{2+} to Mn^{2+} to Cd^{2+}, indicative of crowding in the site. The K_{malate} value increases 10- to 30-fold, suggesting that malate does not bind optimally to the modified enzyme. Deuterium isotope effects on V and V/K_{malate} increase with all three metal ions compared to the native enzyme concomitant with a decrease in the ^{13}C isotope effect, suggesting a switch in the rate limitation of the hydride transfer and decarboxylation steps with hydride transfer becoming more rate limiting. The ^{13}C effect decreases only slightly when obtained with deuterated malate, suggestive of the presence of a secondary ^{13}C effect in the hydride transfer step, similar to data obtained with non-nicotinamide-containing dinucleotide substrates for the native enzyme (see the preceding paper in this issue). The native enzyme is inactivated in a time-dependent manner by Cd^{2+}. This inactivation occurs whether the enzyme alone is present or whether the enzyme is turning over with Cd^{2+} as the divalent metal activator. Upon inactivation, only Cd^{2+} ions are bound at high stoichiometry to the enzyme, which eventually becomes denatured. Conversion of the active-site thiol to thiocyanate makes it more difficult to inactivate the enzyme by treatment with Cd^{2+}.

The mitochondrial NAD-malic enzyme from *Ascaris suum* catalyzes the metal-dependent oxidative decarboxylation of L-malate using NAD[1] as the oxidant. The chemical interconversion of malate to pyruvate and CO_2 has been shown to require the action of a divalent metal ion, presumably to polarize the carbonyl of the oxalacetate intermediate during decarboxylation. In addition, enzyme residues are utilized as a general base to accept a proton from the 2-hydroxyl of malate during hydride transfer and a general acid to donate a proton to enolpyruvate upon tautomerization to pyruvate (Kiick et al., 1986).

A sulfhydryl residue has been identified in or near the malate-binding site with modification by DTNB (Kiick et al., 1984). The thiol is not essential for activity, since cyanolysis of the thionitrobenzoyl-modified enzyme restores activity. The modified enzyme had only 40% of the activity of the unmodified enzyme when assayed at V_{max} conditions for the unmodified enzyme. There are a variety of possible reasons for the decreased activity including modification of only half of the sites with DTNB or a decrease in the rate of one of the steps along the reaction pathway, e.g., the chemical step(s). In order to determine the reason for the decreased activity of the thiocyanate enzyme, it was prepared and characterized with respect to mechanistic differences. This report presents a characterization of the thiocyanate malic enzyme by initial velocity studies and primary deuterium and ^{13}C isotope effects. The results suggest that the conversion of the active-site thiol to thiocyanate likely changes the transition state for hydride transfer. In addition, multiple isotope effect data suggest the presence of a secondary ^{13}C isotope effect in the hydride transfer step as suggested for the native enzyme with alternative dinucleotide substrates (see the preceding paper in this issue).

Materials and Methods

Chemicals. NAD-malic enzyme from *A. suum* was purified according to the procedure of Allen and Harris (1981). The enzyme was homogeneous by the criterion of SDS–polyacrylamide gel electrophoresis (O'Farrell, 1975; Atkins et al., 1975). The enzyme had a final specific activity of 35 units/mg when assayed in the direction of oxidative decarboxylation with 100 mM Hepes, pH 7.0, 1.0 mM DTT, 153.4 mM malate (28 mM uncomplexed), 13.5 mM NAD (2 mM uncomplexed) and

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[1] Abbreviations: 3-APAD, 3-acetylpyridine adenine dinucleotide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide 2′-phosphate, Taps, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Mes, 2-(*N*-morpholino)ethanesulfonic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TNB, thionitrobenzoate; TEA, triethanolamine.

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249 mM $MgSO_4$ (160 mM when corrected for Mg:malate and Mg:NAD chelate complexes). NAD and NADP were purchased from Boehringer Mannheim, while L-malate, DTNB, and DTT were from Sigma. Ethanol-d_6 (99 atom % deuterium) was from Merck.

[^{3}H]NAD (2 μCi/mmol) and L-[^{14}C]malic acid (46 mCi/mmol) were obtained from Amersham. L-Malate-2-*d* was prepared according to the method of Viola et al. (1979) by using ethanol-d_6, NAD and oxalacetate with liver alcohol dehydrogenase, yeast aldehyde dehydrogenase, and malate dehydrogenase. All other reagents, chemicals, and enzymes were of the highest quality available from commercial sources.

Metal Chelate Correction. The concentration of all substrates was corrected for the concentration of the metal–ligand chelate complex according to Park et al. (1984). Dissociation constants used in the calculations are as follows: Mg:malate, 25.1, mM; Mg:NAD, 19.5 mM; Mn:malate, 6.3 mM; Mn:NAD, 12.9 mM; Cd:malate, 4.4 mM; and Cd:NAD, 40 mM. The dissociation constant for metal:3-APAD was assumed to be the same as that for metal:NAD.

Substrate Calibration. All substrate concentrations were calibrated enzymatically by end-point analysis as described by Cook et al. (1980). Concentrations of L-malate-2-(*h*,*d*) were determined by using 2 units of chicken liver malic enzyme with the assay containing the following: NADP, 1 mM; $MgSO_4$, 2 mM; DTT, 0.2 mM; and 100 mM Taps, pH 9.0. Solutions of NAD were calibrated with *A. suum* malic enzyme and 20 mM malate (corrected for Mg-malate), 320 mM $MgSO_4$ (corrected for Mg-malate), and 1 mM DTT, in 100 mM Hepes, pH 7.0.

Initial Velocity Studies. All data were collected with a Gilford 250 spectrophotometer to monitor the appearance of reduced dinucleotide. All assays were run at 25 ± 0.1 °C, and the temperature was maintained with a circulating water bath with the capacity to heat and cool the thermospacers of the Gilford. A typical assay contained 100 mM Hepes, pH 7.0, saturating concentrations of metal (20 K_i), and variable concentrations of malate and dinucleotide. Studies using Cd^{2+} as the divalent metal ion were carried out with enzyme stored under an atmosphere of N_2 at −20 °C (prepared as above). Initial velocity patterns were obtained for the thiocyanate NAD-malic enzyme with three different metal ions including Mg^{2+}, Mn^{2+}, and Cd^{2+}. The apparent K_i values of the metals with the thiocyanate enzyme were determined with NAD or 3-APAD and malate at K_m levels and with variable concentrations of the appropriate metal. The K_i was then calculated according to $K_{i\,app} = K_i(1 + NAD/K_{i\,NAD} + malate/K_{i\,malate})$. All assays reflected initial velocity conditions with less than 10% of the limiting reactant utilized over the time course of the reaction.

Determination of the pH profile for V/K_{malate} with the SCN enzyme was carried out by varying the concentrations of malate at saturating concentrations of $MgSO_4$ and NAD. The pH ranges of buffers used at a final concentration of 100 mM were as follows: Mes, 5–6 and Pipes, 6–7. All buffers were titrated to pH with KOH. The pH of the reaction mixture was measured before and after sufficient data were collected for determination of initial velocities. Negligible pH changes were observed before and after the reaction. Deuterium and ^{13}C isotope effects were obtained as described in the preceding paper in this issue.

Preparation of Thiol-Modified NAD-Malic Enzyme. Purified malic enzyme was stored under an atmosphere of nitrogen at −20 °C in 10 mM TEA-maleate, pH 7.5, with 1 mM DTT and 10% glycerol. An aliquot of the enzyme was dialyzed at 4 °C against the same buffer minus DTT under N_2 to remove the sulfhydryl reagent. There was no loss of specific activity following the dialysis. The DTNB was freshly prepared prior to each experiment. Inactivation of malic enzyme by DTNB was carried out by incubating the enzyme with 0.2 mM DTNB at 25 °C in a total volume of 2 mL. Aliquots were removed at specific time intervals and assayed for activity in the direction of oxidative decarboxylation as described above. At the end of 5–6 h complete loss of activity was observed. The modified enzyme was dialyzed twice overnight against buffer at 4 °C minus DTT. The resultant TNB-enzyme was subjected to cyanolysis by KCN. The TNB enzyme was reacted with 100 mM KCN until maximal activity was restored (10 min), and the time course for appearance of TNB at 412 nm was concomitantly monitored. The thiocyanate enzyme was then again dialyzed against buffer at 4 °C and used without further treatment. A time course for the SCN enzyme was obtained with 100 mM Hepes, pH 7.0, 3 mM Cd^{2+}, 10 mM malate, and 1 mM NAD.

The quantitation of enzyme sulfhydryl groups modified by DTNB was carried out by measuring the absorbance of thionitrobenzoate at 412 nm. The purified native enzyme (0.25 mg) was treated with 0.2 mM DTNB in a total volume of 1.0 mL. The increase in absorbance was measured with time against a control containing buffer and the reagent. The thiol concentration was determined by using a molar extinction coefficient of 13 600 M^{-1} cm^{-1} for the released 5-thio-2-nitrobenzoate (TNB) chromophore at 412 nm.

Cd^{2+} Inactivation. The *Ascaris* malic enzyme (25 μg) was incubated with Cd^{2+} (1 or 6 mM) at 25 °C in 10 mM TEA-maleate, pH 7.0, in a total volume of 1 mL. Protection by Mn^{2+} was tested by adding 20 mM $MnSO_4$ to the incubation mixture. Aliquots of 10 μL were removed at specific intervals and immediately assayed for activity by using 100 mM Hepes, pH 7.0, 25 mM Mg^{2+}, 20 mM malate, and 2 mM NAD (all corrected for the concentration of metal chelate complexes). The log of measured activity was plotted vs time, generating a biphasic plot. The slope gave the first-order rate constant for inactivation.

Stoichiometry of Reactants Bound to NAD-Malic Enzyme upon Cd^{2+} Inactivation. The malic enzyme (500 μg) was incubated with 25 μCi (2 Ci/mmole) of [^{3}H]NAD and 25 μCi (45 mCi/mmole) of [^{14}C]malate followed by the addition of 3 mM Cd^{2+}, 1 mM NAD, 1 mM malate, and 100 mM Hepes, pH 7.0, in a total volume of 25 mL. A 1-mL aliquot of the reaction mixture was monitored spectrophotometrically for the appearance of NADH. The reaction was allowed to proceed until no more increase in absorbance was observed. The reaction mixture was then concentrated on an Amicon concentrator with a YM-10 membrane. The concentrated mixture (2 mL) was then applied to an 80 × 1.5 cm Sephadex G-25 column at 4 °C equilibrated with Tris-HCl, pH 7.5, 1 M KCl, and 1 mM EDTA. The protein was eluted with the same buffer, and the protein peak was detected by the method of Bradford (1976) at 595 nm. A 0.2-mL aliquot of the protein fraction was mixed with 5 mL of Aquasol scintillation cocktail and counted for ^{3}H and ^{14}C to determine the amount of [^{3}H]NAD and [^{14}C]malate bound. An individual control was run for the same enzyme concentration without the metal ion.

The stoichiometry of bound Cd^{2+} was obtained with a Perkin-Elmer model 4000 atomic absorption spectrometer with Halocathode lamp. The enzyme (1 mg) was inactivated as above, passed through the Sephadex G-25 column, dialyzed, and concentrated to 1 mL. The amount of Cd^{2+} detected in this sample was 150 μg/mL on the basis of a standard curve

obtained with a standard Cd^{2+} solution. The triethanolamine–maleate buffer, pH 7.5, had no detectable Cd^{2+}. The final protein concentration was 0.87 mg/mL as determined by the method of Bradford (1976).

Data Processing. Reciprocal initial velocities were plotted vs reciprocal substrate concentrations, and all plots were linear. Data were fitted with the appropriate rate equation and Fortran programs developed by Cleland (1979). Individual saturation curves used to obtain the apparent K_i for the metals, and malate saturation curves for the V/K_{malate} pH profile were fitted with

$$v = VA/(K_a + A) \quad (1)$$

Data conforming to a sequential initial velocity pattern were fitted with

$$v = VAB/[K_{ia}K_b + K_aB + K_bA + AB] \quad (2)$$

Initial velocities obtained by varying the nucleotide concentration at saturating levels of metal and deuterium-labeled or unlabeled L-malate and those obtained by varying the concentration of L-malate-2-(h,d) at saturating metal and nucleotide levels were fitted with

$$v = VA/[K_a(1 + F_iE_{V/K}) + A(1 + F_iE_V)] \quad (3)$$

$$v = VA/[(K_a + A)(1 + F_iE_v)] \quad (4)$$

Data for V/K_{malate} vs pH were fitted with eq 5, which describes a curve with a limiting slope of +1 at low pH.

$$\log y = \log [C/(1 + H/K_1)] \quad (5)$$

Equations 3 and 4 assume independent isotope effects on V and V/K or equal effects on both V and V/K, respectively. In eqs 1–4 A and B are reactant concentrations, K_a and K_b are Michaelis constants for A and B, and K_{ia} is the inhibition constant for A. In eqs 3 and 4, F_i is the fraction of the deuterium label in substrate, while E_V, $E_{V/K}$, and E_v are the isotope effects minus 1 for V, V/K, and both, respectively. In eq 5, H is the hydrogen ion concentration, C is the pH-independent value of V/K_{malate}, y is the value of V/K_{malate} observed at any pH, and K_1 is the acid dissociation constant of an enzyme residue. In all cases, the best fit of the data was chosen on the basis of the lowest values of the standard errors of the fitted parameters and the lowest value of σ. σ is defined as the sum of the squares of the residuals divided by the degrees of freedom, where degrees of freedom is equal to the number of points minus the number of parameters (Cleland, 1979).

Results

Preparation of SCN NAD-Malic Enzyme. A thiol group that is not required for activity is present in or near the malate-binding site of NAD-malic enzyme (Kiick et al., 1984). The thiol was modified to the thiocyanate conjugate by first preparing the thionitrobenzoyl enzyme and then displacing thionitrobenzoate with CN^-. The time course for the reaction of the enzyme with DTNB is shown in Figure 1A. Reaction of the enzyme with DTNB is rapid within the first few minutes after the addition of the reagent with three thiols modified. The total number of thiol groups per enzyme subunit modified with DTNB under the conditions used is six (Figure 1A). Kiick et al. (1984) showed the presence of a total of 10 thiol groups/mole of subunit in the *Ascaris* enzyme as determined by the reaction of the enzyme with 4-PDS (Grassetti & Murray, 1967) in 6 M guanidinium chloride.

Cyanolysis of the TNB-modified enzyme to form an active thiocyanate derivative has been used previously by Kiick et al. (1984) to demonstrate the nonessentiality of the active-site

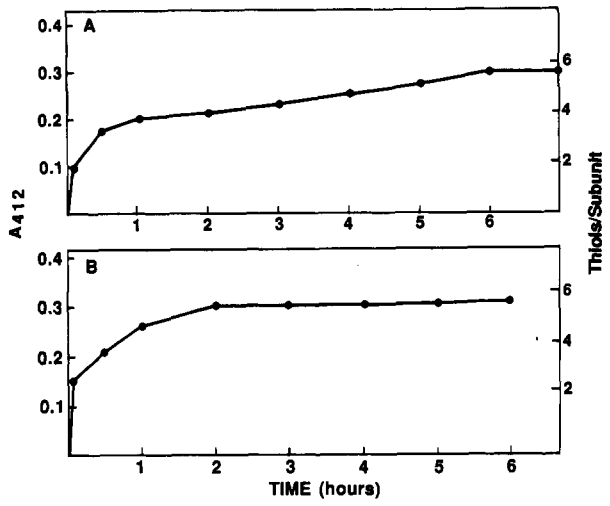

FIGURE 1: Time course for the inactivation of the NAD-malic enzyme by DTNB and subsequent reactivation by cyanide. (A) The inactivation mixture contained the following: 100 mM Tris-HCl, pH 7.0, 0.2 mM DTNB, and 250 μg of the enzyme in a total volume of 1 mL at 25 °C. (B) The reactivation mixture contained the following: 100 mM Taps, pH 9.0, 100 mM KCN, and 250 μg of the TNB-modified enzyme in a total volume of 1 mL at 25 °C.

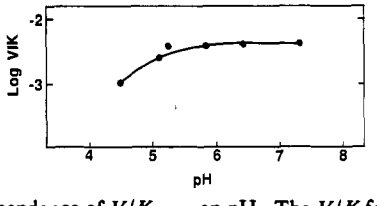

FIGURE 2: Dependence of V/K_{malate} on pH. The V/K for malate was obtained at saturating concentrations of Mg^{2+} (160 mM) and NAD (2 mM) by varying the concentration of malate from 5 to 50 mM. The points shown are experimentally determined values, while the curve is from the fit to the data with eq 5.

cysteine for catalysis. The preparation of the thiocyanate enzyme was carried out as described under Materials and Methods. The restoration of the activity brought about by cyanolysis of the inactive TNB enzyme could not be reversed by the addition of DTT. A total of six TNB moieties per enzyme subunit were released for full restoration of activity with the first three released rapidly with respect to the last three (Figure 1B). Treatment of the E-SCN adduct with DTNB had little effect on its activity, consistent with the reactive cysteine being present as a thiocyanate conjugate.

Characterization of SCN-NAD-Malic Enzyme. The thiocyanate enzyme was characterized by using initial velocity studies and primary deuterium, ^{13}C, and multiple isotope effects with three metal ions including Mg^{2+}, Mn^{2+}, and Cd^{2+}. Initial velocity patterns were obtained for the modified enzyme at saturating concentrations of the divalent metal ion by varying the concentration of nucleotide at several fixed levels of malate. In all cases, the double-reciprocal plots intersect to the left of the ordinate. Kinetic parameters are listed in Table I. Data were also collected with 3-APAD as a substrate and Mg^{2+} as the divalent metal ion activator. The Michaelis constants for 3-APAD and malate are 7.2 ± 0.3 μM and 26 ± 5 mM, respectively, while the maximum velocity is 2.5 relative to that obtained with NAD and native enzyme. The pH dependence of V/K_{malate} over the pH range 5–7 is given in Figure 2.

Primary deuterium isotope effects were determined with L-malate 2-(h,d) and are shown in Table II [$^D(V/K_{DINUC})$ was not obtained as a result in the difficulty of saturating with malate]. Also in Table II are values of the ^{13}C effects obtained

Table I: Values of Kinetic Parameters for the SCN-NAD-Malic Enzyme Reaction[a]

parameter[b]	Mg^{2+}	Mn^{2+}	Cd^{2+}
V/E_t	25	19	11.4
	(0.65)	(0.5)	(0.3)
K_a	0.014 ± 0.030	0.050 ± 0.005	0.30 ± 0.05
K_b	30 ± 6	3.0 ± 0.3	6.0 ± 0.9
V/K_aE_t	1.8×10^6	3.8×10^5	3.8×10^4
	(0.48)	(0.1)	(0.01)
V/K_bE_t	8.5×10^2	6.3×10^3	1.9×10^3
	(0.022)	(0.165)	(0.05)
$K_{i\,metal}$	26	4.0	2.0
K_{Ib}	30	6.7	

[a] All initial velocities were obtained at 25 °C as described under Materials and Methods. Concentration ranges for reactants at 100 mM Hepes, pH 7.0, are as follows: with Mg^{2+} and NAD, 0.01–0.1 mM; Mg^{2+}, 25 mM; malate, 10–100 mM; with Mn^{2+} and NAD, 0.01–0.1 mM; Mn^{2+}, 10 mM; malate, 1–10 mM; with Cd^{2+} and NAD, 0.05–0.5 mM; CD^{2+}, 5 mM; malate, 2–20 mM. Data were fitted with eq 5. For determination of the metal K_i, NAD and malate were fixed at K_m and the concentration of metal and varied. All reactant concentrations are corrected for metal chelate concentration. [b] Units are s^{-1} for V/E_t, mM for K_a, K_b, and K_i and $M^{-1}\,s^{-1}$ for V/K_aE_t and V/K_bE_t with $K_a = K_{DINUC}$ and $K_b = K_{malate}$. Values of K_a, K_b, and K_I are ±SE. Values in parentheses are relative to the native enzyme with Mg^{2+} as the divalent metal.

Table II: Primary Deuterium and ^{13}C Isotope Effects[a]

parameter	Mg^{2+}	Mn^{2+}	Cd^{2+}
^{D}V	2.40 ± 0.04	1.9 ± 0.2	2.60 ± 0.13
$^{D}V/K_{malate}$	2.40 ± 0.04	3.0 ± 0.2	2.60 ± 0.13
$^{13}(V/K_{malate})_H$	1.0191 ± 0.0002	1.0145 ± 0.0002	ND
$^{13}(V/K_{malate})_D$	1.0172 ± 0.0002	1.01028 ± 0.00001	ND

[a] ND is not determined. Data for deuterium isotope effects with Mg^{2+} and Cd^{2+} were fitted with eq 4, while those obtained with Mn^{2+} were fitted with eq 3.

with protium- and deuterium-labeled malate. The deuterium and ^{13}C isotope effects were also obtained with 3-APAD as a substrate. The isotope effects on V and V/K_{malate} are equal with a value of 3.3 ± 0.2, while $^{13}(V/K_{malate})_H$ and $^{13}(V/K_{malate})_D$ are 1.0050 ± 0.0003 and 1.0057 ± 0.0002, respectively.

Cd^{2+} Inactivation. When Cd^{2+} is the divalent metal ion activator, nonlinear time courses are obtained. There is a loss of enzyme activity within the first few minutes as shown in Figure 3A. The lost activity is not regenerated by the addition of more substrates, Mg^{2+}, or Mn^{2+}. In addition, the initial concentration of reactants did not affect the number of turnovers for enzyme inactivation.

The inactivation of NAD-malic enzyme occurred in a biphasic manner, with approximately 50% loss of enzyme activity in first few minutes. The fast inactivation phase gave a rate constant of 0.18 min^{-1}, while the slow phase gave a rate constant of 0.014 min^{-1} with 27 μM enzyme. With the Cd^{2+} increased to 6 mM, inactivation was faster (k = 0.8 min^{-1} for the same enzyme concentration), and 50% of the initial activity was lost in the first minute. The inactivation rate of the second phase was 10-fold slower than that in the first phase. With 20 mM Mn^{2+} included in the inactivation mixture with 1 mM Cd^{2+}, the fast phase inactivation rate is decreased to 0.035 min^{-1}.

A time course for the thiocyanate-modified enzyme was obtained with Cd^{2+} as the divalent metal ion activator (Figure 3B). As can be seen, the modified enzyme turns over at least 10 times more than the native enzyme prior to becoming inactivated. During the reaction, no CN^- was released from enzyme as shown by using $^{14}CN^-$-labeled thiocyanate enzyme. Cyanide release was determined by ultrafiltration of aliquots

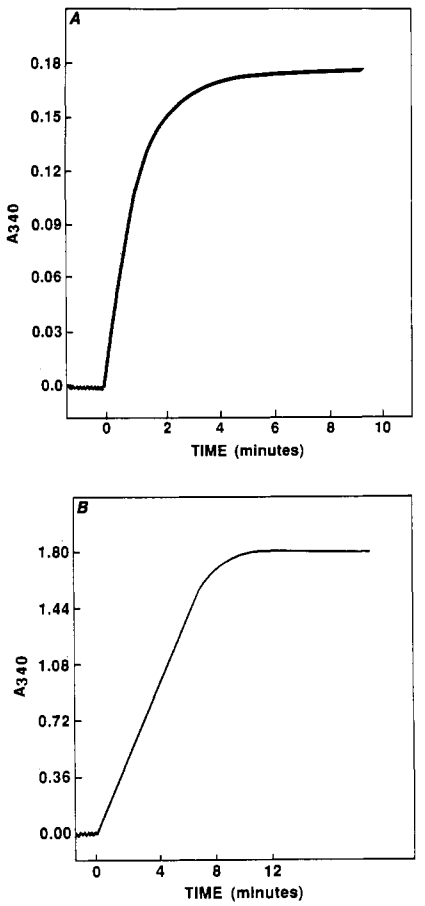

FIGURE 3: (A) Time course for the reaction of malic enzyme with Cd^{2+} as the divalent metal activator. The reaction mixture at 25 °C contained 100 mM Hepes, pH 7, 0.1 mM NAD, 1 mM malate, and 3 mM Cd^{2+}. The reaction was initiated by the addition of the enzyme (1.5 μg) in a total volume of 1 mL. Similar results were obtained with 1 mM NAD. (B) Time course for the reaction of the SCN-enzyme with Cd^{2+} as the divalent metal ion activator. The reaction mixture at 25 °C contained 100 mM Hepes, pH 7, 3 mM Cd^{2+}, 10 mM malate, and 1 mM NAD. The reaction was initiated with 1.5 μg of enzyme.

of the reaction mixture with labeled enzyme and by counting the filtrate with enzyme in the absence of reactants as a control.

The stoichiometry of reactants bound to the enzyme upon Cd^{2+} inactivation was measured. These experiments were carried out with [^{3}H]NAD and [^{14}C]malate as described under Methods. The results indicate that neither NAD nor malate was bound to the enzyme since no radioactivity eluted with the enzyme. However, atomic absorption spectra of the inactive enzyme gave a Cd^{2+} stoichiometry of ca. 90 mol/mole of enzyme subunit. In addition, the enzyme begins to denature after treatment with Cd^{2+}.

Discussion

Characterization of the SCN-NAD-Malic Enzyme. The thiocyanoylated NAD-malic enzyme has been prepared previously (Kiick et al., 1984) but has not been characterized. Treatment with DTNB resulted in the modification of six thiol groups per subunit with the first three modified faster than the last three. Subsequent treatment with cyanide liberated six thionitrobenzoyl moieties with a regain in activity.

It was thought previously (Kiick et al., 1984) that the enzyme had only 40% of the activity of the native enzyme, but the previous studies made use of 28 mM malate to assay the modified enzyme. This is approximately the value of K_{malate}

for the modified enzyme, and thus maximal activity is expected to be about 80–85% (preparations as high as 85% have been obtained). In these studies, a final activity of 65% of V_{max} was obtained, but this was after several dialysis steps.

Initial velocity studies showed that the larger the metal ion, the lower the value for the turnover number. This is in contrast to data obtained with the native enzyme where Mn^{2+} is the best divalent metal activator, being 2-fold better than Mg^{2+} and 4-fold better than Cd^{2+} (unpublished results of S. R. Gavva in this laboratory). The K_i for the metal ion follows the same trend with values for Mg^{2+}, Mn^{2+}, and Cd^{2+} increased 1.6-fold, 2.7-fold, and 13-fold with respect to the native enzyme ($K_{i\,Mg}$, 16 mM; $K_{i\,Mn}$, 1.5 mM; $K_{i\,Cd}$, 0.15 mM; Kiick et al., 1986), while K_{NAD} is the same with Mg^{2+} and increases 8-fold and 5-fold with respect to the native enzyme with Mn^{2+} and Cd^{2+} as metal ion activators. The above data suggest a general crowding in the active site of the modified enzyme that becomes worse the larger the metal ion. However, the value for K_{malate} consistently increases an order of magnitude compared to values obtained with the native enzyme (Mg^{2+}, 30-fold; Mn^{2+}, 10-fold; Cd^{2+}, 35-fold). The K_m values obtained with Mg^{2+} for 3-APAD and malate were not significantly different from those obtained with NAD and Mg^{2+}. Thus, in addition to a general crowding, malate apparently does not bind properly to the modified enzyme, and it is probably this more than anything else that is responsible for the rate changes noted.

A general base has been identified that accepts a proton from the 2-hydroxyl of malate concomitant with hydride transfer (Kiick et al., 1986). To ascertain whether the pK for this enzyme residue is increased, causing the apparent decreased affinity, the V/K_{malate} profile was determined for the SCN-enzyme over the pH range of 5–7. The V/K_{malate} decreases at low pH, giving a pK of 4.9 ± 0.4 (Figure 2). This value is not different from the pK value of 4.7 ± 0.3 obtained for the native enzyme (Kiick et al., 1986). As a result, the decrease in the affinity of malate for the modified enzyme, compared to the native enzyme, is likely the result of substitution at the reactive thiol group present at or near the binding site of malate (Kiick et al., 1984).

The isotope effect data provide both a more qualitative and quantitative assessment of what has occurred upon modification. The deuterium effects with Mg^{2+} as the divalent metal activator increase from 1.5 for the native enzyme to 2.4 for the modified enzyme, and the effects on V and V/K_{malate} are equal. Since the isotope effects are equal on both parameters, it is probable that the rate-determining portion of the reaction is the interconversion of the central complexes whether malate is limiting or saturating. The increase in the value of the deuterium effect suggests that transfer of the hydride from C-2 of malate to C-4 of the nicotinamide ring of NAD has become more rate determining compared to the native enzyme.[2] (This interpretation assumes that the transition state for the hydride transfer step has not changed significantly with the modified enzyme compared to the native enzyme.) In agreement with this, the ^{13}C effect has decreased from a value of 3.6% with the native enzyme to 1.9% for the modified enzyme, suggesting that decarboxylation of the oxalacetate intermediate has become less rate determining overall. The situation is similar for Mn^{2+} with the deuterium effect[3] on V increasing from 1.6 to 1.9 and that on V/K_{malate} increasing from 2.2 to 3. Again, the suggestion is that the same step(s) is (are) slowed down when enzyme is modified. In agreement with this, the ^{13}C effect decreases from 3.5% to 1.4% with Mn^{2+} as the divalent metal ion. This same phenomenon is also apparent with Cd^{2+} since the deuterium effects increase from about 2.2 with the native enzyme to 2.6 with the modified enzyme. Thus, unlike the native enzyme for which decarboxylation of the oxalacetate intermediate is the slowest step with hydride transfer less rate determining, the hydride transfer step has become more rate determining for the modified enzyme. Data obtained with 3-APAD are also in accord with the overall switch in rate-determining steps. The deuterium isotope effect increases from 2.2 to 3.3, while the ^{13}C effect decreases from 0.7% to 0.5%.

As discussed in the preceding paper, multiple isotope effects have been shown to be a powerful tool for obtaining mechanistic information on enzyme reactions (Hermes et al., 1982). A two-step mechanism for oxidative decarboxylation of malate in which hydride transfer precedes decarboxylation has been proposed for the malic enzyme reaction. As shown in the accompanying report, there is some doubt as to whether the simple mechanism holds with the alternative dinucleotide substrates. The preceding report suggests the unmasking of a secondary ^{13}C isotope effect in the hydride transfer step as a result of hyperconjugation of the β-carboxyl of malate as the transition state for hydride transfer is reached. The more rate determining hydride transfer becomes, the more the secondary effect would be observed. In the case of the modified enzyme, the ^{13}C effect decreases only slightly upon deuteration of malate and does not change or increases slightly with 3-APAD and Mg^{2+}. The expected equality for a two-step system in which hydride transfer precedes decarboxylation is given by the following equation (Hermes et al., 1982):

$$[^{13}(V/K)_H - 1]/[^{13}(V/K)_D - 1] = {}^{D}(V/K)/{}^{D}K_{eq} \quad (6)$$

With the values given in Table II for ^{13}C and deuterium isotope effects and a value of 1.18 for ${}^{D}K_{eq}$ (Cook et al., 1980), the equality gives 1.1 = 2 with Mg^{2+} and 1.4 = 2.5 with Mn^{2+}. As a result, although the chemistry appears to occur in two steps since the ^{13}C effect decreases with deuterium compared to protium-labeled substrate, some other step appears to be present and partially compensates for the full expected decrease. This is the same phenomenon observed in the case of the alternative nucleotide reactants with the native enzyme. These data would be consistent with an increase in the ^{13}C effect upon deuteration in the malic enzyme reaction as a result of a ^{13}C effect in the hydride transfer step that becomes more apparent the slower this step becomes. The largest deuterium isotope effect is observed with 3-APAD, and the ^{13}C effect does not change with deuteration of malate. Thus, if the above mechanism holds, the observed value of 0.5% may be the intrinsic secondary ^{13}C isotope effect obtained at C-4 of malate during the hydride transfer step.

Inactivation by Cd^{2+}. A time-dependent loss of the NAD-malic enzyme activity results either from preincubation of malic enzyme with Cd^{2+} or under conditions in which turnover occurs in the presence of Cd^{2+} as the divalent metal ion activator. In the latter case, the concentrations of reactants (NAD^+ and malate) present initially are not important, with the same number of turnovers giving inactive enzyme at all

[2] The value of ${}^{D}k$ is only the intrinsic isotope effect if the isotope-sensitive step is completely rate determining. The ${}^{D}k$ term will otherwise include an internal c_f and any c_r term. A substrate is sticky if it partitions toward product formation faster than it dissociates from enzyme (Cook & Cleland, 1981).

[3] Deuterium isotope effects obtained for the native enzyme are the unpublished results of Dr. S. R. Gavva in this laboratory, while the ^{13}C effects are the unpublished results of Dr. P. M. Weiss of the University of Wisconsin.

concentrations tested. The only material bound to the enzyme once inactivation has occurred is Cd^{2+} at a very high stoichiometry.

As stated above, a thiol is present in or near the binding site for malate on malic enzyme (Kiick et al., 1984). It seemed reasonable that Cd^{2+} inactivated the enzyme by binding to the active-site thiol, no longer being able to perform its normal role in catalysis. Although Cd^{2+} is 10-fold less effective inactivating the thiocyanoyl enzyme, it does still inactivate. Thus, part of the observed inactivation appears to be a result of Cd^{2+} binding to the active-site thiol. That Cd^{2+} binds normally to begin the reaction is shown by turnover of the reaction for a number of cycles prior to inactivation. An explanation consistent with all of the results obtained is that Cd^{2+} binds to a large number of groups on the protein, resulting in denaturation. Thus, it does not matter what is bound at the active site or whether the active-site thiol is modified. The decrease in the inactivation rate in the presence of Mn^{2+} suggests either that Mn^{2+} competes with Cd^{2+} or that it changes the enzyme conformation upon binding to the active site.

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Cysteine-374 of Actin Resides at the Gelsolin Contact Site in the EGTA-Resistant Actin–Gelsolin Complex†

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ABSTRACT: The interaction of pig plasma gelsolin (G) and actin (A) was examined by using photoreactive 4-maleimidobenzophenone–actin (BPM–actin) in which BPM was previously conjugated to Cys-374 of actin through the maleimide moiety. In the presence of micromolar [Ca^{2+}], the major cross-linked product observed after irradiation of the mixture of gelsolin (82 kDa) and actin (42 kDa) had an apparent molecular mass of 130 kDa although gelsolin predominantly existed in the form of an A_2G complex (170 kDa). No cross-linked product was detected in the absence of Ca^{2+}. BPM–actin itself did not give any cross-linked product. By use of fluorescent-labeled gelsolin, the cross-linked 130 kDa was shown to be an AG complex. The cross-linked complex was also formed from the A_2G complex after removal of Ca^{2+} by [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA) followed by irradiation, indicating that it was the EGTA-resistant AG complex that was cross-linked. The results show that Cys-374 at the C-terminal segment of actin in the EGTA-resistant AG complex is 9–10 Å apart from gelsolin. Furthermore, it was shown that the EGTA-resistant actin molecule once incorporated in the A_2G complex did not exchange with free actin in the presence of Ca^{2+}. This was also supported by the effect of phosphatidylinositol 4,5-bisphosphate, which did not dissociate the EGTA-resistant actin molecule from the A_2G complex in the presence of Ca^{2+}, but did after removal of Ca^{2+}.

The intracellular organizations of actin filaments are essential for many forms of cellular motility as well as the structure and mechanical properties of the cytoplasmic matrix. The dynamic nature of actin filament assembly and structure in nonmuscle cells is thought to be explained by a handful of actin-binding proteins (Pollard & Cooper, 1986). However, understanding the mechanism of their interaction with actin at a molecular level is still premature despite its importance in clarifying their functional properties. The problem has been augmented by lack of detailed knowledge of the three-dimensional structure for actin until very recently (Kabsch et al., 1990). To alleviate the problem, many cross-linking studies have been carried out to elucidate the structural feature at the

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