Determination of Dissociation Constants for Enzyme–Reactant Complexes for NAD–Malic Enzyme by Modulation of the Thiol Inactivation Rate†

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ABSTRACT: Incubation of NAD–malic enzyme from *Ascaris suum* with the sulfhydryl reagents *N*-ethylmaleimide (NEM), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), or 4,4′-dithiodipyridine (4-PDS) results in rapid and complete loss of malate oxidative decarboxylase and pyruvate reductive carboxylase activities. With DTNB, this loss of activity occurs concomitantly with the modification of about 1 thiol group per subunit. The majority of the activity is lost when 0.5 thiol per subunit is modified, indicative of possible half-site reactivity with DTNB. Complete restoration of activity follows addition of dithiothreitol to enzyme inactivated by DTNB and 4-PDS but not with NEM. With the DTNB-inactivated enzyme, replacement of the thionitrobenzoate moiety with cyanide restores activity. The presence of a divalent metal ion (Mg^{2+} or Mn^{2+}) results in enhancement of the inactivation rate with all sulfhydryl reagents. However, malate alone or competitors of malate provide protection which is more effective in the presence of Mg^{2+}, while NAD provides only about 25% protection. Thus, the *Ascaris suum* NAD–malic enzyme has a thiol group probably located in or near the malate binding site, which is not essential for enzyme activity. The changes in the rate of inactivation in the presence of reactants were used to determine the dissociation constants for enzyme–reactant complexes. These data suggest that all three possible binary and all three possible ternary complexes form. The binding of malate to free enzyme exhibits negative cooperativity, which is eliminated by the presence of either NAD or Mg^{2+}. The binding of Mg^{2+} to free enzyme is noncooperative and is unaffected by NAD. The partial protection by NAD is similar whether NAD binds to E, E–Mg, or E–malate, and the binding is positively cooperative. Thus, the kinetic mechanism for NAD–malic enzyme is random.

Previous studies (Landsperger et al., 1978) have suggested that the NAD–malic enzyme from *Ascaris suum* possesses a random kinetic mechanism with Mn^{2+} as the divalent metal ion. In the preceding report (Park et al., 1984), extensive evidence was presented for this random mechanism with Mg^{2+} as the divalent metal ion. However, it was also demonstrated that under certain conditions of low substrate concentrations, the enzyme behaved as if it had an ordered mechanism with NAD binding first, followed by Mg^{2+} and malate. In fact, this ordered mechanism is similar to that described for the pigeon liver NADP–malic enzyme (Hsu & Lardy, 1967; Schimerlik & Cleland, 1977). If the mechanism is random, it should be possible to demonstrate the presence of binary complexes between enzyme and substrates. Such complexes have been demonstrated between enzyme–Mn^{2+} and substrates (Landsperger et al., 1978), but they have not been demonstrated for enzyme in the absence of metal or for enzyme–Mg^{2+} and substrates.

Malic enzyme from many sources requires the presence of a sulfhydryl reducing agent for optimal activity, and treatment with sulfhydryl reagents results in inactivation of the enzyme [reviewed in Hsu (1982)]. Although this sulfhydryl group is nonessential for catalytic activity, it appears to be located in or near the malate binding domain since malate analogues protect it from derivatization (Hsu, 1982). The ascarid malic enzyme requires a reducing agent such as dithiothreitol for optimal activity (Fodge et al., 1972). It was thus important to determine if the ascarid malic enzyme possessed a reactive sulfhydryl and to characterize the sulfhydryl as to its essentiality. It was also important to determine if reactivity of the putative sulfhydryl could be modulated by substrates so that this modulation could be used to demonstrate the formation of enzyme–substrate complexes.

The results of these experiments suggest that the ascarid malic enzyme has a reactive sulfhydryl group that is nonessential for catalytic activity but whose reactivity is modulated by the divalent metal ion and both substrates. By use of this modulation of the inactivation rate of the sulfhydryl group, dissociation constants were obtained for several of the enzyme–substrate complexes. The results suggest that all three binary and all three ternary complexes form consistent with a random terreactant mechanism.

Experimental Procedures

Materials. L-Malic acid, DTNB,[1] NEM, 2-mercaptoethanol, and dithiothreitol were from Sigma. 4-PDS was obtained from Aldrich. NAD was from P-L Biochemicals. $K^{14}CN$ was from ICN Pharmaceuticals, Inc. All other chemicals used were of the highest quality commercially available.

Enzyme Assays. Malic enzyme was assayed spectrophotometrically in the forward direction as described previously (Landsperger et al., 1978). The reaction was started by the addition of enzyme, and the production of NADH was monitored at 340 nm. Oxidative decarboxylation was assayed by using 100 mM Hepes, pH 7.3, 154 mM malate (28 mM when corrected for Mg–malate), 13.4 mM NAD (2 mM when corrected for Mg–NAD), and 250 mM $MgSO_4$ (112 mM when corrected for Mg–malate and Mg–NAD). Reductive carboxylation of pyruvate was assayed by using the following reaction mixture in 1 mL total volume: 100 mM Hepes, pH

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[1] Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TNB, thionitrobenzoate; NEM, *N*-ethylmaleimide; 4-PDS, 4,4′-dithiopyridine; DTT, dithiothreitol; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid.

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7.3, 300 mM $MgSO_4$, 50 mM pyruvate (titrated to pH 7 with KOH), 100 mM $NaHCO_3$, 300 μM NADH, and 0.1 μM malic enzyme.

Sulfhydryl Modifications. Purified malic enzyme (Allen & Harris, 1981) (specific activity = 30–35 units/mg using Mg^{2+} as the divalent metal) was stored under an atmosphere of N_2 at −20 °C in a storage buffer containing 15 mM triethanolamine–maleate, pH 7.5, 1 mM EDTA, 10 mM DTT, and 5% glycerol. The enzyme was stable for several months at −20 °C. For use, the enzyme was thawed, an aliquot was removed, and the remaining enzyme was purged with nitrogen and immediately refrozen (freeze–thawing had no apparent effect on activity). The aliquot was dialyzed at 4 °C against storage buffer minus DTT under N_2 to remove the sulfhydryl-reducing agent. There was no loss of specific activity following the dialysis. Incubation with sulfhydryl reagents was carried out in cuvettes at ambient temperature (22–25 °C) or 30 °C in a volume of 0.5–1 mL. Aliquots were removed at specified intervals and assayed for catalytic activity. In cases in which a derivatization process was monitored spectrophotometrically, an aliquot was removed and assayed immediately on a separate spectrophotometer. In these experiments, 100–200 μg of malic enzyme (0.385–0.77 nmol) was utilized, and the derivatization process was followed by the change in absorbance over the specified time period [TNB from DTNB, E_{412nn} = 13 600 M^{-1} cm^{-1} (Ellman, 1959)]. Apparent first-order rate constants of inactivation were calculated from the slopes of log (A_t/A_0) 100 vs. time plots where A_0 = activity at zero time and A_t = activity at time of sampling. The slope of these plots = $k/2.303$, where k is the pseudo-first-order rate constant for inactivation.

Dissociation Constants. For these studies enzyme was prepared in the same manner as for the sulfhydryl modification experiments. Approximately 0.33 unit/mL malic enzyme was incubated at 25 °C with 100 mM buffer (Hepes at pH 7.3 or Ches at pH 9.3) and appropriate concentration of reactants in a final volume of 0.5 mL. The concentration of reactant indicated in the figures is the final concentration in the inactivation mixture. In the case where Mg^{2+} plus either malate or NAD were present, correction was made for chelate complex formation as described in the preceding paper (Park et al., 1984). All incubation times were 10 min prior to addition of DTNB (100 μM at pH 7.3 and 10 μM at pH 9.3). Aliquots (20 μL) were removed at ≃0.5-min intervals and assayed for catalytic activity. Data were plotted as ln v vs. time and were fitted to the equation for a straight line with the absolute value of the slope equal to the pseudo-first-order rate constant. The pH of the incubation mixture was taken directly. At each pH that the inactivation studies were carried out, there was no loss of activity with time when malic enzyme was incubated at the pH for more than the time covered in the inactivation studies.

Theory. The rate of inactivation by chemical reagents specific or partially specific for a certain functional group that can be modulated by the presence of one or more reactants during chemical modification can be used to obtain dissociation constants for the reactant(s) present. The basic assumption made is that the percent change in the rate of inactivation is equal to the percent fractional occupancy of the enzyme site of interest. There are three possible results of including a reactant, inhibitor, or activator in the inactivation reaction mixture. These possibilities are an increase in the rate of inactivation, no change, and a decrease in the rate of inactivation. The decrease may be to zero if complete protection is obtained or some finite lower value if partial protection is obtained, while the increase will always be to some higher finite value.

In the simplest case where reaction is obtained upon collision of reagent with the enzyme functional group of interest, the rate is the bimolecular rate of collision and reaction. Protection can then be very simply described as competition between reagent and reactant for enzyme:

$$\begin{array}{ccc} E & \xrightarrow{k_1 R} & E_{inact} \\ \Big\Downarrow\Big\Uparrow K_s & & \\ ES & & \end{array} \qquad (1)$$

where k_1 is the bimolecular inactivation rate, K_s is the dissociation constant for ES, and R is the reagent. Partial protection, on the other hand, can be described by partial competitive inhibition with the ES complex still reactive, although somewhat less, toward reagent.

$$\begin{array}{ccc} E & \xrightarrow{k_1 R} & E_{inact} \\ \Big\Downarrow\Big\Uparrow K_s & & \\ ES & \xrightarrow{k_1' R} & ES_{inact} \end{array} \qquad (2)$$

In this case k_1' is the bimolecular rate of reaction of ES with R which will be less than k_1, while K_s is still the dissociation constant for ES. This second case will also describe an increase in the rate of inactivation. The only difference is that k_1' will be greater than k_1.

The apparent rate constant for eq 1 will be

$$k_{app} = \frac{k_1 R}{1 + S/K_s} \qquad (3)$$

which can be rearranged to give

$$\frac{k_1 R}{k_{app}} = 1 + \frac{S}{K_s} \qquad (4)$$

so that a plot of $k_1 R/k_{app}$ vs. S will give the dissociation constant as the reciprocal of the slope.

The apparent rate constant for eq 2 will be

$$k_{app} = \frac{k_1 R + k_1' RS/K_s}{1 + S/K_s} \qquad (5)$$

This equation can be rearranged to give

$$\frac{k_1 R}{k_{app}} = 1 + \frac{S}{K_s}\left(1 - \frac{k_1' R}{k_{app}}\right) \qquad (6)$$

This equation describes a hyperbola which starts at 1 and goes to a finite value when $k_1' R$ becomes equal to k_{app}. In this case, the graphical value will be an overestimate unless very low concentrations are used which will probably provide very little protection. Thus, it is preferable to fit the data for k_{app} vs. S directly to eq 5.

The treatment becomes more complex if an enzyme–reagent complex is required prior to inactivation. However, the data presented in this paper conform to the former simple case.

Data Processing. Data were fitted to the appropriate rate equation by using the Fortran programs of Cleland (1979). Data conforming to a straight line were fitted to the equation for a straight line. Data for Mg_f^{2+} saturation curves were fit to eq 7 while all hyperbolic plots of k_{app} vs. reactant concentration were fitted to eq 8. In eq 7, V is the apparent maximum

$$v = \frac{VA}{K + A} \qquad (7)$$

$$y = \frac{a(1 + X/K_{IN})}{(1 + X/K_{ID})} \qquad (8)$$

Table I: Protection against NEM by DTNB

time (min)	% original activity after treatment with[a] DTNB	DTNB	control
0	100	100	100
15	5	7	100
	+buffer	+NEM	+NEM
15	0	0	0
	+DTT	+DTT	+DTT
10	37	41	0
20	79	56	0

[a] Incubation mixture contained in each case 100 μmol of triethanolamine hydrochloride, pH 7.0, 1 μmol of EDTA, and 9 μg of malic enzyme in 1 mL. Concentrations of DTNB, NEM, and DTT when present were 0.255, 0.255, and 2 mM, respectively. Temperature was 24 °C.

rate at saturating Mg_f^{2+}, K is $K_{i\,Mg}$, and A is Mg_f^{2+} concentration. In eq 8, y is k_{app}, a is the value of k_{app} at $X = 0$, $a(K_{ID}/K_{IN})$ is the value of k_{app} at $X = \infty$, X is the reactant varied, K_{ID} is the dissociation constant for the enzyme–reactant complex of interest, and K_{IN} is a ratio of rate constants which results in k_{app} leveling off at a finite value. In all cases, k_{app} is the pseudo-first-order rate constant (k_1R) for reaction of E and reagent, while for cases where k_{app} goes to some finite value K_{IN} is $K_s/k_1'R$.

Results

Characterization of Thiol Inactivation. The total number of thiol groups in the ascarid malic enzyme was determined by reaction of the enzyme with 4-PDS (Grassetti & Murray, 1967) in 6 M guanidinium chloride. The average value obtained in these experiments was 10.1 mol of sulfhydryl/mol of subunit or 40.4 mol of thiol/mol of enzyme. This value for total sulfhydryls is similar to that obtained for the pigeon liver enzyme (Tang & Hsu, 1974).

Loss of malate oxidative decarboxylase activity occurred when the malic enzyme was incubated with three sulfhydryl reagents, NEM, 4-PDS, and DTNB. Each incubation mixture contained identical amounts of malic enzyme and sulfhydryl reagents, and the apparent first-order rate constants for inactivation can be compared directly. These values are the following: NEM, 3.65×10^{-3} min^{-1}; 4-PDS, 1.84×10^{-2} min^{-1}; DTNB, 3.75×10^{-2} min^{-1}. Thus, DTNB and 4-PDS are more effective in inactivating the enzyme than is NEM. Essentially identical values were obtained when the enzyme was assayed by using the reductive carboxylation assay (see Experimental Procedures). In addition, *p*-(chloromercuri)benzoate and iodoacetate also brought about complete inactivation[2] of forward and reverse catalytic activity.

In order to determine if the inactivation by sulfhydryl reagent was actually the result of sulfhydryl modification, enzyme was inactivated by DTNB or 4-PDS and then treated with DTT. The loss of activity brought about by DTNB or 4-PDS could be reversed by the addition of DTT.

While DTNB and NEM have similar effects on the enzyme, their mode of action is different. In order to ascertain if both reagents were causing inactivation by reacting with the same sulfhydryl groups, DTNB-inactivated enzyme was treated with NEM, and the activity could be restored with DTT (Table I). NEM-inactivated enzyme was irreversibly modified, and no activity was regained on treatment with DTT, as is expected with α,β-unsaturated reagents (Jocelyn, 1972). Thus, DTNB protected the sulfhydryl groups from alkylation by NEM.

[2] It is difficult to assess complete inactivation vs. inactivation to a small, but finite, residual activity. We can say that, if there is a small residual activity for NAD–malic enzyme, it must be <0.1%.

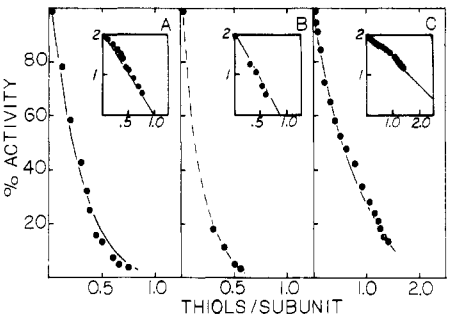

FIGURE 1: Inactivation of enzyme activity and titration of malic enzyme thiol groups by DTNB. Incubation mixtures contained the following: (A) 100 mM triethanolamine hydrochloride, pH 7.0, 55 μM DTNB, and 0.77 nmol of malic enzyme; (B) same as (A) except 10 mM $MnCl_2$ was added; (C) same as (B) except 33 mM malate was added. The maximum number of thiols modified for 99% inactivation were estimated from semilog plots (shown in insets). These values were 1, 1, and 3 for (A), (B), and (C), respectively. Total volume was 1 mL, and the temperature was 24 °C.

Table II: Reaction of TNB–Malic Enzyme with DTT and KCN

time (min)	% initial activity[a] +DTNB	+DTNB
0	100	100
20	0	0
	% initial activity[a]	
time (min)	+DTT	+KCN
5[b]	52	41
10	71	41
	% initial activity[a]	
time (min)		+DTT
15	72	75
20	76	70

[a] All reactions were carried out in a 1 mL volume at pH 7, 26 °C, in 50 mM phosphate and 2% glycerol with 0.2 mM DTNB. The concentration of DTT, when used, was 10 mM, KCN was 40 mM, and 10 μg of malic enzyme was used per experiment. [b] Indicates 5 min after the addition of DTT or KCN; the total reaction time is 25 min.

In order to correlate the number of sulfhydryl groups modified with the loss of catalytic activity, the derivatization process was monitored spectrophotometrically by observing the appearance of thionitrobenzoic acid from DTNB (see Experimental Procedures). Figure 1 shows that, with DTNB as the reactant, about 0.5 SH group was derivatized concomitant with almost total loss of activity. A better estimate of the total number of thiols modified is 1 as discussed in the legend to Figure 1. In addition, increasing the pH to 8 did not result in an increased number of thiols titrated concomitant with activity loss (data not shown).

The results of Tang & Hsu (1974) indicated that the reaction of a sulfhydryl group in the pigeon liver malic enzyme with DTNB resulted in a loss of catalytic activity, but the replacement of the TNB moiety with a cyanonitrile group caused a regain in activity. Thus, the sulfhydryl modified was not essential for activity. A similar experiment was carried out with the ascarid malic enzyme, and the results are depicted in Table II. Reaction of the TNB–malic enzyme with 40 mM KCN resulted in a derivatized enzyme with activity restored to about 40% of control while DTT treatment for the same amount of time gave 70% reactivation. The rapid leveling off in reactivation of TNB–enzyme probably indicates that either the reaction had reached equilibrium or the cyanoylated enzyme is only partially active. When the reaction of TNB–enzyme was carried out in the presence of $K^{14}CN$, radioactivity analysis revealed that 1.46 ^{14}CN groups were incorporated per

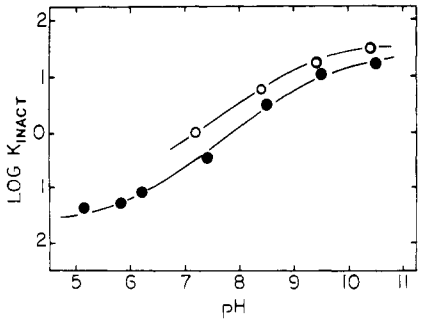

FIGURE 2: pH dependence of the first-order rate of inactivation of malic enzyme by 5,5′-dithiobis(2-nitrobenzoate). (A) Malic enzyme alone (○); malic enzyme plus NAD_f ($20K_{NAD}$) and Mg_f^{2+} ($20\ K_{i\,Mg^{2+}}$) (●). The solid line is from a fit to eq 1. The concentration of 5,5′-dithiobis(2-nitrobenzoate) ranged from 1 mM at pH 5 to 20 μM at pH 9.5. Buffers at 100 mM concentration were the following: 5–6.5, Mes; 7.5, Hepes; 8.5–9.5, Ches; 10.5, Caps.

subunit. Thus, the TNB–CN exchange did occur, and when DTNB was used to inactivate, TNB was probably producing its effect by steric hindrance.

Since the derivatization of about one SH group per subunit was sufficient to completely inactivate the enzyme, it was important to determine if this group was located in the active site. Therefore, derivatization with DTNB was carried out in the presence of substrates. The nucleotide substrate NAD at 2 mM ($25K_{i\,NAD}$) gave 25% protection. In contrast, Mn^{2+} stimulated the inactivation by DTNB by 2-fold. The presence of Mn^{2+}, however, did not increase the number of thiols modified for total activity loss. Essentially identical results could be obtained with Mg^{2+} (data not shown). On the other hand, Mn^{2+} (or Mg^{2+}) plus malate gave protection against DTNB inactivation. When metal plus malate was present, three thiols per subunit were modified concomitant with total loss of activity (Figure 1C). In addition, in the presence of metal, competitive inhibitors of malate such as oxalacetate, mesotartrate, and tartronate (Landsperger et al., 1978) also provided protection. Substrates of the reverse reaction were also tested. Neither NADH at 0.7 mM nor CO_2 at 100 mM (added as $NaHCO_3$) gave protection, but metal plus pyruvate did offer protection against derivatization (data not shown).

pH Dependence of the Thiol Inactivation Rate. The increase in the rate of inactivation effected by metal ion could be the result of a perturbation of the p*K* for the thiol monitored to a lower pH. To ascertain whether this was the case, the first-order rate of inactivation of malic enzyme by DTNB was determined from pH 4.8 to 10.5. These data are shown in Figure 2 in the presence and absence of NAD and Mg_f^{2+}. The inactivation rate decreases below a p*K* of 9.2 ± 0.1 but goes to a constant value below pH 5. The apparent p*K* for the leveling off of this rate is 5.5 ± 0.1. When the pH dependence of the DTNB inactivation rate is obtained in the presence of saturating NAD_f and Mg_f^{2+}, the entire curve is shifted up by about 0.3 log unit (2-fold), with no change in the p*K*. The same experiments were repeated with 4-PDS and iodoacetate (data not shown). The only difference between these data and those discussed above is the absolute value of the inactivation rate obtained with the three agents.

Dissociation Constant for Enzyme–Reactant Complexes from Modulation of the Rate of Inactivation by DTNB. The effect of reactants on the rate of inactivation by DTNB can be used to determine dissociation constants for enzyme–reactant complexes. However, it is important that the rate of inactivation be pseudo first order so that it reflects the concentration of free enzyme. This is only a problem if there is a complex formed between the reagent and enzyme prior

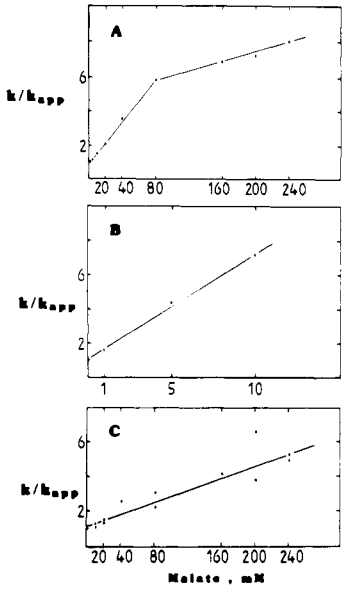

FIGURE 3: Plot of k/k_{app} vs. reactant concentration. All experiments were carried out at pH 7.3, 100 mM Hepes, and 25 °C. The k is the first-order inactivation rate at zero reactant while k_{app} is the first-order inactivation rate obtained at the reactant concentrations indicated. Curves are the theoretical fit by linear regression, while points are experimental values. (A) Dependence of the inactivation rate on malate alone. (B) Dependence of the inactivation rate on malate in the presence of saturating Mg^{2+}. Malate concentratons are the final concentrations in the incubation mixture corrected for the amount of the Mg–malate chelate complex formed by using a dissociation constant of 25.1 mM. The final concentration of uncomplexed Mg^{2+} in the incubation mixture is 100 mM. (C) Dependence of the inactivation rate on malate in the presence of saturating NAD (2 mM).

to modification. Over the range 10–200 μM, reaction of enzyme and DTNB is a second-order process; that is, the E–DTNB complex is not formed and reaction occurs on collision. The second-order rate constant in this case is 47 ± 4 $M^{-1}\ s^{-1}$. For all subsequent experiments reported at pH 7.3, 100 μM DTNB was used.

If different concentrations of malate are included in the preincubation mixture with enzyme and DTNB, the rate of inactivation is decreased as a result of protection by malate. The percent protection by malate as discussed under Theory is a reflection of percent malate bound to enzyme. A series of experiments were carried out with DTNB fixed at 100 μM and malate varied as indicated. A plot of k/k_{app} vs. malate concentration is shown in Figure 3A. As can be seen, although protection is apparently complete, the final plot is biphasic, which may indicate negative cooperativity in the binding of malate to free enzyme. Dissociation constants of 20 mM and 170 mM are estimated from the linear regions of the graph.

The presence of Mg_f^{2+} profoundly effects the binding of malate to enzyme. As shown in Figure 3B, much less $malate_f$ is required for complete protection with saturating Mg_f^{2+}, and a dissociation constant of 1.6 mM is obtained. In the presence of saturating NAD, however, the dissociation constant for malate from E–NAD–malate is very similar to that obtained for the high-affinity site in E–malate (35 mM) (Figure 3C). The only difference is that there is no longer any negative cooperativity apparent.

When the rate of inactivation was determined as a function of Mg^{2+} concentration, a maximal increase of 2–2.5-fold was obtained. However, accurate values of the K_i for Mg^{2+} were difficult to determine. At high Mg^{2+} concentrations, a decrease in the maximum stimulation of the inactivation rate was observed. Thus, only a range of 15–40 mM was obtained

Table III: Dissociation Constants for Enzyme–Reactant Complexes

enzyme form[a]	K_I	pH 7.3 value[b]	pH 9.3 value[b]
E–A–B	K_{IB}	29.3 ± 1.3[c]	10.1 ± 0.6[c]
E–C	K_{ic}	20 ± 2 (high affinity) 177 ± 41 (low affinity)	
E–B–C	K_{IC}	1.6 ± 0.3	
E–A–C	K'_{IC}	35 ± 8	

[a] A = NAD, B = Mg, and C = malate. [b] All values are in millimolar ± SE. [c] From initial velocity studies with saturating NAD_f (20 kM) and $malate_f$ maintained at $^1/_{10}K_{malate}$, varying Mg_f from 1 to 10 mM at pH 9.3 and 10 to 100 mM at pH 7.3.

for the Mg^{2+} K_i. The anomalous behavior at high Mg^{2+} concentrations may be a result of formation of a Mg–DTNB chelate complex which does not react as readily as uncomplexed DTNB. The binding of Mg^{2+} to E–NAD was qualitatively identical with that obtained for free enzyme.

Unlike experiments with malate, NAD provided only partial protection (25%). In addition, the change in the rate with NAD concentration is not hyperbolic, but rather sigmoid. An $S_{0.5}$ of ca. 21 μM is obtained from these data. The partial protection by NAD_f in the presence of saturating Mg_f^{2+} was similar to that obtained for free enzyme (35%).

The dissociation constant of Mg_f^{2+} from E–NAD–Mg was determined kinetically from initial velocity studies by fixing NAD_f at saturating concentration and $malate_f$ at $^1/_{10}$ its K_m value and varying Mg_f^{2+}. In this case, K_{Mg} is equal to $K_{i\,Mg}$. At pH 7.3, the $K_{i\,Mg}$ is 30 mM, while at pH 9.3, the $K_{i\,Mg}$ is 10 mM. Data are included in Table III.

Discussion

Treatment of NAD–malic enzyme from *Ascaris suum* with a variety of sulfhydryl reagents resulted in the complete loss of forward and reverse malic enzyme activities. Complete reactivation of enzyme (previously inactivated by either DTNB or 4-PDS) occurred upon treatment with dithiothreitol. Following DTNB inactivation, exchange of the TNB moiety on enzyme with cyanide produced an enzyme which regained activity. Thus, the thiol group modified is not essential for activity, and replacement of the bulky thionitrobenzoate moiety with the much smaller cyanide group probably relieves a steric effect of the thionitrobenzoate. Therefore, it appears that a sulfhydryl group is not essential for the malic enzyme activity in the *Ascaris suum* enzyme.

The results suggest that DTNB is probably directed toward the thiol and that when half the sites are modified, there is an apparent rearrangement in structure which practically eliminates the activity at the remaining sites. However, total loss of activity required the derivatization of one group per subunit. Half-of-the-site reactivity has been suggested for the pigeon liver malic enzyme cysteinyl residue which reacts with bromopyruvate; this residue is also nonessential (Hsu, 1982).

The presence of divalent metal ion increased the rate at which the thiol reacted with DTNB. It is possible that such a stimulation could be elicited by conformational changes or by metal binding near or to the thiol group, thereby perturbing the pK value of the thiol to a lower value and facilitating its titration. The reduced sulfhydryl which is sensitive to DTNB treatment in *Ascaris suum* malic enzyme has a pK of 9.2 on free enzyme. When NAD and Mg^{2+} are added at a saturating concentration, the pK of this group is not perturbed. Thus, the metal probably increases the inactivation rate by inducing a conformational change.

In addition to the thiol pK of 9 for E and E–NAD–Mg^{2+}, however, another pH dependence is observed which causes the inactivation rate to level off with an apparent pK of 5.5. A pK of 5.5 is also observed for V/K_{malate} vs. pH (data not shown). If the group responsible for this pK is in close proximity to the sulfhydryl group, it could result in a perturbation of the sulfhydryl pK. As the group with a pK of 5.5 becomes protonated as the pH is decreased, it will lose its perturbing effect, and this would result in a constant ratio of S^-/SH over the pH range required to titrate this group. The pK for the group close to the sulfhydryl is not affected by binding of either NAD or Mg^{2+} since it is observed in E and E–NAD–Mg. An alternate possibility is that protonated thiol has a finite reactivity.

The addition of metal plus malate to the DTNB inactivation mixture resulted in an increase in the number of thiol groups modified for complete inactivation. However, metal plus malate also protected against inactivation. Thus, because of the decrease in the rate of inactivation, more of the slower titrating thiol groups were probably derivatized before enzyme was completely inactivated. Oxalacetate, mesotartrate, pyruvate, and tartronate also protected the enzyme from inactivation in the presence of metal but were somewhat less effective than malate.

Other malic enzymes have been studied with respect to their sensitivity to sulfhydryl reagents. These include enzymes from porcine heart mitochondria (NADP) (Lapis & Harrison, 1978), *Escherichia coli* W (NAD) (Yamaguchi, 1979), *E. coli* W. (NADP) (Iwakura et al., 1979), and pigeon liver (NADP) [reviewed in Hsu (1982)]. Of these the best characterized are from *E. coli* and pigeon liver. Complete loss of both malate oxidative decarboxylation and pyruvate reductive carboxylation is obtained upon modification of all four of the above enzymes with a variety of thiol reagents including DTNB and NEM.

For all of the malic enzymes studied so far, including the ascarid enzyme, preparation of the S-cyanoylated enzyme by exchange of the thionitrobenzoate moiety with cyanide restores the malic enzyme activity. Thus, a reduced thiol is most likely located in or near the malate binding site and although it is sensitive to thiol reagents is not essential for activity.

Dissociation Constants for Enzyme–Reactant Complexes. The inactivation of malic enzyme by DTNB is a purely second-order process. The protection obtained with NAD and malate, as well as the stimulation in the inactivation rate obtained with Mg^{2+}, has been utilized to estimate the dissociation constants for a number of enzyme–reactant complexes. The mechanism of NAD–malic enzyme as suggested in the preceding paper (Park et al., 1984) is depicted in Figure 4. Generally, since each of the reaction components affect the inactivation rate, the three binary complexes must form. In addition, NAD eliminates the biphasicity in the k/k_{app} vs. malate curve, indicative that the E–NAD–malate complex forms. The binding of $malate_f$ is enhanced by the presence of Mg_f^{2+} in agreement with formation of E–Mg–malate. Kinetic data presented in the preceding report indicates that both E–malate and E–NAD–malate are dead-end complexes. Thus, data obtained in this study support the kinetic mechanism proposed by Park et al. (1984) and shown in Figure 4.

It is interesting to note that the presence of NAD eliminates the cooperativity in malate binding. Unfortunately, the complexity of NAD binding makes it difficult to assess. In addition, the small change (25–35%) in the inactivation rate makes it difficult to determine whether the data actually reflect NAD binding.[3]

[3] Studies from this laboratory indicate that NAD enhances the intrinsic tryptophan fluorescence of NAD–malic enzyme. A NAD titration making use of this change also suggests positive cooperativity in NAD binding. This aspect is currently being pursued.

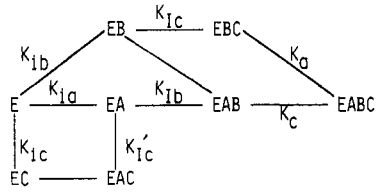

FIGURE 4: Equilibria allowed for the malic enzyme reaction. A, B, and C represent NAD, Mg^{2+}, and malate, respectively. Dissociation constants are K_i for binary complexes and K_I for ternary complexes, with the latter specifying the reactant; K_a and K_c are Michaelis constants.

The dissociation constants for Mg^{2+} from E–Mg could not be obtained with any degree of certainty from enhancement of the thiol inactivation rate. As discussed under Results, this uncertainty may be a result of formation of Mg–DTNB as Mg^{2+} was increased. However, values for the dissociation constant for Mg^{2+} from E–NAD–Mg were obtained kinetically and agree with the range of values obtained from thiol inactivation data. These data suggest that NAD has very little effect on the binding of Mg^{2+}. In addition, there is very little change in the dissociation constant over the 2 pH unit range studied. In fact, if the process is all or none, that is, if Mg^{2+} binds only to unprotonated enzyme, the upper limit of the pK for the group that binds Mg^{2+} would apear to be around 7.

Conclusions. A thiol group that appears to be in or near the malate binding site is sensitive to modification by DTNB and other thiol regents but is not absolutely required for activity as evidenced by regain of activity when the bulky thionitrobenzoyl moiety is exchanged for the much smaller cyanide. The number of thiol groups modified concomitant with activity loss depends somewhat on the nature of the reagent used, but the majority of activity is lost upon modification of a single thiol. Initial velocity studies in the absence of inhibitors (Park et al., 1984) suggest that the kinetic mechanism for NAD–malic enzyme from *Ascaris suum* is random. In support of this mechanism, modulation of the inactivition rate that results from modification of a nonessential thiol group has demonstrated that all possible enzyme complexes form. The kinetic mechanism changes from an ordered sequence at low Mg_f^{2+} concentrations in which the order is NAD_f, Mg_f^{2+}, and $malate_f$ to a random mechanism at high Mg_f^{2+} concentratons in which E–malate and E–NAD–malate are most likely dead end.

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Registry No. NEM, 128-53-0; DTNB, 69-78-3; 4-PDS, 2645-22-9; NAD, 53-84-9; Mg, 7439-95-4; malic acid, 97-67-6; NAD–malic enzyme, 9028-46-0.

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