

Pre-Steady-State Kinetics Reveal a Slow Isomerization of the Enzyme–NAD Complex in the NAD–Malic Enzyme Reaction[†]

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ABSTRACT: Stopped-flow experiments obtained in the pre-steady-state time scale of the NAD–malic enzyme reaction exhibit a lag prior to the attainment of steady state. Previous results from isotope effect studies in which the deuterium isotope effect on $V_{\max}$ decreases to a value of 1 at low pH have been interpreted as suggesting a slow release of NADH [Kiick, D. M., Harris, B. G., & Cook, P. F. (1986) *Biochemistry* 25, 227–236]. The latter, however, requires a burst in the pre-steady-state time course, and thus the previous data have been reinterpreted in view of the observed lag. Preincubation with NAD and/or Mg increases the lag rate, with the latter having the greater effect, while preincubation with Mg and malate (or a malate analog) eliminates the lag. Data suggest a slow isomerization of E:NAD that is increased by addition of malate prior to NAD in the presence of Mg. The lag is also eliminated at low pH as a result of the overall rate being limited by the isomerization; that is, the isomerization is pH-dependent. Fumarate, an activator of the NAD–malic enzyme, when preincubated with enzyme also eliminates the lag, suggesting that the activator preferentially binds the isomerized form of the enzyme or increases the isomerization rate, or both. Stopped-flow data are corroborated by circular dichroism experiments. The unliganded enzyme is approximately 50% α -helix on the basis of secondary structural analysis. Binding of NAD and Mg exhibits a substantial change, with a further change observed upon binding the malate analog tartronate.

The mitochondrial malic enzyme (EC 1.1.1.39) from *Ascaris suum* catalyzes the divalent metal-dependent oxidative decarboxylation of L-malate using NAD as an electron acceptor to produce pyruvate and carbon dioxide. A steady-state random kinetic mechanism has been documented for the malic enzyme in the direction of oxidative decarboxylation in which all three binary (including metal as a pseudoreactant) and all three ternary complexes are allowed and metal must bind prior to malate for formation of a productive E:NAD:Mg:malate complex (Park et al., 1984). This mechanism has been confirmed using substrate protection against inactivation of the enzyme by 5,5'-dithiobis(2-nitrobenzoate) to detect the formation of enzyme–substrate complexes and to determine their dissociation constants (Kiick et al., 1984). The mechanism is also corroborated by the observation of finite and equal deuterium isotope effects on V , V/K_{malate} , and V/K_{NAD} (Kiick et al., 1986) at pH 7, and by isotope partitioning of E:Mg:[¹⁴C]malate and E:[¹⁴C]NAD complexes (Chen et al., 1988). In the direction of reductive carboxylation of pyruvate, there are similarities and differences to that in the opposite reaction direction. Although the mechanism is still steady-state-random (Mallick et al., 1991), there is no evidence for an E:CO₂ complex, suggesting direct attack of enolpyruvate on CO₂ upon collision. However, there is a requirement for the metal bound prior to pyruvate to obtain productive complex formation.

Equal values for deuterium isotope effects on V and the substrate V/K 's at pH 7 were interpreted as indicative of identical rates for release of NAD and malate from E:NAD:Mg:malate and NADH from E:NADH. Further, the release of NADH from the E:NADH complex was suggested to be pH-dependent and solely rate-limiting for the overall reaction at pH values below 5. Substrate activation by malate induced in the presence of the competitive inhibitor oxalate has been observed for the NAD–malic enzyme (Park et al., 1989). These authors further suggested a preference in the binding of malate and oxalate to E:NADH unprotonated and protonated, respectively, at an enzymic general base with a pK of 5. If NADH is released slowly from its binary E:NADH complex, a burst should be observed in the pre-steady-state time course of the oxidative decarboxylation reaction catalyzed by malic enzyme. In addition, although the burst amplitude should represent a fraction of the total enzyme sites at pH 7, it should be stoichiometric to enzyme sites at pH values below 5.

In the present study, stopped-flow kinetic studies have been carried out to obtain information concerning the mechanism of the NAD–malic enzyme in the direction of oxidative decarboxylation of malate. Results suggest a slow isomerization of the E:NAD complex, and not the E:NADH complex. Circular dichroism spectrophotometry confirms a structural rearrangement upon binding of NAD to enzyme.

MATERIALS AND METHODS

Enzyme and Chemicals. Mitochondrial NAD–malic enzyme from *Ascaris suum* was purified according to the procedure of Allen and Harris (1981). Enzyme was >95% pure by the criterion of SDS–polyacrylamide gel electrophoresis (O'Farrell, 1975; Atkins et al., 1975) and had a final specific activity of 20 units/mg ($k_{\text{cat}} = 23 \text{ s}^{-1}$) assayed in the direction of oxidative decarboxylation (Park et al., 1984). Malate dehydrogenase contamination was assayed with 100 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid

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(Hepes),¹ pH 7.5, 0.2 mM NADH, 0.5 mM OAA, 2 mM EDTA, and 1 mM DTT. A contamination of less than 1% of the malic enzyme oxidative decarboxylation activity was found. Purified enzyme was stored at -25 °C in a storage buffer containing 100 mM Hepes, pH 7.5, 10 mM DTT, 1 mM EDTA, and 20% glycerol.

The dinucleotides NAD and NADH were purchased from Boehringer-Mannheim, while L-malate, DTT, fumarate, 1,4-bis-Hep, and Hepes were from Sigma. All other chemicals and reagents were obtained from commercial sources and were of the highest purity available.

Metal Chelate Correction. Concentrations of reactants and inhibitors added to the reaction mixture were corrected for the concentration of metal-ligand chelate complexes according Park et al. (1984). Dissociation constants used in the calculations are as follows: Mg-malate, 25.1 mM; Mg-NAD, 19.5 mM; Mg-fumarate, 350 mM; Mg-oxalate, 1.7 mM (Good et al., 1966; Martell & Smith, 1977).

Enzyme Assays. Malic enzyme was assayed in the direction of oxidative decarboxylation of malate by monitoring the production of NADH at 340 nm ($\epsilon_{340} = 6220 \text{ M}^{-1} \text{ cm}^{-1}$) using a Gilford 250 spectrophotometer, with a Brinkmann Servogor 210 chart recorder equipped with multispeed drive. Reaction cuvettes were 1 cm in path length and 1 mL in volume. All assays were carried out at 25 °C using a circulating water bath to heat and cool the thermospacers of the spectrophotometer cell compartment.

Stopped-Flow Kinetic Experiments. Experiments were performed with a temperature-controlled Hi-Tech SF-51 stopped-flow apparatus equipped with a sample handling unit (SHU-51) and a spectrometer unit (SU-40) and interfaced with a Hewlett-Packard Series 3000 computer. The observation cell of the SHU-51 accommodates a high-efficiency mixer and a 10-mm optical path length. The dead time of the instrument is less than 2 ms. All experiments were carried out with a high concentration of malic enzyme (30 μM) under various premixing conditions at 25 °C, and pH values of 6.9 (100 mM Hepes) and 4.5 (100 mM bis-Hep). Negligible pH changes were observed before and after the reaction. The final concentrations of reactants used were 13.5 mM NAD (2 mM when corrected for Mg-NAD), 50 mM L-malate (27.2 mM when corrected for Mg-malate), and 47 mM MgSO_4 (30.2 mM corrected for Mg-NAD and Mg-malate), and these are considered to be high enough to represent near-saturating conditions. The progress of the reaction was followed by monitoring the NADH absorbance at 340 nm.

Circular Dichroic Spectral Studies. Circular dichroism spectra were recorded on an AVIV Model 62 HDS spectropolarimeter equipped with an RC 6 Lauda refrigerated circulating bath to maintain constant temperature. The enzyme (80 $\mu\text{g}/\text{mL}$) in 20 mM KH_2PO_4 , pH 7.0, was placed in 0.2-cm quartz cuvettes, and spectra were recorded in the far-UV range (250–190 nm), at intervals of 1 nm and a dwell time of 3 s. Each spectrum was the average of two repetitions, and appropriate buffer blanks were determined for each spectrum and subtracted to obtain corrected spectra. In all cases, the dynode voltage over the entire wavelength range sampled did not exceed 500 V. Each experiment was repeated several times (and on different days) with identical results. Ellipticity values recorded by the instrument in millidegrees (obs) were converted to molar ellipticity values according to eq 1 where $[\theta]$ is the molar ellipticity in degrees centimeter

$$[\theta] = [\theta]_{\text{obs}}/10(\text{MRC})l \quad (1)$$

squared per decimole, $[\theta]_{\text{obs}}$ is the ellipticity recorded by the instrument in millidegrees, MRC is the mean residue concentration of the enzyme and is equal to the number of amino acid residues times the concentration of the protein in decimoles per milliliter, and l is the path length in centimeters. Molar ellipticity values were used to obtain an estimation of the secondary structure using the computer program PROSEC (PROtein SECondary structure) supplied by AVIV. The program is based on a binary search algorithm, uses the reference spectra of Chang et al. (1978), and estimates α -helix, β -sheet, β -turn, and coil directly from the instrument data. Values for the secondary structural components obtained from PROSEC were verified using the second program BLEND, which combines pure secondary structural components to produce a synthetic spectrum using multiple regression analysis and the same standard reference spectra used in PROSEC. Spectra thus generated were superimposable with the experimentally recorded spectra in all cases.

Data Processing. The time courses for the rapid kinetic experiments were fitted using the following rate equations and software supplied by Hi-Tech Ltd.:

$$y = 1 + [1/(k_1 - k_2)][k_2 e^{-k_1 t} - k_1 e^{-k_2 t}] \quad (2)$$

$$y = ax + b \quad (3)$$

In these equations, k_1 and k_2 are first-order rate constants, t is time, and e is 2.718. Equation 2 describes a sequential process ($X \rightarrow Y \rightarrow Z$), monitoring Y and Z, respectively. Fits to the data using an equation that describes two parallel first-order reactions were poor as judged by the residuals and a comparison of the theoretical time course derived from the fitted estimates and the experimental time course. Rate constants for stopped-flow experiments are the average of 5–10 runs in all cases.

RESULTS

Stopped-Flow Studies. Kinetic events in the pre-steady-state time range were studied by directly monitoring the spectral changes associated with the production of NADH upon mixing substrates and enzyme. Experiments were carried out with high concentrations of malic enzyme (30 μM), and different preincubation conditions were employed as discussed below.

Figure 1A shows the time course for rapid mixing of enzyme (60 μM) in 100 mM Hepes, pH 6.9, with a solution containing NAD (27 mM), Mg^{2+} (94 mM), and 100 mM malate in the same buffer. A prominent lag is observed at the beginning of the progress curve. The lag rate calculated under these conditions is the lowest observed with a value of 43 s^{-1} . The calculated steady-state rate is 16 s^{-1} and serves as an internal control for all experiments.² Time courses appeared to curve off rapidly, but this was an artifact due to a deviation from Beer's law for this instrument. The latter was corroborated using 1 mM NADH solution which gave an absorbance of 1.8. Final concentrations of reactant and enzyme are half of those given above and are identical for all experiments carried out.

When enzyme is preincubated with NAD and Mg^{2+} at pH 6.9 and rapidly mixed with L-malate, a distinctive lag phase is again observed prior to achieving the steady-state rate (data not shown). The estimated rate constants for the lag phase

¹ Abbreviations: Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; bis-Hep, *N,N'*-bis(2-hydroxyethyl)piperazine; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid.

² The value is about 70% of the actual k_{cat} of 23 s^{-1} obtained from steady-state measurements. The difference is likely due to the difference in instruments used for the two measurements.

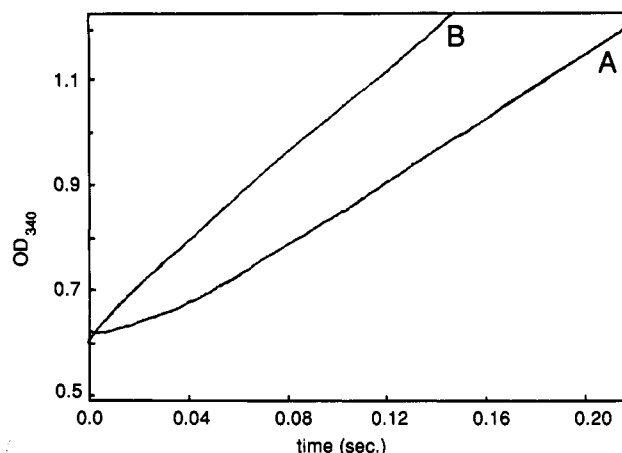


FIGURE 1: (A) Time course for the reaction of malic enzyme with NAD, Mg^{2+} , and malate, when monitored at 340 nm. The final concentrations after dilution are as follows: enzyme, 30 μM ; NAD, 13.5 mM (2 mM corrected for Mg-NAD); Mg^{2+} , 47 mM (27.2 mM corrected for Mg-NAD and Mg-malate); malate, 50 mM (30.2 mM corrected for Mg-malate). (B) Time course for the reaction of malic enzyme preincubated with malate and Mg^{2+} and mixed with NAD. The final concentrations are identical to those given above. Solid curves are drawn through the experimental points generated from the fitted kinetic constants.

Table I: Summary of Rate Constants for the Pre-Steady Lag^a

preincubation	eq	pH	k_1 (s^{-1})	k_{cat} (s^{-1})
none	2	6.93	43	16
NAD ⁺	2	7.0	57	15
Mg^{2+}	2	7.0	160	13
NAD ⁺ , Mg^{2+}	2	6.91, 4.5	65, no lag	16, 4.4
malate, Mg^{2+}	3	6.93	no lag	21
NAD ⁺ , Mg^{2+} , oxalate (0.6 mM)	2	6.88	88.9	16
NAD ⁺ , Mg^{2+} , oxalate (6 mM)	2	6.89	99	15
Mg^{2+} , oxalate (102 mM)	3	7.0	no lag	4
Mg^{2+} , fumarate (10.8 mM)	2	7.0	no lag	14

^a Concentrations of NAD⁺, Mg^{2+} , and malate are 27, 94, and 100 mM, respectively. Data were obtained in 100 mM Hepes, pH 6.9 and 25 °C. Errors on the estimated rate constants are about 5%. ^b Data obtained with L-malate-2-d at pH 6.89 give $k_1 = 69 s^{-1}$ and $k_{cat} = 8 s^{-1}$. Data obtained with L-malate-2-d at pH 4.5 give no lag and $k_{cat} = 4.5 s^{-1}$.

and the apparent steady-state rate are 65 and 16 s^{-1} , respectively. Compared to enzyme alone, a small increase in the lag rate obtained with enzyme alone is observed when enzyme is preincubated with NAD and mixed with Mg^{2+} and malate, while preincubation with Mg^{2+} shows a marked increase in the lag rate. When NAD is mixed with enzyme preincubated with Mg^{2+} and malate, a gradual increase in absorbance is observed, but no lag phase is discernible (Figure 1B). Values of the rate constants estimated from all experiments are summarized in Table I.

Effect of pH on the Transient Phase. When a solution of enzyme (60 μM), NAD (27 mM), and Mg^{2+} (240 mM) in 100 mM bis-Hep buffer at pH 4.5 is mixed with 244 mM malate in the same buffer, no lag phase is observed. The apparent steady-state rate decreases to 4.4 s^{-1} at pH 4.5 compared to a value of 16 s^{-1} at pH 6.9. Data are summarized in Table I.

Isotope Effects. Deuteration of L-malate at C-2 gives no detectable change in the lag rate at pH 6.9 when enzyme is added to a solution containing NAD, Mg^{2+} , and L-malate-2-d. Thus, the rate process that is responsible for the lag in the pre-steady-state is insensitive to isotopic substitution at the position in malate that generates a primary deuterium isotope effect. The latter is not surprising since the hydride-

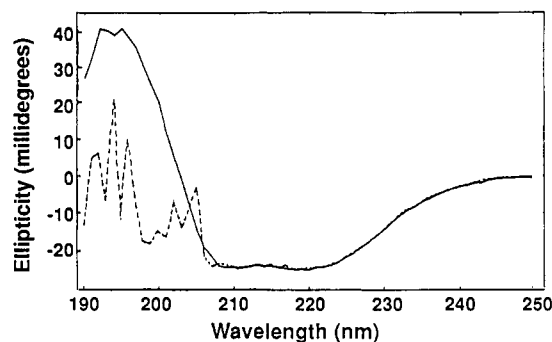


FIGURE 2: Far-UV circular dichroic spectra of *Ascaris suum* NAD-malic enzyme in phosphate and Hepes buffers. Spectra were recorded with 80 $\mu g/mL$ enzyme in 20 mM KH_2PO_4 , pH 7 (—), and in 20 mM Hepes, pH 7 (---). Spectra shown are corrected by subtracting appropriate blanks but are not subjected to polynomial smoothing.

transfer step is the one in which NADH is produced and the step responsible for the lag must occur prior to NADH formation. The k_{cat} value, however, does exhibit a primary deuterium isotope effect of about 2, in reasonable agreement with the effect of about 1.9 measured on the steady-state V/E_t value (Karsten et al., 1992). A repeat of the above isotope effect experiment at pH 4.5 gives no lag, or significant isotope effect (Table I).

Effect of Oxalate. Studies by Kiick et al. (1986) showed that inhibition by oxalate is competitive vs malate at pH 7 and above, but noncompetitive vs malate at pH values below 7. The development of an intercept effect at low pH was attributed to an increase in the steady-state level of E:NADH as a result of the protonation of an enzyme group with a pK of 5. Mixing enzyme preincubated with NAD, Mg^{2+} , and a nonsaturating concentration of oxalate (0.6 mM, 0.02 mM when corrected for Mg-oxalate) at pH 6.9 gives a lag in the time course. The lag rate is increased slightly to 89 s^{-1} , compared to the value of 43 s^{-1} obtained for the reaction without oxalate (Table I). Increasing the concentration of oxalate to 6 mM (0.18 mM corrected for Mg-oxalate) decreases the lag phase, giving a lag rate of 99 s^{-1} , while no lag is observed when the enzyme is preincubated with a saturating concentration of oxalate (102 mM, 3 mM when corrected for Mg-oxalate). The apparent steady-state rate decreases to 4 s^{-1} in the latter case. Data are summarized in Table I.

Effect of Fumarate. It has been reported that fumarate is a positive heterotropic effector of the NAD-malic enzyme from *Ascaris* at low concentrations ($K_{act} = 0.05$ mM) and a competitive inhibitor vs malate at high concentrations ($K_i = 25$ mM) (Lai et al., 1992). When enzyme is preincubated with 10.8 mM fumarate (5 mM when corrected for Mg-fumarate) and Mg^{2+} and then rapidly mixed with NAD and malate, no lag is observed.

Circular Dichroic Spectral Studies. A sensitive physical method for monitoring changes in the secondary structure of proteins is circular dichroism spectroscopy at wavelengths near the absorption band for the amide bond. Thus, far-UV (190–250 nm) circular dichroic spectra of the NAD-malic enzyme in the absence and presence of bound substrate(s) were obtained. The CD spectrum of the native enzyme was recorded in 20 mM KH_2PO_4 , pH 7 (Figure 2). Because of the high dynode voltage at wavelengths below 210 nm, Hepes buffer was not used for CD studies. As shown, the spectrum shows strong negative ellipticity at 208 and 220 nm, indicating a high α -helical content.

The secondary structural composition of the NAD-malic enzyme estimated by the PROSEC program is as follows:

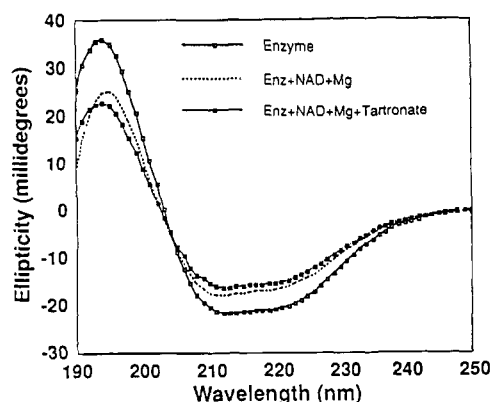


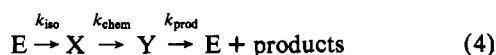
FIGURE 3: CD spectra of the *Ascaris suum* malic enzyme with NAD, Mg^{2+} , and tartronate. Spectra were recorded with 80 μ g/mL enzyme in KH_2PO_4 , pH 7 at 25 °C. Spectra shown are corrected and subjected to polynomial smoothing. Enzyme alone ($\square$ — $\square$); enzyme + 100 μ M NAD + 1 mM Mg^{2+} (---); enzyme + 100 μ M NAD + 1 mM Mg^{2+} + 1 mM tartronate ($\blacksquare$ — $\blacksquare$).

α -helix, 49%; β -sheet and -turn, 31%; coil, 20%. All of the spectra discussed were repeated several times with identical results, and thus a qualitative interpretation is warranted. The binding of NAD and Mg results in a significant structural change in the enzyme (Figure 3). Since it is not possible to obtain the CD spectrum of the enzyme in the presence of all three substrates, the dead-end malate analog tartronate was used in place of malate. Structural changes observed upon addition of malate or tartronate alone to enzyme are similar (data not shown). As shown in Figure 3, a further change in the CD spectrum of the enzyme is observed when tartronate binds to the E:NAD:Mg complex.

DISCUSSION

The lag in the NAD–malic enzyme time course indicates that a slow step occurs prior to the formation of the chromophoric substrate monitored, that is, NADH. The aforementioned slow step is observed under conditions of saturating reactant concentrations and thus must reflect isomerization of either the unliganded enzyme or the E:NAD binary complex. An isomerization of the free enzyme would be observed in initial velocity studies, specifically in the product inhibition patterns, since this conditions defines an isomechanism. However, extensive product and dead-end inhibition studies of the NAD–malic enzyme are consistent with a random kinetic mechanism with no evidence for an isomechanism (Park et al., 1984; Kiick et al., 1984). Thus, the present studies suggest a slow isomerization of the E:NAD binary complex.

As discussed above, pre-steady-state kinetic data suggest a slow isomerization of E:NAD. In addition, steady-state kinetic isotope effects indicate that the oxidative decarboxylation of malate represents the slowest step along the reaction pathway (Weiss et al., 1991; Gavva et al., 1991). As a result, the pre-steady-state data can be interpreted on the basis of the following simplified kinetic scheme:



In eq 4, X represents the productive Michaelis complex E^* :NAD:Mg:malate formed after isomerization of E which represents the E:NAD complex and rapid addition of Mg^{2+} and malate present at saturating concentrations. The second term in eq 4, Y, represents the product complex E:NADH:Mg:pyruvate. The other product, CO_2 , is not present since initial velocity data obtained in the direction of pyruvate

reductive carboxylation suggest that CO_2 has no binding site and reacts upon collision with enolpyruvate (Mallick et al., 1991). The first-order rate constants k_{iso} , k_{chem} , and k_{prod} reflect the isomerization of the E:NAD complex, oxidative decarboxylation of malate, and product dissociation, respectively. The last of these rate processes is not observed directly since it is rapid with respect to the other two (Kiick et al., 1986).

The observed rate constant for the isomerization of E:NAD changes, depending on the conditions of preincubation. The rate of the isomerization under steady-state conditions, with NAD, Mg, and malate saturating, is best modeled by a stopped-flow experiment in which enzyme is preincubated with saturating NAD, Mg, and a malate analog. In the present case, oxalate, an inhibitor competitive vs malate, was used. Preincubation with NAD and Mg gives a slight increase in the lag rate from 43 to 65 s^{-1} compared to enzyme alone. As oxalate is added to the E:NAD:Mg complex, the lag rate becomes progressively larger with values of 89 s^{-1} at 0.6 mM oxalate (0.02 mM corrected for the Mg–oxalate complex) and 99 s^{-1} at 6 mM oxalate (0.18 mM corrected for the Mg–oxalate complex). The latter concentration of 0.18 mM is equal to the dissociation constant for E:NAD:Mg:oxalate at pH 7 (Kiick et al., 1986), and thus the change in the rate constant observed going from 0 to 0.18 mM oxalate represents half the total change expected when oxalate is saturating (99 s^{-1} minus 65 or 34 s^{-1}). At saturating oxalate, then a value of >99 s^{-1} (99 plus 34 s^{-1} , or 133 s^{-1}) is expected, and this agrees with the value of >70 s^{-1} estimated for the slow step from isotope partitioning studies (Chen et al., 1988).

The lag rate measured when enzyme alone is added to a saturating concentration of reactants is about 43 s^{-1} . These conditions reflect mainly the pathway in which NAD adds prior to malate³ and provide an estimate for the lower limit of the rate constant for isomerization of E:NAD. (It is not the lower limit since both pathways, those with NAD adding prior to malate and the opposite, contribute to the pre-steady-state rate.) Preincubation with NAD gives only a slight increase in the lag rate to 56 s^{-1} . The latter result probably indicates that in the presence of NAD an equilibrium exists between E:NAD and E^* :NAD with the former less active form favored. Addition of Mg to the preincubation mixture increases the lag rate constant to 65 s^{-1} , indicating that Mg likely binds to the more active E^* conformation. Interestingly, the rate constant increases significantly to about 165 s^{-1} when enzyme is preincubated with Mg alone prior to mixing with NAD and malate, in agreement with the suggestion that the binding of Mg stabilizes the more active form of the enzyme. Since the effect observed in the presence of NAD and Mg more nearly parallels that observed with NAD alone, there appears to be a more efficient use of the NAD binding energy in stabilizing the E^* conformation of the enzyme. As suggested above, preincubation of the enzyme with Mg^{2+} , NAD, and oxalate and reacting with malate also generate an increase in the lag rate. Thus, the binding of oxalate is preferentially to the E^* form of the enzyme, specifically to H: E^* :NAD:Mg. (As will be discussed below, oxalate must bind to both H:E:NAD:Mg and H: E^* :NAD:Mg in order to be consistent with the observed pH dependence of oxalate inhibition.)

There are several conditions for which no lag is observed in the pre-steady-state. Preincubation of enzyme with Mg and saturating malate (or malate analogs such as oxalate)

³ This is not completely valid since $[NAD]/K_{i,NAD}$ is 25, while $[malate]/K_{i,malate}$ is 12.

eliminates the lag rate and suggests that (1) the equilibrium between E:Mg:malate and E*:Mg:malate lies far toward the latter as opposed to the equilibrium between E:NAD and E*:NAD which lies toward the former or (2) the rate of interconversion of E:Mg:malate and E*:Mg:malate is more rapid than that for interconversion of E:NAD and E*:NAD, or (3) both. Of the three possibilities, the last is the most likely. As indicated above, a steady-state random kinetic mechanism has been proposed for the NAD-malic enzyme from initial velocity studies (Park et al., 1984; Kiick et al., 1986; Mallick et al., 1991), deuterium isotope effect studies (Kiick et al., 1986), and isotope partitioning studies (Chen et al., 1988). The two pathways for formation of the productive E:NAD:Mg:malate complex do not proceed at the same rate according to this class of kinetic mechanism (Park et al., 1984).

When the enzyme is preincubated with fumarate and Mg^{2+} and mixed with malate and NAD, again no lag phase is observed. Landsperger and Harris (1976) reported the activation of malic enzyme by low concentrations of fumarate and suggested the possibility of regulation by fumarate. Activation of the malic enzyme by fumarate was recently corroborated by Lai et al. (1992), on the basis of initial velocity studies, deuterium isotope effects, and isotope partitioning studies. These authors found that the activation by fumarate results in a decrease in the E:Mg:malate dissociation constant and an increase in V/K_{malate} , while the maximum velocity remains constant. Fumarate was found to have two different effects dependent on its concentration, activation at low concentrations ($K_{act} = 50 \mu M$) and inhibition at high concentrations ($K_i = 25 mM$), suggesting binding of fumarate at an allosteric site to cause activation and at the active site to cause inhibition. The present data suggest that fumarate binds to the allosteric site predominantly in the E*Mg form. The effect is thus the same as that observed for malate or oxalate binding to E*:Mg but results from fumarate binding to an allosteric site. Thus, as proposed for malate, fumarate may increase the rate constant for the isomerization of the E:NAD complex, or its equilibrium constant by preferentially binding to the E* form of the enzyme, or both.

Preincubation of enzyme with NAD and Mg^{2+} and reacting with malate at pH 6.9 and 4.5 give a decrease in the steady-state rate at the lower pH compared to that at pH 6.9. On the basis of the decrease in the steady-state rate, a pK of 4.3 is calculated, in agreement within error with the pK of 4.9 recorded by Kiick et al. (1986). The V_{max} for the malic enzyme reaction is pH-independent at high pH and indicates substrate binding to only the correctly protonated form of the enzyme. The decrease in V and $^D V$ observed at lower pH by Kiick et al. (1986) must then be due to a pH-dependent slow step that is not part of the catalytic pathway. On the basis of pre-steady-state studies presented here, this step is likely the isomerization of E:NAD; that is, protonation of the active-site general base preferentially generates the unisomerized H:E:NAD form of the enzyme. The lack of a lag at lower pH indicates rate limitation by isomerization of E:NAD, consistent with a value of 1 for $^D V$ at pH values below 5.

Circular Dichroic Spectral Studies. To determine whether the isomerization could be visualized, circular dichroism experiments for the native enzyme were performed. The secondary structural composition of the *Ascaris* NAD-malic enzyme estimated by the PROSEC program suggests that about 50% of the enzyme is α -helical with the remainder divided among β -structure and coil. Binding of NAD alone causes only slight, if any, changes in the CD spectrum, while preincubation with both NAD and Mg gives a significant

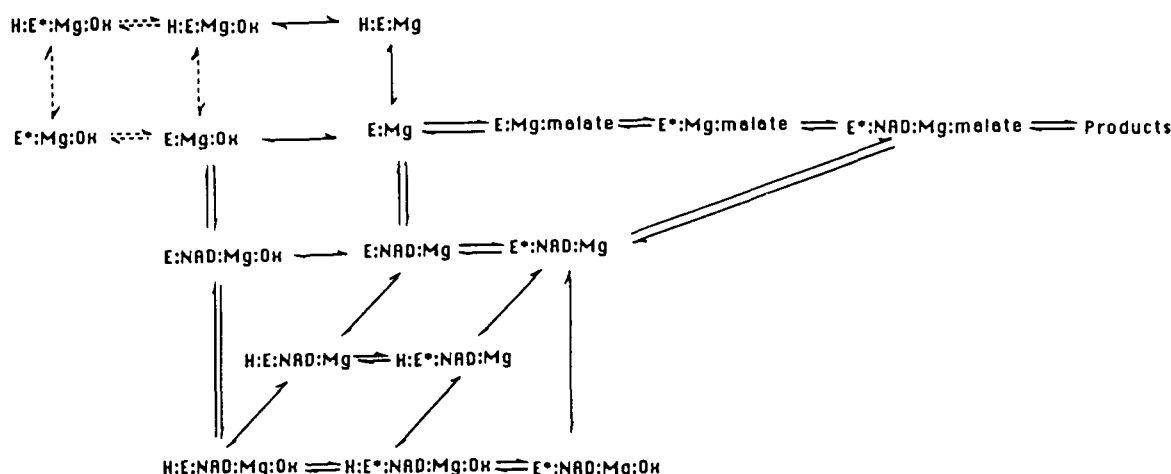
change, in agreement with the rate data (Table I). The binding of malate or the malate analog tartronate alone gives only a slight change in the enzyme structure, and the change is almost identical whether or not NAD is bound (data not shown). A further change in the CD spectrum of the enzyme is then seen when tartronate binds to the E:NAD:Mg complex. When analyzed by the PROSEC program, this spectrum is fitted using 32% α -helix and 68% β -structure and coil. It thus appears that binding of both NAD and malate in the presence of Mg produces changes in enzyme structure that are cumulative. The CD data generally agree with the pre-steady-state rate data presented above in that one of the largest changes estimated in the rate of isomerization of E:NAD is that in the presence of NAD, Mg, and oxalate.

Conformational changes have been observed for a number of dehydrogenases as a result of the binding of NAD (Webb et al., 1973; Eklund et al., 1974). Parker and Holbrook (1977) have suggested that conformational changes in dehydrogenases arise because of a requirement for a nonpolar environment around the active site in order to facilitate hydride transfer from the substrate to C-4 of the nicotinamide ring of NAD.

Kinetic Mechanism of Other Related Enzymes. The pigeon liver NADP-malic enzyme catalyzes the same reactions (Tang & Hsu, 1973) as does the *Ascaris* malic enzyme, but the two enzymes appear to be quite different with respect to their pre-steady-state kinetic behavior. Studies of Reynolds et al. (1978) showed an initial burst of product formation prior to the establishment of the steady-state rate. Their results suggested that hydride transfer is fast ($t_{1/2} < 0.7$ ms) and further suggested that the subsequent slow steady-state rate is due to the slow off-rate for NADPH.

There is evidence to suggest that the pre-steady-state kinetic behavior of the *Ascaris* NAD-malic enzyme is similar to that observed for the isocitrate dehydrogenase (ICDH) from bovine mammary gland tissues. Pre-steady-state studies on ICDH in which enzyme is preincubated with NADP exhibit a distinctive lag, followed by the establishment of a linear steady-state rate (Farrell et al., 1990). Results also showed that the lag disappears when the enzyme is preincubated either with Mn^{2+} and isocitrate or with Mn^{2+} and citrate (a competitive inhibitor; Farrell et al., 1990). On the basis of fluorescence and CD spectral studies of enzyme-ligand complexes, Seery and Farrell (1990) suggested a change in the conformation of the enzyme as a possible mechanism of activation of ICDH by its substrate.

Reinterpretation of Previous Data Obtained for the NAD-Malic Enzyme. The major rate-determining steps along the NAD-malic enzyme reaction pathway are the chemical steps responsible for converting malate and NAD to enolpyruvate and NADH (Weiss et al., 1991). In addition, however, some of the physical steps have been implicated as slow steps. Kiick et al. (1986) showed that oxalate is a competitive inhibitor vs malate at pH values of 6.5 and above, while at low pH the inhibition becomes noncompetitive. These authors also showed via double-inhibition studies that the E:NADH:Mg:oxalate complex could form and that NADH and oxalate bind synergistically in the complex. Deuterium isotope effects on V , V/K_{NAD} , and V/K_{malate} are equal (Kiick et al., 1986), and equal maximal amounts of malate and NAD are trapped in isotope partitioning studies with E:Mg:[^{14}C]malate and E:[^{14}C]NAD (Chen et al., 1988). All of the above suggested an identity in the rate constants for release of NAD and malate from E:NAD:Mg:malate and for release of NADH from the E:NADH complex, and that all of these can contribute to the rate limitation of the reaction (dependent on reactant

Scheme I: Kinetic Mechanism for the *Ascaris suum* NAD–Malic Enzyme in the Direction of Oxidative Decarboxylation of L-Malate^a

^a The scheme is an adaptation of that presented previously (Mallick et al., 1991) based on the present study.

concentration). The noncompetitive inhibition by oxalate is observed as a result of an increase in the concentration of an enzyme form present under $V_{\max}$ conditions (saturating substrate concentrations) as the pH is decreased below 6.5. The pK observed for both slope (interpreted as binding to E:NAD:Mg) and intercept (binding to E:NADH:Mg) inhibition constants for oxalate is 5, and reflects the enzymic general base that accepts a proton from the hydroxyl of malate concomitant with hydride transfer (Kiick et al., 1986). Oxalate binds with higher affinity to H:E:NAD:Mg compared to E:NAD:Mg, and on the basis of the above information, it was suggested that it also bound tighter to H:E:NADH:Mg compared to E:NADH:Mg. The maximum velocity and deuterium isotope effect on V also decrease at low pH, giving a pK of 5 (Kiick et al., 1986). Thus, the release of NADH from E:NADH was thought to be pH-dependent, partially limiting the rate at neutral pH and becoming increasingly slower as the pH is decreased until at pH values below 5 it completely limits the overall rate as a result of forming H:E:NADH. Park et al. (1989) documented substrate activation by malate induced by oxalate in the *Ascaris* NAD–malic enzyme reaction. The activation was suggested to be a result of competition between malate and oxalate for the E:NADH complex at high malate concentration, with malate binding to the form of the complex unprotonated at the enzyme group with a pK of 4.9 and oxalate binding preferentially to the protonated form. Thus, oxalate was thought to decrease the rate of dissociation of E:NADH by shifting the equilibrium toward H:E:NADH as a result of its higher affinity for the latter complex. Since release of NADH from E:NADH was thought to be a slow step even at neutral pH and completely rate-determining at low pH, a pre-steady-state burst of NADH was expected that would be less than stoichiometric at pH 7, but completely stoichiometric at pH 4.5.

As a result of the above reasoning, the observation of a lag instead of a burst in the pre-steady-state time course of the NAD–malic enzyme reaction was surprising. Kiick et al. (1986) suggested a slow release of NADH, reasoning that oxalate will bind to similar E:NAD and E:NADH complexes and based on the observation of double inhibition consistent with an E:NADH:Mg:oxalate complex. The latter, however, is not proof that E:NADH:Mg exists in the steady-state. Another possible complex that might also be expected to be similar to the E:NAD complex with respect to oxalate binding would be an unisomerized form of E:NAD. Thus, the

noncompetitive inhibition by oxalate would result from binding to E:NAD and E*:NAD (where an asterisk represents the active form) with the equilibrium between the two being pH-dependent and in favor of E:NAD at low pH. The induced substrate activation observed by Park et al. (1989) may be explained by the preferential binding of oxalate to H:E:NAD:Mg and the preferential binding of malate to E*:NAD:Mg.

Conclusions. Results from stopped-flow studies suggest a pH-dependent conformational change in the enzyme–dinucleotide complex. It is proposed that during the oxidative decarboxylation of malate by the NAD–malic enzyme the binding of NAD (or NAD plus Mg^{2+}) induces (a) slow conformational change(s) in the enzyme from an inactive to an active catalytic form or that NAD binds with higher affinity to the unisomerized form. Either the latter transition is faster with malate, oxalate, or fumarate and Mg, or these components bind with higher affinity to the isomerized form of the enzyme. In actuality, both of these likely play a role in the isomerization. Circular dichroic spectral studies in the absence and presence of substrates show that different conformational states of the enzyme exist including minimally the unliganded conformation and an isomerized conformation. Thus, it is likely that a conformational change is a slow step in the pre-steady-state. The preincubation of enzyme with Mg and malate activates the enzyme whether or not NAD is present. On the basis of the data presented, a modified kinetic mechanism (Scheme I) is proposed for the *Ascaris* malic enzyme including the conformational changes in enzyme–substrate complexes. The isomerization is shown for both pathways, that is, malate binding prior to NAD and the opposite, but is only slow in the latter pathway. Oxalate is shown binding to both forms (E and E*) and both protonation states of each as suggested by the pH-dependence of inhibition constants (Kiick et al., 1986). The pathways depicted with broken arrows also likely exist, but there is no direct evidence for these species at present.

REFERENCES

- Allen, B. L., & Harris, B. G. (1981) *Mol. Biochem. Parasitol.* 2, 367.
- Atkins, J. F., Lewis, J. B., Anderson, C. W., & Testeland, R. E. (1975) *J. Biol. Chem.* 250, 5688.
- Chang, T. C., Wu, C. C., & Yang, J. T. (1978) *Anal. Biochem.* 91, 13.
- Chen, C.-Y., Harris, B. G., & Cook, P. F. (1988) *Biochemistry* 27, 212.

- Eklund, H., Nordstrom, B., Zeppezauer, E., Soderlund, G., Ohlsson, I., Boiwe, T., & Branden, C.-I. (1974) *FEBS Lett.* 44, 200.
- Farrell, H. M., Jr., Deeney, J. T., Hild, E. K., & Kumosinski, T. F. (1990) *J. Biol. Chem.* 265, 17637.
- Gavva, S. R., Harris, B. G., Weiss, P. M., & Cook, P. F. (1991) *Biochemistry* 30, 5764.
- Good, N. E., Winget, G. D., Winter, W., Connolly, T. N., Izawa, S., & Singh, R. M. M. (1966) *Biochemistry* 5, 467.
- Hsu, R. Y. (1982) *Mol. Cell. Biochem.* 43, 3.
- Karsten, W. E., Harris, B. G., & Cook, P. F. (1992) *FASEB J.* 6, A332.
- Kiick, D. M., Allen, B. L., Rao, J. G. S., Harris, B. G., & Cook, P. F. (1984) *Biochemistry* 23, 5454.
- Kiick, D. M., Harris, B. G., & Cook, P. F. (1986) *Biochemistry* 25, 227.
- Lai, C.-J., Harris, B. G., & Cook, P. F. (1992) *Arch. Biochem. Biophys.* 299, 214.
- Landsperger, W. J., & Harris, B. G. (1976) *J. Biol. Chem.* 251, 3599.
- Landsperger, W. J., Fodge, D. W., & Harris, B. G. (1978) *J. Biol. Chem.* 253, 1868.
- Mallick, S., Harris, B. G., & Cook, P. F. (1991) *J. Biol. Chem.* 266, 2732.
- Martell, A. E., & Smith, R. M. (1979) *Critical Stability Constants*, Vol. 3, Plenum Press, New York.
- O'Farrell, P. H. (1975) *J. Biol. Chem.* 250, 4007.
- Park, S.-H., Kiick, D. M., Harris, B. G., & Cook, P. F. (1984) *Biochemistry* 23, 5446.
- Park, S.-H., Harris, B. G., & Cook, P. F. (1989) *Biochemistry* 28, 6334.
- Parker, D. M., & Holbrook, J. J. (1977) in *Pyridine Nucleotide-Dependent Dehydrogenases* (Sund, H., Ed.) p 458, de Gruyter, Berlin.
- Reynolds, C. H., Hsu, R. Y., Matthews, B., Pry, T. A., & Dalziel, K. (1978) *Arch. Biochem. Biophys.* 189, 309.
- Seery, V. L., & Farrell, H. M. (1990) *J. Biol. Chem.* 265, 17644.
- Tang, C. L., & Hsu, R. Y. (1973) *Biochem. J.* 135, 287.
- Webb, L. E., Hill, E. J., & Benaszak, L. J. (1973) *Biochemistry* 12, 5101.
- Weiss, P. M., Gavva, S. R., Harris, B. G., Urbauer, J. L., Cleland, W. W., & Cook, P. F. (1991) *Biochemistry* 30, 5755.