Multiple Isotope Effects with Alternative Dinucleotide Substrates as a Probe of the Malic Enzyme Reaction†

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ABSTRACT: Deuterium isotope effects and ^{13}C isotope effects with deuterium- and protium-labeled malate have been obtained for both NAD- and NADP-malic enzymes by using a variety of alternative dinucleotide substrates. With nicotinamide-containing dinucleotides as the oxidizing substrate, the ^{13}C effect decreases when deuterated malate is the substrate compared to the value obtained with protium-labeled malate. These data are consistent with a stepwise chemical mechanism in which hydride transfer precedes decarboxylation of the oxalacetate intermediate as previously proposed [Hermes, J. D., Roeske, C. A., O'Leary, M. H., & Cleland, W. W. (1982) *Biochemistry 21*, 5106]. When dinucleotide substrates such as thio-NAD, 3-acetylpyridine adenine dinucleotide, and 3-pyridinealdehyde adenine dinucleotide that contain modified nicotinamide rings are used, the ^{13}C effect increases when deuterated malate is the substrate compared to the value obtained with protium-labeled malate. These data, at face value, are consistent with a change in mechanism from stepwise to concerted for the oxidative decarboxylation portion of the mechanism. However, the increase in the deuterium isotope effect from 1.5 to 3 with a concomitant decrease in the ^{13}C isotope effect from 1.034 to 1.003 as the dinucleotide substrate is changed suggests that the reaction may still be stepwise with the non-nicotinamide dinucleotides. A more likely explanation is that a β-secondary ^{13}C isotope effect accompanies hydride transfer as a result of hyperconjugation of the β-carboxyl of malate as the transition state for the hydride transfer step is approached. The equality of observed deuterium isotope effects on the V/K values for NADP or NAD and malate shows that addition of these substrates is random rather than ordered as has been previously believed for the NADP enzyme.

Multiple isotope effects have been shown to be a powerful tool in obtaining mechanistic information on enzyme-catalyzed reactions. Hermes et al. (1982) used heavy atom effects (^{13}C) with deuterated and unlabeled reactants to determine whether a mechanism occurs via a concerted or stepwise process. The technique has been applied to a number of enzyme-catalyzed reactions including the oxidative decarboxylation of L-malate by the NADP-malic enzyme. The malic enzyme was shown to have a stepwise mechanism in which hydride transfer precedes decarboxylation. These studies allowed the calculation of the intrinsic deuterium (5.5) and ^{13}C (1.051) isotope effects required for a complete quantitative analysis of the enzyme-catalyzed reaction.

Grissom and Cleland (1985) refined the values of the intrinsic effects for the NADP-malic enzyme using the partitioning of oxalacetate between decarboxylation of pyruvate and CO_2 and reduction (with NADPH or NADPD) to malate. The intrinsic deuterium isotope effect on hydride transfer is 5.7, while the intrinsic ^{13}C isotope effect on decarboxylation is 1.044.

The NAD-malic enzyme from *Ascaris suum* has also been shown to catalyze a divalent metal dependent oxidative decarboxylation reaction. The chemical mechanism for the NAD-dependent enzyme was also thought to be stepwise with hydride transfer preceding decarboxylation. There is a requirement for an enzyme residue to act as a general base to abstract a proton from the 2-hydroxyl group of L-malate in the hydride transfer step. Oxalacetate is then produced as a tightly bound intermediate that undergoes decarboxylation to generate the enol of pyruvate. The enol of pyruvate tautomerizes with the aid of a second enzyme residue acting as a general acid to give the product pyruvate.

In the present studies, multiple isotope effects were used as a probe of the malic enzyme reaction. These studies were

Table I: Structures, Redox Potentials, and Spectroscopic Data for the Dinucleotide Substrates Used in the Studies of NAD- and NADP-Malic Enzyme[a]

dinucleotide substrate	structure	ϵ'_o (mV)	λ_{max} (nm)[b]	ϵ at λ_{max} (mM^{-1})[b]
NAD	Figure 1A: R_1 = –H, R_2 = $-CONH_2$	–320	338	6.22
NADP	Figure 1A: R_1 = $-PO_3^{2-}$, R_2 = $-CONH_2$	–320	338	6.22
ϵ-NAD	Figure 1B	–320	338	6.22
thio-NAD	Figure 1A: R_1 = –H, R_2 = $-CSNH_2$	–285	395	11.30
3-PAAD	Figure 1A: R_1 = –H, R_2 = –CHO	–262	358	9.30
3-APAD	Figure 1A: R_1 = –H, R_2 = $-COCH_3$	–258	363	9.10

[a] Refer to Figure 1 for the general structures of the dinucleotides. [b] These values were used to follow the appearance of the corresponding reduced dinucleotide.

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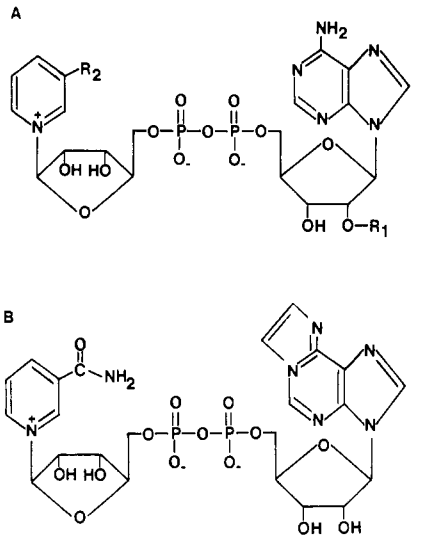


FIGURE 1: Structures of the dinucleotide substrates. (A) Refer to Table I for exact structures of R_1 and R_2 for each dinucleotide. (B) Structure of nicotinamide 1,N^6-ethenoadenine dinucleotide (ϵ-NAD).

carried out with the alternative dinucleotide substrates indicated in Figure 1 and Table I for both the NAD- and NADP-dependent enzymes to determine whether a change in redox potential resulted in a change in transition-state structure. The data suggest that as hydride transfer becomes more rate determining, either the mechanism changes from stepwise to concerted or a large secondary ^{13}C isotope effect at C-4 of malate on hydride transfer becomes expressed.

Materials and Methods

Chemicals and Enzymes. 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., 1983). The enzyme had a final specific activity of 35 units/mg assayed in the direction of oxidative decarboxylation. NAD[1] was purchased from Boehringer-Mannheim. L-Malate, dinucleotide analogues, chicken liver malic enzyme, and DTT were from Sigma. Ethanol-d_6 (99 atom % *d*) was from Merck. L-Malate-2-*d* was prepared and purified according to Viola et al. (1979). All other reagents and chemicals were obtained from commercially available sources and were of the highest quality available.

Substrate Calibration. The precision of V/K isotope effects measured by the direct comparison of initial velocities requires that one accurately know the concentrations of labeled and unlabeled substrates (Cleland, 1982). Therefore, all substrate concentrations were calibrated enzymatically by end-point analysis as described by Cook et al. (1980). Concentrations of L-malate-(*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, 1 mM; and 100 mM Taps, pH 9.0. Solutions of NAD and dinucleotide analogues were calibrated with *A. suum* malic enzyme by using 20 mM malate, 320 mM $MgSO_4$, and 1 mM DTT, in 100 mM Hepes, pH 7.0. The concentrations from several determinations were in agreement within 1%. All reactions went to 100% completion. The 4-cyano adduct of the reduced forms were prepared for each of the analogues. Ultraviolet–visible spectra of both the cyanide adduct and the reduced dinucleotide were obtained with a Gilford 2600, and the results were compared to reported spectra. The reaction mixture for preparation of the cyanide adduct contained 100 mM Hepes, pH 7.0, 10 mM KCN, and 0.1 mM analogue. Reduced dinucleotides were prepared by the NAD-malic enzyme reaction at pH 7.0, with 20 mM malate and 320 mM $MgSO_4$. The dinucleotide was completely converted to its reduced form. In all cases, the dinucleotides were shown to be authentic analogues with no significant contaminating NAD.

Initial Velocity Studies. Malic enzyme was assayed spectrophotometrically in the direction of oxidative decarboxylation by using a Gilford 250 spectrophotometer equipped with a Brinkman Servogor 210 chart recorder with multispeed drive. All assays were run at 25 ± 0.1 °C, and the temperature was maintained by a circulating water bath with the capacity to heat and cool the thermospacers of the Gilford. Reaction cuvettes were 1 cm in pathlength and 1 mL in volume. All cuvettes were incubated for at least 10 min in the water bath prior to initiation of the reaction. Temperatures were routinely monitored with a YSI telethermometer. A typical assay contained 100 mM Hepes, pH 7.0, saturating concentrations of metal ($20K_i$), and variable concentrations of malate and dinucleotide. All reactants were corrected for metal–reactant chelate complexes according to Park et al. (1984). The reaction was initiated by addition of malic enzyme and production of the appropriate reduced dinucleotide was monitored at the corresponding λ_{max} of the reduced dinucleotide.

Chicken liver NADP-malic enzyme was dialyzed twice against 2 L of buffer that contained 10 mM potassium phosphate, pH 6.0, 1 mM DTT, 3 mM EDTA, and 10% glycerol. The resulting enzyme was stored under conditions identical with those given above for *A. suum* malic enzyme and assayed in the direction of oxidative decarboxylation at pH 7.0 and 1.0 mM DTT, 10.8 mM L-malate (6 mM when corrected for Mg:malate), 0.2 mM NADP (0.1 mM when corrected for Mg:NADP), and 25 mM $MgSO_4$ (20 mM when corrected for Mg:malate and Mg:NADP).

Primary deuterium isotope effects with L-malate-2-*d* were obtained by direct comparison of initial velocities. The ^DV and $^DV/K$ for dinucleotides were measured by varying the dinucleotide at saturating levels of Mg^{2+} and L-malate-2-(*h*,*d*), while $^D(V/K_{malate})$ was obtained varying L-malate or L-malate-2-*d* at saturating levels of NAD and Mg^{2+}. Effects were obtained for both NAD- and NADP-malic enzymes.

^{13}C-Isotope Effects. The technique employed for the determination of ^{13}C isotope effects is that of O'Leary (1980) in which the natural abundance of ^{13}C in the C-4 position of L-malate is used. Both high-conversion (100% reaction) and low-conversion (~10% reaction) samples were collected. The $^{12}C/^{13}C$ isotope ratios in the CO_2 produced in the reactions were determined for both samples. From these ratios, the relative rates of reaction for ^{12}C versus ^{13}C, and thus the ^{13}C isotope effect, were calculated (Hermes et al., 1982). Use of this natural abundance method minimizes the errors caused by atmospheric CO_2 contamination. Reaction mixtures for the low-conversion reactions contained the following in 40 mL:

[1] Abbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; NAD, nicotinamide adenine dinucleotide; ϵ-NAD, nicotinamide 1,N^6-ethenoadenine dinucleotide; 3-APAD, 3-acetylpyridine adenine dinucleotide; 3-PAAD, 3-pyridinealdehyde adenine dinucleotide; NADP, nicotinamide adenine dinucleotide 2'-phosphate; thio-NAD, thionicotinamide adenine dinucleotide; Taps, 3-[[tris(hydroxymethyl)methyl]-amino]propane sulfonic acid; Pipes, piperazine-*N*,*N*'-bis(2-ethanesulfonic acid); thio-NADP, thionicotinamide adenine dinucleotide phosphate; 3-APADP, 3-acetylpyridine adenine dinucleotide phosphate. The "+" that is by convention used to represent the charge on the oxidized nicotinanide (or analogue) ring is omitted in the manuscript for convenience.

Table II: Values of Kinetic Parameters for the *A. suum* NAD-Malic Enzyme with Mg^{2+} as the Divalent Metal Ion Activator

parameter[a]	NAD	thio-NAD	3-APAD	3-PAAD	ε-NAD	NADP
V/E_t	38	1.9	91	1.9	27	15
	(1)[b]	(0.05)	(2.4)	(0.05)	(0.7)	(0.4)
K_a	11 ± 1	10 ● 0.3	8 ● 0.5	9 ± 1	8 ± 1	130 ± 50
K_b	1.18 ± 0.06	2.4 ● 0.4	0.48 ± 0.03	0.29 ± 0.03	0.77 ± 0.16	1.2 ± 0.5
V/K_aE_t	3.8×10^6	2.3×10^5	1.3×10^7	2.3×10^5	3.5×10^6	1.5×10^6
	(1)	(0.06)	(3.4)	(0.06)	(0.95)	(0.4)
V/K_bE_t	3.8×10^4	1.1×10^3	2.2×10^5	3.8×10^2	4.2×10^4	1.5×10^4
	(1)	(0.03)	(5.8)	(0.01)	(1.1)	(0.4)
K_{ia}[c]	11	10	2	14	8	130
K_{ib}[c]	1.18	2.4	0.30	0.44	0.77	1.2

[a] Units are s^{-1} for V/E_t, μM for K_a and K_{ia}, mM for K_b, and K_{ib}, M^{-1} s^{-1} for V/K_aE_t and V/K_bE_t. K_a is K_{DINUC}; K_b is K_{malate}. Values of K_a and K_b are ±SE. [b] Values in parentheses are relative to NAD with Mg^{2+} as the divalent metal. [c] Values of dissociation constants from E:DINUC:metal:malate (K_{ia} and K_{ib} for dinucleotide and malate, respectively) calculated as described under Discussion.

20 mM L-malate-2-(*h*,*d*), 13.8 mM NAD(P) or dinucleotide analogue, and 180 mM Mg^{2+} in 100 mM Hepes, pH 7.0.[2] The high-conversion samples used 20 mM Mn^{2+}, 2 mM L-malate-2-(*h*,*d*), and 5 mM NADP. The pH was adjusted with saturated KOH (or NaOD) after sparging with CO_2-free, dry N_2 for 2 h. This was followed by additional sparging for 1 h. The reaction was then initiated by addition of malic enzyme (40 units of chicken liver NADP-malic enzyme added to high-conversion samples and 20 units of *A. suum* NAD-malic enzyme or chicken liver NADP-malic enzyme added to low-conversion samples).

Low-conversion samples were quenched with 1.5 mL of concentrated sulfuric acid at the appropriate time for ~10% reaction. The high-conversion samples were allowed to proceed overnight prior to addition of sulfuric acid and isolation of CO_2. The reproducibility of the ^{13}C content determined for the high-conversion samples used to calculate the ^{13}C isotope effects in Table V was ±0.05% for both malate-2-*h* and malate-2-*d*. Also, if either of the substrates or the enzyme was not added to the reaction, no measurable (<1 μmol) CO_2 was produced. At least four low-conversion and two high-conversion samples were used to calculate each ^{13}C isotope effect in Table V. Isolation and analysis of all samples were carried out on the same day the CO_2 was generated. Isotopic composition of the CO_2 was determined on a Finnigan Delta-E isotope-ratio mass spectrometer. All ratios were corrected for ^{17}O according to Craig (1957).

Each concentration of malate at $t = 0$ and after reactions were quenched was determined in duplicate with the chicken liver NADP-malic enzyme assay. Conditions used were identical with those described above for high-conversion reactions.

Data Processing. Reciprocal initial velocities were plotted vs reciprocal substrate concentrations and all plots were linear.

[2] In previous isotope-effect experiments conducted with chicken liver malic enzyme, oxidized dinucleotide recycling or pyruvate removal systems were used to ensure irreversibility of the malic enzyme reaction (Hermes et al., 1982; Grissom & Cleland, 1988b). During preliminary experiments for the work presented here, an oxidized dinucleotide recycling system was used for both the chicken liver and *A. suum* malic enzymes. Identical results with those later determined without the recycling system were observed with NADP (chicken liver malic enzyme) or NAD (*A. suum* malic enzyme) and malate-2-*h* or malate-2-*d* as substrates, and thus a recycling system was not used for the experiments described herein. The probable reason for identical results being obtained with or without a recycling system is the very high concentration of oxidized dinucleotides used in the experiments (~14 mM). This is in contrast to low levels used in previously reported studies. Since all of the alternative dinucleotides used in these studies had either the same or a less negative redox potential than NAD(P), and thus the equilibrium constant for the oxidative decarboxylation is even greater, recycling systems were not deemed necessary in these experiments either.

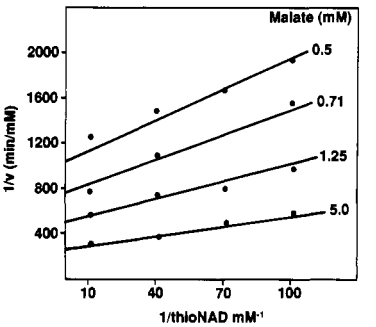


FIGURE 2: Initial velocity pattern for the *A. suum* NAD-malic enzyme with thio-NAD as the dinucleotide substrate. Data were collected at 25 °C, pH 7, in 100 mM Hepes. Concentrations used were as follows: Mg^{2+}, 160 mM; thio-NAD, varied from 0.01 to 0.1 mM at several fixed levels of malate (0.5–5 mM). The solid lines are from a fit of eq 1 to the data, while the points are experimental values. All reactant concentrations were corrected for chelate complex formation.

Data were fitted with the appropriate rate equation and Fortran programs developed by Cleland (1979). Data conforming to a sequential initial velocity pattern were fitted with

$$v = VAB/(K_{ia}K_b + K_aB + K_bA + AB) \quad (1)$$

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

$$v = VA/[K_a(1 + F_iE_{V/K}) + A(1 + F_iE_V)] \quad (2)$$

$$v = VA/[(K_a + A)(1 + F_iE_v)] \quad (3)$$

Equations 2 and 3 assume independent isotope effects on V and V/K or equal effects on both V and V/K, respectively. In eqs 1–3, 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 2 and 3, F_i is the fraction of 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 all cases, the best fit of the data was chosen on the basis of the lowest value 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

Initial Velocity Studies. For the *A. suum* NAD-malic enzyme, initial velocity studies were carried out with ε-NAD,[3]

Table III: Kinetic Parameters for the Chicken Liver NADP-Malic Enzyme with Mg^{2+} as the Divalent Metal Ion

parameter[a]	NADP	thio-NADP	3-APADP
V/E_t	28	14	84
	(1.0)[b]	(0.5)	(3.0)
K_a	0.006 ± 0.0006	0.015 ● 0.003	0.007 ● 0.0006
K_b	0.050 ● 0.002	0.6 ± 0.1	0.06 ± 0.004
V/K_aE_t	7.0×10^6	1.0×10^5	1.2×10^7
	(1.0)	(0.015)	(2)
V/K_bE_t	5.7×10^5	2.0×10^4	1.4×10^6
	(1.0)	(0.035)	(2.5)
K_{ia}	0.015 ± 0.008	0.006 ● 0.001	0.02 ● 0.002
K_{ia}[c]	0.012	0.015	0.011
K_{ib}[c]	0.050	0.60	0.11

[a] Units are s^{-1} for V/E_t, mM for K_a, K_b, K_{ia}, K_{ia}, and K_{ib}, and M^{-1} s^{-1} for V/K_aE_t and V/K_bE_t; $K_a = K_{DINUC}$ and $K_b = K_{malate}$. Values of K_a, K_b, and K_{ia} are ±SE. [b] Values in parentheses are relative to NADP with Mg^{2+}. [c] Values of dissociation constants for E:DINUC:Mg:malate (K_{ia} for DINUC, K_{ib} for malate) are calculated as described in the text.

NADP (K_m higher and V lower than with NAD), 3-APAD, 3-PAAD and thio-NAD (the latter three have a redox potential more oxidizing than that of NAD) (Figure 1 and Table I). Initial velocity patterns were obtained at a saturating concentration of Mg^{2+}; the concentration of dinucleotide was varied at several fixed levels of malate. (An example of an initial velocity pattern obtained with thio-NAD as the dinucleotide substrate is shown in Figure 2). Kinetic parameters are listed in Table II.

For the chicken liver NADP-malic enzyme, initial velocity studies were carried out with NADP, 3-APADP, and thio-NADP with Mg^{2+} as the divalent metal ion. Initial velocity patterns were obtained at saturating concentrations of Mg^{2+}; the concentration of dinucleotide was varied at several fixed levels of malate. Kinetic parameters are listed in Table III.

Isotope Effects. The isotope effects, ^DV, $^D(V/K_{DINUC})$, and $^D(V/K_{malate})$ were determined for the NAD-malic enzyme with either saturating Mg^{2+} and dinucleotide concentrations, varying L-malate 2-(*h*,*d*) or with saturating Mg^{2+} and L-malate 2-(*h*,*d*), varying the dinucleotide substrate. The results are shown in Table IV. Deuterium isotope effect experiments were likewise carried out for the chicken liver NADP-malic enzyme, and the results are shown in Table V.

In addition to the deuterium isotope effects, ^{13}C effects at C-4 of L-malate were obtained with each of the dinucleotides mentioned above for both enzymes. All of these effects were then repeated with L-malate-2-*d* to obtain the multiple isotope effect. Results are presented in Tables IV and V.

Discussion

Chemical Mechanism. The theory and methodology of multiple isotope effects on enzyme-catalyzed reactions developed by Hermes et al. (1982) allows one to distinguish between a concerted and stepwise mechanism and provides estimates of the intrinsic isotope effects on the bond-breaking steps. By this method, isocitrate dehydrogenase (Grissom & Cleland, 1988a), 6-phosphogluconate dehydrogenase (Rendina et al., 1984), and more recently malate synthase (Clark et al., 1988) have been shown to proceed via a stepwise mechanism in which C–H bond cleavage precedes C–C bond cleavage, while formate dehydrogenase (Blanchard & Cleland, 1980; Hermes et al., 1984a), glucose-6-phosphate dehydrogenase (Hermes et al., 1982), and prephenate dehydrogenase (Hermes et al., 1984b) have been shown to catalyze a concerted mechanism.

Hermes et al. (1984a) have shown for formate dehydrogenase that a change in the redox potential of the dinucleotide substrate results in a change in the transition-state structure for hydride transfer. Specifically, as the dinucleotide substrate becomes more oxidizing, the transition state for hydride transfer becomes earlier. Similar studies with alcohol and aldehyde dehydrogenases (Scharschmidt et al., 1984) have resulted in the same suggestion. The results with formate dehydrogenase are especially convincing since hydride transfer has been shown to completely limit the overall reaction.

The multiple isotope effect methodology has also been applied by Hermes et al. (1982) to the NADP-malic enzyme reaction. In the reaction catalyzed by malic enzyme, a carbon–hydrogen bond at C-2 and the C-3 to C-4 bond of malate are broken. This cleavage could occur in either a concerted or a stepwise fashion. The multiple isotope effect data with NADP as the oxidant provided evidence for a two-step oxidative decarboxylation in which hydride transfer precedes decarboxylation. Estimated values of intrinsic isotope effects from these studies are 5.5 for ^Dk and 1.05 for ^{13}k. Grissom and Cleland (1985) obtained more precise estimates of these intrinsic kinetic isotope effects by quantitating the partitioning of oxalacetate between reduction by NADPH(D) to malate and decarboxylation to pyruvate and CO_2. These authors obtained intrinsic ^{13}C and deuterium isotope effects of 1.044 and 5.7, respectively. Experiments were also carried out with 3-APADP, but the ^{13}C effect with deuterated malate was not determined presumably because of the low value of the ^{13}C effect obtained with protium-labeled malate (1.0028).

To determine whether a change in redox potential results in a change in transition-state structure for a redox enzyme that catalyzes multiple chemical steps, multiple isotope effects were obtained with both NAD- and NADP-malic enzymes by using three alternative dinucleotides with redox potentials more oxidizing than NAD(P). In these studies, the kinetic deuterium isotope effect at C-2 of L-malate, the kinetic ^{13}C isotope effect at C-4 of L-malate, and the effect of deuteration on the kinetic ^{13}C isotope effect were measured. In the case of a stepwise mechanism, carbon–hydrogen bond cleavage (transfer of a hydride ion from C-2 of malate to C-4 of the nicotinamide ring) and carbon–carbon bond cleavage (decarboxylation) occur in two different steps. Theoretically, if only the first of these two isotopically sensitive transition states is rate limiting, only one isotope effect will be seen, either deuterium or ^{13}C, depending on which step is rate limiting. If the second step is completely rate limiting, however, the equilibrium isotope effect will be observed for the first step, while the intrinsic isotope effect will be observed for the second step. If both transition states are partially rate limiting, both a primary deuterium and ^{13}C effect will be observed, neither of which is an equilibrium effect. For a concerted mechanism, a single transition state is observed for hydride transfer and decarboxylation since carbon–hydrogen and carbon–carbon bond cleavage occur simultaneously. In this case, deuteration will result in either no change (chemical step completely rate limiting) or an increase (chemical step not completely rate limiting) in the ^{13}C effect. In the case of a stepwise mechanism, deuteration of malate should cause a decrease in the ^{13}C

[3] The ε-NAD analogue was chosen from earlier considerations that the dissociation constant for the dinucleotide analogue was lower than NAD. This would be expected to result in a lower value of the observed isotope effects, assuming that the decrease in dissociation constant was a result of a decrease in the off-rate for the dinucleotide. As can be seen in Table II, however, the kinetic parameters obtained by using ε-NAD are for all intents and purposes identical with those obtained with NAD. The ε-NAD data still serve as a control, however, for reproducibility of the data.

Table IV: Primary Deuterium and ^{13}C Isotope Effects for the *A. suum* NAD-Malic Enzyme with Mg^{2+} as the Divalent Metal Ion Activator

parameter	NAD	thio-NAD	3-APAD	3-PAAD	ε-NAD	NADP
E_o' (V)	−0.32	−0.285	−0.258	−0.262	−0.32	−0.32
^{D}V	1.47 ± 0.04	1.73 ± 0.06	2.85 ± 0.19	2.28 ± 0.11	1.46 ± 0.07	1.64 ± 0.06
$^{D}(V/K_{NUC})$	1.49 ± 0.03	1.69 ± 0.04	1.40 ± 0.16	2.98 ± 0.29	1.44 ± 0.06	1.59 ± 0.06
$^{D}(V/K_{malate})$	1.45 ± 0.04	1.76 ± 0.09	2.15 ± 0.13	2.98 ± 0.21	1.48 ± 0.09	1.67 ± 0.07
$^{13}(V/K_{malate})_H$	1.0342 (0.0002)[a]	1.0102 (0.0002)	1.0070 (0.0001)	1.0028 (0.0002)	1.0344 (0.0008)	1.0399 (0.0007)
$^{13}(V/K_{malate})_D$	1.0252 (0.0001)	1.0180 (0.0003)	1.0120 (0.0002)	1.0053 (0.0004)	1.02488 (0.00009)	1.0279 (0.0004)

[a] Values in parentheses are ±SE for the ^{13}C effects.

Table V: Primary Deuterium and ^{13}C Isotope Effects for the NADP-Malic Enzyme from Chicken Liver with Mg^{2+} as the Divalent Metal Ion[a]

	NADP	thio-NADP	3-APADP
E_o' (V)	−0.32	−0.285	−0.258
^{D}V	1.47 ● 0.03[b]	3.0 ± 0.1	2.0 ± 0.1
$^{D}V/K_{DINUC}$	1.46 ± 0.07	3.2 ± 0.1	2.6 ± 0.3
$^{D}V/K_{malate}$	1.47 ± 0.03[b]	3.0 ± 0.1	2.9 ± 0.1
$^{13}(V/K_{malate})_H$	1.0336 ± 0.0003[c]	1.0090 ● 0.0001	1.0051 ± 0.0001
$^{13}(V/K_{malate})_D$	1.0250 ± 0.0005	1.0147 ± 0.0001	1.0084 ± 0.0001

[a] Data for deuterium isotope effects with NADP and 3-APADP were fitted with eq 2, while those obtained with thio-NADP were fitted with eq 3. [b] Values from Hermes et al. (1982). [c] Values from Grissom and Cleland (1988b).

isotope effect compared to protium-labeled malate. The decrease in the ^{13}C effect upon deuteration is a result of the hydride transfer step becoming slower compared to decarboxylation.

With NAD, ε-NAD, or NADP as the dinucleotide substrate, a decrease in the ^{13}C isotope effect is observed with deuterated compared to protium-labeled malate for the NAD-malic enzyme. These data are suggestive of a stepwise mechanism. Hermes et al. (1982) have shown that similar data obtained for the NADP-malic enzyme from chicken liver (see also Table IV) adhere to a mechanism in which hydride transfer precedes decarboxylation (Scheme I). A chemical description of Scheme I is given in Scheme II. The data obtained with NAD

Scheme I

$$EAB \underset{k_6}{\overset{k_5C}{\rightleftharpoons}} EABC \underset{k_8}{\overset{k_7}{\rightleftharpoons}} (EABC)' \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}} (ERBX) \underset{k_{12}}{\overset{k_{11}}{\rightleftharpoons}} (ERBPQ)' \xrightarrow{k_{15}} ER \xrightarrow{k_{17}} E$$

for the NAD-malic enzyme are nearly identical to those obtained with NADP for the NADP-malic enzyme (Table IV; Hermes et al., 1982; Grissom and Cleland, 1985), and thus are also consistent with the same mechanism. In Scheme I, A, B, C, X, P, Q, and R are $NAD(P)^+$, Mg^{2+}, malate, oxalacetate, CO_2, pyruvate, and NAD(P)H, respectively. The step represented by k_7 and k_8 and one of the steps comprising k_{15} are conformation changes (the latter also contains the steps for release of CO_2 and pyruvate), while k_9 and k_{10} represent hydride transfer and k_{11} and k_{12} represent decarboxylation of the oxalacetate intermediate (X) and carboxylation of enolpyruvate, respectively (this step also includes the tautomerization of enolpyruvate to pyruvate, which is very rapid,[4,5] and is lumped into the decarboxylation step). Intrinsic deuterium and ^{13}C isotope effects on the hydride transfer and decarboxylation steps, respectively, based on Scheme I have been estimated by Grissom and Cleland (1985) as 5.7 and 1.044. Because of the similarities of all observed values for both enzymes, values for the intrinsic isotope effects are also very good if not precise estimates of those for the NAD enzyme (Table IV). As stated above, isotope effects obtained with NADP for the *Ascaris* enzyme are slightly larger as a result of the elimination of a small external forward commitment factor but otherwise also generate very similar estimates of the intrinsic deuterium and ^{13}C isotope effects. Thus, the transition states for hydride transfer and decarboxylation are very similar when the nicotinamide ring is intact.[6]

Table VI: Intrinsic Isotope Effects and Commitment Factors When Concerted Oxidative Decarboxylation Is Assumed

parameter	3-APAD(P)	thio-NAD(P)	3-PAAD
E_o' (V)	−0.258	−0.285	−0.262
	NAD-Malic Enzyme		
$^{D}k_7$	2.6	3.1	4.6
$^{13}k_7$	1.011	1.03	1.014
c_f	0.4	1.8	0.8
	NADP-Malic Enzyme		
$^{D}k_7$	5.1	4.9	
$^{13}k_7$	1.011	1.017	
c_f	1.18	0.94	

With the dinucleotide substrates 3-APAD(P), 3-PAAD, and thio-NAD(P) the ^{13}C effect is increased with deuterated malate compared to protium-labeled malate. It should be noted that the experiments with the APAD(P) were repeated a number of times (each experiment consisting of at least four low-conversion and two high-conversion samples) over a two year period with different batches of enzyme and malate-2-(*h,d*). At face value, these data are consistent with a concerted mechanism. It is possible to write a concerted mechanism for the malic enzyme reaction as shown mathematically in Scheme III and chemically in Scheme IV. In Scheme III all processes and rate constants are identical with those in Scheme I except that k_9 and k_{10} reflect oxidative decarboxylation (and its reverse reaction) in a single step. This would be feasible if, as shown in Scheme IV; malate was bound such that the β-carboxyl is out of the C-1–C-2–C-3 plane and trans to the hydride being transferred to the 4-position of the nicotinamide ring of NAD(P).

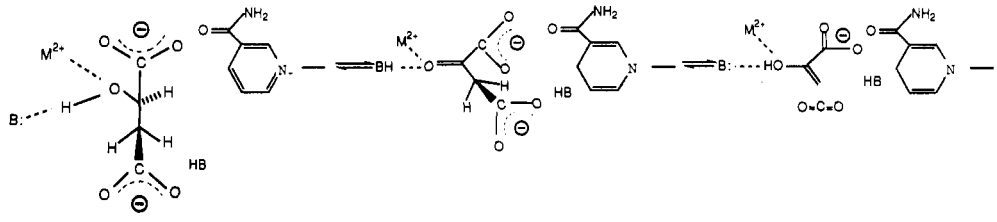
Assuming a concerted mechanism for oxidative decarboxylation, the intrinsic deuterium and ^{13}C isotope effects can be calculated according to the following equations derived on the basis of Scheme III.

[4] The isotope effect obtained with pyruvate-d_3 in the direction of reductive carboxylation is near unity (unpublished work of S. Mallick in this laboratory).

[5] Initial velocity studies in the direction of reductive carboxylation are consistent with reaction of CO_2 upon collision with the E:NADH:Mg:pyruvate complex (unpublished work of S. Mallick in this laboratory). In addition, $K_{pyruvate}$ is 50 mM, and pyruvate is thus also likely to be released rapidly.

[6] In agreement with this, kinetic parameters and isotope effects are also identical with those obtained for NAD, ε-NAD, and NADP when nicotinamide hypoxanthine dinucleotide is used as a substrate (unpublished work of S. R. Gavva and P. M. Weiss).

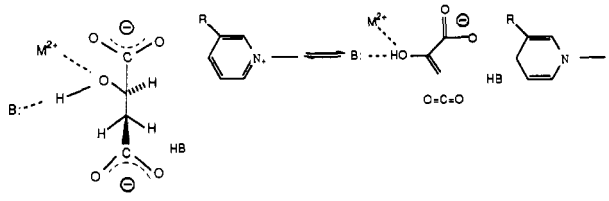
Scheme II: Proposed Stepwise Mechanism for the Malic Enzyme Oxidative Decarboxylation of Malate



Scheme III

$$EAB \underset{k_6}{\overset{k_5C}{\rightleftharpoons}} EABC \underset{k_8}{\overset{k_7}{\rightleftharpoons}} (EABC)' \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}} (ERBPQ)' \xrightarrow{k_{15}} ER \xrightarrow{k_{17}} E$$

Scheme IV: Proposed Concerted Mechanism for the Malic Enzyme Oxidative Decarboxylation of Malate



$$^{D}(V/K_{malate}) = [^{D}k_7 + k_9/k_8(1 + k_7/k_6) + {}^{D}K_{eq}(k_{10}/k_{15})]/[1 + k_9/k_8(1 + k_7/k_6) + k_{10}/k_{15}] \quad (5)$$

$$^{13}(V/K_{malate})_H = [^{13}k_7 + k_9/k_8(1 + k_7/k_6) + {}^{13}K_{eq}(k_{10}/k_{15})]/[1 + k_9/k_8(1 + k_7/k_6) + k_{10}/k_{15}] \quad (6)$$

$$^{13}(V/K_{malate})_D = [^{13}k_7 + k_9/{}^{D}k_9k_8(1 + k_7/k_6) + {}^{13}K_{eq}(k_{10}{}^{D}K_{eq}/{}^{D}k_9k_{15})]/[1 + k_9/{}^{D}k_9k_8(1 + k_7/k_6) + k_{10}{}^{D}K_{eq}/{}^{D}k_9k_1{}^{5}] \quad (7)$$

Since CO_2 is likely released very rapidly,[5] the c_r term (k_{10}/k_{15}) is likely insignificant and eqs 5–7 reduce to three unknowns including $^{D}k_7$, $^{13}k_7$, and c_f $[k_9/k_8(1 + k_7/k_6)]$, and thus an absolute solution is possible. Solution of the above simultaneous equations gives the values shown in Table VI. Note that the trend in the isotope effects for the non-nicotinamide-containing dinucleotides does not follow the redox potential of the dinucleotide substrate.[7] [The transition state for oxidation is expected to become earlier as the redox potential of the dinucleotide becomes more oxidizing (Westheimer, 1961; Hermes et al., 1984a).] There is likely to be a significant tunnel correction and coupled motion contribution as shown by Huskey and Schowen (1983), and thus very little can be stated at this point concerning the chemical nature of the transition state for the oxidation. Very little change is observed in the intrinsic deuterium effect for the NADP-malic enzyme, but again the intrinsic ^{13}C effect has decreased to about 1.01. In both cases, it is not difficult to reconcile the fact that the decarboxylation portion of the transition state has become very early when the oxidative decarboxylation occurs in a single step as opposed to two steps. In fact, since the reaction is stepwise with NAD(P), most likely then with other dinucleotides, if the reaction is concerted, decarboxylation would probably if not certainly lag behind hydride transfer in an asynchronous yet concerted process. This would lead to an earlier transition state as far as the decarboxylation is concerned and to a smaller ^{13}C isotope effect. It is difficult to reconcile this mechanism, however, in the case of a better oxidant that should lead to an earlier transition state for hydride transfer.

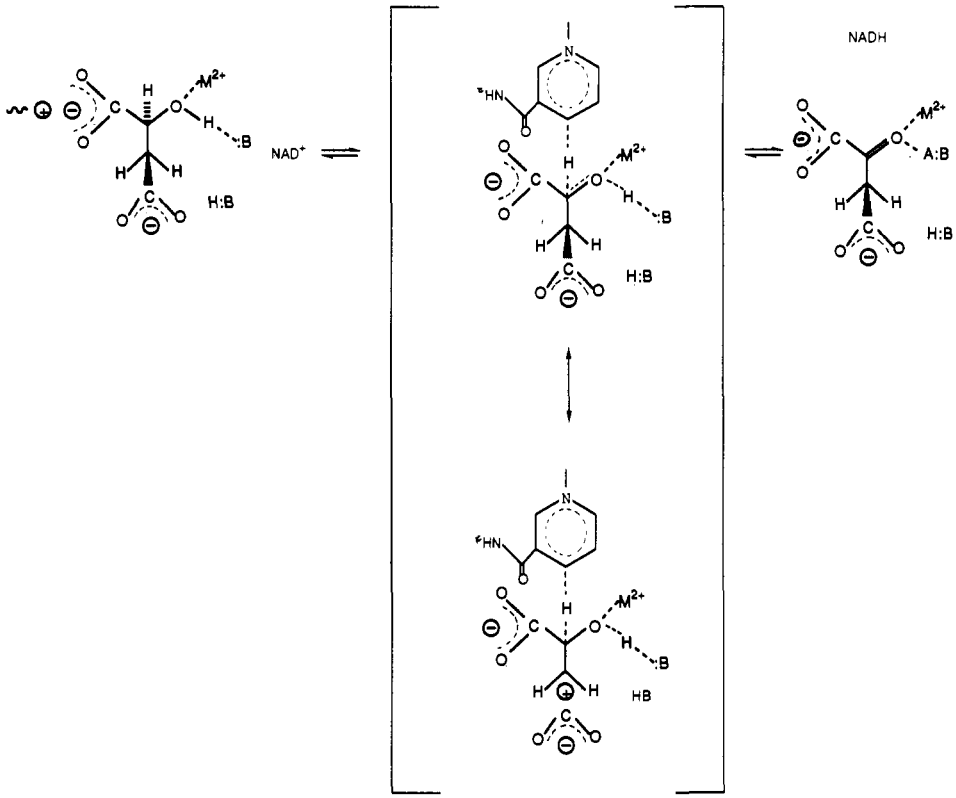
Another possibility is that the mechanism still consists of two steps even when the alternative dinucleotide substrates 3-APAD(P), thio-NAD(P), and 3-PAAD are used. There are several lines of evidence consistent with this hypothesis. First, as stated above, the observed deuterium effect increases smoothly from 1.45 to 3 as the dinucleotide substrate changes from NAD(P) to 3-PAAD while the ^{13}C effect decreases smoothly from 3.4% to 0.3% (the changes in both deuterium and ^{13}C effects are similar for the NADP enzyme). These data are consistent with a two-step mechanism in which, according to Scheme II, there is an increase in the rate limitation of the reverse hydride transfer step with a concomitant loss of rate limitation of the decarboxylation step (Grissom & Cleland, 1985). In addition, the oxalacetate intermediate has been shown to partition between malate and pyruvate production with all of the dinucleotide substrates tested thus far [NAD(P), 3-APAD(P), and thio-NAD], and a substantial deuterium isotope effect on the partition ratio is obtained (2–3) in all cases (Grissom & Cleland, 1988b).[8] These data are also consistent with a two-step mechanism such as that depicted in Scheme II. In fact, the only data inconsistent with a two-step mechanism are the multiple isotope effects, which suggest a change to a concerted mechanism with the alternative dinucleotides that do not contain nicotinamide.

The above quandry of apparently conflicting results, that is, a stepwise vs a concerted mechanism, may be reconciled both mathematically and chemically in terms of a stepwise chemical mechanism. In order to satisfy the data mathematically, a compensatory β-secondary kinetic ^{13}C isotope effect is required to be present on the hydride transfer step. Thus, as the hydride transfer step becomes more rate determining, the ^{13}C effect on that step becomes expressed. In the absence of this secondary ^{13}C effect, a decrease in the observed ^{13}C effect would be expected when L-malate-2-*d* replaces L-malate in the determination of the carbon effect. The presence of the secondary effect in the hydride transfer step would compensate for the decrease expected when L-malate-2-*d* is used and could provide anything from a change smaller than expected to an increase in the ^{13}C effect dependent on the magnitude of the secondary effect. Thus, when NAD is used as a substrate, the hydride transfer step is not very slow overall and as a result the secondary effect would not be expected to contribute to the observed ^{13}C effect. However, with 3-PAAD as the substrate, decarboxylation is not likely to be rate determining and the ^{13}C effect reflects the secondary effect present in the hydride transfer step. Assuming the latter to be correct, the multiple effects should behave as for a

[7] It is possible that the redox potential on the enzyme may be different from that in solution. Moreover, 3-PAAD is present 90% in the hydrated form (personal communication from N. J. Oppenheimer), which can account for the different redox potential than the aldehyde form. Another possible reason the 3-PAAD is out of order is that the enzyme may not bring the reactants as close as it does for other dinucleotide substrates, resulting in an extended reaction coordinate that in turn may be responsible for the higher ^{D}k value. In agreement with this possibility, 3-PAAD is a very slow substrate for malic enzyme.

[8] Unpublished work of S. R. Gavva in this laboratory.

Scheme V: Stepwise Mechanism Taking into Account the Proposed Hyperconjugation of the β-Carboxyl Group of Malate during Hydride Transfer



concerted mechanism, and the intrinsic effects calculated for 3-PAAD should provide an estimate of the intrinsic primary deuterium and secondary ^{13}C effects on the hydride transfer step.[9]

The β-secondary ^{13}C effect could be the result of hyperconjugation of the β-carboxyl of malate as the transition state for hydride transfer is approached, Scheme V. Malate is probably bound reasonably tightly at C-1 and C-2 with the α-carboxyl postulated to be ion paired to an active-site arginine (Rao et al., 1987; Vernon & Hsu, 1983), the 2-hydroxyl hydrogen-bonded to an active-site general base (Kiick et al., 1986; Schimerlik & Cleland, 1977b), and the hydroxyl oxygen coordinated to the active-site divalent metal, consistent with recent evidence from spin echo EPR studies.[10] In addition, as postulated for the concerted mechanism discussed above, the β-carboxyl of malate must be bound with the β-carboxyl out of the C-1–C-2–C-3 plane and trans to the hydride being transferred to the 4-position of the nicotinamide ring of NAD(P). As the transition state for hydride transfer is approached, the electron density that would normally be available to allow formation of the carbonyl of oxalacetate is to a large extent delocalized to the active-site metal. To satisfy this deficiency, electron density is borrowed from the C-3–C-4 bond, that is, the bond to the β-carboxyl group. However, the mechanisms by which the enzyme enforces the out-of-plane configuration do not allow, during initial hydride transfer and until this transition state is reached, colinearization of the oxygens of the β-carboxyl and C-4 to an extent large enough to maintain the original C-4 bond order. This bond-order decrease and concomitant force-constant decrease (between C-3 and C-4) produces the isotope effect. The end result would be that the intermediate oxalacetate to be generated would more closely resemble the transition state for the subsequent decarboxylation step with the geometry correct for removal of the electron density from C-3–C-4 to the π system at C-2–C-3. The decarboxylation is then partially rate limiting as a result of a hindrance to O–C–O linearization at C-4. Currently, using the bond-energy bond-order method [embodied in the program BEBOVIB-IV (Sims et al.)] and optimized structures and force fields from ab initio and semiempirical calculations, we are modeling the proposed chemistry in order to deduce the feasibility of our proposals. Results to date support the existence of a β-secondary ^{13}C isotope effect of the proposed magnitude. Initial results indicate that no more than a 20% reduction in the C-3–C-4 bond order (with proportional force constant decrease) is necessary to produce an isotope effect of ~1%. Additional theoretical studies and experimental studies designed to directly measure the magnitude of this isotope effect are currently underway.

Kinetic Mechanism for NAD-Malic Enzyme. All initial velocity patterns were obtained at a saturating metal concentration. In all cases, the double-reciprocal plots intersected to the left of the ordinate whether plotted with dinucleotide or malate as the variable substrate, indicating that the mechanism remains sequential with all dinucleotides used. Data obtained with NAD and Mg^{2+} adhere to a steady-state random mechanism in which the rates of release of malate and NAD from the E:NAD:Mg:malate complex are equal and these are equal to the rate of release of NADH from the

[9] For the SCN-NAD malic enzyme (see accompanying manuscript) the ^{13}C isotope effect decreases only slightly when deuterated malate is used with the natural substrate NAD. The ^{13}C effect decreases with L-malate-2-D relative to that with malate, but the data no longer adhere to the equation for a stepwise mechanism in which hydride transfer precedes decarboxylation (Hermes et al., 1982). With 3-APAD used as a substrate, the change in the ^{13}C effect when deuterated malate replaces protium-labeled malate is probably not significant, and thus the intrinsic secondary effect may be fully expressed for the SCN-malic enzyme (assuming a stepwise mechanism). In this case the deuterium effect is 3.3, while the ^{13}C effect is 0.5% with both unlabeled and deuterium labeled malate. Thus, the secondary ^{13}C effect may have a value between 0.5% and 1%.

[10] Personal communication from P. Tipton and J. Peisach.

E:NADH complex. The proposed mechanism is based on initial velocity studies (Park et al., 1984; Kiick et al., 1984) and the identity of the deuterium isotope effects on V, V/K_{malate}, and V/K_{NAD} (Kiick et al., 1986) as well as [^{14}C]malate and [^{14}C]NAD isotope-partitioning studies (Chen et al., 1988). Cook and Cleland (1981) have shown that finite isotope effects on the V/K values of all reactants indicate a random mechanism. Further, the difference in the values of the isotope effects reflects differences in the off-rates of the reactants from the central complex with the larger isotope effect indicating a faster off-rate. As can be seen in Tables IV and V, finite isotope effects are observed on both V/K values in all cases indicating that the kinetic mechanism does not change with a change in the dinucleotide substrate. In all cases in which the isotope effects on V/K are not equal, the kinetic mechanism must be steady-state random since at least the substrate with the smallest V/K isotope effect must be sticky. (Stickiness indicates that the reactant, once bound, partitions toward products rather than dissociating from enzyme to give free reactant.) The effects obtained with the 3-acetylpyridine derivative provide an example of such a steady-state random mechanism in which the rate of release of malate from E:dinucleotide:Mg:malate is faster than release of dinucleotide as shown by the larger value of $^{D}(V/K_{malate})$. The general equation to be considered for isotope effects to determine kinetic mechanism is

$$^{D}(V/K) = (^{D}k + c_f)/(1 + c_f) \quad (4)$$

where $^{D}(V/K)$ is the observed isotope effect, ^{D}k is the isotope effect on the sequence of steps from the substrate central complex to release of the first product, and c_f is the external commitment to catalysis or substrate stickiness factor. Equal values for $^{D}(V/K_a)$ and $^{D}(V/K_b)$ may suggest either a steady-state random mechanism in which the off-rates for the two reactants from the central complex are equal or a rapid equilibrium random mechanism. If the V effect is larger than either of the V/K effects, the mechanism must be steady-state random with both substrates exhibiting stickiness. In the case where Mg^{2+} and NAD are used, ^{D}V, $^{D}(V/K_{NAD})$, and $^{D}(V/K_{malate})$ are 1.5, and c_f has been determined to be 1.0 on the basis of isotope-partitioning studies of Chen et al. (1988). The maximum observable isotope effect in the absence of substrate stickiness (^{D}k) is then calculated from eq 4 as 2.0. If this holds for all dinucleotides (and it may not if the intrinsic isotope effect is very different), the mechanism is also likely to be steady-state random when ϵ-NAD, thio-NAD, and NADP are the dinucleotide substrates because of the small isotope effects observed. If the $^{D}(V/K)$ values are equal to ^{D}k, the maximum observable isotope effect, the mechanism is rapid equilibrium random. Thus, the remaining cases in which $^{D}(V/K_{malate})$ and $^{D}(V/K_{DINUC})$ are equal could be either steady-state or rapid equilibrium random since the isotope effects are around 2–3 (the calculated maximum observable value with NAD as the substrate).

There are a number of cases in which the isotope effect on V_{max} is lower than the isotope effect on V/K; probably indicating that the off-rate for reduced dinucleotide partially limits the overall reaction. There is additionally one case, that of 3-APAD, in which the value of ^{D}V is greater than either of the $^{D}(V/K)$ values, indicating that substrates are stickier than any of the products. Interestingly, in this case, the V is 2.4 times that obtained with NAD, which is also consistent with a rapid product off-rate.

The dissociation constants for malate (K_{Ib}) and dinucleotide (K_{Ia}) from E:dinucleotide:Mg:malate can also be calculated from the isotope-effect data and the values for K_{malate} and K_{DINUC} (Klinman & Matthews, 1985). These values are listed in Table II. The dissociation constants for the dinucleotide substrates are all around 10 μM except that for 3-APAD, which is 2 μM. The dissociation constant for malate, however, changes dependent on the nature of the dinucleotide substrate. Thus, it appears that, dependent on the conformation change elicited upon binding of the dinucleotide substrate, malate binds with differing affinity, binding with highest affinity when either 3-APAD or 3-PAAD is bound to enzyme.

Although hydride transfer is a slow step when NAD, ϵ-NAD, and NADP are used as substrates, it is fast relative to decarboxylation. The relative rates are based on a comparison of the observed deuterium and ^{13}C isotope effects with their corresponding intrinsic isotope effects. The observed ^{13}C effect of 3.4% is larger compared to the intrinsic value of about 5% than is the observed deuterium isotope effect of 1.5–1.7 compared to the intrinsic value of >5. As thio-NAD, 3-APAD, and 3-PAAD are substituted for NAD, the relative rates of the reverse hydride transfer and decarboxylation steps are changed with reverse hydride transfer becoming slower than decarboxylation. This change is evidenced by the increase in the observed deuterium effect to a value of 3 with a concomitant decrease in the ^{13}C effect to a value of 1.0028. The change in the relative rates of the two steps is fully consistent with a two-step chemical mechanism in which the two isotope effects reflect different steps. It is likely due to a change in the partition ratio of the oxalacetate intermediate formed after the hydride transfer step with the intermediate partitioning more toward the formation of pyruvate and CO_2 in the case of the more oxidizing dinucleotide substrates.

The increase in the deuterium and ^{13}C isotope effects with NADP as the dinucleotide substrate likely reflects an increase in the rate limitation of the chemical segment of the mechanism, as a result of a decrease in the forward commitment factor c_f. As discussed previously (Cook & Cleland, 1981), c_f can be divided into an internal (c_{fin}) and an external (c_{fex}) portion, where the external commitment is the part of the expression that disappears when the off-rate for reactant becomes infinite. An estimate of the external commitment can be obtained from the change in deuterium isotope effect according to eq 4. The value for $^{D}(V/K_{malate})$, obtained with NADP, can be considered the value observed when c_{fex} is zero. Substituting 1.67 for ^{D}k and 1.45 for $^{D}(V/K)$ in eq 4 gives the calculated value of c_f of 0.5. This value is in good agreement with the value of 0.9 determined from isotope-partitioning studies (Chen et al., 1988).

Kinetic Mechanism for NADP-Malic Enzyme. The kinetic mechanism for the NADP-malic enzyme was postulated to be ordered sequential with NADP adding prior to malate (Hsu et al., 1967), but the data obtained by these workers really only demonstrated ordered release of products and are equally consistent with random addition of NADP and malate. The ordered mechanism was further supported by data of Schimerlik and Cleland (1977a), which showed mesotartrate to be an uncompetitive inhibitor vs NADP. There may be much more synergism in the binding of mesotartrate and NADP than in the case of malate and NADP, as the result of steric hindrance from the extra hydroxyl group in tartrate. In any case, since finite isotope effects are observed on the V/K values for both malate and NADP (Table V), the kinetic mechanism for the addition of these substrates to NADP-malic enzyme is random. In agreement with this, the NADP-malic enzyme has also been shown to catalyze the metal-dependent decarboxylation of oxalacetate in the absence of NADP (Park et al., 1986).

In the case of 3-APADP, the isotope effect on V is lower than the isotope effect on either V/K, probably a result of the off-rate for the reduced dinucleotide partially limiting the overall rate. For thio-NADP, the isotope effects on V and the reactant V/K's are equal. Thus, the rates of release of malate and the dinucleotide from the E:dinucleotide:Mg:malate complex and the rate of release of the reduced dinucleotide from the E:(reduced dinucleotide) complex are all either equal or much greater than the catalytic steps. There is a switch in the relative rates of reverse hydride transfer and decarboxylation as the dinucleotide substrate is changed from NADP to thio-NADP and 3-APADP as was also observed for the NAD-malic enzyme. The deuterium effect increases from about 1.5 to 3 as the ^{13}C effect decreases from 3.4% to 0.5%. Thus, with NADP, reverse hydride transfer is faster than decarboxylation while the opposite is true with the other dinucleotide substrates.

For NADP and 3-APADP, where $^{D}(V/K)$ values are not equal, the dissociation constants for dinucleotide (K_{Ia}) and malate (K_{Ib}) from the E:dinucleotide:Mg:malate complex are calculated according to Klinman and Matthews (1985). Affinities are very similar for all of the dinucleotides studied, but the affinity for malate decreases as the dinucleotide substrate is changed from NADP to 3-APADP to thio-NADP.

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