Biochemistry

Volume 16, Number 4 February 22, 1977

Inhibition and Alternate-Substrate Studies on the Mechanism of Malic Enzyme†

Michael I. Schimerlik‡ and W. W. Cleland*

ABSTRACT: A number of dead-end inhibitors and alternate substrates were examined to gain an understanding of the substrate specificity and mechanism of malic enzyme. Comparison of K_i values for competitive inhibitors suggested that binding of the 1-carboxyl of L-malate is by ion pairing with lysine or arginine, while binding of the 4-carboxyl is weaker, and probably of the induced-dipolar type. The 2-hydroxyl hydrogen bonds to a catalytic group, which, when it is protonated, adsorbs the keto form of oxalacetate. Since the only molecule other than L-malate that is oxidized is L-malate-β-amide, carbon 4 must be trigonal for substrate activity, although a tetrahedral carbon bearing one or two hydroxyl groups gives good binding. Hydroxy groups at carbon 3 contribute to binding, but prevent substrate activity. Hydroxy and ketomalonates are bound more strongly than any of the four carbon acids, suggesting that the latter are bound with some strain. In inhibition studies, pyruvate analogues were competitive vs. pyruvate but noncompetitive vs. malate, while malate analogues were competitive vs. malate and noncompetitive vs. pyruvate. These compounds thus bind to both enzyme-triphosphopyridine nucleotide (E-TPN) and enzyme-reduced triphosphopyridine nucleotide (E-TPNH), but only malate analogues prevent release of TPN, while pyruvate analogues prevent release of TPNH. Ketomalonate and oxalacetate, both of which are slowly reduced by the enzyme in the presence of TPNH and thus must combine in the keto form with E-TPNH, appear to combine with E-TPN mainly in the *gem*-diol (or for oxalacetate, also the enol) form. The substrate for the decarboxylation of oxalacetate at pH 4.5 is the keto form.

Pigeon liver malic enzyme isolated in crystalline form by Hsu and Lardy (1967) catalyzes the following reactions (Hsu, 1970; Tang and Hsu, 1973):

$$\text{TPN}^{1} + \text{L-malate} \overset{Mg^{2+}\text{ or }Mn^{2+}}{\rightleftharpoons} CO_2 + \text{pyruvate} + \text{TPNH} \quad (1)$$

$$\text{(keto)oxalacetate} \xrightarrow[\text{TPN}]{Mg^{2+}\text{ or }Mn^{2+}} CO_2 + \text{pyruvate} \quad (2)$$

$$\text{TPNH} + \alpha\text{-keto acid} \xrightarrow{Mg^{2+}\text{ or }Mn^{2+}} \alpha\text{-hydroxy acid} + \text{TPN} \quad (3)$$

Reaction 1 occurs at neutral and high pH (reported pH optimum, 7.5 (Veiga Salles and Ochoa, 1950)), and on the basis of product inhibition patterns has an ordered kinetic mechanism, with TPN adding before malate, and products released in the order CO_2, pyruvate, and TPNH (Hsu et al., 1967). Reaction 2, on the other hand, occurs only at low pH with a reported pH optimum of 4.5 (Veiga Salles and Ochoa, 1950), and is activated by TPN as well as showing a metal requirement. The best substrate for reaction 3 is oxalacetate (V_{max} about 10% that of the reverse of reaction 1), with pyruvate and ketomalonate an order of magnitude slower, and other keto acids slower yet; the pH optimum is reported to be 6.5 (Tang and Hsu, 1973).

The reciprocal pH behavior of reactions 1 and 2 suggests that reaction 1 may be catalyzed when a catalytic group on the enzyme is ionized, while reaction 2 is catalyzed when it is protonated. The present work was undertaken to test this concept, and, at the same time, to determine the chemical mechanism of the catalytic reaction by careful examination of the pH variation of the kinetic parameters. In order to interpret pH profiles, however, it is necessary not only to know the kinetic mechanism, but also which steps in it are rate limiting under all conditions. In the present paper, we report experiments with alternate substrates and inhibitors which determine the specificity of binding to the enzyme. In the second paper in a series of three in this issue, we present results with inhibitors and isotope effects which determine the rate-limiting steps, and in the third paper in a series of three in this issue, we

† From the Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706. *Received February 19, 1976.* Supported by grants from the National Science Foundation (GB27407) and the National Institutes of Health (GM18938). A preliminary report on a part of this work has been presented (Schimerlik and Cleland, 1975).

‡ Supported in part by National Institutes of Health Training Grant GM00236 BCH from the National Institute of General Medical Sciences.

[1] Abbreviations used are: TPN, triphosphopyridine nucleotide; TPNH, reduced TPN; NMR, nuclear magnetic resonance.

TABLE I: Kinetic Constants at pH 7.2.[a]

Maximum velocity, forward direction (V_1)	26 ± 3 μmol min^{-1} mg^{-1} [b]
Maximum velocity, reverse direction (V_2)	7.8 ± 0.3 μmol min^{-1} mg^{-1} [b]
TPN, Michaelis constant (K_a)	4.1 ± 0.4 μM[b]
TPN, dissociation constant (K_{ia})	5.9 ± 0.5 μM[b]
L-Malate, Michaelis constant (K_b)	0.29 ± 0.02 mM[b]
L-Malate, dissociation constant (K_{ib})	1.6 ± 0.2 mM[c]
CO_2, Michaelis constant (K_p)	0.45 ± 0.10 mM[b]
CO_2, dissociation constant (K_{ip})	2.6 ± 0.5 mM
Pyruvate, Michaelis constant (K_q)	11.2 ± 0.1 mM[b]
Pyruvate, dissociation constant (K_{iq})	52 ± 8 mM[b]
TPNH, Michaelis constant (K_r)	3.3 ± 1.1 μM
TPNH, dissociation constant (K_{ir})	0.83 ± 0.05 μM

[a] 20 mM $MgSO_4$. For buffer used, see Methods. [b] Weighted mean of three experiments. [c] Weighted mean of two experiments.

present the pH profiles and a proposed chemical mechanism for malic enzyme.

Materials and Methods

Pigeon liver malic enzyme was isolated by the procedure of Hsu and Lardy (1967). Substrates and inhibitors were purchased from Sigma, unless noted otherwise. DL-Malate-β-amide was synthesized from bromosuccinate by the method of Lutz (1902), mp 145–149 °C (reported 149 °C). Thin-layer chromatography on cellulose plates (Eastman) in 10:2 1-butanol (saturated with H_2O)–formic acid showed one spot, R_f = 0.1, compared to R_f values of 0.34 and 0.80 for L-malate and bromosuccinate. Methylation with CH_2N_2 followed by gas chromatography showed no trace of the dimethyl ester of L-malate and the NMR spectrum was consistent with the structure for the β-amide. Oxalacetate-β-amide was a gift from Dr. Roland Barden.

L-Malate-β-semialdehyde was synthesized by periodate oxidation of β-glucometasaccharinic acid, which was a gift of Dr. R. M. Rowell of the Forest Products Laboratory, Madison, Wis. Periodate disappearance, followed at 222.5 nm, was complete in 2 h with approximately 2 mol of IO_4 destroyed/mol of β-glucometasaccharinic acid, as expected. An NMR spectrum of the products indicated only a low percentage of aldehydic protons, but the expected amount of formate. 2,4-Dinitrophenylhydrazone (mp 110–4 °C, dec) and semicarbazide (mp 178–81 °C) derivatives formed very slowly, and thus all data suggest that the compound exists in solution mainly as the *gem*-diol (L-2,4,4-trihydroxybutyrate). Both β-glucometasaccharinic acid and the reaction products of $NaIO_4$ plus an excess of ethylene glycol had K_i values as inhibitors vs. malate in excess of 100 mM. L-Malate-β-semialdehyde was reduced with a 50-fold excess of $NaBH_4$ to give L-2,4-dihydroxybutyrate.

All inhibition patterns were run with a buffer 25 mM each in acetic acid, glycine, cacodylic acid, and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes) adjusted to pH with KOH. This buffer, which was chosen for the pH studies reported in the third paper of this series (Schimerlik and Cleland, 1977), chelates metal very weakly (apparent dissociation constant at pH 7 for chelation of Mn^{2+} was 28 mM from an NMR experiment carried out by Dr. W. H. Orme-Johnson, and the binding of Mg^{2+} is certainly less than this), and does not show appreciable inhibition of the reaction. The $MgSO_4$ concentration was 20 mM for all experiments.

The reactions were followed using a Beckman DU monochrometer with deuterium lamp, a Gilford optical density converter and a 10 mV recorder with adjustable zero and multispeed drive. Full scale sensitivity of 0.05 to 0.10 OD and a chart speed of 0.4 to 3.0 in./min were used. The cell compartment was maintained at 25 °C with thermospacers and the reaction was started by addition of enzyme via an adder mixer to the 1.0-cm cuvets, containing 3.0 ml, which were preequilibrated to 25 °C.

Data Processing. The nomenclature used in this paper is that of Cleland (1963a). Reciprocal velocities were plotted vs. reciprocal substrate concentrations and all plots were linear. Data conforming to linear competitive, linear noncompetitive, and linear uncompetitive inhibitions were fitted to eq 4, 5, and 6, while sequential initial velocity data were fitted to eq 7, and single reciprocal plots to eq 8.

$$v = \frac{VA}{K(1 + (I/K_{is})) + A} \quad (4)$$

$$v = \frac{VA}{K(1 + (I/K_{is})) + A(1 + (I/K_{ii}))} \quad (5)$$

$$v = \frac{VA}{K + A(1 + (I/K_{ii}))} \quad (6)$$

$$v = \frac{VAB}{K_{ia}K_b + K_aB + K_bA + AB} \quad (7)$$

$$v = VA/(K + A) \quad (8)$$

In eq 7, K_a and K_{ia} are the Michaelis and dissociation constants for A and K_b the Michaelis constant for B. In the other equations, K, K_{is} and K_{ii} are the apparent Michaelis, and slope and intercept inhibition constants. V is the maximum velocity.

Experimental data were fitted to eq 4–8 by the least-squares method assuming equal variances for the velocities (Wilkinson, 1961), using a digital computer and the Fortran programs of Cleland (1963b, 1967). The points in the reciprocal plots are the experimentally determined values, while the lines are calculated from the fits of these data to the appropriate rate equation.

Data for double-inhibition experiments were fitted to eq 5 by letting I equal TPN reciprocal concentration and A equal the reciprocal of the other inhibitor concentration. $1/K$ then equals the K_i value of the inhibitor in the absence of added TPN and $K_{is}/(K_{ii}K)$ equals the K_i of the inhibitor at infinite TPN.

Results

Kinetic Parameters. In order to have a full set of kinetic parameters with which to make calculations, the kinetic constants were determined at pH 7.1 in the presence of 20 mM $MgSO_4$ (Table I). The Michaelis constant for L-malate (K_b), dissociation and Michaelis constants for TPN (K_{ia}, K_a), and maximum velocity in the forward direction (V_1) were determined from fits to eq 7. TPNH was a competitive inhibitor vs. TPN at 333 μM malate, and a fit of the data to eq 4 gave the dissociation constant for TPNH (K_{ir}). The Michaelis and dissociation constants for pyruvate (K_q, K_{iq}), the Michaelis constant for CO_2 (K_p), and the maximum velocity in the reverse direction (V_2) were determined by fitting initial-velocity patterns for pyruvate and CO_2 at 200 μM TPNH to eq 7. The Michaelis constant for TPNH (K_r) was determined from a fit of the initial velocity pattern for TPNH and pyruvate at 5 mM CO_2 to eq 7. The dissociation constant for CO_2 (K_{ip}) was

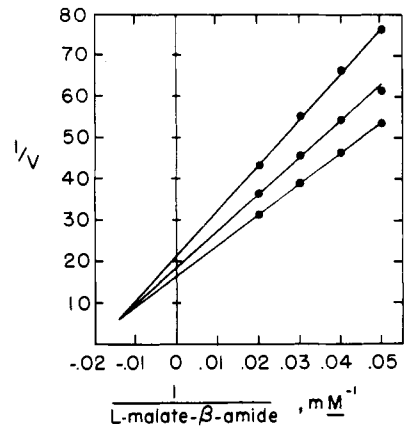


FIGURE 1: L-Malate-β-amide oxidation by TPN at pH 8.0, 5 mM $MgSO_4$, 0.07 unit/ml of malic enzyme. From top to bottom: 30, 60, 200 μM TPN. Data were fitted to eq 7.

calculated from the apparent K_{ii} from a fit of eq 5 of the noncompetitive inhibition of CO_2 vs. malate at 333 μM TPN according to eq 9.

$$K_{ip} = \frac{V_1 K_q (\text{app } K_{ii})}{V_2 K_{iq}} \quad (9)$$

The dissociation constant of L-malate (K_{ib}) was calculated from the apparent K_{ii} for the noncompetitive inhibition of malate vs. CO_2 according to eq 10 (Cleland, 1963a), where Q and R are pyruvate and TPNH concentrations.

$$K_{ib} = \frac{V_2 K_a (\text{app } K_{ii})}{V_1 K_{ia}(K_q K_{ir}/QR + K_r/R + K_q/Q + 1)} \quad (10)$$

The internal consistency of these kinetic constants can be checked using the Haldane relationships for an ordered Bi-Ter mechanism (Cleland, 1963a):

$$K_{eq} = \frac{K_{ip} K_{iq} K_{ir}}{K_{ia} K_{ib}} = \frac{V_1 K_p K_{iq} K_{ir}}{V_2 K_{ia} K_b} \quad (11)$$

The kinetic constants in Table I give values for K_{eq} of 12 ± 0.3 and 45 ± 14 mM, respectively, which agree reasonably well with the experimentally determined value of 30 ± 2 mM (Schimerlik et al., 1975).

Alternate Substrates. The only substrate other than L-malate that was found to be oxidized by TPN was malate-β-amide (eq 12).

$$\text{TPN} + \text{L-malate-}\beta\text{-amide} \overset{Mg^{2+}}{\rightleftharpoons} \text{oxalacetate-}\beta\text{-amide} + \text{TPNH} \quad (12)$$

The initial-velocity pattern was sequential at pH 8 (Figure 1), and gave a K_m for DL-β-amide of 44 ± 4 mM, and a V 16% that for the oxidation of malate.[2] Metal ion was required for this oxidation. The reduction of oxalacetate-β-amide by TPNH was observed at pH 5 (slower than oxalacetate by a factor of 14), but was too slow be observed readily at pH 7.

For reaction 3 with 200 μM TPNH, oxalacetate showed a K_m of 9.8 ± 0.5 mM in the absence and 8.9 ± 0.4 mM in the presence of 2.74 mM CO_2, and nearly identical V values that were 9.3% that for pyruvate and CO_2 in the reverse of reaction 1. Under similar conditions, ketomalonate gave a K_m of 1.3 ± 0.1 mM, and a V equal to 1% of that for pyruvate and CO_2. These results agree with those of Tang and Hsu (1973), and the K_m values agree well with the inhibition constants for these molecules (see below).

TABLE II: Dissociation Constants (mM) at pH 7.2 for Competitive Inhibitors vs. L-Malate.[a]

Succinate	14.2 ± 0.5		
Fumarate	8.4 ± 0.2		
Maleate	36 ± 2		
Hydroxy acids		Keto acids	
L-Malate	1.6 ± 0.2	Oxalacetate	0.41 ± 0.01
L-Malate-β-amide	27 ± 1		
D-Malate	4.5 ± 0.2		
L-2,4,4-Trihydroxybutyrate	5.8 ± 0.2		
L-2,4-Dihydroxybutyrate	3.4 ± 0.1		
L-2-Hydroxybutyrate	11.4 ± 0.3	2-Ketobutyrate	81 ± 3
D-3-Hydroxybutyrate	204 ± 12		
4-Hydroxybutyrate	900 ± 240		
Mesotartrate	1.9 ± 0.1		
L-Tartrate	3.2 ± 0.1		
D-Tartrate	2.9 ± 0.1		
Hydroxymalonate	0.18 ± 0.01	Ketomalonate	0.073 ± 0.003

[a] K_{is} values from fits to eq 4 with 333 μM TPN and 20 mM $MgSO_4$, except for L-malate where the value is from Table I.

Active Form of Oxalacetate. The form of oxalacetate, which acts as substrate for the decarboxylation at pH 4.5 and as an inhibitor of the oxidative decarboxylation of malate at pH 7.2, was investigated by using either solid oxalacetic acid (the *trans*-enol), or a solution which had reached equilibrium among the keto, enol, and *gem*-diol forms (74, 18, and 8%, respectively (Pogson and Wolfe, 1972)). At pH 4.5 (667 μM TPN, 20 mM $MnSO_4$), 1 mM oxalacetate from the solution showed no lag in disappearance of optical density at 281 nm upon addition of enzyme, while an identical reaction mixture prepared from solid material showed a pronounced lag, but the same final velocity. Thus, the keto form appears to be the substrate for the decarboxylation reaction. At pH 7.2 (333 μM TPN, 1 mM L-malate, 20 mM $MgSO_4$), on the other hand, the K_i for oxalacetate from an equilibrated solution was 0.72 mM, while a similar reaction mixture prepared from solid material gave 0.39 mM. The *trans*-enol is thus at least a slightly stronger inhibitor than the keto form.

Inhibitors. The dissociation constants at pH 7.2 of a group of inhibitors that were all competitive vs. malate are shown in Table II. The potassium salts of a number of simple anions were also found to be competitive vs. L-malate. While Cl^-, Br^-, and SO_4^{2-} were rather weak inhibitors (K_i values of 65 ± 2 50 ± 2, and 45 ± 2 mM), NO_3^-, I^-, and CN^- were stronger (K_i values of 6.9 ± 0.3, 6.0 ± 0.5, and 4.1 ± 0.2 mM).

The inhibition by several inhibitors was investigated in more detail. Figure 2 shows the inhibition of mesotartrate vs. TPN, which is uncompetitive as expected for the ordered kinetic mechanism, and gave a K_{ii} value of 2.9 ± 0.1 mM. Bromopyruvate, mesotartrate, oxamate, ketomalonate, hydroxymalo-

[2] We have now prepared pure L- and D-malate-β-amides and shown that the D isomer has almost the same affinity as the active L one. At pH 7.4, the K_m for L-malate-β-amide is 24 mM and the V about 35% that for L-malate (Grimshaw and Cleland, unpublished results).

TABLE III: Inhibition of Forward and Reverse Reactions at pH 7.2.[a]

Inhibitor	Back-Reaction[d] vs. L-Malate[b] K_{is} (mM)	K_{ii} (mM)	vs. Pyruvate[c] K_{is} (mM)	K_{ii} (mM)	Double-Inhibition Study K_i (0 TPN) (mM)	K_i (∞ TPN) (mM)	Calcd[b] K_i (0 TPN) (mM)
Ketomalonate	0.073 ± 0.003[e]	∞	1.5 ± 0.2	2.4 ± 0.4[e]	1.43 ± 0.02	0.21 ± 0.04	0.73 ± 0.05
Hydroxymalonate	0.18 ± 0.01	∞	1.3 ± 0.1[e]	1.4 ± 0.2[e]	1.43 ± 0.04	0.40 ± 0.03	1.32 ± .07
Mesotartrate	1.91 ± 0.04	∞	15 ± 1	40 ± 8	13.5 ± 0.4	2.3 ± 0.1	14.5 ± 2.3
Oxamate	5.2 ± 0.7	28 ± 9	16.3 ± 0.8	∞	21 ± 1	3.6 ± 0.2	24.8 ± 2.0 (40 ± 2)
L-Malate-β-amide	27 ± 1	∞	57 ± 5	418 ± 210	83 ± 3	10 ± 1	99 ± 7
Bromopyruvate	3.2 ± 0.2	6.4 ± 0.4	14.6 ± 0.6	∞	29 ± 2	16 ± 2	18.9 ± 1.0 (36 ± 2)
Oxalacetate	0.41 ± 0.01	∞	7.1 ± 0.3	25 ± 2			

[a] From fits to eq 4 and 5. [b] 333 μM TPN, 20 mM $MgSO_4$. [c] 200 μM TPNH, 3.4 mM CO_2, 20 mM $MgSO_4$. [d] 20 μM TPNH, 3.4 mM CO_2, 20 mM pyruvate, 20 mM $MgSO_4$. [e] Weighted average of two experiments. [f] Values in parentheses assuming K_{ii} vs. L-malate equal to ∞.

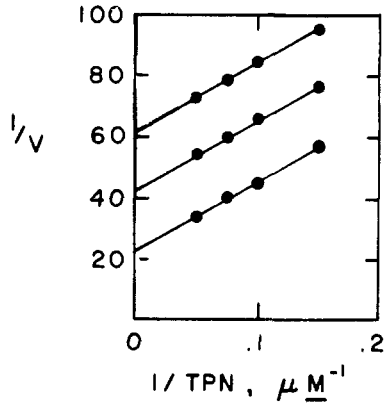


FIGURE 2: Mesotartrate inhibition vs. TPN at pH 7.2. 333 μM L-malate, 20 mM $MgSO_4$, 0.02 unit/ml of malic enzyme. From top to bottom: 5, 2.5, and 0 mM mesotartrate. Data were fitted to eq 6.

nate, and malate-β-amide were tested as inhibitors vs. L-malate and pyruvate in the forward and reverse reactions. Compounds that were competitive vs. malate were noncompetitive vs. pyruvate and vice versa. In addition, these compounds were used at varying levels of TPN in double-inhibition experiments in the back-reaction. The results from these experiments are shown in Table III, along with values for oxalacetate as an inhibitor of forward and reverse reactions.

Discussion

The Binding of Malate. The studies reported here on the binding of competitive inhibitors and alternate substrates allow several conclusions concerning the mode of binding of malate that will be important in formulating the chemical mechanism proposed in the third paper of this series (Schimerlik and Cleland, 1977). First, the fact that the keto, rather than the enol, form of oxalacetate is the substrate for the decarboxylation at low pH agrees with our tentative hypothesis that the state of protonation of the group which hydrogen bonds to the oxygen at C-2 determines whether reaction 1 or 2 occurs. Thus, when this group is ionized, it hydrogen bonds to the OH of malate, and accepts the proton during the hydride-transfer step. When this group is protonated, it binds the keto form of oxalacetate with the same geometry that normally would be present after the oxidation of malate, except that TPN, rather than TPNH, is present. This geometry obviously leads to decarboxylation, regardless which nucleotide is present.

Analysis of the numbers in Table II permits the following conclusions concerning the other binding interactions of malate to the E-TPN-Mg^{2+} complex.

(1) Comparison of succinate, fumarate, and maleate shows that an extended configuration, rather than a cis arrangement, is preferred for binding.

(2) Since the three carbon acids are the best inhibitors, the site appears to be slightly too small for four-carbon acids, and thus they are probably bound with some strain. Such strain would, of course, help promote decarboxylation.

(3) Comparison of 2-hydroxy- and 2-ketobutyrate (which does not enolize or hydrate at neutral pH) shows that a hydroxyl group at C-2 is bound seven- to eightfold more tightly than a keto group. This result is expected, since the pH studies reported in the third paper of this series indicate the group hydrogen bonding to the oxygen on C-2 has a pK of 6 (Schimerlik and Cleland, 1977), and, at pH 7.2, $^1/_{16}$ of it should be protonated, as required to bind a keto group, and $^{15}/_{16}$ should be ionized, as required to bind the hydroxyl group. This hypothesis is supported by the fact that, at pH 5, L-2-hydroxybutyrate is bound less tightly than at pH 7 (K_i of 36 ± 3 mM, as opposed to 11 mM), while 2-ketobutyrate shows the opposite effect (K_i of 10 ± 2 mM, as opposed to 81 mM) (C. E. Grimshaw, unpublished results). Comparison of keto- and hydroxymalonates, however, shows ketomalonate to be bound more strongly. This compound is about 85% hydrated to the *gem*-diol at neutral pH,[3] and thus the *gem*-diol is certainly the inhibitory form of this molecule, although the keto form is a substrate for reaction 3. A similar situation is seen for oxalacetate, which binds fourfold tighter than L-malate. In agreement with the finding that the *trans*-enol is a better inhibitor than the equilibrium mixture of keto, enol, and hydrated forms, it appears that the enol and *gem*-diol, which make up 18 and 8% of the mixture, are the inhibitory forms.

(4) Interaction of the 1-carboxyl with the enzyme appears to contribute a large part of the energy for malate binding. Thus, D-3-hydroxybutyrate, which can be visualized as L-malate with C-1 converted to a methyl group, is bound much more poorly than L-2-hydroxybutyrate, where C-4 has become a methyl group. The strong competitive binding of CN^-, I^-, and NO_3^-, which is linear, and not parabolic, also suggests a

[3] The proton-decoupled ^{13}C NMR spectrum of ketomalonate in H_2O-D_2O at pH 7.2 shows lines corresponding to carboxyl groups with C-2 hydrated or unhydrated (intensity ratio, 5.6:1), and corresponding to C-2 when hydrated. The line for C-2 in the keto form was not seen, possibly because of its long relaxation time. We thank Dr. Philip Hart for running this NMR spectrum.

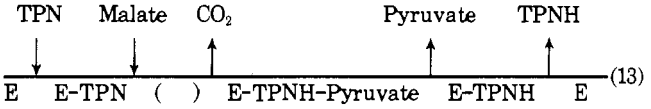
single positively charged pocket which specifically binds anions.

(5) For substrate activity, carbon 4 must be trigonal, but need not be charged (L-malate and its β-amide). When carbons 1 and 2 match L-malate, hydroxyl groups at C-4 provide more binding strength than the planar amide group, suggesting strain in the binding of the latter. This strain is probably responsible for the exact geometry which permits hydride transfer.

(6) Along with the 1-carboxyl, one needs either an L-2-hydroxy group, or a 4-carboxyl to get reasonable binding (L-2-hydroxybutyrate and succinate). The nearly equal contribution of these groups, and the fact that the charged 4-carboxyl in L-malate binds only 17-times tighter than the neutral amide, makes it very unlikely that the binding of this carboxyl is by ion-pair formation with positively charged groups, as is certainly the case for the 1-carboxyl. The most likely form of binding is induced dipolar bonding, as indicated in Figure 3, but, in the absence of further evidence, this must remain conjectural, and hydrogen bonding to hydroxyl groups of serine or threonine must remain a possibility. The amide groups shown in Figure 3 could be from the peptide backbone, or from glutamine or asparagine side chains. Figure 3 also shows the different geometry of binding that would occur with molecules where C-4 was tetrahedral, and could explain their lack of substrate activity.

(7) When both 1- and 4-carboxyls are present, a 3-hydroxy group, as in the tartrates, contributes to binding strength, but prevents catalytic activity. Hydroxyl groups in positions 3 and 4 will not by themselves promote binding, however, and only add to binding if an L-2-hydroxy or 4-carboxyl group is present.

The Kinetic Mechanism. The conclusion by Hsu et al. (1967) that the kinetic mechanism is ordered, as in mechanism 13:



(13)

was based on the product-inhibition patterns, which showed that CO_2 was noncompetitive vs. both substrates, pyruvate was uncompetitive vs. both, and TPNH was competitive vs. TPN and noncompetitive vs. malate. We have extended the studies with pyruvate and find that it is uncompetitive vs. malate over the entire pH range 3.9–9.0. When 5 μM TPNH was added ($= 6 \times K_{iTPNH}$; TPN $= 1.7 \times K_{iTPNH}$), the pattern at pH 7 became noncompetitive with no change in K_{ii}, as predicted by the rate equation for an ordered Bi-Ter mechanism (Cleland, 1963a). Thus, pyruvate seems to combine only with E-TPNH, and not with either free enzyme or E-TPN, and this part of the mechanism is clearly ordered.

Whether malate can combine with E-TPNH and free enzyme is not so clear. Since it shows no substrate inhibition up to 0.5 M when ionic strength and ion concentrations are properly controlled, any combination with E-TPNH must not prevent or slow down TPNH release. Hsu et al. (1976), using NMR techniques, claim that L-malate binds to free enzyme, E-Mn^{2+}, and E-TPNH with a dissociation constant around 1 mM, but to E-TPNH-Mn^{2+} with a K_i of 32 μM. If these data are correct and apply to binding at functional active sites, the tighter binding in the E-TPNH-metal complex must involve for TPNH an increase in the bimolecular rate constant for combination, rather than a decrease in the unimolecular constant for dissociation. Otherwise, malate would slow down

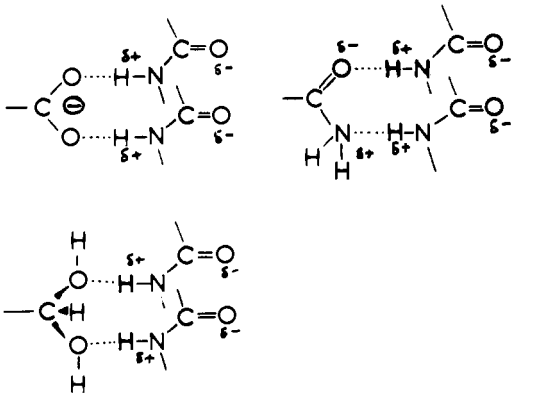


FIGURE 3: Possible mode of binding of carbon 4 of L-malate (upper left), L-malate-β-amide (upper right), and L-2,4,4-trihydroxybutyrate (lower left).

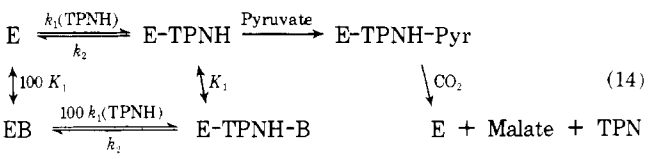
TPNH release, in conflict with the kinetic evidence.

However, the binding studies of Hsu et al. (1976) were done in the presence of only enough Mn^{2+} to saturate half of the subunits, and thus may reflect interactions which do not occur on the other two subunits when metal ions are adsorbed on them also. (Thus, Hsu et al. (1976) reported pyruvate binding to E-TPN and free enzyme, both in the presence and absence of Mn^{2+}; these interactions do not show up in the kinetics.) The kinetics of Mn^{2+} (but not Mg^{2+}) show extreme negative cooperativity (Hsu et al., 1976; Schimerlik et al., 1977), with V_{max} about tenfold higher when all four sites are filled than when just the tight sites are filled, but with a K_m of 4–5 mM for the weak sites and as low as 2 μM for the tight ones. The binding studies indicate that two subunits of the tetramer show the tight binding, and the other two the weak binding. Since the metal-ion kinetics with Mg^{2+} do not show appreciable negative cooperativity, the binding behavior of interest in the presence of Mn^{2+} would be that to the third and fourth subunits when they too contained, or did not contain, Mn^{2+}.

The inhibition patterns seen in the present work bear on these questions. Hydroxy acids (or hydrated keto acids) were competitive vs. malate but noncompetitive vs. pyruvate in the reverse direction. Oxamate and bromopyruvate, on the other hand, were competitive vs. pyruvate, and noncompetitive vs. malate. These patterns are somewhat surprising, since if hydroxy acids combined only with E-TPN, as suggested by the uncompetitive inhibition of mesotartrate vs. TPN, they should be uncompetitive vs. pyruvate. Conversely, if they combined with E-TPNH as well as E-TPN they should give noncompetitive inhibition vs. both malate and pyruvate. Only if the steady-state level of E-TPNH was very low when malate and TPN were saturating would the observed patterns be expected. The rough equality of the K_{is} vs. pyruvate and K_{ii} vs. malate for oxamate and bromopyruvate (Table III) and other more definite data presented in the following paper of this issue (Schimerlik et al., 1977) show, however, that at pH 7.2 TPNH release is solely rate limiting, and the enzyme is all E-TPNH when the substrates are saturating.

We are thus led to conclude that hydroxy acids can combine with E-TPNH, but fail to inhibit the forward reaction at high TPN and malate levels. This could be for two reasons. First, if malate can also combine with E-TPNH, saturating malate would prevent the inhibitors from combining. This explanation then requires that malate not slow down TPNH release. Second, the inhibitors could combine with E-TPNH, but malate could not, and in this case the inhibitors must not slow down

TPNH release. A simple model which explains such a situation is mechanism 14,



(14)

where B is L-malate, or another hydroxy acid inhibitor, and K_i is the dissociation constant of B from E-TPNH-B. As long as the rate of dissociation of EB is considerably greater than k_2, which would certainly be true, the presence of B on the enzyme would not affect the rate of release of TPNH when it was a product. When TPNH was a substrate, however, B would give a slope effect when pyruvate was varied. Since the dissociation constant of EB is 100-fold greater than that of E-TPNH-B, B would appear not to combine with free enzyme.[4]

What then is different when hydroxy acid inhibitors combine with E-TPN, as opposed to TPNH? In the reverse reaction, the noncompetitive patterns vs. pyruvate show that combination with E-TPN does slow down TPN release, although possibly to different extents. The ratio of the K_{ii} vs. pyruvate to the K_{is} vs. malate (Table III) is 8, 15, 21, 33, and 61 for hydroxymalonate, malate-β-amide, mesotartrate, ketomalonate, and oxalacetate. It is possible to calculate the proportion of enzyme present as E-TPN when pyruvate is saturating from the kinetic constants in Table I and the distribution equations given by Cleland (1963a). The resulting value of 0.16 predicts (K_{ii} vs. pyruvate)/(K_{is} vs. malate) ratios of 6.4, but, as discussed by Cleland (1963a), this calculation may not give the correct value if E-TPN isomerization is an obligatory step in the mechanism. The observed ratios for hydroxymalonate and malate-β-amide are certainly consistent with combination of these molecules with E-TPN in a way that prevents TPN release. For the others, the higher ratios may reflect: (a) experimental errors, (b) effects by molecules present at the other subunits on the amount of E-TPN in the steady state as the result of cooperativity between subunits, or (c) slowing down of TPN release, without preventing it totally. In this case, hyperbolic inhibition is predicted for the intercepts, but this has not been observed.

In summary, the inhibition patterns in Table III show that hydroxy- and carbonyl-containing compounds combine with both E-TPN and E-TPNH, but only hydroxy compounds prevent TPN release from the enzyme and only carbonyl compounds prevent TPNH release. This interesting specificity is clearly the basis for the ordered kinetic mechanism. The nature of the conformation changes involved and the reason for the substrate specificity require x-ray work for understanding, and, since the enzyme crystallizes as an E-TPN complex (Hsu and Lardy, 1967), this may be an ideal case for such study.

[4] Although one might expect that the presence of B would alter k_2, and not k_1 (and this is certainly what occurs when B adds to E-TPN), the observed V/K values for the nucleotides calculated from the constants in Table I are not limited by diffusion, and could be increased as postulated here. The increased value of k_1 when B is present would come from B maintaining the conformation of the TPNH site which is exactly complementary to TPNH, with providing any steric hindrance to TPNH combination. When B combines with E-TPN, however, the contact of TPN and B is apparently so intimate that B prevents TPN release, and might, in fact, even make its combination with EB slower than combination with E, although to a lesser extent than it lowers the rate constant analogous to k_2.

The double-inhibition experiments, reported in columns 6 and 7 of Table III, provide a further check on the above conclusions. Since at infinite TPN all of the enzyme should be E-TPN, the values in column 7 should equal those in column 2. This is true within a factor of 2 or 3 for all inhibitors, except bromopyruvate, and, since the two values being compared are determined by entirely different sorts of experiments in forward and reverse directions, the agreement is satisfactory. For comparison with the K_i values in column 6 which correspond to zero added TPN, however, we must consider that the concentrations used were not totally saturating, and thus that the actual distribution among the enzyme forms must be computed and taken into account. The steady-state distribution calculated from the equations on pages 130–131 of Cleland (1963a) with the constants in Table I is: free E, 9%; E-TPNH, 40%; E-TPNH-pyruvate, 6%; central complexes, 37%; E-TPN, 8%. In the presence of an inhibitor, the amount of E-TPN-I will be $[I]/K_{i1}$ times the concentration of E-TPN and the amount of E-TPNH-I will be $[I]/K_{i2}$ times E-TPNH, where K_{i1} and K_{i2} are dissociation constants from E-TPN and E-TPNH. One can then calculate the apparent K_i in millimolar units expected from an inhibitor as:

$$\text{app } K_i = \frac{1}{(1/2.48K_{i2}) + (1/12.4K_{i1})} \qquad (15)$$

where the factors 2.48 and 12.4 are reciprocals of the fraction of enzyme in the E-TPNH and E-TPN form. Apparent K_i's calculated in this way using the K_{is} values in column 2 and 4 of Table III as K_{i1} and K_{i2} are shown in column 8 of Table III. For oxamate and bromopyruvate, the calculation was also made assuming K_{i1} to be infinite, since these molecules are competitive vs. pyruvate and would not be expected to prevent TPN release. The agreement is quite good, and the observed values are equal to or less than the calculated ones in all cases, except possibly for ketomalonate.

References

Cleland, W. W. (1963a), *Biochim. Biophys. Acta 67,* 104.

Cleland, W. W. (1963b), *Nature (London) 198,* 463.

Cleland, W. W. (1967), *Adv. Enzymol. 29,* 1.

Hsu, R. Y. (1970), *J. Biol. Chem. 245,* 6675.

Hsu, R. Y., and Lardy, H. A. (1967), *J. Biol. Chem. 242,* 520.

Hsu, R. Y., Lardy, H. A. and Cleland, W. W. (1967), *J. Biol. Chem. 242,* 5315.

Hsu, R. Y., Mildvan, A. S., Chang, G.-G., and Fung, C.-H. (1976), *J. Biol. Chem. 251,* 6574.

Lutz, O. (1902), *Ber. 35,* 2460.

Pogson, C. I., and Wolfe, R. G. (1972), *Biochem. Biophys. Res. Commun. 46,* 1048.

Schimerlik, M. I., and Cleland, W. W. (1975) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 34,* 495.

Schimerlik, M. I., and Cleland, W. W. (1977), *Biochemistry 16* (third paper in a series of three in this issue).

Schimerlik, M. I., Grimshaw, C. E., and Cleland, W. W. (1977), *Biochemistry 16* (second paper in a series of three in this issue).

Schimerlik, M. I., Rife, J. E., and Cleland, W. W. (1975), *Biochemistry 14,* 5347.

Tang, C. L., and Hsu, R. Y. (1973), *Biochem. J. 135,* 287.

Veiga Salles, J. B., and Ochoa, S. (1950), *J. Biol. Chem. 187,* 849.

Wilkinson, G. N. (1961), *Biochem. J. 80,* 324.