Kinetic Evidence for the Dimerization of the Triphosphopyridine Nucleotide-dependent Isocitrate Dehydrogenase from Pig Heart*

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In agreement with the results of physicochemical studies (Kelly, J. H., and Plaut, G. W. E. (1981) J. Biol. Chem. 256, 330-334), the kinetic properties of crystalline TPN-specific isocitrate dehydrogenase from porcine heart correspond to those of a dimeric enzyme. Investigations of the enzyme, mainly by the Kurganov enzyme dilution approach (Kurganov, B. I. (1967) Mol. Biol. (Mosc.) 1, 17-27), revealed that the dimeric enzyme form is active and the monomer is inactive. These results with TPN-isocitrate dehydrogenase from pig heart concur with those reported for the enzyme from bovine liver (Carlier, M. F., and Pantaloni, D. (1978) Eur. J. Biochem. 89, 371-378). Under conditions where the monomeric form became predominant (e.g. at low enzyme concentrations), the specific activity of the enzyme declined, kinetic properties indicated an ordered rapid equilibrium mechanism with magnesium isocitrate binding to enzyme before TPN+, and, under presteady state conditions, inhibition by dL-threo-a-methylisocitrate (3-hydroxy-1,2,3-butanetricarboxylate) was of the tight binding type with the value of Ki approaching zero when TPN+ was saturating. In double inhibitor experiments with the reaction in the direction of reductive carboxylation of a-ketoglutarate, the results were consistent with mutual facilitation of the binding to monomer of a-methylisocitrate and TPN+. When the dimeric form was prevalent, the specific activity of the enzyme was maximal, the order of addition of substrates was random in accord with previous studies (Uhr. M. L., Thompson, V. W., and Cleland, W. W. (1974) J. Biol. Chem. 249, 2920-2927), and inhibition by a-methylisocitrate was competitive with isocitrate (Ki ~ 0.2 mM) and noncompetitive with TPN+. This monomer-dimer system, with two different kinetic mechanisms, can be described within the confines of Frieden's hysteretic enzyme concept (Frieden, C. (1970) J. Biol. Chem. 245, 5788-5799) in which the monomer-dimer equilibrium is dynamic and responsive to conditions which affect this equilibrium. A model has been proposed in which the rate-limiting step in the ordered rapid equilibrium mechanism is determined by the rate of conversion of the substrate-bound monomer form to the corresponding dimer form before catalysis ensues, and the steady state equilibrium position of the free monomer and dimer forms determines the overall rate of catalysis and the kinetic mechanism. In confirmation of this model, at low enzyme concentrations (monomer prevalent), the addition of a ligand favoring displacement of the monomer-dimer equilibrium toward dimer (e.g. TPNH or EDTA) enhanced the rate of catalysis, shifted the mechanism from the ordered rapid equilibrium to the random mechanism mode, and eliminated the tight binding inhibition by a-methylisocitrate.

The kinetics of the TPN-linked isocitrate dehydrogenase from pig heart has been studied in considerable detail. Uhr et al. (1) and Northrup and Cleland (2) have made extensive kinetic studies of this enzyme including product inhibition, dead-end inhibition, and isotope exchange experiments showing that the enzyme had a random kinetic mechanism. O'Leary and Limburg (3) performed isotope effect experiments which supported the results of Uhr et al. (1) and postulated that TPNH release was the rate-determining step. In contrast to this work, Ehrlich and Colman (4) published direct TPN+ binding studies which showed that the Ki for the coenzyme was at least 50 times the kinetic constant, ruling out a random mechanism or one in which coenzyme was bound before magnesium isocitrate. A preliminary report from this laboratory (5) indicated that the enzyme might follow an ordered rapid equilibrium mechanism, with magnesium isocitrate binding first.

Physical evidence was presented in the accompanying paper (6) that pig heart TPN-dependent isocitrate dehydrogenase is a dimeric enzyme. The results of kinetic experiments presented here are in agreement with these physicochemical studies, showing that the monomer is inactive and that the dimer is the catalytically competent species in the oxidative decarboxylation of isocitrate. A model based on this dimeric structure is presented which accommodates both a random and an ordered rapid equilibrium kinetic mechanism, one or the other of the two mechanisms being favored by the concentration of the enzyme or by the presence of certain ligands, such as TPNH, or EDTA.

EXPERIMENTAL PROCEDURES

Materials—Many of the materials and the preparation of crystalline TPN-linked isocitrate dehydrogenase from porcine heart were as described in the preceding paper (6). Crystalline enzyme was used in the Kurganov type (7) dilution experiments as well as the tight binding studies with inhibitor. A suspension of crystalline enzyme in neutralized 60% saturated (NH4)2SO4 was centrifuged for 2 min in a Brinkmann microcentrifuge (type 3200) and the pellet was dissolved in 1.33 mM MgSO4 and 166 mM Na-Hepes at pH 7.4 and stored in ice. TPN-linked isocitrate dehydrogenase purchased from Sigma Chemical Co. (type IV) was used in the steady state experiments. This enzyme preparation was diluted 1/10 in 166 mM Na-Hepes at pH 7.4 before use and was stored in ice. dL-threo-a-Methylisocitric lactone was synthesized according to Plaut et al. (8). a-Methylisocitric lactone and dL-isocitric lactones were hydrolyzed as described by Chen.

1 The abbreviation used is: Hepes, 4(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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and Plaut (9). Solutions of DL-isocitrate could be stored frozen indefinitely but α-methylisocitrate tends to racemize and was freshly hydrolyzed each day.

Salt-free, lyophilized diaphorase (Worthington Biochemicals) from Clostridium kluyveri was dissolved in 166 mM Hepes at pH 7.4 and stored in ice. Fresh solutions were prepared daily. This enzyme was assayed in a solution of 90 μM TPNH and 29 μM 2,6-dichlorophenol indophenol as recommended by Worthington Biochemicals. A unit of diaphorase, as defined by Worthington, equals the amount which causes an absorbance change of -1.0/min at 25°C in a 1-cm cuvette, monitoring the reaction at 600 nm. Resazurin (K and K/ICN Laboratories) was dissolved in 1 n NaOH, neutralized with an equal amount of 1 n HCl, and passed through a Millipore HA 0.45 μm filter. The filter retained an orange substance, possibly resorufin, which greatly reduced the fluorescence background. Fresh solutions were made daily.

Assays—All steady state and Kurganov (7) type kinetic studies were carried out in thermostated cell blocks at 25°C. The stopped flow, tight binder studies were done at room temperature.

A Gilford model 240 recording spectrophotometer equipped with an automatic sample changer was used in the Kurganov (7) type studies to follow absorbance changes at 340 nm. Steady state experiments were performed in a filter fluorimeter (excitation at 340 nm, emission at 440 nm) built by the machine shop of the Johnson Research Foundation, University of Pennsylvania or in the spectrophotometer described by Harvey et al. (10). Rapid kinetic studies were carried out in a Durrum model D-134 stopped flow spectrophotometer in the fluorescence mode. This particular instrument is equipped with a high intensity light source (75-watt xenon arc lamp) designed and built by the Johnson Foundation machine shop. Experimental velocities were recorded on a Tektronix storage oscilloscope model 564; permanent records were Polaroid photographs. In these experiments, isocitrate dehydrogenase activity was monitored by following the fluorescence change due to the diaphorase-catalyzed reduction by TPNH of resazurin to resorufin (11, 12). All glassware and glass components were treated with Prosil-28 (PCR, Inc.) to prevent nonspecific adsorption and surface denaturation.

Oxalosuccinate reductase activity of TPN-linked isocitrate dehydrogenase was measured by monitoring the decline in absorbance at 340 nm in the presence of 0.15 mM TPNH, 1.33 mM MgSO₄, and 166 mM Hepes at pH 7.4. The oxalosuccinate decarboxylase activity of TPN-isocitrate dehydrogenase was measured in a coupled enzyme assay. The rate of α-ketoglutarate formation from oxalosuccinate by isocitrate dehydrogenase was determined by following the decrease in DPNH fluorescence at 440 nm in reaction mixture containing oxalosuccinate, DPNH, l-aspartate, aspartate aminotransferase, and malate dehydrogenase.

All assays contained 166 mM Na-Hepes at pH 7.4 and 1.33 mM MgSO₄. Other components are described in figure and table legends and in Methods. All experimental points reported are the averages of triplicate determinations. The steady state experiments in Table I were fitted to their respective equation using the computer programs of Cleland (13). Where it was appropriate, data were fitted to a straight line by the method of least squares.

**RESULTS**

Steady State Kinetics—Earlier experiments had shown that inhibition of pig heart TPN-specific isocitrate dehydrogenase by α-methylisocitrate was competitive with isocitrate when TPN⁺ was nearly saturating (8). Additional steady state experiments were done to characterize the action of α-methylisocitrate with other substrates of the enzyme. The inhibition patterns obtained and the various kinetic constants are shown in Table I. Inhibition by α-methylisocitrate was competitive with isocitrate, α-ketoglutarate, or oxalosuccinate with values of Kᵣ which were very similar; inhibition was uncompetitive with CO₂. These results were consistent with the random mechanism proposed for this enzyme by Uhr et al. (1) and Northrup and Cleland (2). The competitive pattern found when TPN⁺ was the varied substrate (Table I and Fig. 1) was at variance with such a mechanism. This pattern should have been noncompetitive if the enzyme had a random mechanism.

The mechanism of the inhibition was investigated further by the double inhibitor approach (2, 14) (Fig. 2). Reaction progress was studied in the direction of reductive carboxylation with α-ketoglutarate, TPNH, and CO₂ as substrates; TPN⁺ and α-methylisocitrate were used as the inhibitors. From the results of this experiment, α, an interaction coefficient for α-methylisocitrate and TPN⁺, could be calculated. When α = ∞, the inhibitors are mutually exclusive. If α > 1, a molecule interferes with the other’s binding. The two compounds do not affect each other’s binding when α = 1; α < 1 indicates mutual facilitation of binding. From the experiment in Fig. 2, α = 0.11 for α-methylisocitrate and TPN⁺. This, coupled with the results in Fig. 1, raised the possibility that under the conditions used, TPN-linked isocitrate dehydrogenase followed an ordered rapid equilibrium mechanism.

The differences between an ordered rapid equilibrium system and a random one are apparent from their respective velocity equations, as described by Cleland (15). A comparison of Equations 1 and 2 shows

\[ v = \frac{VAB}{K_{1a}K_0 + K_0A + AB} \]

\[ v = \frac{VAB}{K_{1a} + K_0 + KA + AB} \]

that there is no \( K_B \) term for the ordered rapid equilibrium system (Equation 2). In the latter, \( K_B \) is not formed, since \( A \) must necessarily bind to the enzyme before \( B \). When the equations are rearranged to their double reciprocal form and terms are included for the inhibitor, Equations 3 and 4 are generated. Equation 4, for the ordered rapid equilibrium mechanism, will give a plot where the lines intersect at the ordinate, as in Fig. 1, whereas Equation 3 will not.

\[ \frac{1}{v} = \frac{K_a}{V} \left[ \frac{1 + \frac{K_a}{A} \left( 1 + \frac{1}{K_1} \right) \frac{1}{B} + \frac{1}{V} \left[ 1 + \frac{K_a}{A} \left( 1 + \frac{1}{K_1} \right) \right] }{B} \right] \]

\[ \frac{1}{v} = \frac{K_a}{V} \left[ \frac{1 + \frac{K_a}{A} \left( 1 + \frac{1}{K_1} \right) \frac{1}{B} + \frac{1}{V} }{B} \right] \]

A kinetic experiment diagnostic for an ordered rapid equilibrium system is described by Equation 5. In this experiment,
the first substrate (A) (DL-magnesium isocitrate) is varied in relationship to the second substrate (B) (TPN⁺). Equation 5 predicts that when the concentration of B becomes saturating, the slope of the line in the double reciprocal plot becomes zero. The results of such an experiment are shown in Fig. 3. At 1.0 μM TPN⁺, the double reciprocal plot has zero slope. This is demonstrated more clearly in Fig. 4 which shows a replot of the slopes of the lines in Fig. 3 versus 1/TPN⁺; this line passes through the origin.

**Tight Binding Inhibitor Studies**—Since TPN-isocitrate dehydrogenase seemed to exhibit an ordered rapid equilibrium mechanism under the conditions used here, it appeared likely that α-methylisocitrate would have some of the properties of a tight binding inhibitor described by Cha (16). The Kᵣ values of such inhibitors approach 1 nM and, consequently, their off rates from the enzyme surface become significant within the time scale of the assay. In an ordered rapid equilibrium mechanism, some step subsequent to substrate binding is rate limiting so that the enzyme and substrates are in equilibrium with respect to binding. As a consequence, increasing the concentration of the second substrate (B) shifts the two coupled equilibria, driving EA to EAB and, in turn, E to EA. This results in the apparent Kᵣ for the first substrate decreasing until, at saturating levels of B, Kᵣ for A approaches zero and the rate of reaction becomes independent of the concentration of A. With an analogue of A which is a dead-end inhibitor, the apparent Kᵣ should approach zero and, at saturating levels of B, the rate of formation of this tight binding complex should be independent of inhibitor concentration. To test for this possibility, an experiment such as that described by Cha (17) was carried out in a Durrum stopped flow spectrophotometer. At time zero, solutions of the enzyme and of substrate plus inhibitor were mixed in equal volumes and the reactions were monitored by fluorescence at 580 nm for 60 s. At each level of isocitrate, a straight line passing through the origin resulted (Fig. 9). This showed that, within the limits of the experiment, the monomer was inactive. One more piece of information could be garnered from these data. Equation 7 was replotted according to Equation 8 (using a₀ determined at each level of isocitrate), a straight line passing through the origin resulted (Fig. 9). This showed that, within the limits of the experiment, the monomer was inactive.

Equation 8, also from Kurganov (7), can be used to determine the specific activity of the monomer. When the data from Fig. 7 were replotted according to Equation 8 (using a₀ determined at each level of isocitrate), a straight line passing through the origin resulted (Fig. 9). This showed that, within the limits of the experiment, the monomer was inactive. One more piece of information could be garnered from these data. Equation 7 was replotted according to Equation 8 (using a₀ determined at each level of isocitrate), a straight line passing through the origin resulted (Fig. 9). This showed that, within the limits of the experiment, the monomer was inactive. One more piece of information could be garnered from these data. Equation 7 was replotted according to Equation 8 (using a₀ determined at each level of isocitrate), a straight line passing through the origin resulted (Fig. 9). This showed that, within the limits of the experiment, the monomer was inactive.

**Effects of Ligands—Sanner and Ingebretsen (20) and Ehrlich and Colman (21) have reported a lag in the attainment of steady state velocity when TPN-isocitrate dehydrogenase was assayed at low divalent metal ion concentrations. Both groups showed that low concentrations of either TPNH or EDTA treatment of polymerizing enzyme systems. This treatment has been successfully applied to lactate dehydrogenase (18) and more recently by Carlier and Pantaloni (19) to the bovine liver TPN-linked isocitrate dehydrogenase. The treatment is based on the fact that the specific activity of a monomeric enzyme will be constant for all concentrations of enzyme, whereas this will not be the case for a reversibly polymerizing system where some form of the protein is more active than another. Kurganov’s equations show that for a monomer-dimer equilibrium, a plot of specific activity versus enzyme concentration will describe a curve from which the specific activity of the monomer and dimer can be calculated. An experiment which measured the specific activity of the TPN-linked isocitrate dehydrogenase at varying enzyme concentrations and several fixed levels of isocitrate is shown in Fig. 7. The series of curves obtained showed a decline of specific activity at low enzyme levels. The specific activity of the dimer can be found by reploting the data in Fig. 7 according to Equation 7, where a₀ is the measured velocity divided by the enzyme concentration (i.e. the specific activity). The specific activity of the dimer is denoted as aₒ₁. Equation 8, also from Kurganov (7), can be used to determine the specific activity of the monomer. When the data from Fig. 7 were replotted according to Equation 8 (using aₒ determined at each level of isocitrate), a straight line passing through the origin resulted (Fig. 9). This showed that, within the limits of the experiment, the monomer was inactive.
could eliminate this lag. In the preceding paper (6), it was demonstrated that either TPNH or EDTA can stabilize the dimer form of the enzyme. Since the latter is the active form, TPNH and EDTA should decrease the decline in specific activity observed with low enzyme concentrations (Fig. 7). When the enzyme dilution experiment was repeated at enzyme concentrations (below 5 nM) where the decline in specific activity with dilution was most pronounced (cf. Fig. 7), activity was completely retained with 1.0 mM TPNH and 10 μM EDTA protected against loss of activity, even though the specific activity with EDTA declined sharply at the lowest enzyme level (Fig. 11).

Fig. 12 shows that TPNH or EDTA also eliminates the tight binding inhibition by α-methylisocitrate. When the enzyme was preincubated with 10 μM TPN+, 10 mM α-methylisocitrate, 1.33 mM MgSO4, 166 mM Hepes at pH 7.4 for 15 min and the reaction was then initiated by addition of 10 μM DL-isocitrate, a lag was seen in the attainment of steady state velocity, as expected with a tight binding inhibitor (18). Preincubation under the same conditions in the absence of α-methylisocitrate showed a linear velocity from time zero. Preincubation with α-methylisocitrate and 1 μM TPNH eliminated the lag; 10 μM EDTA reduced it to a few seconds.

DISCUSSION

Ultracentrifugation and molecular sieving studies have shown that TPN-specific isocitrate dehydrogenase from pig heart is a dimeric enzyme (6). The kinetic observations obtained here, mainly in Kurganov (7) type enzyme dilution experiments, are in agreement with the physical evidence and have shown, in addition, that the dimeric form of the enzyme is catalytically competent and that the monomer is inactive. Displacement of the equilibrium from monomer to dimer is favored by the substrate magnesium isocitrate. This is particularly apparent from the parabolic dependence of the monomer-dimer dissociation constant, 1/KM, on magnesium isocitrate concentration which indicates that at the limits of zero and infinite magnesium isocitrate concentrations, the enzyme will be in the monomer and dimer forms, respectively (Fig. 10). These results, obtained by measurement of enzyme activity, are in accord with ultracentrifugation studies where the presence of dimer and monomer was dependent on the presence and absence of magnesium isocitrate, respectively (6). These properties of TPN-isocitrate dehydrogenase from pig heart agree with the reversible dimerization reported for the enzyme from bovine liver (19).

A number of other properties of the pig heart enzyme have been observed here and elsewhere which may be related to the monomer-dimer equilibrium. (i) Under certain conditions, such as low enzyme concentration and the absence of certain ligands, an ordered rapid equilibrium kinetic mechanism with magnesium isocitrate adding to enzyme before 'TPN' has been observed. The occurrence of this mechanism is in addition to (rather than instead of) the random mechanism demonstrated by Uhr et al. (1) and Northrup and Cleland (2); it is dependent on the conditions of assay. Thus, the assays reported in Table 1, Fig. 1, Fig. 3, and Fig. 4 were done in the presence of 2 to 4 mM isocitrate dehydrogenase and without EDTA or serum albumin at pH 7.4, whereas Uhr et al. (1) used 10 μM EDTA and 1 mg/ml of bovine serum albumin at pH 6.8. When the enzyme was examined in this laboratory in incubation mixtures containing either bovine serum albumin (0.1 mg/ml) or 33 μM EDTA at pH 7.4, the inhibition by α-methylisocitrate with respect to TPN+ was noncompetitive instead of competitive (cf. Table 1) as expected for a random mechanism (1, 2). (ii) The value of Km for α-methylisocitrate was around 0.2 μM under steady state conditions (Table 1) (8), whereas tight binding inhibition could be demonstrated with as little as 1 nM α-methylisocitrate when measurements were made in the pre-steady state and under conditions where TPNH did not accumulate (Fig. 5). Physical measurements have shown that magnesium isocitrate, TPNH but not TPN-, or EDTA favor formation of the dimer (6). A lag in reaching steady state velocity observed at low activating divalent cation could be eliminated by TPNH or EDTA (20, 21). These ligands also protected against the decrease in specific activity at low enzyme concentrations (Fig. 11) and eliminated the tight binding inhibition by α-methylisocitrate (Fig. 12).

Carlier and Pantaloni (19) proposed a model which correlated the effect of isocitrate concentration on the activity of bovine liver TPN-isocitrate dehydrogenase in terms of the monomer-dimer equilibrium. An elaboration of this model is presented (Scheme 1) to account for the present additional observations of two types of inhibition by α-methylisocitrate and the switch in enzyme kinetic reaction mechanisms, both of which are dependent on conditions of incubation.

The model in Scheme I is divided into interactions of substrates or inhibitor with the monomeric form of the enzyme (right side) and the dimer (left side). The binding sequence of substrates to monomer is ordered and random to dimer. The connections between the monomer and dimer forms occur at the stages where the enzyme is free (D + 2 M) and in the substrate-bound forms (DAB + M + MAB), DAB being linear with either [TPN] or [magnesium isocitrate]. This is the situation with high enzyme concentrations or with substrates or inhibitor with the monomeric form of the enzyme (right side) and the dimer (left side). The binding sequence of substrates to monomer is ordered and random to dimer. The connections between the monomer and dimer forms occur at the stages where the enzyme is free (D + 2 M) and in the substrate-bound forms (DAB + M + MAB). Certain simplifications have been made in Scheme 1, mainly to improve the display of the more significant features of the reaction model. Thus, while it has been reported that both substrate addition and product release are random (1), in Scheme I product release from the dimer complex is only shown as ordered in the sequence CO2, α-ketoglutarate, and TPNH, a path which is probably the most favored leg (3). It appears that only 1 molecule of each substrate binds/molecule of dimer since the double reciprocal plots (cf. Table 1, and Figs. 1 and 3) are linear with either [TPNH] or [magnesium isocitrate]. If 2 molecules of substrate-bound/molecule of dimer, these plots would be nonlinear, as discussed below.

Under conditions favoring the monomeric form (e.g. low enzyme concentration), the sequence of reactions is visualized as an ordered addition to monomer (M) of magnesium isocitrate (A) followed by TPN+ (B) to form MAB; another molecule of monomer binds to form DAB and catalysis ensues. Once the products are released, free dimer (D) dissociates into 2 molecules of monomer (M) and the cycle repeats. The final event, dissociation of the dimer, is crucial to the model. If the enzyme remained as a dimer, the ordered rapid equilibrium mechanism would not be seen. The reaction rate would show a lag but would then accelerate and only a random mechanism would be observed. The rate of conversion of MAB to DAB (kr) appears to be the rate-determining step for catalysis in the ordered rapid equilibrium mode. The position of equilibrium (K5) of the free monomer-dimer interconversion (D + 2 M) determines the specific rate (Fig. 10) and mode of catalysis of the enzyme. If the enzyme remains entirely as the dimer after release of products, the reaction mechanism is in the random mode and the release of TPNH will be the rate-limiting step (kr) as reported by O'Leary and Limburg (3). This is the situation with high enzyme concentrations or with ligands which favor the conversion of monomer to dimer (magnesium isocitrate, TPNH, or EDTA). Since the monomer is inactive (Fig. 9) and the dimer is active (Fig. 8), the ordered rapid equilibrium mode is detected only when the ratio of monomer to dimer is relatively large (but not infinite) (e.g.
Figs. 3 and 4) and the enzyme-specific activity \((v/E_s)\) is less than maximal (Fig. 7).

This monomer-dimer system, with two different kinetic mechanisms, can be described within the confines of Frieden's hysteretic enzyme concept (22). A hysteretic enzyme is one which exhibits some slow kinetic change in response to a rapid change in its substrate levels. Although Frieden conceived this change to be a response to some metabolic shift in substrate concentrations, the large concentration differential to which an enzyme is exposed on initiation of an assay in vitro should be equally valid. Equation 9 describes this kinetic response (3), where \(v_0\) is the observed velocity. For the model in Scheme I, \(v_0\)

\[
v_t = v_f + (v_0 - v_f)e^{-k't}
\]

(9)

the velocity when dimerization is rate limiting, can be shown by the usual derivation procedures (23) to be Equation 10, similar to the equation for an ordered rapid equilibrium system (Equation 2) but which incorporates the dimerization step. This equation also predicts the decrease in specific activity upon dilution seen in Figs. 7 and 11. The maximum rate of reaction, \(v_0\) in Equation 10, is defined as \(k_t\), the rate constant for the rate-limiting step, multiplied by \(E_0\), the total enzyme concentration, calculated a monomer of 59,000 daltons. The dependence of the rate on the enzyme concentration can be seen by rearranging Equation 10 to Equation 11. Kurganov (7) has shown

\[
v = \frac{V[MAB]}{k_{13}k_b + k_{13}A + AB}
\]

(10)

that for a monomer-dimer system where only dimer is catalytically active, the concentration of monomer, \(M\), can be calculated from Equation 12, where \(E_0\) is total enzyme concentration calculated as monomer, and \(K_e\) is the monomer-dimer association constant. The

\[
[M] = \frac{(A + 8K_eE_0) - 1}{4K_e}
\]

(12)

combined use of Equations 11 and 12 then predict that at low enzyme concentrations, specific activity should increase as a function of enzyme concentration until a plateau is reached. This is consistent with the experiments shown in Figs. 7 and 11.

The other term in Equation 9, \(v_f\) can be shown to be Equation 1, which can be used to describe a random system. The rate constant \(k'\) in Equation 9 can be defined for Scheme I as Equation 13. In the

\[
k' = \frac{k_1[M]E[A][B]}{k_{13}k_{15}}
\]

(13)

model, therefore, the monomer-dimer equilibrium is dynamic and the steady state position of this equilibrium will determine the kinetic mechanism. Under conditions which favor the monomer, \(k'\) will be a very small number and Equation 9 will reduce to Equation 14, since \(v_0\), the velocity of the dimer, will approach zero. When the dimer is the predominant form of the enzyme, the \((v_0 - v_ne^{-k't})\) term will approach zero, \(v_0 = v_f\), and the random mechanism will be seen. Equation 9 predicts that there should be intermediate stages, where both \(v_0\) and \(v_f\) will be significant and where both will contribute to the observed velocity.

In the model, the tight binding inhibition by \(\alpha\)-methylisocitrate (Fig. 6) is visualized as being the consequence of the ordered rapid equilibrium mechanism. In a bireactant ordered rapid equilibrium mechanism, increasing the concentration of the second substrate \((B)\) lowers the apparent \(K_e\) for the first substrate \((A)\) by shifting the equilibria of the coupled reactions toward \(EAB\); at saturating levels of \(B\), the \(K_e\) for \(A\) appears to be zero. This was demonstrated for magnesium isocitrate \((A)\) and TPN* \((B)\) under the incubation conditions shown in Figs. 3 and 4. By analogy, with \(\alpha\)-methylisocitrate, TPN* would drive the equilibria of the coupled reactions \(M + I \rightleftharpoons M I + B \rightleftharpoons MIB\) toward formation of MIB (Scheme 1).

At saturating levels of TPN*, the \(K_e\) for this dead-end inhibitor should approach zero, leading to the appearance of the tight binding inhibition (Fig. 5). Again, this tight binding inhibition will be seen only under certain assay conditions. The constants reported in Table I were determined from the steady state experiments (Figs. 1 to 4) which were initiated by the addition of enzyme to the assay mixture and which were monitored by the increase in fluorescence due to TPNH accumulation. Previous steady state experiments (8) had shown that isocitrate and \(\alpha\)-methylisocitrate had a \(K_e/K\) ratio of about 150. The kinetic constants in Table I are in reasonable agreement with these earlier values (8). In a steady state experiment under ordered rapid equilibrium conditions, the apparent \(K_e\) and \(K\), will be proportionately lowered at various TPN* concentrations and it seems reasonable to presume that the \(K_e/K\) ratio will be maintained. Therefore, no tight binding is observed under steady state conditions. Tight binding inhibition should be observed only where the enzyme is preincubated with inhibitor or under the pre-steady state conditions described in Figs. 5, 6, and 12.

Sanner and Ingebretsen (20) and Ehrlich and Colman (21) have demonstrated a lag in attainment of steady state velocity at low bivalent metal ion concentrations. Since the concentration of the metal isocitrate complex which promotes dimerization will be low when the metal ion concentration is low, the enzyme will be mainly in the monomer form at zero time. The lag can be predicted from the \((v_0 - v_ne^{-k't})\) term in Equation 8, and the reaction rate should eventually stabilize as a new monomer-dimer steady state is established. The addition of TPNH or EDTA was reported to eliminate this lag (20, 21) and, in fact, the steady state velocity in the presence of EDTA at low metal ion concentration was higher than in its absence (20). This is also consistent with Equation 9 and Scheme I. The addition of EDTA or TPNH stabilizes the dimer (6), which is the catalytically competent species. For the same reason, EDTA or TPNH protected against loss of enzyme activity by dilution (Fig. 11).

In the model, under conditions favoring the presence of monomer, binding of the inhibitor magnesium \(\alpha\)-methylisocitrate to enzyme is depicted to parallel the ordered rapid equilibrium mechanism. Under conditions described in Figs. 7 and 11, where Mg isocitrate, binding of the inhibitor magnesium \(\alpha\)-methylisocitrate should be observed only where the enzyme is preincubated with inhibitor or under the pre-steady state conditions described in Figs. 5, 6, and 12.

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Fig. 1. Double reciprocal plot of velocity versus TPN concentration at fixed levels of NADPH. All assays contained 150 μM NADPH and 0.15 mM substrate. The TPN concentrations were 0.0 and 0.15 mM (solid line), 0.30 mM (dashed line), 0.45 mM (dotted line), and 0.60 mM (dash-dotted line). The data points are the average of triplicate determinations.

Fig. 2. Double reciprocal plot of velocity versus substrate concentration at fixed levels of TPN and NADPH. All assays contained 0.15 mM TPN, 0.05 mM NADPH, and 0.30 mM substrate. The TPN concentrations were 0.0 and 0.05 mM (solid line), 0.10 mM (dashed line), 0.15 mM (dotted line), and 0.20 mM (dash-dotted line). The data points are the average of triplicate determinations.

Fig. 3. Double reciprocal plot of velocity versus concentration of the TPN-isocitrate dehydrogenase complex formation from 0.1 mM TPN and 0.1 mM NADPH. All assays contained 0.1 mM TPN, 0.1 mM NADPH, and 0.1 mM isocitrate. The TPN concentrations were 0.0, 0.05, 0.10, 0.15, and 0.20 mM (solid line), 0.15 mM (dashed line), 0.20 mM (dotted line), and 0.25 mM (dash-dotted line). The data points are the average of triplicate determinations. The complex rate constant, k, determined from the slope of the lines, was 0.9 ± 0.1 min⁻¹.

Fig. 4. Binding of the TPN-Isocitrate dehydrogenase complex to the membrane. The TPN concentration was 0.1 mM (solid line), 0.3 mM (dashed line), 0.5 mM (dotted line), and 0.7 mM (dash-dotted line). The data points are the average of triplicate determinations. The membrane binding constant, Kd, determined from the slope of the lines, was 8.9 ± 0.4 nM.
TPN-Isocitrate Dehydrogenase: Kinetic Evidence for Dimer

Fig. 1. Kinetic type enzyme dilution experiment at different levels of isocitrate. All assays contained 0.5 mM MgCl₂, 50 mM Tris-HCl, pH 7.4, and 100 mM TPN⁺, and were carried out at 25°C. The concentrations of D-isocitrate were: 0.1 mM, 0.5 mM (C), 1.0 mM (D), 5.0 mM (E), and 100 mM (A). Enzyme concentrations were calculated as number of 59,000 daltons.

Fig. 2. Determination of the specific activity of the dimer (ε₄₅) at each level of isocitrate. Applied to the data in Fig. 1 according to equation 1. The concentrations of D-isocitrate were: 0.1 mM (I), 0.5 mM (K), 1.0 mM (L), 5.0 mM (C), and 100 mM (A). The specific activity of the dimer, ε₄₅, extrapolated to infinite isocitrate concentration was 15 mg protein.

Fig. 3. Determination of the specific activity of the monomer (ε₂₃). The data in Fig. 1 were corrected according to equation 2. When the data points were fitted to the line by the method of least squares the intercept was at zero (ε₂₃) with a value of r² = 0.86 for 10 points.

Fig. 4. Determination of the specific activity of the monomer (ε₂₃). The data in Fig. 1 were corrected according to equation 2. When the data points were fitted to the line by the method of least squares the intercept was at zero (ε₂₃) with a value of r² = 0.86 for 10 points.

Fig. 5. Scheme 1. Model for action of TPN-linked isocitrate dehydrogenase. The following symbols are used: A, N-methylsuccinate; B, TPN⁺; C, DAB; D, succinylthioca; E, TPN⁺; D, dimer; H, monomer; I, N-methylisocitrate. See discussion for further details.