Kinetic Studies of Formate Dehydrogenase

By D. PEACOCK* AND D. BOULTER

Department of Botany, University of Durham, Durham City, U.K.

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1. The kinetic mechanism of formate dehydrogenase is a sequential pathway. 2. The binding of the substrates proceeds in an obligatory order, NAD⁺ binding first, followed by formate. 3. It seems most likely that the interconversion of the central ternary complex is extremely rapid, and that the rate-limiting step is the formation or possible isomerization of the enzyme-coenzyme complexes. 4. The secondary plots of the inhibitions with HCO₃⁻ and NO₃⁻ are non-linear, which suggests that more than one molecule of each species is able to bind to the same enzyme form. 5. The rate of the reverse reaction with carbon dioxide at pH 6.0 is 20 times that with bicarbonate at pH 8.0, although no product inhibition could be detected with carbon dioxide. The low rate of the reverse reaction precluded any steady-state analysis as the enzyme concentrations needed to obtain a measurable rate are of the same order as the K_m values for NAD⁺ and NADH.

Formate dehydrogenase (formate-NAD⁺ oxido-reductase; EC 1.2.1.2) catalyses the reaction:

and is known to occur in animals, higher plants and bacteria. The higher-plant enzyme differs from both the animal enzyme, which is activated by ATP (Mathews & Vennesland, 1950), and the bacterial enzyme, which does not require NAD⁺ (Gale, 1939). Although Davison (1951) and Nason & Little (1955) have both reported the effects of various enzyme inhibitors on the activity of the plant enzyme, no kinetic investigation has so far been undertaken.

The present study is an investigation into the mechanism of enzyme action by steady-state kinetics. Various enzyme-substrate/enzyme-inhibitor complexes and their possible pathways of interconversion were determined by using initial-velocity, product-inhibition and dead-end-inhibition analysis.

EXPERIMENTAL

Enzyme. Formate dehydrogenase was purified from *Phaseolus aureus* (mung bean) by a combination of salt fractionation, ion-exchange, hydroxyapatite and alumina-gel chromatography, and assayed as described by Peacock (1970). The molecular weight was estimated by gel filtration as $92\,000 \pm 10\,000$, and the enzyme was not more than 50% pure, as determined by acrylamide-gel electrophoresis.

* Present address: Department of Biochemistry and Chemistry, The Medical College of St Bartholomew's Hospital, Charterhouse Square, London E.C.1, U.K.

Coenzymes. NAD⁺ from Sigma Chemical Co., St Louis, Mo., U.S.A., was purified by the method of Dalziel (1963a). NADH obtained in pre-weighed vials (Sigma Chemical Co.) was estimated as 98% pure enzymically with E_{340}/E_{260} ratio 0.23, and was used without further purification.

Substrates. Analytical-grade ammonium formate and NaHCO₃ were from British Drug Houses Ltd., Poole, Dorset, U.K.

Initial-rate measurements. The reaction was followed by measuring the appearance of NADH at 340 nm by using a Unicam SP.800 spectrophotometer with an attached 10 mV 'slave' recorder. The full-scale deflexion of the recorder was set to correspond to 0.1 extinction unit, and the chart speed adjusted to give recorded traces with an angle of inclination of between 30 and 60°. Silica cells (4 cm light-path) were filled with all the components of the reaction mixture except the enzyme, in a total volume of 9.9 ml, and preincubated at 25°C. The reaction was initiated by the addition of 0.1 ml of suitably diluted enzyme and the cell was immediately placed in the reaction compartment (kept at 25°C). The recorded traces obtained were extrapolated to the time of addition, and a tangent to the curve was drawn at this point and taken as a measure of the initial velocity.

Data processing. Graphs of reciprocal velocities against reciprocals of the various substrate concentrations were plotted by fitting the data for each case to eqn. (1) by using the least-squares method, and assuming equal variances in the velocities (Wilkinson, 1961). In eqn. (1) v is velocity, $[S]$ is substrate concentration and V and K are constants.

$$v = (V[S])/(K + [S]) \quad (1)$$

Calculations were performed with a digital computer (IBM 360/67) with FORTRAN programs, which provided values of K , V , K/V , $1/V$ and the standard error of their estimates. The slopes (K/V) and the intercepts ($1/V$) were

then plotted graphically to determine the type of inhibition (linear, parabolic, competitive etc.) and values for kinetic constants and the standard errors of these values were then obtained by fitting all the data points to the overall equation describing the observed type of inhibition. Data for linear competitive inhibition were fitted to eqn. (2), data for linear non-competitive inhibition to eqn. (3), data for linear uncompetitive inhibition to eqn. (4), data for parabolic competitive inhibition to eqn. (5), data for slope-parabolic, intercept-linear, non-competitive inhibition to eqn. (6), and data for the initial velocity analysis to eqn. (7). All fits assumed equal variances for the observed velocities and used the computer program of Cleland (1963a).

$$v = \frac{V[A]}{K(1 + [I]/K_{is}) + [A]} \quad (2)$$

$$v = \frac{V[A]}{K(1 + [I]/K_{is}) + [A](1 + [I]/K_{ii})} \quad (3)$$

$$v = \frac{V[A]}{K + [A](1 + [I]/K_{ii})} \quad (4)$$

$$v = \frac{V[A]}{K(1 + [I]/K_{i1} + [I]^2/K_{i2}) + [A]} \quad (5)$$

$$v = \frac{V[A]}{K(1 + [I]/K_{i1} + [I]^2/K_{i2}) + [A](1 + [I]/K_{ii})} \quad (6)$$

In eqns. (2)–(6) v is velocity, $[A]$ is substrate concentration, $[I]$ is inhibitor concentration, K and V are constants, and K_{is} and K_{ii} are inhibition constants obtained from secondary plots of slope and intercepts respectively.

RESULTS

Initial-velocity analysis. When NAD^+ was the variable substrate with several different constant concentrations of formate, an intersecting pattern of Lineweaver–Burk plots was obtained (Fig. 1). With formate as the variable substrate and different fixed concentrations of NAD^+ , an intersecting pattern was again obtained. When the slopes and the intercepts of the primary Lineweaver–Burk plots were replotted against the reciprocals of the second fixed substrate (formate), a linear relationship was found. The mechanism is therefore sequential with both substrates binding to the enzyme before any product is released; this mechanism is described by eqn. (7):

$$v = \frac{V[A][B]}{K_{is}K_b + K_{is}[B] + K_b[A] + [A][B]} \quad (7)$$

K_b and K_{is} are Michaelis constants for substrates B and A respectively and K_{is} is an inhibition constant for A.

The values of the kinetic constants were obtained from the appropriate computer program by assigning NAD^+ to A and formate to B. K_{is} , K_b and K_{is} were determined as $7.2 \pm 1.0 \mu\text{M}$, $1.6 \pm 0.3 \text{ mM}$ and $42 \pm 8.0 \mu\text{M}$ respectively, and represent the means of three determinations.

Fig. 1. Initial-velocity analysis of formate dehydrogenase with NAD^+ as the variable substrate for several constant concentrations of formate: $\circ$, 2.5 mM; $\triangle$, 5.0 mM; $\square$, 10.0 mM; $\bullet$, 25.0 mM.

Fig. 2. Product inhibition of formate dehydrogenase by NADH , with NAD^+ as the variable substrate for several constant concentrations of NADH : $\circ$, 10.0 μM ; $\triangle$, 5.0 μM ; $\square$, 2.5 μM ; $\bullet$, 0, with [formate] 2.5 mM.

Product-inhibition analysis. With NAD^+ as the variable substrate, NADH gave linear competitive inhibition (Fig. 2). The replot of the primary slopes against fixed NADH concentrations were linear at

Fig. 3. Secondary plots: slopes of the Lineweaver-Burk plots of NADH product inhibition, with NADH concentrations for two constant concentrations of formate: O, 2.5 mM; Δ, 10.0 mM.

Fig. 4. Product inhibition of formate dehydrogenase by bicarbonate with NAD⁺ as the variable substrate for several constant concentrations of bicarbonate: O, 675 mM; Δ, 500 mM; □, 225 mM; ●, 0, with [formate] 2.5 mM.

Fig. 5. Secondary plot of the slopes and intercepts of the Lineweaver-Burk plots of the bicarbonate product inhibition against NAD⁺. Δ, Slope; O, intercept.

Fig. 6. Product inhibition of formate dehydrogenase by bicarbonate with formate as the variable substrate for several constant concentrations of bicarbonate: O, 675 mM; Δ, 500 mM; □, 225 mM; ●, 0, with [NAD⁺] 6.15 μM.

both fixed concentrations of formate (Fig. 3). With formate as the variable substrate, NADH gave non-competitive inhibition and the secondary plots of both the slopes and intercepts against fixed secondary NADH concentrations were linear.

With NAD⁺ as variable substrate, bicarbonate gave non-competitive inhibition (Fig. 4); secondary plots of both the slopes and the intercepts against several constant concentrations of bicarbonate were non-linear (Fig. 5). With formate as the variable substrate, bicarbonate gave competitive inhibition (Fig. 6) and the secondary plots of the slopes against the fixed concentrations of bicarbonate were non-linear.

Table 1. *Inhibition patterns of formate dehydrogenase activity at pH 8.0*

Inhibitor	Variable substrate	Substrate at constant concentration	Pattern
NADH	NAD ⁺	Non-saturating	Competitive
NADH	NAD ⁺	Saturating	Competitive
NADH	Formate	Non-saturating	Non-competitive
Bicarbonate	Formate	Non-saturating	Competitive*
Bicarbonate	NAD ⁺	Non-saturating	Non-competitive*
ADP-ribose	NAD ⁺	Non-saturating	Competitive
ADP-ribose	Formate	Non-saturating	Non-competitive
Nitrate	Formate	Non-saturating	Competitive*
Nitrate	NAD ⁺	Non-saturating	Uncompetitive*

* Non-linear secondary plots.

The product-inhibition studies described above were carried out at pH 8.0. Similar studies at pH 6.0 showed that the patterns of inhibition produced by NADH were as before with both NAD⁺ and formate as variable substrate. Inhibition studies at pH 6.0 with bicarbonate are not possible, in view of the pK of carbonic acid. A saturated solution of carbon dioxide at concentration of 35 mM gave no inhibition.

Dead-end-inhibition analysis. With NAD⁺ as the variable substrate ADP-ribose was a competitive inhibitor; the secondary plots of the slopes against fixed concentrations of ADP-ribose were linear. With formate as the variable substrate ADP-ribose gave non-competitive inhibition and the secondary plots of both the slopes and the intercepts against fixed concentrations of ADP-ribose were linear.

With formate as the variable substrate, nitrate was a competitive inhibitor and the secondary plots of the slopes against fixed concentration of nitrate were non-linear. With NAD⁺ as the variable substrate, nitrate gave uncompetitive inhibition; the secondary plots of the intercepts against several constant nitrate concentrations were non-linear. The various inhibition patterns obtained are collected together in Table 1.

Reverse reaction. The reverse reaction was followed by measuring the rate of incorporation of [¹⁴C]-bicarbonate (The Radiochemical Centre, Amersham, Bucks., U.K.) into formate at two pH values, approximately equivalent concentrations of enzyme and substrate being used in both cases (Fig. 7). At pH 8.0 with bicarbonate and NADH as substrates an initial rate of formation of 36 pmol of formate/min was obtained. At pH 6.3 with solutions of carbon dioxide (saturated) and NADH, the rate was 710 pmol/min.

However, to obtain these measurable quantities of [¹⁴C]formate the enzyme concentration needed was 60 μ M. This concentration is too high to permit

Fig. 7. Progress curve for the reverse reaction. The concentrations of bicarbonate and NADH were 20 mM and 400 μ M at pH 8.0 (Δ), and 35 mM and 400 μ M at pH 6.3 ($\circ$).

steady-state analysis, which assumes that the rate of change of concentrations of the various enzyme species is very small compared with the rate of change of the substrate concentrations. The value for the ratio of the rates of the forward and backward reaction was calculated as 45 000 at pH 8.0 and 2300 at pH 6.3, but this assumes that the concentration of carbon dioxide was in fact large enough to saturate the enzyme; if this were not so, then the values would be lower.

Effect of ionic strength. There was no effect of increasing the ionic strength with ammonium

sulphate on the initial velocity at non-saturating concentrations of both substrates, at concentrations less than 2M.

DISCUSSION

The initial-velocity data, in conforming with eqn. (7) (intersecting pattern), show that the mechanism is sequential (Cleland, 1963b), in which both substrates bind to the enzyme before any product is released.

The product-inhibition patterns obtained show that the simplest sequential mechanism to fit the data is an ordered Bi Bi mechanism without a rate-limiting central ternary complex. This mechanism was first proposed for liver alcohol dehydrogenase by Theorell & Chance (1951), and the steady-state rate equation for this mechanism can be written in the form of eqn. (8) (Cleland, 1963b):

Eqn. (9) predicts linear non-competitive inhibition, eqn. (10) predicts linear competitive inhibition, eqn. (11) predicts linear competitive inhibition and eqn. (12) linear non-competitive inhibition. These four basic patterns were obtained for formate dehydrogenase under the appropriate set of conditions, except that inhibitions of bicarbonate were in fact non-linear. These results therefore further support a Theorell-Chance mechanism, although the non-linear plots of bicarbonate inhibition indicate a modification of the basic Theorell-Chance mechanism. However, this particular set of patterns could also be explained by another type of sequential mechanism, namely a rapid-equilibrium random mechanism with dead-end EAP and EBQ complexes, with the non-linear bicarbonate inhibition again indicating some modification of the basic mechanism.

It is possible with the use of dead-end inhibitors

$$v = \frac{V_1([A][B] - [P][Q]/K_{eq})}{K_{ia}K_b + K_b[A] + K_a[B] + [A][B] + \frac{K_{ia}K_b[Q]}{K_{iq}} + \frac{K_{ia}K_bK_q[P]}{K_{iq}K_p} + \frac{K_{ia}K_b[P][Q]}{K_pK_{iq}} + \frac{K_bK_q[A][P]}{K_{iq}K_p} + \frac{K_a[B][Q]}{K_{iq}}} \quad (8)$$

where K_p and K_q are Michaelis constants for products P and Q respectively and K_{ip} and K_{iq} are inhibition constants for products P and Q respectively. The relationship between eqns. (7) and (8) is that, in the absence of products, eqn. (8) reduces to eqn. (7).

With either products P or Q set to zero eqns. (9)–(12) are obtained by rearrangement of eqn. (8), and these equations describe the four possible inhibition patterns.

Vary substrate A, inhibit with product P:

$$\frac{1}{v} = \frac{K_a}{V_1} \left(1 + \frac{K_{ia}K_b}{K_a[B]} \right) \left(1 + \frac{[P]}{K_{ip} \left(1 + \frac{K_a[B]}{K_{ia}K_b} \right)} \right) \frac{1}{[A]} + \frac{1}{V_1} \left(1 + \frac{K_b}{[B]} \right) \left(1 + \frac{[P]}{K_{ip} \left(1 + \frac{[B]}{K_b} \right)} \right) \quad (9)$$

Vary substrate A, inhibition with product Q:

$$\frac{1}{v} = \frac{K_a}{V_1} \left(1 + \frac{K_{ia}K_b}{K_a[B]} \right) \left(1 + \frac{[Q]}{K_{iq}} \right) \frac{1}{[A]} + \frac{1}{V_1} \left(1 + \frac{K_b}{[B]} \right) \quad (10)$$

Vary substrate B, inhibit with product P:

$$\frac{1}{v} = \frac{K_b}{V_1} \left(1 + \frac{K_{ia}}{[A]} \right) \left(1 + \frac{[P]}{K_{ip}} \right) \frac{1}{[B]} + \frac{1}{V_1} \left(1 + \frac{K_a}{[A]} \right) \quad (11)$$

Vary substrate B, inhibit with product Q:

$$\frac{1}{v} = \frac{K_b}{V_1} \left(1 + \frac{K_{ia}}{[A]} \right) \left(1 + \frac{[Q]}{K_{iq} \left(1 + \frac{[A]}{K_{ia}} \right)} \right) \frac{1}{[B]} + \frac{1}{V_1} \left(1 + \frac{K_a}{[A]} \right) \left(1 + \frac{[Q]}{K_{iq} \left(1 + \frac{[A]}{K_a} \right)} \right) \quad (12)$$

to distinguish between an ordered and a random sequential mechanism. Thus a dead-end inhibitor that binds only to the free enzyme competes in an ordered mechanism with the first substrate (A), and therefore the inhibition pattern obtained when the second substrate is varied is non-competitive. Conversely a dead-end inhibitor that binds to the EA complex competes in an ordered mechanism with the second substrate (B), and therefore the inhibition pattern produced when the first

substrate is varied is uncompetitive. On the other hand, in a random mechanism, regardless with which substrate the dead-end inhibitor competes, the inhibition obtained by varying the other substrate is non-competitive. Eqn. (13) is the equation relating the reaction rate to the effect of a dead-end inhibitor (ADP-ribose) combining with the free enzyme in an ordered sequential reaction. Eqn. (14) is the equation relating the reaction rate to the effect of a dead-end inhibitor (nitrate) binding to the EA complex (Cleland, 1963c):

$$\frac{1}{v} = \frac{1}{V_1} \left(\frac{K_{is}K_b}{[B]} \right) + K_s \left(1 + \frac{[I]}{K_i} \right) \frac{1}{[A]} + \frac{K_b}{[B]} + \frac{1}{V_1} \quad (13)$$

$$\frac{1}{v} = \frac{1}{V_1} \left(\frac{K_{is}K_b}{[B]} + K_s \right) \frac{1}{[A]} + \frac{1}{V_1} \left(1 + \frac{K_b}{[B]} \right) \left(1 + \frac{[I]}{K_i \left(1 + \frac{[B]}{K_b} \right)} \right) \quad (14)$$

By using the dead-end inhibitor ADP-ribose, which was competitive with NAD^+ and non-competitive with formate, and the dead-end inhibitor nitrate, which was competitive with formate and uncompetitive with NAD^+ , it was shown that the mechanism of formate dehydrogenase was, in fact, ordered sequential with NAD^+ binding to the enzyme followed by formate.

Another feature of ordered mechanisms as opposed to rapid-equilibrium random mechanisms concerns the inhibition of product Q against substrate A. Thus the relationships in eqn. (10) predict that the K_{is} value obtained from the secondary plot of the slope should remain the same regardless of the fixed concentration of B, and this prediction is true for all ordered mechanisms but not for random mechanisms where K_{is} varies with the concentration of B; in fact, the K_{is} value was constant (Fig. 3). However, in random mechanism with dead-end complexes, the variation of K_{is} with the concentration of B is hyperbolic and so difficult to detect experimentally. For this reason the observed constant K_{is} value found cannot unambiguously be interpreted as a clear distinction between the two possible mechanisms for formate dehydrogenase.

The concentrations of bicarbonate needed to produce inhibition (up to 700mM) were so high that there is reason to doubt that bicarbonate acted solely as a product inhibitor (especially in view of the fact that carbon dioxide was 20 times better as a substrate in the reverse reaction). However, bicarbonate did not act simply as a dead-end inhibitor binding to the E-NAD^+ complex, as this would have produced uncompetitive inhibition versus NAD^+ (similar to nitrate). Nor is it likely that it acted as a mixed dead-end inhibitor binding to

both E and E-NAD^+ , since the inhibitions would have been intercept-parabolic slope-linear non-competitive versus NAD^+ , and intercept-linear slope-parabolic non-competitive versus formate, whereas both the slope and intercept secondary plots for bicarbonate were parabolic or of higher order; also the bicarbonate inhibition versus formate was competitive. The possibility that the inhibition by bicarbonate was due to non-specific effects of ionic strength is also unlikely, as sulphate ion had no noticeable effect at concentrations three times

that of the bicarbonate. The most likely mode of action of bicarbonate therefore is that it acted as both a product inhibitor and dead-end inhibitor binding to the E-NAD^+ complex. If this is the case, and if the mechanism is a Theorell-Chance one, then the expected patterns would be as found, i.e. parabolic competitive versus formate, and intercept-parabolic slope-linear non-competitive versus NAD^+ . The Theorell-Chance mechanism is a special form of an ordered Bi Bi mechanism, in which the rate-limiting step is in the addition and release of coenzyme to the free enzyme. Theoretically it is possible in this case to distinguish between a Theorell-Chance and an ordered Bi Bi mechanism by the bicarbonate inhibition pattern. In an ordered Bi Bi mechanism the expected pattern would be intercept-linear slope-parabolic non-competitive versus formate and intercept-parabolic slope-linear non-competitive versus NAD^+ . However, owing to the parabolic nature of the variation in slope, the fact that a linear-intercept variation was not detected could have been due to it having been so small as to have been missed.

Thus the kinetic mechanism of formate dehydrogenase is most likely to be an ordered Bi Bi without a rate-limiting central ternary complex, i.e. Theorell-Chance mechanism (Scheme 1), in which

Scheme 1. Schematic representation of the Theorell-Chance mechanism.

Table 2. *Inhibition constants of formate dehydrogenase, a Theorell–Chance mechanism being assumed*

Kinetic constant	Inhibition	Value from slope	Value from intercept
K_{iq}	NADH versus NAD^+	$2.5 \pm 0.3 \mu\text{M}$	—
K_{iq}	NADH versus formate	$1.2 \pm 0.6 \mu\text{M}$	$2.1 \pm 0.7 \mu\text{M}$
K_{ip}	Bicarbonate versus formate	$1100 \pm 600 \text{ mM}$	—
K_{ip}	Bicarbonate versus NAD^+	$870 \pm 500 \text{ mM}$	$180 \pm 40 \text{ mM}$
$K_i (\text{ADP-ribose})$	ADP-ribose versus NAD^+	$12.1 \pm 1.3 \mu\text{M}$	—
$K_i (\text{ADP-ribose})$	ADP-ribose versus formate	$9.6 \pm 2.5 \mu\text{M}$	$12.5 \pm 3 \mu\text{M}$
$K_i (\text{nitrate})$	Nitrate versus NAD^+	No programs available	
$K_i (\text{nitrate})$	Nitrate versus formate	$1.8 \pm 0.2 \text{ mM}$	—

the molecules of bicarbonate and nitrate bind more than once to the same enzyme forms accounting for the non-linear secondary plots; however, an ordered Bi Bi mechanism with a rate-limiting central ternary complex in which bicarbonate acts both as a product inhibitor as well as a dead-end inhibitor to the E– NAD^+ complex cannot be definitely ruled out.

The kinetic constants (Table 2) were calculated by assuming the Theorell–Chance mechanism as described by eqn. (8). The secondary plots of the nitrate and bicarbonate inhibitions were assumed to be parabolic, although a higher order might describe the inhibitions more accurately.

As the very low rate of the reverse reaction precluded any steady-state analysis, it was not possible to determine all the kinetic constants. For this reason the unique relationships that exist between the kinetic constants in a Theorell–Chance mechanism (Dalziel, 1957, 1963b) cannot as yet be used to test just how close the mechanism of formate dehydrogenase approximates to a Theorell–Chance mechanism.

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