Kinetics for Formate Dehydrogenase of *Escherichia coli*
Formate-Hydrogenlyase*

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(Received for publication, October 25, 1990)

The formate-hydrogenlyase complex of *Escherichia coli* decomposes formate to molecular hydrogen and carbon dioxide under anaerobic conditions (1, 2). Enzymic components of the complex consist of a formate dehydrogenase and a hydrogenase. The formate dehydrogenase, FDHH, has been the centerpoint of studies in recent years which have elucidated the biochemical steps involved in incorporation of selenocysteine into protein (3–5).

Isolation and characterization of the gene, *fdhF*, which encodes FDHH, revealed a TGA codon that directs the cotranslational insertion of selenocysteine into the FDHH polypeptide (6, 7). Oxygen, nitrate, and other electron acceptors negatively regulate *fdhF* transcription, while formate induces expression (8, 9). Transcription of the *fdhF* gene requires the σ^54^ protein, which recognizes and initiates transcription at a distinct subset of bacterial promoters (10).

When *Escherichia coli* is grown anaerobically in the presence of nitrate, FDHH is not produced. Nitrate induces expression of a nitrate reductase-linked formate dehydrogenase (FDHns), while repressing FDHH expression (11). Under these conditions FDHH oxidizes formate, and the resulting electrons are transferred to nitrate reductase. Conservation of metabolic energy occurs in 50 mM Tris-HCl, pH 7.5, 20 mM sodium, and 2 mM benzyl viologen dichloride as the ultimate electron acceptor. This energy may be conserved by the establishment of a proton gradient across the cell membrane due to hydrogen evolution (2).

Purification of FDHH was accomplished recently (12), and this has allowed detailed analysis of the physicochemical characteristics of the enzyme. Although the enzyme is most active at slightly alkaline pH, stability of the enzyme greatly decreases at pH values where it is most active. Oxygen inactivates the enzyme, and azide protects against this inactivation. Low concentrations of nitrate and nitrite salts severely inhibit activity of the enzyme. FDHH contains selenium as selenocysteine, a molybdopterin-guanine dinucleotide cofactor and iron (presumably in an iron-sulfur complex).

In this paper we have extended the characterization of FDHH by examining some of its catalytic properties.

**MATERIALS AND METHODS**

Materials—FDHH was isolated from *E. coli* strain FM911 bearing plasmid pFM20 as described previously (12). All analyses were performed using oxygen-free techniques. Formate-dependent benzyl viologen reduction was followed by measuring the increase in absorbance at 578 nm as a function of time. One unit of activity is defined as the reduction of 1 μmol of benzyl viologen per min at 24 °C. The standard assay solution (1 ml) consisted of 50 mM Tris-HCl, pH 7.5, 20 mM sodium formate, and 2 mM benzyl viologen dichloride. Kinetic analyses contained 0.04 μg of purified FDHH, unless otherwise noted. The effects of alternative substrates or inhibitors were tested by replacement or inclusion in the assay mixture as indicated. The plotted data were subjected to linear least squares regression analysis. Slopes and intercepts were derived from the linear regression results. Protein concentrations were determined from the extinction coefficient, ε_{280} = 2.5 (mg/ml)^{-1} cm^{-1}, established by quantitative amino acid analysis (13).

*Isotope Exchange—Radioisotope exchange between ^14C-labeled so-
dium formate and unlabeled CO$_2$ was measured in 15-mm diameter vials containing 400-μl reaction mixtures stirred gently under a continuous flow of the indicated gas phase. Reactions were initiated by addition of purified FDHH, and the reaction mixtures were incubated at 24 ℃. Aliquots, 10 μl, were removed from the reaction mixtures as a function of time and added to 10% acetic acid, 0.5 ml, contained in standard 20-ml scintillation vials. After addition of the appropriate scintillation mixture, the samples were counted in a Packard 2200CA liquid scintillation analyzer, and the results were corrected to yield dpm. Radioisotope exchange rate was determined by multiplying the formate concentration (mM) by the rate constant (min⁻¹) obtained from the slope of the initial, linear region of semi-logarithmic plots of total dpm versus time. Experiments using 10% CO$_2$/90% N$_2$ employed 50 mM sodium MES buffer initially of pH 6.45, which dropped to pH 6.28 after equilibration with the gas phase. Under 100% CO$_2$ the final pH was 6.14 using 80 mM N-morpholinoethanesulfonate of initial pH 7.07.

Concentrations of CO$_2$ in solution were calculated based on a pK$_a$ of 6.1 for the CO$_2$/bicarbonate equilibrium and the observed pH changes of buffered solutions upon equilibration with the indicated gas mixtures.

Formate concentrations were determined enzymatically. From an isotope exchange reaction conducted as described above, additional aliquots (10 μl) were removed concurrently and added to 490 μl of aerobic water. These mixtures were transferred to cuvettes, adjusted to contain 10 mM benzyl viologen in 25 mM potassium MOPS buffer at pH 7.0 in a final volume of 1.0 ml, and made anaerobic by sparging with argon. Formate-dependent reduction of benzyl viologen was initiated by addition of approximately 2.5 μg of purified FDHH. Absorption increase at 578 nm to a maximal, relatively stable value was complete within 10–15 min. The final absorbance was proportional to the amount of formate added. Formate concentrations were calculated using $ε_{578}$ = 1.1 × 10$^4$ M$^{-1}$ cm$^{-1}$ as the extinction coefficient of reduced benzyl viologen (12) and the stoichiometry of 2 mol of benzyl viologen per mol of formate.

**RESULTS**

**Enzyme Kinetic Mechanism**—Enzyme activity was assayed by measuring the formate-dependent reduction of the chromogen benzyl viologen. Reaction velocity of the enzyme was determined under conditions where concentrations of the two substrates (formate and benzyl viologen) were varied. The plots in Fig. 1 show the dependence of reaction velocity on formate concentration at different, defined concentrations of benzyl viologen. Parallel lines were observed in these plots, which suggests the enzyme undergoes a ping-pong Bi Bi kinetic mechanism. A set of parallel lines is also produced when the data are plotted as varying amounts of benzyl viologen at different fixed concentrations of formate (not shown), as expected for a ping-pong mechanism.

Secondary plots derived from these data are shown in Fig. 2. The data in Fig. 2A reveal that the $K_m$ for formate is 26 mM when benzyl viologen is saturating. As indicated in the figure, this $K_m$ value represents the average of two independent determinations, performed using different enzyme preparations.

At saturating formate, the $K_m$ for benzyl viologen was found to lie in the 1–5 mM range, as seen in Fig. 2B. There appears to be a systematic variation in the determination of the benzyl viologen $K_m$ (Fig. 2B). This may have been due to use of two different lots of benzyl viologen (the method of manufacture was changed between lots), and therefore we report the benzyl viologen $K_m$ as a range.

The $V_{max}$ at saturating levels of both substrates can be extrapolated from these data (not shown). This was used to derive the turnover number, $k_{cat}$, of $1.7 \times 10^3$ min$^{-1}$.

**Deuterioformate as Substrate**—Deuterioformate was used as an alternative substrate in the FDHH reaction replacing (protio)formate. The double reciprocal plots for varying deuterioformate concentrations at two different levels of benzyl viologen are shown in Fig. 3. Also shown are the results of reactions using similar concentrations of protioformate. FDHH exhibited a greater than 3-fold higher apparent $K_m$ for deuterioformate as compared to protioformate. However, the apparent $V_{max}$ differed by only about 30%. Deuterium isotope effects on $V_{max}/K_m$ were calculated to be 4.25 and 4.49 at 2 and 6.2 mM benzyl viologen, respectively.

**Isotope Exchange Analysis**—FDHH was mixed with $^{14}$C-labeled formate in the absence of benzyl viologen or other electron-accepting dyes. Radioisotope remained fixed in solution when the mixture was incubated under an atmosphere of 100% argon, however when carbon dioxide was admitted radioisotope was rapidly lost from the solution, as shown in Fig. 4A. Formate concentration remained unchanged during reaction, although most of the radioactive label was lost from solution, as shown in Fig. 4B. In a separate reaction carried out in the center well of a 20-ml Warburg vessel under a 10%
Kinetics of E. coli Formate Dehydrogenase

The effect of formate concentration on velocity of isotope exchange was analyzed at two different concentrations of CO₂. Parallel lines were found from double reciprocal plots of the data, as shown in Fig. 5. From these data, the dissociation constants of the enzyme complexes with formate (Kₐ) and CO₂ (K₈) were calculated to be 12.2 and 8.3 mM, respectively. These calculations followed the method described by Fronn (14), where the notation used is Kₐ and K₈ for these dissociation constants, respectively.

Inhibition by Nitrate and Azide—By varying the concentrations of sodium nitrate and sodium formate present in the reaction mixture, it was found that nitrate is a competitive inhibitor with respect to formate (Fig. 6A). The Kᵢ for nitrate is calculated to be 7.1 mM, or approximately one-fourth the Kₐ for formate (Fig. 6B). Similar experiments showed that nitrate is not competitive with benzyl viologen (data not shown).

Azide is known to be a potent inhibitor of a number of formate dehydrogenases. The effects of azide concentration on the reaction kinetics are given in Fig. 7A. The observed pattern is diagnostic for noncompetitive inhibition. The secondary plot of slope versus azide concentration disclosed a K(slope) of 75 μM, shown in Fig. 7B. A secondary plot (not shown) in which the y-intercepts of the lines in Fig. 7A were plotted against azide concentration gave a K(intercept) of 88 μM.

**DISCUSSION**

Kinetic analysis of the E. coli enzyme FDH₄ indicates that it undergoes a ping-pong Bi Bi reaction mechanism. This mechanism is substantiated by the demonstration of isotope exchange between the substrate formate and the product carbon dioxide in the absence of the second substrate benzyl viologen.

From these data it is not possible to distinguish between a CO₂ atmosphere all of the radioactivity released from solution was recovered in 1 M hydrazine hydride placed in the peripheral compartment of the flask. In control experiments, the observed exchange rate was proportional to the enzyme concentration, and in the absence of enzyme no exchange was observed (data not shown). These experiments demonstrate that FDH₄ catalyzes carbon exchange between formate and CO₂ in the absence of other electron-accepting molecules.

The rate of isotope exchange catalyzed by FDH₄ under an atmosphere of 100% CO₂ was 1.6 to 1.7 times the rate of formate-dependent benzyl viologen reduction by the enzyme (10 mM benzyl viologen) at pH 6.3 and the same formate concentration (5 mM). These results were found with two separate enzyme preparations.

FIG. 3. Double reciprocal plots with deuterioformate as substrate. FDH₄ activity was determined as described under "Materials and Methods," with varying concentrations of protioformate or deuterioformate at two levels of benzyl viologen, as indicated. The inset chart gives the values of the apparent Kᵢ and Vₘₐₓ for the various plots, as determined from the x- and y-intercepts, respectively. In the chart, the symbols used to represent the results of the various assays are described; TYPE refers to the type of formate used; H, protioformate; and D, deuterioformate. The units for benzyl viologen concentrations and Kᵢ values are millimolar, and the units for Vₘₐₓ values are μmoles/min.

<table>
<thead>
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<th>TYPE</th>
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**FIG. 4.** Isotope exchange between [1⁴C]formate and carbon dioxide. A, effect of gas phase on radioactivity released from formate in the presence of FDH₄. Analysis was made of radioactivity remaining in buffered, anaerobic solutions of sodium [1⁴C]formate (5.0 mM total formate concentration) as a function of incubation time. Purified FDH₄ was present in the reactions as described under "Materials and Methods." Gas phases were either 100% argon or 10% carbon dioxide/90% nitrogen, as indicated. B, constancy of total formate concentration during the course of formate/CO₂ isotope exchange. The reaction was carried out under anaerobic atmosphere containing 10% CO₂, as described in A above, and aliquots were also removed for analysis of formate as described under "Materials and Methods."

**FIG. 5.** Isotope exchange velocity dependence on formate concentration at different levels of carbon dioxide. Total formate concentration was varied in reactions equilibrated with either 10% CO₂/90% N₂ or 100% CO₂, and measurement of initial velocities of formate/CO₂ isotope exchange using approximately 2.5 μg of purified FDH₄ were made as described under "Materials and Methods." The concentrations of CO₂ in solution under these conditions are indicated in parentheses. The values of the slopes and the intercept/slope factors obtained from the double-reciprocal plots were used to solve for the dissociation constants for formate and CO₂ release from their respective enzyme complexes, as described by Fronn (14). The slope of the 100% CO₂ line is 4.261 min, and the intercept/slope is 0.105 mM⁻¹. For the 10% CO₂ line, the slope is 4.180 min, and the intercept/slope is 0.034 mM⁻¹.
one-site and a two-site ping-pong mechanism, i.e. we cannot say whether there is physical separation within the enzyme of the reactions of formate oxidation and viologen reduction. However, a two-site mechanism is consistent with the presence of multiple redox centers on the enzyme, potentially including an ionized selenol, a molybdopterin-guanine dinucleotide cofactor and an iron-sulfur center (12).

The $K_m$ for formate at saturating levels of benzyl viologen was determined to be 26 mM. This suggests formate may be found in similarly high concentrations intracellularly. It is interesting from a physiological standpoint to note that expression of the gene encoding FDHH is induced by formate, and this induction is optimal at 20–30 mM (9). As formate readily diffuses across the cell membrane, formate induction of FDHH gene expression appears to be optimal at concentrations similar to the FDHH $K_m$ for formate.

Some kinetic properties of FDHH have been reported previously (2, 15). Ruiz-Herrera et al. (15), using crude extracts of E. coli, determined the $K_m$ for formate to be 1.5–2.5 mM, and the $K_m$ for benzyl viologen, 0.29–0.56 mM. However, the $K_m$ values reported in that study were determined by varying only a single substrate, and therefore they are apparent $K_m$ values. By extrapolation of the relationships determined by our data (Fig. 2), we would expect the apparent $K_m$ values for formate and benzyl viologen to be 3.3 and 0.55 mM, respectively, at the substrate levels used in that report. Thus, there is excellent agreement between the data presented here and the data of Ruiz-Herrera et al. (15).

Deuterioformate is used by FDHH as a substrate in place of (protoproformate, although at reduced activity. We considered there is likely to be little difference between deuterioformate and protioformate in binding to the enzyme. It was also expected that FDHH would react more slowly with deuterioformate if C-H bond cleavage is involved in the rate-determining step. However, replacement of protioformate by deuterioformate in the coupled reaction caused a greater than 3-fold increase in the apparent $K_m$, with only a 30% reduction in the $V_{max}$. The explanation for these results, which follows, reveals much about the FDHH reaction mechanism.

A representation of a ping-pong reaction is shown in the following diagram.

$$
E \xrightarrow{k_1} EA \xrightarrow{k_2} P \xrightarrow{k_4} E''
$$

$$
E'' \xleftarrow{k_{-4}} E' \xleftarrow{k_{-2}} B \xrightarrow{k_3} E''
$$

where $E$, $E'$, and $E''$ represent FDHH (in the oxidized, one-electron-reduced and doubly-reduced states, respectively). $A$
and B are the substrates (formate and oxidized benzyl viologen, respectively), while \( P \) and \( Q \) are the products (carbon dioxide and reduced benzyl viologen, respectively). The diagram incorporates the stoichiometric requirements for two-electron oxidation of formate and one-electron reduction of benzyl viologen.

The steady-state rate equation for the reaction is as follows.

\[
\frac{1}{v} = \frac{1}{E_0} \left( \frac{k_{c2} + k_2}{k_{c1}} + A \frac{k_{c1}k_{c2} + k_{c1}k_B + k_{c2}k_B + k_c k_B}{k_{c1}k_{c2}k_B} + \frac{k_{c1} + k_{c2} + k_1}{k_{c1}k_B} \right)
\]

Where \( K_m \) for substrate A (formate) is defined as follows.

\[
K_m(A) = \frac{k_{c1}(k_{c1} + k_2)}{k_{c1}(k_{c1} + k_{c2} + k_B)}
\]

In the case where \( k_2 \) (formate oxidation) is much less than \( k_{c1} \) (formate-enzyme dissociation), Equation 2 reduces to the following.

\[
K_m(A) = \frac{k_{c1}k_{c1}}{k_{c1}(k_{c1} + k_{c2} + k_B)}
\]

If substitution of deuterioformate for protioformate results in a decreased rate of hydrogen (or deuterium) atom abstraction, then the effect will be a decrease in \( k_2 \). According to Equation 3 this results in an increased value for \( K_m \) (if the \( k_{c2} \) term is not dominant), and this is consistent with the obtained results.

At the point of intersection between the lines representing the double-reciprocal plots of protioformate and deuterioformate at the same concentration of benzyl viologen, the equations defining the lines are equivalent. Reduction of this equivalence to its simplest terms results in the following.

\[
\frac{1}{v} = \frac{1}{k_2} - \frac{1}{k_{c1}} = \frac{1}{K_d}
\]

Thus, the FDHH dissociation constant \( (K_d) \) for formate can be roughly estimated from Fig. 3 to be approximately 100 mM, which is a rather large dissociation constant.

Examination of Equation 1 reveals that the rate constant \( k_2 \) can be obtained from the slopes of double-reciprocal plots (Fig. 3) using the estimated value of \( K_m \) and \( E_c \) (total enzyme), and the assumption \( k_{c1} \gg k_B \). By this method we find for protioformate \( k_2 = 6.6 \times 10^5 \) min\(^{-1}\) and with deuterioformate \( k_2 = 1.5 \times 10^5 \) min\(^{-1}\). A substantial isotope effect on \( k_2 \) is observed \((k_2^D/k_2^H = 4.4)\).

The influence of a deuterated substrate on the reaction kinetics is often described as the “deuterium isotope effect on \( V_{max}/K_m \)” (16). This expression denotes the ratio of the \((V_{max}/K_m)\) terms for the protio- and deuterio-substrates. Our results indicate that the apparent deuterium isotope effect is largely unaffected by the second substrate. For reactions containing \( 2.0 \) and \( 6.2 \) mM benzyl viologen the magnitude of this effect is \( 4.25 \) and \( 4.49 \), respectively. The measured deuterium isotope effect on \( V_{max}/K_m \) is approximately equal to the deuterium isotope effect on step \( k_2 \).

The FDHH turnover rate constant, \( k_{cat} \), was determined to be \( 1.7 \times 10^5 \) min\(^{-1}\). The equation which defines \( k_{cat} \) follows.

\[
\frac{1}{k_{cat}} = \frac{1}{k_2} + \frac{1}{k_{c1}} + \frac{1}{k_{c2}}
\]

From this equation, limits for the values of \( k_2 \) and \( k_{c1} \) can be estimated. If \( k_2 = k_{c1} \), then both are \( 4.5 \times 10^5 \) min\(^{-1}\). In the case where \( k_2 \gg k_{c1} \) (or vice versa), a minimum value of \( 2.3 \times 10^5 \) min\(^{-1}\) is obtained for the lesser of the two. Thus, reaction steps subsequent to formate oxidation exert rate-limiting effects on the overall process, and this minimizes the effect on \( V_{max} \) when deuterioformate replaces protioformate.

Since benzyl viologen reduction appears to be partially rate-limiting in the reaction, FDHH may have an even higher turnover rate when reacting as part of the formate-hydrogen-lyase complex. From Equation 2 it is apparent that the determined \( K_m \) for formate is related to \( k_2 \) and \( k_{c1} \), the rate constants for benzyl viologen reduction. If the turnover rate differs when FDHH is a component of the formate-hydrogen-lyase complex, then the \( K_m \) for formate might also be changed.

Isotope exchange analysis established that the enzyme catalyzes carbon exchange between formate and carbon dioxide. The \( ^{14} \)C label was released from formate only when carbon dioxide was present in the reaction atmosphere, and the released radioisotope was quantitatively trapped in alkali. Thus, the product was identified as carbon dioxide. The total formate concentration in solution remained unaltered under conditions where extensive release of radioactivity occurred. This demonstrated that reduction of CO\(_2\) to formate was also ongoing, and it was concluded that the release of radiolabel occurred concomitantly with carbon dioxide reduction to formate. Whether the true reactant involved is carbon dioxide or bicarbonate remains to be established.

Under an atmosphere of 100% CO\(_2\), the enzyme catalyzed isotope exchange between formate and carbon dioxide at 1.6 to 1.7 times the rate of formate-dependent benzyl viologen reduction. This suggests that the isotope exchange reaction steps (or components thereof) are kinetically viable portions of the overall reaction.

The isotope exchange reaction can be represented by the following

\[
A + E \rightleftharpoons EA \rightleftharpoons E" + P
\]

where \( A \) (formate) is the initially labeled species, \( P \) represents CO\(_2\), while \( E \) and \( E" \) represent the oxidized and 2-electron-reduced forms of the enzyme, respectively. The exchange reaction velocity was kinetically analyzed as described by Fromm (14), whereby double reciprocal plots at different levels of \( P \) allow estimation of the two dissociation constants for the EA complex in Equation 6. The values determined for \( K_d^1 \) (formate dissociation constant) and \( K_d^2 \) (CO\(_2\) dissociation constant) were 12.2 and 8.3 mM, respectively. These values indicate relatively weak association of both formate and CO\(_2\) with the enzyme, consistent with the high dissociation constant described above. Isotope substitution (deuterioformate) experiments gave an estimation for the formate dissociation constant of roughly 100 mM. The pH, approximately 6.1, chosen for isotope exchange reactions was significantly lower than that of 7.5 used in the isotope substitution experiments. This suggests that the enzyme may bind formate more tightly at the lower pH.

The overall equilibrium constant for reduction of FDHH and oxidation of formate was obtained according to the following.

\[
K_m = \frac{[E"][P]}{[E][A]} = k_{c1}k_{c2} = \frac{K_d^1}{K_d^2} = 0.68
\]

The value of \( K_m \) is not far from 1.0, which indicates very little change of the overall free energy of the system upon reduction of the enzyme. This implies that the large amount of energy available from formate oxidation is conserved in the reduced enzyme. It can be calculated that enzyme reduction occurs with an apparent midpoint reduction potential about 10 mV more negative than the formate/CO\(_2\) couple (\( \Delta G^\circ \approx -RT \ln K_m \approx -n \Delta F^\circ \)).

Nitrate was found to inhibit FDHH in a competitive manner
with respect to formate. The $K_i$ for nitrate is 7.1 mM, or about one-fourth the $K_m$ for formate. Nitrate and nitrite are known to inhibit transcription of the gene which encodes FDHH (8, 9). Interestingly, the concentrations of nitrate necessary to inhibit transcription of the FDHH gene are similar to those which inhibit FDHH enzyme activity. Nitrate concentrations of 5–10 mM reduce transcription of the FDHH gene by about 80% (9). Also, formate concentrations of 20–30 mM can partially alleviate nitrate inhibition of $fdhF$ transcription (9). Thus, the negative effects of nitrate on FDHH enzyme activity and FDHH gene expression are observed in the same concentration range and may be competitive with formate. Nitrate may regulate FDHH activity at the posttranslational level as well as at the transcriptional level.

Azide is a potent inhibitor of most formate dehydrogenases and is thought to be an analog of the transition state (17). We have shown previously that azide inhibition of the enzyme is reversible (12). Noncompetitive inhibition is predicted for a transition-state analog in a ping-pong reaction mechanism. We show here that azide displays noncompetitive inhibition with a $K_i$ of about 80 $\mu$M, which supports the proposal that azide acts as a transition-state analog.

Acknowledgments—We thank P. Boon Chock and Dr. Charles Y. Huang for helpful discussions of the kinetics. Dr. Thressa C. Stadtman provided helpful discussions and support. We also thank Dr. Rodney Levine and Julie Sahakian for performing quantitative amino acid analyses.

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