Analysis of the Kinetic Mechanism of the Bovine Liver Mitochondrial Dihydroorotate Dehydrogenase†

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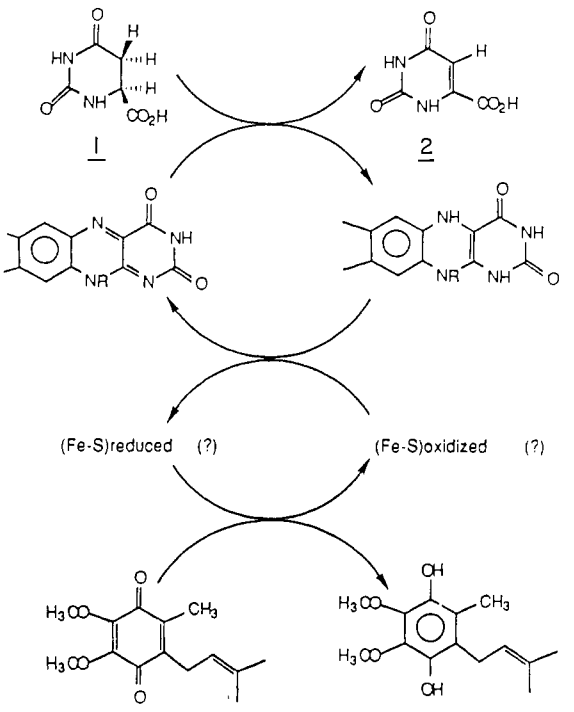
Received October 21, 1987; Revised Manuscript Received August 23, 1988

ABSTRACT: The steady-state kinetic mechanism of highly purified bovine liver mitochondrial dihydroorotate dehydrogenase has been investigated. Initial velocity analysis using *S*-dihydroorotate and coenzyme Q_6 revealed parallel-line, double-reciprocal plots, indicative of a ping-pong mechanism. The dead-end inhibition pattern with barbituric acid and the reactions with alternate cosubstrates methyl-*S*-dihydroorotate and menadione also point to a ping-pong mechanism. However, product orotate was found to be competitive with dihydroorotate and uncompetitive with Q_6. These findings suggest that dihydroorotate dehydrogenase may follow a nonclassical, two-site ping-pong mechanism, typical of an enzyme that contains two nonoverlapping and kinetically isolated substrate binding sites. That these two sites communicate by an intramolecular electron-transfer system involving FMN and perhaps an iron–sulfur center is also suggested by the kinetic behavior of the enzyme.

Dihydroorotate dehydrogenase (DHODase)[1] catalyzes the oxidation of dihydroorotic acid (DHO, **1**) to orotic acid (OA, **2**), the single redox step in de novo pyrimidine biosynthesis. Eukaryotic DHODases occur in both soluble and membrane-associated forms. Cytosolic enzymes have been isolated from the parasitic protozoans *Crithidia fasciculata* and *Trypanosoma brucei* (Pascal et al., 1983; Pascal & Walsh, 1984); both proteins contain flavin as the only redox-active cofactor and utilize molecular oxygen as the cosubstrate electron acceptor. Mammalian DHODases and that isolated from *Neurospora crassa* are mitochondrial membrane proteins; these appear to be linked functionally, and perhaps also structurally, to electron-transport chain components. The *N. crassa* enzyme is an iron-containing flavoprotein that uses long-chain quinones as cosubstrate electron acceptors (Miller & Adams, 1971; Miller, 1975). The homogeneous rat liver DHODase, by contrast, lacks flavin, contains iron and zinc as the two apparent redox-active cofactors, and—like the parasite enzymes—delivers electrons directly to molecular oxygen (Forman & Kennedy, 1977, 1978).

The bovine liver mitochondrial DHODase has been under investigation in our laboratory for some time (Keys & Johnston, 1985; Hines et al., 1986; Hines & Johnston, 1989). The enzyme has optimal activity with ubiquinone (coenzyme Q) as cosubstrate, and cofactor analysis reveals the presence of both FMN and iron—perhaps as an iron–sulfur cluster. Thus, like the fungal enzyme, bovine DHODase classifies as a dihydroorotate:ubiquinone oxidoreductase. Scheme I suggests a potential pathway for the flow of electrons through both the *Neurospora* and bovine enzymes. It seems reasonable that the protein-bound flavin functions as the proximal electron acceptor, experiencing two-electron reduction concomitant with dihydroorotate oxidation. Reduced flavin would then become reoxidized by passing electrons, perhaps one at a time, to the

Scheme I



putative iron–sulfur cluster. Coenzyme Q, in turn, serves as the distal redox-active cosubstrate receiving electrons from the iron center. In situ, the reduced quinone would be expected to be reoxidized by the mitochondrial electron-transport system.

In order to better understand the reactions catalyzed by the various DHODases, we have undertaken a thorough steady-state kinetic analysis of the mammalian enzyme. The kinetic data reported here indicate that the bovine DHODase is among a growing number of enzymes with multiple-substrate binding

† This research was supported in part by National Institutes of Health Grant GM 07197.

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[1] Abbreviations: DHO, dihydroorotate; DHODase, dihydroorotate dehydrogenase; methyl-DHO, dihydroorotate methyl ester; OA, orotate; KP_i, potassium phosphate; Q_6, coenzyme Q_6 (ubiquinone).

0006-2960/89/0428-1222$01.50/0

sites that may be both kinetically and structurally isolated from one another. This finding is at least minimally consistent with the sequential electron flow suggested by Scheme I.

Experimental Procedures

Materials. Coenzyme Q_6 was obtained from Sigma. Menadione, reduced Triton X-100, and barbituric acid were purchased from Aldrich. Barbituric acid was recrystallized twice from methanol prior to use. The quinones were dissolved in ethanol prior to use in the assays. The methyl ester of DHO was prepared as described previously (Hines et al., 1986).

Enzyme. Dihydroorotate dehydrogenase was purified from bovine liver mitochondria as described previously (Hines et al., 1986) with the following modifications. The inclusion of a Sephadex G-200 gel filtration step, which followed chromatography on Red A Dye Matrex, was found to be unnecessary if the specific activity of the enzyme after the Red A chromatography was greater than 10000 μmol of OA formed min^{-1} mg^{-1}. Omission of the gel filtration column under these conditions did not compromise the final purification. Improved recovery from the chromatofocusing column was obtained by incorporating 10% glycerol into all of the column buffers. The enzyme preparations used for these studies were 90–95% homogeneous, with specific activities ranging from 50 to 80 μmol of orotate formed min^{-1} mg^{-1}.

Initial Velocity and Inhibition Analysis. All reaction mixtures were in 0.1 M KP_i buffer, pH 7.8, and 0.1% reduced Triton X-100. The assay was initiated by addition of enzyme to a thermally equilibrated reaction mixture in a 1.0-mL cuvette (37 °C). The rate of the reaction was determined by monitoring orotate formation at 293 nm (Hines et al., 1986). For initial velocity experiments, both DHO and Q_6 were varied from 6 to 60 μM at fixed concentrations of the other substrate. For inhibition studies, the concentration of the varied substrate (DHO or Q_6) ranged from 0.5 to 5 K_M^{app}. The actual substrate concentrations chosen depended upon the K_I of the specific inhibitor used in the assay and on the inhibitor concentration. The concentration of the nonvaried substrate was usually 60 μM. In all of the kinetic studies, the amount of enzyme used in each assay fell within the enzyme concentration range where the initial velocity of the reaction is linearly proportional to enzyme concentration.

Assays with Alternate Substrates. When DHO was the varied substrate, either the reaction contained 30 μM Q_6 and orotate formation was monitored at 293 nm or the reaction contained 400 μM menadione and orotate formation was monitored at 282 nm (ϵ = 6600 M^{-1} cm^{-1}). When Q_6 was the varied substrate, the reaction contained either 18 μM DHO or 200 μM methyl-DHO. The reaction was monitored by following OA or methyl-OA formation at 293 nm. When methyl-DHO was used as an invariant substrate, care was taken to avoid ester hydrolysis prior to kinetic analysis (Hines et al., 1987). Stock substrate solutions were prepared in water, and the reaction was initiated by addition of substrate rather than enzyme to the reaction mixture.

Kinetic Analysis. For each single concentration of fixed substrate or inhibitor, the initial velocities obtained with varying substrate concentrations were fitted to eq 1 by using

$$v = \frac{VA}{K_M + A} \quad (1)$$

a computer program that provides an iterative nonlinear least-squares fit to the best rectangular hyperbola. Statistically significant systematic variations in slope, intercept, or both slope and intercept were used to determine whether inhibition was competitive, uncompetitive, or noncompetitive, respectively. Once an inhibition pattern was established, the entire data set was modeled to the appropriate equation:

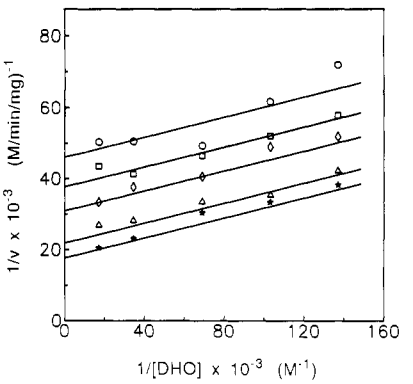

FIGURE 1: Initial velocity pattern for DHO oxidation. Orotate formation was observed at five fixed concentrations of Q_6. The reciprocal velocities are plotted as a function of the DHO concentration. The concentrations of Q_6 used were 7.1 (○), 9.6 (□), 13.4 (◇), 28.6 (△), and 61.6 (★) μM.

competitive inhibition

$$v = \frac{VA}{K_M(1 + I/K_I) + A} \quad (2)$$

uncompetitive inhibition

$$v = \frac{VA}{K_M + (1 + I/K_{II})A} \quad (3)$$

noncompetitive inhibition

$$v = \frac{VA}{K_M(1 + I/K_I) + A(1 + I/K_{II})} \quad (4)$$

The figures presented give the double-reciprocal plots generated from eq 2–4 superimposed upon the experimental data points.[2] Each data point represents an average of duplicate or triplicate determinations.

Results

Initial Velocity Pattern. The classical method for distinguishing between sequential and ping-pong reaction mechanisms is by analysis of the initial velocity patterns obtained by varying the concentration of one substrate at several different fixed concentrations of the second substrate. When data are plotted in double-reciprocal form, ping-pong mechanisms give patterns that consist of parallel straight lines. Sequential mechanisms yield patterns of straight lines that intersect in a common point to the left of the ordinate of the double-reciprocal plot. When initial velocity experiments were carried out with DHODase, no significant differences were observed in the slopes of the double-reciprocal plots, and thus the entire data set was fitted to the equation for a ping-pong mechanism (eq 5). Figure 1 shows the double-reciprocal plots generated

$$v = \frac{VAB}{K_M^A B + K_M^B A + AB} \quad (5)$$

when K_M^{DHO} = 10.2 μM, K_M^{Q6} = 16.6 μM, and V = 72 μM min^{-1} mg^{-1}. This V corresponds to a k_{cat} of 50.4 s^{-1}.

While the data of Figure 1 fit well to a ping-pong model, this experiment alone is not sufficient to eliminate all other kinetic mechanisms. Certain types of sequential mechanisms, wherein the constant term (K_M^A, K_M^B) is very small, can also

[2] Double-reciprocal plots are not given for all experimental sets. Figures 1 and 2 are representative of typical kinetic results. Other data are summarized in the tables.

Table I: Summary of Inhibition Patterns and Inhibition Constants

inhibitor	varied substrate	type of inhibition	K_i (μM)	K_{ii} (μM)
orotic acid	DHO	competitive	5.1 ± 0.4	
	Q_6	uncompetitive		57.6 ± 4.1
barbituric acid	DHO	competitive	92.8 ± 9.1	
	Q_6	uncompetitive		469.3 ± 44.5

Table II: Kinetic Constants for Reaction of DHODase with Alternate Cosubstrates

substrate	K_M (μM)	k_{cat} (s^{-1})
dihydroorotate	9.2 ± 1.3	50.4 ± 1.5
methyl dihydroorotate	154.2 ± 13.5	45.4 ± 1.1
Q_6	14.2 ± 2.3	44.2 ± 2.2
menadione	184.3 ± 28.1	18.3 ± 0.9

give reciprocal initial velocity plots that appear parallel (Segel, 1975). However, these mechanisms can be distinguished from a ping-pong reaction mechanism by product inhibition studies.

Product Inhibition. We have shown that the flavin cofactor of purified DHODase can be reduced by addition of DHO in the absence of any quinone cosubstrate (Hines & Johnston, 1989), which may be interpreted to imply that DHO binding precedes Q_6 binding. Making the assumption that DHO *is* the first substrate bound to DHODase, the expected product inhibition pattern for a ping-pong reaction mechanism using OA as inhibitor would be noncompetitive with respect to DHO and competitive with respect to Q_6. Table I gives the results of product inhibition studies with OA. When DHO was the varied substrate, competitive inhibition was observed; when Q_6 was the varied substrate, uncompetitive inhibition was obtained. This pattern of inhibition by OA is that expected for certain types of sequential reaction mechanisms (Bar-Tana & Cleland, 1974), and these results are in sharp contrast to the initial velocity experiments (Figure 1) that indicate a ping-pong mechanism. Additional studies were therefore carried out to explore further the formal kinetic mechanism. Two approaches that enable one to distinguish between sequential and ping-pong mechanisms are the use of alternate substrates (Huang, 1979) and the use of dead-end inhibitors (Cleland, 1963b).

Alternate Substrates. The bovine DHODase will catalyze the oxidation of both DHO and its methyl ester, although, as shown in Table II, the K_M values for these two dihydropyrimidines differ significantly. Similarly, menadione can replace Q_6 as cosubstrate, but, as shown in Table II, alternate quinones display differences in both K_M and V_{max} values. Inasmuch as all four substrates of Table II have unique kinetic parameters, these differences can be exploited in the determination of the formal kinetic mechanism of DHODase.

For both an ordered and a random rapid equilibrium mechanism, the expression for the slope of the 1/[DHO] plot contains kinetic constants relating to the quinone. Therefore, the slopes of 1/[DHO] plots should vary when structurally distinct quinone cosubstrates are used as the nonvaried substrate. In a ping-pong mechanism, however, the slope term is independent of the quinone and its associated kinetic constants. In this instance, the slopes of 1/[DHO] plots should be independent of the second cosubstrate used. Comparable results are expected when alternate first substrates are used and the quinone is the varied substrate: the slopes of the 1/[Q] plots will be independent of the first substrate in a ping-pong mechanism, whereas the slope will vary with the first substrate in a sequential mechanism.

On the basis of these arguments, two sets of kinetic analyses were performed by using the alternate substrate pairs of Table II. Dihydroorotate was the varied substrate with either Q_6 or menadione as the nonvaried cosubstrate. Alternatively, Q_6 was the varied substrate, and either DHO or methyl-DHO was the nonvaried substrate. In all cases, the nonvaried substrate was at subsaturating concentrations. Figure 2 presents the results of both experiments. With DHO as the varied substrate (Figure 2A), the slopes with Q_6 (0.154 ± 0.045 min mg) and menadione (0.195 ± 0.059 min mg) were nearly identical. Similarly, the slopes with either DHO (0.217 ± 0.037 min mg) or methyl-DHO (0.224 ± 0.048 min mg) were identical when Q_6 was the varied substrate (Figure 2B). Only in the case of a ping-pong mechanism will one obtain invariant slopes with both alternate first and second substrates.

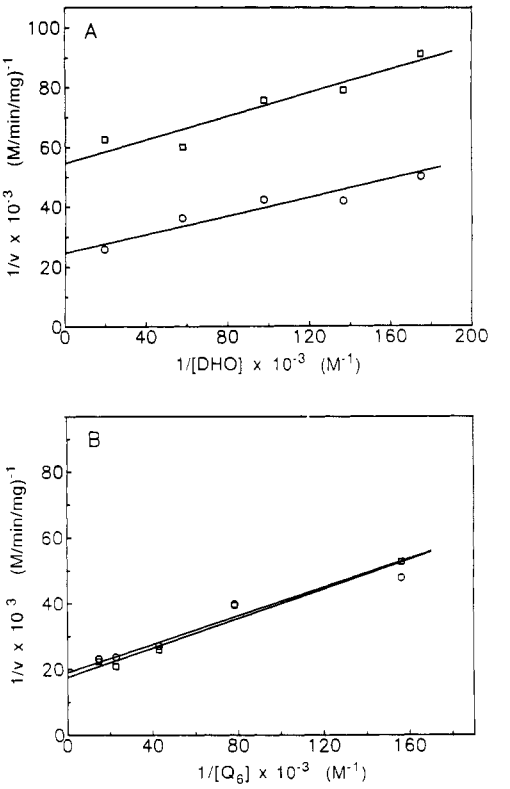

FIGURE 2: Kinetic patterns for reaction of DHODase with alternate substrates. (A) Dihydroorotate was the varied substrate with either 400 μM menadione (□) or 30 μM Q_6 (○) as second substrate. (B) Q_6 was the varied substrate with either 18 μM dihydroorotate (○) or 200 μM methyl dihydroorotate (□) as the first substrate.

Dead-End Inhibition. Final confirmation of the ping-pong mechanism was sought through analysis of dead-end inhibition. On the basis of the rules outlined by Cleland (1963a), a compound that is a competitive inhibitor of DHO should show uncompetitive inhibition with respect to Q_6 in a ping-pong reaction but noncompetitive inhibition with respect to Q_6 in a sequential mechanism. Barbituric acid was used as the dead-end inhibitor, and Table I also includes a summary of the inhibition patterns obtained with varied concentrations of DHO and Q_6. As predicted for a ping-pong mechanism, barbituric acid was competitive with respect to DHO and uncompetitive with respect to Q_6.

Discussion

We found that a number of experimental approaches were required to determine the formal kinetic mechanism of the bovine mitochondrial DHODase. While the results of initial velocity experiments gave evidence for a ping-pong mechanism, other kinetic mechanisms can also produce parallel-line kinetic patterns like those of Figure 1. Thus, we turned to inhibition analysis and experiments using alternate substrates in order

to confirm our initial suspicion that DHODase operates by a ping-pong mechanism.

Table I summarizes the inhibition patterns observed when orotate and barbiturate were used as inhibitors; the K_I values for each inhibitor are also listed in the table. If, as the initial velocity experiments of Figure 1 imply, DHODase catalyzes a classical ping-pong reaction mechanism, one would expect to find that OA is noncompetitive with DHO and competitive with Q_6. However, the observed product inhibition patterns using OA were those expected for a sequential rather than a ping-pong mechanism (competitive with DHO and uncompetitive with Q_6). The dead-end inhibition patterns obtained with barbiturate (competitive with DHO, uncompetitive with Q_6) are consistent with a ping-pong mechanism.

Because of these apparent ambiguities, we examined the reactions of the enzyme with the alternate cosubstrates methyl dihydroorotate and menadione (Figure 2). These compounds have kinetic constants that differ significantly from the cognate values for dihydroorotate and ubiquinone. As a consequence, one is able to use these differences to distinguish formal kinetic mechanisms. As shown in Figure 2, by use of either DHO or Q_6 as the varied substrate, the slopes of double-reciprocal plots were invariant. These results clearly indicate a ping-pong mechanism—albeit a nonclassical ping-pong mechanism—for the DHODase reaction.

The most plausible mechanism that accounts for our experimental data is one characterized by a rapid equilibrium random (two-site) hybrid ping-pong reaction. The essential feature of the hybrid two-site mechanism is that the enzyme contains two distinct catalytic sites, each responsible for half of the ping-pong reaction. Each site is both physically and kinetically isolated from the other; the binding of a substrate or inhibitor at one site does not influence individual rate constants at the second site. These types of two-site reaction mechanisms usually show parallel initial velocity plots, consistent with a classical ping-pong mechanism. However, unique product inhibition patterns are observed. The first product (P) is always competitive with the first substrate (A). Similarly, inhibition by the second product (Q) is competitive with the second substrate (B). Product inhibition by P with respect to B can be either noncompetitive or uncompetitive.

This type of mechanism was first described for proteins that contain a flexible biotin (Northrop, 1969; Barden et al., 1972) or lipoic acid (Tsai et al., 1973) cofactor, which serves to link functionally the structurally independent catalytic sites. More recently, a number of multicofactor proteins have also been characterized as hybrid two-site ping-pong enzymes, including xanthine dehydrogenase (Coughlan & Rajagopalan, 1980), glutamate synthetase (Rendina & Orme-Johnson, 1978), nitrate reductase (Renosto et al., 1981), and two hydrogenases (Arp & Burris, 1981; Livingston et al., 1987). In all of these proteins the separate catalytic sites are linked by internal electron-transferring redox cofactors. If DHODase is also among this group of enzymes, the protein would be expected to contain a pyrimidine binding site for DHO oxidation and a separate quinone binding site for Q_6 reduction. These two sites could be linked by an internal electron-transport system that would include the flavin and an iron-containing, redox-active cofactor. Electrons would be transferred from DHO to flavin and then to the iron center. This iron cofactor, in turn, would be exposed to the quinone binding site but physically separate from the pyrimidine binding site.

Figure 3 outlines the three rapid-equilibrium random segments for the reaction catalyzed by the bovine DHODase as expected for a uni-uni rapid equilibrium random two-site

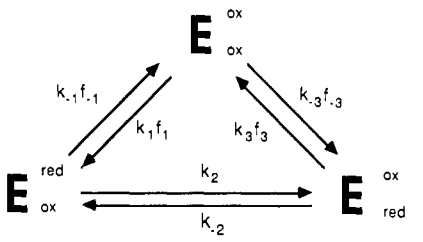

FIGURE 3: Model for the kinetic mechanism of DHODase. The kinetic mechanism consists of three segments. E_{ox}^{ox} represents the fully oxidized enzyme. E_{ox}^{red} represents the reduced flavin/oxidized iron enzyme species. E_{red}^{ox} represents the oxidized flavin/reduced iron enzyme species. f_1, f_{-1}, f_3, and f_{-3} represent the fraction of each rapid-equilibrium segment that participates in the indicated reaction.

hybrid ping-pong mechanism. The three steps of this mechanism include (a) oxidation of DHO with reduction of flavin, (b) intramolecular transfer of electrons from flavin to the iron cofactor, and (c) transfer of electrons to quinone to regenerate fully oxidized enzyme. The complete rate equation for the type of mechanism outlined in Figure 3 has been derived by Coughlan and Rajagopalan (1980)[3] and is presented in eq 6

$$v/V = [A][B]/\{K_{mB}[A] + K_{mA}[B] + [A][B] + (K_{mA}K_{ib}[P]/K_{ip}) + (K_{ia}K_{mB}[Q]/K_{iq})\} + (K_{mA}[B][P]/K_{ip}) + (K_{mB}[A][Q]/K_{iq}) \quad (6)$$

in terms of group rate constants (Cleland, 1963b), where A = DHO, B = Q_6, P = OA, and Q = reduced Q_6.

Examination of eq 6 predicts the following results: (i) parallel initial velocity plots; (ii) competitive inhibition by OA with respect to DHO; and (iii) noncompetitive inhibition by OA with respect to Q_6. While the data presented in this paper are consistent with the first two predictions, a modification of the mechanistic model (Figure 3) is necessary to allow for the observed uncompetitive OA product inhibition with Q_6. This modification involves the elimination of the reversible connection between the DHO and Q_6 binding steps. That is, if the rate constants that govern either reversible oxidation of DHO (k_{-1}) or electron transfer from the reduced iron center back to flavin (k_{-2}) are small compared to all other rate constants of Figure 3, then eq 6 simplifies to eq 7. This is

$$\frac{v}{V} = \frac{[A][B]}{K_{mB}[A] + K_{mA}[B] + [A][B] + (K_{ia}K_{mB}[Q]/K_{iq})} + (K_{mA}[B][P]/K_{ip}) + (K_{mB}[A][Q]/K_{iq}) \quad (7)$$

not an unreasonable modification of the kinetic model for DHODase, given the large equilibrium constant ($K_{eq} \approx 10^{12}$) favoring product formation. Moreover, we have not been able to observe the formation of dihydroorotate upon incubation of reduced quinone and orotate with the enzyme, further supporting the proposition that the reversible steps described by k_{-1} and k_{-2} do not appreciably contribute to the overall kinetic mechanism. Equation 7, in turn, predicts that OA should be uncompetitive with respect to Q_6, as is observed.

Arguments similar to those outlined above have been offered to explain an unusual pattern of product inhibition observed with nitrate reductase (Renosto et al., 1981). This protein is also characterized by a hybrid two-site ping-pong reaction mechanism. The equilibrium constant for the reaction catalyzed is large ($K_{eq} \approx 10^{25}$), and uncompetitive inhibition is

[3] This derivation differs from the original example developed for pyruvate dehydrogenase in that only three rapid equilibrium segments are required. Northrop's original analysis included a fourth segment that represents the migration of the biotin cofactor between the two substrate binding sites (Northrop, 1969).

observed; noncompetitive inhibition is expected for a reversible reaction.

A comparison of the kinetic behavior seen for other DHODases reveals a striking difference between the simple and complex flavoproteins. The simple flavoprotein DHODase from *C. fasciculata*, which lacks a second redox-active cofactor, shows noncompetitive inhibition with OA and DHO (Pascal et al., 1983). While no formal kinetic analysis has been carried out on the *C. fasciculata* enzyme, the demonstration of noncompetitive inhibition between DHO and OA completely excludes a two-site ping-pong mechanism. In contrast, the *N. crassa* enzyme, which contains iron and flavin cofactors, shows parallel initial velocity patterns and competitive inhibition by OA with respect to DHO (Miller & Adams, 1971). These results are identical with our findings for the bovine enzyme and are completely consistent with a two-site ping-pong mechanism.

In summary, bovine DHODase catalyzes a ping-pong reaction with DHO and Q_6 as cosubstrates. This is supported by parallel initial velocity patterns, the use of alternate substrates, and dead-end inhibition analysis. Product inhibition experiments, however, are completely inconsistent with a classical ping-pong mechanism. In fact, these inhibition patterns suggest that DHODase, like other multicofactor enzymes, could be a two-site ping-pong enzyme.

ACKNOWLEDGMENTS

We express our special thanks to Professor John Westley for many helpful discussions.

Registry No. DHODase, 9029-03-2; Q_6, 1065-31-2; DHO, 155-54-4; methyl-DHO, 23903-57-3; OA, 65-86-1; menadione, 58-27-5; barbituric acid, 67-52-7.

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