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ROLE OF HYDROGENASE 1 OF *CLOSTRIDIUM PASTEURIANUM* IN THE REDUCTION OF METRONIDAZOLE

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Abstract—Competition studies between the phosphoroclastic reaction and the metronidazole reduction reaction using dialyzed crude cell-free extracts of *Clostridium pasteurianum* which were essentially devoid of Hydrogenase 1 activity demonstrated that this enzyme plays an important role in the reduction of metronidazole. To determine further the exact function for Hydrogenase 1 in the reduction of the drug, this enzyme was highly purified from *C. pasteurianum*. Metronidazole reduction activity co-purified with Hydrogenase 1 specific activity throughout the purification procedure. Drug reduction required the presence of an electron carrier and could not be accomplished by the enzyme alone. Ferredoxin, and also the low potential electron carrier dyes, methyl and benzyl viologen, and the flavin coenzymes, FAD and flavin mononucleotide (FMN), could couple the reduction of metronidazole. Hydrogenase 1 activity and its metronidazole reduction activity were inactivated irreversibly in the presence of oxygen. Metronidazole could be reduced only by an electron carrier–Hydrogenase 1 mechanism or directly by sodium dithionite.

It has been established by ourselves and other investigators [1–5] that the reduction of metronidazole in *Clostridium pasteurianum* is dependent on ferredoxin, the principal physiological low potential electron carrier in this microorganism [6]. However, confusion exists in the literature as to the exact mechanism of metronidazole's reduction in anaerobic microorganisms. Many investigators besides ourselves believe that the drug is reduced enzymatically by a "nitroreductase(s)" [7–13], similar to the nitroreductases that have been shown to reduce nitrofurans in facultatively anaerobic microorganisms [14]. Other investigators believe that metronidazole is reduced directly by chemical reduction via reduced ferredoxin and methyl viologen [3, 5, 15].

In vitro enzymatic competition studies between the metronidazole reduction system and two other ferredoxin-dependent reactions in *C. pasteurianum* have shown previously that metronidazole is preferentially reduced by siphoning electrons away from reduced ferredoxin used for reduction in the inducible dissimilatory sulfite reductase system [2, 4] and in the phosphoroclastic-Hydrogenase 1 reaction [2, 16]. The results of these biochemical studies suggested that the reduction of metronidazole in *C. pasteurianum* occurred preferentially via a ferredoxin-linked metronidazole reductase(s) enzymatic mechanism [2, 4, 16]. Since both the inducible dissimilatory sulfite reductase system and the phosphoroclastic reaction in *C. pasteurianum* are coupled by ferredoxin and the bidirectional hydrogenase (Hydrogenase 1), we perceived that this enzyme was physiologically important to the reduction of metronidazole and was potentially the "metronidazole reductase" in this microorganism.

Since the function of the oxidizing hydrogenase of *C. pasteurianum* (Hydrogenase 2) which appeared mainly under N_2 fixing conditions is not understood fully [17, 18], we concentrated exclusively on the role of Hydrogenase 1 in the reduction of metronidazole. In this paper we present evidence for the physiological role of *C. pasteurianum* Hydrogenase 1 in the reduction of metronidazole, and discuss the significance of these findings to the mechanism of action of this important antibiotic against *C. pasteurianum*.

MATERIALS AND METHODS

Culture conditions

Clostridium pasteurianum W5 was grown under non-nitrogen fixing conditions in 10 liter batch cultures on a 1% sucrose-synthetic salts medium supplemented with 1 mM SO_4^{2-} plus 10 mM cysteine [19], and the cells were harvested at late log phase as described earlier [4].

Cell-free extracts

Crude cell-free extracts were prepared from the cell pellets as described in Ref. 4 for the purification of Hydrogenase 1 and experiments with the phosphoroclastic reaction. In the latter case, the crude cell-free extract was divided into two fractions. One fraction was dialyzed under aerobic conditions against 0.15 mM potassium phosphate buffer, pH 7.4, at 5° for 12 hr to inactivate Hydrogenase 1

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activity in the presence of air. Hydrogenase 1 activity is inhibited irreversibly by oxygen [20]. This crude cell-free extract was then known as the dialyzed extract. The non-dialyzed crude cell-free extract fraction was stored under H_2 at 5° until used for the experiments in Table 1.

Phosphoroclastic reaction assays

The *C. pasteurianum* phosphoroclastic reaction was assayed with 0.1 ml of 0.1 M methyl viologen in the main compartment of the Warburg flasks by the method described earlier [16], except that a volume equivalent to 12 mg of crude cell-free extract protein was used. Reaction supernatant fractions were immediately analyzed for the reduction of metronidazole in a Perkin–Elmer Lambda 3 spectrophotometer by calculating the amount of drug remaining after the 10-min reaction time using metronidazole's extinction coefficient of $9300\ cm^{-1}\ M^{-1}$ at 320 nm [1].

In the assays using the dialyzed extracts, the contents of the Warburg flask were identical to the control experiments above except that thiamine pyrophosphate (TPP) (1 μg/ml) was added.

Enzyme purification

The bidirectional hydrogenase (Hydrogenase 1) was highly purified from the crude cell-free extracts to the second DE-52 step (see Table 2) by the method of Chen and Mortenson [21]. The molecular weight of the purified enzyme was determined by electrophoresis on sodium dodecyl sulfate–polyacrylamide gel as described by Weber and Osborn [22].

Enzyme assays using purified Hydrogenase 1

All of these assays were done using a Warburg apparatus with a water bath temperature of 30°, and a double sidearm pre-calibrated Warburg flask was gassed out under H_2 for 20 min. The reactions were monitored by standard manometric techniques, and all assays were followed over 6 min. At the end of each experiment, the flask contents were centrifuged at 10,000 *g* for 10 min and the supernatant fractions were analyzed for drug reduction as previously described [2, 4].

H_2 *evolved.* Hydrogenase 1 specific activity during the purification procedure was measured by the H_2 evolution assay with 10 mM methyl viologen acting as the electron carrier and 15 mM $Na_2S_2O_4$ as the reductant in 2 ml of reaction mixture [21, 23]. Bovine serum albumin (1 mg/ml) was added to the 2-ml reaction mixture to help stabilize the more highly purified Hydrogenase 1 fractions as outlined in Ref. 21. One unit of hydrogenase activity was defined as 1 μmol H_2 evolved per min per mg of protein. The metronidazole reduction activity of Hydrogenase 1 could not be quantitated accurately using the H_2 evolution assay for the following reasons. First, we have shown previously that the reduction of metronidazole preferentially consumed the H_2 reducing equivalents being generated by the phosphoroclastic reaction of *C. pasteurianum* whereby electrons for H_2 evolution by the bidirectional hydrogenase (Hydrogenase 1) were diverted towards drug reduction [2, 16]. Second, metronidazole is chemically reduced by sodium dithionite alone [1] (see Table 4).

H_2 *uptake.* The reaction contents in a double sidearm Warburg flask were the same as for the H_2 evolution assay minus sodium dithionite. Hydrogenase 1 specific activity had first-order kinetics within the initial 2 min of the assay. In the presence of metronidazole, Hydrogenase 1 first-order kinetics were extended to 6 min before the enzymatic activity rapidly tapered off. Several different electron carriers were alternatively substituted in the assay system to couple this enzymatic reaction (see Table 3). One unit of Hydrogenase 1 activity was defined as 1 μmol of H_2 uptake per min per mg of protein.

Chemical reduction of metronidazole by reduced methyl viologen. The flask initially contained the following: 0.2 ml of 0.1 M methyl viologen; 0.2 ml of 25 mM metronidazole in the left sidearm; 0.18 ml of 0.05 M Tris–HCl buffer, pH 8.0, in the right side-

Table 1. Evidence for a physiologic role of *C. pasteurianum* Hydrogenase 1 in the reduction of metronidazole (MET)

Assay conditions*	Gas evolved† (μmol)	H_2 evolved‡ (μmol)	Acetyl phosphate produced (μmol)	MET reduced§ (μmol)	H_2:MET
(1) Crude extract	20.8 ± 0.6	9.8 ± 0.3	10.0 ± 0.5		
(2) Crude extract + MET	17.3 ± 0.6	5.3 ± 0.4	13.6 ± 0.6	4.3 ± 0.2	1.9:1
(3) Dialyzed extract	3.0 ± 0.3	0.7 ± 0.1	2.6 ± 0.2		
(4) Dialyzed extract + MET	2.8 ± 0.2	0	3.0 ± 0.2	0.34 ± 0.1	2.1:1

* Assay conditions were as described in the text. A volume equivalent to 12 mg of crude or dialyzed protein was used in these experiments. The reaction assay time throughout was 10 min. Each value is an average of four experiments ± SD.

† Gas evolved = $CO_2 + H_2$.

‡ CO_2 was trapped by NaOH [16].

§ The amount of metronidazole reduced was calculated by subtracting the amount of drug that remained from a starting concentration of 4.4 ± 0.2 μmol.

arm and 1.4 ml of buffer in the main compartment to give a total reaction mixture volume of 2.0 ml. The flask was gassed out before 0.02 ml of Hydrogenase 1 that had been purified to the Sephadex G-100 step (see Table 4) was added via a Hamilton gas-tight syringe into the degassed buffer contained in the rubber-stoppered right sidearm. The methyl viologen was reduced enzymatically by immediately tipping the Hydrogenase 1 into the main compartment of the flask and allowing the system to couple under H_2 gas for a further 10 min. Finally, the flask contents were gassed out for 30 min by carbon monoxide to bring about inhibition of Hydrogenase 1 [20, 24, 25] while leaving the methyl viologen reduced (dark blue). Metronidazole was tipped into the main flask compartment to start the reaction.

Chemical reduction of metronidazole by NADH and NADPH. A double sidearm Warburg flask contained: 1.6 ml of 0.05 M Tris–HCl buffer, pH 8.0, in the main compartment, 0.2 ml of 125 mM NADH or NADPH as the chemical reductants in the right sidearm, and 0.2 ml of 25 mM metronidazole as the substrate in the left sidearm. The reaction was started by simultaneously tipping the drug and NADH/NADPH into the main flask compartment.

Chemical reduction of metronidazole by sodium dithionite. Various concentrations of sodium dithionite from 10 to 75 mM were initially used in the Warburg reaction assay mixture to determine the stoichiometry of the reduction of 25 mM metronidazole. The flask contents were similar to the previous assay system, except that NADH/NADPH was absent. After the flask was gassed out, 0.2 ml of 50 mM degassed sodium dithionite was added into the rubber-stoppered right sidearm. The reaction was started immediately by simultaneously tipping the drug and sodium dithionite into the flask. The flask contents were aerobically shaken at the end of the experiment for 1 hr to oxidize any remaining sodium dithionite which would have spectrophotometrically interfered with metronidazole's absorption peak at 320 nm.

Protein

Protein was measured by either the Biuret procedure as previously described [26], or by the method given in Ref. 27 using dried bovine serum albumin as the standard.

Chemicals

Clostridium pasteurianum type V ferredoxin, coenzyme A, thiamine pyrophosphate (TPP), benzyl viologen, methyl viologen, FAD, flavin mononucleotide (FMN), and sodium dithionite were obtained from the Sigma Chemical Co. DEAE-cellulose (DE-52) was obtained from Whatman Ltd. Hydroxyapatite (Bio-Gel HTP) was obtained from Bio-Rad Laboratories Ltd. Metronidazole was obtained from Rhone-Poulenc Pharma Inc. All other chemicals were of reagent grade quality.

RESULTS

Evidence for a physiological role for C. pasteurianum *Hydrogenase 1 in the reduction of metronidazole*

We have shown previously that the electrons generated by the enzymatic oxidation of pyruvate in *C. pasteurianum in vitro* phosphoroclastic reaction assays were preferentially used to reduce metronidazole instead of being evolved as H_2 via the ferredoxin-Hydrogenase 1 reaction as was the case in the absence of the drug (see Fig. 3 of Ref. 16).

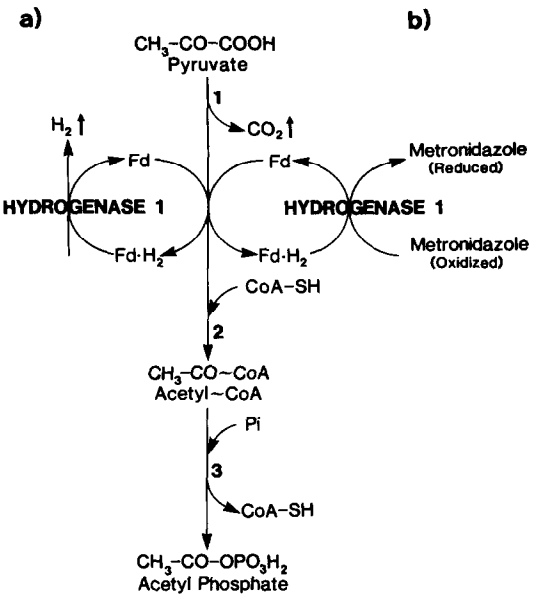

Fig. 1. Role of the phosphoroclastic reaction of *C. pasteurianum* in the reduction of metronidazole. (a) Electron flow of the phosphoroclastic reaction in the absence of metronidazole. (b) Proposed electron flow of the phosphoroclastic reaction in the presence of metronidazole showing no H_2 evolution in which the Hydrogenase 1 is acting as the "metronidazole reductase". Steps 1 and 2 of the reaction involve the pyruvate:ferredoxin oxidoreductase, ferredoxin, thiamine pyrophosphate containing oxidoreductase (TPP-E), and hydroxyethyl-TPP-E (HETPP-E). Step 3 is catalyzed by phosphotransacetylase [28].

The experiments outlined in Table 1 show that the *C. pasteurianum* bidirectional hydrogenase (Hydrogenase 1) was essential for the catalytic reduction of metronidazole. The flow of electrons through the phosphoroclastic system in the absence of metronidazole is shown in Fig. 1a. In the control phosphoroclastic reaction assay using crude cell-free extract [Table 1 (1)], the total amount of gas ($CO_2 + H_2$) evolved in 10 min was twice the amount of H_2 evolved by the assay system containing the CO_2 trapping system which agrees with the expected theoretical $CO_2 + H_2$:H_2 ratio of 2:1 for the phosphoroclastic reaction. Furthermore, the overall stoichiometry of the phosphoroclastic reaction showed that 20.8 μmol of gas ($CO_2 + H_2$) evolved versus 10.0 μmol of actyl phosphate formed gave a ratio of gas : acetyl phosphate of 2.1 : 1. This ratio not only approximates the theoretical 2 : 1 ratio expected for this enzymatic reaction (pyruvate $\rightarrow CO_2 + H_2$ + acetyl phosphate) but also confirms the results of our previous studies [2, 16].

The increase in acetyl phosphate formation which occurred during the reduction of metronidazole [Table 1 (2)] was shown previously to be due to the rate-limiting role of the Hydrogenase 1 in the

complex phosphoroclastic reaction [2, 16]. The lower amount of H_2 gas evolved [Table 1 (2)] in the presence of metronidazole as compared to reaction No. 1 without the drug (Fig. 1a) represented the electron equivalents evolved as H_2 after the initial complete preferential reduction of metronidazole. The flow of electrons through the phosphoroclastic system in the presence of metronidazole is shown in Fig. 1b. The missing 8.3 μmol of H_2 (13.6 μmol of acetyl phosphate minus 5.3 μmol H_2) could be stoichiometrically accounted for in the reduction of 4.3 μmol of metronidazole giving a H_2:metronidazole ratio of 1.9 : 1 which approximates the predicted ratio of 2:1 [1, 4, 29].

When the phosphoroclastic reaction was studied using dialyzed extract in which Hydrogenase 1 was inactivated irreversibly by air (see Materials and Methods), the control reaction [Table 1 (3)] proceeded at a much slower rate than the comparable reaction using crude cell-free extract [Table 1 (1)]. The total acetyl phosphate formed by the phosphoroclastic reaction using dialyzed extract was approximately 25% of the amount formed over the same time period for the non-dialyzed crude cell-free extract. However, this was enough activity to re-reduce the oxidized electron carriers (ferredoxin/methyl viologen) in the assay system if they were involved in the chemical reduction of metronidazole. Although the diminished phosphoroclastic activity in the dialyzed extract may have been due to some instability of the pyruvate dehydrogenase complex, it more likely occurred because of the lower reoxidation rate of reduced ferredoxin by Hydrogenase 1 that is necessary to pull the overall phosphoroclastic reaction [2, 16]. The dialyzed extract contained minimal Hydrogenase 1 activity as only 0.7 μmol of H_2 was evolved during the reaction time which was 14-fold less than that of reaction No. 1 (Table 1). The detection of this minimal Hydrogenase 1 activity was due to the large amount of dialyzed extract which was used (12 mg) in the reaction mixture (see Materials and Methods). Because of the minimal Hydrogenase 1 activity (0.7 μmol of H_2 evolved by the assay containing the CO_2 trapping system), the total gas evolved ($CO_2 + H_2$) [Table 1 (3)] was mainly CO_2 (3.0 μmol). Comparison of the amount of CO_2 evolved to the acetyl phosphate formed (2.6 μmol) yields a CO_2:acetyl phosphate ratio of 0.9:1 which approximates the expected theoretical 1:1 ratio for this reaction.

In experiments using the dialyzed extract in the presence of metronidazole [Table 1 (4)], no H_2 was evolved and the ratio of CO_2 produced to acetyl phosphate formed was 0.9:1 which again approximated the expected 1:1 ratio for this reaction. The amount of acetyl phosphate product formed (3.0 μmol) shows that there were enough reducing equivalents available via reduced ferredoxin/methyl viologen during these experiments to reduce 1.5 μmol of metronidazole. Thus, if metronidazole was being reduced by either direct chemical reduction by these electron carriers or enzymatically via an electron carrier–pyruvate dehydrogenase complex mechanism, then this amount of drug should have been reduced. However, only 23% of this expected amount of drug reduction (0.34 μmol) actually occurred. The amount of metronidazole reduced in the reaction using dialyzed extract could be stoichiometrically accounted for by the minimal Hydrogenase 1 activity expressed in the control reaction [Table 1 (3)]. The ratio of H_2 evolved (0.7 μmol to metronidazole reduced (0.34 μmol) was 2.1:1 which was equivalent to the ratio found in reaction No. 2 (Table 1). Thus, the reduction of metronidazole in this reaction using dialyzed extract (which contained minimal amounts of Hydrogenase 1 activity) clearly demonstrated the dependence of drug reduction upon an electron carrier-linked Hydrogenase 1 mechanism.

Purification of Hydrogenase 1

The previous experiments demonstrated to us that

Table 2. Purification steps for Hydrogenase 1 from *C. pasteurianum* and its comparative specific activity and metronidazole (MET) reduction activity

Step	Protein (mg)	Hydrogenase 1 specific activity H_2 evol.*	Hydrogenase 1 specific activity H_2 uptake*	MET reduction activity†	H_2:MET	Relative activities H_2 evol.	Relative activities H_2 uptake	Relative activities + MET
Crude extract	4567	1.63	1.43	2.30	2.4:1			
Heat (55°)	2218	2.47	2.10	3.80	1.9:1	1.5	1.5	1.7
First DE-52 column	605	6.70	5.10	9.13	2.1:1	4.1	3.6	4.0
Sephadex G-100 column	88	33	28	50.2	2.3:1	20	20	22
Second DE-52 column	10	145	142	223	ND‡	89	99	97

* One μmol H_2 evolved or 1 μmol of H_2 uptake per min per mg of protein (see Materials and Methods).

† Metronidazole reduction activity units are H_2 uptake expressed as 1 μmol H_2 uptake per min per mg of protein (see Materials and Methods).

‡ Not determined.

Table 3. Hydrogenase 1 specific activity and metronidazole reduction activity (MET) using different electron carriers

Electron carrier	Conc. (mM)	Hydrogenase 1 specific activity*	MET reduction activity
None		0	0
Methyl viologen	10	139	224
Benzyl viologen	10	136	177
FMN	10	160	245
FAD	10	147	239
Ferredoxin†	0.03	51‡	120
Ferredoxin + methyl viologen	0.03 + 10	253‡	428
Methyl viologen (Hydrogenase 1 aerated 60 min)	10	0	0

All of the values in this table were derived from experiments using the purification fraction from the second DE-52 column. A protein concentration of 14 μg was used throughout these experiments.

* Hydrogenase 1 specific activity throughout was derived from the H_2 uptake assay (except for the activities outlined in ‡ below), where 1 unit of activity = 1 μmol H_2 uptake per min per mg of protein.

† Ferredoxin concentration was calculated from at. wt 6000.

‡ These values were derived from the H_2 evolution assay (see Material and Methods).

Hydrogenase 1 was playing a key role in the reduction of metronidazole by *C. pasteurianum*. To further delineate the function of the enzyme in the reduction of the drug, it was necessary to purify Hydrogenase 1 for these studies.

Table 2 outlines the purification procedure for the bidirectional hydrogenase (Hydrogenase 1). Hydrogenase 1 specific activity was determined by either the H_2 evolved or H_2 uptake assays which closely paralleled each other throughout the purification procedure and correlated with those originally reported in Refs. 21 and 23. The metronidazole

Table 4. Reduction of metronidazole (MET) by an electron carrier–Hydrogenase 1 mechanism versus chemical methods

MET reduction assay conditions	Concentration of MET remaining* (μmol)	Amount of MET reduced† (μmol)	% MET reduced‡
(1) Control§	4.4	0	0
(2) Hydrogenase 1	4.4	0	0
(3) Hydrogenase 1 + methyl viologen	0.2	4.2	96
(4) Carbon monoxide inhibited Hydrogenase 1 + methyl viologen	4.0	0.4	9
(5) Sodium dithionite (15 mM)	0	4.4	100
(6) NADH	4.0	0	0
(7) NADPH	4.0	0	0

All of the values in this table with the exception of (5) were obtained from experiments using Hydrogenase 1 that had been purified to the Sephadex G-100 column step (see Materials and Methods).

* The metronidazole reduction assay reaction time throughout these experiments was 6 min. The concentration of metronidazole remaining was determined spectrophotometrically using the drug's extinction coefficient of 9300 cm^{-1} M^{-1} [2, 4].

† The amount of metronidazole reduced was calculated by subtracting the amount of metronidazole that remained from a starting drug concentration of 4.4 μmol.

‡ The percentage of metronidazole reduced was derived from a starting drug concentration of 4.4 μmol.

§ Contained neither electron carrier nor enzyme.

reduction activity of Hydrogenase 1 was assayed throughout the purification procedure by the H_2 uptake assay alone (see Materials and Methods for explanation).

The stoichiometry of metronidazole's reduction at each successive stage of Hydrogenase 1 purification approximated an H_2:metronidazole ratio of 2:1 which agrees with the previously reported four-electron reduction of the drug [1, 4, 29].

The metronidazole reduction activity of Hydrogenase 1 was greater than its specific activity for H_2 alone by a factor of 1.6 throughout the purification scheme. However, comparison between the relative activities of Hydrogenase 1 showed nearly identical values for each purification step (Table 2).

SDS–polyacrylamide gel electrophoresis of the enzyme fraction from the second DEAE column revealed one major protein band corresponding to 60,000 M_r (Hydrogenase 1) and a lesser contaminating band (35,000 M_r). This agreed with the original findings for the enzyme after this purification step [21, 23]. Significantly, the band at 60,000 M_r increased throughout the purification procedure with increasing Hydrogenase 1 and metronidazole reduction activity, whereas the 35,000 M_r band steadily diminished. The 35,000 M_r contaminating band could be eliminated by further purification of the enzyme to the final hydroxyapatite step of the original method [21]. Enhanced enzymatic activity of Hydrogenase 1 equal to H_2 evolution/uptake units (182/161) and metronidazole reduction activity units of 358 were obtained after the final hydroxyapatite step, but these enzymatic activities were lower than those previously reported in Ref. 21. We attributed this technical difficulty to the extreme oxygen sensitivity of the enzyme [20, 21, 30]. We were technically able to work with much smaller fraction volumes throughout the purification procedure than those of Chen and Mortenson [21], and thus the strict anaerobic conditions especially required for this final hydroxyapatite column step could not be maintained completely. The second DE-52 enzyme fraction was therefore used to conduct the experiments in Table 3.

Ability of Hydrogenase 1 to reduce metronidazole using different electron carriers

As shown in Table 3, Hydrogenase 1 activity depended on the presence of an electron carrier as no enzymatic activity was obtained in their absence. This enzyme has been shown previously to utilize all of the electron carriers listed in Table 3 for its catalytic activity [1, 31]. Ferredoxin on an atomic weight basis was the most efficient low potential electron carrier studied (Table 3), although it was only 36% as active as methyl viologen in saturating amounts in the assay system. As well, the addition of methyl viologen and ferredoxin to the assay reaction mixture greatly increased the specific activity of Hydrogenase 1 confirming the previous observations of Chen and Mortenson [21].

This Hydrogenase 1 fraction required one of its electron carriers for coupling the metronidazole reduction activity (Table 3). Metronidazole reduction activity of Hydrogenase 1 was greater than its specific activity for H_2 by a factor of between 1.3 and 1.6 for all electron carriers used except for ferredoxin where a greater then 2-fold increase in activity occurred. Ferredoxin on an atomic weight basis was the most efficient electron carrier for coupling Hydrogenase 1 activity including metronidazole reduction activity. When ferredoxin and methyl viologen were used together in the reaction mixture, a substantial increase in activity was noted which paralleled the results found for Hydrogenase 1 activity [21] (Table 3).

Oxygen sensitivity of Hydrogenase 1 and metronidazole reduction activity

As shown in Table 3, the highly purified Hydrogenase 1 was irreversibly inactivated not only for H_2 uptake activity, but also for all metronidazole reduction activity after exposure to oxygen. Aeration for 1 hr completely inactivated Hydrogenase 1, but even a few minutes of exposure to oxygen caused a rapid decrease in enzymatic activity. Hydrogenase 1 has been reported previously to be irreversibly inactivated for H_2 evolved/uptake activity by exposure to oxygen [20, 21, 30]. These results further support the theory that Hydrogenase 1 was not only utilizing H_2 as a substrate but was also potentiating the reduction of metronidazole.

Reduction of metronidazole by an electron carrier-Hydrogenase 1 mechanism versus chemical methods

Table 4 compares the efficiency of reducing metronidazole by Hydrogenase 1 versus several chemical methods. As shown here and in Table 3, Hydrogenase 1 required the presence of an electron carrier to catalyze the reduction of metronidazole. Methyl viologen was used as the electron carrier throughout these experiments because its redox state in the assay system was clearly indicated by a color change. Methyl viologen in its reduced form turns a dark blue color as distinct from its colorless oxidized state [32]. In order to study the chemical reduction of metronidazole via methyl viologen alone, methyl viologen was reduced enzymatically (dark blue) by the purified Hydrogenase 1 fraction and then the enzyme was inhibited with carbon monoxide. Hydrogenase 1 of *C. pasteurianum* and many other hydrogenases are very sensitive to inhibition by carbon monoxide [20]. Carbon monoxide is thought to bind non-competitively at an active site on the enzyme that is distinct from its electron carrier reduction site [1]. In the case of *C. pasteurianum*, carbon monoxide has been shown to be a competitive inhibitor of the H_2 active site of Hydrogenase 1 [20, 24, 25]. Hydrogenase 1 would thus initially reduce methyl viologen prior to the inhibition of the enzyme by carbon monoxide leaving the methyl viologen alone as a chemical reductant in the assay system. Thus, the efficiency of the reduction of metronidazole by a reduced electron carrier alone without interference by either the enzyme or a potent chemical reductant such as sodium dithionite could be studied.

Methyl viologen coupled Hydrogenase 1 enzymatic reduction of metronidazole [Table 4 (3)] was markedly more efficient in the 6-min reaction assay time compared to chemical reduction of the drug by reduced methyl viologen alone [Table 4 (4)]. Enzymatic reduction of metronidazole consumed

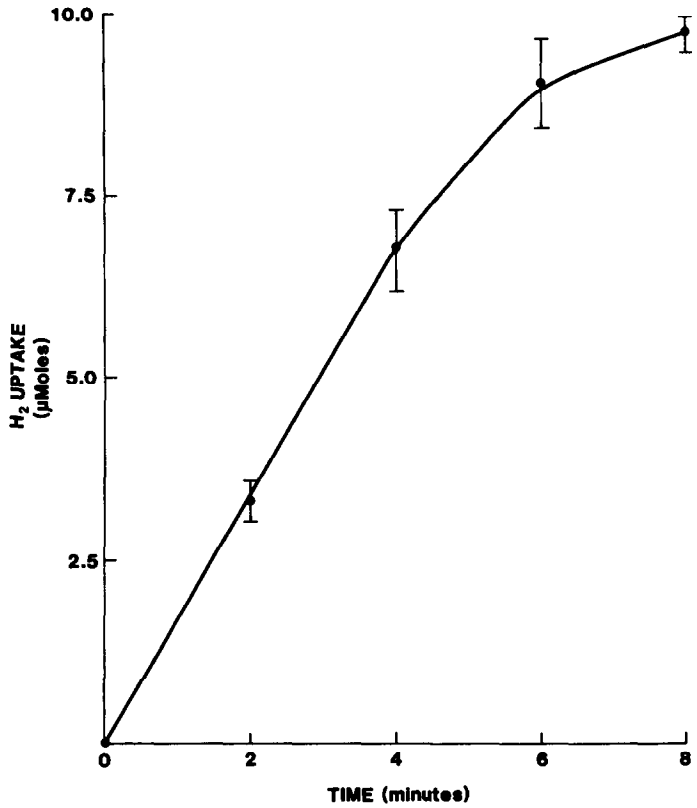

Fig. 2. Stoichiometry of the reduction of methyl viologen by Hydrogenase 1 of *C. pasteurianum*. The stoichiometry of the reduction of 20 μmol of methyl viologen was determined by standard manometric techniques using highly purified Hydrogenase 1 (Sephadex G-100 fraction) in a standard H_2 uptake Warburg assay (see Materials and Methods). Values are means ± SD, N = 3.

96% of the starting amount of the drug, whereas chemical reduction by reduced methyl viologen alone (Hydrogenase 1 inhibited assay system) only reduced 9% of the drug. Erbes and Burris [31] have shown that 1 mole of H_2 gas is consumed by highly purified Hydrogenase 1 in reducing 2 moles of methyl viologen. Thus, as shown in Fig. 2, from the amount of H_2 consumed (10 μmol) it could be calculated that most of the electron carrier in the assay system had been fully reduced (20 μmol). This amount of reduced methyl viologen should have completely reduced all of the metronidazole in the assay system (4 μmol of reduced methyl viologen to reduce 1 μmol of metronidazole). This was clearly not the case [Table 4 (4)], and the reduced methyl viologen in the assay system remained reduced (dark blue) in the presence of the drug. Chemical reduction of metronidazole by this method was insignificant compared to the methyl viologen–Hydrogenase 1 coupled enzymatic reduction of the drug.

As previously shown by other investigators [1], sodium dithionite was also a powerful reductant of metronidazole. Stoichiometric titration studies of the ability of various concentrations of sodium dithionite to reduce approximately 5 μmol of metronidazole demonstrated that it required approximately 2 μmol of sodium dithionite to reduce 1 μmol of metronidazole (Fig. 3). Therefore, the amount of sodium dithionite (10 μmol) used in the reaction assay system (see Materials and Methods) was sufficient to fully reduce the metronidazole [Table 4 (5)].

Finally, the reduced forms of nicotinaminde electron carriers, NADH and NADPH, could not directly reduce metronidazole [Table 4 (6 and 7)].

These experiments (Table 4) support the theory that the nitro-group reduction of metronidazole occurs enzymatically in *C. pasteurianum* by an electron carrier linked-Hydrogenase 1 mechanism, whereas the drug was only chemically reduced by a strong reducing agent, sodium dithionite.

DISCUSSION

In *C. pasteurianum*, ferredoxin is mainly reduced by the phosphoroclastic reaction in which the electron flow is from pyruvate : ferredoxin oxidoreductase to reduced ferredoxin to Hydrogenase 1 (Fig. 1a) and to other metabolic reactions requiring reduced ferredoxin. The enzymatic phosphoroclastic reaction experiments using the non-dialyzed crude cell-free extract showed a decrease in the amount of H_2 evolved in the presence of metronidazole [Table 1 (2)]. This decrease could be stoichiometrically accounted for by the amount of metronidazole that had been preferentially reduced by the siphoning of these electrons (H_2) via reduced ferredoxin confirming our previous results [16] (Fig. 1b). Aerobic dialysis of the crude cell-free extract irreversibly

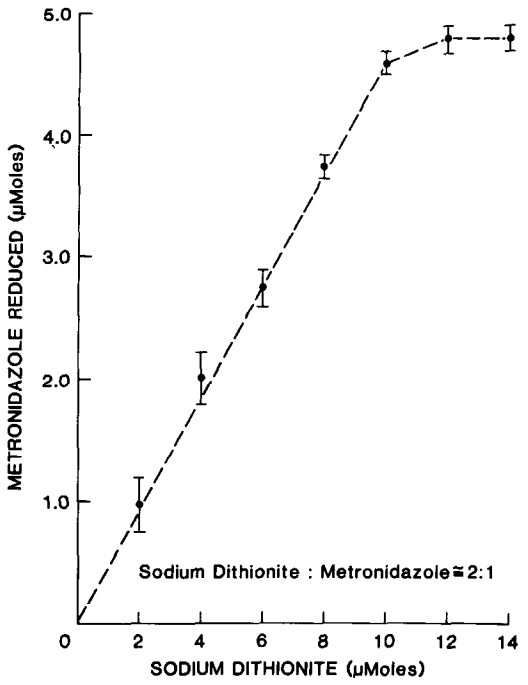

Fig. 3. Stoichiometry of the reduction of metronidazole by sodium dithionite. The ability of sodium dithionite to reduce metronidazole was measured using a standard Warburg assay system (see Materials and Methods). Various concentrations of sodium dithionite were used in the reaction assay mixture to determine the stoichiometry for the reduction of 25 mM metronidazole. Values are means ± SD, N = 3.

inactivated almost all of the Hydrogenase 1 activity [Table 1 (3)], so that the ability of reduced ferredoxin/methyl viologen produced by the pyruvate:ferredoxin oxidoreductase system could be directly evaluated for the chemical reduction of metronidazole. If reduced ferredoxin/methyl viologen was chemically reducing metronidazole, the phosphoroclastic activity should have been nearly the same or slightly higher as compared to the non-dialyzed crude cell-free extract assays [Table 1 (2)]. However, this did not happen, and the stoichiometry of the phosphoroclastic reaction in the presence and absence of metronidazole [Table 1 (3 and 4)] demonstrated that the minimal amount of metronidazole reduced (0.34 μmol) could be attributed to the residual Hydrogenase 1 activity in the dialyzed crude cell-free extract (see explanation in Results). These experiments demonstrate that *C. pasteurianum* Hydrogenase 1 played an essential enzymatic role in the reduction of metronidazole via a ferredoxin-linked mechanism.

Results of experiments using the highly purified Hydrogenase 1 from *C. pasteurianum* further verified this conclusion. The highly purified enzyme could only reduce metronidazole (Table 3) in the presence of its known required electron carriers (ferredoxin, the low potential electron carrier dyes, methyl and benzyl viologen, and the flavin coenzymes, FAD and FMN) [18]. NAD and NADP are not utilized by Hydrogenase 1 as electron couplers [18] and have been shown previously by us to not couple the reduction of metronidazole [4]. Ferredoxin on an atomic weight basis was found to be the most efficient electron carrier for coupling the reduction of metronidazole with the purified enzyme. Further, comparison of the relative activities of Hydrogenase 1 for its natural substrate H_2 and its metronidazole reduction activity throughout the purification procedure were nearly identical (Table 2); the stoichiometry of metronidazole's reduction gave the predicted H_2:metronidazole ratio of 2:1 throughout the various stages of the enzyme purification procedure, and both enzymatic activities were similarly inactivated by oxygen exposure (Table 3), strongly supporting the notion that this enzyme was involved in the reduction of the drug.

Assuming that carbon monoxide had totally inhibited all Hydrogenase 1 activity in the assay system (which could not be verified), then chemical reduction of metronidazole was insignificant compared to the enzymatic reduction of the drug by a reduced methyl viologen–Hydrogenase 1 mechanism (Table 4). Since the nitro-group of metronidazole is a potent electrophile, and an excess of reduced methyl viologen was present in the assay system, the direction of this chemical reaction strongly favored the complete irreversible reduction of metronidazole. However, this was not the case in our *in vitro* assay system with only a minimal amount of metronidazole being reduced chemically by reduced methyl viologen [Table 4 (4)]. Sodium dithionite, a potent reducing agent, completely reduced metronidazole (Table 4). The stoichiometry of this chemical reduction method yielded a sodium dithionite:metronidazole ratio of approximately 2:1. This ratio is equivalent to the stoichiometry for the drug's enzymatic reduction which gave a previously reported ratio of H_2:metronidazole of 2.4:1 [4]. These data confirm that metronidazole's reduction requires four reducing equivalents [4, 29]. In contrast, much weaker reductants such as the reduced forms of nicotinamide, NADH and NADPH could not reduce metronidazole (Table 4).

Using a spectrophotometric assay system, Lindmark and Muller [3] reported the main evidence for the chemical reduction of metronidazole by either reduced ferredoxin or methyl viologen. Thus, it is essential that a detailed outline of the problems we have had with the data of these authors is presented here. Their reported oxidized ferredoxin concentrations used to reduce metronidazole in Fig. 1B of Ref. 3 are incorrect because they have made a calculation error using the Beer–Lambert law (O.D. = Excxl). These authors use a cuvette with a 5-mm optical path for these experiments. However, the ferredoxin concentrations reported (Fig. 1B of Ref. 3) could only be calculated by substituting a 1-cm optical path length instead of 0.5-cm in the Beer–Lambert equation. Thus, the ferredoxin concentrations in Fig. 1B of Ref. 3 should be twice those reported giving a ferredoxin:metronidazole ratio of 4.4:1. Since ferredoxin is a two-electron carrier [6, 28] their data suggest that it requires between 8 and 10 electrons to reduce metronidazole, which

is more than double the electron reduction ratio reported for this reaction (metronidazole's reduction requires four electrons) [1, 4, 29]. This implies that there was non-specific oxidation of the reduced ferredoxin in their assay system to account for this large reduction ratio. Also, these authors did not measure the amount of metronidazole reduced at the end of each assay and have therefore presented no stoichiometric evidence that reduced ferredoxin in fact chemically reduced the drug in these experiments.

In addition, in experiments using reduced methyl viologen [Ref. 3, Fig. 1A], they do not mention the exact amount of sodium dithionite added initially to reduce methyl viologen. As shown in their Fig. 1A, no oxidation of methyl viologen occurred until approximately 10 nmol of nitroimidazole drug had been added. This could be explained by an excess amount of sodium dithionite in their assay system which preferentially reduced metronidazole. Again, these authors did incomplete stoichiometry of this reaction by not measuring the amount of metronidazole reduced at the end of each assay. Therefore, one cannot be sure where the electrons are going in either of these reactions (see Fig. 1A and 1B of Ref. 3). Thus, the discrepancy that has existed in the literature between hypotheses that the reduction of metronidazole is accomplished chemically by a reduced electron carrier alone versus its enzymatic reduction by a "nitroreductase(s)" has been primarily based on a questionable *in vitro* assay system for the chemical reduction of the drug.

We have proposed previously that the preferential siphoning of electrons for metronidazole's reduction away from ferredoxin-linked reactions in *C. pasteurianum*, including the inducible dissimilatory sulfite reductase system [2, 4] and the phosphoroclastic reaction [2, 16], occurs via a ferredoxin-linked metronidazole reductase(s) enzymatic mechanism as illustrated in Fig. 3 of Ref. 16. Our present data suggest that metronidazole also binds to Hydrogenase 1 in close proximity to the electron carrier site in which the nitro-group of metronidazole then acts as a potent electrophile, preferentially oxidizing the electron carrier and thereby enhancing Hydrogenase 1 activity (Tables 2 and 3). Thus, our revised model (Fig. 1b) of the electron flow through the phosphoroclastic reaction in the presence of metronidazole shows Hydrogenase 1 acting as the "metronidazole reductase", catalyzing the reduction of the drug in the presence of ferredoxin.

We now perceive metronidazole's bactericidal mechanism of action against *C. pasteurianum* to proceed as follows: (1) Metronidazole that is taken up rapidly by *C. pasteurianum* whole cells [2] would be immediately reduced by a ferredoxin-linked bidirectional hydrogenase (Hydrogenase 1) reaction, thereby siphoning reducing power from normal metabolic processes in the anaerobic cell causing a rapid cessation of cellular function(s). (2) The end product(s) of metronidazole's reduction would then have a cytotoxic effect(s) upon the host microbial DNA as has been shown previously [33–35]. However, the exact role of Hydrogenase 1 of *C. pasteurianum* in the reduction of metronidazole could only be definitively proven by future binding studies between metronidazole and this enzyme.

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