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Role of the Phosphoroclastic Reaction of *Clostridium pasteurianum* in the Reduction of Metronidazole

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To demonstrate the importance of electron siphoning by the metronidazole reductase system from reduced ferredoxin to the mechanism of action of the drug in *Clostridium pasteurianum*, the effects of the reduction of metronidazole on the phosphoroclastic reaction were studied. Metronidazole concentrations between 0.5 and 5 mM caused a significant increase in acetyl phosphate production by the phosphoroclastic reaction compared to the control system without metronidazole. When this enzymatic reaction was assayed by standard manometric techniques under nitrogen gas, two simultaneous effects of electron siphoning were demonstrated: (i) the electrons from reduced ferredoxin were initially consumed for the reduction of metronidazole instead of being evolved as H_2 via the ferredoxin-linked hydrogenase and (ii) phosphoroclastic activity was stimulated, with augmented production of CO_2 and acetyl phosphate. This work further supports the notion of preferential scavenging of electrons away from ferredoxin-linked enzymatic reactions by metronidazole reductase(s) in *C. pasteurianum*.

We and other investigators (1, 11, 12) have shown that the reduction of metronidazole in *Clostridium pasteurianum* is ferredoxin linked. Also, in competition experiments involving two ferredoxin-coupled reactions in *C. pasteurianum*, the inducible dissimilatory sulfite reductase (8) and the metronidazole reductase systems, metronidazole acted as a powerful electron acceptor, siphoning electrons from enzymatically reduced ferredoxin away from the competitive substrate sulfite (11).

To further demonstrate the importance of electron siphoning by the metronidazole reductase system from reduced ferredoxin to the mechanism of action of the drug in *C. pasteurianum*, it was essential to study the effects of the reduction of metronidazole on the phosphoroclastic reaction. The phosphoroclastic reaction of *C. pasteurianum* has been well studied; it is an important complex ferredoxin-coupled system for catabolic oxidation in this microorganism whereby pyruvate is oxidized by several enzymes to yield H_2, CO_2, and acetyl phosphate (3, 4, 15).

In this study we describe the role of the phosphoroclastic reaction of *C. pasteurianum* in the reduction of the metronidazole nitro group and discuss its physiological significance to the mechanism of action of the drug against anaerobic microorganisms.

MATERIALS AND METHODS

Culture conditions. *C. pasteurianum* strain W5 was grown in 10-liter batch cultures on a 1% sucrose-synthetic salts medium supplemented with 1 mM SO_4^{2-} plus 10 mM cysteine (8), and the cells were harvested at midlog phase as described previously (11).

Cell-free extracts. Crude cell-free extracts were prepared from the cell pellets as described previously (11).

Removal of ferredoxin from crude cell-free extracts. Ferredoxin was removed from the crude cell-free extracts by a DEAE-cellulose titration procedure described previously (9, 13). This crude extract was known as the DEAE-treated extract, which only lacked ferredoxin and possibly some other low-molecular-weight electron carriers.

Enzymatic assay methods. The *C. pasteurianum* phosphoroclastic reaction was assayed by the method of Mortenson et al. (14; see Fig. 1 and Table 1). Acetyl phosphate production was quantitated by the method of Lipmann and Tuttle (10).

To study the effects of the reduction of metronidazole on the stoichiometry of the phosphoroclastic reaction (pyruvate + coenzyme A [CoA] + $P_i \leftrightharpoons$ acetyl phosphate + CoA + H_2 + CO_2), it was necessary to measure not only acetyl phosphate production but also the amounts of gaseous end products (CO_2 + H_2) being evolved. This was done by using crude cell-free extracts in a standard manometric Warburg assay system under nitrogen gas. The complete reaction mixture in the main compartment of a double-side arm Warburg flask contained the following: 0.2 ml of 1 M potassium phosphate buffer, pH 6.5; 0.1 ml of 0.2 M $MgCl_2$; 0.1 ml of 0.1 M methyl viologen; 0.1 ml of 1.3 mM CoA; crude cell-free extract (in a volume equivalent to 3 mg of protein); and distilled water to give a final volume of 2.2 ml in the flask. The right side arm contained 0.2 ml of 0.5 M pyruvate while the left side arm contained either 0.2 ml of distilled water in the control assay or 0.2 ml of 25 mM metronidazole in the experimental assay. When the amount of H_2 being evolved by the phosphoroclastic reaction system was recorded, 0.2 ml of 40% NaOH plus a filter paper wick were added to the center well of the Warburg flask to absorb the evolved CO_2. When the total amount of gas (CO_2 + H_2) being evolved was recorded, the center well of the Warburg flask contained 0.2 ml of distilled water alone. The reaction was initiated by tipping the substrate(s) pyruvate and metronidazole into the main compartment. Incubation was done at 37°C, and the effects of the reduction of metronidazole on gas evolution by this reaction were determined by standard manometric techniques.

The reaction was stopped at 10-min intervals as described

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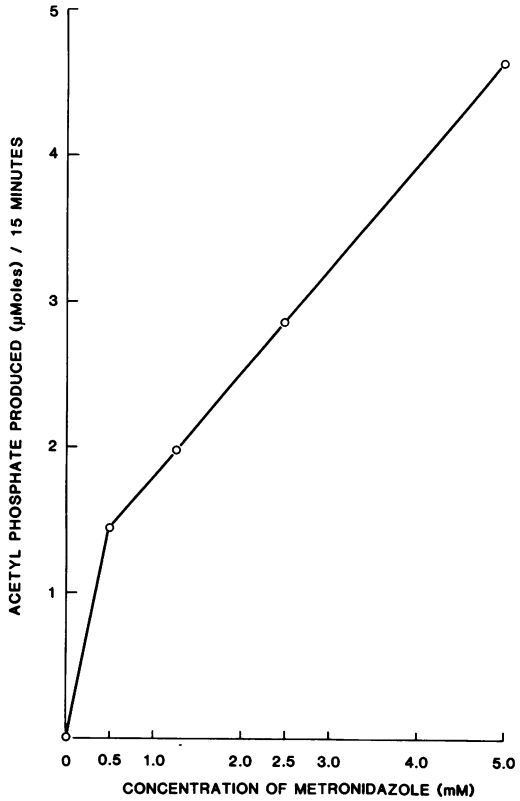

FIG. 1. Effect of various concentrations of metronidazole on the phosphoroclastic reaction in crude cell-free extract (1.6 mg/ml) of *C. pasteurianum*. Endogenous control activity has been subtracted from these data points.

previously (11), and 0.025-ml portions were assayed for acetyl phosphate production by the method of Lipmann and Tuttle (10). The remaining reaction mixture was immediately centrifuged, and the supernatant was examined for the reduction of the metronidazole nitro group by a qualitative assay method described previously (11), in which the disappearance of the drug absorption peak at 320 nm was measured by a Perkin-Elmer Lambda 3 scanning spectrophotometer.

Chemicals. *C. pasteurianum* type V ferredoxin and CoA were obtained from Sigma Chemical Co., and metronidazole was obtained from Rhone-Poulenc Pharma Inc. All other chemicals were of reagent grade quality.

Protein. Protein concentrations were determined by the Biuret procedure of Gornall et al. (6).

RESULTS

Effect of the reduction of metronidazole on the production of acetyl phosphate by the phosphoroclastic reaction. Metronidazole in increasing concentrations from 0.5 to 5.0 mM in the assay system caused a linear increase in acetyl phosphate production compared to the control assay system without metronidazole (Fig. 1).

Table 1 shows that the addition of 0.2 ml of 25 mM metronidazole to the complete crude-extract assay system containing 1.8 mg of enzyme protein increased acetyl phosphate production in the phosphoroclastic reaction approximately threefold.

Further, in the assay system which contained DEAE-treated extracts with the same amount of enzyme protein, no acetyl phosphate was produced, demonstrating that the ferredoxin had been successfully removed (see above). When ferredoxin was restored to the DEAE-treated assay system, acetyl phosphate was then produced in the phosphoroclastic reaction. This reconfirmed the ferredoxin dependence of this reaction in *C. pasteurianum* demonstrated previously (15). The approximate threefold increase in specific activity in the ferredoxin-reconstituted DEAE-treated assay system versus that of the system with complete crude extract occurs because the DEAE-treated assay system contains saturating levels of ferredoxin (11). The addition of metronidazole to the ferredoxin-reconstituted DEAE-treated extract assay system further augmented acetyl phosphate production approximately twofold compared to the ferredoxin-reconstituted DEAE-treated assay system without metronidazole and fivefold compared to the complete crude-extract assay system (Table 1).

Effects of the reduction of metronidazole on the stoichiometry of the phosphoroclastic reaction. The consistently increased production of acetyl phosphate in the enzymatic phosphoroclastic reaction assay system in the presence of metronidazole indicated that the drug was somehow stimulating phosphoroclastic activity (Fig. 1 and Table 1). To delineate how this was occuring, it was necessary to study the effects of metronidazole on the complete stoichiometry of the phosphoroclastic reaction by measuring not only acetyl phosphate production but also the gaseous end products of the reaction (see above). The results of these experiments are shown in Fig. 2a and 2b.

In the control phosphoroclastic reaction assay (Fig. 2a), the total amount of gas (CO_2 + H_2) evolved over 20 min in the system without 40% NaOH was ca. two times the amount of H_2 evolved alone in the system which contained 40% NaOH to absorb CO_2 (see above). This approximates the expected theoretical CO_2 + H_2:H_2 ratio of 2:1 for the phosphoroclastic reaction. Further, the amount of acetyl phosphate produced by the control system at 10 and 20 min was ca. one-half of the total amount of CO_2 + H_2 being evolved by the reaction during these times, giving the expected theoretical phosphoroclastic reaction CO_2 + H_2:acetyl phosphate ratio of 2:1. Acetyl phosphate was also being produced at approximately the same rate as H_2 was

TABLE 1. Effect of the reduction of metronidazole on acetyl phosphate production by the phosphoroclastic reaction of *C. pasteurianum*

Assay conditions[a]	Sp act[b]	% Activity[c]
Crude extract		
Complete	40 ± 0.2	100
Complete + metronidazole	112 ± 0.2	280
DEAE-treated extract		
Complete + ferredoxin[d]	108 ± 0.2	270
Complete + ferredoxin + metronidazole	214 ± 0.3	535

[a] Assay conditions were as described in the text. No activity was observed when crude extract without pyruvate and DEAE-treated extract without ferredoxin were assayed with and without metronidazole.

[b] Measured in nanomoles of acetyl phosphate produced per minute per milligram of protein ± the standard error of the mean from five experiments (1.8 mg of enzyme protein and 0.2 ml of 25 mM metronidazole were used in these experiments).

[c] % Activity is calculated from the crude-extract complete system.

[d] Ferredoxin concentration (0.06 μM) was calculated based on a molecular weight of 6,000.

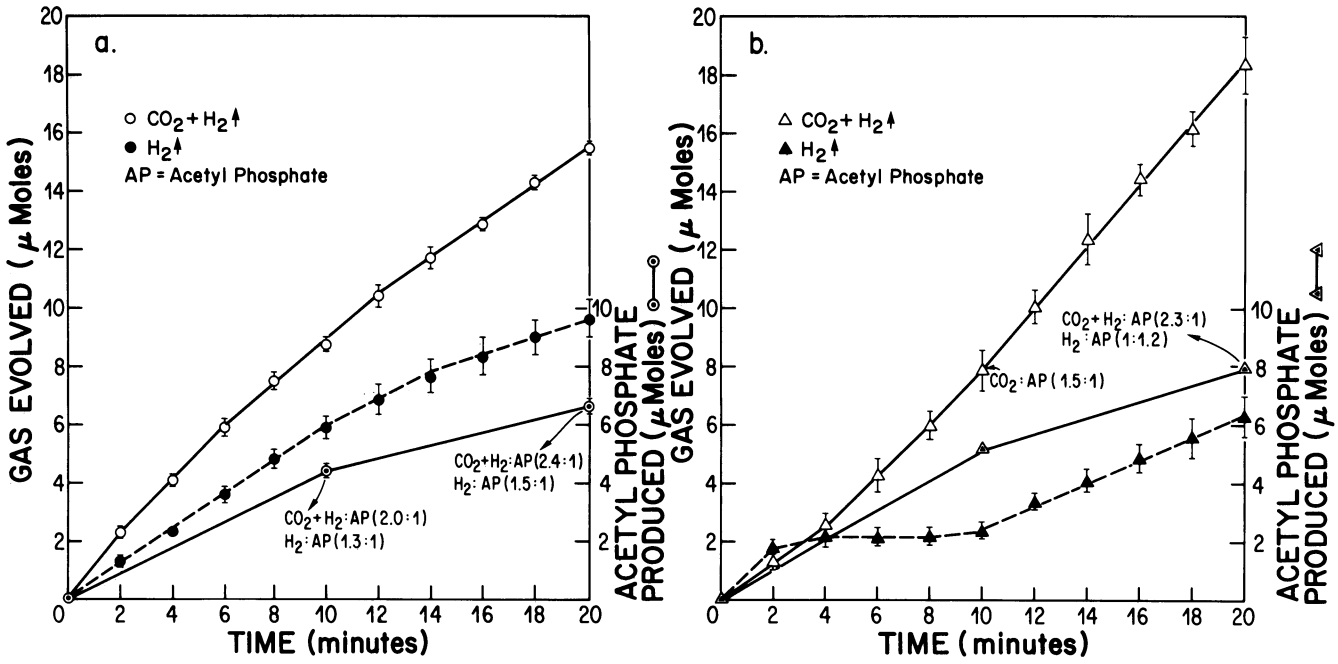

FIG. 2. Effects of the reduction of metronidazole on the stoichiometry of the phosphoroclastic reaction in *C. pasteurianum*. These experiments were done in a Warburg flask under N_2 gas (as described in the text). Panels: a, Data from the control phosphoroclastic-reaction assay system; b, data from the metronidazole-treated phosphoroclastic-reaction assay system. Symbols: ○ and △, Assay without 40% NaOH to absorb CO_2; ● and ▲, assay containing 40% NaOH; ⊙ and ◬, acetyl phosphate (AP) produced in both the CO_2-unabsorbed (○, △) and CO_2-absorbed (●, ▲) assay systems. Metronidazole was entirely reduced within 10 min of the experimental reaction time as shown by qualitative spectrophotometric analysis (see the text). The error bars represent the standard error of the mean from five experiments.

being evolved, yielding the expected theoretical H_2:acetyl phosphate ratio of 1:1 (Fig. 2a). This data for the control phosphoroclastic reaction assay system shows that the method used to absorb CO_2 throughout these experiments was effective.

As shown in Fig. 2b, the experimental assay system containing metronidazole (in which the CO_2 was absorbed by 40% NaOH [see above] evolved H_2 gas for the initial 2 min of the experiment and then ceased to produce H_2 until after 10 min. The metronidazole nitro group was shown by qualitative spectrophotometric analysis (see above) to be entirely reduced within 10 min of the experimental reaction time. The evolution of H_2 in the first 2 min of the experiment occurred because it takes a little time before the flow of electrons from reduced ferredoxin is diverted from hydrogenase evolving H_2 to the reduction of metronidazole. Therefore, because no H_2 is being evolved between 2 and 10 min of the experiment (in the CO_2-absorbed assay system above), the gas being evolved and graphically represented as the cumulative (CO_2 + H_2) evolution in the first 10 min of the metronidazole-treated assay system (which did not contain 40% NaOH) is in fact exclusively CO_2, giving a CO_2:H_2 ratio of 1.5:1 (Fig. 2b).

After 10 min of the experiment, when metronidazole was judged to be fully reduced, H_2 gas was evolved at a linear rate that was approximately 2.4 times slower than the rate at which the total (CO_2 + H_2) gases were being evolved in the assay system (Fig. 2b). At 20 min, the CO_2 + H_2:acetyl phosphate ratio was 2.3:1, and the H_2:acetyl phosphate ratio was 1:1.1, results which approximate the expected theoretical ratios of the control assay system without metronidazole (Fig. 2a and b).

Thus, the metronidazole-treated assay system, in comparison to the control assay system without metronidazole, showed an increase of approximately 25% in the production of CO_2 and an increase of approximately 20% in the production of acetyl phosphate in the phosphoroclastic reaction at 10 min of the experimental reaction time. By 20 min, the experimental assay system had produced approximately 17.5% more acetyl phosphate and approximately 15.8% more gas (CO_2 + H_2) in the phosphoroclastic reaction than had the control system. Thus, the reduction of metronidazole was not inhibiting but actually stimulating phosphoroclastic activity.

DISCUSSION

Metronidazole acts as a powerful electron acceptor, siphoning electrons from reduced ferredoxin away from the phosphoroclastic reaction of *C. pasteurianum* (as depicted in Fig. 3). Two simultaneous effects occur in this reaction as a direct result of this electron siphoning. First, the role of the phosphoroclastic reaction in the reduction of metronidazole is to provide the necessary electrons through reduced ferredoxin that normally would be evolved as H_2 via the ferredoxin-linked hydrogenase reaction (15). As shown in Fig. 2b, the reduction of metronidazole completely consumes the H_2 evolved by the phosphoroclastic reaction system in the first 10 min of the drug-treated experiment. Once the drug is fully reduced (within 10 min), electrons from reduced ferredoxin, which are no longer required for the reduction of metronidazole, then couple in the hydrogenase reaction to evolve H_2.

Second, metronidazole stimulates more phosphoroclastic activity compared to the control system, with augmented yields of CO_2 and acetyl phosphate (Table 1 and Fig. 2a and b). The preferential siphoning of electrons from reduced

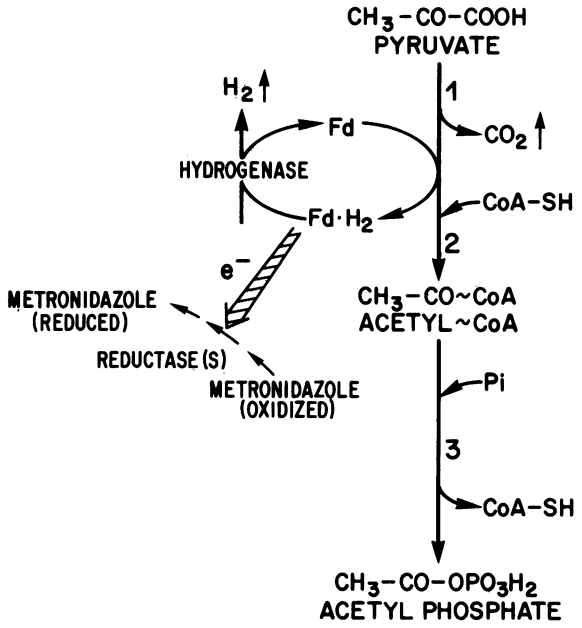

FIG. 3. Role of the phosphoroclastic reaction of *C. pasteurianum* in the reduction of metronidazole. Steps 1 and 2 of the reaction involve the pyruvate:ferredoxin oxidoreductase, ferredoxin, thiamine pyrophosphate containing oxidoreductase (TPP-E), and hydroxyethyl-TPP-E (HETPP-E). Step 3 is catalyzed by phosphotransacetylase (3, 15).

ferredoxin by the metronidazole reductase(s) system increases the rate at which ferredoxin is oxidized and reduced compared to the ferredoxin-linked hydrogenase reaction (Fig. 3), "pulling" the reaction faster to yield more CO_2 and acetyl phosphate than in the enzymatic reaction without metronidazole. Therefore, the hydrogenase activity appears to be the rate-limiting enzyme step of the phosphoroclastic reaction in *C. pasteurianum*.

In a previous in vitro study with *Clostridium acetobutylicum*, O'Brien et al. (16) concluded that metronidazole was temporarily inhibiting H_2 evolution in the face of continuing unaltered CO_2 and acetyl phosphate production by the phosphoroclastic reaction in this microorganism. However, they did not examine the complete stoichiometry of their system (i.e., concurrent measurement of the acetyl phosphate produced and the extent of metronidazole reduction), which makes it difficult to accurately conclude where the electrons (i.e., H_2) are actually going in the presence of metronidazole.

Further, in the previous studies of not only O'Brien et al. (16) but also Edwards et al. with *Trichomonas vaginalis* (5) and *Clostridium* (4), the conclusions regarding the effects of the reduction of metronidazole on the phosphoroclastic reaction are based wholly (4, 5) or in part (16) upon in vivo whole-cell studies, which in our experience have been very difficult to interpret since metronidazole is rapidly bactericidal in concentrations that are even less than therapeutic (i.e., 10 μg/ml). Metronidazole in a concentration of 0.05 mM (8.6 μg/ml) added to a logarithmically growing culture of *C. pasteurianum* causes a 99% loss of cell viability within 5 min (D. L. Lockerby, H. R. Rabin, and E. J. Laishley, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 23rd, Las Vegas, Nev., abstr. no. 169, 1983). Thus, it is not known whether the cessation of H_2 production (and CO_2 production in the studies of Edwards et al. [4, 5]) by the phosphoroclastic reaction after the addition of metronidazole in these in vivo studies is a true metabolic effect of the reduction of the drug or merely occurring because of cell death.

This work demonstrates the unique preferential electron-siphoning ability of the metronidazole reductase(s) via reduced ferredoxin away from another ferredoxin-dependent reaction in *C. pasteurianum*. The effects of electron siphoning via reduced ferredoxin for the reduction of metronidazole on the phosphoroclastic reaction produce a very different result than that previously reported for the inducible dissimilatory sulfite reductase (11). In competition experiments between the latter enzyme and the metronidazole reductase(s) system, the reduction of metronidazole caused a temporary shutdown of the inducible dissimilatory sulfite reductase by diverting the necessary electron flow from the reduced ferredoxin required for its enzyme activity. Once metronidazole was reduced, then the electrons from reduced ferredoxin were available to flow to the inducible dissimilatory sulfite reductase, and its activity was restored to normal levels (11).

The phosphoroclastic reaction is the major system for generating reduced ferredoxin in *C. pasteurianum*, so the preferential siphoning of reducing power from the cell via reduced ferredoxin by the metronidazole reductase(s) over time would have a major effect on the growth and viability of this microorganism by depriving other ferredoxin-linked reduction systems of essential reducing equivalents required for normal metabolic function. We perceive this to be a significant stage in the mechanism of action of metronidazole against *C. pasteurianum* and possibly other anaerobic microorganisms. Furthermore, it has been shown that the intermediate product(s) of reduced metronidazole have cytotoxic effects upon host DNA (2, 7). Finally, this work further supports the concept of a preferentially ferredoxin-linked metronidazole reductase(s) system in *C. pasteurianum*.

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