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Inactivation of Clostridial Ferredoxin and Pyruvate-Ferredoxin Oxidoreductase by Sodium Nitrite†

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Clostridial ferredoxin and pyruvate-ferredoxin oxidoreductase activity was investigated after in vitro or in vivo treatment with sodium nitrite. In vitro treatment of commercially available *Clostridium pasteurianum* ferredoxin with sodium nitrite inhibited ferredoxin activity. Inhibition of ferredoxin activity increased with increasing levels of sodium nitrite. Ferredoxin was isolated from normal *C. pasteurianum* and *Clostridium botulinum* cultures and from cultures incubated with 1,000 μg of sodium nitrite per ml for 45 min. The activity of in vivo nitrite-treated ferredoxin was decreased compared with that of control ferredoxin. Pyruvate-ferredoxin oxidoreductase isolated from *C. botulinum* cultures incubated with 1,000 μg of sodium nitrite per ml showed less activity than did control oxidoreductase. It is concluded that the antibotulinal activity of nitrite is due at least in part to inactivation of ferredoxin and pyruvate-ferredoxin oxidoreductase.

Several iron-sulfur proteins have been isolated from clostridia, including electron transport proteins (i.e., ferredoxins) and oxidation-reduction enzymes including hydrogenase, pyruvate oxidoreductase, nitrogenase, NADH oxidoreductase, CO_2 reductase, and sulfate reductase (for a review, see reference 21). The addition of ascorbate and sodium nitrite ($NaNO_2$) to suspensions of vegetative *Clostridium botulinum* results in the formation of iron-nitric oxide complexes from the iron-sulfur centers of some or all of the iron-sulfur proteins present (11), and it was concluded that since the iron-sulfur proteins are an integral part of many important cellular functions, inactivation by binding of nitric oxide would inhibit growth.

The phosphoroclastic system (oxidative cleavage of pyruvate to acetate) is a major pathway leading to substrate level ATP synthesis in many clostridia and depends on the activity of two iron-sulfur enzymes, ferredoxin and pyruvate-ferredoxin oxidoreductase. Pyruvate-ferredoxin oxidoreductase is a thiamine PP-containing iron-sulfur enzyme that catalyzes the oxidative decarboxylation of pyruvate in the phosphoroclastic reaction (17). Ferredoxin, an electron-transferring iron-sulfur protein first isolated from *Clostridium pasteurianum* (5), has been observed to serve in the transfer of electrons from pyruvate dehydrogenase to hydrogenase in the formation of hydrogen in the phosphoraclastic reaction (6). Ferredoxin also acts as an electron carrier in many other redox-related reactions of the clostridia, including the reactions catalyzed by the iron-sulfur enzymes mentioned above. The presence of ferredoxin in *C. botulinum* had not been reported.

The phosphoroclastic system has been demonstrated in several clostridial species (7, 9, 12). Simmons and Costilow (15) demonstrated the presence of the phosphoroclastic system in *C. botulinum*. Woods et al. (20) reported that nitrite inhibits the phosphoroclastic system in *Clostridium sporogenes* by the reaction of nitric oxide with pyruvate-ferredoxin oxidoreductase but not ferredoxin. Woods and Wood (19) also observed the inhibition of the phosphoroclastic system in *C. botulinum* upon the addition of nitrite by measuring the accumulation of pyruvate in the medium.

The purpose of this study was (i) to determine the presence of ferredoxin in *C. botulinum*, and if present (ii) to separately study the in vivo and in vitro effects of nitrite on the iron-sulfur enzymes of the phosphoroclastic system, ferredoxin and pyruvate-ferredoxin oxidoreductase. Because nitrite inhibition of a clostridial pyruvate-ferredoxin oxidoreductase has previously been demonstrated (20) and because of the importance of ferredoxin to many critical redox reactions of clostridia besides the phosphoroclastic system, the effects of nitrite on ferredoxin from another clostridial species, *C. pasteurianum*, were also investigated.

MATERIALS AND METHODS

Microorganisms and growth conditions. *C. pasteurianum* ATCC 6013 was grown to a volume of 20 liters at 37°C on a nonsterilized medium (13) containing 100 mM glucose, 80 mM NH_4Cl, 90 mM K_2HPO_4, 10 mM KH_2PO_4, 60 mM NaCl, 1.2 mM $MgSO_4$, 200 μM $FeSO_4$, 0.01 μM Na_2MoO_4, 0.1 mg of biotin per liter, and 1 mg of *p*-aminobenzoic acid per liter. Nitrogen was bubbled through the medium for 15 min to remove dissolved oxygen before inoculation.

C. botulinum type A (ATCC 19397) was grown to a volume of 20 liters at 37°C on a sterilized medium (2) containing 1% beef extract, 1% peptone, 0.5% glucose, 0.5% sodium acetate, 0.3% yeast extract, 0.1% soluble starch, 0.05% cysteine, and 0.1% thioglycolate.

In vivo treatment with $NaNO_2$. While the cells were in a vegetative state, as judged by a pH of the medium of 5.5 to 6.0 for *C. pasteurianum* and Gram staining for *C. botulinum*, they were separated into equal 10-liter volumes. To one 10-liter batch was added 10 g of $NaNO_2$ in 100 ml of H_2O. The other batch served as a control. Cells were harvested by centrifugation at 3,000 × *g* for 10 min after a 45-min incubation at 37°C. The packed cells were suspended in distilled water and centrifuged three times to remove traces of growth medium and nitrite and stored at −20°C.

In vitro treatment with $NaNO_2$. Four 0.5-ml samples containing 25 μg of *C. pasteurianum* ferredoxin (Sigma Chemical Co., St. Louis, Mo.) in 0.05 M sodium phosphate (pH

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6.0) were prepared. To each sample was added 0.5 ml of either 0 (control), 1,000, 2,000, or 3,000 μg of $NaNO_2$ per ml. The samples were incubated for 30 min at room temperature and then dialyzed at 4°C against three 2-liter changes of 0.05 M Tris hydrochloride (pH 7.3) for 8 to 10 h each. This procedure was repeated with ferredoxin and pyruvate-ferredoxin oxidoreductase isolated from control *C. botulinum* cultures but only at one level of $NaNO_2$ (final concentration, 1,000 μg/ml).

Ferredoxin isolation. Frozen cells were thawed, suspended in 1.5 volumes of H_2O containing 1 mg of lysozyme per ml of cells and 0.1 mg of DNase per ml of cells, and lysed for 45 min at 37°C. Ferredoxin was isolated by the procedure of Mortenson (4). Final purification and crystallization by ammonium sulfate precipitation was not performed.

Pyruvate-ferredoxin oxidoreductase isolation. Cell extracts of *C. botulinum* were passed through a DEAE-cellulose column to remove ferredoxin by the procedure of Mortensen (4). The unbound fraction containing the oxidoreductase was used for assays of oxidoreductase activity.

Protein determinations. Protein in ferredoxin preparations was determined with Bio-Rad dye reagent (Bio-Rad Laboratories, Richmond, Calif.). Concentrated dye reagent was diluted 1:4 with H_2O, and 2 ml was added to 0.5 ml of the appropriately diluted protein sample. A_{595} was measured after 5 min. Protein concentration was determined by comparison with a standard curve prepared by using crystalline bovine serum albumin.

Ferredoxin activity assay. Ferredoxin activity was assayed by the cytochrome *c* reduction method of Shin (14). To 0.6 ml of H_2O was added 0.1 ml each of 0.1 M Tris hydrochloride (pH 7.8), 0.5 mM cytochrome *c*, ferredoxin-NADP reductase (0.1 U/ml; Sigma), and ferredoxin. The reaction was initiated by the addition of 10 μl of 20 mM NADPH, and the increase in A_{550} was recorded. Ferredoxin activity was expressed as micromoles of cytochrome *c* reduced per minute during the initial linear phase of the assay. Each ferredoxin dilution or treatment was assayed in triplicate.

FAD reduction assay. Pyruvate-ferredoxin oxidoreductase activity was assayed by the flavin adenine dinucleotide (FAD) reduction method of Uyeda and Rabinowitz (18). The reaction mixture (0.8 ml), consisting of 62.5 mM potassium phosphate (pH 6.8), 25 mM 2-mercaptoethanol, 12.5 mM sodium pyruvate, 0.5 mM coenzyme A, and 0.0625 mM FAD, was transferred to a 1-ml quartz cuvette stoppered with a rubber septum. Two syringe needles were inserted through the septum to flush the cuvette with nitrogen before the addition of the enzyme. The reaction was initiated by the addition of 0.1 ml of oxidoreductase through the septum with a 1-ml syringe. The change in A_{450} was determined with a recording spectrophotometer. Each oxidoreductase dilution or treatment was assayed in triplicate.

Acylhydroxamate formation assay. Pyruvate-ferredoxin oxidoreductase activity was also measured by acylhydroxamate formation by the procedure of Raeburn and Rabinowitz (10). A 0.1-ml portion of enzyme was added to 2 ml of the reaction mixture (2 mM sodium pyruvate, 200 mM potassium phosphate [pH 6.8], 0.575 mM coenzyme A, 0.5 mM FAD, 2.5 mM 2-mercaptoethanol), and the reaction was allowed to proceed for 2 min. The reaction was terminated by the addition of 1 ml of neutralized 28% hydroxylamine. Ferric chloride solution (1 ml; 2 M) was added, and after 10 min, denatured protein was removed by centrifugation. The A_{540} was then determined. One micromole of acylhydroxamate gave an optical density reading of 0.17. Each oxidoreductase dilution or treatment was assayed in triplicate.

RESULTS

Estimation of enzyme activity. Dilutions of control ferredoxin or oxidoreductase, isolated from bacteria grown under normal conditions, provided standard curves from which the amount of active enzyme in nitrite-treated samples was estimated. A power function, $y = ax^b$ where y is the enzyme activity and x is the amount of enzyme, provided the best fit as compared with linear, exponential, or logarithmic functions. Separate standard curves were determined for each batch of bacteria. The correlation coefficients (r^2) correspond to the standard curve used to estimate the reported value for inactive enzyme.

In vitro $NaNO_2$ inhibition of ferredoxin. The activity of *C. pasteurianum* ferredoxin (Sigma) in the cytochrome *c* reduction assay was inhibited by the in vitro addition of $NaNO_2$ (Table 1). Higher levels of $NaNO_2$ increased ferredoxin inhibition. The percentages of inactivated *C. pasteurianum* ferredoxin for 0, 500, 1,000, and 1,500 μg of $NaNO_2$ per ml were 4, 41, 70, and 83%, respectively ($r^2 = 0.99$). A duplicate experiment showed an identical pattern: 0, 500, 1,000, and 1,500 μg of $NaNO_2$ per ml inactivated ferredoxin by 14, 36, 68, and 87%, respectively ($r^2 = 1.00$). Bubbling nitric oxide through a ferredoxin solution completely destroyed all ferredoxin activity. Similarly, in vitro treatment of control *C. botulinum* ferredoxin with 1,000 μg of $NaNO_2$ per ml inactivated 45% of the ferredoxin ($r^2 = 0.99$).

In vivo $NaNO_2$ inhibition of ferredoxin. Ferredoxin isolated from cells of *C. pasteurianum* and *C. botulinum* which had been incubated with 1,000 μg of $NaNO_2$ per ml showed decreased activity compared with ferredoxin isolated from control cells. In vivo addition of 1,000 μg of $NaNO_2$ per ml inactivated 94% of the ferredoxin isolated from *C. pasteurianum* ($r^2 = 0.99$). Placement in a boiling water bath for 5 min inactivated 88% of the control ferredoxin. In a second experiment with *C. pasteurianum*, an estimated 95% of the ferredoxin was inactivated after in vivo addition of 1,000 μg of $NaNO_2$ per ml ($r^2 = 0.98$).

In vivo addition of 1,000 μg of $NaNO_2$ per ml inactivated 95% of the ferredoxin isolated from *C. botulinum* ($r^2 = 0.99$). Boiling for 5 min inactivated 98% of the control ferredoxin. When ferredoxin from control and nitrite-treated *C. botuli-*

TABLE 1. Inhibition of clostridial ferredoxin and pyruvate-ferredoxin oxidoreductase by in vivo and in vitro addition of sodium nitrite

Sample	Amt of sodium nitrite (μg/ml)	% Inhibition[a]
C. pasteurianum ferredoxin[b]	500 (in vitro)	38.5
	1,000 (in vitro)	69.0
	1,500 (in vitro)	85.0
C. pasteuriganum ferredoxin[c]	1,000 (in vivo)	94.5
C. botulinum ferredoxin[d]	1,000 (in vivo)	95.0
C. botulinum pyruvate-ferredoxin oxidoreductase[d]	1,000 (in vitro)	78.0[e]
		82.5[f]
	1,000 (in vivo)	74.5[e]
		74.0[f]

[a] Average from two trials.
[b] Commercial ferredoxin from *C. pasteurianum*.
[c] Isolated from *C. pasteurianum*.
[d] Isolated from *C. botulinum*.
[e] FAD reduction assay.
[f] Acylhydroxamate formation assay.

num was mixed in a 1:1 protein ratio and assayed for cytochrome *c* reduction activity, there was 99% of the expected activity of the control. Thus, there was no extraneous inhibition in the cytochrome *c* assay from the nitrite-treated ferredoxin. In a second experiment with *C. botulinum*, an estimated 95% of the ferredoxin was inactivated after in vivo addition of 1,000 μg of $NaNO_2$ per ml (r^2 = 0.99).

In vitro inhibition of pyruvate-ferredoxin oxidoreductase. *C. botulinum* oxidoreductase activity was inhibited by in vitro incubation with 1,000 μg of $NaNO_2$ per ml. Oxidoreductase inactivation was estimated to be 79% (r^2 = 0.98) and 84% (r^2 = 0.99) as assayed by FAD reduction and acylhydroxamate formation, respectively. Oxidoreductase isolated from another batch of *C. botulinum* cells showed 77% (r^2 = 0.95) and 81% (r^2 = 0.97) inactivation after $NaNO_2$ treatment.

In vivo inhibition of pyruvate-ferredoxin oxidoreductase. In vivo addition of 1,000 μg of $NaNO_2$ per ml inactivated 69% (r^2 = 0.98) and 72% (r^2 = 0.99) of *C. botulinum* oxidoreductase as assayed by FAD reduction and acylhydroxamate formation, respectively. Boiling inactivated 95 and 90% of the oxidoreductase. A second experiment with *C. botulinum* showed oxidoreductase inactivation percentages of 80% (r^2 = 0.95) and 76% (r^2 = 0.97).

DISCUSSION

Clostridial ferredoxin and pyruvate-ferredoxin oxidoreductase activity was inhibited by both in vitro and in vivo treatment with $NaNO_2$ (Table 1). Inhibition of these two iron-sulfur enzymes necessary for functioning of the phosphoroclastic system would certainly decrease cell viability since substrate level ATP synthesis would be inhibited. Further, inhibition of ferredoxin activity would interfere with almost every aspect of the redox-related metabolism of clostridia, including hydrogen, nitrogen, purine, sulfur, and one-carbon metabolism (21).

Inhibition of the phosphoroclastic system of clostridia by in vivo treatment with $NaNO_2$ has been previously demonstrated for *C. sporogenes* (20) and *C. botulinum* (19). Woods et al. (20) suggested that nitrite action in vivo occurs only by the inhibition of pyruvate-ferredoxin oxidoreductase and not by inhibition of ferredoxin. However, there was residual $NaNO_2$ in the ferredoxin fraction they used to reconstitute the assay system. They therefore attributed decreased activity in the assay to the reaction of residual nitrite in ferredoxin preparations with pyruvate-ferredoxin oxidoreductase. Ferredoxin fractions assayed in the present study for cytochrome *c* reducing activity were all extensively dialyzed to remove any possible residual nitrite and therefore any possible extraneous inhibition of assay components by nitrite. Additionally, it was found that ferredoxin isolated from nitrite-treated *C. botulinum* did not inhibit assay components. The activity of a mixture of control and nitrite-treated ferredoxin was only slightly lower than that of control ferredoxin.

Tompkin et al. (16) suggested that nitric oxide, formed via nitrous acid from nitrite, reacts with the iron-sulfur proteins of bacteria, thereby destroying enzymatic activity and inhibiting bacterial growth. Previous work in our laboratory (11) demonstrated that $NaNO_2$ reacts with iron-sulfur proteins of *C. botulinum* to form iron-sulfur-nitric oxide complexes. Further, the enzymatic activity of different iron-sulfur clostridial proteins including nitrogenase (3), pyruvate-ferredoxin oxidoreductase (20), and now ferredoxin has been shown to be inhibited by nitrite treatment. Inhibition in each instance is apparently the result of destruction of enzyme iron-sulfer centers due to iron-nitric oxide complex formation. Therefore, it appears likely that sodium nitrite exerts an antibotulinal effect by the destruction of iron-sulfur enzymes within vegetative cells.

Both of the clostridial iron-sulfur proteins analyzed in this study, ferredoxin and pyruvate-ferredoxin oxidoreductase, were inactivated upon $NaNO_2$ treatment. All clostridial iron-sulfur proteins are probably inactivated by similar reaction with $NaNO_2$. Inactivation of a specific enzyme, for example pyruvate-ferredoxin oxidoreductase, as suggested by Woods et al. (20), may be the actual limiting factor to bacterial growth. However, the inactivation of ferredoxin after nitrite treatment would certainly decrease clostridial viability due to inhibition of ATP synthesis and termination of the many critical clostridial redox reactions that require ferredoxin.

There are several possible explanations as to why all bacterial species are not inhibited by $NaNO_2$ via inactivation of iron-sulfur enzymes. First, not all bacteria contain iron-sulfur proteins. Second, all iron-sulfur enzymes are not critical to growth. An example of a noncritical iron-sulfur enzyme is formate dehydrogenase from *Pseudomonas oxalaticus*, which will allow the organism to grow on formate as the sole carbon and energy source (1). Third, many iron-sulfur enzymes contain additional cofactors such as flavin, heme, or molybdenum which may in some instances protect these proteins from reacting with nitric oxide. Meyer (3) found that nitric oxide inhibits the iron-sulfur protein, but not the iron-sulfur-molybdenum protein, of the nitrogenase system in *C. pasteurianum*. However, the clostridial iron-sulfur-thiamine PP enzyme, pyruvate-ferredoxin oxidoreductase, is inactivated by $NaNO_2$ treatment, as reported here and by Woods et al. (20). Finally, bacteria which produce nitric oxide as an intermediate in denitrification systems are believed to contain enzymes which bind nitric oxide (8). The bound nitric oxide is prevented from reacting with other proteins within the cell and is further reduced to less reactive nitrous oxide. Most denitrifiers are themselves sensitive to initially elevated nitrite concentrations or sudden surges in nitrite concentration, probably due to the subsequent production of more nitric oxide than they can bind and inactivate.

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