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Biochemistry

Ferredoxin/flavoprotein-linked pathway for the reduction of thioredoxin

(ferredoxin-thioredoxin reductase/*Clostridium pasteurianum*/FAD/disulfide reduction/fermentative bacteria)

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ABSTRACT **Thioredoxins are small redox proteins, alternating between the S—S (oxidized) and SH (reduced) states, that function in a number of important biochemical processes, including DNA synthesis, DNA replication, and enzyme regulation. Reduced ferredoxin is known to serve as the source of reducing power for the reduction of thioredoxins only in photosynthetic cells that evolve oxygen. In all other organisms, the source of hydrogen (electrons) for thioredoxin reduction is considered to be NADPH. We now report evidence that *Clostridium pasteurianum*, an anaerobic bacterium normally living in the soil unexposed to light, resembles photosynthetic cells in that it uses reduced ferredoxin as the reductant for thioredoxin. Moreover, the transfer of electrons from reduced ferredoxin to thioredoxin is catalyzed by a flavoprotein enzyme that has not been detected in other organisms. Our results reveal the existence of a pathway for the reduction of thioredoxin in which ferredoxin, reduced fermentatively either by molecular hydrogen or by a carbon substrate, provides the reducing power for the flavoprotein enzyme ferredoxin-thioredoxin reductase, which in turn reduces thioredoxin.**

Thioredoxins are small disulfide-containing proteins, present in almost all living cells, that participate in important oxidoreduction reactions. Thioredoxins can act as electron donors to substrates, as in the reduction of ribonucleotides to deoxyribonucleotides (1). In more recent studies, thioredoxin has been shown to function as an essential subunit of a specific DNA polymerase (2). Thioredoxins are also metabolic regulators that act by reducing disulfide groups on enzymes, thereby changing their catalytic activity. In this capacity, thioredoxins are able to modulate key enzymes in chloroplasts and consequently regulate the photosynthetic assimilation of carbon dioxide (3, 4).

To function in these reactions, thioredoxins must be reduced. In many cells, reduction is accomplished by NADPH, which reduces thioredoxin via flavin-containing NADP-thioredoxin reductases (5–7) (Eq. 1).

$$NADPH_2 + \text{thioredoxin}_{ox} \xrightarrow[\text{reductase}]{\text{NADP-thioredoxin}} NADP + \text{thioredoxin}_{red}. \quad [1]$$

By contrast, in oxygen-evolving photosynthetic systems, ferredoxin functions as the reductant for thioredoxin, and an enzyme catalyzing this reductive reaction (Eq. 2), ferredoxin-thioredoxin reductase (FTR), has been purified from chloroplasts and cyanobacteria.

$$2\ \text{Ferredoxin}_{red} + \text{thioredoxin}_{ox} + 2H^+ \xrightarrow{\text{FTR}} 2\ \text{ferredoxin}_{ox} + \text{thioredoxin}_{red}. \quad [2]$$

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This enzyme has been variously reported to be an iron-sulfur protein (8) or to be free of this or other chromophores (9).

The anaerobic bacterium *Clostridium pasteurianum*, a saccharolytic fermenter containing an Fe_4S_4 type ferredoxin, resembles chloroplasts and cyanobacteria, oxygen-evolving photosynthetic structures containing an Fe_2S_2 type ferredoxin, in that ferredoxin plays a central role in its metabolic processes. In an earlier exploratory study (10), we readily demonstrated the presence of thioredoxin in *C. pasteurianum* but were unable to detect a NADP-thioredoxin reductase. Instead, we found evidence that ferredoxin was required for the reduction of thioredoxin by a clostridial preparation that had been freed of these two proteins.

In subsequent studies, we pursued this finding and now report that *C. pasteurianum* contains a type of FTR that, unlike the FTRs of chloroplasts and cyanobacteria, has properties characteristic of a flavoprotein and is highly specific for its own thioredoxin. The present evidence demonstrates that the clostridial FTR flavoenzyme participates in a ferredoxin-linked pathway for the reduction of thioredoxin in this fermentative bacterium.

MATERIALS AND METHODS

Microbial Material. *C. pasteurianum* (American Type Culture Collection no. 6013) was grown on sucrose with $(NH_4)_2SO_4$ as the nitrogen source and stored as a frozen cell paste according to the method of Lovenberg *et al.* (11).

Reagents. Previously described procedures were used for the preparation of *C. pasteurianum* thioredoxin (10), *C. pasteurianum* ferredoxin (12), and corn leaf NADP-malate dehydrogenase (NADP-MDH) (13). *Chromatium vinosum* thioredoxin and NADP-thioredoxin reductase (‡) were kindly supplied by T. C. Johnson of this laboratory. For routine assays, crude *C. pasteurianum* hydrogenase was prepared as described (10) except that steps formerly performed under hydrogen were done under argon. *C. pasteurianum* uptake hydrogenase (14) (500-fold purified) and bidirectional hydrogenase (15) (200-fold purified) were generous contributions from J.-S. Chen (Virginia Polytechnic Institute/State University, Blacksburg, VA). *Escherichia coli* thioredoxin was obtained from H. Follmann (Marburg, Federal Republic of Germany). Thioredoxin from *Rhizobium meliloti* was prepared by modifications of standard procedures (10). Biochemicals were obtained from Sigma. Argon

Abbreviations: FTR, ferredoxin-thioredoxin reductase; NADP-MDH, NADP-malate dehydrogenase; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

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(99.999%) and hydrogen (99.99%) were purchased from Liquid Carbonic (Chicago). Hydroxylapatite was prepared by the method of Tiselius *et al.* (16). Other reagents were of the highest commercially available quality. Buffers were adjusted to the indicated pH at 4°C.

Assays. Thioredoxin was assayed by following its capacity to stimulate corn leaf NADP-MDH with dithiothreitol as the reductant (13).

FTR was assayed by following the activation of corn leaf NADP-MDH in the presence of hydrogen gas and clostridial hydrogenase, ferredoxin, and thioredoxin. The assay mixtures were preincubated at 30°C under hydrogen or argon (1 atm) in stoppered test tubes (1 × 7 cm). The preincubation mixture contained (in 0.11 ml) 10 μmol of Tris·HCl at pH 7.9, 85 μg of *C. pasteurianum* ferredoxin, 8 μg of *C. pasteurianum* thioredoxin, 3 μg of corn leaf NADP-MDH, and ferredoxin-thioredoxin reductase as indicated. Reduction of thioredoxin and activation of NADP-MDH was begun by adding 10 μl of hydrogenase anaerobically with a gastight syringe and incubating for 30–45 min. Assay solution, containing 120 μmol of Tris·HCl at pH 7.9 and 0.15 μmol of NADPH, was then added. After the addition of 2.5 μmol of oxalacetic acid (to give 1.0 ml final reaction volume, 22°C), the mixture was transferred to a cuvette and the change in absorbance at 340 nm was monitored in air.

Crude hydrogenase was used for the routine assay of chromatographic fractions, whereas purified hydrogenase was employed in experiments with purified FTR. For purified uptake hydrogenase experiments, 3.3 units (0.26 μg) was added. For purified bidirectional hydrogenase experiments, 4.6 units (0.79 μg) was added. One unit equals 1 μmol of hydrogen oxidized per min with 5 mM methylene blue as the electron acceptor at pH 8.0 at 30°C.

NADP-thioredoxin reductase was assayed by following the NADPH- and thioredoxin-dependent reduction of 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) as described (17). The thioredoxin and NADP-thioredoxin reductase from *Chromatium* were used as a positive control.

Purification of FTR. All steps were performed at 4°C unless otherwise indicated. Thawed cells (700 g) were suspended in 2 vol of 30 mM potassium phosphate at pH 7.2 (buffer A), several milligrams each of DNase and RNase were added, and the mixture was sonicated in lots of 500 ml (5 min; Branson; power setting, 7). After centrifugation (45 min, 13,700 × *g*), granular $(NH_4)_2SO_4$ was added slowly to the supernatant fraction to give 30% saturation, and the resulting precipitate was removed by centrifugation (15 min, 13,700 × *g*). The supernatant fraction was then adjusted to 90% saturation with $(NH_4)_2SO_4$. The precipitate was collected as above, resuspended in 500 ml of buffer A, and dialyzed against 30 mM sodium acetate buffer (pH 5.5). A small amount of white precipitate formed and was removed by centrifugation. The supernatant fraction was then dialyzed overnight against buffer A, heated to 75°C (8 min) in a boiling H_2O bath, cooled in ice water, and centrifuged to remove the heavy gray precipitate. The heated supernatant fraction was applied to a 4.5 × 20 cm column of hydroxylapatite in buffer A; the column was developed stepwise with 30, 150, and 300 mM potassium phosphate (pH 7.2). The 150 mM eluate, which contained the FTR activity, was concentrated by ultrafiltration (YM5 membrane, Amicon), dialyzed against buffer A, and applied to a 2.6 × 20 cm column of DEAE-cellulose (DE-52, Whatman). This column was developed with a 700-ml linear gradient of NaCl in buffer A (0–300 mM) and 4.0-ml fractions were collected.

Those fractions showing FTR activity were concentrated by ultrafiltration as above, dialyzed against 25% saturated $(NH_4)_2SO_4$ in buffer A (adjusted to pH 7.0), and applied to a 2.5 × 22 cm column of phenyl-Sepharose (Pharmacia) that had been equilibrated beforehand with the same buffer. The column was eluted with a 500-ml descending linear gradient of $(NH_4)_2SO_4$ (25% to 0% saturation) and an increasing linear gradient of ethylene glycol [0% to 50% (vol/vol)] in buffer A at pH 7.0. Fractions (4.0 ml) were collected. The enzyme from this step was dialyzed against 50 mM potassium phosphate (pH 7.2), concentrated by ultrafiltration as above, and applied to a 1.6 × 85 cm column of Sephadex G-100 (superfine; Pharmacia) that had been equilibrated with the same buffer. Fractions (1.5 ml) were collected and frozen separately for further experimentation.

Flavin Analysis. Protein samples to be analyzed were precipitated with 5% (wt/vol) trichloracetic acid. After centrifugation, the supernatant fractions were chromatographed on Florisil columns in 5% (vol/vol) acetic acid followed by 5% (vol/vol) pyridine according to the method of Huennekens and Felton (18). The pyridine eluates were lyophilized, resuspended in 5% pyridine, and spotted on 250 μm × 20 cm silica gel G TLC plates (Analtech, Newark, DE) which were developed either in *n*-butanol/water/glacial acetic acid/methanol, 14:14:1:6 (vol/vol), or in 5% (wt/vol) $Na_2HPO_4 \cdot 12\ H_2O$ (19). Riboflavin, FMN, and FAD standards were run in parallel, and the flavins were then localized by their fluorescence under ultraviolet light.

Affinity Chromatography. *C. pasteurianum* ferredoxin and thioredoxin were bound to CNBr-Sepharose as described in the manufacturer's instructions (Affinity Chromatography, Pharmacia). Ferredoxin (5 mg) and thioredoxin (1 mg) were dialyzed against 0.1 M $NaHCO_3$/0.5 M NaCl, pH 8.3. For each column 0.5 g of CNBr-activated Sepharose 4B was swollen for 15 min in 5 ml of 1 mM HCl. The columns were then poured and each was washed with 125 ml of 1 mM HCl. They were then quickly equilibrated with 0.1 M $NaHCO_3$/0.5 M NaCl and the dialyzed protein samples were added. The stoppered columns were mixed end-over-end for 4 hr at room temperature and then overnight at 4°C. Excess protein was washed out with $NaHCO_3$/NaCl and the columns were treated with 0.2 M glycine (pH 8.0) to block excess reactive groups. For chromatography, protein samples in buffer A were allowed to interact with the column matrix for 20 min at 4°C and then were eluted with 0, 0.5, and 1.0 M NaCl in buffer A.

Other Analytical Methods. FTR was subjected to native gel electrophoresis in 0.75 × 8 cm slab gels of 7.5% and 10% polyacrylamide according to the procedure of Hedrick and Smith (20). The gels were stained for 1 hr in 2.5% (wt/vol) Coomassie blue R250 in 45.5% (vol/vol) methanol/9% (vol/vol) acetic acid, destained in 40% methanol/5% acetic acid until the background was clear, and stored in 7% acetic acid.

The molecular weight of *C. pasteurianum* FTR was estimated by chromatography on a 1.6 × 85 cm column of Sephadex G-100 (superfine) in 50 mM potassium phosphate (pH 7.2). The column was calibrated with horse heart cytochrome *c* (M_r = 12,400), soybean trypsin inhibitor (M_r = 21,000), ovalbumin (M_r = 45,000), bovine serum albumin (M_r = 66,000), and blue dextran.

For estimation of subunit molecular weights, FTR was subjected to electrophoresis in 15% $NaDodSO_4$/polyacrylamide gels by the method of Laemmli (21). The gels were calibrated with lysozyme (M_r = 14,300), β-lactoglobulin (M_r = 18,400), chymotrypsinogen A (M_r = 25,000), ovalbumin, and bovine serum albumin. Dithiothreitol (50 mM) was used rather than mercaptoethanol to reduce the samples. The gels were stained as outlined above.

Absorption spectra were recorded on a Cary 219 spectrophotometer. The effect of reduced thioredoxin on the absorption due to flavin in clostridial FTR was determined as follows. Each cuvette contained, under argon, in a final volume of 1.0 ml: potassium phosphate at pH 7.2, 40 μmol; *C. pasteurianum*

FTR, 14 μg; and, where indicated, dithiothreitol, 2.5 μmol, and thioredoxin, 10 μg. After 5 min, the absorption spectrum was taken and the absorbance at 450 nm was noted. The presence of some oxidized dithiothreitol in the cuvettes caused a high absorbance in the ultraviolet region. This contribution, although minor at 450 nm, was subtracted.

Protein was estimated from the absorption at 280 nm and by the Coomassie blue dye-binding assay of Bradford (22).

RESULTS

In the experiments described here, we coupled FTR, purified from the fermentative bacterium *C. pasteurianum*, to the activation of a target enzyme, chloroplast NADP-MDH (Eq. 3).

$$H_2 \rightarrow \text{hydrogenase} \rightarrow \text{ferredoxin} \rightarrow \text{FTR} \rightarrow \text{thioredoxin} \rightarrow \text{NADP-MDH}. \quad [3]$$

NADP-MDH activation under these conditions was completely dependent on the presence of each component of the assay—i.e., hydrogen gas, hydrogenase, ferredoxin, purified FTR, and thioredoxin (Table 1). Although the hydrogenase used here was of the uptake type, we obtained the same results with a *C. pasteurianum* bidirectional hydrogenase preparation. It was also possible, albeit with low efficiency, to reduce ferredoxin and obtain activation of a target enzyme when hydrogen/hydrogenase was replaced by an electron source consisting of illuminated chloroplast thylakoid membranes and reduced 2,6-dichlorophenolindophenol (23) (data not shown). As far as the target enzyme was concerned, chloroplast fructose-1,6-bisphosphatase could replace NADP-MDH (10). That is, we could assay clostridial FTR with more than one electron donor system as well as with more than one target enzyme.

The requirement of *C. pasteurianum* FTR for the appropriate thioredoxin was absolute. The thioredoxins from *E. coli*, *Chromatium*, and *Rhizobium* did not react with *Clostridium* FTR under our assay conditions (Table 2). By contrast, at the concentrations chosen for this experiment, these "foreign" thioredoxins were more effective than clostridial thioredoxin in the activation of NADP-MDH when they were reduced by the nonphysiological dithiol, dithiothreitol (Eq. 4).

$$\text{Dithiothreitol}_{red} + \text{thioredoxin}_{ox} \rightarrow \text{dithiothreitol}_{ox} + \text{thioredoxin}_{red}. \quad [4]$$

C. pasteurianum FTR required ferredoxin as its reductant. A combination of FTR and thioredoxin from *C. pasteurianum* had no activity in the standard NADP-thioredoxin reductase assay under conditions that gave positive results for the combination of NADP-thioredoxin reductase and thioredoxin from *Chromatium*. In other words, *C. pasteurianum* FTR had a requirement for its substrates—clostridial ferredoxin and thioredoxin—but the other constituents of the assay could be varied. It is also noteworthy that *Chromatium* NADP-thioredoxin reductase had no FTR activity when tested with *Chromatium* thioredoxin (data not shown).

Table 1. Requirements for ferredoxin-dependent thioredoxin reduction in the *C. pasteurianum* system

Treatment	NADP-MDH activity, nmol NADPH oxidized/min
Hydrogen	
Complete	98
Minus:	
Ferredoxin	0
Thioredoxin	0
FTR	0
Hydrogenase	0
NADP-MDH	0
Oxalacetate	0
Argon	
Complete	0

The hydrogenase used in this experiment was of the uptake type; FTR content was 3 μg; preincubation time was 42 min.

Table 2. Effectiveness of bacterial thioredoxins in the *C. pasteurianum* ferredoxin-thioredoxin system

Thioredoxin source	NADP-MDH activity, nmol NADPH oxidized/min	
	Ferredoxin + FTR as reductant*	Dithiothreitol as reductant
E. coli (10 μg)	0	121
R. meliloti (7 μg)	8	116
C. vinosum (8 μg)	0	198
C. pasteurianum (8 μg)	169	68

The hydrogenase used in these experiments was of the bidirectional type; FTR content was 0.3 μg; preincubation time was 42 min.
*With H_2 and purified hydrogenase.

Because FTR previously had been described only for oxygen-evolving photosynthetic cells, the nature of the *Clostridium* enzyme emerged as a principal point of interest. The most purified fractions showed one major band that comprised about 90% of the total protein in both native and NaDodSO$_4$/polyacryamide gel electrophoresis (Fig. 1). These fractions, yellow in color, showed absorption maxima at 450, 375, and 270 nm (Fig. 2). [The A_{450} of a 1 mg/ml solution of FTR (in 50 mM potassium phosphate at pH 7.2; path length = 1 cm) was calculated to be 0.37; A_{450}/A_{280} was equal to 0.22.] This spectrum is typical of a flavoprotein and, in fact, is similar to the absorption spectrum of *E. coli* NADP-thioredoxin reductase (6). The shift of the ultraviolet absorption maximum from its expected position at 280 nm to 270 nm may be attributed to the adsorption of flavins near 260 nm. No evidence for an iron-sulfur group, such as that reported in certain chloroplast FTR preparations, is apparent from this spectrum. *Clostridium* FTR differed again from chloroplast FTR (24) in that it did not form a stable complex with its homologous ferredoxin, as determined by affinity chromatography. There also was no evidence for a complex between clostridial FTR and thioredoxin.

Further evidence that FTR is a flavoprotein came from analyses that demonstrated that the preparation contained a fluorescent substance which showed the same R_f as authentic FAD in both of the TLC solvent systems used. Moreover, when FTR was subjected to Sephadex G-100 (superfine) chromatography, the FTR activity, flavin absorption at 450 nm, and major pro-

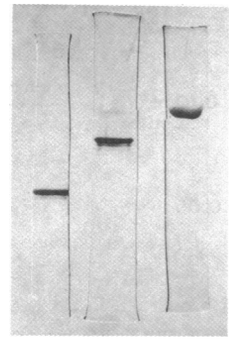


FIG. 1. Behavior of *C. pasteurianum* FTR in native gel electrophoresis in 7.5% polyacrylamide (*Left*), native gel electrophoresis in 10% polyacrylamide (*Middle*), and NaDodSO$_4$/polyacrylamide gel electrophoresis (15% gel) (*Right*).

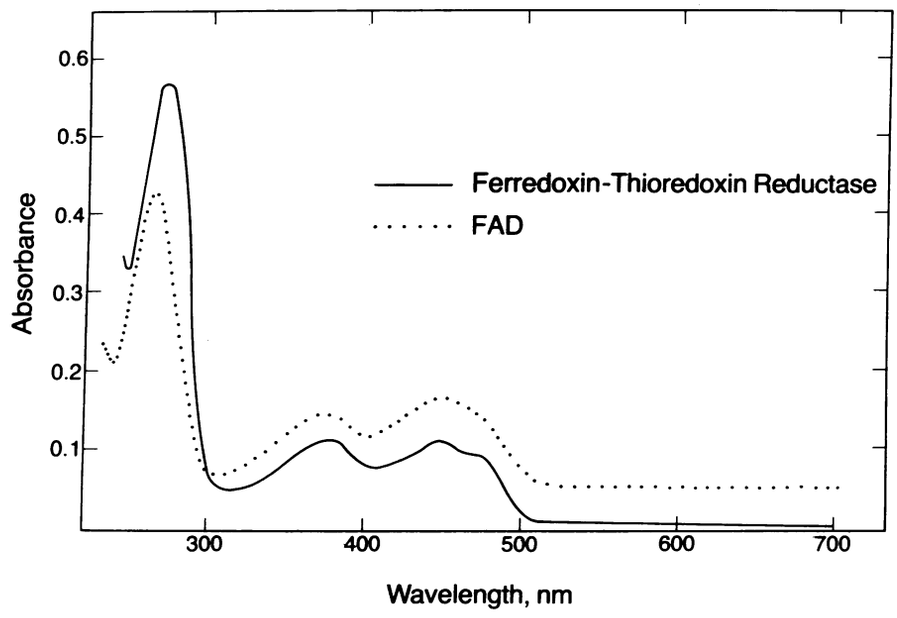


FIG. 2. Absorption spectra of *C. pasteurianum* FTR (0.3 mg/ml) and of FAD (10 nmol/ml). Path length = 1 cm. Base line for FAD is offset by 0.05.

tein component all migrated together as a single peak (Fig. 3). Finally, we observed that the addition of FAD (0.5 nmol) to the FTR assay caused a 50% stimulation of activity whereas addition of flavin without FTR gave no enhancement (data not shown). Such a result, which has been seen with other flavoenzymes (25), may indicate the loss of some flavin during purification. As observed with some other FAD-containing enzymes (26), FMN could replace FAD in causing this stimulation. Quantitatively, clostridial FTR contained one FAD in each of its two subunits (27, 28) (based on a molecular weight of 51,000–53,000 determined by Sephadex G-100 filtration and on a composition of two identical 30,000-dalton subunits determined by $NaDodSO_4$ gel electrophoresis).

Other evidence that *Clostridium* FTR is indeed a flavoprotein was obtained by coupling a redox change in its flavin chromophore to thioredoxin (Table 3). Here, dithiothreitol-reduced thioredoxin was found to bleach the visible absorbance of FTR monitored at 450 nm whereas oxidized thioredoxin was without effect. The addition of dithiothreitol alone caused a slower bleaching of FTR, which we consider to be analogous to its slow activation of certain target enzymes (13, 29, 30). It is particularly significant that dithiothreitol-reduced *E. coli* thioredoxin

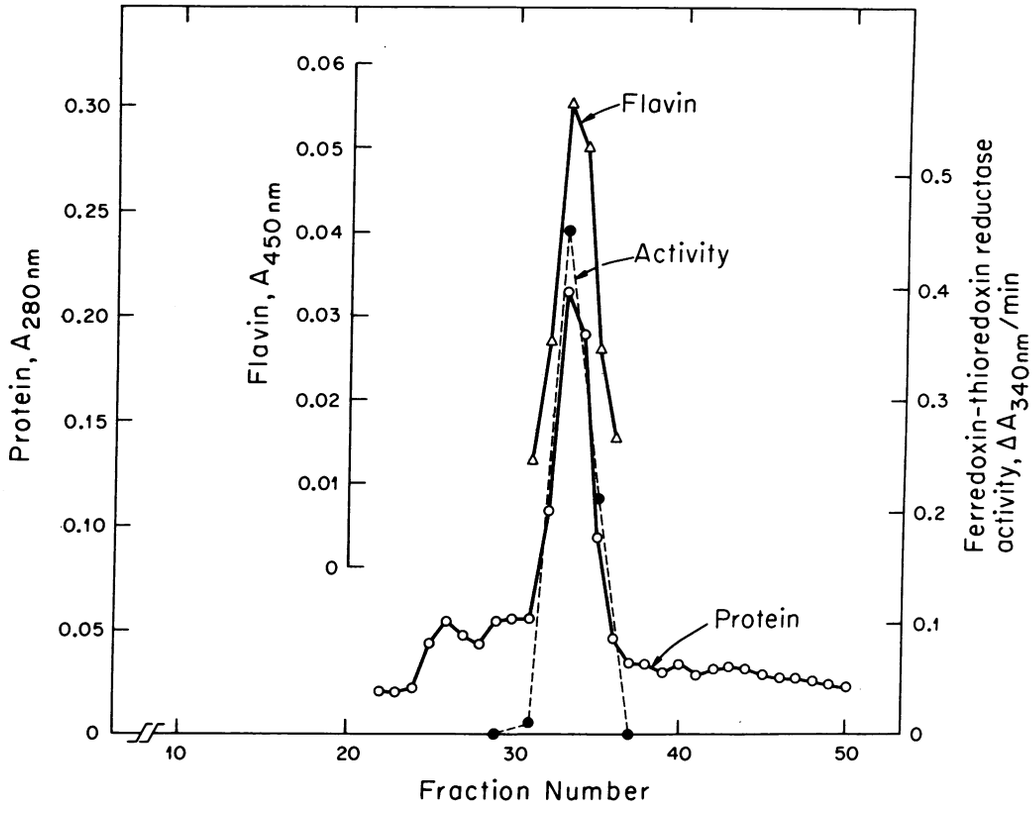


FIG. 3. Chromatography of *C. pasteurianum* FTR on Sephadex G-100 (superfine). The 10-μl samples were assayed for FTR activity; preincubation time was 30 min.

Table 3. Effect of reduced thioredoxin on the visible light absorption due to flavin in *C. pasteurianum* FTR

Addition	A_{450}, % of untreated control
None	100
C. pasteurianum thioredoxin, oxidized	100
Dithiothreitol	55
C. pasteurianum thioredoxin + dithiothreitol	5
E. coli thioredoxin + dithiothreitol	60

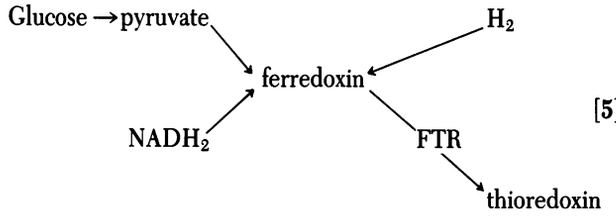
A_{450} of control = 0.005; path length = 1 cm.

was no more active in this test than dithiothreitol alone. That is, both the enzymatic activity of clostridial FTR and the reactivity of its bound flavin showed the same high specificity for clostridial thioredoxin. Such reduction of bound flavin by a reduced substrate is known for many flavoenzymes, including NADP-thioredoxin reductase (5). We concluded, therefore, that bound FAD plays an essential role in catalysis by *C. pasteurianum* FTR.

DISCUSSION

These experiments provide evidence that *C. pasteurianum* contains an FTR with FAD as its prosthetic group. This enzyme, which was highly specific for its own thioredoxin, appeared to be a dimer with 30,000-dalton subunits, each containing one FAD. As determined by bleaching of its visible absorption spectrum, clostridial FTR was reduced specifically by its own thioredoxin. In these respects, *C. pasteurianum* FTR resembled *E. coli* NADP-thioredoxin reductase; however, the clostridial enzyme differed in that it used ferredoxin, not NADPH, as reductant. It is doubtful that clostridial FTR has many attributes in common with the previously discovered chloroplast and cyanobacterial FTRs: these enzymes can reduce thioredoxins from other species and have not been reported to contain flavins (8, 9, 31, 32).

Although the physiological role of thioredoxin in fermentative bacteria remains to be elucidated (10), the present results show that FTR functions in a pathway for the reduction of thioredoxin in *Clostridium* in which ferredoxin is reduced enzymically with pyruvate, $NADH_2$, or hydrogen gas (Eq. 5).



[5]

It remains to be seen whether other fermentative, as well as photosynthetic, bacteria contain FTR and reduce thioredoxin via a ferredoxin-dependent pathway. Recent results have demonstrated that two such photosynthetic organisms—*Rhodopseudomonas*, a purple nonsulfur bacterium, and *Chromatium*, a purple sulfur bacterium—use NADPH to reduce thioredoxin via NADP-thioredoxin reductases in accordance with Eq. 1 (‡, 17). It may be significant that neither of these organisms accomplishes the photoreduction of soluble ferredoxin that one would expect if they contained a functional FTR (cf. refs. 33 and 34). By contrast, a representative of another type of photosynthetic bacterium, the green sulfur bacterium *Chlorobium*, is known to reduce ferredoxin photochemically via a system analogous to that of FTR-containing cyanobacteria and chloroplasts (35–37). The mechanism used by *Chlorobium* to reduce its thioredoxin (38) thus emerges as an interesting problem for the future.

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