THIOREDOXIN AND FERREDOXIN–THIOREDOXIN REDUCTASE ACTIVITY OCCUR IN A FERMENTATIVE BACTERIUM

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1. Introduction

Thioredoxins are low M_r hydrogen carrier proteins, widely distributed in living organisms, which play a major role in numerous metabolic systems. Thioredoxins act by participating in the oxidoreduction of sulfhydryl/disulfide groups in key enzymes, resulting in net reduction of disulfides or in sulfhydryl/disulfide exchange [1–4]. Organisms studied so far can be divided into two categories, depending upon how their thioredoxins are reduced physiologically. In one group, including all heterotrophic cells yet examined, NADPH reduces thioredoxin via the enzyme NADP-thioredoxin reductase [2,5]. In the other category, consisting of oxygenic photosynthetic cells, reduced ferredoxin and ferredoxin–thioredoxin reductase are the active agents [4,6].

One important group which has not been examined with regard to thioredoxins is the fermentative bacteria (i.e. obligate anaerobes). These organisms have been reported to lack glutathione [7], a widely distributed sulfhydryl agent, which in conjunction with glutaredoxin may share some of thioredoxin's capabilities [2]. In the absence of glutathione, thioredoxin might be a particularly important cellular sulfhydryl reductant. Furthermore, if thioredoxin were to occur in the fermentative bacteria, its mode of reduction would present an interesting question, since ferredoxin plays such an important role in many members of this group.

To address these questions, we have investigated the thioredoxin system in an obligate anaerobe, the classical saccharolytic fermenter, *Clostridium pasteurianum*. We now report that *C. pasteurianum* contains thioredoxin, and that reduced ferredoxin rather than NADPH serves as the physiological reductant. In this respect, this anaerobic bacterium resembles oxygenic photosynthetic cells rather than aerobic heterotrophic counterparts. A preliminary account of these findings has been published [8].

Abbreviations: NADP-MDH, NADP-malate dehydrogenase; Fru-P_2ase, fructose 1,6-bisphosphatase; MOPS, 3-(N-morpholino)propanesulfonic acid; M_r, relative molecular mass

2. Methods

2.1. *Purification of* C. pasteurianum *thioredoxin*

Clostridium pasteurianum was grown on sucrose, with $(NH_4)_2SO_4$ as the nitrogen source, and stored as a frozen cell paste, according to [9]. Thioredoxin was prepared as follows: 900 g thawed cells were suspended in 3 vol. 10 mM K_2HPO_4, several mg each of DNase and RNase were added, and the mixture was homogenized by sonication. After centrifugation (15 min, 13 700 × *g*), the supernatant fraction was adjusted to pH 4.5 with 2 N formic acid. The resulting precipitate was removed by centrifugation (15 min, 13 700 × *g*) and the supernatant fraction adjusted to pH 7.0 with 2 N NH_4OH. Cold acetone (3 vol.) was then added slowly with stirring, and the resulting precipitate was collected by centrifugation, suspended in Tris–HCl buffer (30 mM, pH 7.9, 5°C), and dialyzed twice against 50 vol. same buffer (hereafter called 'buffer A'). After removal of insolubles by centrifugation (20 min, 48 000 × *g*), this preparation was applied to a 5 × 7 cm column of DEAE-cellulose pre-equilibrated with buffer A. The thioredoxin activity eluted with the 'passthrough' (300 ml), which was then heated in an 80°C water bath for 6 min. After removal of the resulting precipitate by centrifugation (20 min, 48 000 × *g*), the supernatant fraction was concentrated by precipitation with 90% $(NH_4)_2SO_4$,

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redissolved in a minimum volume of buffer A, and chromatographed on a 2.6 × 90 cm column of Sephadex G-75 in the same buffer. The fractions which showed thioredoxin activity were then pooled, dialyzed against 5 mM Tris–HCl buffer (pH 7.9, 5°C), and applied to a 2.5 × 19 cm column of DEAE-cellulose pre-equilibrated with the same buffer. This column was developed with a 600 ml linear gradient of NaCl (0–0.2 M) in 5 mM Tris–HCl buffer (pH 7.9, 5°C) and 4 ml fractions were collected. The active fractions were pooled, concentrated by ultrafiltration (UM2 membrane, Amicon), and chromatographed twice on a 1.6 × 85 cm column of Sephadex G-50 superfine which had been previously equilibrated with 30 mM buffer A. Fractions (2 ml) were collected and those showing thioredoxin activity were pooled, concentrated by ultrafiltration as above, and frozen.

2.2. *Preparation of hydrogenase/ferredoxin–thioredoxin reductase fraction*

Frozen cells of *C. pasteurianum* (10–15 g) which had been stored in argon-filled vials, were suspended in 2 vol. MOPS buffer (50 mM, pH 7.2, 22°C), broken by sonication, and centrifuged (30 min, 48 000 × *g*). The supernatant fraction was decanted into a glass bottle, stoppered, placed under a hydrogen atmosphere by repeated evacuation and refilling, and heated in a 55°C water bath for 6 min. Following removal of the precipitate by centrifugation under argon (30 min, 17 000 × *g*), 7 ml supernatant fraction were taken up in a syringe and applied to a 2.6 × 25 cm anaerobic column of Sephadex G-50 or G-100 pre-equilibrated with MOPS (50 mM, pH 7.2, 22°C). The eluate was collected in stoppered argon-filled tubes and the active fraction was used to support the hydrogen- and ferredoxin-dependent reduction of thioredoxin.

2.3. *Assay of thioredoxin activity*

For routine determinations, *C. pasteurianum* thioredoxin was assayed by following its capacity to stimulate cornleaf NADP-MDH, using dithiothreitol as the reductant [10–12]. Clostridial thioredoxin was also detected, albeit with less sensitivity, by measuring its stimulation of spinach chloroplast Fru-P_2ase [11,12]. Ferredoxin-dependent thioredoxin reduction was also assayed by following the activation of these enzymes.

2.4. *Other methods*

The M_r of *C. pasteurianum* thioredoxin was estimated by chromatography on 1.6 × 85 cm column of Sephadex G-50 superfine in buffer A. The column was calibrated with *C. pasteurianum* ferredoxin (M_r 6000), horse heart cytochrome *c* (M_r 12 400), myoglobin (M_r 17 800), and chymotrypsinogen A (M_r 25 000).

Clostridial thioredoxin (15 μg) was electrophoresed in 0.5 × 7 cm gels of 10% and 12% polyacrylamide by the method in [13]. The gels were stained with Coomassie blue R250 [14] and scanned in a Gilford spectrophotometer at 560 nm.

Clostridium pasteurianum ferredoxin [15], cornleaf NADP-MDH [16], and spinach chloroplast Fru-P_2ase [17] were prepared as in references indicated. Other proteins and biochemicals were obtained from Sigma. Protein was estimated by measuring the absorbance at 280 nm or by a modified Lowry method [9].

3. Results and discussion

Preliminary experiments, in which crude extracts of *C. pasteurianum* were chromatographed on Sephadex G-75 and DEAE-cellulose, demonstrated the presence of a single peak of thioredoxin activity (not shown). In crude preparations, this thioredoxin did not adhere to DEAE-cellulose, but, following chromatography on Sephadex G-75 as above, it was retained and eluted at ~90 mM salt (fig.1). The thioredoxin could then be separated from remaining contaminants by chromatography on Sephadex G-50 superfine (fig.2).

Clostridium pasteurianum thioredoxin prepared by this procedure appeared homogeneous when electrophoresed on native gels of 10% (fig.3) and 12% (not shown) polyacrylamide. From Sephadex G-50 chromatography, of clostridial thioredoxin M_r was esti-

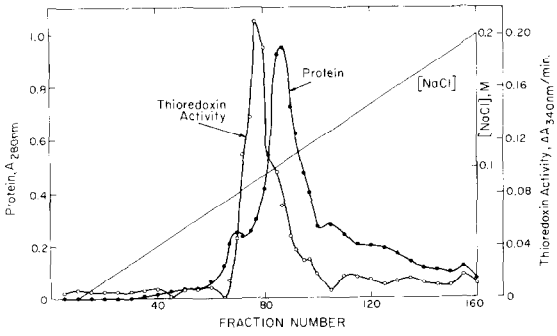


Fig.1. DEAE-Cellulose column profile of *C. pasteurianum* thioredoxin.

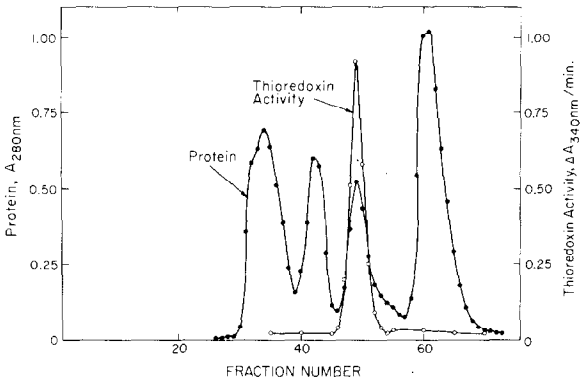


Fig.2. Sephadex G-50 superfine column profile of *C. pasteurianum* thioredoxin. First chromatography of two.

mated to be 10 000 (fig.4). The purified thioredoxin was stable to heat (85°C, 10 min), but was sensitive to trypsin [18] (not shown). These results indicate that *C. pasteurianum* thioredoxin is a heat-stable protein, apparently intermediate in size between the *Escherichia coli* thioredoxin (M_r 12 000) [2] and NADP-MDH-linked chloroplast thioredoxin *m* (M_r 9000) [11]. From the standpoint of size, it appears also that clostridial thioredoxin is more similar to thioredoxin *m* than to the Fru-P_2ase-linked chloroplast thioredoxin (thioredoxin *f*, M_r 16 000) in [11]. Clostridial thioredoxin also showed greater activity/mg protein in the standard thioredoxin *m* assay (3 μg used) than in the established thioredoxin *f* assay (28 μg used) (table 1). A similar conclusion was reached for thioredoxin from

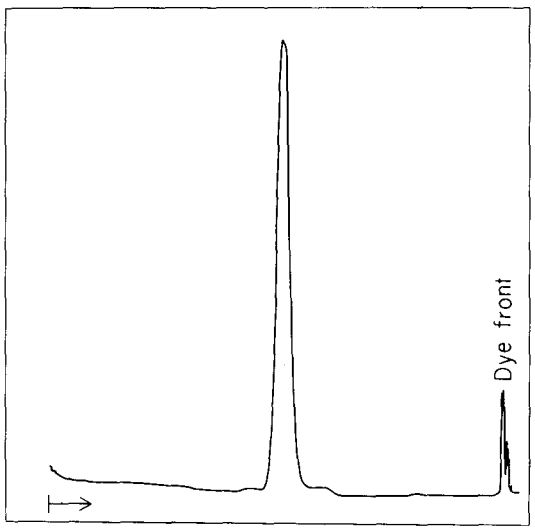


Fig.3. Densitometric trace of *C. pasteurianum* thioredoxin. Peak absorbance at 560 nm is 2.92.

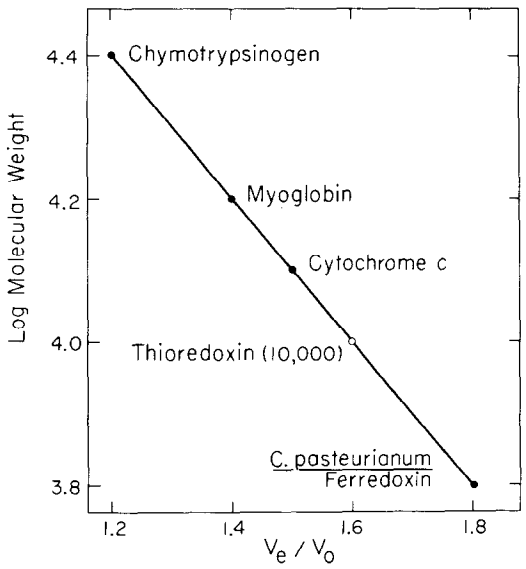


Fig.4. M_r-Value of *C. pasteurianum* thioredoxin estimated by Sephadex G-50 gel filtration.

Table 1
Activity of dithiothreitol-reduced *C. pasteurianum* thioredoxin in the chloroplast NADP-MDH and Fru-P_2ase assays

Treatment	NADP-MDH (nmol NADPH oxidized/min)	Fru-P_2ase (nmol P_i released/min)
Complete	50	166
– thioredoxin	3	28
– dithiothreitol	0	9
– NADP-MDH	0	–
– Fru-P_2ase	–	2
– substrate	0[a]	1[b]

[a] Oxalacetate omitted; [b] fructose 1,6-bisphosphate omitted; a value of 2 nmol/min was obtained when Mg^{2+} was omitted

Both assays were done at 22°C in air. For NADP-MDH, the complete preincubation mixture contained 3 μg *C. pasteurianum* thioredoxin from the Sephadex G-75 step and (μmol): Tris–HCl (pH 7.9) 10; dithiothreitol, 0.5. Preincubation was initiated by adding 1 μg NADP-MDH (0.04 ml final vol.) and continued for 10 min. Assay solution, containing 120 μmol Tris–HCl buffer (pH 7.9) and 0.15 μmol NADPH, was then added. Following the addition of 2.5 μmol oxalacetic acid (1.0 ml final vol.) the change in absorbance at 340 nm was monitored. For Fru-P_2ase, the complete reaction contained (0.5 ml final vol.) 28 μg *C. pasteurianum* thioredoxin from the Sephadex G-75 step, 16 μg chloroplast Fru-P_2ase, and (μmol): Tricine–KOH (pH 7.9) 50; $MgSO_4$, 0.5. Reduction of thioredoxin and activation of Fru-P_2ase was initiated by adding 2.5 μmol dithiothreitol and incubating for 15 min. The reaction was then started by adding 3 μmol fructose-1,6-bisphosphate, continued for 15 min, and stopped with 0.5 ml 10% trichloracetic acid. After removal of the precipitate by centrifugation, P_i was estimated by a modified Fiske-SubbaRow procedure [17]

a photosynthetic purple non-sulfur bacterium, *Rhodopseudomonas capsulata* [19].

The enzyme preparation used to test for thioredoxin reductase activity in the present investigation, while free of interfering low M_r components such as ferredoxin, thioredoxin, and NADPH (see section 2.2), contained a very active hydrogenase. We took advantage of this endogenous hydrogenase activity in experiments that required reduced ferredoxin: by adding both hydrogen and ferredoxin to the assay mixture, it was possible to determine with the clostridial enzyme preparation whether or not reduced ferredoxin could serve as the electron donor for the reduction of thioredoxin, as is the case with chloroplasts.

The results of such assays in which hydrogen and ferredoxin were added, and either NADP-MDH or Fru-P_2ase served as target enzyme, were positive (table 2). A ferredoxin-linked reduction of thioredoxin, measured by the thioredoxin-dependent reductive activation of both of these chloroplast enzymes, was effected by the *C. pasteurianum* enzyme preparation in the presence of hydrogen. Similar experiments, in which hydrogen and ferredoxin were replaced (under argon) with NADPH, showed no evidence of

Table 2
Demonstration of ferredoxin-dependent activation of chloroplast NADP-MDH and Fru-P_2ase using thioredoxin and the hydrogenase/ferredoxin–thioredoxin reductase fraction from *C. pasteurianum*

Treatment	NADP-MDH (nmol NADPH oxidized/min)	Fru-P_2ase (nmol P_i released/min)
Hydrogen		
Complete	20	38
– ferredoxin	1	10
– thioredoxin	1	10
– hydrogenase/ferredoxin–thioredoxin reductase fraction	1	4
– NADP-MDH	0	–
– Fru-P_2ase	–	9
– Substrate	0[a]	4[b]
Argon		
Complete	1	9

[a] Oxalacetate omitted; [b] fructose 1,6-bisphosphate omitted; a value of 8 was obtained when Mg^{2+} was omitted

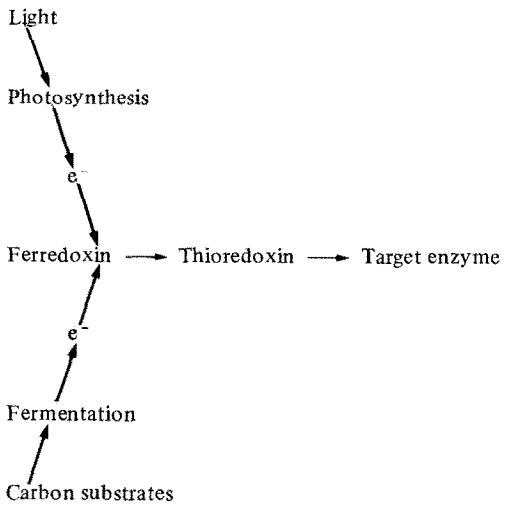
Both assays were preincubated at 30°C under hydrogen or argon in stoppered test tubes. For NADP-MDH, the preincubation mixture contained 36 μg *C. pasteurianum* ferredoxin, 21 μg purified *C. pasteurianum* thioredoxin, 10 μg cornleaf NADP-MDH and 25 μmol Tris–HCl (pH 7.9). Reduction of thioredoxin and activation of NADP-MDH was begun by adding 0.05 ml hydrogenase/ferredoxin–thioredoxin reductase fraction (Sephadex G-100 eluate), to give 0.20 ml final vol. and incubating for 20 min. Assay solution, containing 120 μmol Tris–HCl (pH 7.9) and 0.15 μmol NADPH, was then added. Following the addition of 2.5 μmol oxalacetic acid (1.0 ml final reaction vol. 22°C), the change in absorbance at 340 nm was monitored. For Fru-P_2ase, the complete reaction contained (1.0 ml final vol.) 36 μg *C. pasteurianum* ferredoxin, 33 μg purified *C. pasteurianum* thioredoxin 80 μg chloroplast Fru-P_2ase, and (μmol): Tricine–KOH (pH 7.9) 100; $MgSO_4$, 1. Reduction of thioredoxin and activation of Fru-P_2ase was accomplished by adding 0.20 ml of hydrogenase/ferredoxin–thioredoxin reductase fraction (Sephadex G-50 eluate) and incubating for 30 min. The reaction was then started by adding 5.5 μmol fructose-1,6-bisphosphate, continued for 20 min (30°C), and stopped with 1.0 ml 10% trichloracetic acid. After removal of the precipitate by centrifugation, P_i was estimated as above by a modified Fiske-SubbaRow procedure [17]

thioredoxin reduction (not shown), indicating the absence of the NADP-linked thioredoxin reductase characteristic of other bacteria [2,20]. The 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) assay, which has been used successfully for this enzyme from a number of sources [21], also gave negative results.

Clostridium pasteurianum is the first heterotrophic organism shown to reduce thioredoxin from a source other than NADPH. We have referred to the heated clostridial Sephadex eluate used in this investigation as the 'hydrogenase/ferredoxin–thioredoxin reductase fraction' with recognition that further experiments (in progress) are needed to characterize this activity. In this connection, it will be of interest to determine whether or not *C. pasteurianum* possesses an enzyme similar to the ferredoxin–thioredoxin reductase of chloroplasts [22] and cyanobacteria [6].

4. Concluding remarks

Thioredoxin has been purified from *C. pasteurianum* and its mode of reduction examined. Clostridial preparations supported a ferredoxin-dependent reduction of thioredoxin, but not reduction from NADPH. In view of the central role that ferredoxin plays in the metabolic processes of *C. pasteurianum*, we envisage that a ferredoxin/thioredoxin system, analogous to that found in chloroplasts, may operate in this bacterium:



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References

[1] Buchanan, B. B., Wolosiuk, R. A. and Schürmann, P. (1979) Trends Biochem. Sci. 4, 93–96.
[2] Holmgren, A. (1981) Trends Biochem. Sci. 6, 26–29.
[3] Jacquot, J.-P., Nishizawa, A. N. and Buchanan, B. B. (1980) Plant Physiol. 65 (6S), 67.
[4] Buchanan, B. B. (1980) Annu. Rev. Plant Physiol. 31, 341–374.
[5] Thelander, L. and Reichard, P. (1979) Annu. Rev. Biochem. 48, 133–158.
[6] Yee, B. C., De la Torre, A., Crawford, N. A., Lara, C., Carlson, D. E. and Buchanan, B. B. (1981) Arch. Microbiol. in press.
[7] Fahey, R.C., Brown, W.C., Adams, W. B. and Worsham, M. B. (1978) J. Bacteriol. 133, 1126–1129.
[8] Hammel, K. E. and Buchanan, B. B. (1981) Am. Soc. Microbiol. 81st Annu. Meet., Dallas TX, p. 161.
[9] Lovenberg, W., Buchanan, B. B. and Rabinowitz, J. C. (1963) J. Biol. Chem. 238, 3899–3913.
[10] Wolosiuk, R. A., Buchanan, B. B. and Crawford, N. A. (1977) FEBS Lett. 81, 253–258.
[11] Wolosiuk, R. A. Crawford, N. A., Yee, B. C. and Buchanan, B.B. (1979) J. Biol. Chem. 254, 1627–1632.
[12] Wolosiuk, R. A., Schürmann, P. and Buchanan, B. B. (1980) Methods Enzymol. 69/C, 382–391.
[13] Davis, B. J. (1964) Ann. NY Acad. Sci. 121, 404–427.
[14] Scott, P. G., Telser, A. G. and Veis, A. (1976) Anal. Biochem. 70, 251–257.
[15] Mortenson, L. E. (1964) Biochim. Biophys. Acta 81, 71–77.
[16] Jacquot, J. P., Buchanan, B. B., Martin, F. and Vidal, J. (1981) Plant Physiol. in press.
[17] Buchanan, B. B., Schürmann, P. and Wolosiuk, R. A. (1976) Biochem. Biophys. Res. Commun. 69, 970–978.
[18] Schürmann, P. and Wolosiuk, R. A. (1978) Biochim. Biophys. Acta 522, 130–138.
[19] Jacquot, J. P., Maudinas, B. and Gadal, P. (1979) Biochem. Biophys. Res. Commun. 91, 1371–1376.
[20] Clement-Metral, J. D. (1979) FEBS Lett. 101, 116–120.
[21] Porqué, P. G. Baldesten, A. and Reichard, P. (1970) J. Biol. Chem. 245, 2363–2370.
[22] De la Torre, A., Lara, C., Wolosiuk, R. and Buchanan, B. B. (1979) FEBS Lett. 107, 141–145.