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Thioredoxin System of the Photosynthetic Anaerobe *Chromatium vinosum*

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Chromatium vinosum, an anaerobic photosynthetic purple sulfur bacterium, resembles aerobic bacterial cells in that it has an NADP-thioredoxin system composed of a single thioredoxin which is reduced by NADPH via NADP-thioredoxin reductase. Both protein components were purified to homogeneity, and some of their properties were determined. *Chromatium vinosum* thioredoxin was slightly larger than other bacterial thioredoxins (13 versus 12 kilodaltons) but was similar in its specificity (ability to activate chloroplast NADP-malate dehydrogenase more effectively than chloroplast fructose-1,6-bisphosphatase) and immunological properties. As in other bacteria, *Chromatium vinosum* NADP-thioredoxin reductase was an arsenite-sensitive flavoprotein composed of two 33.5-kilodalton subunits, that required thioredoxin for the NADPH-linked reduction of 5,5′-dithiobis(2-nitrobenzoic acid). *Chromatium vinosum* NADP-thioredoxin reductase very effectively reduced several different bacterial-type thioredoxins (*Escherichia coli*, *Chlorobium thiosulfatophilum* (this name has not been approved by the International Committee of Systematic Bacteriology), *Rhizobium meliloti*) but not others (*Clostridium pasteurianum*, spinach chloroplast thioredoxin *m*). The results show that *Chromatium vinosum* contains an NADP-thioredoxin system typical of evolutionarily more advanced microorganisms.

Thioredoxins are low-molecular-weight disulfide proteins that function in ribonucleotide reduction (21), DNA replication (24), enzyme regulation (5), and sulfate assimilation (3, 28, 30, 34). Thioredoxins are known to be reduced by two routes: an NADP-linked route in which thioredoxin is reduced by NADPH via NADP-thioredoxin reductase (NTR) (the NADP-thioredoxin system) (equation 1) and a ferredoxin-linked route in which thioredoxin is reduced via ferredoxin-thioredoxin reductase (FTR) (the ferredoxin-thioredoxin system) (equation 2).

$$NADPH_2 + \text{thioredoxin}_{ox} \xrightarrow{NTR} \text{thioredoxin}_{red} + NADP^+ \quad (1)$$

$$2\ \text{Ferredoxin}_{red} + \text{thioredoxin}_{ox} + 2H^+ \xrightarrow{FTR} \text{thioredoxin}_{red} + 2\ \text{ferredoxin}_{ox} \quad (2)$$

in which ox and red denote the oxidized and reduced forms, respectively. The NADP-thioredoxin system is typical of aerobic bacteria (25) and animal (15, 16) and heterotrophic plant cells (32; R. Q. Cao, T. C. Johnson, and B. B. Buchanan, Int. Congr. Photosynth. 6th, Brussels, Belgium, abstr. 1, p. 60, 1983), whereas the ferredoxin-thioredoxin system is functional in chloroplasts (5), cyanobacteria (36; N. A. Crawford, C. W. Sutton, B. C. Yee, T. C. Johnson, D. C. Carlson, and B. B. Buchanan, Arch. Microbiol., in press), and, as found recently, fermentative bacteria (14).

One group of bacteria that has been neglected in thioredoxin studies is the obligate photosynthetic anaerobes. Thus, although thioredoxin has been partially purified from various types of aerobic and facultative bacteria, including facultative photosynthetic (*Rhodopseudomonas*) representatives (8, 19), little is known about the properties of the thioredoxin that has been shown to occur in obligate photosynthetic anaerobes (6). We have, therefore, undertaken a study to purify thioredoxin from a representative of this group and now report that *Chromatium vinosum*, an anaerobic photosynthetic purple sulfur bacterium, resembles its aerobic and facultative counterparts in that it contains a single type of thioredoxin which is reduced by NTR, a typical flavoprotein enzyme, that we also have purified and characterized. Furthermore, the immunological properties of *Chromatium vinosum* thioredoxin reveal a close similarity to other bacterial thioredoxins, which, in general, are similar to thioredoxin *m* from chloroplasts and cyanobacteria. A preliminary account of these findings has been published (T. C. Johnson, K. L. Cornwell, B. B. Buchanan, W. R. Mathews, H. Hartman, and K. Biemann, Fed. Proc. **42**:2175, 1983).

MATERIALS AND METHODS

Growth of cells. *Chromatium vinosum* was grown autotrophically on the carbonate medium of Arnon et al. (1) and stored as a frozen cell paste (−20°C) for thioredoxin or used fresh for NTR.

Reagents and coupling enzymes. Previously described methods were used for the preparation of corn NADP-malate dehydrogenase (NADP-MDH) (18) and spinach fructose-1,6-bisphosphatase (9, 35). *Escherichia coli* thioredoxin antibody was a gift of V. Pigiet (Johns Hopkins University), and spinach thioredoxin *m* antibody was a gift of N. H. Chua (Rockefeller University). *E. coli* thioredoxin was a kind gift of D. Le Master (Yale University). Thioredoxins from *Rhizobium meliloti* and *Chlorobium thiosulfatophilum* (this name has not been approved by the International Committee of Systematic Bacteriology) were supplied by K. Cornwell, and *Clostridium pasteurianum* thioredoxin was a gift of K. Hammel (both from this laboratory). Previously described methods were used to purify thioredoxins *f* and *m* from spinach (35) and *Nostoc muscorum* (36). Horseradish peroxidase color development reagent was purchased from Bio-Rad Laboratories. Biochemicals, including goat anti-rabbit immunoglobulin G (whole molecule) peroxidase conjugate, were obtained from Sigma Chemical Co. DEAE-cellulose (DE52) was purchased from Whatman, Inc., and all other column materials were purchased from Pharmacia Fine

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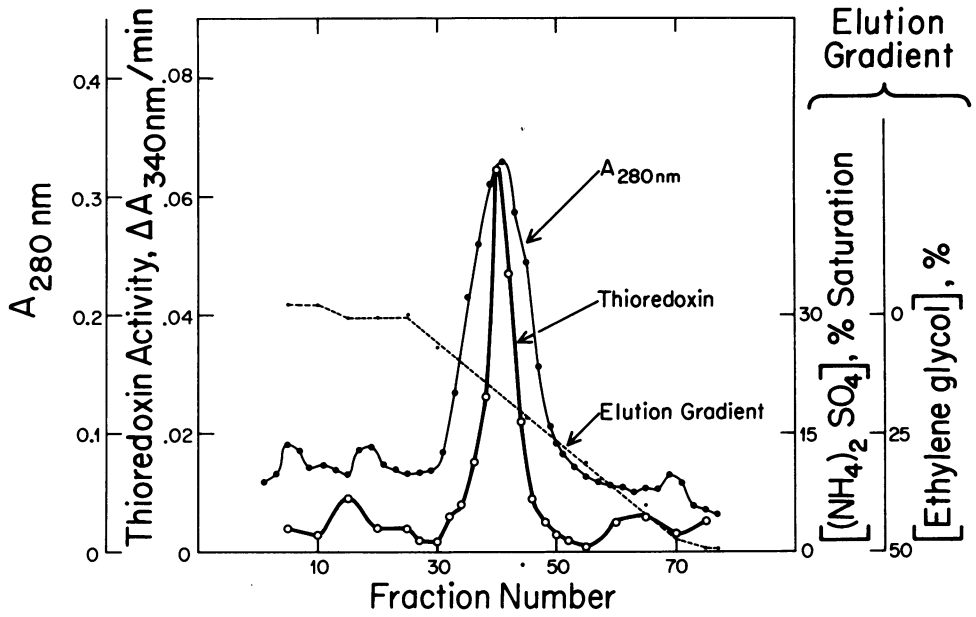

FIG. 1. Hydrophobic chromatography of *Chromatium vinosum* thioredoxin on phenyl-Sepharose CL-4B. A_{280nm}, Absorbancy at 280 nm.

Chemicals. Other reagents were obtained from commercial sources and were of the highest quality available. Except for buffers used for protein purification, all buffers were adjusted to the indicated pH at 22°C.

Analytical methods. An enzyme-linked immunosorbent assay described previously was used to determine the immunological relatedness between *Chromatium vinosum* thioredoxin and thioredoxins from other sources (26). Protein concentrations were routinely determined by the dye binding assay as described by Bradford (4) with either ovalbumin (enzyme-linked immunosorbent assays) or bovine serum albumin (protein purification) as standard proteins. Sodium dodecyl sulfate (SDS) and native polyacrylamide slab gel electrophoresis were performed as described by Laemmli (20) and Davis (10), respectively. As indicated, SDS-polyacrylamide gradient gels (10 to 16%) were prepared and developed according to Chua (7). Protein was stained with Coomassie blue R-250 (0.4% [wt/vol] in 40% [vol/vol] methanol and 7% [vol/vol] acetic acid) and destained with a solution containing 5% (vol/vol) methanol and 7% (vol/vol) acetic acid.

Western blot analysis of electrophoretic gels. The purity of the different thioredoxins used in immunological studies was confirmed by Western blot procedures. Gradient SDS gels (10 to 16%) were developed and probed for thioredoxin by a modification of the procedures previously described (25a). Upon completion of electrophoresis, the gels were washed three times in distilled water (10 min per wash). Transfer of proteins to nitrocellulose or cyanogen bromide paper was accomplished by applying a current of 0.9 A for 2 h toward the anode in 50 mM sodium acetate buffer (pH 7.0). After transfer was complete, the paper (blot) was blocked by soaking in a solution of 1 M glycine and 1% bovine serum albumin for 30 min. The blot was then washed for 30 min in a solution containing 10 mM sodium phosphate buffer (pH 7.3), 130 mM NaCl, 0.1% bovine serum albumin, and 0.1% Nonidet P-40 detergent (buffer A). The blot was first incubated for 24 h with rabbit antisera generated against thioredoxin from either *E. coli* or spinach chloroplasts (thioredoxin *m*) in 10 ml of buffer A and then washed for 30 min in buffer A alone.

In the case of nitrocellulose paper transfer, anti-rabbit immunoglobulin G (whole molecule) peroxidase conjugate was used to probe the blot. The peroxidase conjugate (100 μl) was diluted to 10 ml with buffer A and allowed to incubate for 3 h. The blot was then washed in buffer A as before. The peroxidase label was developed by incubating the paper in 100 ml of a solution containing 20 mM Tris-hydrochloride buffer (pH 7.5) and 500 mM NaCl for several minutes. The reaction was started by adding 60 μl of 30% H_2O_2 and 20 ml of a solution containing 60 mg of horseradish peroxidase color development reagent in absolute methanol (both solutions were at 4°C; the horseradish peroxidase solution also was protected from light). Incubation was continued at room temperature until blue bands became distinct. The cyanogen bromide paper treatment was as previously described (25a) except that the ^{125}I-labeled staphylococcal protein A (a gift of S. Mayfield of our division) was incubated with the blot in 10 ml of buffer A.

TABLE 1. Purification of *Chromatium vinosum* thioredoxin

Step	Total activity (10^3)[a]	Sp act (10^3)[b]	Recovery (%)
Sonic extract	7,400	0.77	100.0
Acid supernatant	2,100	0.61	28.0
Acetone	1,600	0.92	22.0
DE52 chromatography	1,400	2.4	19.0
Sephadex G-75 filtration	920	26.0	12.0
Sephadex G-75 superfine filtration	384	91.4	5.2
Phenyl-Sepharose hydrophobic chromatography	280	136.0	3.7[c]

[a] Micromoles of DNTB reduced per minute.

[b] Micromoles of DNTB reduced per minute per milligram of protein.

[c] Represents 2 mg from 250 g of cell paste.

Assays. (i) Thioredoxin assays. *Chromatidium vinosum* thioredoxin was routinely assayed by the chloroplast NADP-MDH assay (9, 18) in which dithiothreitol-reduced thioredoxin (equation 3) reduces, and thereby activates, NADP-MDH (equation 4), which, when added to the assay mixture, catalyzes the reduction of oxalactate by $NADPH_2$ (equation 5).

$$\text{Dithiothreitol}_{red} + \text{thioredoxin}_{ox} \rightarrow \text{thioredoxin}_{red} + \text{dithiothreitol}_{ox} \quad (3)$$

$$\text{Thioredoxin}_{red} + \underset{\text{(inactive)}}{\text{NADP-MDH}_{ox}} \rightarrow \underset{\text{(active)}}{\text{NADP-MDH}_{red}} + \text{thioredoxin}_{ox} \quad (4)$$

$$\text{Oxalacetate} + NADPH_2 \xrightarrow{\text{NADP-MDH}} \text{malate} + NADP^+ \quad (5)$$

In this assay, excess NADP-MDH (3 μg) was preincubated for 5 min with thioredoxin fractions in 0.2 ml (final volume) of a solution containing (micromoles): Tris-hydrochloride buffer (pH 7.9), 100; dithiothreitol, 2. The preincubation mixture (100 μl) was then injected into a 1-ml cuvette containing the following in 0.85 ml (micromoles): Tris-hydrochloride buffer (pH 7.9), 100; NADPH, 0.25. The reaction was started by the addition of 50 μl of 50 mM oxalacetic acid, and the NADP-MDH activity was followed by measuring the change in absorbance at 340 nm. The dithiothreitol-linked thioredoxin *f* assay was carried out with spinach fructose-1,6-bisphosphatase as described previously (9, 35). In both of these assays, reduced thioredoxin increases the activity of the target enzyme by a factor of 5 to 10.

Although the NADP-MDH assay worked well with the various column fractions, it was ineffective in crude extracts due to interfering oxidase activities. Consequently, to generate a purification table for thioredoxin, we adopted the 5,5′-dithiolbis(2-nitrobenzoic acid) (DTNB) assay described below (the DTNB assay was also used throughout for the following NTR purification).

(ii) NTR assay. NTR activity was determined by the DTNB assay (15, 25). In this assay procedure, NTR which is reduced by NADPH (equation 6) reduces thioredoxin (equation 7) which, in turn, reduces DTNB (equation 8). The advantages and disadvantages of the DTNB assay have been discussed by Holmgren (15).

$$NADPH_2 + NTR_{ox} \rightarrow NTR_{red} + NADP^+ \quad (6)$$

$$NTR_{red} + \text{thioredoxin}_{ox} \rightarrow \text{thioredoxin}_{red} + NTR_{ox} \quad (7)$$

$$\text{Thioredoxin}_{red} + DTNB_{ox} \rightarrow \text{2(2-nitro-5-mercaptobenzoic acid)} + \text{thioredoxin}_{ox} \quad (8)$$

Activity in the DTNB assay was followed at 25°C by measuring the increase of absorbance at 412 nm during the first minute. The reaction was performed in air in 1-ml cuvettes and started by the addition of 8 μg of *Chromatium vinosum* thioredoxin to an NTR fraction and the following in a final volume of 1 ml (micromoles): potassium phosphate buffer (pH 7.1), 100; EDTA, 10; NADPH, 0.25; DTNB, 0.02. The DTNB solution was prepared fresh daily by dissolving 4 mg of DTNB in 1 ml of 95% ethanol and then diluting to 10 ml with 500 mM Tris-hydrochloride buffer (pH 7.9). One unit of enzyme activity was defined as the formation of 1 μmol of the reaction product, 2-nitro-5-mercaptobenzoic acid, per min, corresponding to an increase in absorbance at 412 nm of 27.9 (15).

Purification procedures. All preparative procedures were carried out at 4°C unless otherwise stated. Spectrapore dialysis membrane with a molecular weight cutoff of 6 to 8 kilodaltons (kDa) was used throughout for dialysis steps. Unless indicated otherwise, ultrafiltration was accomplished with an Amicon Ym-5 membrane (molecular weight cutoff, 5 kDa).

***Chromatium vinosum* thioredoxin. (i) Preparation of cell extract.** Frozen *Chromatium vinosum* cells (250 g) were thawed and suspended in 4 volumes (wt/vol) of 30 mM Tris-hydrochloride buffer (pH 7.9) (buffer B) and disrupted by sonic oscillation in lots of 500 ml (3-min continuous duty cycle at power setting 7 with a Branson Sonifier model no. 200 fitted with a large tip). The temperature was maintained at 4°C with a salt-ice bath during sonication. The sonic extract was clarified by centrifugation (15 min, 13,000 × *g*); the supernatant fraction was adjusted to pH 4.7 with 2 N formic acid and then again centrifuged (15 min, 13,000 × *g*). The pellet was discarded, and the supernatant solution was adjusted to pH 7.9 with 2 N NH_4OH. Cold (−20°C) acetone (3 volumes) was then added slowly while stirring, and the suspension was allowed to stand for 1 h at −20°C. The resulting precipitate was collected by centrifugation (3 min, 1,500 × *g*), suspended in 120 ml of buffer B, and dialyzed for 3 days against 50 volumes of buffer A (three buffer changes).

(ii) DE52 chromatography. Insoluble material formed during dialysis was removed by centrifugation (45 min, 48,000 × *g*), and the supernatant fraction was collected and applied to a DE52 column (2.5 by 19 cm) that had been equilibrated with buffer B. The column was developed by eluting sequentially with 2 volumes of buffer B, with a linear salt gradient of 5 volumes from 0 to 500 mM NaCl in buffer B, and finally with two volumes of 500 mM NaCl in buffer B. Fractions of 5.5 ml were collected. Thioredoxin was recovered as a single peak. The active fractions were combined and concentrated to 10 ml by ultrafiltration. The thioredoxin and residual NTR present in the preparation copurified on DE52 and were both eluted at 200 mM NaCl.

(iii) Sephadex G-75 filtration. The pooled concentrated thioredoxin fraction from the DE52 step was chromatographed on a Sephadex G-75 column (2.5 by 85 cm) equilibrated with buffer B. Fractions of 3.8 ml were collected.

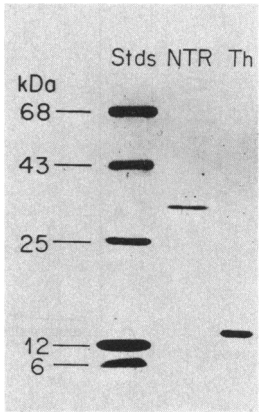

FIG. 2. Photograph of gradient SDS-polyacrylamide gel of thioredoxin and NTR from *Chromatium vinosum*. The standards (Stds) were: bovine serum albumin (68 kDa); ovalbumin (43 kDa); chymotrypsinogen (25 kDa); cytochrome *c* (12 kDa); and bovine trypsin inhibitor (6 kDa). The *Chromatium vinosum* NTR and thioredoxin (Th) samples contained 2 and 3 μg of protein, respectively.

Fractions containing thioredoxin activity were combined, concentrated by overnight dialysis versus solid sucrose, dialyzed for 12 h against 800 ml of buffer B, and finally concentrated to 2 ml by ultrafiltration. The Sephadex G-75 step separated thioredoxin from residual NTR in the preparation.

(iv) Sephadex G-75 superfine filtration. Further purification of *Chromatium vinosum* thioredoxin was achieved by a second gel filtration accomplished by application of the ultrafiltrate from above to a Sephadex G-75 superfine column (1.6 by 85 cm) that had been equilibrated and developed with buffer B. Fractions of 2 ml were collected, and those showing thioredoxin activity were combined and concentrated to 2.4 ml by ultrafiltration.

(v) Phenyl-Sepharose CL-4B hydrophobic chromatography. The pooled concentrated fractions from the Sephadex G-75 superfine column were brought to 30% ammonium sulfate saturation by the addition of an equal volume of 60% saturated ammonium sulfate in buffer B. The precipitate was removed by centrifugation (1 h, 145,000 × *g*), and the supernatant solution was applied to a phenyl-Sepharose CL-4B column (1.0 by 7.6 cm) that had been equilibrated with 30% saturated ammonium sulfate in buffer B. The column was developed by eluting with 3 column volumes of equilibration buffer, followed by a linear gradient in buffer B of 13 column volumes decreasing in ammonium sulfate concentration (from 30 to 0% saturation) and simultaneously increasing in ethylene glycol concentration (from 0 to 50% [vol/

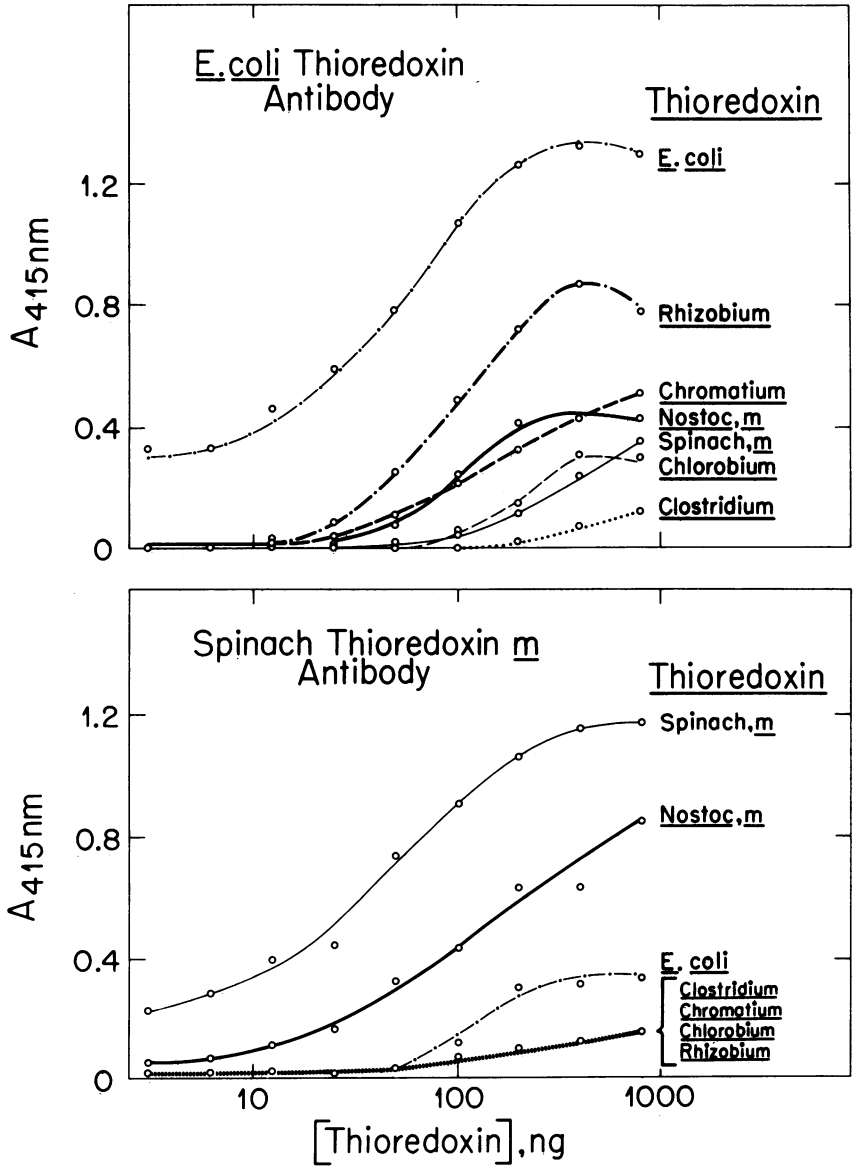

FIG. 3. Immunological cross-reactions of different thioredoxins probed by *E. coli* thioredoxin and spinach thioredoxin *m* antibodies with the enzyme-linked immunosorbent assay. The *E. coli*, *R. meliloti*, *Chromatium vinosum*, and *Chlorobium thiosulfatophilum* thioredoxins and spinach thioredoxin *m* samples were pure. The other thioredoxin samples (*N. muscorum* and *Clostridium pasteurianum*) were at least 50% pure. The thioredoxin content of all samples was determined by a combination of gradient SDS-polyacrylamide analytical gel electrophoresis and Western blot procedures. Change in A_{415nm} (absorbancy at 415 nm) reflects the amount of antibody bound to the antigen detected by a coupled peroxidase assay.

vol]). The column was finally eluted with 3 volumes of 50% ethylene glycol in buffer B. Fractions of 1.8 ml were collected, and those showing thioredoxin activity were pooled, concentrated by dialysis versus solid sucrose, and then dialyzed for 2 days versus 1 liter of buffer B (two buffer changes). The *Chromatium vinosum* thioredoxin was finally concentrated by ultrafiltration and stored at −20°C.

***Chromatium vinosum* NTR. (i) Preparation of cell extract.** *Chromatium vinosum* cells (100 g; fresh cell paste) were suspended in 4 volumes of buffer B and disrupted by sonication as above. The sonic extract was then centrifuged (20 min, 13,000 × *g*), the pellet was discarded, and the supernatant fraction was brought to 30% ammonium sulfate saturation by the addition of solid ammonium sulfate (the pH was maintained at 7.5 with NH_4OH). The precipitate was removed by centrifugation (20 min, 48,000 × *g*), and the supernatant fraction was collected, brought to 60% ammonium sulfate saturation (pH maintained as before), and stirred for 1 h at 4°C. The resulting precipitate was collected by centrifugation (20 min, 48,000 × *g*), resuspended in 70 ml of buffer B, and dialyzed for 12 h versus 4 liter of buffer B. Insolubles formed during dialysis were removed by ultracentrifugation (2 h, 300,000 × *g*), and the supernatant fraction was collected.

(ii) DE52. The clarified dialyzed 30 to 60% ammonium sulfate fraction was applied to a DE52 column (3.1 by 13.8 cm) that had been equilibrated with buffer B. The column was eluted sequentially with 2 volumes of buffer B, then with 6 volumes of a linear salt gradient from 0 to 500 mM NaCl in buffer B, and finally with 2 volumes of 500 mM NaCl in buffer B. Fractions of 6 ml were collected. Fractions showing NTR activity were combined, concentrated by dialysis against solid sucrose, and then dialyzed overnight versus 1 liter of buffer B. The final volume was 7.8 ml. The NTR and thioredoxin copurified on DE52 and were both eluted by 200 mM NaCl (see above).

(iii) Sephadex G-100. The pooled concentrated fractions from the DE52 column were applied to a Sephadex G-100 column (2.5 by 85 cm) that had been equilibrated and developed in buffer B. Fractions of 2.3 ml were collected. Fractions showing NTR activity were pooled and concentrated to 4 ml by ultrafiltration. The Sephadex G-100 step separated NTR from residual thioredoxin.

(iv) Hydrophobic chromatography, octyl-Sepharose CL-4B. The NTR from the Sephadex G-100 step was brought to 25% ammonium sulfate saturation by the addition of solid ammonium sulfate, and the precipitate was removed by centrifugation (10 min, 17,000 × *g*). The supernatant fraction was applied to an octyl-Sepharose CL-4B column (1.5 by 8.2 cm) equilibrated with 25% saturated ammonium sulfate in buffer B. The column was developed by eluting first with 2.8 volumes of equilibration buffer and then with a linear gradient of buffer B (7 volumes) containing decreasing ammonium sulfate concentrations (25 to 0% [wt/vol]) and simultaneous increasing ethylene glycol concentrations (0 to 50% [vol/vol]). The column was then finally eluted with 2.8 volumes of buffer A containing 50% ethylene glycol. Fractions of 2 ml were collected. At this step, thioredoxin reductase was resolved into a major and a minor peak, which were combined separately, concentrated by dialysis versus solid sucrose, and then dialyzed versus buffer B. The major peak was subjected to further purification as described below. The minor peak, which was not seen in all preparations, was not dealt with further. Significantly, the flavin chromophore that is characteristic of NTR from other sources was apparent in both octyl-Sepharose peaks.

(v) 2′-5′-ADP–Sepharose. The pooled concentrated major NTR peak from the octyl-Sepharose column was applied to a 2′-5′-ADP–Sepharose column (1 by 5.5 cm) that had been equilibrated with buffer B. Fractions of 1.8 ml were collected. The column was washed with 9.3 volumes of buffer B, and the thioredoxin reductase activity was then eluted with buffer B (19 volumes) containing 5 mM NADP. The active fractions were pooled, concentrated by ultrafiltration with an Amicon PM-10 membrane, and stored at −20°C. The enzyme was stored at a concentration of 0.02 mg/ml and under these conditions lost no activity after 1 year.

RESULTS AND DISCUSSION

Purification of *Chromatium vinosum* thioredoxin. Preliminary experiments, in which crude extracts of *Chromatium vinosum* were chromatographed on DE52 and Sephadex G-75 columns, demonstrated the presence of a single heat-sensitive peak that showed thioredoxin activity in the DTNB, NADP-MDH, and fructose bisphosphatase assays (data not shown). As monitored by the NADP-MDH and the DTNB assays, *Chromatium vinosum* thioredoxin could be highly purified by a variant of a previous procedure devised for the purification of a clostridial thioredoxin (13), i.e., acid fractionation, acetone precipitation, DE52 chromatography, and Sephadex G-75 and G-75 superfine gel filtration steps. The *Chromatium vinosum* thioredoxin purified by this procedure could be separated from the remaining contaminants by the introduction of a hydrophobic chromatography step in which the enzyme was applied to a phenyl-Sepharose column and eluted with an ascending ethylene glycol and a descending ammonium sulfate gradient (Fig. 1). A summary of the purification and yield of *Chromatium vinosum* thioredoxin in each of these steps is shown in Table 1. The thioredoxin was purified 136-fold with a recovery of 3.7% (activity based on the DTNB assay). There was a substantial loss of activity in the acid precipitation step (Table 1). This apparent loss was at least partly attributed to an inactivation of the NTR in the preparation that, before its inactivation by the acid treatment, increased the apparent DTNB reduction rate.

Properties of *Chromatium vinosum* thioredoxin. *Chromatium vinosum* thioredoxin purified as described above was homogeneous in electrophoresis in both native (15%), SDS (15%)-, and gradient SDS (10 to 16%)-polyacrylamide gels (Fig. 2). When calibrated with protein standards, SDS gel electrophoresis (15% polyacrylamide) and gel filtration (Sephadex G-75 superfine) revealed a molecular weight of 13 kDa for *Chromatium vinosum* thioredoxin, a value ca. 1 kDa

TABLE 2. Purification of *Chromatium vinosum* NTR

Step	Total activity (10^3)[a]	Sp act (10^3)[b]	Recovery (%)
Sonic extract	7,620	1.0	100
Ammonium sulfate concn (30 to 60% saturation)	3,050	1.8	40
DE52 chromatography	2,800	7.8	37
Sephadex G-100 filtration	3,400	46.0	31
Octyl-Sepharose CL-4B hydrophobic chromatography	1,500	460.0	19
2′-5′-ADP–Sepharose 4B affinity chromatography	720	4,600.0	9[c]

[a] Micromoles of DTNB reduced per minute.

[b] Micromoles of DTNB reduced per minute per milligram of protein.

[c] Represents 0.2 mg from 100 g of cell paste.

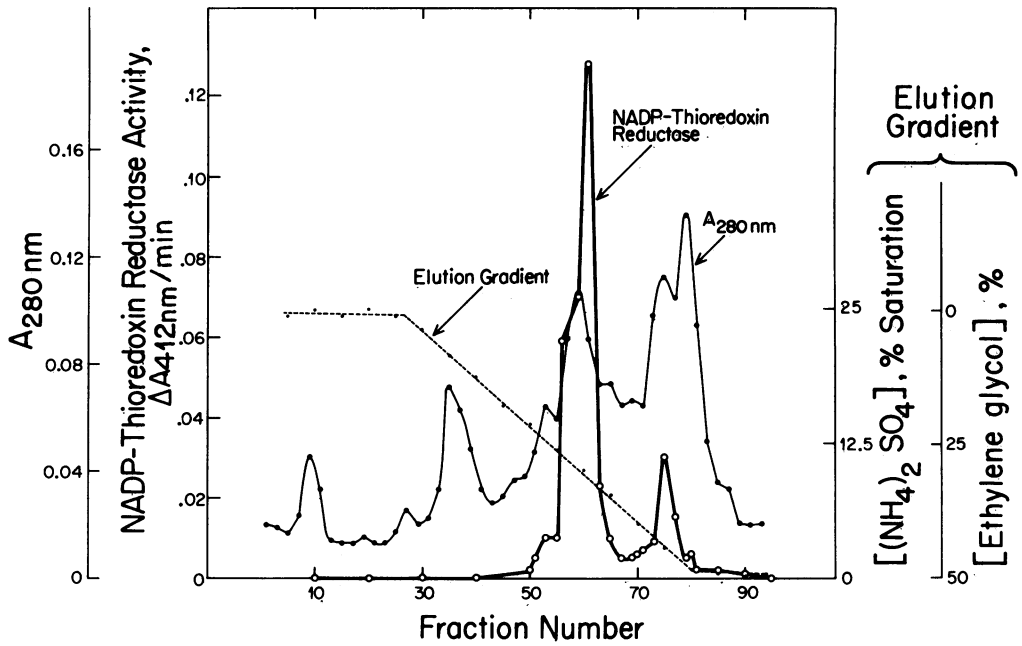

FIG. 4. Hydrophobic chromatography of *Chromatium vinosum* NTR on octyl-Sepharose CL-4B. A_{280nm}, Absorbancy at 280 nm.

higher than typical thioredoxins such as those from *E. coli* (21) and spinach chloroplasts (31).

Like its counterpart from other bacteria (13, 19, 33), *Chromatium vinosum* thioredoxin was more active in the chloroplast NADP-MDH assay (originally devised for thioredoxin *m*) than in the chloroplast fructose bisphosphatase assay (originally devised for thioredoxin *f*) (data not shown). Based on enzyme specificity, it seems that the structure of *Chromatium vinosum* thioredoxin is similar to other bacterial-type thioredoxins.

The similarity of *Chromatium vinosum* thioredoxin to other bacterial-type thioredoxins was also evidenced by immunological studies. Thus, when tested in the enzyme-linked immunosorbent assay, an antibody prepared against thioredoxin from *E. coli* showed a cross-reaction with thioredoxin from *Chromatium vinosum*, as well as from other types of bacteria (aerobic, *R. meliloti*; photosynthetic, *Chlorobium thiosulfatophilum*), as well as a substantial reaction with thioredoxin *m* from chloroplasts (spinach) and a cyanobacterium (*N. muscorum*) (Fig. 3). By contrast, an antibody prepared against spinach chloroplast thioredoxin *m* was less versatile: although cross-reacting with its *N. muscorum* and *E. coli* counterparts, it showed only marginal reactivity with the other bacterial thioredoxins, including that from *Chromatium vinosum*. The antibody results thus support the conclusion that *Chromatium vinosum* thioredoxin is of the bacterial type. The results also show that *E. coli* thioredoxin is immunologically more similar to chloroplast thioredoxin *m* than are the other bacterial thioredoxins tested. It is significant to note that Scheibe and Anderson previously observed an immunological similarity between *E. coli* thioredoxin and a thioredoxin from chloroplasts that seemingly was of the *m* type (29). It is also worth noting that thioredoxin from the fermentative bacterium *Clostridium pasteurianum* reacted very weakly with both antibodies tested.

Properties of *Chromatium vinosum* NTR. Early in this study, NTR was identified as a constituent of *Chromatium vinosum* enzyme preparations, separable from thioredoxin by gel filtration. NTR was purified 4,600-fold with a recovery of 9% by the techniques summarized in Table 2 (ammonium sulfate fractionation, DE52 chromatography, Sephadex G-100 gel filtration, octyl-Sepharose hydrophobic chromatography, and 2′-5′-ADP–Sepharose chromatography). The final preparation was judged to be homogeneous by electrophoresis in 12.5% native, 12.5% SDS-, and gradient SDS (10 to 16%)-polyacrylamide gels (Fig. 2).

Hydrophobic chromatography on octyl-Sepharose CL-4B was an especially effective step in freeing the preparation of contaminating proteins (Fig. 4). The octyl-Sepharose fractions, yellow in color, showed absorption maxima at 450, 375, and 270 nm as is typical of an NTR flavoprotein (Fig. 5) (21). The *Chromatium vinosum* flavin component was markedly enriched in the final 2′-5′-ADP–Sepharose 4B affinity chromatography step. However, as observed by others (27), the affinity step permanently altered the flavin spectrum exhibited by the NTR preparation through the introduction

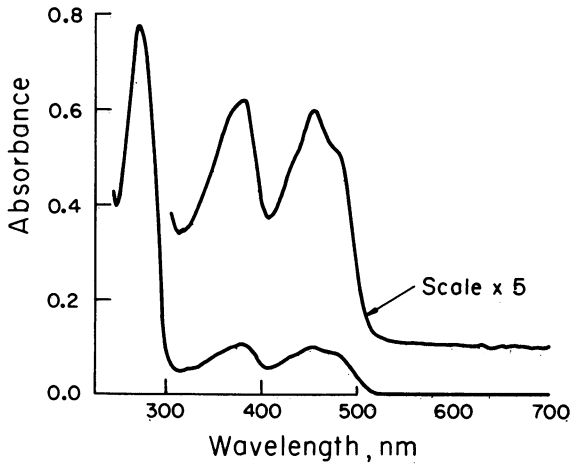

FIG. 5. Visible absorption spectrum of *Chromatium vinosum* NTR.

TABLE 3. Effect of reduced thioredoxin on the visible flavin absorption of *Chromatium vinosum* NTR[a]

Treatment	A_{450}
No additions	0.018
Dithiothreitol	0.011
Chromatium vinosum thioredoxin	0.015
Chromatium vinosum thioredoxin + dithiothreitol	0.001

[a] Each assay contained (in a final volume of 1.0 ml) 30 mM Tris-hydrochloride (pH 7.9) and 400 μg of 460-fold-purified NTR. The reaction was carried out in 1-ml cuvettes under argon. As indicated, 2.5 mM dithiothreitol and 10 μg of *Chromatium vinosum* thioredoxin were added, and A_{450} (absorbancy at 450 nm) was measured after 5 min, at which time it was found to be stable.

TABLE 4. Activity of different thioredoxins in the *Chromatium vinosum* NTR assay

Thioredoxin[a]	DTNB reduction (ΔA_{412}[b] per min)
Chromatium vinosum	0.032
Chlorobium thiosulfatophilum	0.112
Rhizobium meliloti	0.041
Escherichia coli	0.065
Clostridium pasteurianum	0.002
Chloroplast thioredoxin *m*	0.003
None	0.000

[a] Thioredoxins showed equal activity in the chloroplast NADP-malate dehydrogenase assay with dithiothreitol as the reductant.
[b] ΔA_{412}, Change in absorbancy at 412 nm.

of a highly absorbant constituent, presumably NADP. Subsequent attempts to remove the interfering NADP constituent were unsuccessful.

The flavin component associated with the *Chromatium vinosum* preparation was shown to be a part of the NTR enzyme by spectroscopic methods. Thus, as found for *E. coli* NTR (21), the visible flavin spectrum shown in Fig. 5 for an octyl-Sepharose fraction was reduced under anaerobic conditions (argon) by the addition of $NADPH_2$ (data not shown) or, alternately, by the addition of thioredoxin in the presence of dithiothreitol (Table 3) (see reference 14). Like its counterpart from other sources (16), *Chromatium vinosum* NTR was arsenite sensitive; i.e., its DTNB reduction activity was strongly inhibited by the addition of 1 mM sodium arsenite to the assay (data not shown).

Purified NTR showed molecular weights of 60 kDa by filtration on a calibrated Sephadex G-100 column and of 33.5 kDa by SDS-polyacrylamide gel electrophoresis (12.5% gels), thus indicating that the enzyme is a dimer of ca. 60,000 molecular weight in *Chromatium vinosum*, as in other cells (16, 22, 27).

Chromatium vinosum NTR showed a specificity between that of the NTRs typical of microbial cells and that of the NTRs of animal cells (12, 15, 17). Thus, like typical bacterial NTRs, the *Chromatium vinosum* enzyme required thioredoxin and NADPH for the reduction of DTNB (Fig. 6), but like mammalian NTR, the *Chromatium vinosum* enzyme reduced several target thioredoxins (Table 4). *C. vinosum* NTR reduced several thioredoxins in addition to the native one, i.e., that of *Chlorobium thiosulfatophilum*, *R. meliloti*, and *E. coli*. Interestingly, the *Chromatium vinosum* NTR did not reduce the thioredoxins of *Clostridium pasteurianum* or the bacterial type of thioredoxin from spinach chloroplasts (thioredoxin *m*). The acceptor specificity of *Chromatium vinosum* NTR thus falls between that of mammalian tissues (which reduces DTNB directly without an added thioredoxin) and that of bacterial cells (which typically are highly thioredoxin specific) (17).

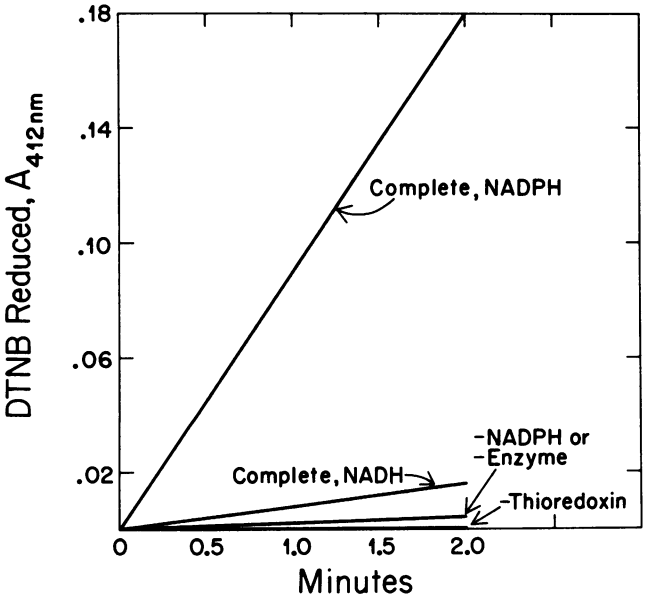

FIG. 6. DTNB reduction by *Chromatium vinosum* NTR. A_{412nm}, Absorbancy at 412 nm.

Conclusion. The present results extend the occurrence of the NADP-thioredoxin system to include the anoxygenic photosynthetic anaerobe, *Chromatium vinosum*. This representative of the purple sulfur bacteria appears to resemble facultative purple nonsulfur bacteria (*Rhodopseudomonas*-type) in the mode of thioredoxin reduction and to differ from fermentative bacteria that utilize a variant of the ferredoxin-thioredoxin system typical of chloroplasts and cyanobacteria (Fig. 7). Furthermore, the thioredoxin and NTR components of *Chromatium vinosum* are similar to their counterparts in other organisms containing the NADP-thioredoxin system. This latter point is of considerable interest in view of the recognition of *Chromatium vinosum* as a primitive purple photosynthetic bacterium (11). Accordingly, on the basis of current evidence, it would seem that the NADP-thioredoxin system developed relatively early in photosynthetic cells, and that once developed, it changed little during evolution; i.e., the system consists of a single thioredoxin and a 60-kDa flavoprotein NTR enzyme composed of two identical subunits.

The adoption of the NADP-thioredoxin system by *Chromatium vinosum* seems appropriate in view of evidence (23) that the organism utilizes ATP-driven reverse electron transport, rather than direct photoreduction, to reduce electronegative acceptors such as NAD^+ ($E_0 = -320$ mV) and presumably ferredoxin ($E_0 = -490$ nm) (2). Accordingly, because of the more oxidizing redox potential, thioredoxin reduction via NADP would be energetically less costly than via ferredoxin and would have the added advantage of lying closer to the main photosynthetic electron transport pathway should there be a need to relate electron transport status to the extent of thioredoxin reduction. Apropos this point, it should be noted that, in contrast to oxygenic organisms, the role of thioredoxin in anoxygenic procaryotes such as *Chromatium vinosum* seems to lie outside the regulation of

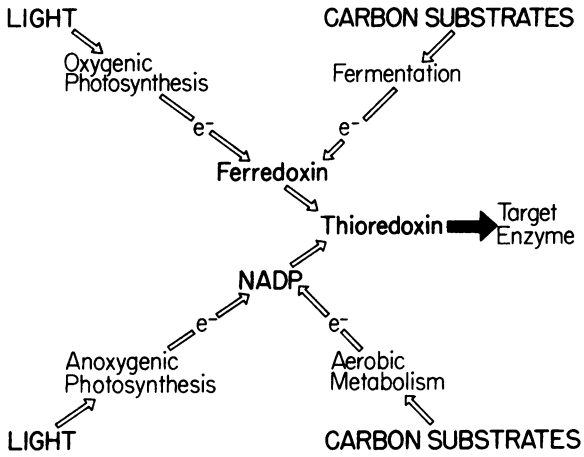

FIG. 7. Mechanism of thioredoxin reduction by different cellular processes. The ability of certain unicellular oxygenic organisms (green algae) to reduce thioredoxin via NADP is not indicated (Cao et al., 6th Inter. Congr. Photosynth., 1983). Until additional representative bacteria are studied, it is also possible that a ferredoxin-linked reduction of thioredoxin occurs in anoxygenic photosynthesis.

photosynthetic carbon dioxide assimilation in an area as yet unknown (Crawford et al., in press).

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