Thioredoxin is essential for *Rhodobacter sphaeroides* growth by aerobic and anaerobic respiration

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To investigate the biological role of thioredoxin in the facultative photosynthetic bacterium *Rhodobacter sphaeroides*, attempts were made to construct a thioredoxin-deficient mutant by site-specific mutagenesis, using the Tn903 kanamycin resistance gene for selection. *In situ* and Southern hybridization analyses have demonstrated that the TrxA⁻ mutation is lethal for *R. sphaeroides* growth under anaerobic conditions with DMSO as terminal electron acceptor and under aerobic conditions. In addition, the DNA region upstream of the *trxA* initiation codon is essential for aerobic growth of *R. sphaeroides*. An ORF of unknown function was identified in this region and is suggested to encode a product essential for aerobic metabolism of *R. sphaeroides*. The mechanism of thioredoxin action was also analysed by using the procedure for gene replacement to introduce a Cys33 to Ser mutation into the *trxA* chromosomal copy. The strain carrying this mutation produced a thioredoxin impaired in its protein-disulfide reductase activity and was also not viable. These data suggest that the physiological function of *R. sphaeroides* thioredoxin is redox-dependent. Thioredoxin purified from *R. sphaeroides* was shown to have a glutathione-disulfide oxidoreductase activity typical of glutaredoxins. This unexpected finding suggests that *R. sphaeroides* thioredoxin, in contrast to *Escherichia coli* thioredoxin, has the potential to act in GSH-dependent processes. Thus, the fundamental role of *R. sphaeroides* thioredoxin in cell growth probably originates from the multiple functions it can serve *in vivo*.

Keywords: *Rhodobacter sphaeroides*, thioredoxin, site-specific mutagenesis, disulfide oxidoreductase activity

INTRODUCTION

Thioredoxins are widely distributed small proteins containing an extremely reactive dithiol-disulfide in their active site. These proteins are assumed to regulate many biological processes by thiol redox control of target proteins, but also have non-redox functions in some protein-protein interactions (Gleason & Holmgren, 1988; Buchanan *et al.*, 1994).

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In plants, the ferredoxin/thioredoxin system (including chloroplast thioredoxins f and m) is responsible for light-mediated enzyme regulation in oxygenic photosynthesis by a selective thiol redox control (Buchanan, 1980, 1991; Cséke & Buchanan, 1986). In addition, the chloroplast m-type thioredoxin is essential for photosynthetic growth of the cyanobacterium *Anacystis nidulans* (Muller & Buchanan, 1989).

Thioredoxin-negative mutants (TrxA⁻) previously isolated in *Escherichia coli* were viable (Holmgren *et al.*, 1978; Russel & Model, 1984), and the only known *in vivo* role of *E. coli* thioredoxin is its hydrogen donor function for methionine-sulfoxide reductase (Russel & Model, 1986). In addition, the reduced form of *E. coli* thioredoxin is an essential subunit of phage T7 DNA

polymerase (Mark & Richardson, 1976) and is indispensable for maturation of the filamentous viruses f1 and M13 (Russel & Model, 1985; Lim *et al.*, 1985).

The NADP/thioredoxin system is also implicated in control of the division and development of eukaryotic cells. Indeed, thioredoxin is required for normal DNA synthesis in *Saccharomyces cerevisiae* (Muller, 1991, 1994) via a redox-dependent function (Muller, 1995) and is involved in the earliest embryonic divisions in both *Drosophila* (Salz *et al.*, 1994) and *Xenopus* (Hartman *et al.*, 1993). Other sites of thioredoxin action are suggested by its redox regulation of the mammalian transcription factors NF- κ B (Toledano & Leonard, 1991; Sorachi *et al.*, 1992; Okamoto *et al.*, 1992; Schenk *et al.*, 1994) and AP-1 (Abate *et al.*, 1990; Schenk *et al.*, 1994), and of the translational activator modulating the synthesis of photosystem II reaction centre D1 protein in *Chlamydomonas reinhardtii* (Danon & Mayfield, 1994).

In addition to its regulatory activity, thioredoxin is suspected to be involved in defence against oxidative stress by its ability to reduce hydrogen peroxide (Spector *et al.*, 1988; Mitsui *et al.*, 1992; Tomimoto *et al.*, 1993; Nakamura *et al.*, 1994) and by its thiol redox control in repair of oxidatively inactivated proteins (Fernando *et al.*, 1992; Natsuyama *et al.*, 1992). Thioredoxin has also been reported to act as the physiological hydrogen donor for the antioxidant activity of yeast peroxidase (Chae *et al.*, 1994).

The purple non-sulfur bacterium *Rhodobacter sphaeroides* is an extremely versatile organism capable of growth by aerobic and anaerobic respiration in the dark, by fermentation, and by anoxygenic photosynthesis in the light. Synthesis of its photosystem is known to be tightly regulated by oxygen tension (Kiley & Kaplan, 1988). In *R. sphaeroides*, thioredoxin was originally characterized by its ability to regulate aminolaevulinic acid synthase (the first enzyme of the branched bacteriochlorophyll and haem biosynthetic pathway) activity *in vitro* by a dithiol-disulfide exchange (Clément-Métal, 1979), suggesting that thioredoxin may be involved in oxygen regulation of bacteriochlorophyll biosynthesis at a post-translational level by thiol redox control (Clément-Métal, 1986). The single thioredoxin-encoding gene (*trxA*) of *R. sphaeroides* has been isolated and sequenced (Pille *et al.*, 1990). Expression analyses have demonstrated that *R. sphaeroides* thioredoxin can replace *E. coli* thioredoxin as hydrogen donor for methionine-sulfoxide reductase, as a subunit of phage T7 DNA polymerase and in assembly of filamentous M13 phage (Pille *et al.*, 1990). More precisely, Gly74 of *R. sphaeroides* thioredoxin was shown to have a direct role in the binding of T7 gene 5 protein (Pille *et al.*, 1996). In addition, we have recently demonstrated that transcription of the *R. sphaeroides trxA* gene is oxygen-regulated, with increased expression under high oxygen tension, suggesting possible involvement of thioredoxin in defence against oxidative stress in this organism (Pasternak *et al.*, 1996).

To investigate the biological function of thioredoxin in *R. sphaeroides*, we have attempted to construct a thioredoxin-deficient mutant. Our results indicate that this mutation is lethal for *R. sphaeroides* growth by anaerobic respiration in the presence of DMSO or by aerobic respiration. In addition, the DNA region upstream of the *trxA* gene is essential for aerobic growth but not for anaerobic respiration. Surprisingly, homogeneous *R. sphaeroides* thioredoxin exhibits some GSH-disulfide oxidoreductase activity, which may explain why it is essential for growth of *R. sphaeroides*.

METHODS

Materials. 2-Hydroxyethyl disulfide was obtained from Aldrich; NADPH, GSH and yeast GSH reductase from Sigma; and *E. coli* thioredoxin from Promega. *E. coli* glutaredoxins (Grx1 and Grx3) were a gift from Professor Arne Holmgren (Karolinska Institute, Stockholm, Sweden). *R. sphaeroides* thioredoxin was expressed and purified as described previously (Assemet *et al.*, 1995).

Bacterial strains, plasmids and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *R. sphaeroides* strains were grown in YCC rich medium (Sistrom, 1977), supplemented with appropriate antibiotics when necessary. Anaerobic dark growth was achieved using 20 mM dimethyl sulfoxide as the terminal electron acceptor and 0.25% (w/v) glucose in completely filled screw-cap tubes for liquid cultures and by using Anaerocults (Merck) for agar plates. *E. coli* strains were grown aerobically at 37 °C in Luria-Bertani (LB) medium (Maniatis *et al.*, 1982). Antibiotics were added to growth media at the following concentrations: ampicillin (Ap) 200 μ g ml⁻¹ in LB; tetracycline (Tc) 10 μ g ml⁻¹ in LB; 1.5 μ g ml⁻¹ in YCC; kanamycin (Km) 100 μ g ml⁻¹ in LB, 20 μ g ml⁻¹ in YCC; streptomycin (Sm) 25 μ g ml⁻¹ in YCC.

Bacterial mating. Plasmid DNA was mobilized into *R. sphaeroides* strains by diparental conjugation with *E. coli* SM10 as the donor (Simon *et al.*, 1983). Filter matings were performed on YCC plates by incubating for 15 h at 37 °C [the optimal temperature of conjugation for the *R. sphaeroides* WS8 recipient strain (Table 1)], using a cell ratio of $\approx 1 \times 10^9$ late-exponential-phase chemoheterotrophically grown recipients to $\approx 5 \times 10^7$ early-exponential-phase donors per mating. The mating mixtures were removed from the filters, washed once in YCC medium and spread on YCC plates containing appropriate antibiotics.

Molecular biological techniques. Plasmids and DNA fragments were isolated, treated with modifying enzymes and electrophoretically analysed by standard techniques (Maniatis *et al.*, 1982). Sequencing was performed by the dideoxy technique using the T7 sequencing kit (Pharmacia). Site-directed mutagenesis was performed as described elsewhere (Pille *et al.*, 1996).

Chromosomal DNA from *R. sphaeroides* was isolated by an alkaline lysis procedure (Davis *et al.*, 1980). Samples of DNA were electrophoresed on a 0.7% (w/v) agarose gel, blotted onto nylon membranes (NY 13, Schleicher & Schull) by vacuum pressure (Vacuum Blotting, Pharmacia) following the manufacturer's instructions and used for Southern hybridization. For *in situ* hybridization, *R. sphaeroides* colonies were picked from appropriate antibiotic-supplemented plates and smeared onto a nylon membrane. After incubating the membranes for 24 h at 32 °C, these membranes were placed on 3MM Whatman paper presoaked in lysis buffer (Davis *et al.*,

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
<i>E. coli</i>		
HB101	<i>supE44 hsdS20</i> (r_B^- , m_B^-) <i>recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5mtl-1</i>	Bolivar & Backman (1979)
SM10	<i>recA thi thr leu</i> ; chromosomal RP4-2 (Tc::Mu) <i>Tra</i> ⁺ Km ^R	Simon <i>et al.</i> (1983)
<i>R. sphaeroides</i>		
WS8	Wild-type	Sistrom (1977)
WSm	WS8 derivative, Sm ^R (spontaneous)	This study
Plasmids		
pUC18	Ap ^R , MCS from M13mp19 into <i>lacZ'</i>	Yanisch-Perron <i>et al.</i> (1985)
pSUP203	pBR325 derivative, Mob ⁺ Ap ^R Cm ^R Tc ^R	Simon <i>et al.</i> (1983)
pUC4KSAC	Ap ^R , source of the Km ^R cassette	Barany (1985)
pUTC3	421 bp <i>NcoI</i> – <i>PstI</i> <i>trxA</i> fragment in pKK233-2	Pille <i>et al.</i> (1990)
pUTC66	2.9 kb <i>EcoRI</i> <i>trxA</i> fragment in pUC18	This study
pUTC76	2.1 kb <i>EcoRI</i> <i>trxA</i> fragment in pUC18	This study (Fig. 1)
pUTC84	pUTC76::Km ^R cassette ligated into <i>Bgl</i> III site of <i>trxA</i> fragment	This study
pUTC78	pUTC76::Km ^R cassette ligated into <i>Sac</i> I site of <i>trxA</i> fragment	This study
pUTC92	pUTC76::Km ^R cassette ligated into <i>Bam</i> HI site of <i>trxA</i> fragment	This study
pUTC98	pUTC76, <i>trxA</i> Cys33 to Ser mutation	This study
pUTC100	pUTC98::Km ^R cassette ligated into <i>Bam</i> HI site of <i>trxA</i> fragment	This study
pUTC80	pSUP203::3.4 kb <i>EcoRI</i> <i>trxA</i> ΩKm ^R fragment from pUTC78	This study (Fig. 1)
pUTC86	pSUP203::3.4 kb <i>EcoRI</i> <i>trxA</i> ΩKm ^R fragment from pUTC84	This study (Fig. 1)
pUTC97	pSUP203::3.4 kb <i>EcoRI</i> <i>trxA</i> ΩKm ^R fragment from pUTC92	This study (Fig. 1)
pUTC101	pSUP203::3.4 kb <i>EcoRI</i> <i>trxA</i> ΩKm ^R fragment from pUTC100	This study (Fig. 1)

Fig. 1. Km resistance cartridge insertions into the *trxA* region. Lines indicate DNA fragments in the *trxA* region which were ligated into pUC18 or pSUP203 to form the designated plasmids (Table 1). Insertions of the Tn903 cartridge (Barany, 1985) conferring Km resistance are shown and the *trxA* genotype corresponding to the resulting plasmid is indicated. The Cys33 to Ser mutation of *trxA* gene on pUTC101 was introduced by site-directed mutagenesis as described in Methods. Restriction enzymes used were *Bam*HI (B), *Bgl*III (Bg), *Eco*RI (E) and *Sac*I (S).

1980). After 15 min, membranes were transferred into 0.5 M NaOH, 1.5 M NaCl for 2 × 15 min and neutralized with 1 M Tris/HCl pH 7.5, 1.5 M NaCl for the same time. Filters were

then washed in 2 × SSC (Maniatis *et al.*, 1982) for 5 min and dried. DNA was cross-linked onto nylon membranes by UV irradiation (Stratalinker, Stratagene). Unbound material was removed by washing in 3 × SSC, 0.1% (w/v) SDS at 50 °C for 3 h. Non-radioactive DNA probes using DIG⁺-dUTP were made and utilized according to the manufacturer's instructions with the random-primed DNA Labeling Kit (Boehringer Mannheim). The labelled probes were detected either by colorimetric reaction or preferentially by chemiluminescent reaction when membranes needed to be reprobed.

Insertions of the Km resistance cartridge into the *trxA* gene region. The 2.9 kb *Eco*RI fragment containing the *trxA* gene region was first isolated from the *R. sphaeroides* λEMBL3 genomic library constructed previously (Pille *et al.*, 1990) and cloned into pUC18, yielding the plasmid pUTC66 (Table 1). To facilitate subsequent genetic analyses, several subcloning steps were performed to generate plasmid pUTC76, a pUC18 derivative containing a 2.1 kb *Eco*RI *trxA* fragment (Table 1; Fig. 1). The Tn903 Km resistance cartridge of pUC4KSAC (Barany, 1985) was then inserted into the *trxA* fragment of pUTC76 at three different sites: the *Bgl*III site located 174 bp upstream of the *trxA* initiation codon, the *Sac*I site of the *trxA* coding region and the *Bam*HI site located 90 bp downstream of the *trxA* stop codon, generating pUTC84, pUTC78 and pUTC92, respectively. In addition, the 300 bp *Bgl*III–*Sac*I *trxA* fragment from pUTC76 was replaced by the corresponding restriction fragment containing the mutation Cys33 to Ser to produce pUTC98. The same Km resistance cartridge was then inserted into the *Bam*HI site of pUTC98 as described above, generating pUTC100 (Table 1). For subsequent transfer to *R. sphaeroides*, the four resulting insertions were then isolated as 3.4 kb *Eco*RI fragments and subcloned into the unique *Eco*RI site of the mobilizable pSUP203 vector (Simon *et al.*, 1983),

generating the plasmids pUTC86, pUTC80, pUTC97 and pUTC101, respectively (Fig. 1).

GSH-disulfide oxidoreductase assay. The assay was performed as described by Holmgren (1979), by measuring the reduction of 2-hydroxyethyl disulfide by GSH at the expense of NADPH recorded at 340 nm. One unit (U) of activity was defined as 1 μ mol of NADPH oxidized per min. Purified *E. coli* Grx1 and Grx3 were used in each experiment as positive controls. The crude extracts used in this assay were prepared as described elsewhere (Klug, 1991). Protein concentration was determined as described by Bradford (1976).

RESULTS

Disruption of the chromosomal *trxA* gene is lethal for both aerobic and anaerobic respiration of *R. sphaeroides*

The biological function of *R. sphaeroides* thioredoxin was investigated by site-specific mutagenesis, using several insertions of the Tn903 Km resistance cassette into the cloned *trxA* gene region as described in Methods and shown in Fig. 1. Homologous recombination events with the genome of the Sm^R *R. sphaeroides* strain WSm (Table 1) were the result of using the mobilizable ColE1 pSUP203 plasmid (Simon *et al.*, 1983) and directly selected by the Km resistance of the exconjugants.

Given that the phenotype of the TrxA⁻ mutant was totally unknown, and since the expression of the *trxA* gene is regulated by oxygen (Pasternak *et al.*, 1996), the Km^R exconjugants were isolated either under aerobic conditions or under anaerobic dark conditions in the presence of DMSO as terminal electron acceptor.

Southern hybridization analyses demonstrated that the double cross-over required for integration of the Km resistance cassette upstream of the *trxA* gene (corresponding to the construct pUTC86, Fig. 1) occurred in 8.1% of exconjugants selected for growth under anaerobic dark conditions (data not shown).

In contrast, gene exchange was not observed among the 614 exconjugants resulting from transfer of the pUTC80 construct (containing the disrupted *trxA* gene, Fig. 1) and isolated under anaerobic dark conditions. This result strongly suggests that mutants in which the chromosomal *trxA* gene had been disrupted were not viable and that *R. sphaeroides* thioredoxin is essential for cell growth by anaerobic respiration.

Isolation of *R. sphaeroides* exconjugants under aerobic growth conditions did not allow growth of double recombinants obtained using either pUTC80 (476 Km^R exconjugants were examined) or pUTC86 plasmids. In contrast, conjugative transfer of the pUTC97 plasmid (Table 1; Fig. 1) which contains the Km resistance cartridge downstream of the *trxA* gene, provided gene replacement in 2.5% of exconjugants isolated under aerobic conditions, as deduced from both *in situ* and Southern hybridization analyses (data not shown). These data suggest that both the *trxA* gene and the DNA region upstream of the *Bgl*III site (Fig. 1) are essential for *R. sphaeroides* aerobic growth.

Sequence analysis of the DNA region upstream of the *trxA* coding region

To define the DNA region flanking the Km resistance cartridge in the control plasmid pUTC86 (Fig. 1), the *trxA* upstream region was sequenced as described in Methods. DNA sequence analysis of this region indicated the presence of an ORF, named ORF993 (Fig. 2) which has a remarkably high G+C content of 74.6 mol%, given that the typical G+C content of *R. sphaeroides* chromosomal DNA is 68% (Pfennig & Truper, 1974). ORF993 shows a codon usage typical of *R. sphaeroides*, as deduced by codon preference computer program analysis, strongly suggesting that it codes for a protein. The predicted product contains 331 amino acid residues, has a predicted molecular mass of 35771 Da and is essential for *R. sphaeroides* aerobic growth since disruption of ORF993 at the *Bgl*III site (Fig. 2) is lethal for aerobic respiration. This predicted protein has a remarkably high content (43.5%) of hydrophobic residues. A putative Shine-Dalgarno ribosome-binding site (AGGTG) is located 10 nucleotides upstream of the presumed ATG initiation codon. We have not identified sequences in the GenBank database either at the nucleotide or protein level that bear significant resemblance to ORF993.

The essential biological role of *R. sphaeroides* thioredoxin may involve its oxidoreductase activity

To determine whether the biological role of thioredoxin involves the redox properties of its active site or redox-independent functions, we attempted to construct a *R. sphaeroides* mutant which expresses solely a thioredoxin impaired in its oxidoreductase activity, by using a gene replacement technique as described above. The Cys33 (the second cysteine of the active site which corresponds to Cys35 of *E. coli* thioredoxin) to Ser substitution was introduced by site-directed mutagenesis and the resulting mutant *trxA* gene was used to generate the mobilizable pUTC101 plasmid as described in Methods. In this construct, the Cys33 to Ser mutation would block the protein in reduced form and should therefore abolish the oxidoreductase activity of thioredoxin. pUTC101 was transferred to the Sm^R *R. sphaeroides* strain WSm and selection of exconjugants was performed as described above.

In situ hybridization analyses of 200 exconjugants with the Km resistance cassette or linearized pSUP203 vector as probes demonstrated that all the colonies isolated under aerobic or anaerobic dark conditions displayed a positive signal with both probes (data not shown), indicating that they had integrated the Tn903 gene by plasmid addition. In the control plasmid pUTC97, the Km resistance cartridge was inserted into the same *Bam*HI site as in pUTC101 (Fig. 1). Since the Km resistance cassette of pUTC97 had integrated by gene replacement at a frequency of 2.5% in aerobic selection and of 3.0% in anaerobic dark selection (data not shown), the lack of double cross-over among all the exconjugants resulting from pUTC101 transfer was not

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1 aggcgtgctgcttacgagcggcagcgtgcccagcctcagggccttctgacatggcttcagaccgaggtaggctcgaggt 80
81 gaagcggcag ATG GAG GCC GCG GGG CGC ATC CGG GTG ATG ACG GTG CAT GGC TCG AAA GGG 141
1 M E A A G R I R V M T V H G S K G 17
142 CTC GAG GCG CCC ATC GTG ATC CTG CCC GAC ACG GCC GAC CGC AAG CCG CGC AGC GAC GAG 201
18 L E A P I V I L P D T A D R K P R S D E 37
202 CTG TTC ACG TTG GAG GGG GCG GCG GTC TGG AAG ACG CCC GCC GAA AGC CCG CCG 261
38 L F T L E G G A R V W K T P A A E S P P 57
262 CAG ATT GCC GCC GCG GAG CGG CGG CGC GCA CGC GAT GCG GCC GAG AAC CTG CGG CTG 321
58 Q I A A A R E R R R A R D A A E N L R L 77
322 CTC TAT GTC GCC ATG ACG CGG GCC CAG TGC TGG CTG ATC GCG GCG GCG GCC GGC AAG CTC 381
78 L Y V A M T R A Q C W L I A A A A G K L 97
382 GAG ACG GGG GGC GCC TGG CAC GAC CTG ATC CGG CAG GGG ATG GAG GCC GCG GTG GCG AGC 441
98 E T G G A W H D L I R Q G M E A A V A S 117
442 GGC GTG ACG ACC GGT GCG CTG GTG CTC GAC GAA GGC CTC TGC CCC CGC CGC GCG CAG GCC 501
118 G V T T G A L V L D E G L C P R R A Q A 137
502 TCG GGC GCC GCC GCG CGG CCC GTC CTC CCG GGT GGG CTC TGG CCC GCG GCC GAC GTC CGG 561
138 S G A A A R P W L P A G L W P A A D V R 157
562 CGG CCC GAG GTC TGT CCC TCG GCG CTC GCG GGG GCC AAG CGC TGC CCG GCG ACG CGA TGC 621
158 R P E V C P S A L G G A K R C P A T R C 177
622 CCA CCG AGG AAG GCG TGG CGA TGG CTC GCC GTC CAC CGG CTG CTC GAG CAT CTG CCG CTC 681
178 P P R K A W R L A V H R L L E H L P L 197
682 CAT CCG CCC GAG GAG TGG GAG GCG GCG GCG GAG CTT CTG CCC GAC CTT CCC GAT GTG 741
198 H P P E E W E A A A R E L L P D L P D V 217
742 GCC CCC GTT CTG GCG GAG GCC CGG GCG GTG CTC TCG GCC GAA CCT CTG GCC GAG GCG CTG 801
218 A P V L A E A R A V L S A E P L A E A L 237
802 GTG CCG CAT GCG CTG CTC GAG GTG GCG GTG ACG GCC GAG GTG GCG GGG CGG CGG CTT TCC 861
238 V P H A L L E V A V T A E V A G R R L S 257
862 GGC ACC ATC GAC CGG CTG GTG GTG GAG CCC GAC CGG GTG CTG GCG GTC GAT TAC AAG ACG 921
258 G T I D R L V V E P D R V L A V D Y K T 277
922 AAC CGC GTG GTC CCG GAC CGT CCC GAA GAG GTG CCC GAG GGC ATC CTG CGG CAG ATG GGC 981
278 N R V V P P E E V P E G I L R Q M G 297
982 GCC TAT GCC GAG GCG CTG GCG CAG ATC TAC CCC GGA CGG ACG ATC GAG ACG GCG ATC CTC 1041
298 A Y A E A L A Q I Y P G R T I E T A I L 317
1042 TGG ACG CAG GCA GCC GCG CTG ATG GCG TGC CGC GCG ATA TCG TGA ggtcgcccttggtccggggc 1106
318 W T Q A A R L M R C R A I S * 332
1107 acgacaacttgacggcgccggggccatccttactttccaagcaagcctcaggtcaggagaattgaat 1176

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Fig. 2. Nucleotide and deduced amino acid sequence of ORF993, preceding the *trxA* gene in the chromosome of *R. sphaeroides*. The possible ribosome-binding site is indicated in bold type. The stop codon is indicated by an asterisk. The *Bgl*II restriction site in which the Km resistance cartridge was inserted in the construct pUTC86 (Fig. 1) is shown. The *trxA* coding region starts at position 1177.

due to physical constraint of homologous DNA flanking the cassette present in this construct. More likely, cells in which the Cys33 to Ser mutation has been introduced into the chromosomal *trxA* copy by gene replacement are not viable, suggesting that the physiological function of *R. sphaeroides* thioredoxin is redox-dependent.

***R. sphaeroides* thioredoxin has GSH-disulfide oxidoreductase activity**

GSH-disulfide oxidoreductase (or transhydrogenase) activity is an inherent property of glutaredoxin (Holmgren, 1979). *E. coli* contains three species of GSH transhydrogenase activity corresponding to Grx1, the first glutaredoxin discovered (Holmgren, 1979) and two other proteins that were identified more recently and therefore called Grx2 and Grx3 (Åslund *et al.*, 1994, 1996). To examine whether or not *R. sphaeroides* contains glutaredoxin-like system(s), we tested crude extracts from aerobic cultures of the *R. sphaeroides* WS8 wild-type strain in a GSH oxidoreductase assay. The results of these experiments are summarized in Table 2. *E. coli* Grx1 and Grx3 were used as positive controls and were found to have a specific activity of 106 and 156 U (mg protein)⁻¹, respectively. These results are in agreement with the values previously reported (Holmgren, 1979; Åslund *et al.*, 1994) and therefore argue for the validity of our experimental conditions.

Surprisingly, *R. sphaeroides* thioredoxin purified to homogeneity was demonstrated to have an inherent GSH transhydrogenase activity of 10 U (mg protein)⁻¹ (Table 2), whereas *E. coli* thioredoxin lacked such activity (Table 2) as previously reported (Holmgren, 1978). However, most of the total GSH-disulfide oxidoreductase activity in the crude extract of *R. sphaeroides* was apparently unrelated to thioredoxin (Table 2). Indeed, given that the intracellular amount of thioredoxin in *R. sphaeroides* was previously estimated to be 0.24 µg (mg protein)⁻¹ (Clément-Métal *et al.*, 1986), approximately 1.8% of the GSH transhydrogenase activity in a crude extract of *R. sphaeroides* can be attributed to thioredoxin. Analysis of heated (80 °C for 10 min) crude extracts after separation of heat-denatured proteins by centrifugation has shown that the specific GSH transhydrogenase activity of *R. sphaeroides* was enriched by approximately fivefold, while it was almost unchanged for *E. coli* (Table 2). These results indicate that the major species responsible for this activity in *R. sphaeroides* behave as heat-stable products (a property which is common to thioredoxin and Grx1), but that some of the species responsible for this activity in *E. coli* are denatured by heat. In addition, purified thioredoxin of *R. sphaeroides* submitted to this heat treatment retained all its GSH transhydrogenase activity (Table 2). The bacterial extracts previously heated were then submitted to an overnight dialysis

Table 2. Assay of GSH-disulfide oxidoreductase activity

Material	GSH-disulfide oxidoreductase activity [U (mg protein) ⁻¹]*
Purified protein†	
<i>E. coli</i> Grx1	106
<i>E. coli</i> Grx3	156
<i>E. coli</i> Trx	0
<i>E. coli</i> Trx‡	0
<i>E. coli</i> Trx§	0
<i>R. sphaeroides</i> Trx	10
<i>R. sphaeroides</i> Trx‡	10
<i>R. sphaeroides</i> Trx§	0
Crude extract	
From <i>E. coli</i>	
1¶	1.5
2‡	1.42
3‡§	0.58
From <i>R. sphaeroides</i>	
1¶	0.133
2‡	0.633
3‡§	0.094

* GSH transhydrogenase activity was determined as described in Methods. In the assays, three different concentrations of proteins or extracts were used and all experiments were performed at least twice.

† Grx, glutaredoxin; Trx, thioredoxin.

‡ Crude extracts or purified proteins were heated to 80 °C for 10 min. In crude extracts the resulting precipitate was removed by centrifugation to give a clear supernatant.

§ Crude extracts or purified proteins were dialysed overnight against 25 mM Tris/HCl pH 8, 5 mM EDTA, 15 mM Na₂S₂O₃, 50 mM NaCl, so that compounds of molecular mass equal to or smaller than 12–14 kDa were removed.

|| The thioredoxin of *R. sphaeroides* was purified as described in Methods.

¶ Crude extracts were prepared as described in Methods.

(which removes components of molecular mass equal to or less than 12–14 kDa). The resulting samples retained approximately 15% of the specific GSH transhydrogenase activity for *R. sphaeroides* and 40% for *E. coli* (Table 2). In control experiments, purified thioredoxin of *R. sphaeroides* (10.8 kDa) and *E. coli* (11.7 kDa) were submitted to the same dialysis treatment. Determination of the protein concentrations of the resulting samples (data not shown) indicated that almost all of the thioredoxin protein from both microorganisms is removed through dialysis and no more GSH-disulfide oxidoreductase activity was detected for the dialysed sample of *R. sphaeroides* thioredoxin (Table 2). These results provide evidence that most of the components with a molecular mass less than 12–14 kDa were also removed from the cell-free extracts after dialysis. The remaining (40%) GSH transhydrogenase activity in *E. coli* after dialysis indicates

that the major species responsible for GSH-disulfide oxidoreductase activity has a molecular mass greater than 14 kDa. This finding is consistent with the recent identification of an atypical glutaredoxin in *E. coli*, Grx2 (27 kDa) with a relative contribution to the GSH transhydrogenase activity of 81.4% (Åslund *et al.*, 1994). In contrast, 85% of the specific activity in *R. sphaeroides* was lost after dialysis, indicating that most of the GSH transhydrogenase activity is due to species with a molecular mass less than 12–14 kDa.

DISCUSSION

To investigate the contribution of thioredoxin to the regulation of photosynthetic metabolism, we attempted to construct a *R. sphaeroides* TrxA mutant by gene replacement. From a series of experiments which examined integration of the Tn903 Km resistance gene into different sites of the *trx*A region, we have demonstrated that disruption of the unique chromosomal *trx*A copy with a Km resistance cartridge is a lethal mutation. Thus, although dispensable in *E. coli* (Russel & Model, 1984), thioredoxin is absolutely required for both aerobic and anaerobic respiration of the facultative photosynthetic bacterium *R. sphaeroides*.

To examine whether the mechanism of thioredoxin action is redox-dependent, we attempted to produce a mutant strain which expresses a thioredoxin altered in its protein-disulfide reductase activity due to a Cys33 to Ser substitution. This experiment showed that such a mutant is also not viable, suggesting that the biological involvement of *R. sphaeroides* thioredoxin requires its redox properties. The lack of double cross-over in the DNA region downstream of the Cys33 codon after conjugative transfer of the pUTC101 construct (which would generate a wild-type *trx*A chromosomal copy followed by Km resistance cassette insertion into the downstream *Bam*HI site as observed for 2.5–3.0% of exconjugants provided by transfer of the pUTC97 control construct) is most probably due to the length of the sequence between the Cys33 codon and the *Bam*HI site of Km insertion (only 300 bp), for which the gene exchange frequency is expected to be very low.

In addition, the fact that insertion of the Km resistance cassette into the *Bgl*II site at 174 bp upstream of the *trx*A coding region (pUTC86 construct; Fig. 1) was obtained by gene exchange only after selection under anaerobic dark conditions (with a frequency of 8.1%) but not after selection under aerobic conditions suggests that this region is essential for aerobic metabolism but not for anaerobic metabolic pathways using DMSO as terminal electron acceptor. Since the regulatory sequences required for oxygen-regulated expression of *trx*A are localized downstream of the *Bgl*II site (Pasternak *et al.*, 1996) and since a construct containing only 100 bp upstream of *trx*A coding region is sufficient to replace *E. coli* thioredoxin for *in vivo* hydrogen donor function for methionine-sulfoxide reductase (data not shown), it is unlikely that insertion of the Km resistance cassette into *Bgl*II would interrupt *cis*-acting regulatory sequences

involved in oxygen regulation of *trxA* gene expression. More likely, the insertion of the Km resistance gene into the *Bgl*III site could disrupt another gene encoding a product essential for aerobic respiration but not for anaerobic respiration. Indeed, an ORF (ORF993) which flanks the Km resistance cassette in the pUTC86 construct has been identified, but this shows no significant homology to any known genes and its function remains to be determined.

In other respects, *R. sphaeroides* thioredoxin purified to homogeneity was shown to have an inherent GSH-disulfide oxidoreductase activity, which is a feature of glutaredoxins (Holmgren 1979; Åslund *et al.*, 1994). This unexpected property of *R. sphaeroides* thioredoxin differentiates it from *E. coli* thioredoxin which lacks such activity (Holmgren, 1978) and may indicate that *R. sphaeroides* thioredoxin has some function(s) that it does not have in *E. coli* (i.e. participation in GSH-dependent processes).

In *E. coli*, the viability of the double mutant lacking thioredoxin and Grx1 (Russel *et al.*, 1990; Miranda-Vizuete *et al.*, 1994) provided evidence of a functional redundancy of the enzyme systems for reduction of the ribonucleotide reductase, and the recently discovered Grx3 is likely to be an alternative hydrogen donor in the ribonucleotide reduction reaction in the double mutant (Åslund *et al.*, 1994). The apparent level of total GSH oxidoreductase activity is 10-fold lower in *R. sphaeroides* than in *E. coli* (Table 2) and is exclusively due to small species having a molecular mass lower than 14 kDa, while in *E. coli*, about 40% of the total activity involves species with a molecular mass greater than 14 kDa. These results suggest a lower abundance of hypothetical alternative redox activity such as glutaredoxin systems in *R. sphaeroides*. However, most of the GSH transhydrogenase activity of *R. sphaeroides* is not related to thioredoxin, suggesting that additional glutaredoxin-like systems are also present in *R. sphaeroides*. *In vitro*, thioredoxin and glutaredoxins serve as hydrogen donors for ribonucleotide reductase (Gleason & Holmgren, 1988), which is essential for DNA synthesis. Thus, thioredoxin may be essential in *R. sphaeroides* for deoxyribonucleotide synthesis. Given the reactivity of *R. sphaeroides* thioredoxin with the GSH-system as shown by our experiment (Table 2), the thioredoxin may use either thioredoxin reductase or GSH as reduction pathways to couple oxidation of NADPH to reduction of ribonucleotides in *R. sphaeroides*.

In other respects, by its general disulfide oxidoreductase activity, thioredoxin may also be essential for keeping the cysteines of cytoplasmic proteins reduced, as a control mechanism of enzyme activity like the thiol redox control exercised by thioredoxin on chloroplast enzymes of the Calvin cycle (Cséke & Buchanan, 1986).

While the present data do not allow us to define precisely the physiological role of *R. sphaeroides* thioredoxin, they suggest several hypotheses. First, the absolute requirement of thioredoxin in oxidative metabolism of *R. sphaeroides* may reflect its implication in defence

against oxidative stress, by acting in regeneration of proteins oxidatively inactivated and/or by its cellular antioxidant role as has been previously shown in other organisms. Selection of exconjugants under aerobic conditions in the presence of 1 mM DTT did not allow us to isolate double recombinants (data not shown), indicating that the essential function of *R. sphaeroides* thioredoxin cannot be replaced by a low-molecular-mass reducing agent.

During anaerobic dark growth, DMSO causes oxidation of methionine residues in methionine sulfoxide (Brot & Weissbach, 1982). In *E. coli*, such damage can be repaired by methionine-sulfoxide reductase (Moskovitz *et al.*, 1995) for which the physiological hydrogen donor is thioredoxin (Russel & Model, 1986). Thus, the lethality of *R. sphaeroides* TrxA⁻ mutants when selected under anaerobic dark conditions suggests that thioredoxin may also be the *in vivo* hydrogen donor for methionine-sulfoxide reductase in *R. sphaeroides*. In other respects, based on the previous hypothesis that *R. sphaeroides* thioredoxin may be the *in vivo* regulator of aminolaevulinic acid (ALA) synthase activity by a thiol redox control (Clément-Métal *et al.*, 1986), the thioredoxin negative mutants could be auxotrophic for ALA. Given that *R. capsulatus* mutant strains auxotrophic for ALA are not viable under anaerobic growth conditions in the presence of DMSO (Yen & Marrs, 1977), we attempted to isolate the thioredoxin-deficient mutant under anaerobic dark conditions with DMSO in the presence of 1 mM ALA (data not shown). These experiments provided the same results as those obtained for selection without supplementation with ALA, indicating that the essential function of *R. sphaeroides* thioredoxin is not related to the activation of ALA synthase.

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