A new member of membrane-anchored periplasmic thioredoxin-like proteins was identified in *Bradyrhizobium japonicum*. It is the product of *cycY*, the last gene in a cluster of cytochrome *c* biogenesis genes. Muta
tional analysis revealed that *cycY* is essential for the biosynthesis of all *c*-type cytochromes in this bacterium. The CycY protein was shown to be exported to the periplasm by its N-terminal signal sequence-like do
main. Results from Western blot analyses of membrane and soluble fractions indicated that the CycY protein remains bound to the membrane. A soluble version of the protein devoid of its N-terminal membrane anchor (CycY*) was expressed in *Escherichia coli* and purified to homogeneity from the periplasmic fraction. The protein showed redox reactivity and properties similar to other thioredoxins such as fluorescence quenching in the oxidized form. Its equilibrium constant with glutathione was determined to be 168 mM, from which a standard redox potential of $-0.217$ V was calculated, suggesting that CycY might act as a reductant in the otherwise oxidative environment of the periplasm. This is in agreement with our hypothesis that CycY is re
quired, directly or indirectly, for the reduction of the heme-binding site cysteines in the CXXCH motif of *c*-type apocytochromes before heme attachment occurs.

A key step in the post-translational maturation of *c*-type cytochromes is the covalent ligation of the heme cofactor to the reduced apocytochrome (1). In bacteria, this reaction occurs in the periplasm in a rather oxidative environment. Thus, a re
ductive step in the cytochrome *c* maturation pathway may be postulated that would ensure that the cysteines in the heme-binding site motif (CXXCH) of the apocytochrome are in the reduced dithiol form before heme is attached. Bacterial cytochrome *c* biosynthesis is known to depend on the products of at least nine different genes. These encode an ABC transporter, a putative cytochrome-*c*-heme lyase complex, and a thioredoxin-like protein (1–7). The gene for the latter has been identified in *Rhodobacter capsulatus* (helX) (8) and *Rhizobium leguminosarum* (*cycY*) (9) because of the presence of two conserved redox-active cysteines in the derived amino acid sequence that are typically present in thioredoxins. Mutations in these genes were found to affect the maturation of *c*-type cytochromes. In contrast to conventional cytoplasmic thiore
oxins, the predicted HelX and CycY polypeptides carry an N-terminal extension resembling a typical signal sequence. A topological analysis with the *R. capsulatus* HelX protein, using alkaline phosphatase (PhoA) fusions, suggested its periplasmic location. It was postulated that HelX might function as a periplasmic reductant of the heme-binding site in apocytochromes (10).

In the soybean root nodule symbiont *Bradyrhizobium japoni
cum*, an open reading frame (*orf194*) with a high degree of similarity to *helX* and *cycY* has been identified downstream of the cytochrome *c* biogenesis gene *cycX* (11). A transposon (Tn5) insertion detaching the last eight codons of the gene led to a partial loss of cytochrome *c* biosynthesis, suggesting that the gene was essential for this process. Surprisingly, however, a kanamycin resistance cassette insertion (mutant 98) disrupting the gene in the presumptive fourth codon had no effect on cytochrome *c* biogenesis. It was concluded that the gene started downstream of the site of the cassette insertion in mutant 98. The next possible start codon, a TTG at position 3216 in the published nucleotide sequence, was therefore predicted to rep
resent the start of the gene, which was therefore called *orf132* (11). However, the *orf132* product lacked the hydrophobic N-terminal domain present in HelX and CycY. Unfortunately, a true null mutant of this gene was not obtained.

Intrigued by the high degree of similarity of *B. japonicum* *orf132* to genes for other bacterial thioredoxin-like proteins, we further investigated its function. Here we present an extensive molecular analysis of this gene, now called *cycY*, which shows unequivocally that it codes for a membrane-anchored periplasmic thioredoxin and is essential for cytochrome *c* maturation. We overexpressed and purified a periplasmic soluble variant of the protein (CycY*) from *Escherichia coli* and determined its redox potential. This allowed us to make several important predictions with respect to the role of this peculiar thioredoxin in cytochrome *c* maturation.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions—** *B. japonicum* 110spc4 (12) is called the wild type throughout this work. *E. coli* DH5α (13) was used as a host for DNA cloning. *E. coli* S17-1 (14) was used as donor strain in bacterial conjugation experiments. *E. coli* BL21(DE3) (15) was used for overexpression of CycY* from the phage T7 promoter. *B. japonicum* strains were grown aerobically at 28 °C in PSY medium (12) or anaerobically in YEM medium supplemented with 10 mM KNO₃ (16). Antibiotics were added at the following final concentrations: kanamy
cin, 100 μg/ml; spectinomycin, 100 μg/ml; tetracycline, 60 μg/ml; and chloramphenicol, 10 μg/ml. *E. coli* cells were grown at 30 or 37 °C in LB medium to which antibiotics were added at the following concentra-

---

* This work was supported by grants from the Swiss National Foundation for Scientific Research and the Federal Institute of Technology, Zürich (to H. H. and L. T.-M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

† To whom correspondence should be addressed: Mikrobiologisches Inst., Eidgenössische Technische Hochschule, ETH-Zentrum, Schmelz
bergstrasse 7, CH-8092 Zürich, Switzerland. Tel.: 41-1-632-44-19; Fax: 41-1-632-11-48; E-mail: lthoeny@micro.biol.ethz.ch.

‡ From the Mikrobiologisches Institut and the Institut für Molekularbiologie und Biophysik, Eidgenössische Technische Hochschule, CH-8092 Zürich, Switzerland.

§ From the Institut für Molekularbiologie und Biophysik, Eidgenössische Technische Hochschule, CH-8092 Zürich, Switzerland.

¶ To whom correspondence should be addressed: Mikrobiologisches Inst., Eidgenössische Technische Hochschule, ETH-Zentrum, Schmelz
bergstrasse 7, CH-8092 Zürich, Switzerland. Tel.: 41-1-632-44-19; Fax: 41-1-632-11-48; E-mail: lthoeny@micro.biol.ethz.ch.

This paper is available on line at http://www-jbc.stanford.edu/jbc/
Thioredoxin-like Protein CycY for Cytochrome c Maturation

...ions: ampicillin, 100–200 μg/ml; tetracycline, 10 μg/ml; and carbeni-
cillin, 400 μg/ml.

Recombinant DNA Work—Molecular cloning procedures were car-
rried out according to Sambrook et al. (17). DNA sequence analysis was performed using the chain termination method and the equipment for automatic DNA sequencing (Sequenase version 2.0 model 370A and fluorescent dye terminators, Applied Biosystems, Inc., Foster City, CA).

Plasmid and Mutant Constructions—The lacZ gene of pNM480X (18) was fused in frame to the sixth codon of cycY at a SalI site, resulting in plasmid pRJ2801. The lacZ-containing plasmid pNM482 (19) was mod-
ed first by inserting an XhoI linker (5′-CGGCTGAGGCGG-3′); from Pro-
metabolic (Switzerland) into the unique Stul site, and sub-
sequently, the lacZ gene was fused in frame to the sixthtieth codon of cycY at an XhoI site, which gave plasmid pRJ2802. The phoA gene lacking the coding region for its own signal sequence was fused to the sixthtieth codon of cycY by inserting the 2.9-kb PsI fragment of pCH40 (20), yielding pRJ2806. All fusions contained the entire cycY gene (21). DNA sequence was confirmed to be in frame by sequencing of the fusion site. The plasmids were subsequently transferred into the broad host range plasmid pRK290 (21), giving plasmids pRJ2803 (cycYlacZ), pRJ2804 (cycYlacZ), and pRJ2757 (cycYlacZ; phoA). These plasmids were conjugated into B. japonicum wild type. The cycYphoA fusion was created by digesting the 3′-end of cycY with Bal-31 nuclease and by inserting a PsI linker at codon 127 plus the 2.9-kb PsI fragment of pCMC1 (22). The resulting plasmid pRJ2808, whose 3.7-kb XhoI fragment was subcloned into the suicide plasmid pSUP202X (52) and cointegrated into the B. japonicum wild-type chromosome after conjugation.

The cycY mutant was constructed by first inserting the 1.3-kb Smal fragment containing the kanamycin cassette of pUC4-KIX into the blunt-end cycY internal SalI site and subsequently subcloning the disrupted gene into pSUP202 (14). After conjugation, the mutant was obtained by double crossover events, yielding strain BJ2746.

To generate a CycY antigen for antibody production, a plasmid encod-
ing a soluble version of the protein with a C-terminal His tag was created by digesting the 3′-end of cycY with Bal-31 nuclease and by inserting a PsI linker at codon 127 plus the 2.9-kb PsI fragment of pCMC1 (22). The resulting plasmid pRJ2808, whose 3.7-kb XhoI fragment was subcloned into the suicide plasmid pSUP202X (52) and cointegrated into the B. japonicum wild-type chromosome after conjugation.

The CycY* mutant was constructed by inserting the 3.1-kb cycY fragment containing the kanamycin cassette of pUC4-KIX into the blunt-end cycY internal SalI site and subsequently subcloning the disrupted gene into pSUP202 (14). After conjugation, the mutant was obtained by double crossover events, yielding strain BJ2746.

To generate a CycY antigen for antibody production, a plasmid encod-
ing a soluble version of the protein with a C-terminal His tag was created. For this purpose, a DNA fragment was amplified by poly-
merase chain reaction that contained an NcoI site at codon 38 of cycY and six additional histidine codons at the end of the gene. The fragment was cloned using the following primers: primer 1, 5′-GCATCCAT- GGATCTTCCGCGTTACCTCCTG-3′; and primer 2, 5′-GCAGATG-GAGAAAAGGCCTGAGCACCACAACACACCAACCACTAGATGATCCGG-3′. The polymerase chain reaction product was cloned as an NcoI-BamHI fragment into pUCBM21 (Boehringer, Mannheim, Germany) and sequen-
ced. From this plasmid (pRJ2760), the NcoI-BamHI fragment was cloned into pET22b (Novagen, Madison, WI), resulting in pRJ2762. On the other hand, the NcoI-BamHI fragment was ligated to the N-terminal fusion of the signal sequence from the Erwinia carotovora PeB protein with the soluble part of cycY (residues 38–194) carrying a C-terminal His tag. This protein variant was called CycYlacZp. A similar CycYlacZp version with the original B. japonicum-derived N-terminal hydrophilic CycY segment instead of the PeB signal sequence was encoded by plasmid pRJ2764. This plasmid was constructed by cloning the 0.44-kb XhoI-EcoRI fragment from pRJ2762 (cycYlacZp) into pRJ2764 (23), yielding pRJ2765. Subsequently, the adjacent 3.1-kb wild-type XhoI fragment containing the DNA up-
stream of the cycY internal XhoI site was cloned into pRJ2763, resulting in pRJ2764. This plasmid was conjugated into mutant BJ2746 and tested for complementation.

CycY*, the soluble form of CycY lacking the C-terminal His tag, is encoded by plasmid pRJ2766, in which the XhoI fragment of pRJ2762 (containing codons 61–194 plus the extra histidine codons; see above) was replaced by the corresponding 471-base pair wild-type XhoI frag-
ment. The correct orientation of the insert and the intact fusion site were confirmed by sequencing.

Expression and Purification of CycY*—E. coli BL21(DE3) cells were transformed with the expression plasmid pRJ2766 and grown in 10 liters of LB medium containing ampicillin (200 μg/ml) at 30 °C for 36 h. (Induction of gene expression by isopropyl-β-D-thiogalactopyranoside was not required for efficient expression of CycY* under these condi-
tions.) The cells were harvested by centrifugation at 5000 × g and washed in cold buffer A (10 mM MOPS/NaOH, 150 mM NaCl, and 5 mM EDTA, pH 7.0). After centrifugation at 12,000 × g for 10 min, the cell pellet was suspended in cold extraction buffer (buffer A with 1 mM sodium polyoxymethylene B sulfate (Sigma), 2 mg/l of cells, wet weight). The suspend-

The abbreviations used are: kb, kilobase pair(s); MOPS, 3-(N-mor-
pholino)propanesulfonic acid; PAGE, polyacrylamide gel electrophore-
sis; HPLC, high performance liquid chromatography; DTT, 1,4-dithio-
atrithreitol.
Thioredoxin-like Protein CycY for Cytochrome c Maturation

4469

...the true start of the gene downstream of the thioredoxin-like motif are in boldface letters. The hydrophobic domain that functions as a membrane anchor and periplasmic target sequence is underlined. The consequence of the insertion in mutant 98, an altered N-terminal sequence, is shown at amino acid 4. The open arrowheads designate the sites of translational lacZ and phoA fusions. The closed arrowhead marks the position of the pelB'-cycY fusion that was constructed to overexpress a soluble version of the protein (CycY*). The vertical arrow indicates the site of the kanamycin resistance cassette insertion in mutant B2746. The asterisk marks the previously described start of orf132 (11).

The rate of the reaction between CycY* and DTT was determined by recording the change in fluorescence intensity at 335 nm at an excitation wavelength of 295 nm. Ten μl of different DTT stock solutions (1 mM to 1 M) were added to 1 ml of oxidized CycY* (1 μM) in degassed 100 mM potassium phosphate, pH 7.0, and 2 mM EDTA, and the change in fluorescence intensity was recorded for 15 min. The apparent second-order rate constant of CycY* reduction was determined using the measured pseudo first-order rate constant.

Production of CycYN*-Antigen and Antiserum—E. coli BL21(DE3) cells were transformed with the expression plasmid pRJ7682 and grown in 2 liters of LB medium at 37 °C to an absorbance (550 nm) of 0.5. After induction by isopropyl-β-D-thiogalactopyranoside (final concentration: 0.2 mM) the cells were grown at 37 °C for 2 h. The cells were harvested by centrifugation and washed in cold buffer C (10 mM Tris-HCl, pH 7.9, 150 mM NaCl, and 5 mM EDTA). The preparation of the periplasmic extract was done as described above, except that buffer D (buffer C with 1 mg/ml polymyxin B sulfate, 2 ml/g of cells, wet weight) was used. The purification by nickel affinity chromatography (His-Bind metal chelation resin, Novagen) was performed according to the instructions of the manufacturer. The purified fraction was analyzed by SDS-PAGE, and the protein was stained with 0.2% Coomassie Brilliant Blue R-250. The gel piece containing the pure protein was excised from SDS-PAGE, and the protein was stained with 0.01% Coomassie Blue R-250. The gelpiece containing the pure protein was excised and used to immunize a New Zealand White rabbit. The antiserum was measured using the Bio-Rad assay with bovine...
Expression of the B. japonicum cycY gene and subcellular localization of its product

TABLE I

| Fusion*          | β-Galactosidase activity,† whole cells | Alkaline phosphatase activity |
|------------------|----------------------------------------|------------------------------|
| cycY<sup>a</sup>-lacZ | 101 ± 3                                | ND                           |
| cycY<sup>a</sup>-phoA | 13.5 ± 0.5                             | ND                           |
| cycA<sup>a</sup>-phoA | ND                                     | ND                           |
| No fusion        | 6.6 ± 0.3                              | 7.7 ± 0.7                    |

a The amino acid at which the CycY and CycA proteins are fused to the reporter proteins is indicated by a subscript.

† Miller units; values are means of duplicate measurements from four independent cultures.

‡ 10<sup>−3</sup>Δ<sub>OD<sub>590</sub></sub> min<sup>−1</sup> OD<sub>590</sub>; values are means of duplicates from three independent cultures.

§ mol of 4-nitrophenol min<sup>−1</sup> mg<sup>−1</sup>; values are means of four to six measurements.

ND, not determined.

Fig. 2. Analysis of e-type cytochromes in B. japonicum strains. A, heme stain of proteins from membranes (lanes 1 and 2) and soluble fractions (lanes 3 and 4) of the wild type (lanes 1 and 3) and the cycY<sup>a</sup>-mutant (lanes 2 and 4) separated by SDS-PAGE (15%). B, Western blot of proteins from the soluble fractions of the aerobically grown wild type and the cycY<sup>a</sup>-mutant using a cytochrome <i>c</i><sub>550</sub>-specific antisera.

Fig. 3. Identification of CycY as a membrane-bound protein. A Western blot of membrane and soluble fractions is shown. Each lane contains 50 μg of protein from the wild type (+ lanes), cycY<sup>a</sup>-mutant Bj2746 (− lanes), or mutant Bj2746 plus pRJ2764 (cycY<sup>a</sup> complemented with cycY<sub>mut</sub>) (H lanes). The samples were separated by SDS-PAGE (15%), blotted onto a nitrocellulose membrane, and probed with anti-CycY<sub>mut</sub>* polyclonal serum.

Characterization of Redox Properties—CycY<sup>a</sup>* contains two cysteine residues separated by only two amino acids in the proposed active site (CVCPC) (Fig. 1). We investigated the protein by SDS-PAGE under reducing and nonreducing conditions. Fig. 4B shows that the oxidized form of CycY<sup>a</sup>* migrated slightly faster on the gel than the reduced form. This confirmed that the two cysteine residues are capable of forming a disulfide bond.

It is known that many proteins of the thiol-disulfide oxidoreductase family such as thioredoxin and DsbA of <i>E. coli</i> (34, 35) or TpA of <i>B. japonicum</i> (26) show an increase in fluorescence intensity upon reduction of their active-site cystines. Therefore, the fluorescence spectra of oxidized and reduced CycY<sup>a</sup>* were compared. When oxidized CycY<sup>a</sup>* was excited at 295 nm (selective excitation of tryptophan residues), the addition of 1 mM DTT led to a 2.3-fold increase in the fluorescence intensity and to a shift of the emission maximum from 327 to 332 nm (Fig. 5A). We assume that, like in thioredoxin, the fluorescence of at least one of the two homologous tryptophans adjacent to the cysteines is quenched by the disulfide bond (Fig. 1). In the presence of 7 M guanidinium chloride, almost identical spectra for unfolded oxidized and unfolded reduced CycY<sup>a</sup>* were obtained with emission maxima of 354 nm (Fig. 5A), which are typical for denatured proteins. The minor difference in fluorescence intensity between unfolded oxidized and unfolded reduced CycY<sup>a</sup>* may reflect the fact that the quenched tryptophan residues are located in the immediate vicinity of the active-site.
disulfide in the primary sequence of the protein.

Using the fluorescence properties of CycY*, the CycY*/glutathione redox equilibrium constant $K_{eq}$ was determined, which is given by Equations 6 and 7.

$$\text{CycY}^* \text{SH} + \text{GSSG} \rightleftharpoons \text{CycY}^* \text{S} + 2 \text{GSH} \quad \text{(Eq. 6)}$$

$$K_{eq} = \frac{[\text{CycY}^* \text{S}][\text{GSH}^2]}{[\text{CycY}^* \text{SH}][\text{GSSG}]^{2/3}} \quad \text{(Eq. 7)}$$

Oxidized CycY* was incubated in the presence of 10 mM GSSG and increasing concentrations of GSH. The fraction of reduced CycY* at equilibrium was measured by the intrinsic fluorescence of oxidized and reduced CycY*. Purified CycY* was applied to the SDS gel either with (+ lanes) or without (- lane) the addition of 2-mercaptoethanol (2-ME) to the sample. The reduced (red) and oxidized (ox) forms of CycY* are indicated.

Fig. 4. Purification of CycY*. A, steps during purification of CycY*, analyzed by SDS-PAGE (15% separating gel) and silver staining. Lane 1, whole cell extract from E. coli cells expressing pBJ2766; lane 2, periplasmic extract; lane 3, pooled fractions after chromatography on DE52- and CM52-cellulose; lane 4, purified CycY* after chromatography on phenyl-Sepharose. B, SDS-PAGE (15%) analysis of the oxidized and reduced forms of CycY*. Purified CycY* was applied to the SDS gel either with (+ lanes) or without (- lane) the addition of 2-mercaptoethanol (2-ME) to the sample. The reduced (red) and oxidized (ox) forms of CycY* are indicated.

The redox potential of CycY* is closer to that of cytoplasmic thioredoxins ($-0.23$ to $-0.27$ V) (37–39) than to that of eukaryotic protein disulfide-isomerase ($-0.11$ V) (40) and DsbA ($-0.124$ V) (35), indicating that CycY* is a rather reducing enzyme. (For direct comparison, a redox potential of $-0.240$ V was used for the glutathione redox potential, and the published value for DsbA was recalculated with that value.) The redox mixtures had to be incubated for at least 3 days to reach equilibrium, which is much longer than in the case of DsbA (35) or TlpA ($< 20$ h) (26).

To measure the CycY*/glutathione equilibrium by an independent method, it was quenched by acid. Oxidized CycY*, reduced CycY*, and CycY*/glutathione mixed disulfides were separated by reversed-phase HPLC. Quantification of the peak areas allowed determination of the equilibrium constants $K_1$ and $K_2$, whose product corresponds to $K_{eq}$ (Equation 2). Under any redox conditions, the fraction of the CycY*/glutathione mixed disulfide was below 16%. The values for $K_1$, $K_2$, and $K_{eq}$ are $3.8$ and $4.7 \times 10^{-2}$ M and $0.182$ M, respectively, the latter of which is in good agreement with the value of $K_{eq}$ obtained by fluorescence spectrometry.

**DISCUSSION**

The results of this work show that *B. japonicum* contains a cytochrome c biogenesis gene that codes for a periplasmically oriented membrane-anchored protein with a thioredoxin-like function. The sequence of cycY has been published before (11); however, at that time, it was not clear where the translation of the open reading frame started and whether or not the gene was involved in maturation of c-type cytochromes. The results presented here have clarified both points. First, the lacZ and phoA fusion analyses unambiguously showed that the hydrophobic segment at the N terminus, encoded by what was previously called orf194 (11), is translated and thus part of the protein. However, the identity of orf194 as the bona fide cycY gene was previously questioned due to the inconspicuous wild-type phenotype of *B. japonicum* mutant 98, which carries a kanamycin resistance cassette inserted between codons 4 and 5. How can this be explained? We sequenced the border fragments of the pUC4-KIXX-derived kanamycin cassette and found that by inserting the XhoI fragment of the cassette into the SauI site overlapping codons 4 and 5 of cycY, an in-frame fusion of cycY to a sequence encoding a newly created N-terminal tripeptide (MRI) is obtained (Fig. 1). Interestingly, the ATG start codon of this tripeptide is preceded by the sequence AAG at a distance of nine nucleotides, which might serve as a ribosome-binding site (data not shown). Thus, assuming there is promoter activity within the kanamycin cassette, the mutant might express an almost normal CycY protein in which only the first four amino acids (MSEQ) are replaced by MRI (Fig. 1). Hence, strain 98 might produce an artificial CycY protein that is normally exported and functional, leading to a wild-type phenotype.

**FIG. 5. Redox properties of CycY***. A, fluorescence emission spectra of oxidized and reduced CycY* under native and denaturing conditions. Spectra were recorded at protein concentrations of 1 mM in 100 mM sodium phosphate, pH 7.0, and 1 mM EDTA. Samples of reduced CycY* additionally contained 1 mM DTT, and samples of unfolded CycY* contained 7 mM guanidinium chloride. Fluorescence spectra of native oxidized (open circles), native DTT-reduced (open squares), unfolded oxidized (closed circles), and unfolded reduced (closed squares) CycY* are shown. The excitation wavelength was 295 nm. B, redox equilibrium of CycY* with glutathione. The relative amount of reduced CycY* at equilibrium (R) was measured using the specific CycY* fluorescence at 335 nm (excitation at 295 nm). Oxidized CycY* (1 mM) was incubated for 3 days at 30 °C in 100 mM sodium phosphate, pH 7.0, and 1 mM EDTA. Open and closed circles show the results from two independent experiments.
Some mutations in *B. japonicum* cytochrome c biogenesis genes lead to small colonies on plates, probably because (micro)aerobic respiration is affected when c-type cytochromes are not synthesized (43). We now succeeded in constructing a true cycY* mutant by screening for small colonies after conjugation. The slow-growing exconjugants proved to be the correct mutants. Therefore, although cycY is important, it is not essential for (micro)aerobic growth of *B. japonicum* due to the presence of an alternative quinol oxidase (44). However, anaerobic growth with nitrate, which depends on certain c-type cytochromes such as cytochrome *c*$_{550}$ (45), was affected. The absence of all c-type cytochromes in the cycY* mutant indicated that a general step of the maturation pathway was affected by the mutation.

The subcellular localization of the CycY protein was investigated by two different approaches. The result with the cycY*-phoA fusion concurred very well with the direct immunological localization of the protein to the membrane fraction. This is a new aspect in view of some indirect evidence for the periplasmic location of the *R. capsulatus* CycY homologue, HelX. *helX*-phoA fusions were expressed in *E. coli* and gave high alkaline phosphatase activities in the periplasmic fraction, suggesting that the hybrid protein was cleaved after translocation (8). Indeed, several putative signal sequence cleavage sites were identified in the HelX presequence. Nevertheless, there is no direct evidence for the precise subcellular location of HelX in *R. capsulatus*. In the N-terminal leader sequence of the *B. japonicum* CycY protein, the sequence GSG (positions 36–38; see Fig. 1) following a hydrophobic stretch of 19 amino acids might serve as a signal peptidase cleavage site. However, our results support the idea that CycY is anchored in the cytoplasmic membrane and faces the periplasmic space, which is reminiscent of another membrane-bound thioredoxin-like protein identified in this organism, TlpA (28). The latter is necessary for the biosynthesis of the aa$_{3}$-type cytochrome oxidase.

What might be the biological function of the CycY protein? Its resemblance to thioredoxins suggests that it is a periplasmic protein thiol-disulfide oxidoreductase. Knowing that CycY is required for one of the steps in the cytochrome c maturation pathway, it is tempting to speculate that its function is to keep the cysteine residues in the heme-binding motif of apocytomnes c reduced before heme is attached. This hypothesis predicts that CycY is a reductant, with a possible intramolecular disulfide in apocytomne c being the target. CycY*, the soluble version of CycY expressed in *E. coli*, was tested for its redox reactivity, and its redox potential was determined. The only two cysteines of the polypeptide are located in the WCVPC motif (Fig. 1) that presumably forms the active site and is thus reminiscent to that of *E. coli* thioredoxin (WCGPC), supporting the view that CycY is a thioredoxin-like, redox-active enzyme.

The spectroscopic characterization of CycY* also revealed strong similarities to thioredoxin. The fluorescence of CycY increased by a factor of 2.3 upon reduction of its disulfide, compared with a 1.3-fold increase in fluorescence observed for the reduction of thioredoxin (34). These fluorescence properties of CycY* were used to determine its intrinsic redox potential. The obtained value of $-0.217$ V is similar to that of *E. coli* glutaredoxin, a protein that complements thioredoxin deficiency in ribonucleotide synthesis, and is closer to that of the reductant thioredoxin ($-0.23$ to $-0.27$ V) than to that of the oxidant DsbA and protein disulfide-isomerase ($-0.124$ and $-0.11$ V, respectively). This indicates that CycY* may indeed act as a reductant in the otherwise oxidizing environment of the periplasm. The determination of the equilibrium constant between CycY* and glutathione by fluorescence measurements was based on the assumption that the population of CycY*/glutathione mixed disulfides at equilibrium was negligible.

This was proven by HPLC analysis of acid-quenched equilibrium mixtures. Since the fraction of the mixed disulfides was <16% even under oxidizing conditions, the fluorescence analysis yields a reliable value for $K_{eq}$. This result was confirmed independently by evaluating the HPLC elution profiles under different redox conditions, which also allowed the determination of the “microscopic” equilibrium constants $K_1$ and $K_2$ (see Equations 4 and 5).

In conclusion, there is now genetic and biochemical evidence for CycY being a membrane-bound thioredoxin involved in a redox reaction required for cytochrome c maturation in the periplasm. However, the natural substrate of CycY, which could be apocytomne c, heme, or yet another periplasmic molecule that is required for cytochrome c maturation, has not yet been identified. Furthermore, we do not know how CycY, once oxidized in the course of a reaction cycle, becomes recycled as a reductant in the oxidative environment of the periplasm. In this context, it is interesting to note that another cytochrome c biogenesis protein of *B. japonicum*, the cycl gene product, also contains a periplasmically oriented CXXC motif, but otherwise does not resemble thioredoxins (3). This protein might also catalyze disulfide reduction in apocytomnes c or CycY.

In *E. coli*, several periplasmic protein thiol-disulfide oxidoreductases have been shown to be required for cytochrome c maturation, among which are DsbA, DlpZ, and CcmG (5, 7, 46, 47). CcmG shares a high degree of sequence similarity with HelIX and CycY. Apart from the extra N-terminal hydrophobic stretch in this class of thioredoxins, an additional sequence of 27 highly conserved amino acids in the C-terminal third of these polypeptides is remarkable, which might be essential for substrate recognition (isoleucine 149 to proline 175).

A relevant observation in this context may be that, in contrast to other thiol-disulfide oxidoreductases including TlpA (26), CycY* was not capable of mediating the reduction of insulin disulfides by DTT, one of the standard assays for thiol-disulfide oxidoreductase activity (41). Measurement of the parent second-order rate constant of the reduction of CycY* by DTT (3.5 $\text{m}^{-1}\text{s}^{-1}$ at pH 7.0) showed that this reaction is $\sim 3$ orders of magnitude slower compared with thioredoxin ($-10^3$ $\text{m}^{-1}\text{s}^{-1}$) (41) and $-6$ orders of magnitude slower compared with DsbA ($-10^6$ $\text{m}^{-1}\text{s}^{-1}$) (Ref. 42 and data not shown). It follows that generation of reduced CycY* by DTT as catalytic reductant of insulin disulfides becomes rate-limiting under the conditions used, which explains why CycY* does not act as a catalyst in the insulin reduction assay (48). The slow disulfide exchange reactions of CycY* were also reflected by the fact that 3 days of incubation were necessary to reach equilibrium with glutathione redox buffers. The slow reactions between CycY* and small organic thiol compounds suggest that the enzyme may specifically act on a single substrate molecule in *vivo*. It is known from studies on the polypeptide specificity of DsbA and protein disulfide-isomerase (42, 49–51) that a specific substrate-binding site next to the active-site disulfide increases the rate constants of the disulfide exchange between the enzyme and its substrates. To investigate the participation of CycY in redox reactions in a more general way, two further possibilities of CycY reactivity were explored: (i) the interaction between oxidized DsbA from *E. coli* and reduced CycY* was investigated, revealing that DsbA was not capable of oxidizing CycY* in *vitro*; (ii) the alkaline phosphatase activity of the cycA*-phoA fusion was compared in the *B. japonicum* wild type and in the cycY* mutant. The activity was identical in both strains, indicating that, in contrast to DsbA (22), CycY is not involved in disulfide bond formation of alkaline phosphatase (data not shown). Our main goal for future experiments is now the identification of the target molecule of CycY.
Acknowledgments—We thank J. Fleschke for construction of the cyca’-phoA fusion plasmid, R. Zafferey for analysis of microaerobically expressed c-type cytochromes, P. James for mass spectrometry and N-terminal sequence analyses, R. Fischer for assistance with antibody production, H. Loferer and K. Maskos for helpful technical advice, and J. Hennecke for fruitful discussions.

REFERENCES

1. Thöny-Meyer, L., Ritz, D., and Hennecke, H. (1994) in Mol. Microbiol. 15, 307–318
2. Thöny-Meyer, L., Fischer, F., Kunzler, P., Ritz, D., and Hennecke, H. (1995) J. Bacteriol. 177, 4321–4326
3. Ritz, D., Thöny-Meyer, L., and Hennecke, H. (1995) Mol. Microbiol. 15, 307–318
4. Page, M. D., and Ferguson, S. J. (1995) in Methods for Evaluating Biological Nitrogen Fixation (Bergersen, F. J., ed) pp. 111–138; John Wiley & Sons Ltd., Chichester, United Kingdom
5. Thöny-Meyer, L., Kunzler, P., and Hennecke, H. (1996) Eur. J. Biochem. 235, 754–761
6. Holmgren, A. (1979) J. Biol. Chem. 254, 3927–3932
7. Dyksterhuis, E. J., and Jansen, A. H. (1972) in Experiments in Molecular Genetics, Cold Spring Harbor, NY
8. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
9. Thöny-Meyer, L., Ritz, D., Bott, M., and Hennecke, H. (1993) Mol. Microbiol. 12, 392–398
10. Minton, P. N. (1984) in Methods for Evaluating Biological Nitrogen Fixation (Bergersen, F. J., ed) pp. 111–138; John Wiley & Sons Ltd., Chichester, United Kingdom
11. Ramseier, T. M., Winteler, H. V., and Hennecke, H. (1991) J. Biol. Chem. 266, 7793–7803
12. Regensburger, B., and Hennecke, H. (1983) Arch. Microbiol. 135, 103–109
13. Hanahan, D. (1983) J. Mol. Biol. 166, 557–563
14. Simon, R., Prieler, U., and Puhler, A. (1983) in Molecular Genetics of the Bacteria-Plant Interaction (Puhler, A., ed) pp. 98–106, Springer-Verlag, Berlin
15. Studier, F. W., and Moffat, B. A. (1986) J. Mol. Biol. 189, 113–130
16. Daniel, R. M., and Appleby, C. A. (1972) Biochim. Biophys. Acta 275, 347–354
17. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1988) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
18. Ritz, D., Bott, M., and Hennecke, H. (1993) Mol. Microbiol. 9, 729–740
19. Minton, P. N. (1984) Gene (Amst.) 31, 269–273
20. Hohensee, C. S., and Wright, A. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 5107–5111
21. Ditta, G., Stanfield, S., Corbin, D., and Helinski, D. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 7474–7481
22. Bardwell, J. C. A., Mc Govern, K., and Beckwith, J. (1991) Cell 67, 581–589
23. Alvarez-Morales, A., Betancourt-Alvarez, M., Kaluza, K., and Hennecke, H. (1986) Nucleic Acids Res. 14, 4207–4227
24. Gill, S. C., and von Hippel, P. H. (1989) Anal. Biochem. 182, 319–326
25. Ellman, G. L. (1959) Arch. Biochem. Biophys. 82, 70–77
26. Loferer, H., Wunderlich, M., Hennecke, H., and Glockshuber, R. (1995) J. Biol. Chem. 270, 26178–26183
27. Smith, D. E., and Fisher, P. A. (1984) J. Cell Biol. 99, 20–28
28. Loferer, H., Bott, M., and Hennecke, H. (1993) EMBO J. 12, 3373–3383
29. Thöny-Meyer, L., Stax, D., and Hennecke, H. (1989) Cell 57, 683–697
30. Miller, J. H. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
31. Hahn, M., and Hennecke, H. (1984) Mol. & Gen. Genet. 193, 46–52
32. Turner, G., and Gibson, A. H. (1980) in Methods for Evaluating Biological Nitrogen Fixation (Bergersen, F. J., ed) pp. 111–138; John Wiley & Sons Ltd., Chichester, United Kingdom
33. Thöny-Meyer, L., Kunzler, P., and Hennecke, H. (1996) Eur. J. Biochem. 235, 754–761
34. Holmgren, A. (1979) J. Biol. Chem. 254, 9627–9632
35. Wunderlich, M., and Glockshuber, R. (1993) Protein Sci. 2, 717–726
36. Rost, J., and Rapoport, S. (1964) Nature 201, 185
37. Krause, G., Lundstrom, J., Barea, J. L., de la Cuesta, C. P., and Holmgren, A. (1991) J. Biol. Chem. 266, 9494–9500
38. Berglund, O., and Sjöberg, B.-M. (1979) J. Biol. Chem. 245, 6030–6035
39. Gleason, P. K. (1992) Protein Sci. 1, 609–616
40. Hawkins, H. C., de Nardi, M., and Freedman, R. B. (1991) Biochem. J. 275, 341–348
41. Holmgren, A. (1979) J. Biol. Chem. 254, 9627–9632
42. Wunderlich, M., Otto, A., Seekler, R., and Glockshuber, R. (1993) Biochemistry 32, 12251–12256
43. Preisig, O., Anthamatten, D., and Hennecke, H. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 3309–3313
44. Berglund, O., and Sjöberg, B.-M. (1979) J. Biol. Chem. 245, 6030–6035
45. Holmgren, A. (1979) J. Biol. Chem. 254, 9627–9632
46. Wunderlich, M., Otto, A., Seekler, R., and Glockshuber, R. (1993) Biochemistry 32, 12251–12256
47. Preisig, O., Anthamatten, D., and Hennecke, H. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 3309–3313
48. Surpin, M. A., Moshiri, F., Murphy, A. M., and Maier, R. J. (1994) Biochemistry 33, 73–77
49. Bott, M., Thöny-Meyer, L., Loferer, H., Rossbach, S., Tully, R. E., Keister, D., Appleby, C. A., and Hennecke, H. (1995) J. Biol. Chem. 270, 2214–2217
50. Metheringham, R., Griffith, L., Croke, H., Forsythe, S., and Cole, J. (1995) Arch. Microbiol. 164, 301–307
51. Sambongi, Y., Croke, H., Cole, J. A., and Ferguson, S. J. (1994) FEBS Lett. 344, 207–210
52. Loferer, H., and Hennecke, H. (1994) Eur. J. Biochem. 223, 339–344
53. Darby, N. J., and Creighton, T. E. (1995) EMBO J. 14, 392–398
54. Zapan, A., Bardwell, J. C. A., and Creighton, T. E. (1993) Biochemistry 32, 5083–5092
55. Thöny-Meyer, L., and Kunzler, P. (1996) J. Bacteriol. 178, 6166–6172