A PERIPLASMIC MOLECULAR CHAPERONE-LIKE PROTEIN PROTECTING UNFOLDED DMSO REDUCTASE AGAINST AGGREGATION IN *RHODOBACTER SPHAEROIDES* F. SP. *DENITRIFICANS*

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CONTENTS

ABBREVIATIONS

BSA	bovine serum albumin
DEAE	dietylaminoethyl
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
IAA	iodoacetamide
kDa	kilodalton
min	minute(s)
Mo	molybdenum
PAGE	polyacrylamide gel electrophoresis
PDI	protein disulfide isomerase
PPI	peptydyl prolyl cis-trans isomerase
s	second(s)
SDS	sodium dodecylsulfate
Tris	tris(hydroxymethyl)aminomethane

INTRODUCTION

When bacteria such as *Escherichia coli* are exposed to heat shock and several other stressful conditions, they respond by inducing the synthesis of several proteins known as stress proteins, some of which are molecular chaperones. These include the molecular chaperons DnaK and GroEL (Hartl et al. 1994). The chaperonin GroEL is a member of the Hsp60 class of molecular chaperones which recognize and bind unfolded or misfolded proteins in the cell (Robinson et al. 1994, Zahn et al. 1994, Lin et al. 1995). DnaK, the Hsp70 homologue, cooperates functionally with two additional heat shock proteins, DnaJ and GrpE (Langer et al. 1992). DnaK and GroEL seem to interact with different structural elements of unfolded polypeptides. Consistent with their binding to nascent chains, DnaK interacts with synthetic peptides and with polypeptides in extended conformations. In contrast, GroEL may recognize secondary structure elements and stabilize bound proteins as conformational intermediates formed early folding, like the 'molten globule'. These two proteins protect the cell against stressing injuries because they prevent the aggregation of damaged proteins and mediate their refolding to native state. Members of the chaperone families are also expressed in normally growing cells, facilitating the transport of proteins across cell membranes, cotranslational protein folding, proper protein assembly and other

normal functions of cells (Hartl et al. 1994).

The periplasm is the region between the inner and outer membranes of Gram-negative bacteria, and the compartment can be compared to the lumen of the endoplasmic reticulum (ER) in eukaryotic cells. Many proteins are found in the periplasm, but the physiological functions of only a few such proteins are known. Furthermore, the periplasm of *E. coli* has been of great interest with respect to the functional expression of a wide variety of recombinant proteins from different sources. However, the periplasmic folding of proteins has not been studied in any great detail and its mechanism(s) is unknown (Wulfing and Pluckthun 1994).

Recent observations indicate that protein folding *in vivo* is facilitated by a class of proteins other than molecular chaperones. This class of proteins includes some enzymes that modify the structures of amino acids within polypeptides. This class includes protein disulfide isomerase (PDI), first isolated in the ER, which can shuffle disulfide bonds (Bulleid 1993, Novia and Lennarz 1992), and proline isomerase, which catalyzes the *cis-trans* isomerization of proline (Harding et al. 1989, Takahashi et al. 1989). A protein designated DsbA (Bardwell et al. 1991) or PpfA (Kamitani et al. 1992), with disulfide isomerase activity, has been identified in the periplasm of *E. coli*. DsbA is required for the correct formation of disulfide bonds of periplasmic proteins *in vivo* and *in vitro* (Akiyama et al. 1992, Akiyama and Ito 1993, Bardwell et al. 1991, Bardwell and Beckwith 1993). It is also required for the production

of recombinant eukaryotic proteins in the periplasm of *E. coli*, such as serine proteases (Bardwell and Beckwith 1993), antibody fragments (Knappik et al. 1993) and fragments of T-cell receptor (Wulfing and Pluckthun 1994).

However, no general molecular chaperone is known in the periplasm of Gram-negative bacteria. In the cytoplasm of Gram-negative bacteria, the GroEL, GroES and DnaK proteins appear to act as molecular chaperones, but there is no evidence that such cytoplasmic molecular chaperones have a direct effect on protein-folding processes in the periplasm.

The physiological conditions required for the protein folding in the periplasm are obviously important. It has been argued that the presence of ATP in the periplasm is highly improbable. Moreover, the periplasm is assumed to maintain an oxidative environment to allow the formation of disulfide bonds (Wulfing and Pluckthun 1994).

A denitrifying phototrophic bacterium, *Rhodobacter sphaeroides* f. sp. *denitrificans* (Satoh et al. 1976) is capable of dimethyl sulfoxide (DMSO) respiration as one of its anaerobic respiration systems. The terminal reductase of the respiration, DMSOR, is a periplasmic soluble protein with a molecular mass of 84,903 consisting of a single polypeptide and contains one molecule of molybdenum (Mo) cofactor and nine cysteine residues per molecule (Satoh and Kurihara 1987, Yamamoto et al. 1995). Recently, its three-dimensional structure has been determined by x-ray

crystallography (Schindelin et al. 1996). We have been studying the way in which incorporation of the Mo cofactor into the polypeptide is associated with folding of the polypeptide in the periplasm. In a previous paper, spheroplasts prepared from a mutant strain deficient in the Mo cofactor secrete both unfolded and folded apo-DMSOR into the medium and we suggested that the unfolded form might be a stable intermediate generated during the folding that yields the native form (Masui et al. 1994).

Many experiments have been carried out *in vitro* to determine the mechanism by which proteins attain their native conformations, for example, analysis of refolding of a denatured enzyme. Since the periplasmic folding of proteins has not been studied well and its mechanism is unknown although there are so many proteins in the compartment, I addressed in this study to elucidate the mechanism of folding and identification of proteins with molecular chaperone-like activity in the periplasm. DMSOR of *R. sphaeroides* f. sp. *denitrificans* is produced by about 10% of total periplasmic protein when induced with DMSO. Formation of native form of DMSOR appears closely correlated with Mo cofactor available in cells. Then I thought DMSOR is a very good tool for such a study. In this paper, I write these results in three chapters.

In chapter 1, I studied the refolding of acid-unfolded DMSOR *in vitro* in an effort to understand the mechanism of the folding of the protein in the periplasm. I describe that acid-unfolded DMSOR is stabilized in the presence of GroEL or a protein(s) in the periplasm

and that DTT reduces the acid-unfolded reductase to a fully reduced form and helps in the refolding that yields to the native conformation.

In chapter 2, I examined the redox nature of DMSOR, and describe that there exists no disulfide bond in native conformation of DMSOR. A new folding intermediate with a similar conformation to the native DMSOR is also described. It was inferred that DMSOR is secreted into the periplasmic space first as an unfolded form and then folds to yield the native conformation via an intermediate without involving formation of any disulfide bonds.

In chapter 3, isolation of a periplasmic protein which have an ability of protecting the unfolded DMSOR against aggregation is described. This provides the first experimental system to identify a protein(s) which can take a role of molecular chaperones in the periplasm. The protein has a molecular weight of 58,000 and has no subunit. The sequence of 14 amino-terminal residues of the protein was completely identical to those of a periplasmic dipeptide transport protein (DppA) of *Escherichia coli*. This protein represents the first example of a periplasmic protein that has molecular chaperone-like activity.

CHAPTER 1.

Stabilization by GroEL, a Molecular Chaperone, and a Periplasmic Fraction, as Well as Refolding in the Presence of Dithiothreitol, of Acid-Unfolded Dimethyl Sulfoxide Reductase, a Periplasmic Protein of *Rhodobacter sphaeroides* f. sp. *denitrificans*.

ABSTRACTS

The mechanisms of folding of a periplasmic protein was studied *in vitro* using dimethyl sulfoxide reductase (DMSOR), a periplasmic enzyme of *R. sphaeroides* f. sp. *denitrificans*. When DMSOR was denatured by acidification to pH 2 at 30℃, the molybdenum cofactor was immediately released and unfolded forms of DMSOR appeared within 2 min. When the acid-unfolded DMSOR has been incubated in refolding buffer (pH 8.0) at 20℃ for 2 h, it became almost undetectable after electrophoresis on a non-denaturing gel. This result suggests that the acid-unfolded DMSOR might have aggregated after incubation. The aggregation was suppressed by incubation in the presence of commercial GroEL, a molecular chaperone. When reduced dithiothreitol (DTT) was added to the acid-unfolded forms in the presence of GroEL, some of the DMSOR was converted to the native form, which had the same mobility on a non-denaturing gel as the active emzyme. Non-reducing SDS-polyacrylamide gel electrophoresis of the acid-unfolded forms of DMSOR indicated that the unfolded forms were a mixture of heterogeneously folded or misfolded forms and that their forms were converted by DTT to the fully reduced form. The periplasmic fraction of the phototroph was also able to suppress the aggregation of the acid-unfolded DMSOR, and a protein(s) with a molecular mass of about 40 kDa in the periplasm was revealed to have stabilizing activity. It appears that there exists a mechanism whereby the unfolded DMSOR that is secreted into

the periplasm is maintained in a non-aggregated and reduced form during folding to the native form.

The periplasm is the region between the inner and outer membranes of Gram-negative bacteria, and the compartment can be compared to the lumen of the endoplasmic reticulum (ER) in eukaryotic cells. Many proteins are found in the periplasm, but the physiological functions of only a few such proteins are known. Furthermore, the periplasm of *E. coli* has been of great interest with respect to the functional expression of a wide variety of recombinant proteins from different sources. However, the periplasmic folding of proteins has not been studied in any great detail and its mechanism(s) is unknown (Wulfing et al. 1994).

Recent observations indicate that protein folding *in vivo* is facilitated by two classes of proteins. One class includes some enzymes that modify the structures of amino acids within polypeptides. This class includes protein disulfide isomerase (PDI), first isolated in the ER, which can shuffle disulfide bonds (Bulleid 1993, Novia et al. 1992), and proline isomerase, which catalyzes the *cis-trans* isomerization of proline (Harding et al. 1989, Takahashi et al. 1989). A protein designated DsbA (Bardwell et al. 1991) or PpfA (Kamitani et al. 1992), with disulfide isomerase activity, has been identified in the periplasm of *E. coli*. DsbA is required for the correct formation of disulfide bonds of periplasmic proteins *in vivo* and *in vitro* (Akiyama et al. 1992, Akiyama et al. 1993, Bardwell et al. 1991, Bardwell et al. 1993). It is also required for the production of recombinant eukaryotic proteins in the periplasm of *E. coli*, such as serine proteases

(Bardwell et al. 1993), antibody fragments (Knappik et al. 1993) and fragments of T-cell receptor (Wulfing et al. 1994). The second class of proteins are the molecular chaperones (for reviews, see Frydman and Hartl 1994, Hightower et al. 1994, Randall et al. 1994) that play roles in the correct folding of proteins by preventing side-reactions. However, no such molecular chaperones are known in the periplasm of Gram-negative bacteria. In the cytoplasm of Gram-negative bacteria, the GroEL, GroES and DnaK proteins appear to act as molecular chaperones, but there is no evidence that such cytoplasmic molecular chaperones have a direct effect on protein-folding processes in the periplasm.

The physiological conditions required for protein folding in the periplasm is are obviously important. It has been argued that the presence of ATP in the periplasm is highly improbable. Moreover, the periplasm is assumed to maintain an oxidative environment to allow the formation of disulfide bonds (Wulfing et al. 1994).

Rhodobacter sphaeroides f. sp. *denitrificans* (Satoh et al. 1976) is a phototrophic bacterium that also is capable of denitrification and dimethyl sulfoxide (DMSO) respiration. The terminal reductase of such respiration, DMSO reductase (DMSOR), is a soluble periplasmic protein consisting of a single polypeptide and containing one molecule of a molybdenum (Mo) cofactor per molecule (Satoh and Kurihara 1987). DMSOR is synthesized as a precursor, with a molecular mass of 89,206 Da. It has a signal peptide of 42

amino acids and is processed to the mature form that has a molecular mass of 84,903 Da (Yamamoto et al. 1995). We have been studying the way in which incorporation of the Mo cofactor into the polypeptide is associated with folding of the polypeptide in the periplasm. We found that spheroplasts prepared from a mutant strain deficient in the Mo cofactor secrete both unfolded and folded apo-DMSOR into the medium and we suggested that the unfolded form might be a stable intermediate generated during the folding that yields the native form (Masui et al. 1994).

Many experiments have been carried out *in vitro* to determine the mechanism by which proteins attain their native conformations, for example, analysis of refolding of a denatured enzyme. Since the periplasmic folding of proteins has not been studied well and its mechanism is unknown although there are so many proteins in the compartment, I addressed in this study to elucidate the mechanism of folding and identification of proteins with molecular chaperone-like activity in the periplasm. DMSOR of *R. sphaeroides* f. sp. *denitrificans* is produced by about 10% of total periplasmic protein when induced with DMSO. Formation of native form of DMSOR appears closely correlated with Mo cofactor available in cells. Then I thought DMSOR is a very good tool for such a study.

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and that DTT reduces the acid-unfolded reductase to a fully reduced form and helps in the refolding that yields to the native conformation.

In this chapter, I studied the refolding of acid-unfolded DMSOR *in vitro* in an effort to understand the mechanism of the folding of the protein in the periplasm. I describe here that acid-unfolded DMSOR is stabilized in the presence of GroEL or a protein(s) in the periplasm and that DTT reduces the acid-unfolded reductase to a fully reduced form and helps in the refolding that yields to the native conformation.

MATERIALS AND METHODS

Bacteria and growth conditions

A green mutant strain of *Rhodobacter sphaeroides* f. sp. *denitrificans* IL106 (Satoh et al. 1976) was used in this study. The medium and conditions for growth of the photodenitrifier were described previously (Yoshida et al. 1991).

Preparation of DMSOR

DMSOR was purified by the published method (Satoh et al. 1987).

Preparation of spheroplasts and of periplasmic, cytoplasmic and membrane fractions

Cells were grown photosynthetically in the absence of DMSO in a 150-ml screw-capped bottle and harvested by centrifugation. Cells were suspended in 5 ml of 500 mM sucrose-1.3 mM EDTA-50 mM Tris-HCl buffer, treated with lysozyme (600 μg/ml), and centrifuged at 10,000-$\times g$ for 10 min. The supernatant and pellet were obtained as the periplasmic fraction and spheroplasts, respectively (Yoshida et al. 1991). The spheroplasts were suspended in 5 ml of 50 mM Tris-HCl buffer (pH 7.5), sonicated for 15 sec, and centrifuged at 10,000 $\times g$ for 10 min to remove intact cells. The supernatant was centrifuged at 100,000$\times g$ for 1 h and the supernatant and pellet were obtained as the cytoplasmic and membrane fractions, respectively.

Assay for Mo cofactor

The Mo cofactor was quantitated as described previously (Masui et al. 1992, Matsuzaki et al. 1993).

Acid denaturation

A solution of purified DMSOR (80 μg/ml) in 50 mM Tris-HCl buffer (pH 7.5) was supplemented with an equal volume of 20 mM HCl (final pH, 2.0) and incubated at 30℃ for indicated times. The solution of denatured DMSOR was then diluted 2.5-fold with the refolding buffer (125 mM Tris-HCl, pH 8.0, and 12.5 mM KCl) and immediately placed on ice. The preparation was kept on ice prior to analysis.

Electrophoresis on non-denaturing gels and non-reducing SDS-gels and immunoblotting analysis

Samples were subjected to electrophoresis on a non-denaturing gel (7.5% polyacrylamide) at 4℃ as described by Davis (1964). DMSOR on the gel was transferred to a nitrocellulose membrane after the gel had been immersed in buffer [250 mM Tris-HCl (pH 8.0), 2% SDS, 10% 2-mercaptoethanol] and heated in a microwave oven for 20 sec to improve the sensitivity of detection of DMSOR (Masui et al. 1994), and proteins were analyzed immunologically as described elsewhere (Yoshida et al. 1991). Non-reducing SDS-polyacrylamide gel electrophoresis was carried out as described by Ostermeiyer and Georgiou (1994) at room temperature on a 7.5% polyacrylamide gel,

with the exception that the sample was not boiled after addition of 2% SDS. DMSOR on the gel was transferred to a nitrocellulose membrane after denaturation.

Materials

GroEL and GroES were purchased from Takara Biochemicals (Kyoto, Japan). Dithiothreitol (DTT) and bovine serum albumin (fraction V) were obtained from Sigma Chemical Company (St. Louis, MO, USA). Sephacryl S-200 was obtained from Pharmacia Biotech (Uppsala, Sweden).

RESULTS

Release of the Mo cofactor and unfolding of DMSOR by acid

We reported previously that spheroplasts from a Mo cofactor-deficient mutant of *R. sphaeroides* f. sp. *denitrificans* secrete apo-DMSOR of the maturesize with a conformation distinct from that of the native enzyme, into the medium, and we suggested that this apo-DMSOR is an unfolded intermediate generated *en route* to the native form (Masui et al. 1994). We prepared a refolding system *in vitro* using DMSOR that has been unfolded by releasing the Mo cofactor with acid. The results of an analysis of refolding should enhance our understanding of the nature of the unfolded form secreted by spheroplasts. Figure 1 shows the time course of release of the Mo cofactor from DMSOR into solutions and the formation of unfolded DMSOR, as analyzed by electrophoresis on a non-denaturing gel and immunoblotting. Electrophoresis and the quantitation of the Mo cofactor were performed with a sample that has been placed on ice immediately after a solution of acid-treated DMSOR had been diluted in the refolding buffer. Almost all of the Mo cofactor was released from the enzyme within 30 sec and its release was followed by formation of unfolded DMSOR, the more slowly migrating species designated U in Figure 1. The unfolded DMSOR (U) appeared to migrate mainly as two bands on the non-denaturing gel. Figure 1 shows that DMSOR was completely unfolded within 2 min. However in some experiments, native DMSOR remained detectable even after 10

min. Therefore, the acid-unfolded DMSOR used in subsequent experiments was prepared by treating DMSOR with acid at 30℃ for 20 min.

We tried to denature DMSOR by incubation with 1% SDS, 8M urea and 6M guanidine-HCl, respectively, at 30℃ for 20 min. The enzyme was stable in SDS and urea. Guanidine-HCl denatured the enzyme, but the denatured enzyme was not used in the following experiments because refolding of the denatured enzyme was unsuccessful.

Effect of GroEL on the stability of acid-unfolded DMSOR

The acid-unfolded form of DMSOR became undetectable after electrophoresis on a non-denaturing gel when it had been incubated at 20 ℃ for 2 h after dilution in the refolding buffer (Fig. 2, lane 3). However, when the acid-unfolded DMSOR in the refolding buffer was incubated in the presence of GroEL, the best-characterized molecular chaperone from *E. coli*, unfolded DMSOR became visible on the non-denaturing gel (Fig. 2, lanes 4). When a commercial preparation of GroES was included in addition to GroEL, a clearly unstained band in the region of the unfolded form was observed (Fig. 2, Lanes 5). The purchased GroES was found, in this study, not to be pure. It yielded at least four protein bands when analyzed on a non-denaturing gel and one band migrated exactly to the position that corresponded to U (data not shown). We assumed that the decolored band was caused by the one of the bands of GroES. A combined effect of GroEL and GroES was not observed. Bovine serum

albumin (BSA) also had a stabilizing effect, but its effect was always small or, at least, less than that of GroEL (Fig. 2, lane 6). These results suggest that GroEL might protect acid-unfolded DMSOR against aggregation. ATP had no additive effect when added in combination with GroEL and GroES (data not shown). When acid-unfolded DMSOR that had been incubated for 2 h in the refolding buffer was boiled in the sample buffer for denaturing SDS-gel electrophoresis, DMSOR appeared on the non-denaturing gel after electrophoresis as single, sharp band (Fig. 2, lane 1). This result suggests that U was a mixture of heterogeneously folded or misfolded forms and that the acid-unfolded DMSOR in the refolding buffer might have aggregated during incubation for 2 h.

Refolding of acid-unfolded DMSOR in the presence of DTT

GroEL appeared to stabilize the unfolded DMSOR, but GroEL alone was unable to convert the unfolded form to a native form. When a high concentration of reduced DTT was present together with GroEL, the broad band (U) of the acid-unfolded DMSOR was mainly converted to one band and the native form (N) was formed even in the absence of the Mo cofactor (Fig. 3, lane 4). DTT alone was able to convert only a small amount to the native form (Fig. 3, lane 3). In the presence of BSA, as a control, DTT converted U to the native form (Fig. 3, lane 6). GroES in addition to GroEL had almost no additive effect (Fig. 3, lane 5). Figure 4 shows the time course of the conversion to the native form in the presence of DTT and GroEL

together. The amount of the native form increased with time during the incubation. These results indicate that DTT helps in the refolding of the acid-unfolded DMSOR to a native form.

Analysis of DTT-reduced acid-unfolded DMSOR by non-reducing SDS-gel electrophoresis

In the absence of DTT, the acid-unfolded DMSOR, when protected against aggregation, migrated on non-denaturing gels as a broad band, which probably consisted of heterogeneous forms of DMSOR. To clarify the nature of the unfolded form and to understand how DTT might affect the acid-unfolded DMSOR, we performed non-reducing SDS-gel electrophoresis (Fig. 5). SDS was added to the acid-unfolded DMSOR in refolding buffer either without (Fig. 5, lanes 3 to 6) or with DTT (Fig. 5, lanes 7 to 10), and the mixture was subjected, without boiling, to electrophoresis on an SDS-containing gel. In the presence of GroEL, of GroEL plus GroES, and of BSA, the acid-unfolded DMSOR appeared heterogeneous when DTT was absent (Fig. 5, lanes 4 to 6). However, when DTT was present, only one band, corresponding to the fully reduced form, was observed (Fig. 5, lanes 7 to 10). Even in the absence of GroEL, of GroEL plus GroES, or of BSA, the faint band that was observed in the absence of DTT (Fig. 5, lane 3) changed to a distinct band of the fully reduced form in the presence of DTT (Fig. 5, lane 7). These results indicate that U consists of heterogeneous forms with disulfide bonds that are reduced by DTT.

Effect of the periplasmic fraction on the stabilization of acid-unfolded DMSOR

To investigate whether molecular chaperone-like proteins exist in the periplasm, we studied the effects of periplasmic, cytoplasmic and membrane fractions on the stabilization of the acid-unfolded DMSOR (Fig. 6). Total protein from the periplasm stabilized the acid-unfolded DMSOR, and DMSOR migrated on non-denaturing gels in the same way as U (Fig. 6, lane 4), as observed also in the presence of GroEL. The native form of DMSOR visible on the gel was due to the presence of the periplasmic fraction (Fig. 6, lane 7). This result suggests that a protein(s) exists in the periplasm that is able to stabilize the acid-unfolded DMSOR as does GroEL. During incubation with the cytoplasmic fraction, by contrast, no bands of DMSOR corresponding to U (Fig. 6, lane 5) were observed. The result was probably due to digestion by proteases in the cytoplasmic fraction because the bands of DMSOR corresponding to U also disappeared when the acid-unfolded DMSOR was incubated in the presence of both periplasmic and cytoplasmic fractions (data not shown). The native form of DMSOR in the cytoplasmic fraction (Fig. 6, lanes 5 and 8) was due to the periplasmic contamination of the cytoplasmic fraction. The membrane fraction appeared to have no effect on the acid-unfolded DMSOR (Fig. 6, lane 7) .

Fractionation of a protein with stabilizing activity from the periplasmic fraction by gel chromatography

In order to examine whether the stabilizing activity is attributable to a specific protein and not merely to the high level of proteins in the periplasmic fraction, we precipitated the proteins in the periplasmic fraction of cells that has been grown under photosynthetic conditions with ammonium sulfate at 80% saturation. The proteins were dissolved in a minimum amount of 50 mM Tris-HCl (pH 7.5) and chromatographed on a column of Sephacryl S-200. The eluate was assayed for stabilizing effects on the acid-unfolded DMSOR, as described in the legend to Figure 2 (Fig. 7). Although the data in the inset are not quantitatively exact, the fraction eluted in the void volume (fraction 34), which has high absorbance at 280 nm, had no stabilizing activity. The peak of activity appeared around fractions 50 to 52, differing from the peak of absorbance at 280 nm by about two fractions. The molecular mass of proteins in fraction 51 was determined to be about 40 kDa. These results indicate that a specific protein(s) with stabilizing activity had a molecular mass of about 40 kDa and that the stabilizing effect of the periplasmic fraction was not due to cytoplasmic molecular chaperones, such as GroEL (molecular mass of the native form, 802 kDa) and GroES (73 kDa) that might have contaminated the periplasmic fraction during its preparation. This result suggests that a protein with a molecular chaperone-like role exists in the periplasm.

DISCUSSION

In this study we showed that commercially purchased GroEL protected the acid-unfolded DMSOR from unfavorable interactions *in vitro*, and that a protein(s) with a molecular mass of about 40 kDa in the periplasm also had such a stabilizing effect. Since GroEL is a cytoplasmic molecular chaperone, it is uncertain how GroEL affect to the unfolded DMSOR. However, there seems to be general agreement that the major role of GroEL in protein folding is to prevent of formation of aggregates or unfolded or partially folded proteins (Murai et al. 1995). The stabilizing effect of GroEL on the acid-unfolded DMSOR could involve such prevention. GroES also is a cytoplasmic molecular chaperone and it is not known to bind to any proteins other than those to which GroEL binds (Martin et al. 1993). The GroES that we purchased gave several bands on a non-denaturing gel (and on an SDS-polyacrylamide gel) and one of the bands migrated similarly to the unfolded DMSOR (Fig. 2). Therefore, the meaning of the results obtained with GroES are unclear. We found that a periplasmic protein with a molecular mass of about 40 kDa had a stabilizing effect on the acid-unfolded DMSOR. Thus, a specific protein in the periplasm could act to protect against aggregation periplasmic proteins in an unfolded form that have just been processed and secreted in to the periplasm. We are now isolating the 40-kDa protein.

Reduced DTT (10 mM) in the presence of GroEL converted the

heterogeneous forms of acid-unfolded DMSOR to a protein that migrated as one band, merely, the fully reduced, native form. Even in the presence of a 10-fold higher concentration of DTT (100 mM), the efficiency of the formation of the native form was the same as that in the presence of 10 mM DTT (data not shown). DTT probably reduced the misfolded DMSOR with incomplete or "incorrect" disulfide bonds to a fully reduced form. Our results suggest that the periplasm provides a reducing environment and that the fully reduced form could be necessary for correct folding. Wunderlich and Glockshuber (1993) reported that the addition of reduced glutathione to the growth medium resulted in an up to 14-fold increase in the yield of native RBI [α-amylase/trypsin inhibitor from *Ragi* (*Eleusine coracana Gaertneri*)] as a consequence of shuffling or disulfide bonds by DsbA in the periplasm of *E. coli*. Further, Ostermeier and Georgiou (1994) showed that oxidized glutathione and oxidized DTT had no effect on the folding of BPTI (preOmpA-bovine pancreatic trypsin inhibitor) in the periplasm of *E. coli*. These results are consistent with our present hypothesis. Therefore, the redox environment in the periplasm might be different from that in the ER, where protein folding has been found to depend strongly on the presence of oxidized glutathione. The mechanism for oxidation or sulfhydryl groups might differ between eukaryotic and prokaryotic cells, as suggested by Ostermeier and Georgiou (1994). It is unclear, however, how disulfide bonds are formed in such a reducing environment. DsbA protein, which has disulfide isomerase activity,

exists as a homodimer of a protein with a molecular mass of 21 kDa and has been shown to be necessary for protein folding in the periplasm of *E. coli* (Akiyama et al. 1992, Akiyama et al. 1993, Bardwell et al. 1991, Bardwell et al. 1993, Knappik et al. 1990). We tested the effect of PDI from bovine liver (final concentration, 100 μg/ml) on the refolding of the acid-unfolded DMSOR in the presence of DTT, but the efficiency of formation of the native form was almost the same as that in the presence of BSA. However, it remains possible that the DsbA protein might have stabilizing activity. We are now isolating the 40-kDa protein to examine its properties.

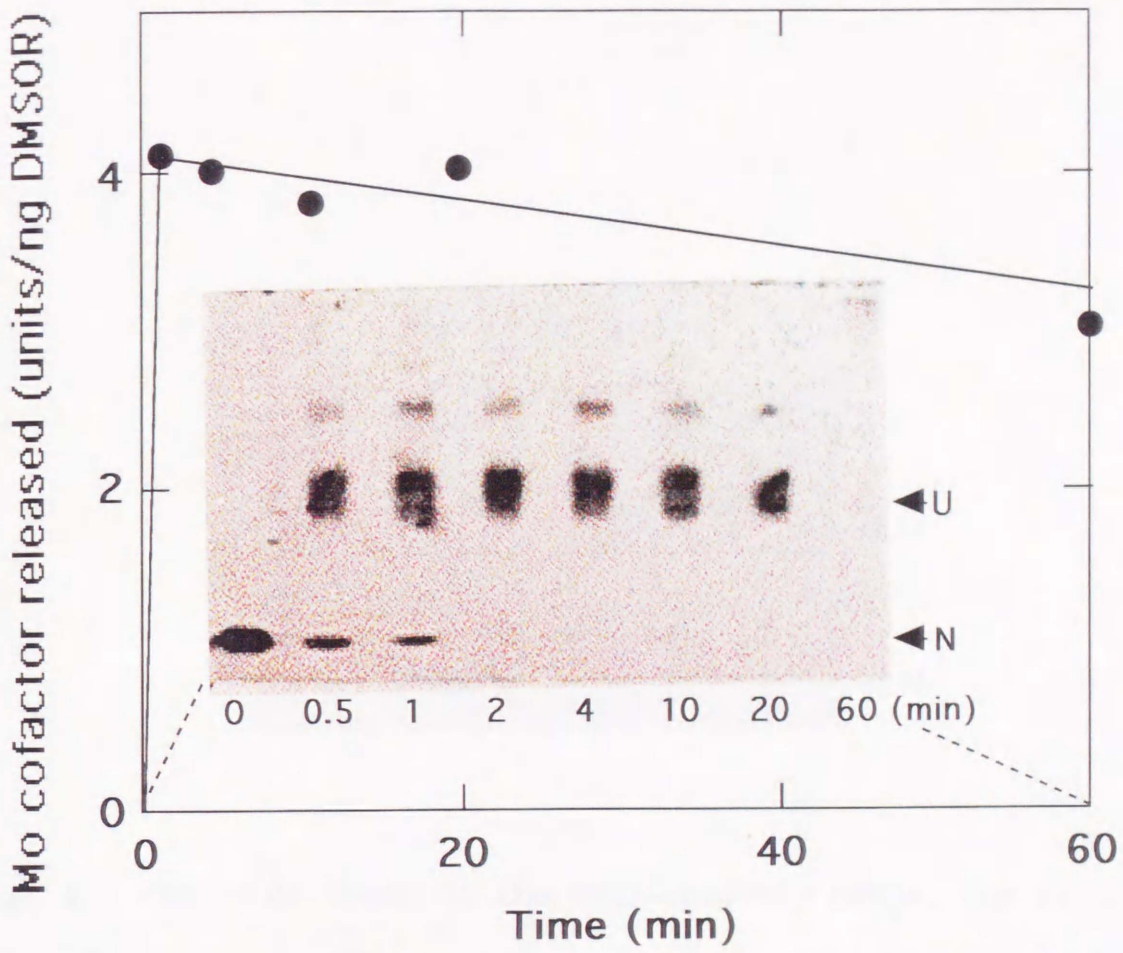

Fig. 1. Time course of the acid-induced unfolding of DMSOR. A solution of DMSOR (80 μg/ml) in 50 mM Tris-HCl buffer (pH 7.5) was supplemented with an equal volume of 20 mM HCl and incubated at 30℃. At the times indicated, an aliquot (3 μl) of the solution was diluted 2.5-fold with refolding buffer and placed immediately on ice. Samples (10 μl) were subjected to the assay of Mo cofactor activity, which is expressed in units per ng of DMSOR and to non-denaturing polyacrylamide gel electrophoresis and immunoblotting. Inset: non-denaturing gel electrophoresis. N, Native form of DMSOR. U, unfolded form.

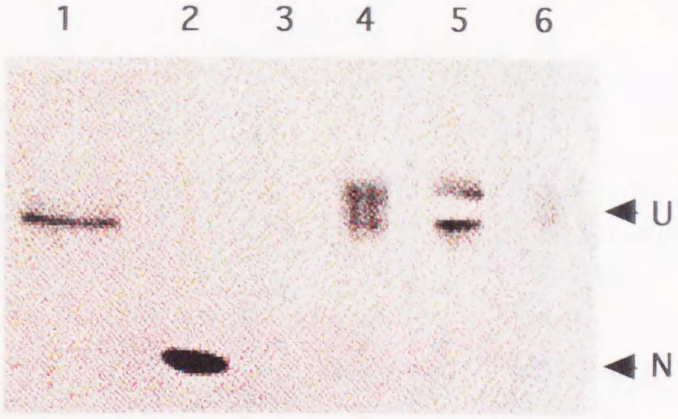

Fig. 2. Effect of GroEL on the acid-unfolded DMSOR. An aliquot (15 μl) of the acid-unfolded DMSOR in the refolding buffer was mixed with an equal volume of a solution of GroEL (a final concentration of 100 μg/ml), of GroEL plus GroES (100 μg/ml each), or of BSA (100 μg/ml). Mixtures were incubated at 20℃ for 2 h and 20 μl of each sample were subjected to electrophoresis on a non-denaturing polyacrylamide gel. Lane 1, acid-unfolded DMSOR after incubation at 20℃ for 2 h was completely reduced by boiling in the sample buffer (2% SDS, 5% 2-mercaptoethanol and 20% glycerol). Lane 2, purified active DMSOR. Lane 3, no additions. Lanes 4 to 6, GroEL, GroEL plus GroES, and BSA were added, respectively. For U and N, see the legend to Fig. 1.

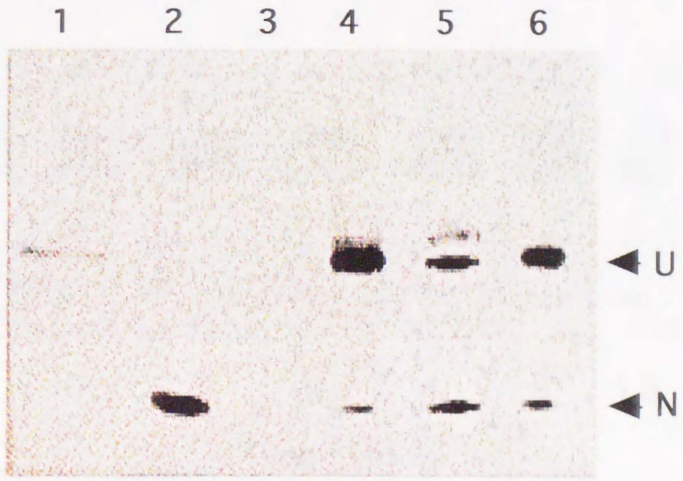

Fig. 3. Effects of DTT on the acid-unfolded DMSOR. The same experiment as described in the legend to Fig. 2 was performed except that the incubated samples included 10 mM reduced DTT. For lanes and symbols, see the legend to Fig. 2.

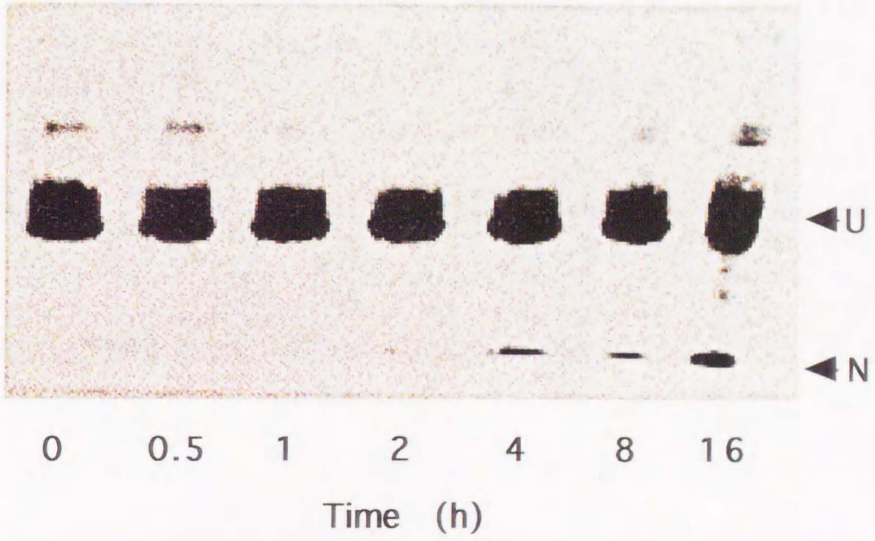

Fig. 4. Time course of refolding of the acid-unfolded DMSOR in the presence of DTT. The acid-unfolded DMSOR in the refolding buffer was incubated at 20℃ in the presence of GroEL and DTT as described in the legend to Fig. 3. At the times indicated, an aliquot (25 μl) of the mixture was withdrawn and placed immediately on ice, it was then subjected to electrophoresis on a non-denaturing gel. For symbols, see the legend to Fig. 1.

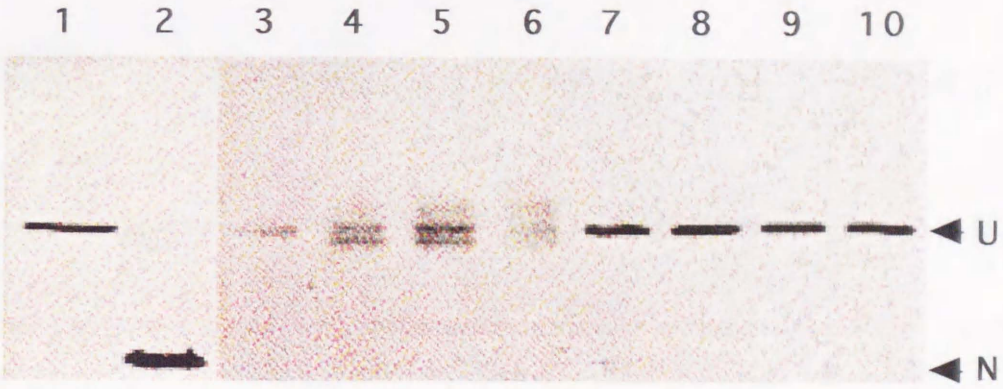

Fig. 5. Non-reducing SDS-polyacrylamide gel electrophoresis of the acid-unfolded DMSOR that has been incubated without or with DTT. The acid-unfolded DMSOR was incubated without (lanes 3 to 6) or with (lanes 7 to 10) DTT in the presence of various additions as described in the legends to Figs. 2 and 3. SDS was added to the samples at a final concentration of 2% and they were subjected to non-reducing SDS-polyacrylamide gel electrophoresis without boiling. Lanes 1 and 2 are the same as those in Fig. 2. Lanes 3 and 7, no additions. Lanes 4 to 6 and 7 to 10, GroEL, GroEL plus GroES, and BSA, respectively. For symbols, see the legend to Fig. 2.

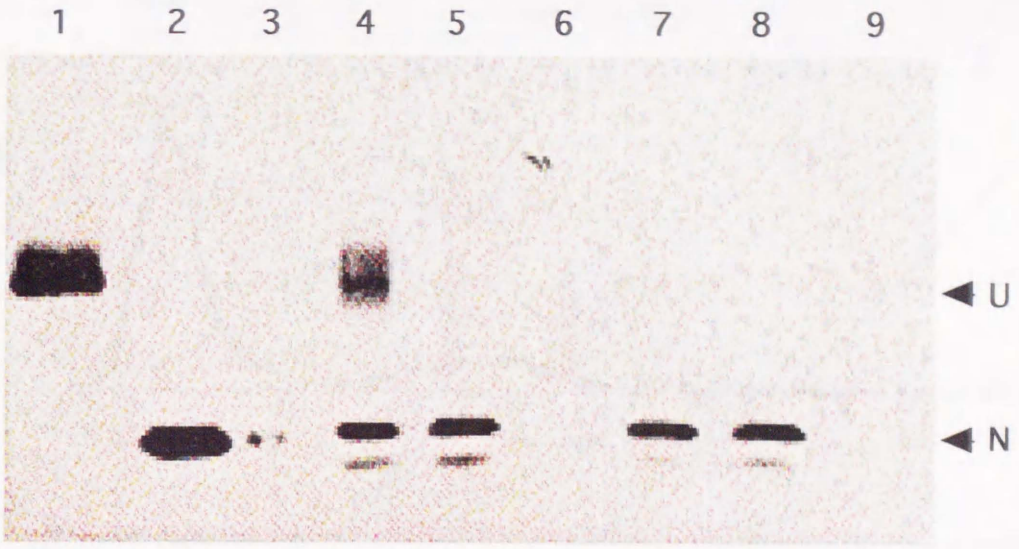

Fig. 6. Effect of the periplasmic, cytoplasmic and membrane fractions. Cells were grown photosynthetically in 150 ml of medium and periplasmic, cytoplasmic and membrane fractions were obtained as described in Materials and Methods. The final volume of each fraction obtained was adjusted to 5 ml. An aliquot (15 μl) of the acid-unfolded DMSOR in the refolding buffer was mixed with 15 μl of each fraction and incubated for 2 h at 20℃. Lanes 1 to 3 are the same as those in Fig. 2. Lanes 4 to 6, the periplasmic, cytoplasmic and membrane fractions were added to the acid-unfolded DMSOR, respectively. Lanes 7 to 9, the periplasmic, cytoplasmic and membrane fractions alone were subjected to electrophoresis on a non-denaturing gel, respectively. For symbols, see the legends to Fig. 1.

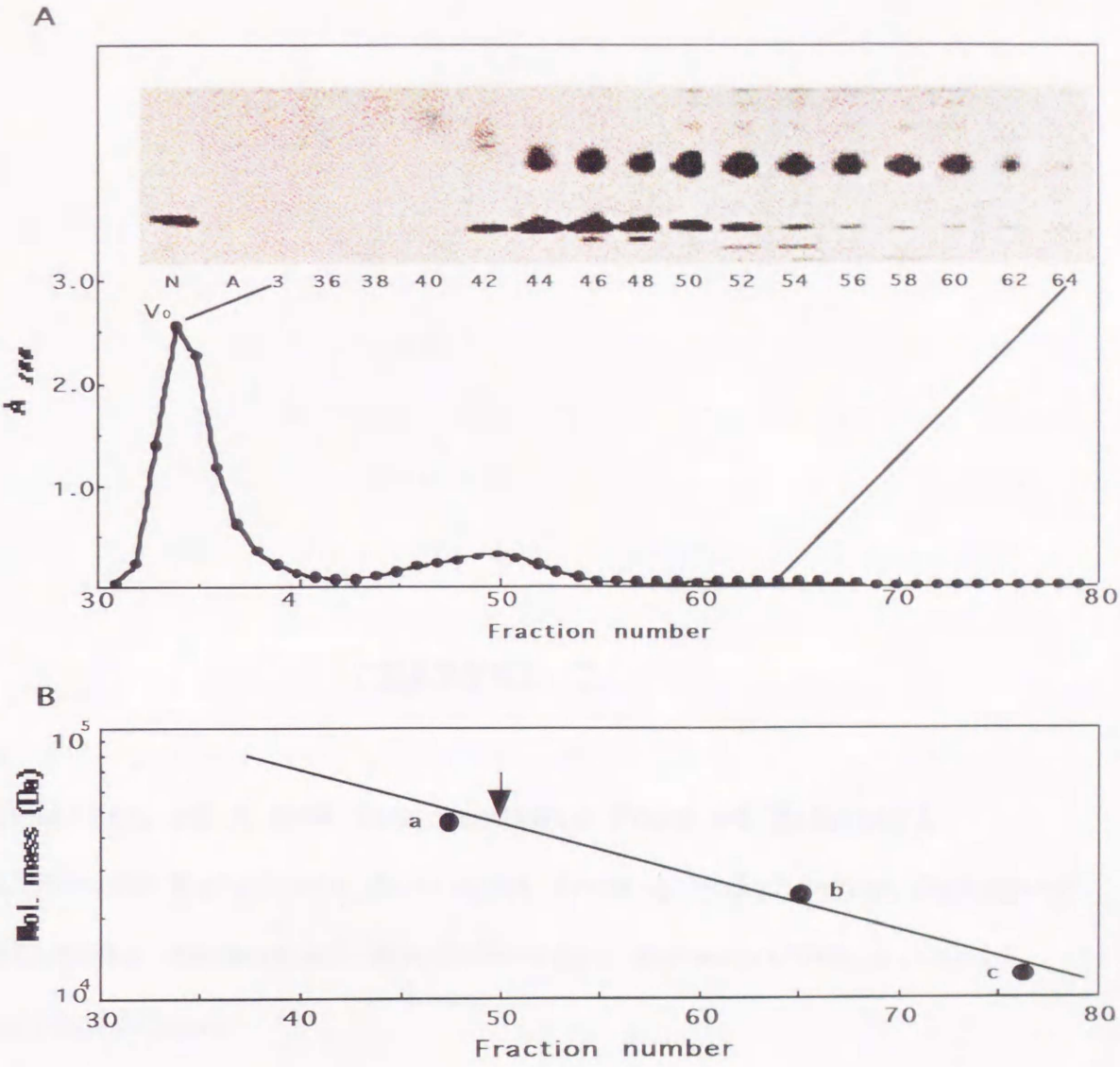

Fig. 7. Gel-filtration profiles of the periplasmic fraction. (A) Cells grown photosynthetically to exponential phase in 2 l of medium were harvested and the periplasmic fraction was obtained. The proteins were precipitated with 80% ammonium sulfate, dissolved in a minimum quantity of 50 mM Tris-HCl buffer (pH 7.5), and then fractionated (2-ml fractions) by chromatography on a column of Sephacryl S-200 (1 cm i.d. $\times$90 cm). Absorbance at 280 nm of each fraction was measured (Fig. 7A) and an aliquot (15 μl) of each fraction was used to determine its stabilizing activity, as described in the legend to Fig. 2 (Fig. 7A, inset). In the inset: numbers, fraction numbers; N, purified DMSOR; A, acid-unfolded DMSOR in the refolding buffer; V_0, void volume. (B) The molecular mass of the proteins in fraction 51 (arrow) was determined using the same column and molecular mass standards: a, ovalbumin (44.0 kDa); b, chymotrypsinogen A (24.5 kDa); c, cytochrome *c* (12.4 kDa).

CHAPTER 2.

Detection of a New Intermediate Form of Dimethyl Sulfoxide Reductase Secreted from a Molybdenum Cofactor-Deficient Mutant of *Rhodobacter sphaeroides* f. sp. *denitrificans*

ABSTRACTS

All of 9 cysteine residues in dimethyl sulfoxide reductase (DMSOR), the terminal reductase of DMSO respiration of *Rhodobacter sphaeroides* f. sp. *denitrificans*, existed as reduced forms, implying the absence of disulfide bond in DMSOR. We reported previously that spheroplasts prepared from a molybdenum cofactor-deficient mutant secreted both unfolded and folded forms of DMSOR into the medium (Masui et al. 1994). The unfolded form was also detected in DMSOR proteins secreted by spheroplasts from a wild type strain when iodoacetamide was present, suggesting that DMSOR is once secreted into the periplasm as the unfolded form. Another intermediate form of DMSOR was detected on non-denaturing gel between the unfolded form and one with native conformation in spheroplasts from a Mo cofactor-deficient mutant. This form was presumed to be a folding intermediate which has a similar configuration to the native one.

The periplasmic space is the region between inner and outer membrane of Gram-negative bacteria. Recently, the periplasm of *Escherichia coli* has been shown to play an important role in the expression of a wide variety of recombinant proteins from different sources. However, folding of proteins in the periplasm has not been studied in any great detail and its mechanism is unknown (Wulfing and Pluckthun 1994). The periplasm contains several enzymes that facilitate protein folding. DsbA (Bardwell et al. 1991, Kamitani et al. 1992, Akiyama et al. 1992, Alksne et al. 1995) that has disulfide isomerase activity, and PPI (peptidyl prolyl isomerase) that catalyzes *cis-trans* isomerization of proline (Harding et al. 1989, Takahashi et al. 1989, Hayano et al. 1991, Schonbrunner and Schmid 1992) are enzymes which can modify the structure of amino acid residues within polypeptides. However, no proteins in the periplasm are known to function as a general molecular chaperone. As to the physiological conditions required for protein folding in the periplasm, it has been argued that the periplasm maintains an oxidative condition to allow the formation of disulfide bonds, and that the presence of ATP which is required for the action of molecular chaperones is highly improbable (Wulfing and Pluckthun 1994).

Secretory proteins are believed to be secreted in a reduced and at least partially unfolded state to the secretory compartment, and the formation of disulfide bonds in the proteins is considered to take place concomitant with the secretion (Bardwell and Beckwith

1993, Randall 1992). In *E. coli*, for example, the disulfide bond formation proceeds so rapidly in wild type cells that the reduced forms of periplasmic proteins (e.g. β-lactamase, alkaline phosphatase, and OmpA) cannot be observed *in vivo*. They can be observed only in *dsbA* mutant cells (Bardwell et al. 1991, Akiyama and Ito 1993).

A denitrifying phototrophic bacterium, *Rhodobacter sphaeroides* f. sp. *denitrificans* (Satoh et al. 1976) is capable of dimethyl sulfoxide (DMSO) respiration as one of its anaerobic respiration systems. The terminal reductase of the respiration, DMSOR, is a periplasmic soluble protein with a molecular mass of 84,903 consisting of a single polypeptide and contains one molecule of molybdenum (Mo) cofactor and nine cysteine residues per molecule (Satoh et al. 1987, Yamamoto et al. 1995). Recently, its three-dimensional structure has been determined by x-ray crystallography (Schindelin et al. 1996). We reported previously that spheroplasts prepared from a Mo cofactor-deficient mutant secreted into the medium an unfolded form of DMSOR which was sensitive to trypsin and urea, in addition to the native conformation enzyme. The unfolded form was interpreted to be a stable folding intermediate (Masui et al. 1994). The unfolded form, however, could not be detected in spheroplasts from wild type cells. In an *in vitro* refolding experiment (Matsuzaki et al. 1996), we showed that acid-unfolded DMSOR prepared by releasing the Mo cofactor from DMSOR by acidification was stabilized in the presence of GroEL or a

protein(s) in the periplasm. Dithiothreitol (DTT) reduced the heterogeneous acid-unfolded forms to the fully reduced form and helped its refolding to the native conformation. Since DMSOR contains 9 cysteine residues per molecule, we thought at first that the fully reduced DMSOR would take the unfolded form, whereas the oxidized form with disulfide bonds the native conformation.

In chapter 2, I examined the redox nature of DMSOR, and describe that there exists no disulfide bond in native conformation DMSOR. A new folding intermediate with a similar conformation to the native DMSOR is also described. It was inferred that DMSOR is secreted into the periplasmic space first as an unfolded form and then folds to yield the native conformation via an intermediate without involving formation of any disulfide bonds.

MATERIALS AND METHODS

Bacteria and growth conditions

A green mutant strain of *R. sphaeroides* f. sp. *denitrificans* IL106 (Satoh et al. 1976) and its Mo cofactor-deficient derivative F182 (Masui et al. 1992) were used. The medium and conditions for growing the photodenitrifier were described previously (Yoshida et al. 1991).

Preparation of spheroplasts and secretion of DMSOR into medium by spheroplasts

Spheroplasts were prepared by the lysozyme-EDTA method, and secretion of DMSOR into the medium by spheroplasts was detected as described before (Masui et al. 1994).

Non-denaturing PAGE. non-reducing SDS-PAGE and immunoblotting analysis

DMSOR was separated by non-denaturing PAGE, and subjected to immunoblotting analysis after denaturing the protein on the gel by exposing to microwave (Masui et al. 1994). Non-reducing SDS-PAGE was done as described by Matsuzaki et al. (1996) by reference to the method by Ostermeier and Georgiou (1994) except that samples were not boiled after the addition of 2% SDS if not indicated.

Two-dimesional electrophoresis

Two-dimensional PAGE was carried out as described before (Masui et al. 1994) except that the non-denaturing gel in the first PAGE was immersed in the denaturing buffer containing 2% SDS but no 2-mercaptoethanol, and was incubated for 1 h at room temperature or was heated in a microwave oven for 20 s.

Determination of sulfhydryl groups

Sulfhydryl groups were titrated with 5,5' -dithiobis(2-nitrobenzoate), using an extinction coefficient of 13,600 M^{-1} cm^{-1} at 412 nm (Pollitt and Zalkin 1983). An aliquot (1-ml) of the incubation mixture containing 100 mM Tris-HCl (pH 7.5), 3% SDS, 5 mM EDTA, and 12 nmol of DMSOR was heated in the presence or absence of 50 mM DTT to 100℃ for 10 min. Protein in the incubation mixture was precipitated by the addition of 170 mg of trichloroacetic acid and precipitated by centrifugation. The precipitate was dissolved in the incubation mixture and precipitated again to remove traces of DTT. The amount of DMSOR protein was estimated from the total amino acid content after protein determination by the Lowry method using a corrected factor; the amount of DMSOR determined on the basis of total amino acid content was 1/1.26 of that determined by the Lowry method (Satoh et al. 1987).

RESULTS AND DISCUSSION

DMSOR contains no disulfide bond

The amino acid sequence of DMSOR indicates that there exists 9 cysteine residues at positions 12, 125, 219, 268, 297, 399, 615, 669, and 753 (Yamamoto et al. 1995). To determine whether or not these residues form disulfide bonds, sulfhydryl groups were titrated in denatured enzyme before and after reduction. Purified DMSOR was denatured by boiling in SDS in either the absence or presence of DTT, and free sulfhydryl content was assayed. DMSOR denatured in the absence and presence of DTT gave a sulfhydryl content of 9.07 eq/mol and 9.32 eq/mol, respectively, showing almost the same values irrespective of the presence of reducing agent. These results clearly indicate that native DMSOR contains no disulfide bond.

Susceptibility to IAA and DTT of DMSOR

This was further confirmed by use of non-reducing SDS-PAGE of IAA-modified DMSOR protein with IAA (Fig. 1). This method was first used to resolve disulfide bonded folding intermediates of a serum retinol binding protein within the endoplasmic reticulum (Kaji and Lodish 1993), or those of preOmpA-bovine pancreatic trypsin inhibitor in the periplasm of *E. coli* (Ostermeier and Georgiou 1994). When an SDS-unfolded polypeptide is modified with IAA in the absence of reducing agent, it is expected that the polypeptide molecules would retain some of the disulfide bonds, and migrate

faster than those having no disulfide bond. In contrast to this expectation, DMSOR protein boiled with SDS in the absence of IAA did not show such difference in electrophoretic mobility, but migrated to the same position (Fig. 1, lane 3) as that of DMSOR protein denatured in the denaturing buffer (U) (Fig. 1, lane 1). The DMSOR protein boiled with SDS in the presence of IAA in which all the exposed thiols groups were carbamoylmethylated, also exhibited the same migration (Fig. 1, lane 5) as the denatured DMSOR (U). These results further indicate the absence of disulfide bond in DMSOR. DMSOR incubated with SDS at room temperature remained in its folded form (Fig. 1, lane 2) and the addition of IAA had no effect (Fig. 1, lane 4).

We previously reported that both unfolded and native conformation forms of DMSOR protein were present in the suspension medium of spheroplasts prepared from a Mo cofactor-deficient mutant (Masui et al. 1994). On the other hand, only DMSOR with native conformation could be detected in the suspension medium of spheroplasts prepared from wild type cells. This is probably because the unfolded form had folded too rapidly, when the prosthetic group was present, to be detected. Since we did not find any disulfide bond in DMSOR with native conformation, we may consider naturally that the unfolded form contains no disulfide bond. It may also be assumed that, if IAA was present in the medium, an unfolded form would be detected in the suspension of spheroplasts from wild type cells.

Trap of the unfolded DMSOR with IAA

Figure 2 shows non-reducing SDS-PAGE of DMSOR proteins secreted into the medium containing IAA by spheroplasts prepared from either a Mo cofactor-deficient mutant or a wild type strain (A), and effect of DTT on the secreted DMSOR proteins (B). In spheroplasts from wild type cells, a sharp band of the unfolded DMSOR was detected in the presence of IAA (Fig. 2A, lane 6), whereas it was not in the absence of IAA (Fig. 2A, lane 5), suggesting that DMSOR is once secreted into the periplasm as the unfolded form in which all the thiol groups are exposed. In spheroplasts from the mutant both the unfolded and native conformation forms, although the band for the former was somewhat broadened, were detected in the absence of IAA (Fig. 2A, lane 3). The broadened band was converted to a sharp band (Fig. 2B, lane 2) when the medium was incubated with DTT before the electrophoresis. This suggests the formation of heterogeneous disulfide bonds sensitive to reduction by DTT with other proteins or in a molecule. Thus, in the presence of IAA, all the exposed thiol groups of the unfolded DMSOR were carbamoylmethylated and it migrated to make a sharp band (Fig. 2A, lane 4).

Strange to say was that the unfolded form secreted by spheroplasts from the mutant existed stably, being easily recognized on non-reducing SDS-PAGE. It means that the unfolded form escaped from its aggregation or digestion by some proteases. This might suggest that there exists a mechanism by which the unfolded DMSOR secreted into the periplasm is maintained in a reduced and non-

aggregated form. We previously showed in an *in vitro* experiment that acid-unfolded DMSOR was stabilized in the presence of a protein(s) in the periplasm (Matsuzaki et al. 1996). The protein(s) might participate in maintaining the unfolded form.

A large amount of DMSOR with native conformation present irrespective of the presence of IAA came from DMSOR produced in large quantities in the periplasmic space of wild type cells from which spheroplasts had been prepared.

The intermediate has a configuration similar to the native form

Figure 3 shows two-dimensional electrophoresis of the medium containing DMSOR proteins secreted by spheroplasts from the mutant. On the first non-denaturing gel, we found another band (I) between the broad band corresponding to U and the fastest migrating one corresponding to N (Fig. 3, A and B). U and N forms were identified as the unfolded form and one with native conformation, respectively (the second non-reducing SDS-PAGE, Fig. 3A). I form migrated to the same position as N and U in the second SDS-PAGE when the first gel was heated, indicating that it is truly DMSOR protein (Fig. 3B). When it was not heated, however, the mobility of I form was the same as N, but not U (Fig. 3A). This suggests that I form has a similar configuration to the native one. The I form might be a folding intermediate during folding of DMSOR.

Free Mo cofactor existing in cells of this phototrophic bacterium can be assayed by monitoring its ability to reconstitute

in vitro the activity of NADPH-dependent nitrate reductase present in cell extracts of the *nit-1* mutant of *Neurospora crassa* which lacks the ability of Mo cofactor biosynthesis (Miller and Amy 1983, Masui et al. 1992). It indicates that the Mo cofactors of DMSOR and the fungus nitrate reductase are exchangeable. Further, it suggests that the nitrate reductase of the Mo cofactor-deficient fungus exists in a native conformation. Thus, it does not seem strange that DMSOR with native conformation is formed in the medium after secreted by spheroplasts from the Mo cofactor-deficient mutant of *R. sphaeroides*, although the reconstitution of the DMSOR activity by the external addition of Mo cofactor has not been succeeded yet. We assume that a slow folding from the unfolded form to the native conformation of DMSOR might occur in the medium via I form when Mo cofactor is not present. Further characterization of I form could serve to elucidate the mechanism of protein folding in the periplasm.

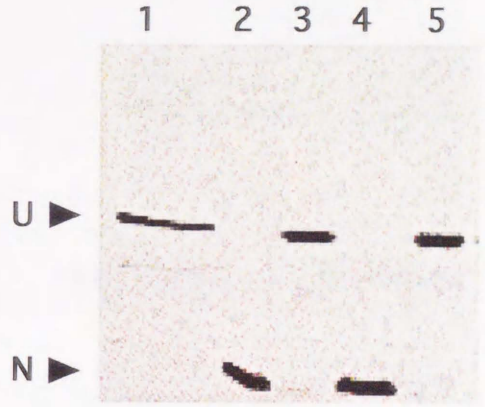

Fig. 1. Effect of SDS and IAA on DMSOR. The purified DMSOR (0.2 μg) was incubated with 2% SDS for 4 min at room temperature (lanes 2 and 4) or at 100℃ (lanes 3 and 5) in the absence of 30 mM IAA (lanes 2 and 3) or presence of IAA (lanes 4 and 5). Lane 1 is the purified DMSOR denatured by boiling in the denaturing buffer containing 4% SDS and 10% 2-mercaptoethanol. These samples were then applied to non-reducing SDS-PAGE. N, Native DMSOR. U, denatured DMSOR.

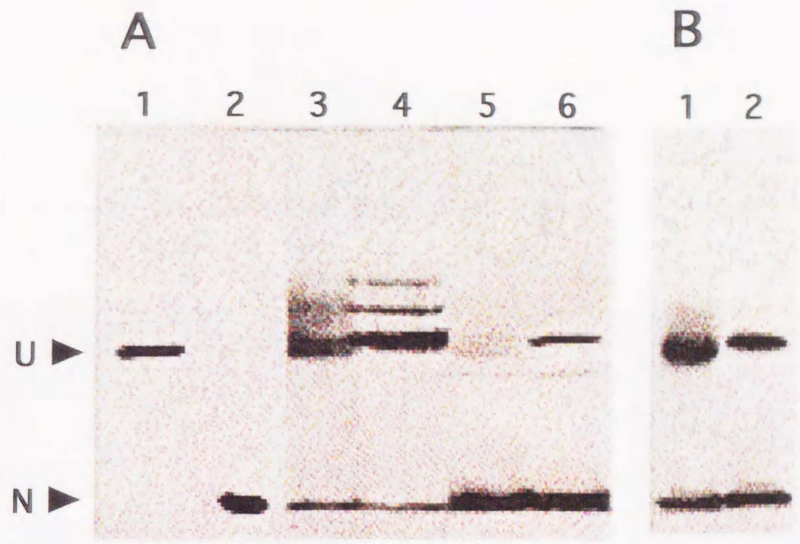

Fig. 2. DMSOR proteins secreted by spheroplasts in the presence of IAA (A), and effect of DTT on the secreted DMSOR proteins (B). A: Spheroplasts prepared from Mo cofactor-deficient mutant cells (lanes 3 and 4), or wild-type cells (lanes 5 and 6) were incubated in hypertonic medium for 2 h in the absence (lanes 3 and 5) or presence of 30 mM IAA (lanes 4 and 6). The medium was analyzed by non-reducing SDS-PAGE. B: The medium incubated with spheroplasts from Mo cofactor-deficient mutant cells in the absence of IAA was incubated without (lane 1) or with 10 mM DTT (lane 2) for 10 min at room temperature before being analyzed by non-reducing SDS-PAGE. Lanes 1 and 2 in Fig. 2A are denatured DMSOR and native DMSOR, respectively. Symbols, U and N, are the same as those in Fig. 1.

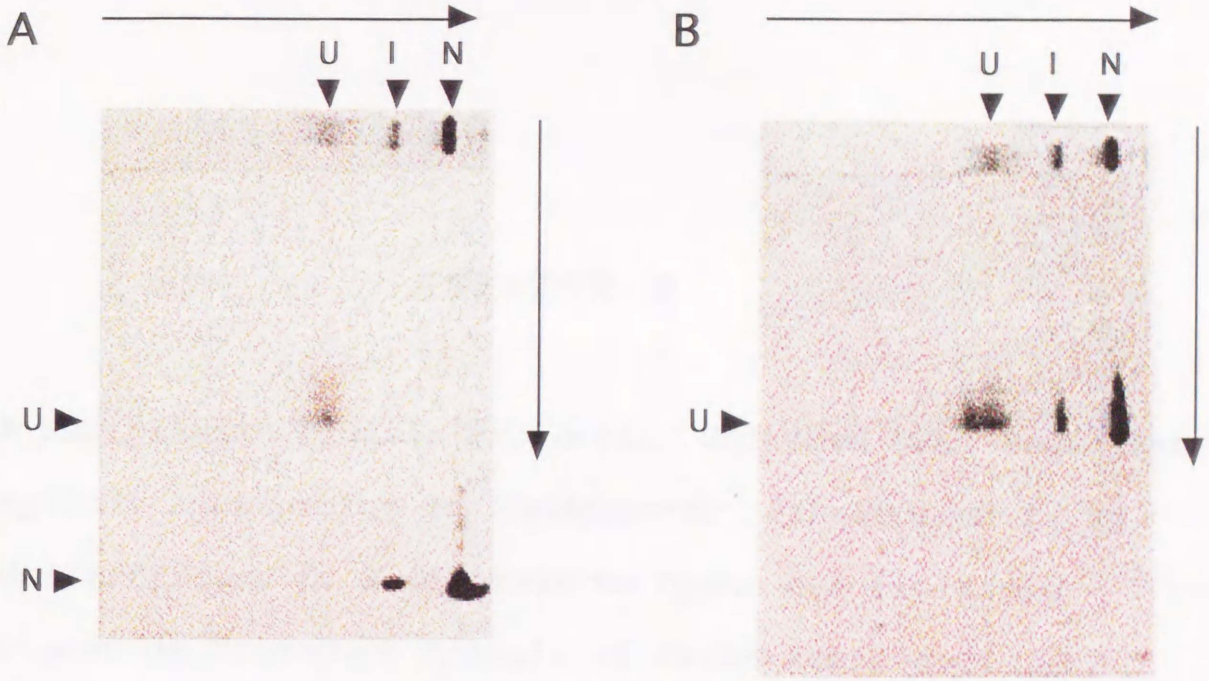

Fig. 3. Two-dimensional electrophoresis of secreted DMSOR. Medium (20 μl) incubated with spheroplasts from Mo cofactor-deficient mutant cells was first subjected to non-denaturing PAGE. The gel was immersed in the buffer containing 2% SDS but no reducing agent and incubated at room temperature (Fig. 3A) or heated in a microwave oven (Fig. 3B), and then subjected to non-reducing SDS-PAGE. Symbols, U and N, are the same as those in Fig. 1. I, Intermediate form.

CHAPTER 3.

A Periplasmic Protein Protecting Unfolded DMSO Reductase against Aggregation in *Rhodobacter sphaeroides* f. sp. *denitrificans* is Homologous to DppA, a Periplasmic Dipeptide Transport Protein of *Escherichia coli*

ABSTRACTS

Many proteins require assistance of protein such as molecular chaperones in order to maintain their unfolded forms during protein folding. However, such proteins have not yet been identified in the periplasm of Gram-negative bacteria. There exist a protein in the periplasm of *Rhodobacter sphaeroides* f. sp. *denitrificans* which can protect dimethyl sulfoxide reductase (DMSOR), a periplasmic terminal reductase of DMSO respiration, against aggregation. The protein was purified by monitoring *in vitro* the ability of suppressing aggregation of the acid-unfolded DMSOR. The protein had a molecular mass of 58 kDa and had no subunit. The sequence of 14 amino-terminal residues of the protein was completely identical to those of a periplasmic dipeptide transport protein (DppA) of *Escherichia coli*. The DppA-like protein was electrophoresed to made a broad band on non-denaturing gel. This protein represents the first example of a periplasmic protein that has molecular chaperone-like activity.

The periplasmic space is the region between inner and outer membrane of Gram-negative bacteria, and the compartment can be compared to the lumen of the endoplasmic reticulum (ER) in eukaryotic cells. Recently, the periplasm of *E. coli* has been shown to play an important role in the expression of a wide variety of recombinant proteins from different sources. However, the periplasmic folding of proteins has not been studied in any great detail and its mechanism(s) is unknown (Wulfing and Pluckthun 1994). The periplasm contains some enzymes that facilitate protein folding. DsbA (Bardwell et al. 1991, Kamitani et al. 1992, Akiyama et al. 1992, Alksne et al. 1995) which is required for the correct formation of disulfide bonds of periplasmic proteins *in vivo* and *in vitro* and PPI (peptidyl prolyl isomerase) which catalyzes *cis-trans* isomerization of proline are enzymes which modify the structures of amino acids within the polypeptides. However, no general molecular chaperone is known in the periplasm of Gram-negative bacteria. In the cytoplasm of Gram-negative bacteria, the GroEL, GroES and DnaK proteins appear to act as molecular chaperones, but there is no evidence that such cytoplasmic molecular chaperones have a direct effect on protein-folding processes in the periplasm.

The physiological conditions required for the protein folding in the periplasm are obviously important. It has been argued that the presence of ATP in the periplasm is highly improbable. Moreover, the periplasm is assumed to maintain an oxidative environment to allow the formation of disulfide bonds (Wulfing and

Pluckthun 1994).

A denitrifying phototrophic bacterium, *Rhodobacter sphaeroides* f. sp. *denitrificans* (Satoh et al. 1976) is capable of dimethyl sulfoxide (DMSO) respiration as one of its anaerobic respiration systems. The terminal reductase of the respiration, DMSOR, is a periplasmic soluble protein with a molecular weight of 84,903 consisting of a single polypeptide and contains one molecule of molybdenum (Mo) cofactor and nine cysteine residues per molecule (Satoh Kurihara 1987, Yamamoto et al. 1995). Recently, its three-dimensional structure has been determined by x-ray crystallography (Schindelin et al. 1996). We reported previously that spheroplasts prepared from a Mo cofactor-deficient mutant secreted into the medium an unfolded form of DMSOR which was sensitive to trypsin and urea, in addition to the native conformation of the enzyme, although the unfolded form could not be detected in spheroplasts from wild type cells (Masui et al. 1994). We examined the redox nature of DMSOR, and found that there exists no disulfide bond in native conformation of DMSOR (Matsuzaki and Satoh submitted). We also found that the unfolded form was detected in spheroplasts from wild type cells when iodoacetamide was present in the secretion medium and that a new folding intermediate was identified in the secretion by spheroplasts from Mo cofactor-deficient cells. It was inferred that DMSOR is secreted into the periplasmic space first as an unfolded form and then folds to yield the native conformation via a folding intermediate without involving formation of any disulfide

bonds. In an *in vitro* refolding experiment (Matsuzaki et al. 1996), we showed that acid-unfolded DMSOR prepared by releasing the Mo cofactor from DMSOR by acidification was stabilized in the presence of GroEL or a protein(s) in the periplasm. Dithiothreitol (DTT) reduced the heterogeneous acid-unfolded forms to the fully reduced form and helped its refolding to the native conformation. This provides the first experimental system to identify a protein(s) which can take a role of molecular chaperones in the periplasm.

In chapter 3, isolation of a periplasmic protein which have an ability of protecting the unfolded DMSOR against aggregation is described. The protein has a molecular mass of 58 kDa and has no subunit. The sequence of 14 amino-terminal residues of the protein was completely identical to those of dipeptide transport protein (DppA), a periplasmic protein of *E. coli* . This protein represents the first example of a periplasmic protein that has molecular chaperone-like activity.

MATERIALS AND METHODS

Bacteria and growth conditions

A green mutant strain of *Rhodobacter sphaeroides* f. sp. *denitrificans* IL106 (Satoh et al. 1976) was used. The medium and conditions for growing the photodenitrifier have been described previously (Yoshida et al. 1991).

Preparation of DMSOR

DMSOR was purified according to the published protocols (Satoh and Kurihara 1987).

Acid denaturation

DMSOR was denatured by eliminating Mo cofactor by acidification as described elsewhere (Matsuzaki et al. 1996).

Measurement for stabilizing ability

An aliquot (15 μl) of the acid-unfolded DMSOR in the refolding buffer was mixed with an equal volume of each fraction to be determined for the ability. Mixtures were incubated at 30℃ for 3 h and an aliquot (20 μl) of the mixture was subjected to electrophoresis on a non-denaturing polyacrylamide gel.

Protein determination

Protein concentration was determined using protein assay kit (Bio-Rad Laboratories, Inc.) with bovine serum albumin as a standard.

Purification of a protein with stabilizing ability

Purification of the protein was started with preparation of the periplasmic fraction, since the stabilizing factor was found to be located in the periplasmic space (Matsuzaki et al. 1996). The periplasmic fraction obtained from 10-liter bacterial cultures was fractionated with ammonium sulfate at 34-85% (0.2-0.65 g/ml) saturation, after which the prepicitate was dissolved in a minimum quantity of 50 mM Tris-HCl buffer (pH 7.5) and applied to a column (3.0 ×100 cm) of Sephacryl S-200 equilibrated with 50 mM Tris-HCl buffer (pH 7.5) containing 0.1 M NaCl. The proteins were eluted with the same buffer and the eluate was collected in 4.7-ml fractions. The stabilizing activity was recovered in fractions between 57 and 72. These fractions were pooled and applied to a column (1.5 ×10 cm) of DEAE-Sephacel equilibrated with 50 mM Tris-HCl buffer (pH 7.5) containing 0.1 M NaCl. The protein was eluted with linear NaCl gradient of 0.1-0.3 M in 80 ml of buffer. Stabilizing activity was recovered in fraction eluted at NaCl concentrations of 0.17-0.21 M. These fractions were pooled, concentrated by ultrafiltration, and resuspended in 50 mM potassium phosphate buffer (pH 7.0) containing 2 M ammonium sulfate. It was

applied to a column (1.5 ×6 cm) of Phenyl Sepharose CL-4B equilibrated with 20 mM potassium phosphate buffer (pH 7.0) containing 2 M ammonium sulfate. The proteins were eluted with linear ammonium sulfate gradient of 2 - 0 M in 80 ml of buffer. A protein with stabilizing activity was not eluted in ammonium sulfate concentration in the region of 2 -0 M. Then the protein was eluted with 10 mM potassium phosphate buffer (pH 9.0).

Non-denaturing gel electrophoresis and immunoblotting analysis

Samples were subjected to non-denaturing gel electrophoresis at 4℃ with 7.5% acrylamide using the Davis system (1964). DMSOR on the gel was transferred to a nitrocellulose membrane after the gel had been immersed in a buffer (250 mM Tris-HCl (pH 8.0), 2% SDS, 10% 2-mercaptoethanol) and heated in a microwave oven for 20 s to improve the sensitivity of detection of DMSOR (Masui et al. 1994), and was analyzed immunologically as described elsewhere (Yoshida et al. 1991).

Two-dimensional electrophoresis

Two-dimensional PAGE was carried out as described before (Masui et al. 1994).

Amino acid sequence analysis

A fraction from the ion exchange chromatography which had the highest stabilizing ability was electrophoresed on a SDS-polyacrylamide gel and blotted onto an ProBlott™ (Applied Biosystems) membrane. The membrane was stained with 0.2% Coomassie Blue R-250 and destained in 50% methanol. The most thick band was cut off out of the membrane and offered to analyze amino acid sequence with a protein sequencer (Applied Biosystems Inc., Model 473A).

Materials and chemicals

Sephacryl S-200, DEAE-Sephacel and Phenyl Sepharose CL-4B were obtained from Pharmacia Biotech (Uppsala, Sweden). GroEL was purchased from Takara Biochemicals (Kyoto, Japan) and bovine serum albumin (fraction V) was from Sigma Chemical Company (St. Louis, MO, U.S.A.).

RESULTS

Identification and Purification of the protein protecting the unfolded DMSOR against aggregation

In a previous work (Matsuzaki et al. 1996) we showed that acid-unfolded DMSOR aggregated during incubation in refolding buffer and that the aggregation was suppressed by incubation with GroEL, the best characterized cytoplasmic molecular chaperone from *E. coli*, or a protein(s) with a molecular mass of about 40 kDa in the periplasm from *R. sphaeroides*. Then, the periplasmic protein was further purified. The proteins were precipitated with ammonium sulfate at 34-85% saturation, and chromatographed on a column of Sephacryl S-200. The fractions with stabilizing effects on the acid-unfolded DMSOR were then chromatographed on a column of DEAE-Sephacel (Fig. 1). Although the data in the inset are not quantitatively exact, the peak of activity appeared around fractions 36 to 44, differing from the peaks of absorbance at 280 nm of 23, 52, 57, and 64. This means a specific protein is involved in the stabilizing activity. The recovered active fraction (fractions 36-44) had a slight amount of contamination of other proteins (Fig. 2). By further purification with Phenyl Sepharose CL-4B hydrophobic interaction chromatography (data not shown) the protein was purified to almost one polypeptide (Fig. 2). The protein consists of a single polypeptide with a molecular mass of 58 kDa, with consideration that the molecular mass was about 40 kDa by gel filtration. Figure 2

shows SDS-PAGE of proteins at various purification steps, showing that all the fractions contain a protein with a molecular mass of 58 kDa. The 58 kDa protein is clearly visible in the protein profile of crude periplasmic extracts, showing that it is abundant periplasmic protein in *R. sphaeroides* under the conditions used.

Specific stabilizing activity

We previously showed that GroEL had a strong activity of stabilizing the acid-unfolded DMSOR and that BSA also had a stabilizing effect, although its effect was always small. Then, the specific stabilizing activity of the purified protein was determined comparing with those of GroEL and BSA (Fig. 3). At protein concentrations of 1.0 and 0.5 μg/20 μl of the reaction mixture GroEL appeared to have the most intense activity among three, but the purified protein had a little weaker activity to GroEL. BSA had the activity but low. At a concentration of 0.2 μg/20 μl of the reaction mixture, the 58 kDa protein appeared to have rather stronger activity than GroEL. These results indicate that the 58 kDa protein had the activity comparable to GroEL. Since GroEL is an oligomeric protein composed of 14 subunits, the activity per monomer protein of GroEL is indicated to have more than 14-fold stronger than that of the 58 kDa protein.

Amino terminal amino acid sequence of the 58 kDa protein

The amino terminal sequence of the 58 kDa protein was

determined (Fig. 4). A comparison of the sequence with proteins in the DNA Data Base of Japan revealed that the sequence of 14 N-terminal amino acid residues is completely identical with DppA, the periplasmic dipeptide transport protein of *E. coli*. DppA was reported that it has a weak similarity to OppA, the oligopeptide-binding protein from *Salmonella typhimurium* but that it is more similar to OppA than any other protein of ORF in the data base (Olson et al. 1991). Though the N-terminal sequence of OppA is also aligned in Figure 4, only 5 residues were identical between the 58 kDa protein and OppA in 20 residues in N-terminal sequence. Then, we referred tentatively the 58 kDa protein to as DppA-like protein.

Association of DppA-like protein

We found that the DppA-like protein migrated to make a broad band on non-denaturing gel. We first questioned if the purified DppA-like protein preparation was contaminated with heterologous proteins or if the protein associated during PAGE in the absence of detergent. To address these questions, two-dimensional electrophoresis was carried out (Fig. 5). On the first non-denaturing gel, DppA-like protein migrated as the broadened band (Fig. 5A). The protein migrated to a position in the second SDS-PAGE after the first gel was heated by microwave oven in the denaturing buffer containing SDS and 2-mercaptoethanol. The result indicates that the purified protein is composed of truly only DppA-like protein and that the protein has a tendency to associate.

DISCUSSION

There exist a lot of proteins in the periplasm of Gram-negative bacteria. The periplasm of *E. coli* has been of great interest with respect to the functional expression of a wide variety of recombinant proteins from different sources. However, the periplasmic folding to make a three dimensional structure of unfolded proteins after secreted through the cytoplasmic membrane has not been studied in any detail and its mechanism is unknown. In the periplasm of *E. coli*, a class of proteins is reported which modify the structures of amino acids within polypeptides such as DsbA, which has protein disulfide isomerase activity and is required for the correct formation of disulfide bonds, and PPI, which catalyzes the *cis-trans* isomerization of proline. However, the molecular chaperones, which play roles in the correct folding of proteins by preventing side-reactions, are not known in the periplasm. I have thought that there should exist a mechanism which facilitates correct folding of periplasmic proteins.

DMSOR of *R. sphaeroides* f. sp. *denitrificans* is a good tool for the study about protein folding in the periplasm by the following reasons. First, it is produced by 10% of periplasmic proteins when it is induced with DMSO. Second, in spheroplasts from Mo cofactor-deficient cells, folding of DMSOR is such a slow event that we can easily identify an unfolded form and an intermediate form in the proteins secreted by spheroplasts into the medium. The unfolded

form has the same configuration as completely denatured DMSOR. The intermediate form has almost the same configuration as native DMSOR. Third, DMSOR has no disulfide bond although it contains 9 cysteine residues. So it folds without any help of proteins which take part in disulfide bond formation, indicating that participation of such proteins is not necessary to take into consideration. Fourth, DMSOR is easily unfolded by elimination of Mo cofactor by acidification. We found that the unfolded form aggregated during incubation in the refolding buffer and that GroEL and a periplasmic protein prevent the aggregation. This provides the first experimental system to identify a protein(s) which can take a role of molecular chaperones in the periplasm.

The purified protein had a sequence similarity to DppA protein of *E. coli* which is composed of 507 amino acid residues (Olson et al. 1991). It was surprising that 14 N-terminal amino acid residues of the purified protein were completely identical with DppA. DppA has regions of a amino acid similarity with a peptide-binding protein (OppA) from *S. typhimurium* which is composed of 497 amino acid residues (Hiles et al. 1987). Although DppA has a similar physiological role to OppA in the periplasm, the deduced amino acid sequence of DppA has an only weak similarity to that of OppA (24% identity). Therefore, the complete identity of 14 amino acid residues of the purified protein from *R. sphaeroides* with DppA is surprising, because both *E. coli* and *S. typhymurium* has similar nature taxonomically, belonging to the same family,

Enterobacteriaceae. Recently the structure of the unbound form of DppA has been determined (Nickitenko et al. 1995). DppA consists of two distinct domains (I and II) connected by two "hinge" segments which form part of the base of the wide groove between the two domains. The relative orientation of the two domains gives the protein a pearlike shape, with domain I and domain II forming the larger and smaller apical ends, respectively. Domain I consists of two integrated subdomains and domain II is more compact. This structure reminds us that of a molecular chaperone Hsp70. The three dimensional structure of the N-terminal fragment of bovine Hsc70 reveals that the ATPase domain consists of two lobes with the nucleotide bound at the base of a deep cleft between them (Gething and Sambrook 1992). The C-terminal domain of Hsc70, is proposed to be the specificity domain that binds target proteins. The C-terminal domain is deduced to have a consensus secondary structure and has a pattern of α-helices and β-strands that could be aligned with that of the α-1 and α-2 domains of the human MHC class I antigen HLA, suggested that there exists a peptide binding cleft in Hsp70. Therefore, it is very likely that the polypeptide chain which binds to the Hsp70 would bind in a extended conformation. In fact, it is reported that DnaK, *E. coli* Hsp70, binds a 13-residue synthetic peptide in a conformation that lacks any defined structural features. Then, it is likely that the wide groove between the two domains of DppA could bind the peptide. Although I have no evidence yet showing that newly isolated protein from *R.*

sphaeroides has the same three-dimensional structure as DppA protein, the new protein would have an ability of binding the unfolded form of DMSOR. Recently, it has been reported that proteins originally having other activities exhibit chaperone-like activity. For example, PDI promotes the in vitro folding of various proteins and is suggested to be involved in the quality control system, in which misfolded proteins are destined to degradation in the cell. It is very likely that the newly isolated protein would have such a chaperone-like activity.

I am now isolating the gene encoding the DppA-like protein from *R. sphaeroides* f. sp. *denitrificans*. Comparing the deduced amino acid sequence with DppA and OppA will provide advantageous information for understanding its three-dimensional structure, the binding region to the unfolded DMSOR, and mechanism of protecting the unfolded DMSOR from aggregation. Further, mutations causing defects in the gene will provide its physiological functions in the photodenitrifier.

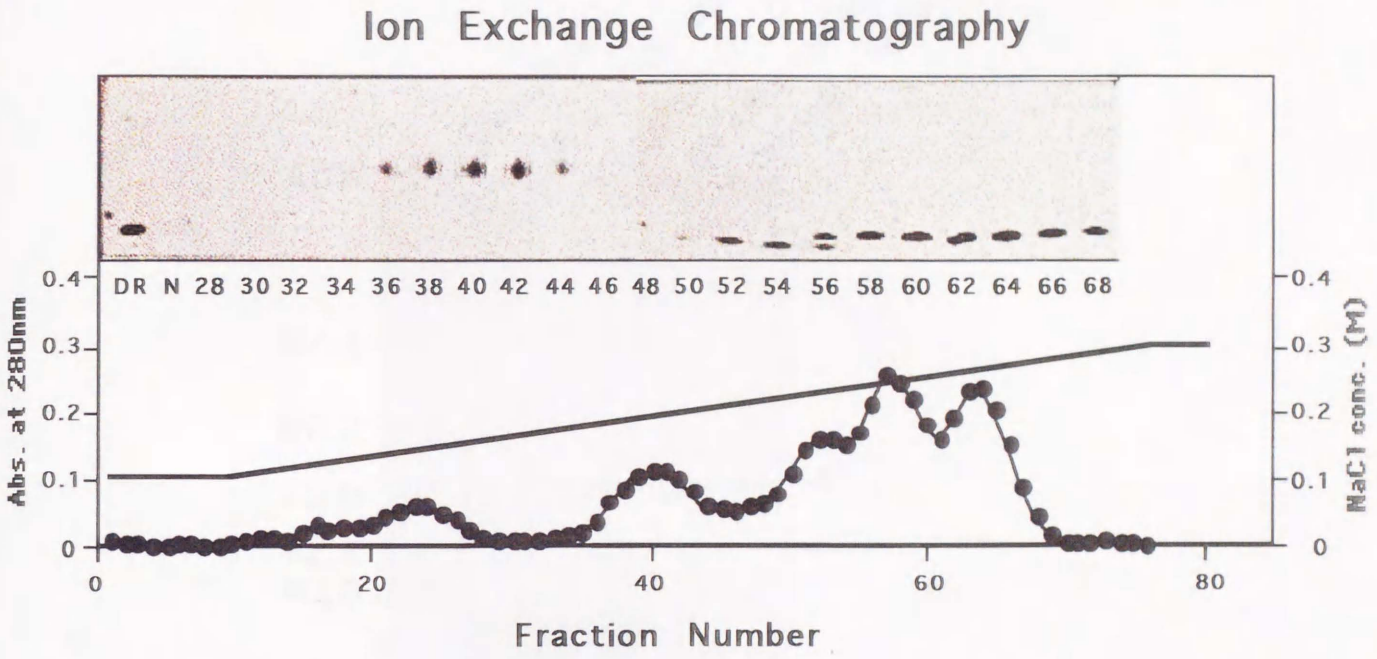

Fig. 1. Ion exchange chromatography profiles after gel filtration. The fractions with the stabilizing activity after gel filtration applied to a column (1.5 ×10 cm) of DEAE-Sephacel and eluted (2.1-ml fractions) with 50 mM Tris-HCl (pH 7.5) containing NaCl at concentrations from 0.1 to 0.3 M. Absorbance at 280 nm of each fraction was measured and an aliquot (15 μl) of each fraction was used to determine its stabilizing activity (inset).
DR, native DMSOR. N, acid-unfolded DMSOR with no sample.

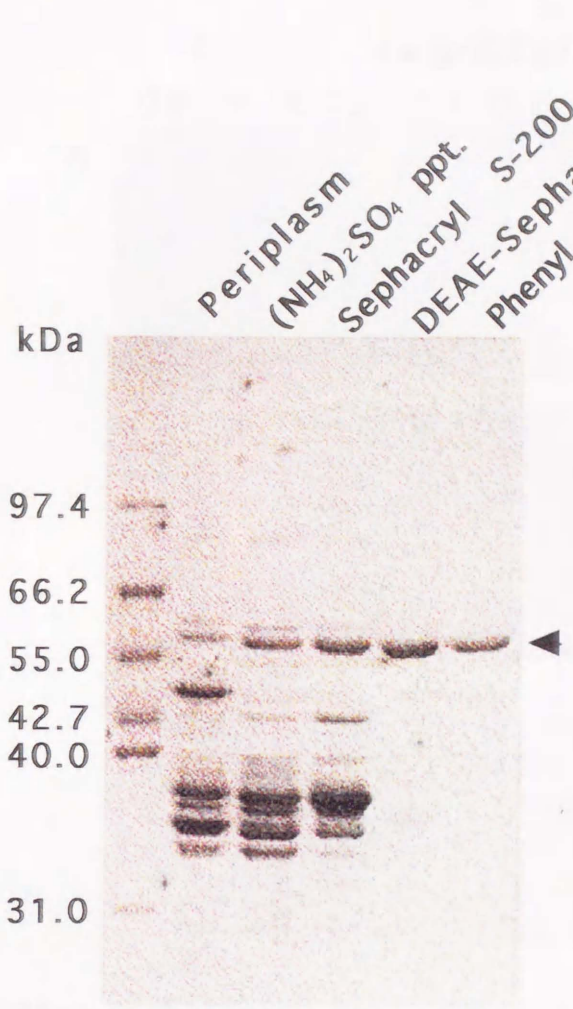

Fig. 2. Purification of the protein with stabilizing activity. Samples at each purification step were analyzed by 10% SDS-PAGE, and then stained with Coomassie Brilliant Blue. The amount of proteins applied to the lanes of the gel, from the left (periplasm) to the right (Phenyl Sepharose CL-4B) were, 20, 15, 15, 5 and 5 μg respectively. The migration positions of molecular weight marker proteins are shown to the left of the gel.

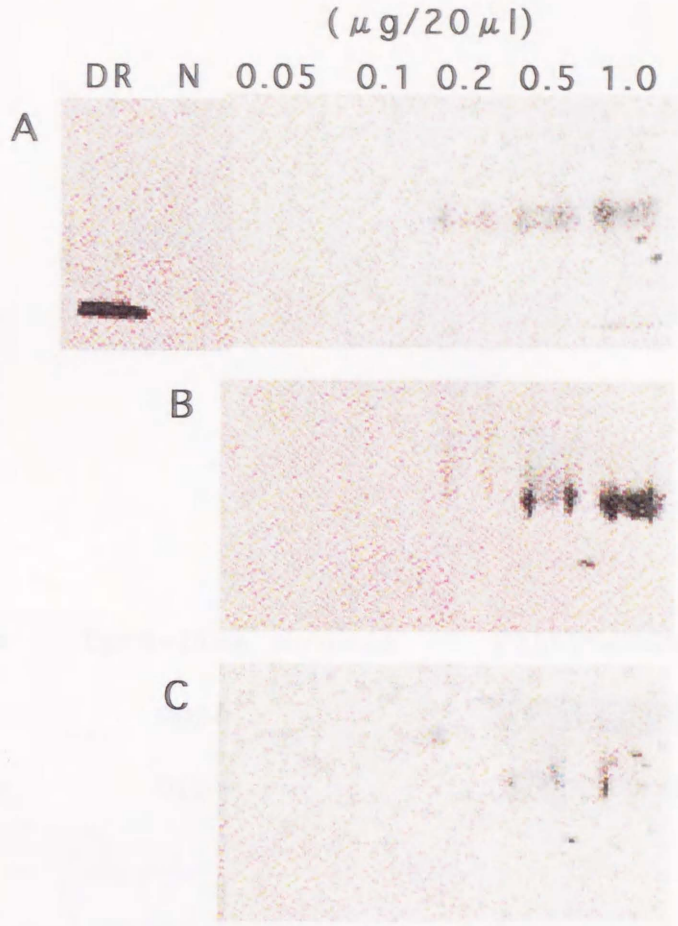

Fig. 3. Specific stabilizing activity of the protein. An aliquot (15μl) of the acid-unfolded DMSOR in the refolding buffer was mixed with an equal volume of the purified protein (A), GroEL (B) and BSA (C) at final concentrations of 0.05, 0.1, 0.2, 0.5 and 1.0 μg per 20 μl of the reaction mixtures each, respectively. The mixtures was incubated at 30℃ for 3 h to determine the stabilizing activity as Fig. 1. An aliquot (20 μl) was subjected to electrophoresis on a non-denaturing polyacrylamide gel.

DR, native DMSOR. N, acid-unfolded DMSOR with no sample.

R. sphaeroides	DppA-like protein	KTLVYCSEGSPEGFDPAPYT
E. coli	DppA	KTLVYCSEGSPEGFNPQLFT
S. typhimurium	OppA	QTLVRNNGSEVQSLDPHKIE

Fig. 4. N-terminal amino acid sequence of the isolated protein (DppA-like protein) of *R. sphaeroides*. Reverse contrast letters show identities of DppA or OppA to *R. sphaeroides* DppA-like protein.

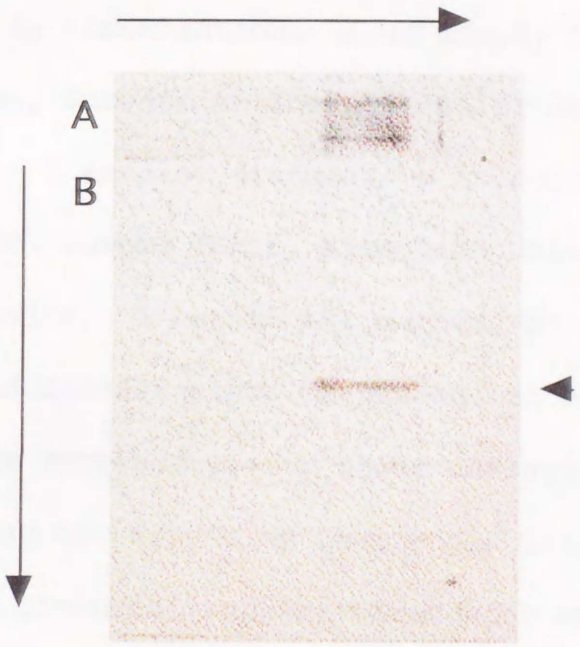

Fig. 5. Two-dimensional electrophoresis of DppA-like protein. DppA-like protein was first subjected to non-denaturing PAGE (A) and then electrophoresed on SDS polyacrylamide gel (B). The gels stained with Coomassie Brilliant Blue. The amount of proteins applied to the gel were 10 μg. The arrowhead indicates the position of DppA-like protein.

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