JOURNAL OF BACTERIOLOGY, Mar. 1994, p. 1624–1629
0021-9193/94/$04.00+0

Vol. 176, No. 6

Secretion of Both Partially Unfolded and Folded Apoproteins of Dimethyl Sulfoxide Reductase by Spheroplasts from a Molybdenum Cofactor-Deficient Mutant of *Rhodobacter sphaeroides* f. sp. *denitrificans*

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Spheroplasts prepared from a molybdenum cofactor-deficient mutant of *Rhodobacter sphaeroides* f. sp. *denitrificans* secreted dimethyl sulfoxide (DMSO) reductase which had no molybdenum cofactor and therefore no activity, whereas those from wild-type cells secreted the active reductase. The inactive DMSO reductase proteins were separated by nondenaturing electrophoresis into two forms: form I, with the same mobility as the native enzyme, and form II, with slower mobility. Both forms had the same mobility on denaturing gel. Form I and active DMSO reductase had the same profile on gel filtration chromatography. Form II was eluted a little faster than the native enzyme, suggesting that DMSO reductase form II was not an aggregated form but a compactly folded form very similar to the native enzyme. Form II was digested by trypsin and denatured with urea, whereas form I was unaffected, like native DMSO reductase. These results suggested that form II was a partially unfolded but compactly folded apoprotein of DMSO reductase.

Newly made proteins must be translocated to their specific place in a cell and then form highly ordered folded structures to function. Many in vitro experiments have been carried out to determine the mechanism by which proteins attain their native conformations, such as analysis using biophysical techniques of refolding of the denatured enzyme (1, 3, 6, 9, 12). Such experiments suggest that formation of stable secondary structures and covalent interactions such as disulfide bonds seem to occur during early stages of refolding, and then subsequent folding seems to occur through distinct compact intermediates called molten globules (13, 23, 24) or a collapsed form (12). The rate-limiting step in the refolding process occurs at a late stage, just before the protein adopts its final native conformation. A question, however, exists as to whether in vitro experiments accurately reflect the process of protein folding in vivo, since refolding in vitro is frequently much less efficient and often requires protein concentrations and physicochemical conditions very different from those that occur in a cell. Further, to obtain the denatured form a high concentration of urea or guanidinium chloride is often used (7, 12).

These in vitro studies were carried out with proteins such as RNase (1), lysozyme (10), cytochrome *c* (5, 14, 23, 27), α-lactalbumin (10, 24), and rhodanese (9). There has been little investigation, however, of whether the conformations are the same or different between the apoenzymes and holoenzymes in enzymes which contain prosthetic groups such as those involved in energy conservation and how incorporation of the prosthetic groups into apoprotein is coupled with the formation of native, physiologically active conformations.

Rhodobacter sphaeroides f. sp. *denitrificans* (25) is a phototrophic bacterium which possesses a versatile energy conservation system. The phototroph is capable of dimethyl sulfoxide (DMSO) respiration, the terminal reductase of which, DMSO reductase, is a periplasmic soluble protein consisting of a single polypeptide with a molecular mass of 82 kDa and containing one molecule of an Mo cofactor per molecule (26). We have been studying when and where the Mo cofactor is incorporated into the apo-DMSO reductase: whether from the cytoplasmic side during its translocation through the cytoplasmic membrane or at the periplasmic side after its translocation and processing are completed. We isolated Mo cofactor-deficient mutant strains and found that they accumulated the precursor form of DMSO reductase in the cytoplasm and cytoplasmic membrane as a result of partial inhibition of the processing and suggested that the Mo cofactor is incorporated into apo-DMSO reductase from the cytoplasmic side in connection with its translocation (17).

The DMSO reductase of the photodenitrifier has an advantage as an experimental system for investigation of the question described above in that the apo-DMSO reductase which does not contain the prosthetic group can be easily obtained under physiological conditions by using Mo cofactor-deficient mutants. In this study, we analyzed the apo-DMSO reductase secreted into the medium by spheroplasts with a newly developed method of nondenaturing polyacrylamide gel electrophoresis and found that the mutants secreted a mature-sized apo-DMSO reductase with a conformation distinct from that of the native enzyme in addition to the enzyme in the native conformation. We suggest that this is a stable folding intermediate form of native DMSO reductase.

MATERIALS AND METHODS

Bacteria and growth conditions. A green mutant strain of *R. sphaeroides* f. sp. *denitrificans* IL106 (25) and its chlorate-resistant derivative F182 (17) were used in this study. The medium and conditions for growth of the microorganisms were previously described (28).

Preparation of spheroplasts and the periplasmic fraction. Spheroplasts and the periplasmic fraction were prepared by using the lysozyme-EDTA method as described by McEwan et al. (20).

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spheroplasts. DMSO reductase was anaerobically induced for 3 h in the light in a 250-ml culture containing 0.2% DMSO, and spheroplasts were obtained. Spheroplasts (1 g [wet weight]) were suspended in 4 ml of malate basal salt medium containing 0.5 M sucrose and incubated in the light in the absence of DMSO. Every hour, 100 μl of the suspension was withdrawn and centrifuged to obtain medium. Then, 40 μl of the medium was subjected to nondenaturing polyacrylamide gel electrophoresis and immunoblotting analysis.

Assay for DMSO reductase activity and the Mo cofactor. DMSO reductase activity was assayed as previously described (26), and the Mo cofactor was also determined as previously described (17).

Nondenaturing polyacrylamide gel electrophoresis and immunoblotting analysis. Samples were subjected to nondenaturing gel electrophoresis at 4°C with 7.5% acrylamide by using the Davis system (4). Since the native form of DMSO reductase was difficult to transfer from the polyacrylamide gel to a nitrocellulose membrane and also to cross-react with anti-DMSO reductase serum raised in rabbits, DMSO reductase on the nondenaturing gel was denatured prior to transfer to the membrane. The gel was immersed in a buffer (250 mM Tris-HCl [pH 8.0], 2% sodium dodecyl sulfate [SDS], 10% 2-mercaptoethanol) and heated in a microwave oven for 20 s. The proteins on the gel were then transferred to a nitrocellulose membrane and analyzed immunologically as described elsewhere (28). This method improved the sensitivity of detection of the reductase 10 times or more.

Two-dimensional electrophoresis. The sample was subjected to nondenaturing polyacrylamide gel electrophoresis, and the gel was heated in the presence of both SDS and 2-mercaptoethanol as described above. The lane containing the sample was cut off, mounted on the top of an SDS-polyacrylamide gel, and secondarily electrophoresed by using the Laemmli system (15).

RESULTS

Secretion of DMSO reductase proteins by spheroplasts. To obtain DMSO reductase which contains no Mo cofactor, we developed a system of secretion by spheroplasts into the medium. We first unsuccessfully tried to induce and secrete DMSO reductase into the medium in spheroplasts that were prepared from cells that had not been induced with DMSO. We then found that spheroplasts prepared from cells in which DMSO reductase had been previously induced with DMSO secreted the reductase efficiently. Cells of Mo cofactor-deficient mutant F182 were cultured with DMSO for 3 h to induce apo-DMSO reductase, and the spheroplasts were prepared. The spheroplasts were suspended in hypertonic medium containing no DMSO and incubated. Every hour, an aliquot of the spheroplast suspension was withdrawn and the medium was subjected to nondenaturing polyacrylamide gel electrophoresis and immunoblotting analysis (Fig. 1B). Figure 1 shows that two types of DMSO reductase proteins with different mobilities on nondenaturing gel were secreted by the spheroplasts (Fig. 1B) and that both accumulated with time (Fig. 1D); band I has the same mobility as the purified enzyme, and band II has slower mobility. The protein quantity in band II was estimated to be approximately 50% of that in band I (Fig. 1D). In addition to these two main bands, another band with mobility between those of bands I and II appeared. This band was, however, too faint to be analyzed further. The spheroplasts prepared from the wild-type strain secreted a large amount of DMSO reductase (band I) and little band II protein (Fig. 1A). Figure 2B is the second SDS-gel electrophoresis after the nondenaturing

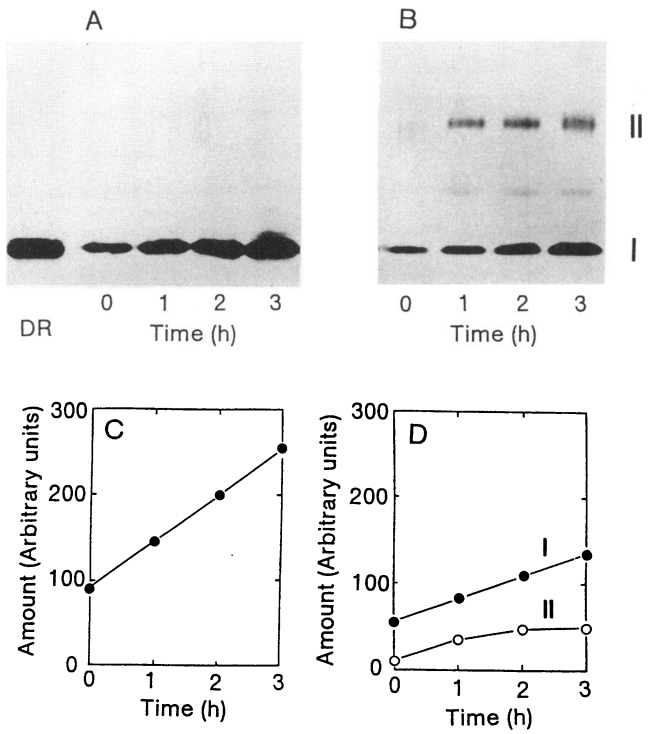

FIG. 1. Secretion of DMSO reductase (DR) proteins by spheroplasts. Spheroplasts prepared from wild-type and Mo cofactor-deficient mutant cells were incubated in hypertonic medium to induce secretion of DMSO reductase proteins. Every hour, an aliquot of the spheroplast suspension from the wild-type and Mo cofactor-deficient mutant cells was withdrawn and centrifuged to obtain medium. DMSO reductase proteins in the medium were subjected to nondenaturing polyacrylamide gel electrophoresis and analyzed by immunoblotting. (A) Secretion by spheroplasts from wild-type cells. (B) Secretion by spheroplasts from Mo cofactor-deficient mutant cells. (C and D) Quantitative analyses of immunoblots on the nitrocellulose membranes in panels A and B, respectively. Blots were analyzed with a personal computer program (Rasband), NIH Image 1.44, by using an Apple Macintosh personal computer. I and II, DMSO reductase protein forms I and II, respectively.

electrophoresis, showing that band II has the same mobility on SDS-gel as either band I or DMSO reductase. Since only one band of DMSO reductase was detectable with the antibody at the electrophoretic mobility corresponding to a molecular mass of 82 kDa (28), the band II protein was certified to be truly DMSO reductase protein.

Enzyme activity and Mo cofactor content of secreted DMSO reductase. To confirm that DMSO reductase protein secreted by the spheroplasts from the Mo cofactor-deficient mutant was inactive and contained no Mo cofactor, the activity and Mo cofactor contents in the medium after 3 h of incubation were measured (Table 1). The medium of spheroplasts from wild-type cells contained active DMSO reductase and high Mo cofactor activity. It is known that the Mo cofactor occurs in two forms: one is bound tightly to molybdoproteins and is able to be assayed only after release with heat treatment, and the other occurs in a free form or bound to a carrier protein (19). The Mo cofactor activity of the medium from wild-type cells could be measured only after heating it, indicating that most of the cofactor was probably bound to DMSO reductase. The amounts of bands I and II from the mutant cells in the medium after 3 h of incubation were calculated to be about 52 and 20%,

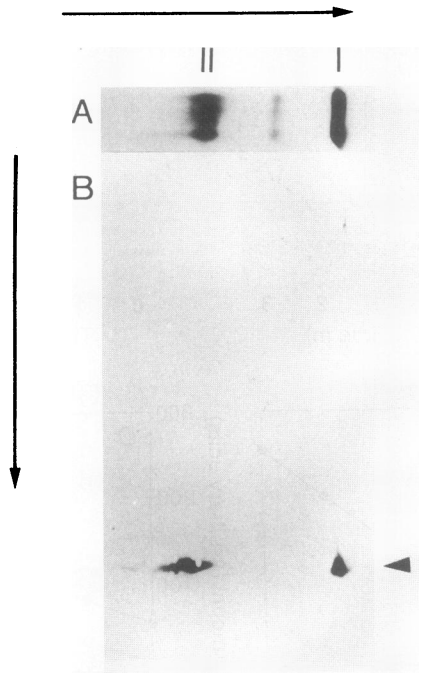

FIG. 2. Two-dimensional electrophoresis of secreted DMSO reductase proteins. Spheroplasts from Mo cofactor-deficient mutant cells were incubated for 3 h as described in the legend to Fig. 1. Medium (40 μl) was first subjected to nondenaturing polyacrylamide gel electrophoresis (A) and then electrophoresed on SDS-polyacrylamide gel (B). DMSO reductase proteins were analyzed by immunoblotting. The arrowhead indicates the position of native DMSO reductase. I and II, DMSO reductase protein forms I and II, respectively.

respectively, of that of band I from wild-type cells (Fig. 1C and D). On the other hand, the Mo cofactor content and DMSO reductase activity from the mutant cells in the medium after 3 h of incubation were 4 and 8% of those from wild-type cells, respectively. Thus, DMSO reductase forms I and II secreted by spheroplasts from the mutant cells seemed to contain little of the Mo cofactor. These results also indicate that the DMSO reductase protein into which the prosthetic group is not incorporated forms at least two kinds of conformations; one is the same as the active DMSO reductase, and the other has slow mobility on nondenaturing gel.

Secretion of band II by molybdenum-depleted spheroplasts

TABLE 1. Mo cofactor and DMSO reductase activities of medium[a]

Cell type and induction time (h)	Mo cofactor activity (U/ml of medium)	DMSO reductase activity (μmol of reduced methyl viologen/min/ml of medium)
Wild type		
0	320	0.63
3	841	1.20
Mutant		
0	8	0.02
3	32	0.09

[a] Spheroplasts from wild-type and Mo cofactor-deficient mutant cells were incubated as described in the legend to Fig. 1, and the activities of the medium after 3 h of induction were assayed. Mo cofactor activity was measured after heat treatment of the medium.

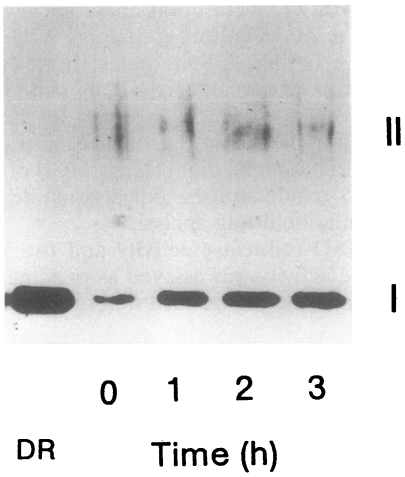

FIG. 3. DMSO reductase (DR) secretion by spheroplasts from wild-type cells in molybdate-depleted medium. Wild-type cells were anaerobically grown in the light with 0.2% DMSO in the absence of molybdate, and spheroplasts were prepared. Secretion of DMSO reductase by the spheroplasts was induced as described in the legend to Fig. 1. I and II, the DMSO reductase protein forms I and II, respectively.

from the wild-type strain. To confirm that the band II protein was produced only as a result of an Mo cofactor deficiency, the effect of removal of molybdate from the culture medium on the formation of this form by the spheroplasts from wild-type cells was studied (Fig. 3). Wild-type cells were cultured with DMSO in the absence of molybdate for 3 h, and then spheroplasts were prepared. Secretion of DMSO reductase by the spheroplasts was carried out under the molybdenum-depleted conditions. Band II was clearly formed and appeared on the nondenaturing gel as a smeared band.

Gel filtration chromatography of DMSO reductase secreted by spheroplasts. It was conceivable that band II was an aggregated form of band I and therefore moved slowly on nondenaturing gel. To examine this possibility, medium containing band I and II proteins secreted by spheroplasts of Mo cofactor-deficient mutant was subjected to gel filtration chromatography with Sephacryl S-300 (Fig. 4A), and the DMSO reductase proteins in the fractions were analyzed with nondenaturing gel electrophoresis (Fig. 4B). The elution peak of band II appeared to be between fractions 17 and 18, whereas both band I and the purified DMSO reductase were at fraction 18. Thus, band II is not an aggregated form. It is a single polypeptide with a compactly folded form very similar to that of active DMSO reductase.

Susceptibility to trypsin and urea. In *Escherichia coli*, precursor forms of β-lactamase (22), alkaline phosphatase (11), and maltose-binding protein (18) are easily digested by trypsin, whereas their mature forms are stable. Susceptibility to trypsin is believed to be a reflection of the unfolding of polypeptides. We examined the digestion of two forms of DMSO reductase proteins by trypsin (Fig. 5A). Medium containing band I and II proteins was treated with a quite low concentration of trypsin (10 μg/ml) at 0°C for 2 min, 200 μg of soybean trypsin inhibitor per ml was added, and the mixture was analyzed by nondenaturing gel electrophoresis. Band II was digested by trypsin, although the active DMSO reductase remained unchanged.

Urea is an agent which unfolds proteins, so the difference in sensitivity to urea among bands I and II and the active DMSO

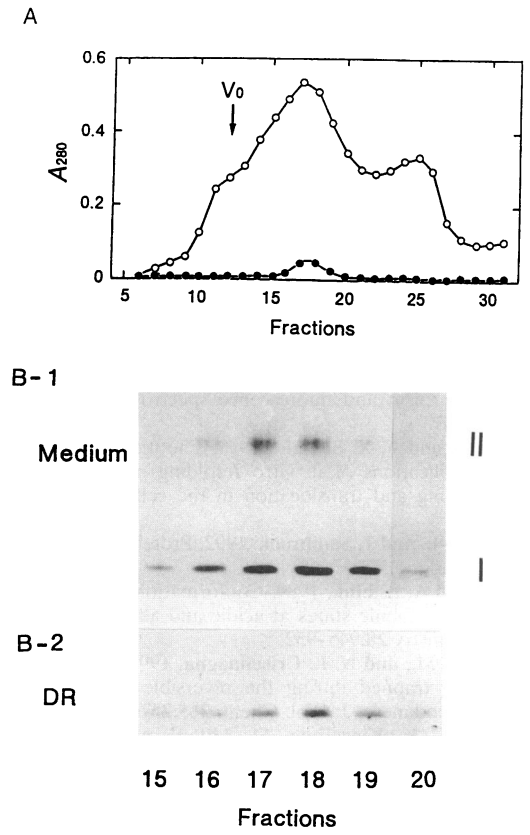

FIG. 4. Gel filtration chromatography of DMSO reductase (DR) proteins secreted by spheroplasts from Mo cofactor-deficient mutant cells. (A) Medium (200 μl) was fractionated (200 μl for each fraction) by chromatography on a column (0.5 cm [inside diameter] by 15 cm) of Sephacryl S-300 (Pharmacia) equilibrated with malate basal salt medium–0.5 M sucrose, and A_{280} was determined. Symbols: ○, medium of spheroplasts from Mo cofactor-deficient mutant cells; ●, chromatographed native DMSO reductase. V_0, voided volume. Each chromatography fraction (40 μl) was subjected to nondenaturing polyacrylamide gel electrophoresis and immunoblotting as for Fig. 1. (B-1) Medium. (B-2) Native DMSO reductase.

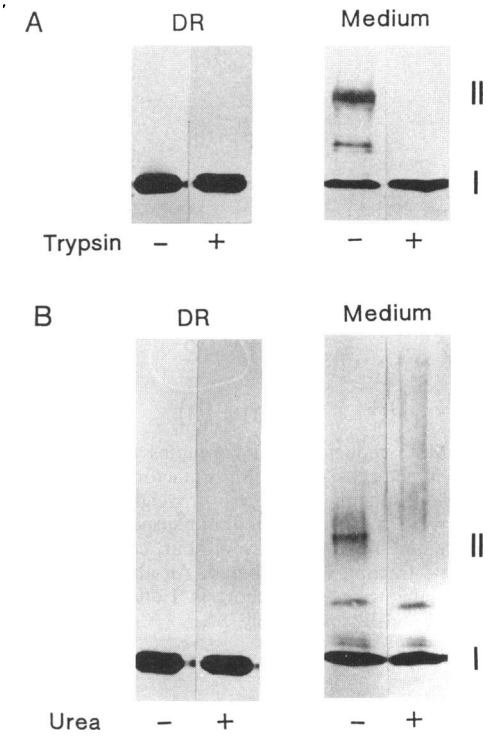

FIG. 5. Susceptibility of DMSO reductase (DR) proteins to trypsin and urea. (A) Trypsin (10 μg/ml) was added to the medium or to native DMSO reductase in 50 mM Tris-HCl (pH 7.2) and incubated at 0°C for 2 min, and then 200 μg of soybean trypsin inhibitor per ml was added. An aliquot (40 μl) was immediately subjected to nondenaturing polyacrylamide gel electrophoresis and analyzed immunologically as described in the legend to Fig. 1. (B) Urea (5 M) was added to the medium or native enzyme solution as described for panel A and incubated at 0°C for 10 min. Both samples were subjected to electrophoresis as described in the legend to Fig. 1. I and II, DMSO reductase protein forms I and II, respectively.

reductase was examined (Fig. 5B). The medium was incubated for 10 min at 0°C after addition of 5 M urea and analyzed by nondenaturing gel electrophoresis. Band II changed into a broadly spread band with yet slower mobility, although both band I and the active DMSO reductase were unchanged. These results suggest that the band II protein is compact but lacks a well-defined tertiary structure. That band I is insensitive to trypsin and urea although it contains no prosthetic group suggests that its structure is almost the same as that of the active enzymes. This means that a protein is able to form its native form even if its prosthetic group is depleted.

Spheroplast-specific secretion of the band II protein. A question arose as to whether the band II protein is produced in the periplasm of intact cells of the mutant. Cells of the mutant strain were incubated with DMSO for 3 h to induce DMSO reductase as described for the spheroplast experiment, and cells were harvested and suspended in fresh medium containing no DMSO. Every hour, cells were harvested and the periplasmic fractions were prepared and subjected to nondenaturing electrophoresis and immunoblotting (Fig. 6). It took about 2 h to obtain the periplasmic fractions from the cells. Although band II was observed at 0 h (Fig. 1), it had disappeared at 2 to 3 h.

DISCUSSION

Periplasmic proteins translocated across the cytoplasmic membrane are processed and folded into their catalytically active forms. The active forms generally possess tightly folded structures and are resistant to proteases, including trypsin, whereas incompletely folded or partially unfolded proteins are easily digested by trypsin. Therefore, trypsin susceptibility is widely used in biochemical studies on protein structure as an indicator of protein unfolding (2, 11, 16, 18, 21, 22). The nondenaturing electrophoresis method described here was also an efficient way to differentiate the native and other conformations. By using this method, we found that spheroplasts prepared from Mo cofactor-deficient mutant cells secreted two forms, I and II, of mature-sized DMSO reductase apoprotein (Fig. 1 and 2) which had little of the Mo cofactor and therefore little activity (Table 1). Form II, which had slower mobility than form I on nondenaturing gel, was easily digested by trypsin, as expected, and was also sensitive to 5 M urea. Thus, this method seems to reflect the conformation of a protein correctly.

The form II DMSO reductase which we found in this experiment is suggested to be partially unfolded because of its

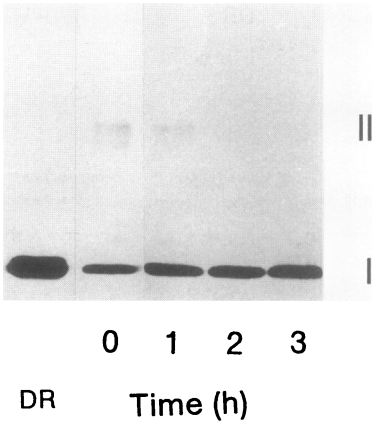

FIG. 6. DMSO reductase (DR) proteins in the periplasm after induction in intact cells of the Mo cofactor-deficient mutant. Mo cofactor-deficient mutant cells (1 g [wet weight]) previously induced with DMSO for 3 h were collected and suspended in 4 ml of fresh medium containing no DMSO. Every hour, cells were collected and the periplasmic fraction was prepared. An aliquot (40 μl) was subjected to nondenaturing electrophoresis. I and II, DMSO reductase protein forms I and II, respectively.

sensitivity to trypsin and urea. However, it has quite a compact conformation because it appeared on gel filtration chromatography to have a molecular mass similar to that of native DMSO reductase. This led to Ptitsyn's interpretation of form II as a molten globule or collapsed form (12), a compact folding intermediate. Collapsed forms are reported to occur commonly but under restricted conditions, such as in acid or alkaline pH and under high-salt conditions (8), and to have a strong tendency to aggregate (8, 13). Therefore, it is strange that form II exists in the medium in a stable form for at least 3 h even under physiological conditions. Collapsed forms are suggested to expose nonpolar side chains to water (8). If this is true for form II, it seems compatible with its slow mobility on nondenaturing gel. Form II existed stably in the periplasm of intact cells but seemed to disappear during incubation in the absence of DMSO (Fig. 6). This suggested that it changed to form I during incubation. Further, form I increased linearly with time, whereas the increase of form II halted at 2 h (Fig. 1D). These considerations suggest that form II is a kind of collapsed form or molten globule and a folding intermediate on the pathway of folding to form I. It is expected that further characterization with physicochemical methods of the form II protein will contribute to an understanding of the folding process.

This report shows that a protein possessing prosthetic groups is able to acquire the correct native structure even if the prosthetic groups are not incorporated into it. This may not be so strange; the Mo cofactor is assayed on the basis of its ability to reconstitute the activity of NADPH-dependent nitrate reductase from cells of a *Neurospora crassa nit-1* mutant which lack the cofactor, suggesting that the nitrate reductase has a native structure which accepts the cofactor to make an active enzyme. This is the case for DMSO reductase formed in the periplasm of intact cells of the Mo cofactor-deficient mutant of this phototroph. The question of why the partially unfolded DMSO reductase (form II) is formed in spheroplasts from the mutant at the higher rate arises. It can be said, however, that when the cofactor is absent, folding to a native form progresses at a slower pace than when it is present. This correct folding in vivo may be mediated by proteins known as members of the group of heat shock proteins (7), which recognize and stabilize form II to facilitate formation of the native conformation. It is conceivable, therefore, that lack of such heat shock proteins in the protein secretion system with spheroplasts gives rise to formation of the form II type of protein.

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