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Redox states of *Desulfovibrio vulgaris* DsrC, a key protein in dissimilatory sulfite reduction

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ABSTRACT

Dissimilatory reduction of sulfite is carried out by the siroheme enzyme DsrAB, with the involvement of the protein DsrC, which has two conserved redox-active cysteines. DsrC was initially believed to be a third subunit of DsrAB. Here, we report a study of the distribution of DsrC in cell extracts to show that, in the model sulfate reducer *Desulfovibrio vulgaris*, the majority of DsrC is not associated with DsrAB and is thus free to interact with other proteins. In addition, we developed a cysteine-labelling gel-shift assay to monitor the DsrC redox state and behaviour, and procedures to produce the different redox forms. The oxidized state of DsrC with an intramolecular disulfide bond, which is proposed to be a key metabolic intermediate, could be successfully produced for the first time by treatment with arginine.

1. Introduction

In prokaryotic sulfur-based energy metabolism, the dissimilatory sulfite reductase, DsrAB, is an essential enzyme that catalyzes the reduction of sulfite to sulfide. This reaction also involves the small protein DsrC, which contains two redox-active conserved cysteines in a flexible C-terminal arm. The electron transfer chain to DsrAB, and the mechanism and products of sulfite reduction have long been the subject of controversy. The determination of the crystal structure of *Desulfovibrio vulgaris* DsrAB in complex with DsrC was a major achievement towards understanding the mechanism of sulfite reduction and how energy conservation may be obtained in this process [1]. In this crystal structure, the C-terminal arm of DsrC projects inside DsrAB in a way that brings its last Cys right next to the active site, pointing to the direct involvement of DsrC in sulfite reduction. A solution structure of DsrC showed that its C-terminal arm is disordered [2,3], whereas in a crystal structure it adopted a more compact conformation where the two conserved Cys are close enough to allow a disulfide bond [4]. This closed conformation is also observed when DsrC is bound to DsrAB [5]. We proposed that the oxidized form of DsrC with an intramolecular disulfide bond is a key metabolic intermediate and the substrate for the membrane DsrMKJOP complex [1].

The *dsrC* gene is present in all organisms that contain DsrAB, be it sulfate/sulfite reducing organisms [6], sulfur-oxidizing bacteria [3] or organosulfonate reducers. In sulfate reducing bacteria *dsrC* is one of the most highly transcribed genes [7]. It is also one of the most abundantly expressed genes among sulfur metabolism genes in environmental metagenomic samples [8], and in intracellular symbionts [9]. A *dsrC* deleted strain could not be stably produced in the phototrophic sulfur oxidizer *Allochromatium vinosum* suggesting an essential role [3]. Hence, DsrC seems to be a key protein in dissimilatory sulfur metabolism.

DsrC is homologous to a larger family of proteins widespread in bacteria, such as *Escherichia coli* TusE in which only the last C-terminal cysteine is conserved. This cysteine is involved in sulfur-transfer reactions for the biosynthesis of thio-modifications of bacterial tRNAs [10]. In *A. vinosum*, the last cysteine of DsrC has also been shown to receive sulfur from persulfurated DsrE of the DsrEFH complex [11].

DsrC was initially considered as a third subunit of DsrAB [12], but this seems unlikely because their expression is not co-ordinately regulated [13] and several DsrAB proteins, like the one from *Archaeoglobus fulgidus* [14], do not include DsrC when isolated from the host organism. Instead, it was proposed that DsrC is directly involved in the sulfite reduction process [1,3,4], and that it may also interact with other proteins such as the DsrMKJOP membrane complex [1,15] and soluble HdrABC proteins [6]. In *Desulfovibrio* spp. the DsrAB protein is mostly associated with DsrC

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[1,12], but it has not been established if the reverse is true, i.e., if most DsrC in the cell is associated with DsrAB.

To further study the physiological function of DsrC and its interaction with possible partners, it is essential to be able to generate DsrC in its different redox states. The oxidized state with an intramolecular disulfide bond is not straightforward to produce due to the tendency of DsrC to dimerize through the last C-terminal cysteine [3]. In addition, there is no easy method to determine the redox state of DsrC in solution. Here, we report a convenient gel-shift assay to monitor this redox state and describe studies aimed at selective oxidation of the protein. In addition, we show that most DsrC is not associated with DsrAB.

2. Materials and methods

2.1. Cloning and expression of DsrC from D. vulgaris

The *D. vulgaris* Hildenborough *dsrC* gene (DVU2776) was amplified by PCR using genomic DNA and the following oligonucleotides: 5′-GTCCAAGGAGAAACATATGGCTG-3′ and 5′-CTTCCGCCT CGAATTCTCTTGT-3′ with NdeI and EcoRI restriction sites, respectively. The PCR product was cloned into pET-28a(+) vector (Novagen), which allows insertion of a 6×-His tag at the N-terminus. The recombinant plasmid was transformed in *E. coli* BL21Gold(DE3) cells (Stratagene) that were grown at 37 °C in M9 minimal medium with kanamycin (30 μg/mL) until an OD_{600} of 0.4. At this stage 100 μM of isopropyl-β-D-thiogalactopyranoside was added and growth was continued for another 4 h.

2.2. Protein purification

Cells were harvested by centrifugation, resuspended in 25 mM potassium phosphate, 300 mM NaCl, 30 mM imidazole pH 7.5 (buffer A), and disrupted in a French Press in the presence of DNase. The extract was centrifuged for 20 min at 20,000×*g*, followed by ultracentrifugation for 2 h at 140,000×*g*. The supernatant was loaded into a HiTrap Chelating HP column (GE Healthcare) charged with $NiCl_2$ and equilibrated with buffer A. The protein was eluted with the same buffer but containing 100 mM imidazole, and was concentrated and dialyzed to 25 mM potassium phosphate pH 7.5. The protein purity was analyzed by SDS–PAGE and the concentration was determined at 280 nm using the absorption coefficient of 18.6 mM^{-1} cm^{-1}.

2.3. Western blot analysis

D. vulgaris Hildenborough soluble cell extract was prepared as previously described [15], applied into a FF Q-Sepharose column equilibrated with 20 mM Tris–HCl pH 7.6, and a stepwise gradient of NaCl was performed. The protein fractions were subjected to 10% Tricine–SDS–PAGE, followed by transfer to 0.45 μm PVDF (polyvinylidene difluoride) membranes (Roche) at 100 V in a Mini Trans-Blot wet cell (Bio-Rad). The membranes were dried overnight and then blocked with TBS (20 mM Tris–HCl pH 7.5, 150 mM NaCl) containing 5% dry milk. Western blot analysis was conducted using the primary antibody (DsrC antiserum, 1:7,000 and DsrB antiserum, 1:10,000) for 1 h in TBST (TBS with 0.025% Tween 20), followed by incubation with the secondary antibody, anti-rabbit IgG conjugated with alkaline phosphatase (1:15,000, Sigma). Detection was performed using a solution of nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate toluidine salt.

2.4. MalPEG gel-shift assay and redox treatments

DsrC redox state was analysed by a gel-shift assay using MalPEG (methoxy-polyethylene glycol maleimide, MW 5000 g/mol, Fluka) as labelling reagent. The protein was treated with 1 mM MalPEG at 30 °C for 15 min (adapted from [16]). The reaction was stopped by addition of an equal volume of 2× SDS loading buffer (38 mM Tris–HCl buffer pH 6.8 with 10% glycerol, 6% SDS, 0.05% bromophenol blue) and subjected to 10% Tricine–SDS–PAGE without boiling the sample, under non-reducing conditions, and stained with Coomassie Blue.

To obtain reduced DsrC, the protein was treated with 5 mM dithiothreitol (DTT) during 30 min at 30 °C. Oxidation of DsrC was tested at 20 °C using: (i) 20 mM H_2O_2 for 24 h [3]; (ii) 10 mM TBHP for 30 min [4]; (iii) air oxidation for up to two weeks; and (iv) 1 M L-arginine for 2 h at 30 °C. Excess of reductant/oxidant was removed by centrifugal gel filtration (Micro Bio-Spin 6, Bio-Rad).

2.5. Mass spectrometry

DsrC was incubated with 10 mM iodoacetamide for 1 h at 30 °C. Samples were directly infused into a 12 tesla Bruker Solarix FTICR-MS (Fourier transform ion cyclotron resonance mass spectrometer) and on a platform coupling an ion mobility spectrometry separation with a time-of-flight mass spectrometer (IMS-TOF MS), in order to obtain structural information. The IMS-TOF MS platform built in-house couples a 1 m IMS drift cell with an Agilent 6224 TOF MS upgraded with a 1.5 m flight tube to attain a resolution of ~25,000. Further details on this platform can be obtained from [17].

3. Results and discussion

D. vulgaris DsrC was heterologously expressed in *E. coli*, with an N-terminal His-tag, and purified by metal affinity chromatography. SDS–PAGE showed that recombinant DsrC was pure, which was then used to produce polyclonal antibodies.

3.1. Association of DsrC with DsrAB

D. vulgaris soluble cell extract was fractionated in an ion-exchange chromatography column. The fractions obtained were analyzed by Western blot against DsrC and DsrB. DsrC was detected in two distinct and well separated regions (Fig. 1): fraction 6/7 (eluted at ~150 mM NaCl) and fraction 11–13 (eluted at 300–350 mM NaCl), which also contains the DsrAB protein. The majority of DsrC is present in fraction 6/7, which does not contain any DsrAB, as revealed by Western blot and by the absence of its characteristic absorbance at ~628 nm. Therefore, the majority of DsrC is not stably associated with DsrAB, and is thus free to interact with other proteins. In *D. vulgaris* and *Desulfomicrobium norvegicum*, most DsrAB is associated with DsrC, but different oligomeric forms can be present with one or two DsrC molecules per DsrAB dimer [18]. Our Western blot analysis indicates that the expression level of DsrC is higher than that of DsrAB, in agreement with previous reports [7].

3.2. A gel-shift assay to monitor the DsrC redox state

To monitor the redox state of the DsrC cysteines, a gel-shift assay was developed using MalPEG, which selectively labels free thiol groups covalently [16]. This modification can be detected by SDS–PAGE, since the molecular mass of the protein is increased

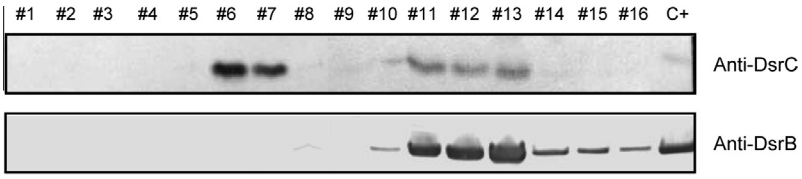

Fig. 1. Western blot analysis of fractions obtained from ion-exchange fractionation of *D. vulgaris* soluble cell extract. Fractions (30 μg) were resolved with a 10% Tricine–SDS–PAGE and further analysed by Western blot with anti-DsrC and anti-DsrB antibodies. *D. vulgaris* DsrABC complex was used as positive control (C+).

by ~10 kDa per SH group modified. After optimization, the method was tested with recombinant DsrC from *A. vinosum*, since this protein contains only the two C-terminal Cys (Cys100 and Cys111), and single and double Cys mutants are available [3]. Treatment of the recombinant wild type protein with MalPEG results in several band-shifted forms (Fig. 2, lane 2). The most intense band at ~50 kDa is assigned to the labelled dimeric species formed by an intermolecular disulfide bond between two terminal Cys111. This dimer has two MalPEG groups bound to the two free Cys100 (2×15 kDa [dimer] + 2×10 kDa [MalPEG] = 50 kDa). The other minor bands correspond to the dimer binding only one MalPEG group (30 + 10 = 40 kDa), and the monomer binding one (15 + 10 = 25 kDa) or two MalPEG groups ($15 + 2 \times 10 = 35$ kDa) (Fig. 2, lane 2).

MalPEG labelling of the *A. vinosum* DsrC Cys111Ser mutant produces a single 10 kDa band shift indicating it is mostly in monomeric form, as expected (Fig. 2, lane 5). In contrast, the Cys100Ser mutant is mostly in a dimeric form, which shows no shift with MalPEG due to the absence of free Cys (Fig. 2, lanes 7 and 8). The much higher concentration of dimer in the Cys100Ser mutant *versus* the wt protein reveals that Cys100 can promote the intramolecular reduction of the Cys111-Cys111 disulfide bond of the dimer, as previously proposed [3]. The double mutant does not react with MalPEG, as predicted (Fig. 2, lanes 10 and 11). Pre-reduction of DsrC with DTT followed by MalPEG labelling (Fig. 2, lanes 3, 6, 9 and 12) led to the expected results: a band shift corresponding to two labelled cysteines for the wt protein, one labelled cysteine for both single mutants, and no band shift for the double mutant. Thus, the study with *A. vinosum* DsrC confirms that the MalPEG gel-shift assay is a suitable method to monitor the solution redox state of this protein.

3.3. Redox states of *D. vulgaris* DsrC

D. vulgaris DsrC has three cysteine residues: the conserved C-terminal Cys93 and Cys104 (equivalent to *A. vinosum* Cys100 and Cys111), and Cys26 close to the N-terminus (Fig. S1). Thus, the gel-shift pattern is predicted to be more complex, and to facilitate interpretation a schematic representation is provided (Fig. 3).

MalPEG labelling of freshly purified *D. vulgaris* DsrC produced three main bands with ~35, 45 and ~70 kDa (Fig. 4A, lane 2). The 35 and 45 kDa bands are assigned to the reduced DsrC ($DsrC_{red}$) binding two and three MalPEG groups. The weaker 70 kDa band is assigned to the dimer ($DsrC_{dimer}$) binding four MalPEG groups. Hence, the heterologous expression of *D. vulgaris* DsrC produces mainly $DsrC_{red}$. Pre-reduction with DTT followed by MalPEG gives rise mainly to the 45 kDa band of $DsrC_{red}$, as expected, and also a 35 kDa band for the two most accessible Cys (Fig. 4A, lane 4).

Next, we tested conditions to obtain the oxidized form with an intramolecular disulfide bond ($DsrC_{ox}$). Previous studies reported that TBHP, H_2O_2 and air exposure could produce this form, at least partially [3,4]. For *D. vulgaris* DsrC we observed that treatment with TBHP or H_2O_2 led mainly to $DsrC_{dimer}$ (Fig. 4B, lanes 2 and 4 *versus* lane 1). Furthermore, MalPEG labelling after TBHP or H_2O_2 results in no band shifts (Fig. 4B, lanes 3 and 5). This indicates that the thiol groups of the Cys were converted into higher oxidation species that no longer react with MalPEG, such as sulfenic (R-SOH), sulfinic (R-SO(OH)) and sulfonic acid (R-SOH(=O_2)) forms. Thus, TBHP and H_2O_2 did not produce the $DsrC_{ox}$ form.

We also tested prolonged air exposure (2 weeks), as reported for *A. vinosum* DsrC [3], but in the case of *D. vulgaris* DsrC this resulted in very slow oxidation and a lot of protein precipitation.

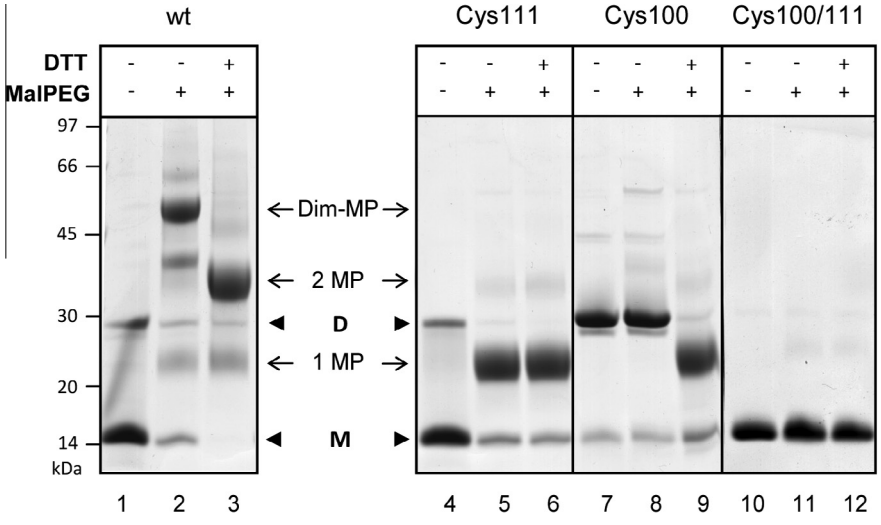

Fig. 2. Analysis of *A. vinosum* DsrC cysteines with MalPEG gel-shift assay, for the wild type (wt), single mutants (Cys111Ser and Cys100Ser) and double (Cys100/111Ser) mutant, in as-isolated state (lanes 1, 4, 7 and 10), after MalPEG treatment (lanes 2, 5, 8 and 11), and after DTT reduction followed by MalPEG treatment (lanes 3, 6, 9 and 12) (5 μg/lane). The arrow-heads indicate unlabelled DsrC monomer (M) and dimer (D), and the arrows indicate DsrC labelled with one or two MalPEG (MP) groups, and dimeric DsrC binding 2 MalPEG groups (Dim-MP).

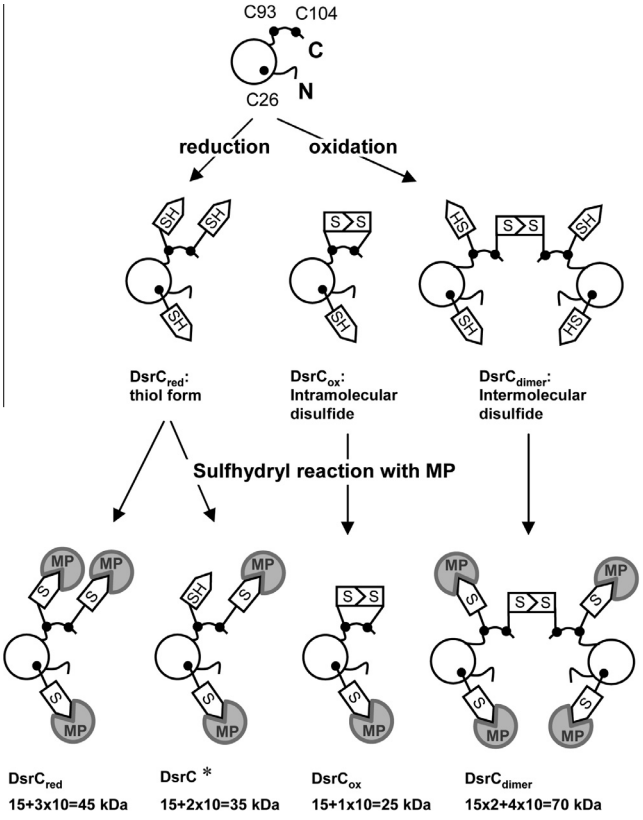

Fig. 3. Schematic representation of *D. vulgaris* DsrC redox states, and corresponding MalPEG (MP) modifications. This scheme does not depict the conformational rearrangement of the C-terminal arm.

The gel-shift assay of air-exposed protein revealed a series of bands resulting from one to three MalPEG additions to the DsrC monomer, plus the band corresponding to the dimer (Fig. 4B, lane 7). These results indicate that non-specific oxidation is occurring together with both intra and intermolecular disulfide bond formation. Thus, air oxidation is also not a suitable method to generate the DsrC$_{ox}$ form, since it leads to a mixture of states. These results confirmed that it is not straightforward to produce *in vitro* the intramolecular disulfide bond in DsrC.

Finally, we tested treatment with L-arginine, a low-molecular-weight additive that can suppress protein aggregation, and which has been reported to facilitate *in vitro* formation of disulfide bonds through a still unknown mechanism [19]. Incubation of DsrC with arginine, followed by MalPEG treatment yielded a protein giving a single band with ~25 kDa (Fig. 4B, lane 9), corresponding to the DsrC$_{ox}$ form. To confirm the formation of the intramolecular disulfide bond, the arginine-oxidized DsrC (Fig. 4B, lane 9) was treated with DTT followed by a second MalPEG treatment, resulting in the DsrC$_{red}$ pattern (Fig. 4B, lane 10). Thus, arginine treatment is the first method described that is able to achieve the specific oxidation of DsrC into DsrC$_{ox}$, in a complete and clean reaction. The guanidinium group of arginine has a pK_a of about 12.5, generating an alkaline pH that promotes the formation of the thiolate anion (R–S$^-$) and disulfide bond formation. However, incubating DsrC at the same final pH (11), in the absence of arginine, led only to partial formation of DsrC$_{ox}$.

We also tested the stability of the intramolecular disulfide bridge on DsrC$_{ox}$ by incubation at room temperature. With time, the DsrC$_{ox}$ gradually reverted back to a state with two and three Cys available for MalPEG modification (Fig. 4B, lane 11), suggesting that the DsrC$_{ox}$ form is unstable. In fact, a disulfide bond was never observed in the crystal structures, even when the two Cys are close together [4,5]. This may indicate that the formation of the intramolecular disulfide bond brings tension to the C-terminal arm and with time the S–S bond is disrupted, which agrees with the idea that the oxidized form of the protein might be a transient metabolic intermediate.

To further confirm the results obtained by the gel-shift assays, the samples were analyzed with mass spectrometry. Freshly prepared DsrC was treated with the cysteine-alkylating agent iodoacetamide that adds a mass of 57 Da per thiol group. The as-isolated untreated DsrC presented a major mass peak of 13,903 Da (for an expected mass of 13,905 Da, lacking the terminal Met), indicating that it is mostly in monomeric form. The DTT reduced, iodoacetamide-treated protein showed a mass peak of 14,133 Da that corresponds to the fully reduced protein. The arginine-oxidized, iodoacetamide-treated DsrC gave a mass peak of 13,961 Da, which corresponds to a single iodoacetamide group, confirming the production of the DsrC$_{ox}$ species. Samples were also analyzed with a platform that couples ion mobility spectrometry with time-of-flight mass spectrometry (IMS-TOF MS). The IMS separation is able to distinguish between separate conformers even if

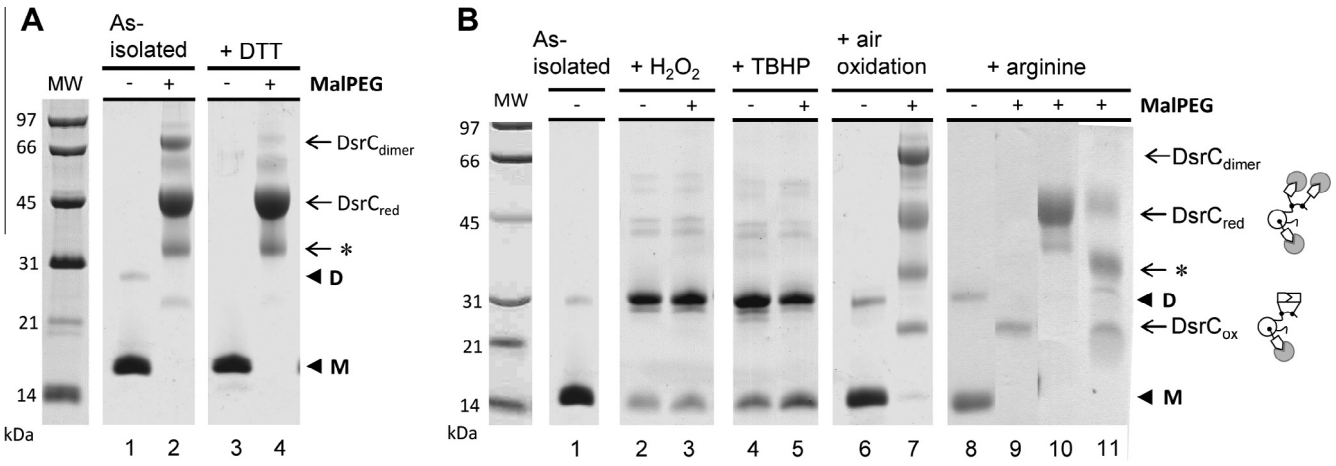

Fig. 4. Gel-shift assay of *D. vulgaris* DsrC. (A) DsrC as-isolated or reduced previously with 5 mM DTT. (B) DsrC after treatment with different oxidizing agents, namely with 20 mM H_2O_2, 10 mM TBHP, air oxidation, or 1 M arginine. In the case of arginine-oxidized DsrC (lane 8 and 9), this was followed by reduction with DTT and MalPEG treatment (lane 10), or a two-week incubation at room temperature (lane 11). Samples (5 μg/lane) were treated (+) or not (–) with MalPEG. The arrow-heads indicate unlabelled DsrC monomer (M) and dimer (D), and the arrows indicate DsrC binding one (DsrC$_{ox}$), two (*), three (DsrC$_{red}$), or four MalPEG moieties (DsrC$_{dimer}$).

they occur at the same mass as illustrated in a nested two-dimensional plot of IMS drift time *versus* mass. IMS-TOF MS analysis of the samples showed that the arginine-treated sample was different from the as-isolated and reduced forms (Fig. S2), in that the $DsrC_{ox}$ form had two distinct conformations with multiple charge states, while only one conformation was observed in the other forms. The more extended conformation of the arginine-treated sample appears similar to the other sample treatments, whereas the new distribution travelled through the IMS drift cell faster (higher mobility). This reflects a more compact form, as expected for $DsrC_{ox}$ since the C-terminal arm is retracted originating a more globular conformation. Another interesting observation was that the compact form was present in the mass spectrum at a lower charge state distribution (or higher m/z) than the extended conformer indicating a distinct structural packing and charge placement, further suggesting the presence of a different structure.

In conclusion, DsrC was shown to be a highly abundant protein in *D. vulgaris* cell extracts, with the majority not being tightly associated with DsrAB. We were successful in producing for the first time DsrC in the physiological oxidized state with an intramolecular disulfide bond, and showed that other oxidizing conditions lead to a heterogeneous mixture of redox states. The production of $DsrC_{red}$ and $DsrC_{ox}$ forms is an essential step towards future functional and interaction studies with physiological partners, such as the DsrMKJOP membrane complex, in order to investigate the energy-conservation mechanism associated with the reduction of sulfite. The optimized MalPEG protocol is likely to be useful for the study of other proteins containing redox-active cysteines.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.10.116.

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