

Characterization and Reconstitution of a 4Fe-4S Adenylyl Sulfate/Phosphoadenylyl Sulfate Reductase from *Bacillus subtilis**

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Carsten Berndt‡, Christopher H. Lillig§, Markus Wollenberg‡, Eckhard Bill¶, María C. Mansilla||, Diego de Mendoza||, Andreas Seidler‡, and Jens D. Schwenn‡**

From the ‡Biochemie der Pflanzen, Fakultät für Biologie, Ruhr Universität, Bochum 44780, Germany, §Medical Nobel Institute for Biochemistry, Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm, Sweden, ¶Max Planck Institut für Bioorganische Chemie, Mülheim-Ruhr, ||Instituto de Biología Molecular y Celular de Rosario and Departamento de Microbiología, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Rosario, Argentina

CysH1 from *Bacillus subtilis* encodes a 3'-phosphoadenosine-phosphosulfate-sulfonucleotide reductase (SNR) of 27 kDa. Recombinant *B. subtilis* SNR is a homodimer, which is bispecific and reduces adenylylsulfate (APS) and 3'-phosphoadenylylsulfate (PAPS) alike with thioredoxin 1 or with glutaredoxin 1 as reductants. The enzyme has a higher affinity for PAPS (K_m PAPS 6.4 μ M Trx-saturating, 10.7 μ M Grx-saturating) than for APS (K_m APS 28.7 μ M Trx-saturating, 105 μ M Grx-saturating) at a V_{max} ranging from 280 to 780 nmol sulfite $mg^{-1} min^{-1}$. The catalytic efficiency with PAPS as substrate is higher by a factor of 10 (K_{cat}/K_m 2.7 $\times 10^4$ –3.6 $\times 10^4$ liter $mol^{-1} s^{-1}$). *B. subtilis* SNR contains one 4Fe-4S cluster per polypeptide chain. SNR activity and color were lost rapidly upon exposure to air or upon dilution. Mössbauer and absorption spectroscopy revealed that the enzyme contained a 4Fe-4S cluster when isolated, but degradation of the 4Fe-4S cluster produced an inactive intermediate with spectral properties of a 2Fe-2S cluster. Activity and spectral properties of the 4Fe-4S cluster were restored by preincubation of SNR with the iron-sulfur cluster-assembling proteins IscA1 and IscS. Reconstitution of the 4Fe-4S cluster of SNR did not affect the reductive capacity for PAPS or APS. The interconversion of the clusters is thought to serve as oxygen-sensitive switch that suppresses SO_3 formation under aerobiosis.

Plants, fungi, and many bacteria reduce inorganic sulfate to sulfide to cover their need for the element sulfur. This assimilatory type of sulfate reduction has two reductive steps in which sulfate is reduced to sulfite and sulfite is reduced further to sulfide. The reduction of sulfate to sulfite requires two electrons at a redox potential of -517 mV, which is considered too high for physiological electron carriers. Chemical activation of the sulfate by forming a mixed anhydride between phosphoric and sulfuric acid as in adenylyl sulfate (APS)¹ or 3'-phosphoad-

enylyl sulfate (PAPS) lowers the potential to $mE_0 = -60$ mV, which is covered by thiols or pyrimidine nucleotides. The reduction is catalyzed by APS- and PAPS reductases. These proteins have a similar core protein of ~ 30 kDa in which the reactive center is located in a conserved ECG(L/I)H motif. Small redox proteins like thioredoxin (Trx) and glutaredoxin (Grx) are the physiological reductants for this group of reductases, whereas the APS reductases from higher plants also use monothiols like glutathione. Plant APS reductases that can use glutathione are larger than the Trx/Grx-dependent reductases because of an extension at their C terminus of ~ 16 kDa. This extension with its YAPWCXXC motif shows structural and functional similarities to thioredoxins or glutaredoxins (1–3). The core protein of the plant APS reductase and many of the PAPS reductase homologues found in different bacteria of the phytosphere contain a second CXXC and a Cys-Cys double cysteine motif. APS reductases of the plant type differ from PAPS reductases of the Trx-Grx type in that they contain a 4Fe-4S group (4). Kopriva *et al.* (5) reported that the loss of this chromophore had detrimental effects on the enzyme activity from *Arabidopsis thaliana*, although APS reductase from *Catharanthus roseus* still retained considerable catalytic activity in its absence (3). In view of the structural similarity between plant APS reductase and the reductases from *Rhizobium* (6) or *Pseudomonas aeruginosa* (7), it appeared plausible to suggest that these bacteria also reduce APS instead of PAPS. Williams *et al.* (8) support this view most recently. They concluded that the *Bacillus* gene product was an APS-specific sulfonucleotide reductase because *Bacillus subtilis* *cysH1* complemented an APS kinase-deficient strain of *Escherichia coli* (JM81A *cysC*). Based on a comparison of the primary structure of sulfonucleotide reductases from higher plants and bacteria, Kopriva *et al.* (9) propose that the 4Fe-4S reductases represent a new class of APS-specific reductases in which the occurrence of this 4Fe-4S group distinguishes the specificity for APS as substrate from the specificity for PAPS.

The molecular mechanism for assimilatory sulfate reduction is barely characterized in *Bacillus subtilis* and other Gram-positive bacteria. In this study, we analyzed *B. subtilis* sulfonucleotide reductase (SNR), the *cysH* gene product as model enzyme to explore the substrate specificity, and the reaction kinetics under steady state conditions using a recombinant sulfonucleotide reductase. The enzyme was isolated and analyzed as a tag-free protein obtained by using the intein-cata-

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** To whom correspondence should be addressed: Lehrstuhl Biochemie der Pflanzen ND2–149, Fakultät für Biologie, Ruhr-Universität-Bochum, Postfach 102148, 44780 Bochum, Germany. Tel.: 49-234-322-3657; Fax: 49-234-321-4396; E-mail: jens.schwenn@ruhr-uni-bochum.de.

¹ The abbreviations used are: APS, adenylylsulfate; EPR, electron paramagnetic resonance; Grx, glutaredoxin; IMPACT, intein-mediated purification with an affinity chitin binding tag; IscA and IscS, iron-

sulfur cluster (assembling) proteins; PAPS, 3'-phosphoadenylyl sulfate; SNR, sulfonucleotide reductase; Trx, thioredoxin; DTT, dithiothreitol; *Bs*, *B. subtilis*.

lyzed self-cleavage expression system. Data from ultraviolet-visible absorption, EPR, and Mössbauer spectroscopy were collected to elucidate the nature and function of the prosthetic group. The Mössbauer data presented here show that the enzyme from *B. subtilis* is a 4Fe-4S protein. The function of the 4Fe-4S group in the reduction of APS and PAPS was investigated using *Bs* apoprotein in which the iron-sulfur group was first removed by treatment with an oxidant in combination with a chelator and then reassembled using the iron-sulfur cluster-synthesizing proteins IscA1 and IscS. The effect of this treatment on substrate specificity for APS or PAPS was tested with either substrate in combination with recombinant thioredoxin or glutaredoxin from *E. coli* as reductants.

MATERIALS AND METHODS

Bacteria Strains, Plasmids, and Oligonucleotides—*E. coli* BL21(DE3) used for expression was obtained from Novagen (Bad Soden), and plasmid pTYB12 was from New England Biolabs. Plasmid pBscysH is a derivative of pTYB12 harboring a 732-base pair *cysH* PCR fragment from *B. subtilis*. The *cysH* fragment was amplified from a pET16b*cysH* subclone² of pBS170 (10) using NdeI, 5'-GAGAGACATATGTTAACGTATGATAATTGGGAAGAACC-3', and SmaI, 5'-CTCTCTCCGGGTTTCATGCGAGTCCCGATTCTGTTTTCGC-3', primers. NdeI/SmaI restriction sites (underlined) were introduced to enable in-frame cloning in pTYB12. Correct insertion and error-free amplification of the *BscysH1* gene cloned in pTYB was verified by DNA sequencing.

Growth of Bacteria—Transformed *E. coli* BL21(DE3) cells were grown in mineral medium (11) at a constant pH of 7.6 in a chemostat. The medium was supplemented with 2% glycerol, 0.5% glucose, 1% mineral Pfennig-medium SL4 (12), 20 μ M thiamin, and 100 μ g/ml ampicillin. The fermenter (Mercedes GmbH, Göttingen, Germany) was inoculated with a mid-log phase culture of *E. coli* BL21(DE3) at a concentration of 2% (v/v) harboring plasmid pTYB12*BscysH*. Cells were grown under vigorous aeration at 35 °C. When the cells had reached a density of A_{600} 0.7–0.8, adding isopropyl- β -D-thiogalactoside to a final concentration of 0.5 mM induced 0.8 expression of *cysH*. The temperature was reduced to 12 °C, and the cells were grown for 16 h until harvest by centrifugation at 10,000 $\times g$ for 20 min (yield 3–3.5 $\times g \times \text{liter}^{-1}$). They were washed once in 20 mM HEPES-NaOH, 200 mM NaCl, pH 8.0, and kept frozen at –28 °C until use.

Preparation of SNR—Pelleted bacteria were resuspended in 20 mM HEPES buffer/NaCl to give 0.1 g of wet weight/ml. The cells were disrupted by two consecutive passages through a French press (Ribi Cell Fractionator) applying 14.2 megapascals at 5–10 °C. The homogenate was clarified by centrifugation at 30,000 $\times g$ for 45 min and loaded to a column (24-mm-inner diameter \times 35 mm) packed with chitin beads (IMPACT-CN system, New England Biolabs). Self-cleavage of the chitin affinity intein tag was induced by on-column reaction of the bound protein with 50 mM cysteamine at 12 °C for 48 h. Tag-free protein was eluted with HEPES buffer/NaCl. The protein was concentrated by pressure dialysis under N_2 in an Amicon-type of a concentrator using a YM3 membrane. The average yield per liter of broth was 5 mg of SNR.

Protein intended for Mössbauer spectra was isolated from bacteria grown under virtually identical conditions in a medium containing ⁵⁷Fe(NH₄)₂SO₄ as the only source of iron. The protein (in HEPES buffer/NaCl) was purified as described above and enriched by ultrafiltration and pressure dialysis to give a final concentration of approximately 0.7 mM SNR.

SNR Assay, Preparation of ³⁵S-labeled Substrates, and Auxiliary Enzymes—Initial rates of SNR were measured as the formation of [³⁵S]sulfite from either [³⁵S]APS or [³⁵S]PAPS as described previously (13) and modified (for details see Ref. 14). The assay for kinetic constants contained 100 mM Tris-HCl, pH 8.0, 0.3 μ g of SNR, 10 mM dithiothreitol (or 10 mM reduced glutathione), thioredoxin 1 varied from 1.3 to 100 μ M (or glutaredoxin 1 varied from 1.3 to 80 μ M), [³⁵S]APS (1.3–60 μ M) (or [³⁵S]PAPS (1.3–50 μ M)), and 10 mM Na₂SO₃. [³⁵S]PAPS was prepared as described previously (15) using recombinant APS kinase (16). [³⁵S]APS was made from PAPS by nuclease treatment and purified by chromatography on a Fractogel/DEAE 650 (S) ion-exchanger.

Recombinant IscS and IscA1 gene products from *Synechocystis* PCC 6803 were prepared as described previously (17). His-tagged Trx1 and Grx1 were expressed as described in Ref. 14. Concentrations of Trx1

and Grx1 were determined using molar absorption coefficients at 280 nm (15,222 M⁻¹ cm⁻¹ and 10,810 M⁻¹ cm⁻¹), whereas soluble protein was routinely measured as Coomassie Brilliant Blue R dye complex (18) using bovine serum albumin as reference. The purity was examined by SDS-PAGE replacing DTT for β -mercaptoethanol.

Determination of Acid-labile Sulfide, Iron, and UV-visible Spectra—Hydrogen sulfide was measured colorimetrically as methylene-blue complex at 650 nm adapting the method of Beinert (19) as modified previously (20).

The content of iron in *Bs* SNR was determined by atom absorption spectroscopy in a Zeiss AAS3 spectrometer using a solution of Fe(NH₄)₂(SO₄)₂ in HEPES buffer/NaCl as reference. Column effluents were measured by the rapid colorimetric micromethod of Fish (21).

Optical spectra during SNR reconstitution were recorded on a Kontron Uvikon 810 two-beam spectrophotometer under anaerobic conditions using a slit width of 2 nm and 1-cm light path. The reference cuvette contained the reaction mixture without SNR. Spectra under aerobic condition were recorded using a diode array spectrophotometer (Beckmann DU 7400).

Reconstitution of *Bs* SNR—The Fe-S cluster of *Bs* SNR was completely removed by treatment with ferricyanide and extraction of the metal with EDTA (22). In a typical set up, 15 μ M SNR in 100 mM HEPES-NaOH, pH 8.0, were incubated on ice with 0.3 mM K₃Fe(CN)₆ and 0.75 mM EDTA for 10 min (ratio of enzyme, EDTA:K₃Fe(CN)₆, approximately 1:50:20). The apoprotein was then rapidly separated from ionic contaminants by gel filtration on PD10 (Amersham Biosciences) equilibrated with HEPES buffer. For reassembly of the 4Fe-4S cluster, approximately 200 pmol of SNR were incubated with 800 pmol of IscA1 precharged by IscS with 2Fe-2S cluster equivalents in HEPES buffer and DTT as described previously (17). Uncharged IscA1 or Fe(NH₄)₂(SO₄)₂ and Na₂S were used as control. The progress of reassembly of the 4Fe-4S cluster was tested measuring SNR activity with samples of 0.3 μ g. IscA1 or IscS did not exhibit any SNR activity.

The transition from 4Fe-4S to 2Fe-2S cluster was analyzed following the changes in activity and spectra of 20 μ M SNR incubated in HEPES buffer with 0.2 μ M IscA1, 0.2 μ M IscS, 80 μ M Fe(NH₄)₂(SO₄)₂, 200 μ M cysteine, 5 mM DTT, and 10 μ M pyridoxal phosphate. The kinetics of the 4Fe-4S cluster decomposition were measured at 35 °C under aerobic conditions following the changes in activity and spectra of 30 μ M SNR.

Mössbauer and EPR Spectra—Mössbauer data were recorded with a spectrometer of the alternating constant acceleration type. The minimum experimental line width was 0.24 mm s⁻¹ (full width at half-height). The sample temperature was maintained constant either in an Oxford Instruments Variox or an Oxford Instruments Mössbauer Spectromag cryostat. Isomer shifts are quoted relative to iron metal at 300 K.

EPR Spectra—X-band EPR spectra were recorded with a Bruker Elexsys E500 spectrometer equipped with a helium flow cryostat (Oxford Instruments ESR 910), an NMR gaussmeter, and a Hewlett-Packard frequency counter.

RESULTS

B. subtilis is the best characterized member of the Gram-positive bacteria. The wealth of available information regarding this bacterium has made *B. subtilis* the paradigm for analysis of the physiology of Gram-positive bacteria, of bacterial differentiation, and of useful application of bacterial products. However, the mechanism of sulfate reduction and cysteine biosynthesis remains poorly understood (23). *B. subtilis* PAPS reductase was originally discovered in a Tn917-mutagenized strain of BD170 screened for lipoic acid auxotrophy. Nutritional and complementational analysis of the transposon-treated strain indicated that the resultant auxotrophs were impaired for cysteine biosynthesis. Restoration to prototrophy of an *E. coli cysH*-deficient strain showed that the DNA region affected by insertional mutagenesis represented an ortholog of *E. coli cysH* (10). We subcloned *cysH* from *B. subtilis* in the vector/host system required for the intein-mediated purification with a chitin binding affinity tag. Because of its inducible self-cleavage activity, this expression system produces a gene product without affinity tag in a single chromatographic step. Yet, the main reason for using the intein system was because recombinant APS and PAPS reductases with a polyhistidine tag were observed to bind nonspecifically divalent metal ions.

² A. Prior, unpublished data.

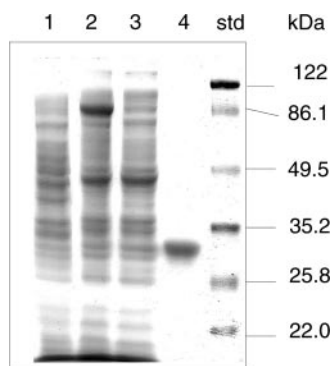


FIG. 1. **Expression of *B. subtilis* sulfonucleotide reductase.** SDS-polyacrylamide gel electrophoresis of cell extracts (10 μ g) from *E. coli* BL21 (DE3) stained with Coomassie Brilliant Blue R is shown. Lane 1, untransformed host; lane 2, transformed with pTYB12BscysH; lane 3, waste from the chitin-affinity column; lane 4, purified *B. subtilis* SNR (2.5 μ g); std, molecular weight markers and size of reference proteins as indicated.

Recombinant *Bs* SNR obtained with the intein expression system was homogeneous and migrated to a position corresponding to 29 kDa when examined by SDS-PAGE. This size is in good agreement with the mass predicted from the amino acid sequence (Fig. 1, $M_r = 26.974$).

Fe-S Cluster Sensitivity Toward Oxygen—The *cysH* gene product was eluted from the affinity matrix as brownish protein. The color faded rapidly under low ionic strength (e.g. gel filtration) and under aerobic conditions. In the early stages of protein purification, we observed that SNR recovered from column effluents had lost its activity together with most of its iron and the brownish color. As dilution and aerobic conditions appeared to induce de-coloration and loss of enzymatic activity, the purified protein intended for kinetic analysis was concentrated by pressure dialysis under nitrogen immediately after elution from the affinity column and kept in the highly concentrated form in 50% glycerol until use.

UV-visible absorption spectroscopy showed in addition to the absorption maximum for polypeptides at 280 nm a broad absorption band between 350 and 500 nm with a shoulder at around 410 nm. The brownish color faded rapidly upon exposure to air. Difference spectra of un-oxidized *versus* oxidized protein showed that the bleaching at 410 nm was accompanied by appearance of a new maximum at 468 nm (Fig. 2). This transition very strongly resembled the oxygen-induced conversion of the 4Fe-4S cluster of fumarate nitrate reduction protein reported by Koroshilova *et al.* (24). The absorption spectra obtained from native (and from reconstituted protein) were taken as evidence that the enzyme contains an iron-sulfur cluster. The occurrence of a Fe-S center was not unexpected because the primary structure of the homologous plant-type APS reductase was already described to contain a 4Fe-4S cluster (5). The plant-type APS reductase is 33% identical with a similarity of the core protein of approximately 53% also containing the typical SIGCAPC and Cys-Cys motifs in addition to the ECGL-catalytic center cysteine. To gain information regarding the atomic composition of an iron-sulfur cluster in *Bs* SNR, the content of iron was measured by atomic absorption spectroscopy, whereas the content of acid-labile sulfur was determined colorimetrically (19, 20). When purified *Bs* SNR was tested, 3.9 ± 0.03 equivalents of Fe and 4.8 ± 0.5 equivalents of sulfur as acid-labile sulfide were obtained per monomer of SNR (concentration of 0.21 mM per assay, three independent determinations). These data were taken as a stoichiometry of 4 irons and 4 sulfurs per monomer presumably originated from a 4Fe-4S cluster.

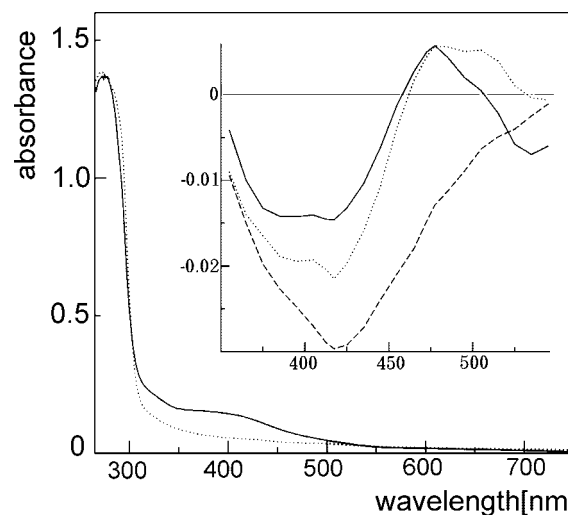


FIG. 2. **Optical spectrum of the *B. subtilis* sulfonucleotide reductase.** UV-visible spectrum of 0.1 mM *B. subtilis* SNR in HEPES buffer/NaCl as isolated with shoulder at 410 nm (solid line) and cluster-removed by oxidative treatment (dotted line) is shown. Inset, difference spectra of oxidized *versus* reduced enzyme. ϵ_{\min} 425 nm and ϵ_{\max} 468 nm as formed after exposure to air for 15, 45, and 205 min.

Mössbauer and EPR Spectroscopy—The presence of a cubane 4Fe-4S cluster could be nicely corroborated by Mössbauer measurements of a sample of SNR enriched in ^{57}Fe (concentration of protein 0.7 mM, Mössbauer-spectra taken from tag-free protein as isolated and concentrated without reconstitutive treatment described above). The spectrum recorded at 80 K is depicted in Fig. 3A. It shows an asymmetric doublet that could be deconvoluted in two independent symmetric quadrupole doublets with Lorentzian line shapes and isomer shifts and quadrupole splittings as follows: $\delta = 0.44 \text{ mm s}^{-1}$, $\Delta E_Q = 1.07 \text{ mm s}^{-1}$ (subspectrum I, $\Gamma_{\text{fwhm}} = 0.33 \text{ mm s}^{-1}$, 87% abundance) and $\delta = 0.32 \text{ mm s}^{-1}$, $\Delta E_Q = 0.44 \text{ mm s}^{-1}$ (subspectrum II, $\Gamma_{\text{fwhm}} = 0.30 \text{ mm s}^{-1}$, 13% relative abundance).

The Mössbauer parameters of the major “subspectrum I” are typical for $[\text{4Fe-4S}]^{2+}$ clusters with an average iron oxidation state $\text{Fe}^{2.5}$ that arises from valence delocalization of formally two Fe(II) and two Fe(III) (25). Particularly, the isomer shift, which is a measure of the charge density at the iron nucleus site, is very close to the average limiting values found for typical tetrahedral Fe(III)S_4 moieties found in oxidized $[\text{2Fe-2S}]^{2+}$ clusters ($\delta = 0.27 \text{ mm s}^{-1}$) and the valence localized Fe(II)S_4 site of the reduced $[\text{2Fe-2S}]^+$ clusters ($\delta = 0.64 \text{ mm s}^{-1}$) as expected for oxidized $[\text{4Fe-4S}]^{2+}$ clusters. The experimental spectrum of SNR shown in Fig. 3A does not show indication of magnetic broadening, which is in accord with the $S = 0$ ground state of the $[\text{4Fe-4S}]^{2+}$ cluster.

The low isomer shift and quadrupole splitting of “subspectrum II” resemble the values of iron(III) as it appears in $[\text{2Fe-2S}]^{2+}$ clusters. Although the archetypical $[\text{2Fe-2S}]^{2+}$ clusters in plant type ferredoxins (with all-cysteine coordination) show somewhat lower isomer shifts (0.27 mm s^{-1}), the value for subspectrum II in the SNR sample matches that of the $\text{His}_2\text{-Cys}_2$ -coordinated ferric ions in Rieske-type $[\text{2Fe-2S}]^{2+}$ centers ($\delta = 0.32 \text{ mm s}^{-1}$, $\Delta E_Q = 0.91 \text{ mm s}^{-1}$ (26)). Therefore, it is tempting to assign subspectrum II to an oxidized $[\text{2Fe-2S}]^{2+}$ cluster, which are probably $\text{Cys}_2\text{-Xaa}_2$ -coordinated units, because they would be formed by conversion of $[\text{4Fe-4S}]^{2+}$ centers in Cys_4 coordination.

Alternatively, however, we cannot readily exclude an interpretation of subspectrum II as a contribution from iron-hydroxide contaminations (e.g. $\gamma\text{-FeOOH}$, which would have had $\delta = 0.30 \text{ mm s}^{-1}$, $\Delta E_Q = 0.55 \text{ mm s}^{-1}$ (at 291 K)). It might be not unusual

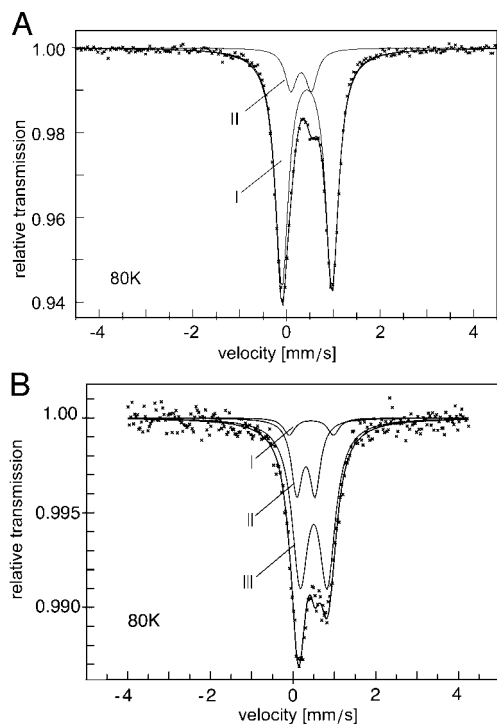


FIG. 3. Mössbauer spectrum of *B. subtilis* sulfonucleotide reductase. *A*, spectrum of *Bacillus subtilis* SNR (0.7 mM) taken at 80 K from the tag-free protein as isolated is shown. SNR protein was not treated for Fe-S cluster reconstitution as in Fig. 5. The solid lines are fits with Lorentzian doublets. Parameter for subspectrum I and subspectrum II are as given in the text. Spectrum I is taken as a clear indication of a 4Fe-4S cluster in *B. subtilis* SNR, whereas spectrum II indicates either the presence of $[2\text{Fe-2S}]^{2+}$ clusters or traces of unspecifically bound iron oxide. *B*, spectrum of *B. subtilis* SNR (0.3 mM) taken at 80 K from the tag-free protein exposed to air for 120 min at 35 °C. The solid lines are fits with Lorentzian doublets. Parameter for subspectra I, II, and III are as given in the text. Subspectra I and II represent the same species as in Fig. 3*A*, and subspectrum III accounts for Fe^{III} in oxygen or nitrogen coordination.

to find precipitations of iron (oxi)hydroxides attached to the protein. Small particles sizes of such oxidic contaminations would show superparamagnetic behavior with fast spin relaxation, which leads to apparently pure quadrupole spectra with completely collapsed magnetic hyperfine splitting. Such superparamagnetic contaminations can be virtually EPR-silent.

Bs SNR similar to the plant APS reductase is EPR-silent (*e.g.* SNR spectra measured at 20, 10, and 4.2 K). Reduction by dithionite ($\text{S}_2\text{O}_4^{2-}$) or by the physiological reductant thioredoxin (plus DTT) failed to produce a signal that would indicate a reduced $[\text{4Fe-4S}]^{1+}$ cluster. Quite similarly, oxidation by PAPS of a Trx-reduced SNR did not induce new EPR signals. Purified SNR, however, displayed a very weak Fe^{III} signal (at $g = 4.267$). This high spin Fe^{III} signal is believed to represent an artifact of the enzyme preparation. It is possibly related to the ferric iron contamination observed in the Mössbauer spectrum. In this respect, it is noteworthy that *E. coli* PAPS reductase, which lacks a CXXC and Cys-Cys motif, does not bind extra iron Fe^{III} and lacks this g signal.

Decomposition and Reassembly of the Fe-S Clusters—Enzymatic activity in *Bs* SNR was correlated with the presence of a 4Fe-4S cluster. Destruction of this group affected the reduction of APS and PAPS alike (Fig. 5). Half-life of the enzyme under air in the Tris-buffered assay system was $\tau/2 = 12$ min with glycine betaine at 2.5 M as the compatible solute lifetime was increased slightly to $\tau/2 = 22$ min. When *Bs* SNR inactivation was monitored by absorbance spectroscopy, an intermediate between 4Fe-4S and fully decomposed cluster was observed,

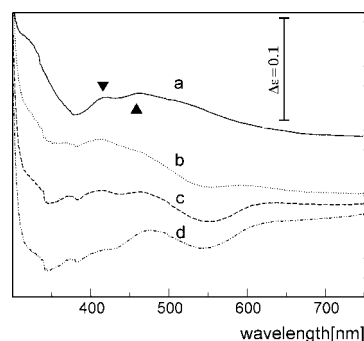


FIG. 4. Repair and reoxidation of the 4Fe-4S cluster. Spectral changes of partly oxidized 20 μM SNR as observed after introduction of iron-sulfur cluster proteins IscA1 (0.2 μM) and IscS (0.2 μM) from *Synechococcus* (17, 27) under conditions of Fe-S cluster repair (*a*, 1 min; *b*, 30 min; *c*, 115 min; and *d*, 145 min) after mixing of the components, supporting Isc repair as described under “Materials and Methods” (arrowheads at ϵ_{max} 410 and ϵ_{max} 468 nm).

which displayed spectral properties of a 2Fe-2S cluster ($\epsilon_{\text{max}} = 468$ nm) (Fig. 4). Mössbauer spectra taken from this nearly fully decomposed form support the view that the 4Fe-4S cluster may have decomposed into a 2Fe-2S cluster before complete degradation. The 80-K Mössbauer spectrum of the oxidatively degraded SNR sample is an asymmetric pattern that could be deconvoluted in three symmetric quadrupole doublets as shown in Fig. 3*B*. Quadrupole splittings and isomer shifts of subspectra I and II were constrained to the values found in the native sample (as shown in Fig. 3*A*). Under this condition, a third subspectrum (subspectrum III) is found with $\delta = 0.50$ mm s^{-1} , $\Delta E_{\text{Q}} = 0.65$ mm s^{-1} , and 72% relative abundance. The values are typical of iron(III) in oxygen or nitrogen 6 coordination and exclude tetrahedral sulfur coordination for that contribution. The relative intensities of the previously known subspectra I and II were dropped to 6 and 22%, respectively. The first and solid conclusion was that the $[\text{4Fe-4S}]^{2+}$ centers of native SNR were largely (>90%) destroyed by oxidative degradation. Secondly, the possible presence of subspectrum II might be taken as an indication for conversion of $[\text{4Fe-4S}]^{2+}$ centers into $[\text{2Fe-2S}]^{2+}$ centers to some degree. However, the relative intensity of 22% for subspectrum II has to be taken more as an upper limit for that abundance because of the excessive overlap with the other spectral species.

Because spectral data in combination with the Mössbauer spectrum may have indicated an interconversion of the 4Fe-4S cluster into a 2Fe-2S form that could serve as mechanism to control enzyme activity as described for the FNR factor (23, 25), we investigated the possibility to restore the enzyme activity together with its Fe-S cluster by adding recombinant iron-sulfur cluster-synthesizing proteins IscA1 and IscS from *Synechocystis* (17) When added to partly oxidized *Bs* SNR (Fig. 4, trace *a*), IscA and IscS led to an increase of absorption at 410 nm (trace *b*, 30 min), whereas the absorption at 468 nm was decreased. Yet, upon prolonged storage under air, the signal at 468 nm returned (trace *c*, 115 min; trace *d*, 145 min). These spectral changes were paralleled by similar changes in enzyme activity, rising from 0.19 (in trace *a*) to 0.23 units/ mg^{-1} (in trace *b*), respectively, decreasing finally to 0.07 units/ mg^{-1} in (trace *d*). In a second type of experimental set up that could restore the iron-sulfur cluster, the enzyme was at first stripped completely of the remaining Fe-S clusters using the technique as described for beef heart aconitase (22) and then incubated in a second step with $[\text{2Fe-2S}]$ -preloaded IscA1 protein as a repair protein for Fe-S groups (24). The residual SNR activity of affinity-purified untreated enzyme was 0.06 ± 0.003 units/ mg^{-1} with PAPS and 0.05 ± 0.004 units/ mg^{-1} with APS. The activity remaining after removal of the iron-sulfur cluster was

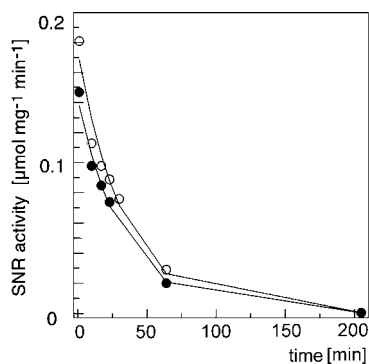


FIG. 5. Kinetics of SNR inactivation due to aerobiosis. SNR oxidized in the presence of 2.5 M glycine betaine at 35 °C under air (two independent measurements, samples of 0.5 μg of SNR taken at intervals as indicated and tested for sulfite formation as described under "Materials and Methods"). Inactivation affects reduction of APS (●) and PAPS (○) alike.

0.004 units/mg⁻¹ with PAPS, whereas activity with APS was below detection. For comparison, PAPS reductase from *E. coli* treated likewise was only marginally affected ($89 \pm 7\%$ remaining activity compared with untreated PAPS reductase). The activity of *Bs* SNR was restored when the protein was treated with IcsA1 from *Synechocystis* PCC6803 that was preloaded with 2Fe-2S groups (17). SNR was allowed to react with preloaded IcsA1 for the time intervals as indicated (Fig. 6) and assayed for reduction of APS or PAPS. Maximal rates displayed after reconstitution of the cluster were $0.18 \mu\text{mol SO}_3^{2-} \text{mg}^{-1} \text{min}^{-1}$ with PAPS and $0.14 \mu\text{mol SO}_3^{2-} \text{mg}^{-1} \text{min}^{-1}$ with APS as substrate. Control experiments contained the same amount of SNR, which was incubated in the presence of uncharged IcsA1 protein (apo-IcsA1, Fig. 6) or with sulfide and $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$. These conditions did not support SNR activity.

Substrate Specificity and Catalytic Constants—We investigated the substrate specificity of *Bs* SNR for either sulfonucleotide in combination with thioredoxin and glutaredoxin as reductants under steady state conditions. The data are summarized in Table I. Recombinant *Bs* SNR was catalytically active with both sulfonucleotides reducing either APS or PAPS. With *E. coli* thioredoxin 1 or glutaredoxin 1 as reductants, a significantly higher rate was observed with PAPS than with APS. Especially with glutaredoxins, the preference for PAPS was more pronounced. For the determination of $K_{m\text{APS}}$ or $K_{m\text{PAPS}}$, both substrates were varied while the concentration of thioredoxin (200 μM) or glutaredoxin (180 μM) was kept constant. The enzyme exhibited a higher affinity for PAPS (K_m 6 μM and 11 μM) when compared with APS (29 and 106 μM), resulting in a higher catalytic efficiency with PAPS (27×10^3 to 36×10^3) than with APS (5.2×10^3 to 3.2×10^3) as substrate. The K_m values for thioredoxin and glutaredoxin were determined with saturating non-varied concentrations of APS and PAPS. With $K_{m\text{TRX1}}$ between 54 μM (APS as second substrate) and 68 μM (PAPS as second substrate) and $K_{m\text{GRX1}}$ between 23 μM (with APS as second substrate) and 61 μM (with PAPS), the affinity toward both redoxins in *B. subtilis* was ~ 10 -fold lower than the affinity for thioredoxin or glutaredoxin in the PAPS reductase of *E. coli* (14). Also noteworthy is the finding that glutaredoxin 1 as a reductant of PAPS gave the highest rates *in vitro* of 0.6 – $0.8 \mu\text{mol mg}^{-1} \text{min}^{-1}$, which compares to $25.6 \mu\text{mol mg}^{-1} \text{min}^{-1}$ of the PAPS reductase system of *E. coli*. Yet, the physiological significance of glutaredoxin as reductant is not clear because *B. subtilis* whose genome is completely sequenced seems to lack an ortholog of glutaredoxin. A comparison of the kinetic constants shows that V_{max} was always higher when glutaredoxin was used as reductant. At saturating

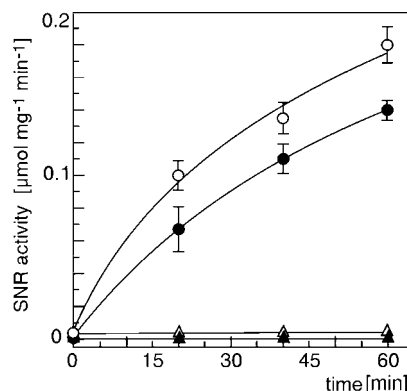


FIG. 6. Reconstitution of the *B. subtilis* sulfonucleotide reductase activity. SNR activity of a protein treated for removal of its 4Fe-4S cluster (see "Materials and Methods") was restored by incubating the SNR apoprotein (200 pmol) together with the recombinant protein IcsA1 (800 pmol) from the cyanobacterium *Synechocystis* PCC 6803 precharged *in vitro* with a 2Fe-2S cluster by IcsS to give a holo-IcsA1 (17). After intervals as indicated, samples containing 0.3 μg of SNR were removed and assayed for SNR activity with either substrate. Control experiments contained the same amount of SNR incubated in the presence of uncharged IcsA1 (apo-IcsA1) or with sulfide and $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$. Shown are the SNR activity with APS (●) and PAPS (○) as substrate and activity of control experiments (△).

TABLE I
Kinetic constants of *Bacillus subtilis* sulfonucleotide reductase
Recombinant *trx1* or *grx1* from *E. coli* was used as electron donor.

Substrate		K_m	V_{max}	K_{cat}	$K_{\text{cat}} K_m^{-1}$
Varied	Saturated				
		$\mu\text{mol liter}^{-1}$	$\text{nmol mg}^{-1} \text{min}^{-1}$	s^{-1}	$\text{liter mol}^{-1} \text{s}^{-1}$
APS	<i>trx</i>	28.7	280	0.149	0.52×10^4
APS	<i>grx</i>	105.4	628	0.335	0.32×10^4
PAPS	<i>trx</i>	6.4	322	0.172	2.69×10^4
PAPS	<i>grx</i>	10.7	716	0.382	3.57×10^4
Trx	APS ^a	54.3	327	0.174	0.32×10^4
Trx	PAPS ^a	67.5	411	0.219	0.32×10^4
Grx	APS ^b	22.5	274	0.146	0.65×10^4
Grx	PAPS ^b	60.6	789	0.421	0.69×10^4

^a 100 μM .

^b 60 μM .

PAPS, glutaredoxin 1 produced a V_{max} of $0.8 \mu\text{mol mg}^{-1} \text{min}^{-1}$ compared with $0.3 \mu\text{mol mg}^{-1} \text{min}^{-1}$ with APS saturating. The catalytic efficiency was not altered in this case (ranging from 0.69 to $0.65 \times 10^4 \text{ liter mol}^{-1} \text{s}^{-1}$). In general, it appears higher with PAPS as substrate, which displays the lowest K_m values for thioredoxin or glutaredoxin with a catalytic efficiency in the range of 2.7×10^4 (Trx1) to 3.6×10^4 (Grx1) $\text{l mol}^{-1} \text{s}^{-1}$.

DISCUSSION

The data presented here show that the *Bs* enzyme will have to be described as bispecific, reducing APS and PAPS alike. In addition, these data exclude the proposal that a 4Fe-4S cluster defines the specificity for APS as substrate. It appears more likely that the 4Fe-4S cluster represents an oxygen-sensitive device that seems have a regulatory function.

Catalytic Properties of the Redoxin-dependent PAPS or APS Reductase—The reduction of adenylyl sulfates as in APS or PAPS requires two electrons, which in enterobacteria, fungi, and some species of cyano and purple sulfur bacteria are supplied by thioredoxin or glutaredoxin. These redoxins are low molecular mass proteins of ~ 12 kDa whose deletion or mutation causes cysteine auxotrophy in *E. coli* (28). They transfer two electrons to PAPS reductase, which upon reduction is converted into a stable reduced state (29). The reduced enzyme then reacts with PAPS-liberating sulfite and adenosine 3',5'-biphosphate. Biochemical properties and mutational analysis

of the protein from *E. coli* led to the proposal that the single cysteine, Cys-239, located in a highly conserved ECGLH motif at the C terminus serves as the reactive thiol group. In the dimeric form of the enzyme the two cysteinyl groups form a redox active dithiol-disulfide couple. Cysteine 239 is located at a highly flexible part of the C terminus, which can get into direct contact with the redoxins as illustrated by a stable enzyme-reductant complex with the Grx1 mutant C14S (30). Moreover, when cysteine 239 reacts with oxidized glutathione, the enzyme forms a mixed disulfide, which is enzymatically inactive (31).

The mono cysteine PAPS reductases define a new class of ATP hydrolases, which have a modified type of nucleotide binding pocket (*i.e.* a highly altered version of the P-loop) formed by two conserved serine residues (Ser-53, Ser-55) and a DTG motif (32). A second type of sulfonucleotide reductase from plants and bacteria of the phytosphere was described more recently. This type is also found in *B. subtilis* whose polypeptide contains additional cysteines in a SIGCXXC and a Cys-Cys motif upstream of the C-terminal ECGLH center but shows also high degree of similarity ($\geq 50\%$) with the monocysteine protein from *E. coli*. These additional cysteinyl groups seem to have little effect on the secondary structure because modeling of *Bs* SNR results in an arrangement of α -helices and β -sheets that is virtually identical with the secondary structure of the bacterial PAPS reductase. When the three-dimensional structure of *E. coli* PAPS reductase (Protein Data Bank code 1sur) is used for comparative protein modeling, the *Bs* SNR displays a basically identical structure with a root mean square deviation of 0.51 Å over 214 equivalent α C atoms. The additional cysteinyl groups in the Cys-Cys and SIGCAPC motifs, however, can be arranged to form the ligands of a mononuclear metal center.³

4Fe-4S and 2Fe-2S in *Bs* SNR—*Bs* SNR contains one iron-sulfur cluster of the 4Fe-4S type per polypeptide chain. The function of this group is not known, but considering the lack of chromophores in the monocysteine PAPS reductases, it is tempting to speculate that these Fe-S groups serve as electron storage buffer. However, although it appears plausible to assume a function in the electron transfer or storage, it should not be overlooked that the clusters were not reduced by dithionite or by thioredoxin under experimental conditions that would otherwise fully support the reduction of PAPS. So far, we have no experimental evidence that the Fe-S clusters are involved in the reduction of PAPS. A different function of the cluster seems feasible pointing toward a control of enzymatic activity. Exposure to air converts the 4Fe-4S cluster into a 2Fe-2S cluster. The 2Fe-2S form is enzymatically inactive; however, activity and the 4Fe-4S cluster was restored using pre-charged IscA as repair protein. Hence, the cluster could serve as the oxygen-sensitive element that serves as a switch between active and inactive form of the enzyme. A physiological background for this type of regulation could result from the finding that sulfite as reaction product readily reoxidizes to sulfate in the presence of oxygen. It remains to be shown that this damage by oxygen of the 4Fe-4S cluster leads to the formation of a 2Fe-2S cluster and that the reversal of the damage by an iron-sulfur cluster repair protein *in vitro* has any meaning *in vivo* as it has been shown for the FNR transcription factor from *E. coli* (24). FNR is also only active as dimer where the protein function is controlled via two 4Fe-4S clusters. Oxidized FNR with its 2Fe-2S clusters is inactive because it loses its structural integrity, which is required for the binding to DNA. Aconitase is yet another example of control of activity via

apoenzyme and 4Fe-4S cluster containing holoenzyme (33).

Until now, the mechanism of electron transfer from thioredoxin to the catalytic center thiols of the homodimer remains unknown. If the cysteinyl-groups of the ECGLH motif are used as reaction center as in the *E. coli* PAPS reductase forming a reactive dithiol-disulfide redox couple, the two 4Fe-4S clusters would have no direct function in the transfer of electrons. This raises the question of whether these Fe-S clusters help to stabilize the dimeric structure in a charge transfer complex. A high resolution three-dimensional structure of the *Bs* protein including the two Fe-S clusters in a homodimer will be of immense value in this context. The second question that merits further investigations will have to address the lack of substrate specificity in *Bs* SNR. At least in *Bs* SNR, the 4Fe-4S cluster does not determine the specificity for APS as suggested for higher plants (5). In view of the similarity between the polypeptide chains of SNRs from different sources (6–8), it will be necessary to test whether APS is truly preferred over PAPS in enzymes that contain Fe-S clusters.

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³ C. H. Lillig, unpublished data.