Biophysical Characterization of the Entire Bacterial Surface Layer Protein SbsB and Its Two Distinct Functional Domains*

Received for publication, August 11, 2003, and in revised form, November 17, 2003 Published, JBC Papers in Press, November 17, 2003, DOI 10.1074/jbc.M308819200

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The crystalline bacterial cell surface layer (S-layer) protein SbsB of Geobacillus stearothermophilus PV72/p2 was disassembled into its N-terminal part defined by the three consecutive S-layer homologous motifs and the remaining large C-terminal part. Both parts of the mature protein were produced as separate recombinant proteins (rSbsB1–178 and rSbsB177–889) and compared with the full-length form rSbsB1–889 (rSbsB). Evidence for functional and structural integrity of the two truncated forms was provided by optical spectroscopic methods and electron microscopy. In particular, binding of the secondary cell wall polymer revealed a high affinity dissociation constant of 3 nM and could be assigned solely to the soluble rSbsB1–178, whereas rSbsB177–889 self-assembled into the same lattice as the full-length protein. Furthermore, thermal as well as guanidinium hydrochloride induced equilibrium unfolding profiles monitored by intrinsic fluorescence, and circular dichroism spectroscopy allowed characterization of rSbsB1–178 as an α-helical protein with a single cooperative unfolding transition yielding a ΔG value of 28.5 kJ mol⁻¹. The C-terminal rSbsB177–889 could be characterized as a β-sheet protein with typical multidomain unfolding, which is partially less stable as stand-alone protein. In general, the truncated forms showed identical properties compared with the full-length rSbsB with respect to structure and function. Consequently, rSbsB is characterized by its two functionally and structurally separated parts, the specific secondary cell wall polymer binding rSbsB1–178 and the larger rSbsB177–889 responsible for formation of the crystalline array.

Crystalline bacterial cell surface layers (S-layers), representing the outermost cell envelope component of many bacteria and archaea, are composed of a single species of identical protein or glycoprotein subunits forming a lattice with either oblique, square, or hexagonal symmetry (1–3). S-layer subunits can be isolated from cell wall fragments by disintegration with high concentrations of hydrogen bond-breaking agents (e.g., GdmHCl). The intrinsic ability of isolated S-layer subunits to recrystallize into their original regular arrays either in suspension or on surfaces (e.g., noble metals, lipid films) makes S-layer proteins unique building blocks in molecular nanobiotechnology and biomimetics (5–9). The mature S-layer protein SbsB (10) of Geobacillus stearothermophilus PV72/p2 (11) is processed from a 920-amino acid-long preprotein by cleavage of a 31-residue-long signal peptide and assembles into an oblique (p1) lattice type. Recently, SbsB has been used to generate chimeric S-layer proteins by fusion of SbsB with streptavidin either on the N-terminal or on the C-terminal end of SbsB (12). These chimeric S-layer proteins combine both the ability to form monomolecular protein lattices on different surfaces and the binding of biotinylated compounds. Such a functional biomolecular matrix with repetitive features in the subnanometer range allows a unique approach for immobilizing compounds with defined spacing on a surface.

The second feature of S-layer proteins is their specific anchoring to the cell wall, which has frequently been assigned to S-layer homologous (SLH) motifs. SLH motifs (13) consist of a conserved sequence of 50–60 amino acids and are found in triplicate at the N terminus of many S-layer proteins (10, 14–19). They have also been identified at the C-terminal end of cell-associated exoenzymes and other exoproteins of Gram-positive bacteria (20), where they occur in single copies. SbsB possesses three N-terminal SLH motifs that recognize a secondary (accessory) cell wall polymer (SCWP) in the rigid cell wall as specific binding partner (21, 22). This glycan is mainly composed of N-acetylglycosamine and N-acetylmannosamine in a molar ratio of 2:1 and contains pyruvic acid residues (22).

Despite their high physiological expense (S-layer protein synthesis can occupy up to 15% of the whole capacity in protein synthesis of the cell (23)) and the widespread occurrence in all major phylogenetic groups of bacteria, no structural model at atomic resolution of any S-layer protein is available. This fact may be explained by the molecular mass of the subunits being too large for NMR analysis and by their high tendency to form two-dimensional lattices preventing the formation of isotropic three-dimensional crystals required for x-ray crystallography. In addition, the very low solubility of isolated subunits is a general hindrance for both methods. Attempts like sequence-structure alignment are hindered by the absence of significant sequence similarities to proteins with known structure (1, 24). Thus, even recognition of further domains other than those formed by SLH motifs remains problematic. According to their molecular mass ranging from 40 to 200 kDa S-layer proteins...
are large multidomain proteins. Thus, direct determination of domain properties is not only more challenging than for small proteins, but often impossible for wild-type proteins.

In the present study, the "dissection approach," frequently applied to cytoskeleton proteins, e.g. titin (25–27), was applied to an S-layer protein. The aim of the study was to dissect the multidomain protein SbsB into its two functional domains, one binding the substrate and the other one comprising the self-assembly ability. The maintenance of function and structure of the isolated parts compared with their properties in the context with the wild-type protein. This work is considered as a first step toward resolving the three-dimensional structure of an S-layer protein.

**EXPERIMENTAL PROCEDURES**

**Materials**—Ultrapure GdmHCl (AA grade) was obtained from Niglu (Waldkraburg, Germany), and concentrations of GdmHCl solutions were calculated from refractive index measurements (28). All other reagents were of analytical grade.

**Plasmid Construction, Protein Production and Purification**—The plasmids for gene expression were cloned by inserting PCR fragments of sbsB (10), which were generated with Pwo DNA polymerase (Roche Applied Science), into the vector pET-28a (+) (Novagen). The template DNA for PCR was chromosomal DNA of *G. stearothermophilus* ATCC 5208 that was prepared from biomass grown in continuous culture (11). DNA for PCR was chromosomal DNA of *G. stearothermophilus* ATCC 5208 that was prepared from biomass grown in continuous culture (11) using a genomic DNA isolation kit (Qiagen). The gene fragments for p2, which was prepared from biomass grown in continuous culture (11), were amplified with the primer pair F2/R3SLH and truncated SbsB (rSbsB1–77–889) and N-terminally truncated SbsB (rSbsB1–77–889) were amplified using the primer pairs F2/R1 and F3/R1, respectively, and inserted into the Neo and BamHI sites of pET-28a (+). For production of the SLH domain (rSbsB1–77–889), a gene fragment was amplified with the primer pair F2/R3SLH and inserted into the Neo and XhoI sites of pET-28a (+). PCR and cloning steps were performed according to standard procedures (29). *Escherichia coli* TG1 was used as a cloning host and transformed by electroporation. The following oligonucleotides were used as PCR primers (restriction sites are italicized, start and reverse complementary stop codons are underlined, and complementary regions are bold): F2, 5'-CGGAATTCAGTCCGGAACGCTACGATGGTCG-3'; F3, 5'-CGGATATCCATGGATATGCGGTTCGTTTGC-3'; F1, 5'-GCCCCTGCCCTTATTTTGGTCAAGTCACATTTTAGC-3'; R1, 5'-GACGCCCTTGCGTTATCCTAACATTTCTGCAGC-3'.

*E. coli* HMS174(DE3) carrying an expression vector was used as a host for SbsB gene fragment expression. Protein production and purification were performed as described previously (30). For spectroscopic measurements protein solutions were prepared by dissolving 1 mg of lyophilized protein in 1 ml of 8 M GdmHCl and subsequent dialysis in a dialysis membrane (Biomol, cut-off: 12–16 kDa) against aqua purificata at 4 °C. Dialysis was stopped after 1 h because of the refractive index difference between the protein solution and water was smaller than 0.001, indicating a residual GdmHCl concentration below 10 mM. After removing self-assembly products by centrifugation (16,000 g, 15 min, 4 °C), the supernatant was dialyzed against 10 mM CaCl2 in aqua purificata and incubated with uranylacetate (2% in aqua purificata) for 10 min at room temperature. After drying on a filter paper, the grids were examined in a Philips CM 100 transmission electron microscope at 80 kV using a 30-μm objective aperture. The lattice orientation was confirmed by Fourier processing (12).

**Proof of Complete Absence of Any Interaction between rSbsB1–178 and rSbsB177–889**—To show that rSbsB1–178 and rSbsB177–889 do not associate to form the complete S-layer protein SbsB when they are denaturated and renaturated together, rSbsB1–178 and rSbsB177–889 were dissolved in 8 M GdmHCl in 50 mM Tris/HCl buffer (pH 7.2), and the solution was dialyzed against 10 mM CaCl2 and against aqua purificata for 24 h. For negative staining, 20 μl of the suspensions were incubated with glow-discharged, Formvar-coated copper grids for 15 min at room temperature. After that, fixation with 2.5% glutaraldehyde (Pierce) in 0.1 M sodium cacodylate buffer (pH 7.2) for 20 min was done. Then the grids were washed at least five times with aqua purificata and incubated with uranylacetate (2% in aqua purificata) for 10 min at room temperature. After drying on a filter paper, the grids were examined in a Philips CM 100 transmission electron microscope at 80 kV using a 30-μm objective aperture. The lattice orientation was confirmed by Fourier processing (12).

**Secondary Structure Prediction**—Far-UV CD spectra were measured in the range from 190 to 260 nm at 0.2-nm intervals in a cuvette with an optical path length of 2 mm at 25 °C using an Applied Photophysics P-180 spectropolarimeter. Protein solutions were prepared immediately after centrifugation as described above by dilution to 0.04–0.08 mg/ml to prevent any formation of subunit and/or self-assembly products. Buffer base lines were measured under identical conditions and subtracted from the spectra.

**Predictions of Protein Secondary Structures Based on far-Uv CD Data**—The far-UV CD spectra of SbsB were analyzed using the neural network program CDNN (35) and the algorithms SELCON, CDSTR, and CONTIN, which were accessed through the DichroWeb website (36).

**Predictions of Protein Secondary Structures Based on far-Uv CD Data**—The far-UV CD spectra of SbsB were analyzed using the neural network program CDNN (35) and the algorithms SELCON, CDSTR, and CONTIN, which were accessed through the DichroWeb website (36).
scribed above were diluted at least 10-fold for spectroscopic measurements adding buffer solutions containing 20 mM sodium phosphate (pH 7.4) and the indicated concentration of GdmHCl. Reversibility of unfolding was tested using an equilibrated protein sample with a high concentration of GdmHCl, which was diluted to 1/10 of the original denaturant concentration. The spectroscopic properties of the diluted sample were compared with a protein sample prepared from the native state at the same diluted GdmHCl concentration. Denaturation curves were obtained by two different methods: First, samples with fixed mixtures of protein in buffer and GdmHCl were measured. Sample solutions were prepared at least 4 h before measurements. There were neither time-dependent changes in fluorescence intensity after 1 min of sample preparation nor detectable differences in measurements taken at 4 and 24 h. Second, titrations were performed either on a sample of native protein in buffer that was mixed stepwise with a sample of the same protein concentration but containing 8 M GdmHCl or vice versa. After each addition of denatured protein the same volume of the mixed solution was removed and used for determination of GdmHCl concentration by refractive index measurements. The concentration steps were spaced at 0.15 x GdmHCl intervals in the range from 0.2 to 7 M. Fluorescence emission was measured using a PerkinElmer Life Sciences LS50B spectrofluorometer equipped with a magnetic stirrer in a 10- x 10-mm quartz cell after a 1-min setting that allowed the protein solution to come to chemical equilibrium after each change in denaturant concentration. The protein concentration was 1 μM, with an absorbance lower than 0.05 at the excitation wavelengths. The samples were excited at 275 nm and 295 nm, and emission was measured from 285 (or 305) to 450 nm at 0.5-nm intervals. The band widths of the excitation and emission monochromator were both set at 5 nm. Each spectrum was corrected by subtraction of the corresponding blank sample obtained by a second titration without protein.

Far-UV CD spectra were taken alternating with fluorescence emission measurements on the same sample solutions in the 10-nm quartz cell using both methods of sample preparation as described above. Because of the strong absorption of GdmHCl, far-UV spectra were recorded only for wavelengths longer than 217 nm by averaging three wavelength scans, each obtained by signal averaging for 2.5 s at intervals of 0.5 nm. For an accurate determination of the molality of GdmHCl at 222 nm, an additional measurement at 222 nm with 30-s integration time was performed. Appropriate blank spectra were recorded on the buffer components and subtracted from spectra obtained on protein solutions.

Models of Unfolding—Spectroscopic data obtained in unfolding experiments were analyzed by a simple two-state model (N ↔ U) (38) considering the native protein (N) and the unfolded species (U) as described by Equation 3.

\[
F = \frac{F_N + s_N[D]}{1 + e^{-m_N[D]/RT}} = \frac{F_U + s_U[D]}{1 + e^{-m_U[D]/RT}} \tag{3}
\]

Equation 3, which includes base-line slopes, was used for determination of ΔG* (the Gibbs free energy change of unfolding in the absence of denaturant), and m, the rate of change in free energy with respect to denaturant concentration. F_N and F_U are the fluorescence intensities of the native and unfolded states in the absence of denaturant, s_N and s_U are the base-line slopes for the native and unfolded regions, respectively, and D is the concentration of denaturant. Fitting procedures were performed by the Marquardt nonlinear least squares algorithm using the program Origin (Microcal).

The second scheme for unfolding includes an intermediate species (N ↔ I ↔ U) and is given by Equation 4.

\[
F = \frac{F_N + s_N[D]}{1 + e^{-m_N[D]/RT}} + \frac{F_I + s_I[D]}{1 + e^{-m_I[D]/RT}} \tag{4}
\]

with the additional terms ΔG_I, ΔG_J, m_I, and m_J for the free energies and m values for the N ↔ I and I ↔ U transitions, respectively. The denaturation concentration at the transition midpoint \( c \) is defined by the respective ratio of ΔG to m. An analogous scheme including two intermediate species was used as a four-state model.

\textbf{Thermal Stability Studies by Far-UV CD Spectroscopy—Thermally induced protein denaturation was monitored by far-UV CD spectroscopy. Protein concentrations used were 0.5 μM in 5 mM potassium phosphate buffer (pH 7.4). A quartz cuvette with 2-mm path length was placed into a Peltier controlled sample holder unit. Full spectra were recorded from 190 to 260 nm at 1-nm intervals and for 2 °C increments with a heating rate of 1 °C/min. Additionally, temperature profiles at}

\[\theta_0 = \left[\frac{(m_p^G T - b_p) - (m_p^E T - b_p)}{1 + \left(\frac{T}{T_m}\right)^{n_p}}\right] + m_p^T T - b_p \tag{5}\]

where \( \theta_0 \) is the ellipticity at temperature \( T \), \( m_p \) is the slope of the curve within the transition region, and the inflection point of the curve the melting temperature \( T_m \). At each temperature \( \theta_0 \) and \( \theta_p \) can be extrapolated from the pre- and post-transition baselines, \( (m_p^G T - b_p) \) and \( (m_p^E T - b_p) \), respectively.

\textbf{RESULTS}

Design and Production of Proteins

Both the genes encoding the three SLH motifs (rSbsB1–178) and the SLH-truncated part (rSbsB177–889) were expressed as isolated recombinant proteins and compared with the mature full-length protein SbsB (residues 1–889), termed rSbsB. The design of these truncated forms was based on a domain boundary as determined by sequence analysis. Because SLH motifs have a conserved sequence of about 55 residues (13), the boundary between the SLH part (amino acid residues 1–178) and the large SLH-truncated part (residues 177–889) was considered as being defined by the end of the three consecutive SLH motifs (residue range 5–61, 62–119, 120–178) at the N terminus (Fig. 1).

Maintenance of Function

Comparison of Binding to SCWP by Fluorescence Anisotropy—The SCWP binding activity was investigated in solution by a fluorescence-based binding assay to characterize quantitatively the interaction between the protein and its target carbohydrate polymer. F-SCWP was employed as ligand and its binding affinity to rSbsB, the two truncated forms and an equimolar mixture of them was studied. Fig. 2 shows a compilation of anisotropy-based binding isotherms of F-SCWP with rSbsB1–178, rSbsB177–889, an equimolar mixture of the former ones, and full-length rSbsB at 25 °C. For both rSbsB and rSbsB1–178, an increase in anisotropy could be observed resulting from complex formation, which was in contrast to the SLH-truncated rSbsB177–889 which did not bind at all. The anisotropy profiles for rSbsB and rSbsB1–178 upon binding to F-SCWP were characterized by an initial plateau for the unbound F-SCWP followed by an anisotropy increase over a range of approximately 2 orders of magnitude in concentration, which is typical of binding in a 1:1 stoichiometry. A plateau for saturated binding concluded this increase. Because of the lower molecular mass compared with rSbsB, the final anisotropy value was lower for rSbsB1–178. However, the slopes for the increase in anisotropy were identical (Fig. 2), thus reflecting equal affinity to F-SCWP. The calculated K_d values, 2.2 nm for rSbsB and 3.1 nm for rSbsB1–178, can be considered identical within the S.D. ± 0.5 obtained from the fit. The binding isotherm of the equimolar mixture of rSbsB1–178 and rSbsB177–889 was completely superimposable on the rSbsB1–178 isotherm (Fig. 2). In addition, adding aliquots of rSbsB177–889 to a F-SCWP solution saturated with rSbsB1–178 did not lead to any

\textbf{FIG. 1. Schematic drawing of the primary structure of the mature SbsB. Boxes from left to right indicate the SLH part (carbohydrate binding region) with the boundaries between the three SLH motifs marked by dotted lines, and the self-assembly part. The black triangles mark the positions of the three tryptophan residues; black bars indicate all positions of the 21 tyrosine residues.}

\textbf{FIG. 2. Maintenance of Function}

Comparison of Binding to SCWP by Fluorescence Anisotropy—The SCWP binding activity was investigated in solution by a fluorescence-based binding assay to characterize quantitatively the interaction between the protein and its target carbohydrate polymer. F-SCWP was employed as ligand and its binding affinity to rSbsB, the two truncated forms and an equimolar mixture of them was studied. Fig. 2 shows a compilation of anisotropy-based binding isotherms of F-SCWP with rSbsB1–178, rSbsB177–889, an equimolar mixture of the former ones, and full-length rSbsB at 25 °C. For both rSbsB and rSbsB1–178, an increase in anisotropy could be observed resulting from complex formation, which was in contrast to the SLH-truncated rSbsB177–889 which did not bind at all. The anisotropy profiles for rSbsB and rSbsB1–178 upon binding to F-SCWP were characterized by an initial plateau for the unbound F-SCWP followed by an anisotropy increase over a range of approximately 2 orders of magnitude in concentration, which is typical of binding in a 1:1 stoichiometry. A plateau for saturated binding concluded this increase. Because of the lower molecular mass compared with rSbsB, the final anisotropy value was lower for rSbsB1–178. However, the slopes for the increase in anisotropy were identical (Fig. 2), thus reflecting equal affinity to F-SCWP. The calculated K_d values, 2.2 nm for rSbsB and 3.1 nm for rSbsB1–178, can be considered identical within the S.D. ± 0.5 obtained from the fit. The binding isotherm of the equimolar mixture of rSbsB1–178 and rSbsB177–889 was completely superimposable on the rSbsB1–178 isotherm (Fig. 2). In addition, adding aliquots of rSbsB177–889 to a F-SCWP solution saturated with rSbsB1–178 did not lead to any

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where \( \theta_0 \) is the ellipticity at temperature \( T \), \( m_p \) is the slope of the curve within the transition region, and the inflection point of the curve the melting temperature \( T_m \). At each temperature \( \theta_0 \) and \( \theta_p \) can be extrapolated from the pre- and post-transition baselines, \( (m_p^G T - b_p) \) and \( (m_p^E T - b_p) \), respectively.
further increase in anisotropy (not shown).

Comparison of Self-assembly Product Formation by Electron Microscopy—The ability of rSbsB and rSbsB}_{177–889} to form self-assembly products was investigated by transmission electron microscopic analysis of negatively stained preparations of suspensions obtained after dialysis of the GdmHCl denatured rSbsB forms. As shown in Fig. 3, rSbsB and the N-terminal truncation rSbsB}_{177–889} were capable of assembling into flat sheets, which clearly exhibited the oblique p1 lattice structure. Fourier processing confirmed that the lattice constants ($a = 10.4$ nm, $b = 7.9$ nm, base angle $\gamma = 81^\circ$) were the same for self-assembly products formed by either rSbsB or rSbsB}_{177–889}. In contrast to self-assembly products obtained with rSbsB which were mostly monolayers, those formed by rSbsB}_{177–889} were double layers, indicating that the individual monolayers had bound to each other with inner surface where the N-terminal region had been deleted. The missing SLH domain and the formation of double layers would explain the different staining behavior as observed for the self-assembly products formed by rSbsB and rSbsB}_{177–889} (Fig. 3). In contrast, the C-terminally truncated form rSbsB}_{1–178} was water-soluble and not capable of self-assembling (not shown).

Proof of Complete Absence of Any Interaction between rSbsB}_{1–178} and rSbsB}_{177–889}—The two S-layer protein forms rSbsB$_{1–178}$ and rSbsB$_{177–889}$ were dissolved in 8 M GdmHCl in 50 mM Tris/HCl buffer (pH 7.2). After renaturation by dialysis, the suspension was centrifuged, and the pellet and the supernatant were subjected to SDS-PAGE analysis. As shown in Fig. 4, almost the whole amount of rSbsB$_{177–889}$ was detected in the pellet, whereas rSbsB$_{1–178}$ was found in the fraction of the supernatant. These findings excluded that both truncated rSbsB forms had associated.

Maintenance of Structure

Comparison of Secondary Structure by Far-UV CD—The far-UV CD spectra of rSbsB and of the truncated forms rSbsB$_{1–178}$ and rSbsB$_{177–889}$ were measured in their native state between 190 and 260 nm for each of the proteins and for an equimolar mixture of rSbsB$_{1–178}$ and rSbsB$_{177–889}$ (Fig. 5). The shape of the far-UV CD spectrum of rSbsB$_{1–178}$ pointed already at a high content in $\alpha$-helical structure according to the occurrence of two minima at 207 and 225 nm, which are typical of $\alpha$-helices. Deconvolution of the spectra by the algorithms CDNN, SELCON, CDSSTR, and CONTIN yielded $\alpha$-helical fractions of 32–34% and values for $\beta$-sheet structure of 15–17%. The spectrum of rSbsB$_{177–889}$ displayed only one minimum at 215 nm, which is characteristic of $\beta$-sheets. The obtained $\beta$-sheet fraction of 38–41% could be assigned mainly to antiparallel $\beta$-sheets using the neuronal network program CDNN.

The values for $\alpha$-helical structure of 4–6% were almost negligible. The spectrum of the full-length protein SbsB showed a broad minimum extending from 210 to 220 nm and therefore indicated a summation of the spectra of the two truncated forms. Indeed, both the algebraically summed spectra and the measured spectrum of an equimolar mixture of rSbsB$_{1–178}$ and rSbsB$_{177–889}$ (Fig. 5) were the same for secondary structure were in good agreement with results of the GORIV algorithm based upon primary sequence data.

Comparison of Tertiary Structure by Intrinsic Fluorescence—Fluorescence spectroscopy was used as a method for reporting differences in tertiary structure by intrinsic fluorescence because intensity and wavelength of the emission maximum of tryptophan residues.
phosphorylation residues are strongly related to their microenvironment in the protein (40). SbsB possesses three tryptophan residues whose distribution is shown in Fig. 1: 1 tryptophan (Trp127) is located in the third SLH motif of rSbsB178, and the 2 others (Trp260 and Trp802) in rSbsB178. In Fig. 6A fluorescence emission spectra of rSbsB, rSbsB178, and rSbsB178–889 in their native state recorded after excitation at 295 nm were compared with an equimolar mixture of the two truncated proteins. The spectrum of the mixture was superimposable on the algebraically summed spectra of rSbsB178 and rSbsB178–889. The difference in intensity between the mixture and the full-length SbsB was attributed to quenching effects, which occur when the two functional domains are linked together. All spectra showed an emission maximum at ~342 nm which is typical for partially solvent exposed tryptophan residues (40). Although rSbsB178 contains only 1 tryptophan residue, the fluorescence intensity in the native state was 4.4 times higher than that of rSbsB178–889 harboring 2 tryptophan residues. However, in the denatured state the ratio of fluorescence intensity was equal to the number of tryptophans. To excite both tyrosine and tryptophan residues the excitation wavelength was set to the absorption maximum of the former at 275 nm (Fig. 6B). Although tyrosine emission at 303 nm was predominant over tryptophan fluorescence in case of rSbsB178–889 it was completely absent in the spectrum of rSbsB178, even though half of the 21 tyrosine residues are concentrated in this part (Fig. 1). This is a clear indication of a high resonance energy transfer rate from tyrosine to tryptophan residues (40). The shape of the spectrum of rSbsB178–889 exhibited a 3.5-fold decrease (Fig. 7C), whereas Trp260 and Trp802 of rSbsB178–889 exhibited a 2.4-fold overall increase in intensity (Fig. 7D). Full-length SbsB showed a 1.5-fold overall decrease in intensity (Fig. 7A) as a consequence of the paramount quantum yield of Trp127. The same overall decrease was seen for an equimolar mixture of rSbsB178 and rSbsB178–889 (Fig. 7C).

Additional information on the compactness of the proteins was obtained by investigating alterations in the distance related fluorescence resonance energy transfer from tyrosine to tryptophan residues (40) upon unfolding. All spectra upon denaturation with GdmHCl after excitation at 275 nm are displayed in Fig. 8. In Fig. 8B the difference in energy transfer from tyrosine to tryptophan residues is clearly visible by appearance of a second fluorescence emission maximum at 303 nm. In contrast, the observed decrease in the predominant tyrosine emission of rSbsB178–889 upon unfolding is solely a consequence of a red-shift of the underlying tryptophan emission instead of a significant change in fluorescence resonance energy transfer efficiency (Fig. 8D). The emission spectra of the equimolar mixture of rSbsB178 and rSbsB178–889 in Fig. 8C demonstrated again additivity as shape and intensity of all spectra were identical to those of full-length SbsB (Fig. 8A).

Comparison of Stability

Thermodynamic Stability of SbsB—To characterize the stability of SbsB and of the two truncated forms, equilibrium unfolding data derived from fluorescence emission of tryptophan residues acting as local probes of tertiary structure were combined with data on global unfolding of secondary structure provided by far-UV CD spectroscopy.

Equilibrium Unfolding by Intrinsic Fluorescence—Figs. 7 and 8 show all spectra obtained per incremental addition of 0.15 GdmHCl between 0.2 and 7 M GdmHCl as described under “Experimental Procedures.”

The tryptophan emission spectra of the full-length SbsB (Fig. 7A) reflected three phases of unfolding. In a first phase (0–2 M GdmHCl) the emission maximum shifted from 342 to 348 nm with a decrease in intensity, whereas in a second phase the intensity increased in two steps yielding plateaus in intensity at ~3–4.5 and 5.5–7 M GdmHCl accompanied by a red-shift to 352 and 355 nm. The emission spectra obtained exciting both tryptophan and tyrosine residues at 275 nm showed the same...
three-step unfolding (Fig. 8A). However, the first change (0–2 M GdmHCl) was a larger decrease in intensity with a concomitant transformation of the shoulder at 303 nm into a maximum of intensity in tyrosine emission, which has to be explained as decrease in energy transfer because of loss in tertiary structure. The following increase in intensity left the tyrosine signal nearly unchanged but red-shifted the tryptophan emission in two steps again separated by plateaus at 3–4.5 and 5.5–7 M GdmHCl from 342 to 348 and finally to 353 nm.

In contrast, the emission intensity of the single tryptophan in rSbsB178 decreased in one step accompanied by a simultaneous red-shift from 342 to 355 nm (Fig. 7B), and the occurrence of the distinct tyrosine emission at 303 nm when the excitation wavelength was set to 275 nm (Fig. 8D).

The tryptophan emission spectra (Fig. 7D) of rSbsB177–889 showed only the stepwise increase phase of the full-length SbsB. The first intensity increase was simultaneous with a red-shift of the emission maximum from 342 to 350 nm leading to a plateau at 2.5–3 M GdmHCl. Thereafter the intensity raised again accompanied by a further red-shift to 355, ending in a plateau at 4–5 M GdmHCl. In contrast, the following increase in intensity was devoid of a red-shift in tryptophan fluorescence. The emission spectra recorded after excitation at 275 nm were dominated by tyrosine fluorescence (Fig. 8D) before the first phase of unfolding. The small shoulder of the tryptophan emission at ~340 nm increased afterward in three steps with an isomissive point at 335 nm during the first unfolding phase and became dominant in the second and third steps with each increase separated by a short plateau. Again, the final increase in intensity was devoid of a red-shift in tryptophan fluorescence.

Fluorescence emission spectra (Fig. 7C and 8C) upon GdmHCl denaturation for an equimolar mixture of rSbsB1–178 and rSbsB177–889 gave essentially the same spectra compared with the full-length SbsB. The plateaus of the increase phase were, however, at the lower GdmHCl concentrations characteristic of rSbsB177–889.

Equilibrium Unfolding by Far-UV Circular Dichroism—Spectra displayed in Fig. 9 were recorded for all three SbsB forms and an equimolar mixture of rSbsB1–178 and rSbsB177–889 in the wavelength range 217–260 nm on the same sample solutions alternating with the fluorescence measurements described above. In contrast to rSbsB178, whose changes in ellipticity upon denaturation were uniform over the whole wavelength range (Fig. 9B), rSbsB177–889 showed an increase in negative ellipticity above 230 nm in addition to the decrease in negative ellipticity at wavelengths lower than 225 nm (Fig. 9D). The resulting isoelectric region near 230 nm was also observed for the denaturation of rSbsB (Fig. 9A), although changes in ellipticity were more uniform over the whole wavelength range. The same situation was observed for the equimolar mixture (Fig. 9C) of the two truncated proteins.

Comparison of Denaturation Profiles obtained by Fluorescence and Far-UV CD Spectroscopy—A selection of denaturation profiles obtained by fluorescence spectroscopy to those obtained by far-UV CD spectroscopy is compared in Fig. 10. The unfolding profiles of the full-length SbsB revealed several distinct transition phases almost over the whole concentration range of denaturant agent. The profile monitored by means of fluorescence emission at 355 nm with excitation at 295 nm (Fig. 10A) started with a slight linear decrease in intensity (0.25–1.25 M) followed by a sharp sigmoidal decrease ending in a minimum (2 M). The following rise in intensity reached a plateau (3–4.5 M), which was followed by a final increase (4.5 and 5.5 M). Thus, the profiles of rSbsB reflected at least three apparent transition steps with midpoints at 1.6, 2.6, and 5.0 M GdmHCl determined by global fitting using the four-state unfolding model. These transitions could also be seen at other emission wavelengths except for 320 nm, which reflected only the first transition (not shown). However, different intensity ratios at all emission wavelengths indicated the involvement of several species during unfolding. The profiles after 275 nm excitation reflected the same three-step pattern but were more pronounced in the first sigmoidal decrease (Fig. 10A). Far-UV CD unfolding profiles for the full-length SbsB at wavelengths below 230 nm (displayed for 222 nm in Fig. 10A) showed a sharp decrease in negative ellipticity between 1 and 2 M GdmHCl. The further decrease at higher concentrations was smaller and ended at 3 M in a plateau. This was in contrast to the ellipticity change between 232 and 245 nm, which showed an additional two-step increase in negative ellipticity at GdmHCl concentrations higher than 3 M GdmHCl (shown for 236 nm). Thus, two transitions could also be observed at 1.6 and 2.6 M GdmHCl, respectively, could be assigned by global fitting using the three-state model.

In contrast, rSbsB178 showed only one single sigmoidal decrease in intensity with a transition midpoint at 1.6 M

![Fig. 8. Tyrosine and tryptophan fluorescence emission spectra in dependence of GdmHCl concentration.](Image)

![Fig. 9. Far-UV CD spectra obtained by GdmHCl titration.](Image)
GdmHCl for all fluorescence excitation/emission wavelength pairs and for all far-UV CD profiles at different wavelengths (Fig. 10B shows profiles at wavelengths corresponding to Fig. 10A). Evaluation of the unfolding profiles by global fitting to the two-state model yielded a ΔG of 26.5 ± 1.2 kJ mol⁻¹ and an m value of 16.5 ± 0.7 kJ mol⁻¹ m⁻¹.

Again, a complex unfolding behavior was observed for rSbsB177–889 extending over the whole region between 0.5 and 7 M GdmHCl. In Fig. 10D the global unfolding of rSbsB177–889 is contrasted by the fluorescence emission profiles regarding mainly the two tryptophan-carrying domains. The fluorescence emission profiles for rSbsB177–889 are characterized by a three-step evolution. Because of the appearance of isoemissive points in the fluorescence emission spectra (Fig. 8D), the first two unfolding steps could be considered separately as a function of the emission wavelength chosen. After excitation at 275 nm the emission profile at 320 nm reports the first and last unfolding step, whereas the 335 nm emission profile is devoid of the first step (not shown). At 355 nm all three steps could be detected, which was also the case for emission profiles obtained at 355 nm after excitation at 295 nm (Fig. 10D). However, because the last step was devoid of a red-shift in tryptophan fluorescence and failed to reach a plateau, only the first two transitions were evaluated using the three-state model yielding transition midpoints at 1.6 and 3.5 M GdmHCl. Distinct steps in the far-UV CD unfolding profiles could be also observed as a function of the wavelength chosen. The ellipticity at 222 nm after excitation at 295 nm (Fig. 10D) showed a two-step unfolding profile which was also the case for emission profiles obtained at 355 nm after excitation at 295 nm (Fig. 10C). The midpoints obtained from the far-UV CD profile at 236 nm collapsed into one broad transition with a midpoint at 3.0 M GdmHCl.

According to these findings, analysis of the unfolding profiles yielded only apparent transition midpoints for comparison of stability but no meaningful ΔG and m values except for rSbsB178.

Comparison of Thermal Denaturation by Far-UV CD—Far-UV CD spectra obtained for rSbsB, rSbsB1–178, and rSbsB177–889 in a temperature range between 20 and 95 °C are shown in Fig. 11.

In case of rSbsB178 (Fig. 11B) the major amount of secondary structure remained intact, as evidenced by significant negative ellipticity at 222 nm and the unchanged positive ellipticity at 190 nm. The two minima at 207 and 225 nm collapsed into one minimum at 215 nm. Although the thermal denaturation of rSbsB178 was irreversible, the melting profile was highly reproducible and independent on the heating rate. Thus, the melting temperature Tm can be used as a semiquantitative indicator of thermostability.

In contrast, rSbsB177–889 showed a large spectral change ending in a minimum at 200 nm typical of random coil conformation (Fig. 11D). Precipitation of unfolded protein could be prevented, and reversibility was up to 80% as long as the concentration did not exceed 0.5 μM.

The thermal denaturation of the full-length SsbB (Fig. 11A) reflected both the smaller decrease in negative ellipticity at lower temperatures in the wavelength range from 205 to 240 nm and the large increase at higher temperatures. Because of remaining structure in the N-terminal part corresponding to rSbsB1–178, denaturation was only partially reversible, and the final minimum in negative ellipticity was shifted to 205 nm. However, melting profiles were again reproducible and independent of the heating rate.

As a consequence of the irreversible thermal denaturation of rSbsB1–178, corresponding measurements of a mixture of the two truncated proteins were not meaningful and are replaced in Fig. 11C by temperature profiles of the former proteins recorded at 206 and 222 nm. The profiles of rSbsB1–178 yielded a midpoint thermal transition (Tm) of 64 ± 2 °C and were rather broad, indicating low cooperativity because of irreversible denaturation. In contrast, both melting profiles of
rSbsB\textsubscript{177–889} showed a sharp increase in negative ellipticity resulting in a \(T_m\) of 71 ± 1 °C followed by a more flat increase, which resulted probably from a superposition with irreversible denaturation at higher temperatures. The melting profile recorded at 222 nm for the full-length SbsB showed a slight decrease in negative ellipticity at a temperature corresponding to the \(T_m\) of rSbsB\textsubscript{1–178} followed by a stepwise increase after 75 °C, which can be explained by the superposition of the melting profiles of the two truncated proteins and their comparable change in negative ellipticity at this wavelength. However, the profile recorded at 206 nm reflected mainly the transition of rSbsB\textsubscript{177–889}, and yielded a \(T_m\) of 72 ± 2 °C for the full-length SbsB, which is significantly higher than for rSbsB\textsubscript{177–889}.

**DISCUSSION**

To open the way for structure determination at atomic resolution, the dissection approach to the S-layer protein SbsB of *G. stearothermophilus* PV72/p2 was applied. The hypothesis that SbsB is a composite protein consisting of two independent functional domains could be confirmed because the C-terminal end of the three consecutive SLH motifs defined exactly the domain boundary between the N-terminal SCWP binding SLH domain and the C-terminal self-assembly domain. Accordingly, both domains could be produced as stable recombinant proteins, which were termed rSbsB\textsubscript{1–178} and rSbsB\textsubscript{177–889}. The specific function of both domains was maintained when they were produced as stand-alone proteins.

In particular, maintenance of the SCWP binding capacity was confirmed by a solution based fluorescence anisotropy assay enabled by fluorescence end labeling of SCWP. Because binding isothersms could be obtained at submicromolar concentrations of proteins, interference with the self-assembling ability of SbsB, which occurs only at micromolar concentrations, was completely prevented. The experiments performed with SbsB demonstrated that the specific SCWP binding activity of SbsB relied only upon the three N-terminal SLH motifs without any involvement of the C terminus because rSbsB\textsubscript{1–178} bound the SCWP with affinity equal to that of the full-length SbsB, whereas SLH-truncated rSbsB\textsubscript{177–889} showed no binding. Furthermore, because the equimolar mixture of the two truncated proteins showed the same binding isotherm as the SLH domain alone, an association of the two isolated proteins can be excluded.

Previous studies have shown that SLH motifs are responsible for cell wall anchoring (13), and SLH-truncated proteins did not interact with purified cell walls as demonstrated with the S-layer proteins Sap and EA1 of *Bacillus anthracis* (41). Furthermore, Mesnage et al. (41) showed that the isolated three-motif SLH domain could not bind cell walls in vitro when the SCWP was removed by extraction with hydrofluoric acid. Accordingly, Ries et al. (21) have demonstrated that the SCWP is responsible for anchoring the S-layer protein SbsB to the cell wall. This was the reason why the isolated SCWP was used for binding assays in the present study. Because this true equilibrium binding assay employed both interaction partners in an isolated form, the obtained \(K_d\) values in the low nanomolar range demonstrate an interaction of high specificity between SLH domain and the SCWP.

Maintenance of the self-assembly ability was demonstrated by electron microscopy of negatively stained preparations. Only rSbsB\textsubscript{1–178} but not rSbsB\textsubscript{1–889} retained the ability to self-assemble into the lattice characteristic of SbsB. Therefore, the SLH-truncated part was termed the “self-assembly” domain of SbsB. An equimolar mixture of rSbsB\textsubscript{1–178} and rSbsB\textsubscript{177–889} was easily separated when rSbsB\textsubscript{177–889} but not rSbsB\textsubscript{1–178} was removed by centrifugation of formed self-assembly products, which provided further evidence for the complete absence of any interaction between the two truncated proteins.

The proven independence of the two functional domains permitted us to compare the structure of the isolated domains with their structure in context of the full-length protein by spectroscopic techniques, which report changes of the secondary and tertiary structure. In particular, far-UV CD spectroscopy was applied to monitor global changes in secondary structure, whereas fluorescence spectroscopy was used for monitoring local changes in tertiary structure. Each of the methods revealed an additivity of the spectroscopic properties of the two parts.

In particular, deconvolution by different algorithms of the far-UV CD spectrum of the three SLH motifs yielded a considerably high \(\alpha\)-helical content of 32–34% (in contrast to 7–11% for the full-length SbsB), supporting the model proposed by Engelhardt and Peters (24). From multiple sequence alignments of 77 complete and 14 incomplete SLH motif sequences a structure for a single SLH motif was deduced which consists of two \(\alpha\)-helices flanking a short \(\beta\)-strand motif followed by an intermediate loop region. Thus, the data obtained with rSbsB confirmed the model suggested by Engelhardt and Peters (24). Furthermore, GdmHCl-induced unfolding studies allow us to address some important aspects concerning the structural organization of the three-motif SLH domain. The proposition that a single SLH motif of 50–60 amino acid residues already comprises the SLH domain has to be considered in context with the singly occurring SLH motifs in exoenzymes. However, because these enzymes are often found to be active as trimers, the conclusion was drawn that oligomeric SLH structures represent the apparent functional unit in vivo (24). In addition, Mesnage et al. (16) found that hybrid proteins consisting of only one or two SLH motifs fused to an enzyme were unstable and did not tightly bind to the cell surface. The present study revealed that GdmHCl-induced unfolding of the three-motif SLH domain occurred with a complete loss in both secondary and tertiary structure in a highly cooperative single transition. Thus, the SLH part must either consist of a single folding unit or of three independent folding units (folding domains) of equal stability. Thermodynamic analysis of GdmHCl-induced unfolding revealed a stability of 26.5 kJ mol\(^{-1}\) for the SLH part. In contrast, thermal denaturation yielded an irreversible noncooperative transition with a high fraction in residual secondary structure.

The SLH-truncated part of SbsB, rSbsB\textsubscript{177–889} allowed us for the first time to address the self-assembly properties of an S-layer protein without any disturbance by parts of other functionalities. Although many S-layer proteins have been isolated, sequenced, and investigated with respect to their self-assembly properties, biophysical data concerning structural properties of S-layer proteins remain scarce. Up to now, only a few far-UV CD spectra on isolated S-layer protein samples have been reported (42–44), and all of them pointed mainly to a \(\beta\)-sheet structure. Thus, the far-UV CD spectrum of SbsB is not only the first one exploiting a recombinant S-layer protein, but the SLH-truncated protein allowed a closer characterization of the structural basis of the self-assembly property. This is illustrated by the enhanced \(\beta\)-sheet character of about 40% compared with 36% of the full-length SbsB. Because the fraction of \(\alpha\)-helical structure was not higher than 5%, the self-assembly part of SbsB can be classified as an all-\(\beta\)-sheet protein. Moreover, the predominance of antiparallel \(\beta\)-sheets in rSbsB\textsubscript{177–889} is in agreement with the proposed structural scaffold of the self-assembly function. The large self-assembly domain showed a complex unfolding behavior as expected for a protein consisting of more than 700 amino acid residues. Although this func-
tional domain has to be considered as a multidomain protein in terms of folding, analysis of the GdmHCl-induced unfolding transitions was facilitated considerably by the locally resolved fluorescence emission profiles of the 2 tryptophan residues in this part. Because of their large distance of 540 residues in the primary sequence, they reside most probably in different folding domains. Thus, at least two distinct unfolding events could be distinguished from the remaining unfolding events traced by far-UV CD signals. Accordingly, the analyzed unfolding profiles yielded only apparent transition midpoints for comparison of stability but no $\Delta G$ and $n$ values, which were attributable to particular folding domains. Nevertheless, comparing the transition midpoints of the full-length rSbsB with those of the equimolar mixture of both parts revealed a partially reduced stability of the isolated self-assembly part detected by both denaturant and thermal unfolding. Furthermore, the comparison of global unfolding profiles with profiles based on local probes pinpoints the great potential of single tryptophan mutants for performing “domain-resolved” unfolding experiments.

Generation of a set of single tryptophan mutants of the SLH-truncated SbsB protein has to be considered as a promising way for identification and characterization of folding units and would allow further extension of the dissection approach on S-layer proteins.

In summary, the present study demonstrates that the S-layer protein SbsB is composed of two distinct functional domains, namely a SCWP binding SLH domain at the N terminus and a large C-terminal self-assembly domain. Their diversity in function is confirmed at the structural level because a highly $\alpha$-helical metastable unit cooperatively folding contrasts an all $\beta$-sheet multidomain folding protein with higher stability. Although the isolated self-assembly domain appeared to be partially less stable than in the full-length protein, both domains remain functionally and structurally intact and can be studied as isolated proteins.

Thus the dissection approach might be accessible for the whole class of S-layer proteins leading to truncated forms, which are accessible for direct structure determination at atomic resolution.

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