A Reversibly Unfolding Fragment of P22 Tailspike Protein with Native Structure: The Isolated β-Helix Domain†

Stefan Miller,‡,§ Benjamin Schuler,‡,§ and Robert Seckler*,‡,||

Institut für Biophysik und Physikalische Biochemie, Universität Regensburg, D-93040 Regensburg, Germany, and Physikalische Biochemie, Universität Potsdam, Im Biotechnologiepark, D-14943 Luckenwalde, Germany

Received January 26, 1998; Revised Manuscript Received April 27, 1998

ABSTRACT: The homotrimeric tailspike endorhamnosidase of phage P22 has been used to compare in vivo and in vitro folding pathways and the influence of single amino acid substitutions thereon. Its main structural motif, which contains the known folding mutation sites, consists of three large right-handed parallel β-helices. A thermodynamic analysis of the stability of tailspike is prevented by the irreversibility of unfolding at high temperatures or high concentrations of denaturant, probably due to interdigitation of the domains neighboring the β-helix. We therefore expressed and isolated a tailspike fragment comprising only its central β-helix domain (residues 109−544). As shown by equilibrium ultracentrifugation, the isolated β-helix is a monomer at concentrations below 1 μM and trimerizes reversibly at higher protein concentrations. Both the similarity of fluorescence and CD spectra, compared to the complete protein, and the specific binding and hydrolysis of substrate suggest a nativelike structure. Moreover, urea denaturation transitions of the β-helix domain are freely reversible, providing the basis for a future quantitative analysis of the effects of the folding mutations on the thermodynamic stability of the domain and of structural features responsible for folding and stability of the parallel β-helix motif in general.

The tailspike endorhamnosidase of *Salmonella* bacteriophage P22 has been used to approach the protein folding problem with the aid of genetic techniques *(1, 2)*. It is one of very few proteins for which the pathway of folding and assembly in vivo, upon translation in intact cells, has been compared with the reaction occurring in vitro, upon dilution of the unfolded polypeptide from denaturant solutions *(3−6)*. The tailspike protein constitutes the adhesion organelle by which the virus attaches to its receptor, the *O*-antigen polysaccharide at the surface of its bacterial host *(7, 8)*. Upon infection of *Salmonella* with phage mutants defective in capsid assembly, or upon recombinant expression, respectively, the tailspike protein accumulates as a soluble homotrimer of 666-residue (71 kDa) polypeptides. Crystals suitable for X-ray diffraction have become accessible upon dissection of the protein into head-binding and receptor-binding fragments, comprising residues 1−124 and 109−666, respectively, and atomic structures of both parts and of complexes of the receptor-binding fragment with *O*-antigen oligosaccharides have been determined *(9−11)*. The main structural feature in the central part of the tailspike protein (residues 143−540) is a right-handed, parallel β-helix of 13 complete turns (Figure 1). On its solvent-exposed face, several longer loops inserted into the β-helix make up the walls of a long groove that constitutes the oligosaccharide-binding site *(10)*. The β-helices are associated side-by-side in parallel orientation in the trimer. The amino termini of the three receptor-binding fragments form a bundle of short α-helices that is connected—apparently by flexible linker peptides—to the concave side of the dome-shaped structure made up from the head-binding domains of the tailspike protein *(11)*. In contrast to the β-helical part, where polar side chains dominate at the subunit interface, the contact surfaces between the carboxy-terminal segments (residues 556−666) of the polypeptides are very hydrophobic, and the three polypeptides are highly interdigitated *(9)*. In agreement with the crystal structure, biochemical data suggest that major structural rearrangements in this carboxy-terminal part of the protein are responsible for the rate-limiting folding reaction occurring after subunit association during tailspike assembly in vivo and in vitro *(4, 12)*. The trimer maturation reaction can be blocked by thiol reagents and may involve the intermittent formation of disulfides *(13)*.

The motif of a multiturn, right-handed parallel β-helix was first found in the crystal structure of the bacterial pectate lyase PelC *(14)* and, besides the tailspike protein, has since been observed in three other pectate and pectin lyases *(15−17)*, in an endogalacturonase from *Aspergillus (18)*, and in pertactin, a surface protein from the human pathogen *Bordetella pertussis* involved in cell adhesion *(19)*. The ligand of pertactin is unknown, but the substrates of pectate lyases, pectinases, and the endogalacturonase are long-chain carbohydrates, and the polysaccharides are thought to bind in a groove along one face of the β-helix, as has been demonstrated for the tailspike endorhamnosidase *(10, 20)*. The β-helices vary considerably in their length between 7

† Supported by the Deutsche Forschungsgemeinschaft (Se 517/10-1) and by the Fonds der Chemischen Industrie.

* Address correspondence to this author at Universität Postdam.
‡ Universität Regensburg.
§ These authors contributed equally to this work.
|| Universität Postdam.

S0006-2960(98)00190-1 CCC: $15.00
Published on Web 06/05/1998

and 16 helical turns in pectate lyase C and pertactin, respectively, but their L-shaped cross sections with 3 β-sheets made up from multiple short parallel strands are very similar. Despite this structural similarity and the repetitive nature of the structural motif, there is no detectable sequence homology between the tailspike protein, pertactin, and the pectate lyases, and, in contrast to the left-handed β-helices described recently *(21, 22)*, there are no extensive internal sequence repeats in the right-handed parallel β-helices *(14, 23)*.

Although there are obvious structural features, such as stacks of hydrophobic residues and hydrogen-bonded "ladders" of polar side chains *(23)*, little is known about the contributions of such interactions to the stability of the β-helix motif and about their role in the folding pathway. In the β-helix of phage P22 tailspike protein, numerous mutation sites have been identified, where single amino acid substitutions affect the folding efficiency *(9, 24−26)*. To date, the effects of such folding mutations on the stability of the β-helix domain could be inferred only indirectly from kinetic experiments *(12, 27, 28)*, because unfolding of the tailspike protein is irreversible at elevated denaturant concentrations *(4)*.

We considered this irreversibility to be due to the strong interactions between the subunits at their carboxy termini, and therefore attempted to isolate the parallel β-helix domain. The present paper reports on the preparation and biophysical characterization of this central part of the tailspike polypeptide comprising residues 109−544. The construct was produced as a soluble protein upon recombinant expression in *Escherichia coli*, and was purified to apparent homogeneity making use of a carboxy-terminal hexahistidyl tag. As shown by analytical ultracentrifugation, the isolated domain was a monomer at protein concentrations below 1 μM; its reversible association at higher concentrations can be described as a monomer−trimer equilibrium. Circular dichroism, fluorescence spectroscopy, oligosaccharide binding, and the presence of endorhamnosidase activity proved the conformation of the monomer to be very similar to that of the β-helix domain in the complete tailspike trimer. Its unfolding induced by urea was freely reversible at low temperature, making the isolated domain suitable for the investigation of mutational effects on the folding and thermodynamic stability of the parallel β-helix.

MATERIALS AND METHODS

Materials. Purified oligosaccharide fragments derived from *Salmonella enteritidis O*-antigen and an aminomethylcoumarin-labeled dodecasaccharide *(8)* were kindly provided by Andrej Weintraub and Ulrich Baxa. UV[1] transparent plastic fluorescence cells (Kartell 4.5 mL PMMA cuvettes) were obtained from Semadeni, Ostermundingen, Switzerland. Ultrapure urea and guanidinium chloride were purchased from ICN Biomedicals, Aurora, OH.

Plasmid Construction and Protein Expression. The plasmid coding for the isolated β-helix domain of P22 tailspike was generated by oligonucleotide-directed mutagenesis *(29, 30)* of pTSF1, the expression plasmid for the amino-terminally shortened tailspike protein *(5, 9, 12, 31)*. The oligonucleotide used to delete the 3′-end of the structural gene and to introduce a sequence coding for a hexahistidine tag followed by two stop codons has the sequence 5′-GCTATCATAGCCGAATTACTAGTGGTGATGG-TGATGGTGCCCTTCTTCTGCCAA-3′ and introduces a singular *Spe*I restriction site. The resulting plasmid pBHX codes for residues D109−G544 preceded by methionine and followed by six histidine residues. The coding sequence, verified by chain-termination sequencing, is under the control of the lacUV5 promoter, and the plasmid, a derivative of pASK40 *(32)*, contains the colE1 replication origin, the *bla* and *lacI* structural genes, and the f1 intergenic region. For protein expression, pBHX was introduced into *E. coli* BL21 or JM83 *(33, 34)*, cells were grown in rich media containing 0.1 mg/mL ampicillin, aerated by shaking at 30 °C to an optical density at 550 nm of 1.0, expression from the lacUV5 promoter was induced by adding IPTG to a final concentration of 1 mM, and incubation was continued overnight.

Protein Purification. Cells from a 2 L culture were harvested by centrifugation in the cold, resuspended in water, and disrupted by high-pressure lysis. The supernatant of a 100000*g* centrifugation (30 min in a Beckman Ti60 rotor) was brought to 35% saturation by addition of solid ammonium sulfate at 0−4 °C; precipitated protein was collected (20 min at 38000*g*), resuspended in buffer A (50 mM Tris/HCl, 0.5 M NaCl, pH 7.5), cleared by centrifugation, and applied at 4 °C to a column (1.5 × 15 cm) of Chelating Sepharose Fast Flow (Pharmacia) loaded with Zn^{2+} ions according to the procedure recommended by the manufacturer. The column was eluted with a gradient of 0−0.3 M imidazole in buffer A. Fractions containing protein were detected by their absorbance at 280 nm, immediately supplemented with EDTA (∼10 mM) and either directly frozen in liquid nitrogen or first concentrated by ultrafiltration to ∼1 mg/mL. Around 10 mg of purified protein was obtained from 1 L of bacterial culture. Protein was stored at −70 °C and was dialyzed against buffer A containing 50 mM EDTA before use.

Analytical Ultracentrifugation. Sedimentation velocity and sedimentation equilibrium runs were performed in a Beckman Model E analytical ultracentrifuge equipped with high-energy light source and photoelectric scanner using double-sector cells with 12 mm optical path length and sample volumes of 0.36 mL (column height 1.6 cm). Sedimentation velocity runs were done at 4 °C and a rotor speed of 40 000 rpm. Sedimentation coefficients were corrected to the viscosity and density of water at 20 °C using a partial specific volume of 0.74 mL/g estimated from the polypeptide sequence of Bhx[1] *(35)*. Ultraviolet absorbance scans were registered on a 10-in. chart recorder. For the analysis of sedimentation equilibria, a linear magnification of 94 was used, recorder traces were digitized using a Summasketch II plus graphics tablet, and data were evaluated by nonlinear regression using the program MULTEQ3B kindly provided by Alan Minton.

Oligosaccharide Binding and Hydrolysis. Binding of octasaccharide and dodecasaccharide fragments derived from *Salmonella enteritidis O*-antigen was measured by fluorescence titration essentially as described *(8)*. Briefly, 3 μL aliquots of oligosaccharide solutions (133 μM−10 mM) were added to a solution of Bhx (200 nM in 50 mM sodium

[1] Abbreviations: Bhx, isolated β-helix domain of phage P22 tailspike protein; CD, circular dichroism; tsf, temperature-sensitive folding; TSPΔN, phage P22 tailspike protein lacking the amino-terminal, head-binding domain; UV, ultraviolet.

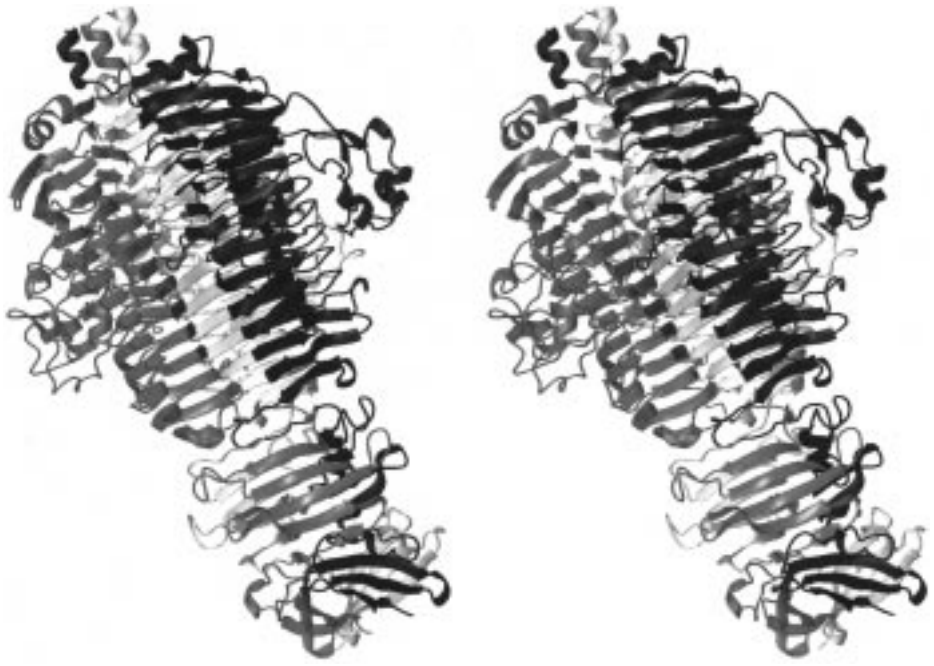

FIGURE 1: Tailspike crystal structure. In the stereo ribbon diagram, prepared with MOLMOL (*50*) for wall-eyed observation, the subunits of the amino-terminally shortened tailspike trimer (TSPΔN) are traced in three shades of gray. Note that the parallel β-helices are covered by short α-helices at their amino-terminal ends (top) and by extended chain segments from the neighboring subunits at the carboxy-terminal ends.

phosphate, pH 7) in a stirred cell (Hellma 119.004F-QS) at 20 °C. After equilibration, tryptophan fluorescence was measured at 347 nm (6 nm bandwidth) with excitation at 295 nm (4 nm bandwidth) in a Spex Fluoromax spectrofluorometer. Volume-corrected fluorescence readings were fit by nonlinear regression to a binding isotherm for a single class of binding sites. To measure the endorhamnosidase activity of Bhx, aminomethylcoumarin-labeled dodecasaccharide (20 μM) was incubated with Bhx (0.01−1.0 mg/mL) for 20 min−20 h at 37 °C in 50 mM sodium phosphate, 50 mM EDTA, 0.5 M NaCl, pH 7, the reaction was stopped by addition of acid and cooling to 0 °C, and the substrate and product oligosaccharides were quantified by reversed-phase HPLC *(8)*.

Folding and Unfolding. For refolding kinetics, protein was denatured in a freshly prepared solution of 4 M ultrapure urea, 50 mM sodium phosphate, pH 7.0, for at least 10 min at room temperature. Refolding was initiated at 10 °C by injection of 24 μL of this solution into a stirred, thermostated cuvette containing 1.18 mL of 50 mM sodium phosphate, pH 7.0, to a final protein concentration of 10 μg/mL. Fluorescence was recorded in a Spex Fluoromax spectrofluorometer at 345 nm with an excitation wavelength of 280 nm.

Folding transitions were measured at 10 °C in a Perkin-Elmer MPF3 spectrofluorometer with a thermostated cell holder using disposable plastic fluorescence cuvettes. For unfolding transitions, cold 50 mM sodium phosphate, pH 7.0, was first added to a small sample of concentrated protein solution (generally 10−50 μL at 1 mg/mL); then a concentrated urea solution of known molarity containing 50 mM sodium phosphate, pH 7.0, was added to give a final volume of 1.2 mL and an accurately known concentration of denaturant. For refolding transitions, urea solution was added first, and refolding was started by dilution with 50 mM sodium phosphate, pH 7.0. To avoid aggregation, mixing was carried out on ice, and the samples were homogenized quickly by multiple pipetting without introduction of air bubbles. All samples were incubated at 10 °C for more than 16 h to ensure that equilibrium was reached.

RESULTS

In the fragment (residues 109−544) chosen for recombinant expression, polypeptide segments capping both ends of the β-helix were included, in addition to the parallel β-helix motif and its inserted loops (residues 143−540 of the tailspike protein). The segment included at the amino-terminus forms two short α-helices α1 (residues 114−120) and α2 (residues 133−139), and the first strand of β-sheet A (residues 124−126, see Ref. *9*). Helix α2 docks against the hydrophobic interior of the parallel β-helix (Figure 1). Very similar amino-terminal caps are observed in other parallel β-helices *(17, 18, 23)*. At its carboxy-terminal end, the tailspike β-helix narrows and merges into a long loop (residues 541−555) wrapping around the 3-fold axis of the trimer. The backbone of the first segment of this loop caps the β-helix of the neighboring subunit in the trimer in an extended conformation. To allow for similar capping interactions in either a monomer or a trimer of the tailspike β-helix domain, residues 541−544, followed by a hexahistidyl tag for rapid purification, were included in the recombinant fragment.

A plasmid coding for the tagged β-helix domain (Bhx) was obtained by oligonucleotide-directed mutagenesis of a construct coding for the amino-terminally shortened tailspike protein *(5, 12)*. When expression from the *lac* promoter was induced at 24−37 °C, Bhx was found mainly in the soluble fraction of cell lysates. The amount found in the insoluble fraction became significant only at the upper end of this temperature range. Upon expression at 30 °C, Bhx was purified from an ultracentrifugation supernatant of the cell

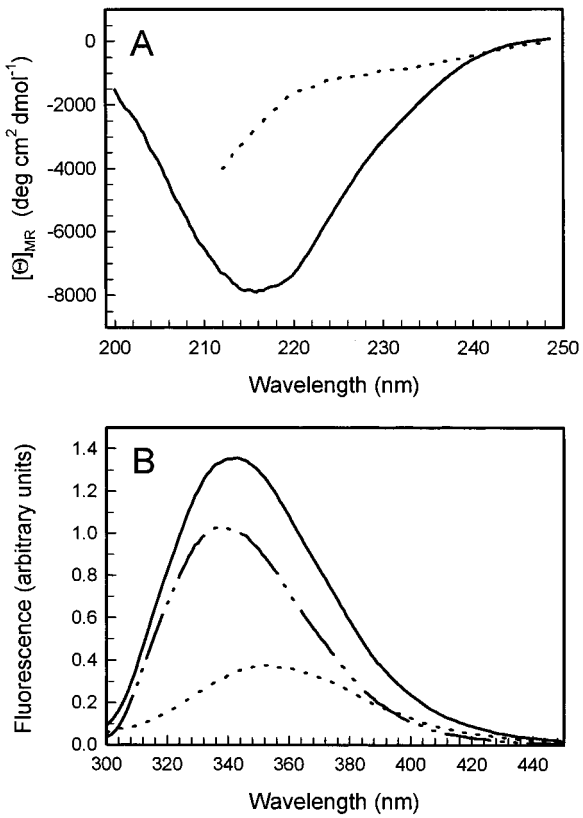

FIGURE 2: Spectroscopic characterization of the isolated β-helix. CD spectra (A) and fluorecence emission spectra (B) of Bhx in its native state (solid line) and in its urea-denatured state (dotted line) were recorded in 50 mM sodium phosphate, 1 mM EDTA, pH 7, containing no denaturant or 4 M guanidinium chloride, respectively. The dash−dotted line in panel B represents the difference spectrum between the native and the denatured protein. CD spectra are averages over 5 scans taken in an Aviv 62A-DS instrument at a spectral bandwidth of 2 nm with 2 s integration time using rectangular fused silica cells with an optical path length of 1 mm (>220 nm) or 0.1 mm cylindrical cells with detachable windows. Uncorrected fluorescence emission spectra were recorded in a Spex Fluoromax with excitation at 280 nm and spectral bandwidths of 2 nm (excitation) and 4 nm (emission). All spectra were recorded at 10 °C and are buffer-corrected. The protein concentrations were 0.5 mg/mL for CD and 10 μg/mL for fluorescence.

extract by ammonium sulfate precipitation and metal chelate chromatography, yielding electrophoretically homogeneous material.

Spectroscopic Characterization. The conformation of Bhx was probed by circular dichroism (CD) and fluorescence spectroscopy (Figure 2). CD spectra recorded in the far-ultraviolet are sensitive to the secondary structure content of a protein. Spectra of Bhx exhibited a single minimum around 216 nm, as observed for the complete tailspike protein *(36)* and considered typical for β-sheet polypeptides *(37)*. The amplitude of this band was -7850 ± 200 deg·cm^2·dmol^{-1}, not unusual for β-sheet proteins, but somewhat less negative than the values reported for the β-helix proteins PelC and PelE from *Erwinia (38)*. It was independent of total protein concentration between 70 μg/mL and 1 mg/mL. The minimum around 216 nm disappeared upon addition of the denaturants urea or GdmCl at high concentrations, indicating unfolding of Bhx.

Fluorescence emission in the near-ultraviolet reflects the environment of tryptophan and, to a lesser degree, of tyrosine residues, and thus the tertiary structure of a protein *(39)*. Upon excitation of Bhx at 280 nm, maximal emission was observed at 342 nm (Figure 2B). This wavelength is identical to the emission maximum of the native tailspike trimer. Upon addition of denaturant, the band was shifted to 353 nm, the emission maximum of solvent-exposed Trp, and a small shoulder around 304 nm indicated the loss of energy transfer from Tyr to Trp residues. Bhx contains six of the seven Trp residues present in tailspike, and the fluorescence of the missing Trp640 may be quenched in the native tailspike trimer *(12)*. The very similar shape of the emission spectra and the identity of the emission maxima therefore indicate very similar molecular environments of the aromatic residues of the β-helix domain in Bhx and in the complete tailspike protein.

State of Association. Analytical ultracentrifugation was used to probe the oligomerization state of Bhx. Sedimentation velocity runs at a protein concentration of 0.3 mg/mL (6.25 μM) resulted in a sedimentation coefficient of 5.8 S after correction to the density and viscosity of water at 20 °C. This value falls between the sedimentation coefficients expected for a monomer (~4 S) and a trimer (~8 S) of a protein with the size and shape of Bhx *(40)*. Sedimentation equilibrium data acquired at a rotor speed of 16 000 rpm at starting concentrations of 2, 6, and 8 μM Bhx are depicted in Figure 3. A global fit of the data with a model for a monodisperse sample with a single molecular mass resulted in bad residuals and $M = 71$ kDa, i.e., 1.5-fold the mass of the Bhx polypeptide calculated from the sequence. A model assuming a monomer−trimer equilibrium produced a much better fit. Similar results were obtained at a rotor speed of 12 000 rpm. The association equilibrium constant determined from the fits was $(7 \pm 2) \times 10^9$ M^{-2}, corresponding to a standard free energy of association per subunit of $\Delta G^\circ \approx -18$ kJ/mol. Sedimentation equilibrium and sedimentation velocity data are consistent with a weak and reversible trimerization of Bhx. At low protein concentrations, Bhx is almost exclusively monomeric (>99% monomer below 0.5 μM); protein concentrations of around 0.5 mg/mL are required for half-maximal trimerization (Figure 3C).

Receptor Oligosaccharide Binding. The phage P22 tailspike protein recognizes the repeating oligosaccharide structure of the *O*-antigen portion of *Salmonella* lipopolysaccharide. Making use of the receptor-destroying hydrolytic activity inherent in the protein, defined oligosaccharide fragments corresponding to two or more *O*-antigen repeats can be isolated and used in quantitative binding experiments *(8)*. Binding of the oligosaccharides to the isolated tailspike β-helix was measured by fluorescence titration at 20 °C. The octasaccharide and dodecasaccharide fragments were isolated from *Salmonella enteritidis* lipopolysaccharide and correspond to the smallest product and substrate of the endorhamnosidase reaction, respectively. The binding experiment was done at a protein concentration of 10 μg/mL, where Bhx is around 99.99% monomeric. Upon titration of Bhx with oligosaccharide, a decrease in the intrinsic protein fluorescence was observed which was well described by equilibrium binding to a single set of sites with dissociation constants of 5 ± 1 μM for the octasaccharide and 3 ± 1 μM for the dodecasaccharide, respectively. Both dissociation constants are around 3-fold higher than those measured with the complete trimeric tailspike protein under identical conditions.

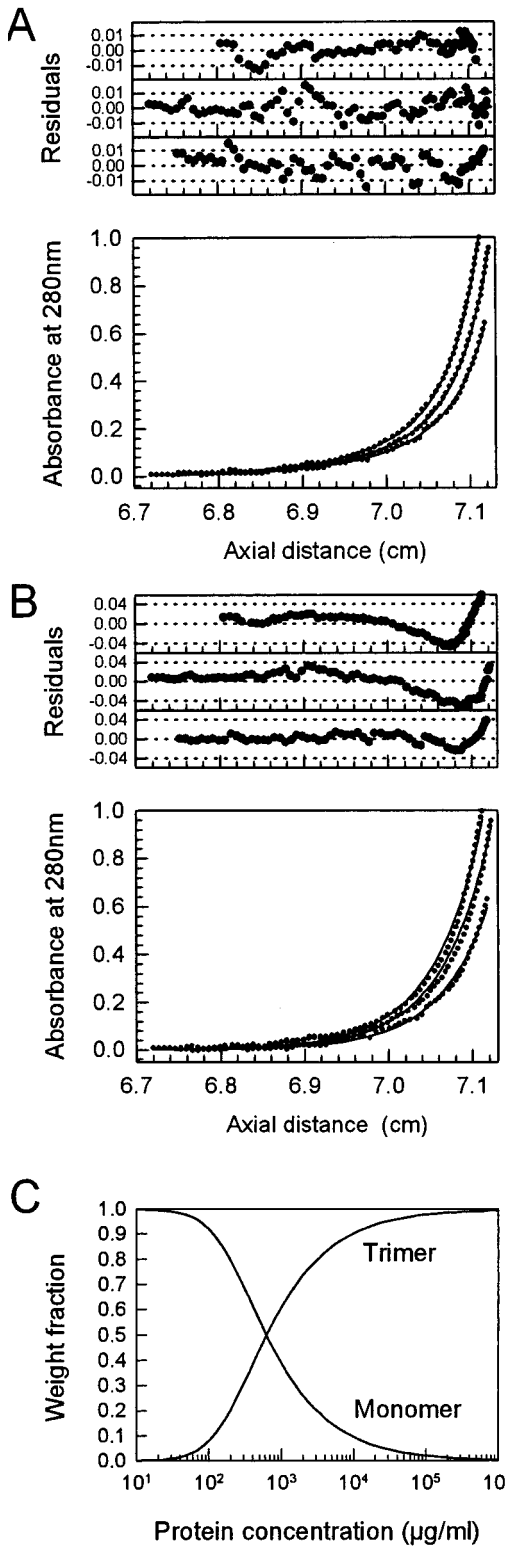

FIGURE 3: Association state of the isolated β-helix. Equilibrium analytical ultracentrifugation of Bhx was done at initial protein concentrations of 0.4, 0.3, and 0.1 mg/mL (top to bottom in panels A and B). The solid lines in panels A and B are fits of the data to a monomer−trimer equilibrium of 48 kDa subunits (A) and to a distribution for a homodisperse sample (B), the latter yielding a molecular mass of 71 kDa. Panel C illustrates the monomer−trimer equilibrium as a function of total protein concentration, calculated with the association equilibrium constant of 8×10^9 M^{-2} as determined in panel A.

To determine whether the oligosaccharide binding affinity of Bhx increases with its reversible trimerization, equilibrium ultracentrifugation was performed at a saturating oligosaccharide concentration of 100 μM along with samples lacking oligosaccharide. A fit of the data to a monomer−trimer equilibrium with the calculated subunit molecular mass of 49 230 Da for the complex of Bhx and octasaccharide resulted in an association equilibrium constant of 2.6×10^{10} M^{-2}, only slightly higher than the trimerization constant determined in the absence of ligand. As oligosacharide binding and subunit association are linked equilibria, the dissociation constant for octasaccharide binding to the trimer can be calculated from the binding constant at low protein concentration and the increase in the trimerization constant induced by the ligand (*41*). It results to 3 ± 1 μM, assuming independent binding to the three sites in the trimer, no sedimentation of free ligand, and the absence of significant concentrations of dimer. Thus, the increase in ligand binding affinity upon trimerization is hardly significant, and an influence of trimerization on the oligosaccharide binding analysis described above can be excluded. Together with the spectroscopic analysis, in particular the invariance of the specific far-UV CD signal over the protein concentration range where the association occurs (cf. above), the oligosaccharide-binding and sedimentation results prove that Bhx folds into a conformation closely resembling the structure of the β-helix domain in the native, trimeric tailspike protein.

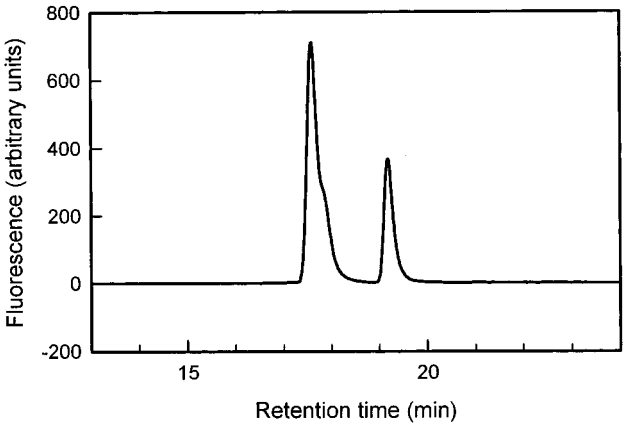

FIGURE 4: Endorhamnosidase activity of the isolated β-helix. A sample of dodecasaccharide fragment, corresponding to three repeats of the repetitive *O*-antigen portion of *Salmonella enteritidis* lipopolysaccharide and labeled at its reducing end with aminomethylcoumarin, was incubated with Bhx and analyzed by reversed-phase HPLC. The peaks eluting at 17.6 and 19.2 min represent labeled dodecasaccharide substrate and labeled tetrasaccharide product of the P22 tailspike endorhamnosidase, respectively. The shoulder at 17.8 min corresponds to some undecasaccharide representing the three outermost *O*-antigen repeats.

Endorhamnosidase Activity. Three carboxylate residues (Asp392, Asp395, and Glu359) at the active site of the tailspike protein are required for its receptor-destroying endorhamnosidase activity *(8)*. As the enzymatic activity is expected to be very strongly dependent on the proper positioning of these side chains in the vicinity of the scissile bond *(10, 11)*, it should provide a very sensitive signal for the native conformation. A sensitive quantitative assay of the endorhamnosidase activity is based on the separation of fluorescence-labeled dodecasaccharide substrate and tetrasaccharide product by reversed-phase HPLC *(8)*. Figure 4 depicts a chromatogram of labeled oligosaccharides extracted after incubation of 20 μM coumarin-labeled dode-

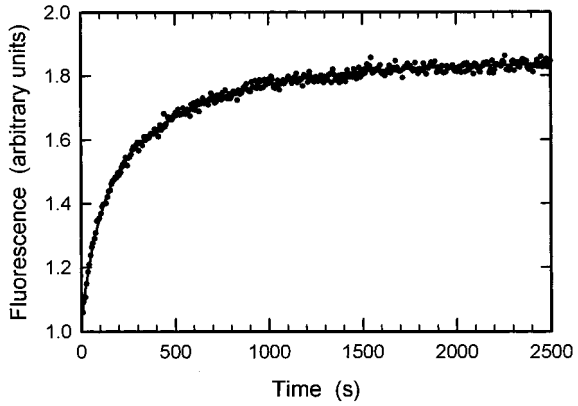

FIGURE 5: Folding kinetics of the isolated β-helix. Bhx, unfolded by urea, was diluted into neutral buffer at 10 °C (final protein concentration 10 μg/mL), and the resulting change in fluorescence was recorded at an emission wavelength of 345 nm. The solid line represents a fit to the sum of two exponentials, resulting in rate constants of $k_1 = 1.0 \times 10^{-2}$ s^{-1} and $k_2 = 1.8 \times 10^{-3}$ s^{-1} and amplitudes of 20% and 23% of the final signal, respectively.

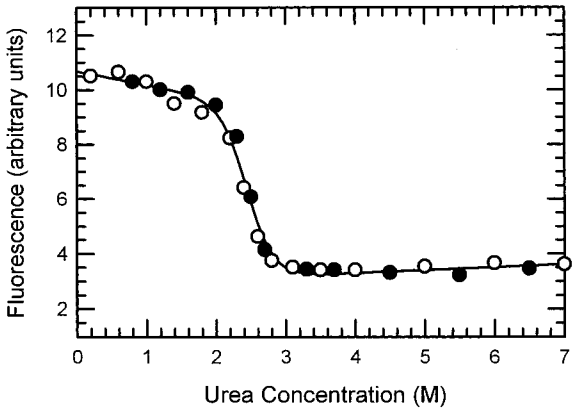

FIGURE 6: Equilibrium unfolding of the isolated β-helix. Native Bhx (○) or urea-unfolded Bhx (●) was diluted to the denaturant concentration indicated and kept at 10 °C overnight, and the fluorescence of the samples was read at an emission wavelength of 345 nm. The solid line represents a nonlinear fit of unfolding and refolding data to a two-state equilibrium unfolding transition *(51)*. The protein concentration was 10 μg/mL.

casaccharide with Bhx (1 mg/mL) for 1 h at 37 °C. Only two peaks corresponding to labeled dodecasaccharide and tetrasaccharide were observed, indicating that Bhx specifically cleaved the substrate into the expected products. Bhx is largely trimeric under these conditions, but a series of assays using protein concentrations of 0.01−1 mg/mL revealed little dependence of the specific endorhamnosidase activity on protein concentration. The turnover numbers estimated from such experiments for the trimeric and monomeric forms of Bhx are identical within experimental error at approximately $(5 \pm 2) \times 10^{-5}$ s^{-1}, corresponding to 0.2% of the activity of the complete, native tailspike trimer.

Folding Kinetics. Spectroscopic signals, substrate binding, and enzymatic activity indicated Bhx to be close in conformation space to the corresponding domain in the complete, native tailspike protein. Prompted by these results, we analyzed the folding and unfolding of Bhx and compared it to the observations made previously on the folding of the complete tailspike *(4, 27, 36)* and of TSPΔN, the tailspike protein lacking the amino-terminal head-binding domain *(5)*. Folding kinetics of Bhx, as measured by fluorescence spectroscopy, are depicted in Figure 5. Following denaturation at high urea concentrations, where the CD signal indicated complete unfolding of the Bhx polypeptide, Bhx folding was initiated by rapid dilution into neutral buffer at 10 °C. After a burst in fluorescence in the dead-time of manual mixing, the fluorescence signal at the emission maximum of the native protein (342 nm) increased 1.8-fold with a half-time of around 4 min. This signal rise reflected the fluorescence change observed during subunit folding of the tailpike protein and of TSPΔN, both in its amplitude (45% of the final fluorescence) and in the half-time of the signal change. Furthermore, a fit of the data to a biexponential rise (solid line in Figure 5) resulted in amplitudes of 20% and 23% of the final signal and rate constants of $k_1 = 1.0 \times 10^{-2}$ s^{-1} and $k_2 = 1.8 \times 10^{-3}$ s^{-1}, respectively. These values are identical within experimental error to those observed for TSPΔN subunit folding *(5)*.

Equilibrium Unfolding. Unfolding−refolding transitions of the complete tailspike protein exhibit apparent hysteresis; i.e., the native protein is stable for many days in saturated urea solutions and at GdmCl concentrations of up to 2 M, but efficient renaturation is only observed below 0.2 M GdmCl or 0.6 M urea *(4)*. Similar observations have been made for TSPΔN *(12)*. In contrast, the unfolding of Bhx induced by urea was freely reversible; i.e., samples diluted to a given intermediate urea concentration from 0 and 5 M urea, respectively, exhibited identical fluorescence after overnight incubation. With increasing urea concentration, the fluorescence decreased in a single cooperative transition, and the transitions for unfolding and refolding were superimposable (Figure 6, ●, and ○). A fit of the data to a two-state model, as indicated in Figure 6, resulted in a free energy of folding extrapolated to denaturant-free buffer of $\Delta G_{fold} = -32$ kJ·mol^{-1} with a denaturant dependence of $m = d\Delta G/d[\text{urea}] = 12.7$ kJ·mol^{-1}·M^{-1}.

DISCUSSION

The results presented above prove our recombinant protein construct Bhx comprising residues 109−544 of the phage P22 tailspike protein to fold into a conformation very closely resembling the β-helix domain in the native trimeric tailspike. The intrinsic fluorescence emission maximum of Bhx is identical to that of the native tailspike protein, Bhx binds receptor oligosaccharide with an affinity close to that of native tailspike, and it specifically cleaves the endorhamnosidase substrate, albeit at a significantly reduced rate. The approximately 50-fold reduction in specific activity compared to a subunit in the native tailspike trimer is not unusual, when it is compared to subunits of other homooligomeric enzymes *(42−44)*. The loops inserted into the β-helix and flanking the bound oligosaccharide on both sides are also involved in intersubunit contacts in the tailspike trimer *(9)*. As observed with triosephosphate isomerase, local distortions upon dissociation merely affecting the conformations of loops in the vicinity of the binding site may be sufficient to greatly reduce the turnover number *(45)*.

What is the minimum size of a tailspike fragment polypeptide to form a stable β-helix? A number of amino-terminal tailspike fragments have been synthesized previously as products of *amber* chain termination mutants *(46)*. Several of these have been tested in crude bacterial lysates for their

ability to react with monoclonal antibodies specific for natively folded rather than denatured tailspike polypeptides *(47)*. The largest *amber* fragment comprising residues 1−488 exhibited weak reactivity with antibodies specific for native tailspike protein [note that *am*H1014 and *am*H703 are identical (Jonathan King, personal communication)]. The same fragment was shown to sediment as a monomer when the soluble fraction of radioactively pulse-labeled cell extracts was analyzed by sucrose gradient centrifugation *(47)*. In the absence of a crystal structure of tailspike, recombinant polypeptides terminating after Asn488 were our first attempts at producing a monomeric tailspike fragment. Plasmid-encoded polypeptides comprising residues 1−488 and 109−488, respectively, were both detectable in the soluble fraction of *E. coli* lysates, but the bulk of the material either was found in the cell debris pellet (at high expression temperature) or was degraded by cellular proteases (at low expression temperature). Attempts to purify these fragments have remained unsuccessful. Residue Gln489, the site of the *am*1014 chain-termination mutation, is located in the 11th β-helical turn, which comprises 26 residues. Chain termination at this site is expected to leave the surface of a number of large hydrophobic side chains (Leu465, Phe472, Ile475, Ile477, Ile478) uncovered at the carboxy-terminal end of the β-helix. The lack of success with these constructs thus points to the importance of capping elements at the ends of parallel β-helices. Capping of the β-helix ends by α-helices or extended segments is observed in all proteins containing right-handed parallel β-helices *(9, 17, 18, 23)*. It should be noted, however, that Bhx is not a well-behaved protein, either. Under conditions other than low temperature, moderate salt concentration, and presence of chelating agents (to prevent interactions of the hexahistidine tails), the protein aggregates upon prolonged storage or when concentrated by ultrafiltration using N_2 at elevated pressure. Again, this is reminiscent of observations made with folded subunits of other oligomeric proteins *(42)*.

The CD spectrum of Bhx in the far-UV is not unusual for a protein consisting largely of β-structure. The molar ellipticity of Bhx is 75% of the molar ellipticity of a subunit in the complete tailspike protein. Considering the expected contributions of the β-sheets at the carboxy and amino termini, this is an expected result. The negative band around 216 nm is similar in shape to those observed in the spectra of other proteins containing right-handed parallel β-helices *(38)*. The amplitude of the minimum, however, is significantly smaller. Compared to the spectra of PelC and PelE, it amounts to 73% and 80%, respectively, although both pectate lyases have much smaller β-helix contents than Bhx. This result casts some doubt on the conjecture that there is a diagnostic CD signature of right-handed parallel β-helices *(38)*.

The similarity of the tryptophan fluorescence emission spectra of Bhx to those of native tailspike trimers reflects the fact that none of the six Trp residues in the tailspike β-helix domain are located at the subunit interface. Two of them (W365 and W391) are highly solvent-exposed in the active site, and their fluorescence is quenched upon receptor oligosaccharide binding to both Bhx and the tailspike trimer. The other four Trp residues are located in the dorsal fin (W202, W207, W213) and in a loop region (W315), and are partly solvent-exposed, in agreement with their average fluorescence emission maximum of 338 nm, as apparent from the spectrum taken at saturation with oligosaccharide. The only tryptophan of the tailspike not located in the β-helix domain is W640. It is largely buried at the subunit interface in the carboxy-terminal "caudal fin" part of the tailspike *(9, 12)*. As the subunit polypeptide chains are highly interdigitated in this domain, which is missing in Bhx, this part of the protein can fold into the native secondary and tertiary structure only after subunit association. A significant fluorescence decrease concomitant with trimer maturation thus suggests that the emission from Trp640 becomes largely quenched upon completion of tailspike folding at the trimer level *(12)*. The quenching of Trp640 and the virtual absence of fluorophores at the subunit interface between the β-helix domains explain the similarity in the fluorescence spectra of Bhx and the tailspike trimer. The paucity of large hydrophobic residues at the interface between the β-helices also provides an explanation for the weak trimerization tendency of Bhx.

At protein concentrations insufficient for significant trimerization of Bhx (<50 nM), the complete tailspike polypeptides, as well as the amino-terminally shortened TSPΔN polypeptides, readily associate during refolding to form the protrimer species, before completing the folding reaction at the trimer level *(5, 27)*. Protrimers, although lacking the thermostability and detergent-resistance of the native protein, do not exchange subunits over a time span of many hours *(3, 4)*. Apparently, tailspike protrimers are stabilized by more than just the interactions between the β-helical domains. This is in agreement with the finding that the kinetics of protrimer formation are governed by a first-order reaction rather than second-order association *(27)*. Furthermore, the observations of Robinson and King *(13)*, who found interchain disulfides after radioactive pulse-labeling of phage-infected bacteria and isolation of protrimers trapped in the cold, appear to suggest that the interactions between subunits in the protrimer are strong enough to force the formation of a disulfide bond in the reducing environment of the *Salmonella* cytoplasm.

In contrast to protrimer formation, which is not connected with a spectroscopic signal change, the subunit folding reaction preceding it is readily detected as a large increase in tryptophan fluorescence intensity *(4)*. On the basis of the observed fluorescence and CD signal changes and of the effects of *temperature-sensitive folding (tsf)* and *tsf*-suppressor mutations on the kinetics of these changes, we have proposed that this step corresponds to the folding of the β-helix domain *(28)*. The present results nicely confirm this suggestion, as both the amplitude and the kinetics of the fluorescence increase during refolding of Bhx upon dilution from urea correspond exactly to the fluorescence change observed during the folding of TSPΔN. The oligosaccharide binding activity of Bhx then would also imply that tailspike folding and assembly intermediates should be able to bind receptor fragments. Indeed, quenching of tryptophan fluorescence by *O*-antigen fragments (but not by unrelated oligosaccharides) is observed when these are added early during tailspike folding, and fluorescence titrations of the protrimer trapped at 5 °C indicate that its *O*-antigen binding affinity is similar to that of native tailspike protein (Ulrich Baxa and R. Seckler, unpublished results).

Attempts to establish equilibria between folded and unfolded conformations of the complete tailspike protein or of TSPΔN have not been successful. Folding−unfolding transitions of the proteins induced by varying the concentration of denaturants exhibit apparent hysteresis; i.e., the transition curves for unfolding and refolding do not coincide, even after incubation for many days *(4, 12)*. Both proteins aggregate upon unfolding at high temperature, rendering thermal unfolding transitions irreversible *(48)*. Thus, it has not been possible to quantify the thermodynamic stability of the folded structures of wild-type and mutant tailspike proteins.

In contrast, the unfolding of Bhx is freely reversible under conditions preventing aggregation as a side-reaction. The unfolding equilibrium transition measured by fluorescence spectroscopy is cooperative and occurs around 2.5 M urea at 10 °C. As a first approximation, the fluorescence transition can be described by a two-state model, but the validity of this concept will have to be tested with other spectroscopic or hydrodynamic techniques.

All the known sites of *tsf* mutations and the two sites of globally acting *tsf*-suppressor mutations are located within the sequence of Bhx *(25, 26)*. Thus, the near-native conformation of Bhx and the reversibility of its unfolding will open a way to a quantitative analysis of the effects of the two types of mutations on the thermodynamic stability of the major structural domain of the tailspike protein. Destabilization and stabilization of a folding intermediate containing a largely structured β-helix domain has been proposed as the cause of the phenotypes of *tsf* and *tsf*-suppressor mutations *(27, 28, 49)*.

In contrast to most other β-helix proteins, in which disulfides cross-link individual turns of the β-helix, Bhx does not contain disulfide bonds. This makes it a suitable model for the analysis of the role of structural features such as polar and nonpolar side-chain stacks and of turn formation in the folding and stabilization of this new folding motif.

REFERENCES

1. King, J., Haase-Pettingell, C., Robinson, A. S., Speed, M., and Mitraki, A. (1996) *FASEB J. 10*, 57−66.
2. Betts, S. D., Haase-Pettingell, C., and King, J. (1997) *Adv. Protein Chem. 50*, 243−264.
3. Goldenberg, D., and King, J. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 3403−3407.
4. Fuchs, A., Seiderer, C., and Seckler, R. (1991) *Biochemistry 30*, 6598−6604.
5. Danner, M., Fuchs, A., Miller, S., and Seckler, R. (1993) *Eur. J. Biochem. 215*, 653−661.
6. Seckler, R. (1997) in *Molecular Chaperones in the Life Cycle of Proteins* (Fink, A. L., and Goto, Y., Eds.) pp 391−413, Marcel Dekker, New York.
7. Israel, V., Rosen, H., and Levine, M. (1972) *J. Virol. 10*, 1152−1158.
8. Baxa, U., Steinbacher, S., Miller, S., Weintraub, A., Huber, R., and Seckler, R. (1996) *Biophys. J. 71*, 2040−2048.
9. Steinbacher, S., Seckler, R., Miller, S., Steipe, B., Huber, R., and Reinemer, P. (1994) *Science 265*, 383−386.
10. Steinbacher, S., Baxa, U., Miller, S., Weintraub, A., Seckler, R., and Huber, R. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 10584−10588.
11. Steinbacher, S., Miller, S., Baxa, U., Budisa, N., Weintraub, A., Seckler, R., and Huber, R. (1997) *J. Mol. Biol. 267*, 865−880.
12. Miller, S., Schuler, B., and Seckler, R. (1998) *Protein Sci.* (submitted for publication).
13. Robinson, A. S., and King, J. (1997) *Nat. Struct. Biol. 4*, 450−455.
14. Yoder, M. D., Keen, N. T., and Jurnak, F. (1993) *Science 260*, 1503−1507.
15. Lietzke, S. E., Yoder, M. D., Keen, N. T., and Jurnak, F. (1994) *Plant Physiol. 106*, 849−862.
16. Pickersgill, R., Jenkins, J., Harris, G., Nasser, W., and Robert-Baudouy, J. (1994) *Nat. Struct. Biol. 1*, 717−723.
17. Mayans, O., Scott, M., Connerton, I., Gravesen, T., Benen, J., Visser, J., Pickersgill, R., and Jenkins, J. (1997) *Structure 5*, 677−689.
18. Petersen, T. N., Kauppinen, S., and Larsen, S. (1997) *Structure 5*, 533−544.
19. Emsley, P., Charles, I. G., Fairweather, N. F., and Isaacs, N. W. (1996) *Nature 381*, 90−92.
20. Kita, N., Boyd, C. M., Garrett, M. R., Jurnak, F., and Keen, N. T. (1996) *J. Biol. Chem. 271*, 26529−26535.
21. Raetz, C. R., and Roderick, S. L. (1995) *Science 270*, 997−1000.
22. Kisker, C., Schindelin, H., Alber, B. E., Ferry, J. G., and Rees, D. C. (1996) *EMBO J. 15*, 2323−2330.
23. Jurnak, F., Yoder, M. D., Pickersgill, R., and Jenkins, J. (1994) *Curr. Opin. Struct. Biol. 4*, 802−806.
24. Yu, M.-H., and King, J. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 6584−6588.
25. Villafane, R., and King, J. (1988) *J. Mol. Biol. 204*, 607−619.
26. Haase-Pettingell, C., and King, J. (1997) *J. Mol. Biol. 267*, 88−102.
27. Danner, M., and Seckler, R. (1993) *Protein Sci. 2*, 1869−1881.
28. Beissinger, M., Lee, S. C., Steinbacher, S., Reinemer, P., Huber, R., Yu, M.-H., and Seckler, R. (1995) *J. Mol. Biol. 249*, 185−194.
29. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) *Methods Enzymol. 154*, 367−382.
30. Yuckenberg, P. D., Witney, F., Geisselsoder, J., and McClary, J. (1991) in *Directed mutagenesis: A practical approach* (McPherson, M. J., Ed.) pp 27−48, IRL Press, Oxford, U.K.
31. Miller, S. (1995) D.Sc. Thesis, University of Regensburg.
32. Skerra, A., Pfitzinger, I., and Plückthun, A. (1991) *BioTechnology 9*, 273−278.
33. Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) *Gene 33*, 103−119.
34. Studier, F. W., and Moffatt, B. A. (1986) *J. Mol. Biol. 189*, 113−130.
35. Cohn, E. J., and Edsall, J. T. (1965) *Proteins, Amino Acids and Peptides as Ions and Dipolar Ions*, 686 pp, Hafner, New York.
36. Seckler, R., Fuchs, A., King, J., and Jaenicke, R. (1989) *J. Biol. Chem. 264*, 11750−11753.
37. Woody, R. W. (1985) *Peptides (N.Y.) 7*, 15−114.
38. Sieber, V., Jurnak, F., and Moe, G. R. (1995) *Proteins*: *Struct., Funct., Genet. 23*, 32−37.
39. Schmid, F. X. (1997) in *Protein Structure, A Practical Approach* (Creighton, T. E., Ed.) 2nd ed., pp 261−297, Oxford University Press, New York.
40. Smith, M. H. (1970) in *CRC Handbook of Biochemistry* (Sober, H. A., Ed.) pp C3−C35, CRC Press, Cleveland, OH.
41. Wyman, J., and Gill, S. J. (1990) *Binding and linkage: functional chemistry of biological macromolecules*, 330 pp, University Science Books, Mill Valley, CA.
42. Jaenicke, R. (1987) *Prog. Biophys. Mol. Biol. 49*, 117−237.
43. Borchert, T. V., Abagyan, R., Jaenicke, R., and Wierenga, R. K. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 1515−1518.
44. Beernink, P. T., and Tolan, D. R. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 5374−5379.

45. Borchert, T. V., Kishan, K. V., Zeelen, J. P., Schliebs, W., Thanki, N., Abagyan, R., Jaenicke, R., and Wierenga, R. K. (1995) *Structure 3*, 669−679.
46. Fane, B., and King, J. (1987) *Genetics 117*, 157−171.
47. Friguet, B., Djavadi-Ohaniance, L., Haase-Pettingell, C. A., King, J., and Goldberg, M. E. (1990) *J. Biol. Chem. 265*, 10347−10351.
48. Sturtevant, J. M., Yu, M.-H., Haase-Pettingell, C., and King, J. (1989) *J. Biol. Chem. 264*, 10693−10698.
49. Lee, S. C., and Yu, M.-H. (1997) *Biochem. Biophys. Res. Commun. 233*, 857−862.
50. Koradi, R., Billeter, M., and Wüthrich, K. (1996) *J. Mol. Graphics 14*, 51−55.
51. Santoro, M. M., and Bolen, D. W. (1988) *Biochemistry 27*, 8063−8068.

BI980190E