Levitt, M., Sander, C., & Stern, P. S. (1985) *J. Mol. Biol. 181*, 423-447.
Liddington, R., Derewenda, Z., Dodson, G., & Harris, D. (1988) *Nature 331*, 725-728.
Luisi, B., & Shibayama, N. (1989) *J. Mol. Biol. 206*, 723-736.
Makinen, M. W., & Eaton, W. A. (1973) *Ann. N.Y. Acad. Sci. 206*, 210-222.
Makinen, M. W., & Eaton, W. A. (1974) *Nature 247*, 62-64.
Marden, M. C., Hazard, E. S., III, & Gibson, Q. H. (1986) *Biochemistry 25*, 7591-7596.
Marshall, A. G., Lee, K. M., & Martin, P. W. (1983) *J. Chem. Phys. 78*, 1528-1532.
Martino, A. J., & Ferrone, F. A. (1989) *Biophys. J. 56*, 781-794.
Mauer, R., Vogel, J., & Schneider, S. (1987) *Photochem. Photobiol. 26*, 255-262.
McCalley, R. C., Shimshick, E. J., & McConnell, H. M. (1972) *Chem. Phys. Lett. 13*, 115.
Monod, J., Wyman, J., & Changeux, J.-P. (1965) *J. Mol. Biol. 12*, 88-118.
Murray, L. P., Hofrichter, J., Henry, E. R., Ikeda-Saito, M., Kitagishi, K., Yonetani, T., & Eaton, W. A. (1988a) *Proc. Natl. Acad. Sci. U.S.A. 85*, 2151-2155.
Murray, L. P., Hofrichter, J., Henry, E. R., & Eaton, W. A. (1988b) *Biophys. Chem. 29*, 63-76.
Nagle, J. F., Bhattacharjee, S. M., Parodi, L. A., & Lozier, R. H. (1983) *Photochem. Photobiol. 38*, 331-339.
Oncley, J. L. (1938) *J. Am. Chem. Soc. 60*, 1115-1123.
Perutz, M. F. (1970) *Nature (London) 228*, 726-734.
Perutz, M. F. (1987) in *The Molecular Basis of Blood Diseases* (Stamatoyannopoulos et al., Eds.) pp 126-162, W. B. Saunders, Philadelphia.
Perutz, M. F., Fermi, G., Luisi, B., Shaanan, B., & Liddington, R. C. (1987) *Acc. Chem. Res. 20*, 309-321.
Ross, P. D., & Minton, A. P. (1977) *Biochem. Biophys. Res. Commun. 76*, 971-976.
Sawicki, C. A., & Gibson, Q. H. (1976) *J. Biol. Chem. 251*, 1533-1542.
Sawicki, C. A., & Gibson, Q. H. (1977) *J. Biol. Chem. 252*, 5783-5788.
Shrager, R. I. (1986) *Chem. Intell. Lab. Systems 1*, 59-70.
Shulman, R. G., Hopfield, J. J., & Ogawa, S. (1975) *Q. Rev. Biophys. 8*, 325-420.
Srajer, V., Schomacker, K. T., & Champion, P. M. (1986) *Phys. Rev. Lett. 57*, 1267-1270.
Steinfeld, J. I., Francisco, J. S., & Hase, W. L. (1989) *Chemical Kinetics and Dynamics*, Prentice Hall, Englewood Cliffs, NJ.
Stryer, L. (1965) *J. Mol. Biol. 13*, 482-495.
Su, C., Park, Y. D., Lui G.-Y., & Spiro, T. G. (1989) *J. Am. Chem. Soc. 111*, 3457-3459.
Venable, R. M., & Pastor, R. W. (1988) *Biopolymers 27*, 1001-1014.
Wyman, J., Gill, S. J., Noll, L., Giardina, B., Colosimo, A., & Brunori, M. (1977) *J. Mol. Biol. 109*, 195-205.

In Vitro Folding Pathway of Phage P22 Tailspike Protein†

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ABSTRACT: The intracellular chain folding and association pathway of the thermostable, trimeric phage P22 tailspike endorhamnosidase has been the subject of a previous detailed study employing temperature-sensitive folding mutants. Recently, reconstitution of native tailspikes from completely unfolded polypeptides has been accomplished, providing a model system to compare protein folding pathways in vivo and in vitro. The in vitro reconstitution pathway of the protein after dilution from guanidine hydrochloride or acid–urea solutions at 10 °C was characterized by spectroscopic and hydrodynamic techniques, and may be summarized as an ordered sequence of folding, association, and folding reactions. Multiphasic folding of monomers was indicated by changes in circular dichroism and fluorescence, with a rate constant of $k = 1.6 \times 10^{-3}$ s^{-1} for the slowest phase observed spectroscopically. Trimerization of structured monomers was followed by size-exclusion HPLC and was completed within 1.5 h at a protein concentration of 20 μg/mL. Although at this time trimers did not exchange subunits, they were readily dissociable by dodecyl sulfate in the cold. Formation of native, detergent-resistant trimers was only completed after 3 days of reconstitution at 10 °C. The reconstitution pathway of the tailspike protein closely resembles its intracellular maturation path. Thus, the in vitro reconstitution system, as a valid model of chain folding and association in vivo, should provide the tools to localize the steps or intermediates on the pathway that are the targets of temperature-sensitive folding mutations.

A number of cellular proteins have been described in recent years to function as folding helpers increasing the efficiency or the rate of protein folding and assembly (Ellis, 1990; Fischer & Schmid, 1990). Nevertheless, it remains generally accepted that the final three-dimensional structure a polypeptide chain assumes in a functional protein is determined by its amino acid sequence, although the rules of such structure formation have yet to be elucidated (Kim & Baldwin, 1982, 1990; Jaenicke, 1987; Kuwajima, 1989). Clues toward determining such rules may be obtained from studying the refolding and reassociation

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pathway of purified proteins after denaturation in vitro. However, because in the cell polypeptides begin to fold while they are synthesized on the ribosome (Bergman & Kuehl, 1979), and without having ever been folded before, such in vitro observations have to be complemented by experiments on intracellular protein folding and association. One approach to explore protein folding in vivo has been the isolation of conditionally lethal point mutations affecting the folding efficiency of a protein (King & Yu, 1986). An extensive study along these lines has focused on the tailspike protein of *Salmonella typhimurium* phage P22 (Smith et al., 1980; Smith & King, 1981; Goldenberg & King, 1981; Goldenberg et al., 1983). P22 tailspikes are homotrimers of a 666 amino acid polypeptide (M_r = 71 759) of known sequence (Goldenberg et al., 1982; Sauer et al., 1982). The purified protein (Berget & Poteete, 1980; Sauer et al., 1982; King & Yu, 1986) consists largely of β-sheet, as determined by Raman spectroscopy (Sargent et al., 1988) and circular dichroism (R. Seckler, unpublished results). Although the tailspike protein is stable to thermal denaturation up to 80 °C, many temperature-sensitive point mutations in the phage gene *9* coding for the tailspike polypeptide have been isolated and characterized by DNA sequencing (Smith et al., 1980; Yu & King, 1984; Villafane & King, 1988). Such mutants display a common temperature-sensitive folding (*tsf*) phenotype. Upon biosynthesis at the permissive temperature (28 °C), mutant tailspike polypeptides fold and associate into native trimers. Such native mutant tailspikes, like wild type, are thermostable, resistant to denaturation by the ionic detergent sodium dodecyl sulfate (SDS),[1] and display endorhamnosidase activity (Goldenberg & King, 1981; Sturtevant et al., 1989). At the restrictive temperature (39 °C), no native tailspike molecules are formed in cells infected with the mutant phage (Smith & King, 1981), and mutant tailspike polypeptides do not associate to form the "protrimer" folding intermediate (Goldenberg & King, 1982; Goldenberg et al., 1983), but rather precipitate intracellularly (Haase-Pettingell & King, 1988).

According to a working model (King & Yu, 1986; Haase-Pettingell & King, 1988; Mitraki & King, 1989), the mutations may destabilize an essential intermediate on the folding and assembly pathway of the tailspike protein. Since refolding and reassociation of detergent-resistant, enzymatically active tailspike trimers from polypeptides unfolded by acid–urea has been accomplished recently (Seckler et al., 1989), the P22 tailspike system provides a unique opportunity to compare an intracellular protein folding and association pathway with observations made in vitro.

In the present study, folding and association reactions on the reconstitution pathway of the tailspike protein are characterized. The pathway of tailspike reconstitution closely parallels the in vivo maturation path of the protein, indicating that in vitro reconstitution may be used as a legitimate model of tailspike folding and association in the cell, and to locate the targets of *tsf* mutations on the maturation pathway.

Experimental Procedures

Materials. Bacterial and phage strains were kindly provided by Dr. J. King (MIT, Cambridge, MA). Tailspike protein was purified from *S. typhimurium* LT2-DB7136 (Winston et al., 1979) infected with phage P22 [5^-_{am}, 13^-_{am} (Botstein et al., 1973)] essentially as described (King & Yu, 1986). Tailspike protein concentrations were determined spectroscopically using a specific absorbance of $A_{278nm}^{1mg/mL} = 1.01$ (Sauer et al., 1982). Ultrapure GdnHCl and urea were obtained from ICN/Schwarz-Mann, Cleveland, OH. DTE was from Roth, Karlsruhe, Germany, SDS and acrylamide were from Serva, Heidelberg, Germany. Other chemicals were analysis-grade from E. Merck, Darmstadt, Germany. Quartz-bidistilled water was used throughout.

Denaturation and Reconstitution. Tailspikes were unfolded by incubation at 25 °C in 5 M urea, 63 mM sodium phosphate, and 0.6 mM EDTA, pH 2.9 for 30 min, or in 6 M GdnHCl, 50 mM sodium phosphate, and 0.5 mM DTE (or 3 mM β-mercaptoethanol), pH 7.0 for 4 h. Reconstitution was initiated by rapid dilution into neutral buffer [50 mM sodium phosphate, 1–2 mM EDTA, and 0.5 mM DTE (or 3 mM β-mercaptoethanol), pH 7.0] preequilibrated at 10 °C. Dilutions were 50-fold and 120-fold for reconstitution from acid urea and GdnHCl, respectively.

Electrophoresis. For separation of native, SDS-resistant tailspikes from SDS-sensitive folding intermediates and aggregate byproducts, 350 μL of 50 mg/mL SDS, 0.16 M Tris-HCl, 250 mg/mL glycerol, and 0.25 mg/mL bromophenol blue, pH 6.8, was added to 500-μL samples of reconstituting tailspike protein, and aliquots of 15 μL were subjected to polyacrylamide gel electrophoresis in the presence of 1 mg/mL SDS. Electrophoresis was performed in 0.75-mm gels (Midget electrophoresis units, Pharmacia) with tap water cooling at less than 1.5 VA per gel. Gels were silver-stained (Heukeshoven & Dernick, 1988) and subjected to densitometry employing an LKB 2202 densitometer interfaced with an Apple II computer. Tailspike protein concentration standards revealed a linear dependence of band intensity on protein concentration.

Size-exclusion HPLC was performed on a TSK 3000 SW gel filtration column (30 × 0.75 cm) with a TSK G SWP precolumn (Pharmacia/LKB) at a flow rate of 0.5 mL/min and at 20 °C. The running buffer, in addition to 0.1 M sodium phosphate and 1 mM EDTA, contained 0.8 M urea, to suppress adsorption of folding intermediates to the column material. Eluted protein was detected by its fluorescence at 335 nm (λ_{exc} = 280 nm) employing a 12-μL flow cell in a Hitachi F 1000 fluorescence detector (E. Merck). Fluorescence signals were registered on a strip chart recorder, and peak areas were determined by planimetry. Marker proteins (Boehringer Mannheim Biochemicals) used to calibrate the column were bovine α_2-macroglobulin ($M_r \approx$ 725 000, t_R = 12.6 min), *Escherichia coli* β-galactosidase (465 000, 14.4 min), sheep IgG (150 000, 18.1 min), F_{ab} fragment from sheep IgG (50 000, 23.0 min), and horse myoglobin (17 800, 25.1 min).

Miscellaneous Methods. The equipment and conditions employed for fluorescence and CD spectroscopy, as well as analytical ultracentrifugation, have been described (Seckler et al., 1989). Thermostated cylindrical cells (Hellma, 5-mm path length, total volume 650 μL) were used for far-UV CD kinetics.

Results

Denaturation and Conditions of Reconstitution. The high thermostability of phage P22 tailspike protein is also reflected in its resistance to unfolding by chemical denaturants. At neutral pH, the tailspike protein trimer withstands urea up to the limit of solubility of the denaturant at 20 °C. By use of a combination of urea and acid pH (5 M urea at pH 3),

[1] Abbreviations: DTE, dithioerythritol; EDTA, ethylenediaminetetraacetic acid; GdnHCl, guanidine hydrochloride; HPLC, high-performance liquid chromatography; SDS, sodium dodecyl sulfate; t_R, retention time; Tris, tris(hydroxymethyl)aminomethane; $[\theta]_{MRW}$, mean-residue-weight ellipticity; λ, wavelength; F_t, fluorescence intensity at time t; θ_t, ellipticity at time t.

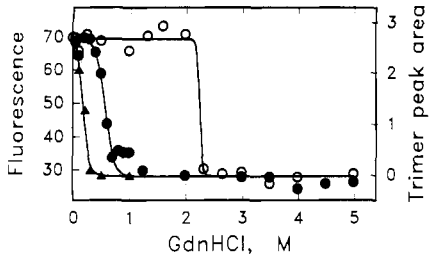

FIGURE 1: Denaturation (○) and reconstitution (●, ▲) transitions as monitored by fluorescence emission at 335 nm (○, ●) and size-exclusion HPLC (▲). Tailspike protein, native (○) or unfolded by guanidine (●, ▲), as described under Experimental Procedures, was diluted to the indicated final concentrations of GdnHCl and incubated for 4 days (○, ●) or 7 days (▲) at 10 °C. The protein concentration was 10 μg/mL. Fluorescence emission spectra were recorded (λ_{exc} = 280 nm), or samples were analyzed for trimer formation by HPLC as described under Experimental Procedures.

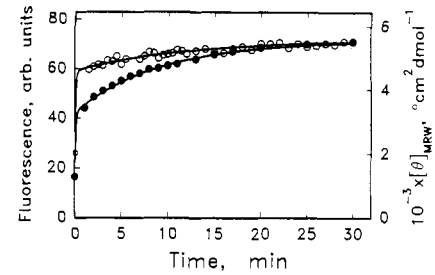

FIGURE 2: Refolding of tailspike protein as observed by fluorescence emission at 340 nm (●) or dichroic absorption at 220 nm (○). Reconstitution was initiated by dilution from acid–urea solutions, as described under Experimental Procedures. Symbols correspond to data from experiments at protein concentrations of 10 μg/mL (fluorescence) and 58 μg/mL (CD). Solid lines correspond to biphasic unimolecular reactions with rate constants for the slow phases in fluorescence and CD of 0.0018 and 0.0014 s^{-1}, respectively. Ellipticities and fluorescence amplitudes for the unfolded polypeptide at t = 0 min were determined from denaturation transitions by extrapolation to zero denaturant concentration. The fast, probably multiphasic changes in CD and fluorescence, which could not be resolved by manual mixing, were modeled with rate constants arbitrarily set to 10 min^{-1}, only to demonstrate that these changes occurred in the deadtime of the experiment.

tailspike polypeptides have been dissociated and completely unfolded, and by dilution of such solutions into neutral buffer at 10 °C, native tailspike trimers have been reconstituted with high yield (Seckler et al., 1989). To induce denaturation of phage P22 tailspikes at neutral pH, high concentrations of GdnHCl (>2 M) were employed. Unfolding, as observed by changes in the fluorescence emission or far-UV circular dichroism, exhibited first-order kinetics with $k_{unf} = 1.3 \times 10^{-3}$ s^{-1} at 25 °C in 6 M GdnHCl. This was concluded from plots of log $(F_t - F_{t=\infty})$ vs t, and log $(\theta_{t=\infty} - \theta_t)$ vs t, which were linear over ≥45 min (five half-times, correlation coefficients of linear regression >0.999). Tailspike polypeptides at 6 M GdnHCl were monomeric and unfolded, as judged by analytical ultracentrifugation (M_r = 74000), circular dichroism at 220 nm ($[\theta]_{MRW}$ = −2000 deg cm^2 $dmol^{-1}$), fluorescence emission maximum (λ_{max} = 347 nm), and fluorescence quantum yield ($F_{335,denat} \approx 0.3 \times F_{335,native}$). Upon long-term incubation at varied GdnHCl concentrations (72–96 h at 25 °C), denaturation transitions were observed with midpoints around 2.5 M GdnHCl. Denaturation profiles were essentially independent of protein concentration between 5 μg/mL and 1 mg/mL.

As frequently observed with oligomeric proteins (Jaenicke & Rudolph, 1986; Jaenicke, 1987), denaturation/renaturation profiles of the tailspike endorhamnosidase exhibited hysteresis; i.e., unfolding of the tailspike protein was not reversible upon dilution to intermediate guanidine concentrations. At 10 °C, denaturation occurred in a sharp transition around 2.2 M GdnHCl, while, upon dilution of unfolded polypeptides, fluorescence spectra indicated nativelike tertiary structure only below 0.5 M residual guanidine (Figure 1). Tailspike trimers were only formed upon reconstitution at less than 0.2 M residual GdnHCl. Possible reasons for this unusually high degree of hysteresis are (i) high ionic strength due to residual guanidine, favoring aggregation, or (ii) destabilization of a marginally stable essential intermediate on the tailspike folding pathway by GdnHCl, again leading to aggregation as a side reaction [cf. Mitraki et al. (1987)]. To distinguish between these alternative explanations, P22 tailspike polypeptides were refolded at 10 °C in the presence of 50 mM residual GdnHCl and varied amounts of neutral salts [$(NH_4)_2SO_4$ or NaCl] or urea, a denaturant not contributing to the ionic strength of the medium. While mimicking the ionic strength of 0.25 M residual GdnHCl by the addition of $(NH_4)_2SO_4$ to 0.2 M or NaCl to 0.5 M had no effect, yields of reconstitution were strongly reduced in the presence of ≥0.6 M urea. No formation of native tailspikes was observed in the presence of 1 M urea, indicating that the effect of GdnHCl at moderate concentrations preventing tailspike reconstitution was due to destabilization of an essential intermediate rather than due to elevated ionic strength.

The product of reconstitution at pH 7, 10 °C, and ≤50 mM residual GdnHCl of the tailspike endorhamnosidase from polypeptides unfolded in 6 M GdnHCl was identical with native tailspikes by the criteria used to characterize phage P22 tailspikes reconstituted from acid urea [cf. Seckler et al. (1989)]. Fluorescence emission maxima (λ_{max} = 335.5 ± 1.5 nm), far-UV ellipticities ($[\theta]_{MRW}$ = −6300 ± 300 deg cm^2 $dmol^{-1}$), retention times on a TSK 3000 GW size-exclusion column (17.65 ± 0.25 min), and electrophoretic mobilities of the SDS-resistant trimers were identical for native and reconstituted tailspikes. Yields and kinetics of tailspike reconstitution from polypeptides unfolded by guanidine at pH 7 or by urea at pH 3 were indistinguishable, and determined only by the composition of the refolding buffer and the protein concentration during refolding. Conditions resulting in maximal reconstitution yields (10 °C, ≤50 mM GdnHCl or ≤0.1 M urea) were employed to characterize the folding and association pathway of the tailspike protein in vitro. When spectroscopic and hydrodynamic methods were applied, four kinetic phases corresponding to secondary structure formation, formation of association-competent monomers, association, and maturation of trimers could be detected during reconstitution of tailspikes from guanidine or acid urea.

Monomer Folding. By fluorescence and far-UV CD spectroscopy, biphasic folding kinetics were observed (Figure 2). In the deadtime of manual mixing (<1 min), the amplitude of fluorescence emission at 340 nm increased from 27 ± 5% to 60 ± 5% of the fluorescence observed after complete refolding. At the same time, the negative ellipticity at 220 nm increased from 35 ± 5% to 82 ± 5% of the final value, indicating that most of the secondary structure was formed within seconds. A second kinetic phase of folding could be well resolved by manual mixing; 40 ± 5% of the final fluorescence amplitude at 340 nm was recovered with first-order kinetics (k = 0.0017 ± 0.0003 s^{-1}, Figure 3), and at the same time, 18 ± 3% of the final CD signal reappeared (k = 0.0014 ± 0.0003 s^{-1}, Figure 2). Beyond 30 min after the onset of reconstitution, further changes in both fluorescence and CD were negligible. As the rates of the slower phase observed spectroscopically were independent of protein concentration

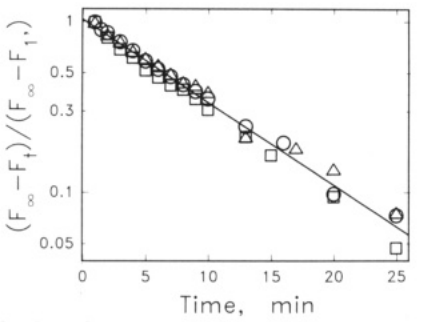

FIGURE 3: Linearization of the slower phase of folding observed by fluorescence emission at 340 nm. Tailspike protein was unfolded by urea at pH 3, and reconstitution at 5 (○), 10 (△), and 20 μg/mL (□) protein was initiated by dilution into neutral buffer at 10 °C as described under Experimental Procedures. Fluorescence amplitude changes ($F_\infty - F_t$) were normalized by the differences between fluorescence amplitudes at infinity (1–2 h) and $t = 1$ min ($F_\infty - F_{t'}$). The slope of the straight line corresponds to a first-order rate constant of 0.0017 s^{-1}.

(Figure 3) and preceded association (cf. below), both fast and slow changes observed in fluorescence and CD must correspond to polypeptide folding reactions at the level of the monomeric polypeptide. The ratio of the amplitudes of the fast (<1 min) and slow ($k \approx 0.0016$ s^{-1}) folding phases was different in CD ($A_{fast}/A_{slow} \approx 2.5$) vs fluorescence ($A_{fast}/A_{slow} \approx 0.9$). This indicates that the two kinetic phases did not arise by parallel reactions of fast (U_F) and slow folding (U_S) isomers of unfolded polypeptides but rather correspond to formation of structured monomers via partially folded intermediates.

Subunit Association. Upon biosynthesis in the *Salmonella* cell, maturation of phage P22 tailspikes to detergent- and protease-resistant trimers is preceded by subunit association into a protease-sensitive, detergent-labile trimer species (Goldenberg et al., 1982; Goldenberg & King, 1982). Association of tailspike polypeptides during reconstitution in vitro was analyzed by size-exclusion HPLC, and by following the formation of hybrids between wild-type and mutant polypeptides. When samples of reconstituting tailspike protein were submitted to HPLC on a TSK 3000 SW size-exclusion column, four different tailspike protein species could be separated and detected by their tryptophan fluorescence (Figure 4A). Upon reconstitution for 48–120 h, one major, nearly symmetrical peak was observed at a retention time of 17.6 min characteristic for the native tailspike trimer and corresponding to an apparent molecular weight of 200 000, when the column was calibrated with globular proteins (cf. Experimental Procedures). A small amount of protein eluted in the void volume of the column ($t_R \approx 13$ min) and probably corresponded to large aggregates formed in an off-pathway reaction. When tailspike protein samples were submitted to HPLC immediately after the onset of reconstitution, a single peak eluted at a retention time of 20.0 min. As revealed by comparison with molecular weight marker proteins, this protein peak contained incompletely folded monomeric polypeptides (apparent M_r = 90 000). Samples reconstituted at 10 °C and 20 μg/mL tailspike protein for 20–120 min were separated on the sizing column into monomers (t_R = 20 min), aggregates (t_R = 13 min), and a broad peak at t_R = 16.7 min exhibiting a distinct shoulder at a position corresponding to the native trimer (t_R = 17.5 min). During the first half hour of reconstitution, the monomer peak in subsequent chromatograms increased in area. This period corresponds to the slower phase of folding observed spectroscopically (cf. above). Thus, the increase in peak area was probably due to the formation of structured monomers which exhibited decreased unspecific binding to the column, and eluted with better efficiency. After 30 min, the amount

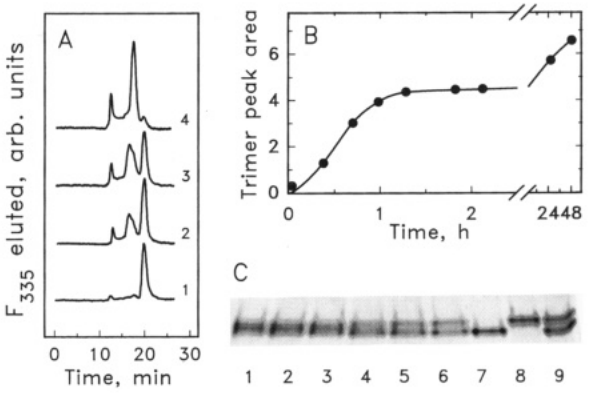

FIGURE 4: Association of tailspike polypeptides (20 μg/mL) as observed by size-exclusion HPLC (A, B) and by formation of mutant/wild-type hybrid trimers (C). (A) Elution profiles from a TSK 3000 SW size-exclusion column. Tailspike protein was unfolded by GdnHCl and reconstituted at 10 °C by dilution to 50 mM GdnHCl. Profiles 1–4 correspond to samples taken 2 min, 42 min, 77 min, and 48 h after the onset of reconstitution. (B) Areas of the peaks at $t_R \approx 17$ min in elution profiles as in (A) were determined by planimetry and are plotted vs time of reconstitution. The ordinate units are arbitrary. (C) Tailspike protein from wild-type phage and mutant tailspikes carrying an additional positive charge (mutant H304, Gly244 → Arg) were dissociated and unfolded by acid–urea. Refolding of wild-type and mutant polypeptides was initiated in separate vessels by dilution into neutral buffer at 10 °C. At varied times after the onset of reconstitution (lane 1, 1 min; lane 2, 10 min; lane 3, 20 min; lane 4, 40 min; lane 5, 60 min; lane 6, 2 h; lane 9, 120 h), samples of reconstituting wild-type and mutant proteins were mixed, and reconstitution of tailspikes was completed during further incubation of the mixtures at 10 °C; 144 h after initiation of reconstitution, SDS (20 mg/mL) was added to the samples, and protein species were separated by electrophoresis and were visualized by silver staining (cf. Experimental Procedures). Only the trimer region of the gel is shown (cf. Figure 5). Lanes 7 and 8, reconstituted wild-type and Gly244 → Arg mutant tailspike homotrimers.

of monomers decreased, while the peak at t_R = 16.7 min increased in area. Aggregate byproducts (t_R = 13 min) were formed rapidly and, in contrast to all other species, were visible only when reconstitution was performed at protein concentrations above 10 μg/mL, or at elevated temperatures.

Upon reconstitution at 10 °C for many hours, or upon raising the temperature of reconstitution to 20 °C, the species at t_R = 16.7 min appeared only as a shoulder on the increasing peak of native tailspike trimers. Obviously, the species eluting at 16.7 min from the sizing column was formed by association from the monomeric polypeptides, and represented a precursor of the native tailspike protein trimer. Its retention time on the sizing column corresponded to the hydrodynamic radius of a globular protein of M_r = 230 000, slightly larger than that of the native tailspike trimer, strongly suggesting that it represented an incompletely folded trimer species. The combined peak area of the species eluting at 16.7 and 17.5 min was therefore taken to represent trimerization of the tailspike polypeptides, and was plotted against time of reconstitution (Figure 4B). During the first hour of reconstitution at 10 °C and 10–20 μg/mL protein, trimer peak areas increased, and plateaued after ≈80 min. At this time of reconstitution, tailspike protein was still completely SDS-sensitive (cf. below). As a further slow increase of trimer peak areas by ≈45% over the next 48 h of reconstitution paralleled the formation of SDS-resistant trimers, it was probably due to native, SDS-resistant tailspikes eluting from the column with higher yield than the primary association product, SDS-sensitive, partially folded trimers. The shape of the broad trimer peak with a shoulder at t_R = 17.5 min remained essentially unchanged during the first 80 min of reconstitution (Figure 4A), indicating that a constant fraction of the trimers matured to native

tailspikes at 20 °C during injection or chromatography (cf. below).

Association was preceded by a short *lag* period (Figure 4B) of approximately 1 half-time of the slow phase of monomer folding (7 min). No species eluting between native trimers and monomers were detected in any chromatogram, indicating that dimers, if present at the time of injection, either were in rapid equilibrium with monomers or were dissociated in the column buffer. Because moderate concentrations of urea had to be included in the column buffer (see Experimental Procedures), and because a detailed analysis of association rates at varied protein concentrations was hampered by strongly reduced yields of polypeptide elution at tailspike protein concentrations below 10 μg/mL, the results of the HPLC analysis did not provide unequivocal evidence regarding the kinetics of reconstitution. Thus, association kinetics were followed by a second, independent method. As attempts to chemically cross-link native or reconstituting tailspike polypeptide oligomers using bifunctional reagents [cf. Jaenicke and Rudolph (1986)] were unsuccessful, hybridization of mutant and wild-type proteins was employed.

Polypeptides from the tailspike folding mutant H304 carry a single Gly → Arg substitution at amino acid residue 244. Native tailspike trimers from this mutant display altered mobility on polyacrylamide electrophoresis gels irrespective of the presence of SDS (Goldenberg et al., 1982; Yu & King, 1988, cf. Figure 4C, lane 8). Upon co-infection of *Salmonella* cells with Gly244 → Arg mutant and wild-type phage, hybrid tailspikes with intermediate electrophoretic mobility are formed (Goldenberg et al., 1982). At 10 °C, Gly244 → Arg mutant tailspikes can be reconstituted in vitro from unfolded polypeptides with yields and kinetics identical with those of wild-type tailspike protein (C. Seiderer and R. Seckler, unpublished results). To determine the kinetics of trimer formation, reconstitution of Gly244 → Arg mutant and wild-type tailspike protein from acid–urea unfolded polypeptides was initiated in separate vessels. After varied time of reconstitution (1–120 min), samples of mutant and wild-type protein were mixed, reconstitution was allowed to come to completion (140 h), and the trimers formed were separated by polyacrylamide electrophoresis in the presence of SDS (Figure 4C). In samples mixed immediately after the onset of reconstitution, most of the tailspike protein was detected as hybrids of intermediate mobility (lane 1). The ratio of the band intensities agreed qualitatively with the 1:3:3:1 ratio expected statistically for nonpreferred formation of homo- and heterotrimers. With increasing time of separate reconstitution before mixing (1–60 min), intensities of heterotrimer bands decreased and those of homotrimer bands increased on subsequent electrophoresis gels (Figure 4C, lanes 2–5), until in samples mixed 2 h after the onset of reconstitution no heterotrimers were formed during subsequent coreconstitution for 6 days. The time corresponding to 50% association estimated from the hybridization experiment (≈30 min) agreed closely with the results of the HPLC analysis at identical protein concentration (cf. Figure 4B,C).

Maturation of Tailspike Trimers. As native phage P22 tailspikes are quite resistant to SDS in the cold, while folding intermediates and off-pathway aggregates are dissociated by the detergent, SDS–polyacrylamide gel electrophoresis may be employed to follow maturation of tailspike trimers (Goldenberg et al., 1982, 1983). Upon biosynthesis of tailspike protein in phage-infected cells, tailspike polypeptides can be trapped in a detergent-sensitive state by reducing the temperature to 0 °C (Goldenberg et al., 1982; Goldenberg & King, 1982), and form native, SDS-resistant tailspikes only very slowly at temperatures below 20 °C (Goldenberg, 1981). Similarly, formation of native tailspikes during reconstitution from unfolded polypeptides occurs during many hours at 10 °C (Seckler et al., 1989). To determine maturation kinetics, tailspike protein was allowed to refold and reassociate at 10 °C for 0–96 h, and reconstitution was quenched by addition of SDS. Samples were separated by SDS gel electrophoresis (Figure 5A), and bands were analyzed by densitometry after silver-staining (Figure 5B). After a *lag* period of ≥1 h, during which no SDS-resistant trimers were detectable, the fraction of detergent-resistant tailspikes increased during the following 48 h, and saturated at ≈65% of the total protein. The half-time of maturation estimated from the densitometry data was $T_{1/2} \approx 12$ h.

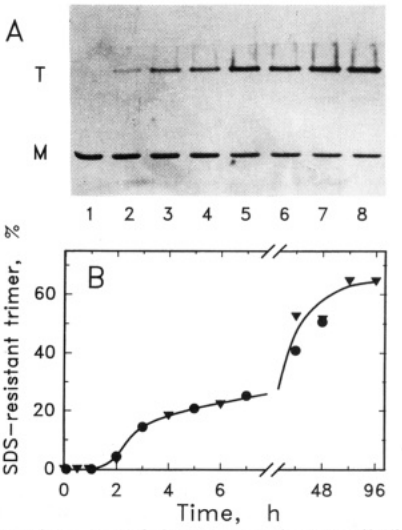

FIGURE 5: Reconstitution of detergent-resistant tailspike trimers followed by SDS gel electrophoresis. Tailspikes were unfolded by acid–urea and allowed to refold and associate at 10 °C for the times indicated below. The protein concentration was 20 μg/mL. Reconstitution was quenched by the addition of SDS; detergent-sensitive and detergent-resistant tailspike protein species were separated by SDS gel electrophoresis. (A) SDS gel of samples reconstituted for 1 (lane 1), 2 (lane 2), 4 (lane 3), 6 (lane 4), 24 (lane 5), 48 (lane 6), 72 (lane 7), and 96 h (lane 8). The positions of trimer (T) and monomer (M) bands are indicated. (B) Quantitative analysis of trimer formation by densitometry. Data are from the gel in (A) (▼) and from a second, completely independent experiment (●).

Discussion

Purified phage P22 tailspike polypeptides dissociated and unfolded by either 6 M GdnHCl or 5 M urea, pH 3, equally efficiently refolded and reassociated upon dilution into neutral buffer at low temperature. Although >2.5 M GdnHCl was required for the denaturation of tailspikes, and no denaturation by urea was observed at neutral pH, efficient reconstitution was limited to <0.1 M residual GdnHCl or ≤0.6 M urea. The strong apparent hysteresis can be explained by destabilization of an essential folding intermediate at moderate denaturant concentrations leading to off-pathway aggregation.

On the basis of observations on reconstitution kinetics made by applying spectroscopic and hydrodynamic techniques, the in vitro reconstitution pathway of the phage P22 tailspike protein may be summarized as an ordered sequence of folding, association, and folding reactions (Scheme I) where U denotes unfolded polypeptides, M structured monomers, T_p partially folded "pro"trimers, and N native, detergent-resistant tailspikes.

Scheme I

$$3U \rightarrow\rightarrow 3M \rightarrow\rightarrow T_p \rightarrow N$$

As commonly observed for large polypeptides comprising more than a single domain (Jaenicke, 1987), folding of the tailspike protein monomer (U → M in Scheme I) was at least biphasic, when detected by fluorescence or CD spectroscopy. A large fraction of the amplitude changes (70% of $\Delta[\theta_{220}]$, 45% of ΔF_{340}) was observed in the deadtime of manual mixing, indicating that within seconds, most of the secondary structure is formed and the tryptophans become partially shielded from the solvent. The remaining fractions of the changes in both CD and fluorescence could be attributed to a single unimolecular reaction with a rate constant of $k = 1.6 \times 10^{-3}$ s^{-1} at 10 °C, corresponding to the rate-limiting step in the formation of structured, association-competent monomers (M). This folding rate, which increases to 7×10^{-3} s^{-1} at 25 °C (C. Seiderer and R. Seckler, unpublished results), is comparable to those observed during reconstitution of other oligomeric proteins, where rates of structured monomer formation are generally on the order of 10^{-3}–10^{-2} s^{-1} (Jaenicke, 1987). Somewhat surprisingly, association of monomers to form the trimeric quaternary structure of the tailspike did not contribute measurably to the spectroscopic signals, indicating that the seven tryptophan residues in the tailspike polypeptides are not exposed on the subunit contact interface in the native trimer. This is in agreement with the fluorescence emission maximum of native tailspikes at 335 nm, and with recent results from Raman spectroscopy (Thomas et al., 1990). Both indicate that most of the tailspike tryptophans are in a relatively hydrophilic environment.

The association reaction following monomer folding was monitored by size-exclusion HPLC and by hybrid formation between wild-type and mutant tailspike polypeptides. Both techniques bore identical results. After a short *lag* attributable to monomer folding, association to trimers at 10 °C and 20 μg/mL protein was completed within 90 min. At this time of reconstitution, essentially all tailspike protein was still dissociable by SDS in the cold. No dimers were detected during size-exclusion HPLC. With the given precision of the data, the results are consistent with either of two models. Tailspike polypeptide dimers may be in a rapid preequilibrium with monomers; in this case, the bimolecular association reaction between a dimer and a monomer should determine the rate of trimer formation, and the dimers should be dissociated, and elute as monomers under the conditions of chromatography. Alternatively, the rate-limiting step of association may be the bimolecular association of two monomers to form a dimer, which should then rapidly bind a third monomer to form the trimer. In the latter case, the dimer concentration during reconstitution may not become large enough to be detected by HPLC. Further studies are in progress to validate one of the two models.

A folding reaction at the trimer level ($T_p \rightarrow N$) is rate-determining for reconstitution of native P22 tailspikes. The kinetics of trimer maturation to form native, SDS-resistant tailspikes were observed by SDS–polyacrylamide gel electrophoresis. Maturation at 10 °C was extremely slow, and only completed after ≥3 days of reconstitution. As maturation of trimers can be essentially stopped for many days by lowering the temperature further to 0 °C, but completed within 2 h upon subsequently increasing the temperature to 20 °C (C. Seiderer and R. Seckler, unpublished results), the activation energy of the maturation reaction must be high. Folding of the collagen triple helix also occurs after registration-peptide-mediated association with a high activation energy, probably determined by cis–trans isomerizations of X–Pro peptide bonds (Bruckner & Eikenberry, 1984). However, as the bulk of the secondary and tertiary structure of the phage P22 tailspike subunits is formed within minutes, and before association takes place, the slow process of tailspike maturation does not resemble collagen folding but rather comprises reshuffling of preformed structural elements. The pathway of reconstitution of phage P22 tailspikes in vitro exhibits close similarity to the folding and association path of newly synthesized tailspike polypeptides in the *Salmonella* cell, as observed after pulse-labeling with radioactive amino acids (Goldenberg & King, 1982; Goldenberg et al, 1983). Upon dilution from concentrated GdnHCl or acid–urea solutions, or upon biosynthesis on the ribosome, tailspike polypeptides rapidly fold into structured monomers and associate to form a nonnative trimer intermediate (T_p). The properties of the trimer intermediate observed in vitro are identical with those of the in vivo "protrimer" intermediate described by Goldenberg and King (1982). Both in vivo and in vitro protrimers are readily dissociated by the ionic detergent SDS in the cold, but individual protrimer molecules do not exchange subunits. The hydrodynamic radius of the protrimer is enlarged compared to the native tailspike trimer, as observed by size-exclusion HPLC of reconstituting tailspike protein, or by nondenaturing gel electrophoresis after pulse-labeling of phage-infected *Salmonella* cells. Conversion of the protrimer intermediate into native SDS-resistant tailspikes is the rate-determining step, both during reconstitution and during biosynthesis of tailspikes. Furthermore, a single amino acid exchange in the tailspike protein leading to a temperature-sensitive folding phenotype in vivo has been observed to also affect the efficiency of tailspike reconstitution at elevated temperatures (C. Seiderer and R. Seckler, unpublished results).

Our results indicate that in vitro reconstitution of phage P22 tailspikes may be employed as a model for in vivo folding and association of the phage protein during biosynthesis. Further work is in progress to dissect folding and association steps on the reconstitution path of the protein, and to localize the steps or intermediates on the pathway that are the targets of folding mutations.

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REFERENCES

Berget, P. B., & Poteete, A. R. (1980) *J. Virol. 34*, 234–243.
Bergman, L. W., & Kuehl, W. M. (1979) *J. Biol. Chem. 254*, 8869–8876.
Botstein, D., Waddell, C. H., & King, J. (1973) *J. Mol. Biol. 80*, 669–695.
Bruckner, P., & Eikenberry, E. F. (1984) *Eur. J. Biochem. 140*, 391–395.
Ellis, R. J. (1990) *Semin. Cell Biol. 1*, 1–19.
Fischer, G., & Schmid, F. X. (1990) *Biochemistry 29*, 2205–2212.
Goldenberg, D. P. (1981) Thesis, MIT, Cambridge, MA.
Goldenberg, D. P., & King, J. (1981) *J. Mol. Biol. 145*, 633–651.
Goldenberg, D. P., & King, J. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 3403–3407.
Goldenberg, D. P., Berget, P. B., & King, J. (1982) *J. Biol. Chem. 257*, 7864–7871.

Goldenberg, D. P., Smith, D. H., & King, J. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 7060-7064.
Haase-Pettingell, C. A., & King, J. (1988) *J. Biol. Chem. 263*, 4977-4983.
Heukeshoven, J., & Dernick, R. (1988) *Electrophoresis 9*, 28.
Jaenicke, R. (1987) *Prog. Biophys. Mol. Biol. 49*, 117-237.
Jaenicke, R., & Rudolph, R. (1986) *Methods Enzymol. 131*, 218-250.
Kim, P. S., & Baldwin, R. L. (1982) *Annu. Rev. Biochem. 51*, 459-489.
Kim, P. S., & Baldwin, R. L. (1990) *Annu. Rev. Biochem. 59*, 631-660.
King, J., & Yu, M.-H. (1986) *Methods Enzymol. 131*, 250-266.
Kuwajima, K. (1989) *Proteins: Struct., Funct., Genet. 6*, 87-103.
Mitraki, A., & King, J. (1989) *Bio/Technology 7*, 690-697.
Mitraki, A., Betton, J.-M., Desmadil, M., & Yon, J. (1987) *Eur. J. Biochem. 163*, 29-34.
Sargent, D., Benevides, J. M., Yu, M.-H., King, J., & Thomas, G. J., Jr. (1988) *J. Mol. Biol. 199*, 491-502.
Sauer, R. T., Krovatin, W., Poteete, A. R., & Berget, P. B. (1982) *Biochemistry 21*, 5811-5815.
Seckler, R., Fuchs, A., King, J., & Jaenicke, R. (1989) *J. Biol. Chem. 264*, 11750-11753.
Smith, D. H., & King, J. (1981) *J. Mol. Biol. 145*, 653-676.
Smith, D. H., Berget, P. B., & King, J. (1980) *Genetics 96*, 331-352.
Sturtevant, J. M., Yu, M.-H., Haase-Pettingell, C., & King, J. (1989) *J. Biol. Chem. 264*, 10693-10698.
Thomas, G. J., Becka, R., Sargent, D., Yu, M.-H., & King, J. (1990) *Biochemistry 29*, 4181-4187.
Villafane, R., & King, J. (1988) *J. Mol. Biol. 204*, 607-619.
Winston, F., Botstein, D., & Miller, J. H. (1979) *J. Bacteriol. 137*, 433-439.
Yu, M.-H., & King, J. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 6584-6588.
Yu, M.-H., & King, J. (1988) *J. Biol. Chem. 263*, 1424-1431.

Carbon-13 NMR Study of Switch Variant Anti-Dansyl Antibodies: Antigen Binding and Domain-Domain Interactions†

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ABSTRACT: A ^{13}C NMR study is reported of switch variant anti-dansyl antibodies, which possess the identical V_H, V_L, and C_L domains in conjunction with highly homologous but not identical heavy-chain constant regions. Each of these antibodies has been selectively labeled with ^{13}C at the carbonyl carbon of Trp, Tyr, His, or Cys residue by growing hybridoma cells in serum-free medium. Spectral assignments have been made by following the procedure described previously for the switch variant antibodies labeled with [1-^{13}C]Met [Kato, K., Matsunaga, C., Igarashi, T., Kim, H., Odaka, A., Shimada, I., & Arata, Y. (1991) *Biochemistry 30*, 270-278]. On the basis of the spectral data collected for the antibodies and their proteolytic fragments, we discuss how ^{13}C NMR spectroscopy can be used for the structural analyses of antigen binding and also of domain-domain interactions in the antibody molecule.

Immunoglobulin G (IgG),[1] which is a multifunctional glycoprotein with a molecular weight of 150K, consists of two identical heavy chains and two identical light chains. The heavy chains are composed of four homology units, V_H, C_H1, C_H2, and C_H3, whereas the light chains are divided into two homology units, V_L and C_L. Each of these homology units forms a characteristic domain structure known as an *immunoglobulin fold* that is rich in antiparallel β-sheets.

In the expression of a variety of antibody functions, domain-domain interactions play a crucial role. The antigen-binding site is constructed by V_H and V_L domains, whereas a variety of effector functions are carried out by the Fc region that is composed of two C_H2 and two C_H3 domains. Therefore, it is essential to understand how each of these domains interacts and behaves in the process of the expression of a variety of antibody functions.

We have recently reported a ^{13}C NMR study of a mouse monoclonal antibody specifically labeled with [1-^{13}C]Met (Kato et al., 1989). It has been shown that, even with the intact IgG with a molecular weight of 150K, the line widths of methionyl carbonyl carbon resonances are sufficiently narrow, and therefore, a double-labeling method developed by Kainosho and Tsuji (1982) can be applicable to site-specific resonance assignments. In the double-labeling method the carbonyl carbon of one type of amino acid (X) is labeled with ^{13}C and the α-nitrogen of another type of amino acid (Z) is

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[1] Abbreviations: C_L, constant region of the light chain; C_H1, C_H2, C_H3, constant regions of the heavy chain; CDR, complementarity-determining region; CDR1(H), CDR2(H), CDR3(H), CDR in the V_H domain; CDR1(L), CDR2(L), CDR3(L), CDR in the V_L domain; DNS-Lys, ϵ-dansyl-L-lysine; Fab, antigen-binding fragment; Fab*, a three-domain fragment composed of V_H, V_L, and C_L; Fc, fragment composed of the C-terminal halves of the heavy chains; FR, framework region; FR1(H), FR2(H), FR3(H), FR in the V_H domain; FR1(L), FR2(L), FR3(L), FR in the V_L domain; Fv, antigen-binding fragment composed of V_H and V_L; IgG, immunoglobulin G; IgG2a(s), a short-chain mouse IgG2a monoclonal antibody that lacks the entire C_H1 domain; [W]IgG, IgG labeled with [1-^{13}C]Trp (similar notations are used for other ^{13}C-labeled analogues); [H, Y]IgG, IgG that is doubly labeled with [1-^{13}C]His and [^{15}N]Tyr (similar notations are used for other doubly labeled analogues); NMR, nuclear magnetic resonance; V_H, variable region of the heavy chain; V_L, variable region of the light chain.

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