Biophysical Characterization of Refolded Drosophila Spätzle, a Cystine Knot Protein, Reveals Distinct Properties of Three Isoforms*1

The Drosophila Spätzle protein, involved in the embryonic development of the dorsal-ventral axis and in the adult immune response, is expressed as a preprotein and is activated by the serine proteinases Easter or Spätzle-processing enzyme. Proteolytic cleavage generates a 106-amino acid COOH-terminal fragment, C106, homologous to the mature form of nerve growth factor NGF, a cystine knot protein. Through alternative splicing, the Spätzle gene encodes for several isoforms that (with one exception, the “prepeptide isoform”) share C106 but differ in the prosequence. Three isoforms have been expressed recombinantly in Escherichia coli strains. The prepeptide isoform could be expressed in soluble form and is unstructured according to CD and NMR measurements. Dimeric full-length Spätzle isoforms have been refolded from insoluble inclusion bodies and are able to rescue Spätzle-deficient embryos. Although the two full-length isoforms exhibit similar far-UV CD spectra, large differences in tryptophan fluorescence quenching by the respective pro-parts are observed. Both full-length isoforms exhibited highly cooperative folding transitions. Proteolytic digestion using trypsin resulted in C106, whose unfolding exhibits lower thermodynamic stability and cooperativity compared with the full-length proteins. The structure of C106 reveals a T-shaped dimer with significant differences to NGF and a deep internal cavity. Substantial β-sheet formation is observed between the two monomers, whereas a long loop containing the single tryptophan residue is disordered in the crystals. Our results suggest that the prepeptides stabilize the tertiary structure of the “mature” Spätzle cystine knot.

The Spätzle protein is the precursor of a nerve growth factor-like ligand in Drosophila melanogaster (1). It defines the dorsal-ventral axis in Drosophila embryos and acts in the initiation of immune response to fungal and bacterial infection in adult flies (2). Sequence homology to coagulogen and human nerve growth factor (NGF),2 together with the spacing of cysteine residues, suggests a cystine knot motif, in which two disulfide bridges form a ring through which a third disulfide bridge is threaded (3).

Extracellular binding of mature Spätzle to its receptor Toll is thought to lead to receptor dimerization and autophosphorylation of the cytoplasmic Toll/interleukin-1 receptor domains (4, 5). Although Toll is distributed uniformly within the embryonic perivitelline membrane, a concentration gradient of Spätzle results in dorsal-ventral asymmetry. The extracellular domain of the transmembrane receptor Toll consists of two leucine-rich repeat domains that bind the ligand. The cytoplasmic domain of Toll shares sequence similarity with the vertebrate Toll-like receptors, such as interleukin-1 receptor (6, 7), also involved in innate immunity.

Activation of Spätzle proceeds via one of two extracellular proteolytic cascades. In the developmental pathway, Spätzle is activated by Easter, generating a 12-kDa COOH-terminal fragment, C106, that is capable of activating Toll and thereby initiating a gradient of the transcription factor Dorsal, resulting in dorsal-ventral differentiation. In the innate immune response, however, full-length dimeric Spätzle is activated by the Spätzle-processing enzyme (SPE) (8), ending ultimately with the production of the antibiotic Drosomycin. SPE is activated by the serine proteinase Spirit, which in turn is activated by either Grass (Gram-positive bacterial infection) or Persephone (fungal infection) (9).

The Spätzle gene encodes several proteins, ranging in size from 20 to 70 kDa, that are secreted in the perivitelline space of Drosophila embryos. Alternative splicing generates different pre-pro isoforms (10, 11) that share the signal sequence, Easter/SPE cleavage site, and NGF-like domain. The majority of the isoforms, which vary only in their propeptide regions, are expressed during early stages of Drosophila embryogenesis (11, 12). Although the COOH-terminal NGF-like domain is

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2 The abbreviations used are: NGF, nerve growth factor; GdnHCl, guanidinium hydrochloride; DSC, differential scanning calorimetry; C106, cystine knot domain of Spätzle; C106pro, C106 derived from Spz11.7 with COOH-terminal His tag; SPE, Spätzle-processing enzyme; BMP, bone morphogenic protein.
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Expression Vectors, Constructs, and Strains—Spätzle cDNAs, kindly provided by Dr. Robert DeLotto, were amplified by polymerase chain reaction using the following primers: Spz11.7, 5'-GGC AGG CAT ATG AAG GAG TAC GAA CGT ATC ATC-3' (forward) and 5'-AAA CTC GAG CCC AGT TCT CAA CGC GCA CTG CT-3' (reverse); Spz8.19, 5'-GGG AAC CAG ATG ATG ATG TTG AGC AGT ATC ATC ATC-3' (forward) and 5'-AAA GATCC TCA CCC AGT CTG CCG GCC GCA CTG CT-3' (reverse); and Spz8.24, 5'-GGGC AGG CAT ATG AAG GAG TAC GAA CGT ATC ATC ATC-3' (forward) and 5'-AAT GGAATCC TTA TTG ACA ATG ACC GAG CAG ATG CCC ATC-3' (reverse). Spz11.7 was cloned into the pET21a vector (COOH-terminal His6 tag) using restriction sites NdeI/BamHI. Spz11.7 was cloned into the pET15b vector (NH2-terminal His6 tag) using restriction sites NdeI/XhoI, and Spz8.19 and Spz8.24 were cloned into the pET15b vector (NH2-terminal His6 tag) using restriction sites NdeI/BamHI. Spz11.7 was cloned into the pET21a vector (NH2-terminal His6 tag) using restriction sites NdeI/XhoI, and Spz8.19 and Spz8.24 were cloned into the pET15b vector (NH2-terminal His6 tag) using restriction sites NdeI/BamHI. Positive vectors were sequenced and transformed in Ca2+ competent E. coli cells. Spätzle isoforms were expressed in E. coli strains BL21 (DE3), BL21 pUBS (DE3), and Rosetta (DE3) (Novagen).

Recombinant Protein Expression—Test expressions were carried out in 250-ml shake flasks with 50 ml of lysogenic broth medium at 37 °C and 125 rpm. Cultures were induced with 1 mM isopropyl-β-D-thiogalactopyranoside at A600 of ~0.5, and proteins were expressed for 4 h. For purification strategy development, larger amounts were produced under the same conditions in 5-liter shake flasks with 1.5 liters of lysogenic broth medium. Large scale expression was carried out in bioreactors with an 8-liter working volume and temperature and oxygen control in yeast extract medium. After inoculation, the cultures were fed with yeast extract and glucose starting at A600 of ~10 and induced with 1 mM isopropyl-β-D-thiogalactopyranoside at A600 of ~50. Cells were harvested 4 h after induction by centrifugation and stored at -80 °C.

Inclusion Body Purification and Solubilization—After thawing, cells were resuspended in 0.1 M Tris/HCl, 1 mM EDTA, pH 7.0, buffer and disrupted by high pressure using a French press (Gaulin). Bacterial DNA was removed by digestion with Benzonase (Sigma) for 30 min at 4 °C. A buffer of 6% Triton X-100, 60 mM EDTA, 1.5 M NaCl was added at a ratio of 1:2 (v/v), and the solution was centrifuged for 10 min at 31,000 × g to clarify the solution. The pelleted inclusion bodies were resuspended in 0.1 M Tris/HCl, 20 mM EDTA, pH 7.0, buffer and centrifuged a second time. The last step was repeated three times until no further soluble protein was washed out. Subsequently, the inclusion bodies were solubilized in 6 M guanidinium hydrochloride (GdnHCl), 0.1 M Tris/HCl, 1 mM EDTA, pH 8.0, buffer with fresh 100 mM dithiothreitol. After shifting the pH to 4, the protein was dialyzed against 4 M GdnHCl, 10 mM EDTA, pH 4.0 (15).

In Vitro Folding—The solubilized inclusion bodies were rapidly diluted between 1:200 and 1:300 to filtered, degassed, and
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chilled folding buffer at 8 °C to a concentration of 150 mg/liter and renatured for at least 48 h. Two folding buffers were developed: either 1 M L-arginine, 0.1 M Tris/HCl, 5 mM EDTA, 1 mM GSSG, 5 mM GSH, pH 8.5, or 1 M Tris/HCl, 5 mM EDTA, 1 mM GSSG, 5 mM GSH, pH 9.5. After in vitro folding, the protein solution was concentrated 10-fold by cross-flow filtration (Vivaflow). The folding buffer additives L-arginine and the oxidized shuffling system were removed by dialysis against 0.1 M Tris/HCl, 5 mM EDTA, pH 7.4 (15, 16).

Purification—The full-length isoforms were purified further by size exclusion chromatography with a Superdex 75 or 200 preparation grade column (GE Healthcare) in 20 mM Tris/HCl, 5 mM EDTA, pH 7.4. The soluble expressed propeptide isoform Spz2.24 was purified by affinity, ionic exchange, and size exclusion chromatography. Affinity chromatography using an Ni<sup>2+</sup>-nitrilotriacetic acid matrix (GE Healthcare) was performed in 0.1 M Tris/HCl, 20 mM imidazole, pH 7.4, binding buffer with stepwise elution up to 500 mM imidazole. For the ion exchange step, a HiTrap SP FF column (GE Healthcare) with 50 mM sodium citrate, 100 mM sodium phosphate, pH 5.5, was used. Elution was achieved using a 20 column volume gradient to 1 M NaCl. The final size exclusion step was carried out as described for the full-length isoforms.

Limited Proteolysis by Trypsin—The Spätzle full-length isoforms were processed by incubation with bovine trypsin (Sigma) at a ratio 100:1 (w/w) in 0.1 M Tris/HCl, pH 7.4, at 20 °C (4). In order to visualize intermediates during limited proteolysis, digestion was also performed at 4 °C, slowing down the rate of the reaction. For analytical samples, digestion was stopped by heating in SDS loading buffer for 5 min at 95 °C. For preparative samples, the cystine knot domain with a COOH-terminal His<sub>6</sub>-tag (C106His<sup>+</sup>) was purified by affinity chromatography with an Ni<sup>2+</sup>-nitrilotriacetic acid column in 0.1 M Tris/HCl, 20 mM imidazole, pH 7.4, binding buffer with stepwise elution up to 500 mM imidazole. For C106 from Spz2.19, which lacked a COOH-terminal His<sub>6</sub>-tag, the cystine knot domain was purified using a heparin affinity chromatography. To remove any traces of active trypsin, a HiTrap Benzamidin FF column (GE Healthcare), equilibrated in 0.1 M Tris/HCl, 0.15 M NaCl, pH 7.4, was applied, allowing collection of the cystine knot domain in the flow-through.

Cysteine Modification and Mass Spectrometry—The free cysteine content was determined by iodoacetamide modification; a molecular mass increase of 57 Da for each free cysteine should be detected using mass spectrometry. 1M iodoacetamide in 1M molecular mass increase of 57 Da for each free cysteine should be detected using mass spectrometry. 1M iodoacetamide in 1M molecular mass increase of 57 Da for each free cysteine should be detected using mass spectrometry.

Fluorescence Spectroscopy—Fluorescence measurements were carried out on a FluoroMax-2 spectrometer. Protein samples with ~2 µM concentrations in 20–50 mM sodium phosphate, 20 mM NaCl, pH 7.4, at 20 °C in 1-cm cuvettes were studied under native and varying GdnHCl-denatured conditions. Fluorescence was excited at 295 nm, and the emission scanned from 310 to 400 nm. Sample intensities were subtracted from buffer intensities and normalized to the protein concentration.

Differential Scanning Calorimetry (DSC)—Calorimetric measurements were performed using a VP-DSC Micro Calorimeter (MicroCal) with a 0.5-ml sample volume. Thermal denaturation was measured in a temperature range from 25 to 105 °C with a temperature slope of 9 °C/h.

NMR Spectroscopy—One-dimensional 1H spectra were recorded on a Bruker Avance II 600 spectrometer (Spz117, C106His<sup>+</sup>) and Bruker Avance 900 spectrometer (Spz2.24) at 298 K. Samples were measured in 20 mM sodium phosphate, 20 mM NaCl, pH 7.4, containing 10% (v/v) D<sub>2</sub>O. Water suppression was achieved by presaturation and/or WATERGATE. Spectra were processed and analyzed using Felix 2000.

In Vivo Activity Assay in D. melanogaster Embryos—Recombinant Spätzle protein was injected into embryos produced from spz null Df(3R)ED6255/spz<sup>2 ca</sup> females crossed to wild type males. Df(3R)ED6255 (96D2-F1) uncovers the spz gene (19), and spz<sup>2</sup> is a null allele (10). Df(3R)ED6255/spz<sup>2 ca</sup> transheterozygotes were produced by a cross of Df(3R)ED6255/ TM6c, Sb females with spz<sup>2 ca</sup>-/TM6c, Sb males. The spz<sup>2 ca</sup>-/ TM1, Me strain obtained from the Bloomington Drosophila Stock Center was balanced over the TM6c, Sb balancer chromosome to achieve the more visible dominant marker Sb (Stubble). Due to maternal gene effects, embryos from Df(3R)ED6255/spz<sup>2 ca</sup> females lack functional Spätzle, resulting in interruption of the dorsoventral axis development and the production of dorsalized embryos.

Flies were kept on freshly yeastfed plates and embryos collected at 30-min intervals. These were washed in 1 × phosphate-buffered saline (10 mM sodium phosphate, 150 mM NaCl, pH 7.2), dechorionated in 12% hypochloride solution, again washed in 1 × phosphate-buffered saline, dried for 7 min at 30 °C, covered with halocarbon oil, lined up on agar blocks, transferred to heptane-glued coverslips, and covered with 10S Voltalef oil. Injection of protein at a concentration of 5 g/liter in 0.1 mM sodium phosphate, 5 mM KCl, pH 7.4, into the ventral perivitelline space in embryos at developmental stage 2 (1) was carried out using drawn out capillaries and monitored under a
microscope. After injection, embryos on the coverslip were placed in a moist environment for 22 h at 25 °C. To detect rescued embryos, superfluous oil was removed by heptane, and the coverslip with embryos was inverted and mounted in Hoy-
er’s medium (20) with lactic acid (1:1) for permanent mounting. After clearing overnight at 65 °C, the embryos were visualized in dark field and counted for successful development of dorsal-
ventral patterning.

Crystalization and Structure Determination—Crystalliza-
tion of C106His is described elsewhere (21). A data set from a single C106 crystal was collected at beam line BL 14.1 (BESSY, Berlin) equipped with a fast scanning 225-mm CCD-mosaic detector (Marresearch). Oscillation photographs were inte-
grated, merged, and scaled using the XDS package (22). The structure of C106 was determined by molecular replacement with data between 20 and 3.0 Å. An assembly of five truncated NGF monomers (Protein Data Bank codes 1BTG (23), 1SG1 (24), 1BET (25), 1SGF (26)) was used as search model with PHASER (27). The asymmetric unit comprises two monomers (24), 1BET (25), 1SGF (26)) was used as search model with PHASER (27). The asymmetric unit comprises two monomers linked by an intermolecular disulfide bond. The structure was manually rebuilt and verified against simulated annealing com-
posite omit maps as well as sαA weighted difference Fourier maps using the programs O and Coot (28, 29). Refinement was car-
ried out using CNS (30) against a maximum likelihood target based on slow cooling simulated annealing (both torsion angle and Cartesian dynamics) combined with standard minimiza-
tion and individually restrained B-factor refinement. Both overall anisotropic B-factor and bulk solvent corrections were applied. Strong NCS restraints between equivalent residues in the two monomers were applied during the initial steps of the refinement; these were later relaxed and finally abandoned as a result of noticeable differences between the two monomers. Final refinement steps were carried out using alternate cycles of PHENIX (31) and CNS refinement, both using the same R-free set, to model anisotropic displacements of the C106 monomers. The PHENIX refinement was performed with six TLS groups (3 groups/monomer). The final model, consisting of residues Asp5–Glu108 in monomer A and Ser4–Gly106 in monomer B, one glicerol, and 37 water molecules, has been refined at 2.4 Å resolution to R and Rfree factors of 0.2219 and 0.2651, respecti-
vely. No electron density was observed for residues Leu23 to 41BET, 589.8, 62.2, 2.40, and 5.5 (63.1)4

| Parameter                          | Value       |
|-----------------------------------|-------------|
| Resolution range (Å)              | 19.83–2.40  |
| Completeness (%)                  | 99.2 (99.7) |
| No. of reflections (F > 0)        | 7919        |
| Wilson B (Å²)                     | 59.22       |
| R cryst (%)                      | 22.2 (37.1) |
| R free (%)                       | 26.5 (46.0) |
| Nonhydrogen atoms                |             |
| Total                             | 1462        |
| Protein                           | 1419        |
| Water                             | 37          |
| Glycerol                          | 6           |
| Root mean square deviation from ideality | 0.007 |
| Bond lengths (Å)                  | 1.40        |
| Dihedrals (degrees)               | 27.30       |
| Improper (degrees)                | 0.73        |
| Ramachandran statistics           |             |
| Percentage of residues in favored regions | 91.7 |
| Percentage of residues in allowed regions | 8.3 |
| B-factor (Å)²                    | 66.78       |
| Average                           |             |
| Protein atoms/monomer A/monomer B | 67.27/60.34 |
| Main chain/monomer A/monomer B    | 62.87/54.44/71.39 |
| Water                             | 50.62       |
| Glycerol                          | 73.96       |
| Protein Data Bank deposition      | 3E07        |

Table 1

| Crystallographic data statistics for C106His |
|---------------------------------------------|
| Parameters                                | Values |
|---------------------------------------------|
| Wavelength (Å)                             | 0.91840 |
| Space group                                | P2, 2, 2 |
| a (Å)                                      | 52.80  |
| b (Å)                                      | 58.98  |
| c (Å)                                      | 62.21  |
| Maximum resolution (Å)                     | 2.40   |
| Rmerge (%)                                 | 5.5 (63.1)4 |
| Completeness (%)                           | 99.1 (99.9) |
| No. of reflections (F > 0)                 | 37,360 |
| No. of unique reflections (i/oF(i))        | 7919   |
| R free (%)                                 | 16.49 (2.55) |

According to far-UV CD spectroscopy (Fig. 2), Spz8.24 possesses little if any secondary structure, with a minimum ellip-
spectra measured at 220 nm (Fig. 2B). The one-dimensional 1H NMR spectrum revealed an absence of peaks upfield of 0.5 ppm and a lack of dispersion of the amide resonances between 7.8 and 8.5 ppm (Fig. 3A), indicative of an unstructured protein (35). Thus, we conclude that the propeptide isoform Spz8.24 is natively unfolded, as shown previously for the propeptide of the Spätzle isoform HL01462 (4).

E. coli strain Rosetta (DE3) (supplemental Fig. 1) and purified using affinity, ion exchange, and size exclusion chromatogra-
phy, the last indicating a monomeric state for Spz8.24. The absence of covalently linked dimers was confirmed using mass spectrometry, which also revealed removal of the NH2-terminal methionine residue (Table 2). Spz8.24 contains three cysteine residues; iodoacetamide treatment and subsequent mass spectrometry showed that all three are modifiable, indicating that they are not involved in disulfide formation.

Expression of Spz8.24 in E. coli Results in Soluble, Monomeric Protein That Lacks Tertiary Structure—The propeptide iso-
form Spz8.24 could be expressed as a soluble protein in the
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TABLE 2
Determination of molecular mass and modifications of different isoforms

Free cysteines were modified by iodoacetamide to carbamidomethylated (CAM) cysteines, so that a shift of molecular mass for each modified cysteine of 57 Da is expected. One GSH molecule from the refolding buffer increased the mass of the monomer by 305 Da. A missing N-terminal Met reduces the mass by 131 Da. We have been unable to assign the mass difference of −840 Da in two of the cysteine knot forms.

| Isoform and species | Detected mass | Expected mass | Mass difference | Interpretation |
|---------------------|---------------|---------------|-----------------|----------------|
|                     | Da            | Da            | Da              | Disulfide bridges |
| Spz8.24             |               |               |                 |                |
| Monomer             | 19,026        | 19,028        | −2              | −1 Met         | 0              |
| Monomer-modified    | 20,428        | 20,428        | 0               | 3 CAM          | 0              |
| Spz11.7             |               |               |                 |                |
| Monomer             | 27,501        | 27,500        | 1               | 1 GSH          | 3              |
| Monomer-modified    | 27,554        | 27,557        | −3              | 1 CAM + 1 GSH  | 3              |
| Dimer               | 54,386        | 54,388        | −2              | 1 CAM          | 3              |
| Dimer-modified      | 54,555        | 54,559        | −4              | 3 CAM          | 7              |
| Cystine knot        | 25,958        | 25,959        | −1              | 2 CAM          | 8              |
| IB                  | 27,201        | 27,201        | −837            | 0 CAM          | 8              |
| IB-modified         | 27,713        | 27,714        | −1              | 9 CAM          | 0              |
| Spz8.19             |               |               |                 |                |
| Monomer             | 36,636        | 36,638        | −2              | −1 Met         | 3              |
| Dimer               | 73,271        | 73,274        | −3              | −2 Met         | 7              |
| Dimer-modified      | 73,444        | 73,445        | −1              | 3 CAM          | 7              |
| Cystine knot        | 22,987        | 23,829        | −842            | 7              |
| IB                  | 36,644        | 36,644        | 0               | −1 Met         | 0              |

Folding of Spz11.7 and Spz8.19 from Inclusion Bodies Results in Soluble Monomer and Dimer Fractions—The protein Spz11.7 was expressed as insoluble inclusion bodies in the E. coli strain Rosetta (DE3) (supplemental Fig. 1) with a COOH-terminal His6 tag. Following inclusion body preparation and GdnHCl solubilization, in vitro folding was achieved via rapid dilution into refolding buffer supplemented with a redox shuffling system. Subsequent size exclusion chromatography revealed the presence of both monomeric and dimeric Spz11.7 species. The monomer/dimer ratio could be influenced by the buffer conditions chosen for refolding; although 1 M arginine, 0.1 M Tris/HCl yields monomers and dimers in roughly equal amounts, the dimeric form is clearly favored in the presence of 1 M Tris/HCl (data not shown).

Mass spectrometry of the monomeric Spz11.7 fraction revealed the presence of a bound glutathione molecule (Table 2), which we assume binds to the intradimeric disulfide-forming cysteine residue. Iodoacetamide treatment revealed up to three free cysteines in the monomeric fraction, which can be interpreted in terms of two free cysteines in the prodomain, one free cysteine in the “mature” part, and the remaining six cysteines involved in a disulfide knot. A lack of suitable accessible proteinase cleavage sites precludes a more detailed assignment of the disulfide connectivity (11, 12). Since the monomeric fraction undergoes rapid and complete degradation upon limited trypsin proteolysis (data not shown), we assume the monomeric Spz11.7 to be an in vitro folding intermediate.

The Spz11.7 dimer, with a molecular mass of 54 kDa, was also subjected to iodoacacetamide treatment (Table 2), revealing masses corresponding to the modification of 0–3 cysteines. This is also consistent with cysteine knot formation together with an intermonomeric disulfide bridge, as predicted. Disulfide formation of the four cysteines in the pro-part could however be neither affirmed nor negated. As a control, the solubilized inclusion body material was also investigated; in this case, all nine cysteines were modified.

The longer full-length Spätzle isoform (Spz8.19), expressed with an NH2-terminal His6 tag and refolded similar to Spz11.7, produced corresponding results following mass spectrometric analysis (Table 2). Both in vitro folded dimeric Spätzle isoforms Spz11.7 and Spz8.19 are stable in acetate buffer, pH 4.5, and for 5 min at 100 °C without precipitation. These properties of native Spätzle have previously been applied to purify Spätzle from Drosophila cell extracts using low pH and high temperature (6, 36).

Refolded Spz11.7 Dimer Is Functional and Can Rescue spz-deficient Drosophila Embryos—Microinjection of recombinantly expressed dimeric Spz11.7 in the perivitelline space of spz-deficient D. melanogaster embryos was carried out to test the functionality of the in vitro folded protein. A significant number of the injected embryos (8 of 183) developed clearly visible denticle bands (Fig. 4); this is probably a lower limit due to the difficulties in detecting small numbers of denticles. A control experiment with 238 noninjected embryos resulted in 0 embryos with any ventral structures. Previous microinjection experiments using Spz mRNA yielded rescue rates between 40 and 60% (10); although the RNA concentration was 10 times lower than that used here for the recombinant protein, mRNA is able to undergo multiple cycles of translation. For protein microinjection experiments, neither rescue rates, protein concentrations, nor concentration dependence have been reported (1, 6, 12). The partial or complete doroventral patterning
observed here provides qualitative evidence that the Spz11.7 protein is correctly folded and biologically active.

**In Vitro Folded Full-length Spätzle Isoforms Are Stable Proteins, Exhibiting Highly Cooperative Folding Transitions and Strong Tryptophan Quenching**—The far-UV CD spectrum of full length Spz11.7 shows a negative ellipticity with a minimum around 203 nm (Fig. 5). The CD spectrum is atypical (37) but is reminiscent of that of NGF (38), another cysteine knot protein. Chemical denaturation results in a significant loss in signal in the wavelength range 215–225 nm. The GdnHCl-induced denaturation transition suggests a highly cooperative two-state model (Fig. 5B), with a transition midpoint of 2.1 M GdnHCl, indicating moderate stability of Spz11.7 against GdnHCl. Dispersion of the resonances of the amide protons and high field-shifted methylene/methyl groups in the proton NMR spectrum of Spz11.7 confirms a defined tertiary structure of the protein (Fig. 3B).

Spätzle full-length isoforms possess only one tryptophan (in the cystine knot domain) (Fig. 1), whose fluorescence properties can be used to follow conformational changes in the structure (3). Excitation at 295 nm results in a tryptophan fluorescence emission maximum at \( \lambda_{\text{max}} \) of \( \sim 345 \) nm for dimeric Spz11.7, with a substantial red shift (\( \lambda_{\text{max}} \) of \( \sim 360 \) nm) and increase in fluorescence intensity upon GdnHCl denaturation (Fig. 6A). Chemical unfolding of Spz11.7 monitored by fluorescence has a single transition midpoint of 2.1 M GdnHCl (Fig. 6B), comparable with that measured by far-UV CD spectroscopy.

For the larger of the Spätzle isoforms Spz8.19, the tryptophan fluorescence signal is extinguished almost entirely (Fig. 6A). The fluorescence spectrum of chemically denatured Spz8.19 is similar to that of denatured Spz11.7, as is the unfolding transition monitored by fluorescence (see also Fig. 7, C and D). Thus, both full-length isoforms undergo a highly cooperative unfolding transition that can be depicted in terms of a two-state model, with similar values for \( \Delta G_U \) and \( m_U \). Furthermore, the strong fluorescence quenching observed indicates that the single tryptophan residue of the cystine knot domain is buried in the full-length molecules and becomes exposed during unfolding. On the other hand, the stark differences in the degree of quenching in Spz11.7 and Spz8.19 provide strong evidence for divergent structural properties of the two isoforms.
The thermal stability of Spz8.19 was further investigated with DSC (Fig. 6). A temperature scan from 25 to 105 °C resulted in a reversible, symmetric peak with a melting temperature \( T_m \) of 72 °C. The high symmetry of the heat capacity peak indicates an ideal two-state model of thermal unfolding. These results could be confirmed for thermal denaturation monitored by tryptophan fluorescence (data not shown).

Limited Trypsin Digestion of Full-length Spa¨tzle Isoforms Releases the Cystine Knot Domain C106

—Limited proteolysis of dimeric Spz11.7 with bovine trypsin at 20 °C leads to rapid degradation, resulting in a single stable fragment when analyzed by reducing and nonreducing SDS gel electrophoresis (Fig. 7A). Mass determination of the purified product revealed a ~26-kDa dimeric protein (Table 1), corresponding to the cystine knot domain C106His that would be expected from Easter or SPE cleavage as well as an explained species with decreased mass of ~840 Da. Similar results have been obtained with recombinant Spätzle isoform HL01462 expressed in Sf9 cells (4), confirming a native structure of in vitro folded dimeric Spätzle. Reduction of the temperature to 4 °C led to slower degradation, revealing a variety of intermediates (Fig. 7A). Limited proteolysis proceeds for the most part via sequential cleavage of the two prodomains.

The far-UV CD spectrum of the cystine knot domain C106His at 4 °C shows a minimum that is blue-shifted from 203 to 197 nm for full-length Spz11.7, with lower ellipticity (Fig. 7B). The one-dimensional \(^1\)H NMR spectrum of C106His displays the key signals for a folded protein, with methylene/methyl peaks upfield of 0.5 ppm and a good dispersion of amide proton resonances (Fig. 3C). Comparison of the far-UV CD spectra for Spz11.7 and C106His suggests a higher \( \beta \)-sheet content in the full-length protein.

C106 derived from trypsin treatment of Spz8.19, differing from Spz11.7-derived C106His as a result of the absence of the COOH-terminal His\(_6\) tag, was indistinguishable from C106His both in terms of far-UV CD and fluorescence spectra. Thermally induced denaturation of C106 monitored by far-UV CD spectroscopy displayed an isosbestic point for the temperature range 20–90 °C at 210 nm, indicative of two...
starting at 25 °C with a nominal $T_m$ of 58 °C (data not shown).

The fluorescence properties of C106 were also investigated (Fig. 7C). Excitation of C106 at 295 nm results in an emission maximum $\lambda_{\text{max}}$ of ~350 nm and slightly decreased fluorescence intensity ($\lambda_{\text{max}}$ of ~360 nm) in the presence of denaturing concentrations of GdnHCl (i.e. no quenching is observed). The fluorescence spectrum of denatured C106 is indistinguishable from those of denatured Spz11.7 and Spz8.19, supporting the thesis that the strong tryptophan quenching observed for the full-length forms is the result of tertiary interactions of the cystine knot domain with the prodomain.

GdnHCl denaturation of C106 monitored by tryptophan fluorescence gives a transition midpoint of 2.4 M GdnHCl (Fig. 7D). In accordance with the DSC measurements, the chemical unfolding of C106 shows a broad transition, starting at low GdnHCl concentrations, with lower free energy of unfolding and lower cooperativity than observed for the full-length Spätzle isoforms.

Crystal Structure of C106His Reveals a T-shaped Molecule with a Large Internal Cavity—As predicted, C106 forms a parallel dimer, covalently linked by an intermolecular disulfide bond CysA98-CysB98 (Fig. 8). The cystine knot itself bears a strong resemblance to that of NGF, with the predicted intramolecular disulfide connectivity Cys99–Cys47, Cys10–Cys68, and Cys101–Cys54. The main body of each monomer consists of a highly twisted four-stranded mixed β-sheet.

In contrast to NGF, however, the regions of the molecule distal to the cystine knot are splayed apart, leading to a three-stranded intermolecular β-sheet almost perpendicular to the dimer axis. The undersides of these “wings,” which give the molecule an overall T-shape, are noteworthy for the exposure of an extensive hydrophobic surface (provided by the side chains of Ala78, Ile80, Leu86, and ValB16, TyrB18, and AlaB41 from the second monomer; see Fig. 9). Within the crystals, this surface is largely responsible for crystal contacts between symmetry related molecules (supplemental Fig. 3). The wings are flanked by a disordered loop segment LeuB23–AspB36 of the second monomer, which contains the single tryptophan residue TrpB29 (the “Trp-loop”). Residues Asn91–Phe93, which show significant differences in Cα positions of the two C106 monomers, may act as a hinge between the disulfide-linked core and the wings.

In addition to the significant intermonomer contacts caused by the wing β-sheet formation, the dimeric interface is characterized by extensive burial of large hydrophobic surfaces in the vicinity of the disulfide knots (Fig. 9). These interactions are supplemented by a network of charged/polar interactions. The side chain carboxylate of Glu45 is in contact with Arg14 and Lys8100 and His871 of the second monomer; Arg14 in turn contacts the side chain of Gln14 and the main chain carbonyl oxygen of Tyr872, and Asp6 interacts with Lys8100 of the second monomer.

3 The C106 numbering scheme is used here, with Val1 being the first residue after the Easter cleavage site. This differs from the Spz11.7 numbering scheme used in the previously published crystallization report (21), where the corresponding residue is Val124 (i.e. shifted by the 123 residues of the Spz11.7 propeptide).
tein (10) have been shown previously to rescue the spz null phenotype. We have demonstrated that full-length dimeric Spätzle variant Spz11.7 folded in vitro is capable of in vivo rescue of spz null Drosophila. This is seemingly in contradiction with the results reported recently for B. mori Spätzle (39), in which only the mature refolded BmSpz was able to induce expression of antimicrobial peptides. Although this may reflect (i) a species difference in molecular behavior or (ii) a fundamental difference in the processing pathways for dorsal-ventral development and innate immunity, the fact that the pro-BmSpz used in that study was monomeric suggests that the full-length protein may not have been correctly refolded.

Combining the results of far-UV CD, DSC, NMR, fluorescence, tryptic digestion, and mass spectrometry provides an insight into the overall structure of different Drosophila Spätzle isoforms. The propeptide of full-length Spätzle appears to be structured in the context of the proprotein, yet it lacks tertiary contacts in isolation. This finding is in agreement with in vitro folding studies of other members of the cystine knot protein superfamily: in both pro-NGF (16, 38, 41, 42) and pro-BMP-2 (43), the propeptides yield measurable far-UV CD signals in the presence of the (covalently bound) disulfide knot domain. Since this phenomenon is now also observed for the invertebrate Spätzle, it is conceivable that this is a general property of cystine knot proproteins.

Surprisingly, although the chemical and thermal stability of the “mature” C106 is relatively high, it is considerably lower than that of other members of the superfamily; NGF unfolds at a GdnHCl concentration of 3.5 M (41), as does BMP-2 (43), whereas a melting temperature of 108 °C has been reported for vascular endothelial growth factor (44). Furthermore, the denaturation of Spätzle is fundamentally different to that of pro-NGF (42) and pro-BMP-2 (43). For the latter two proteins, two distinct unfolding transitions are observed during chemical denaturation, with the prodomain unfolding at denaturant concentrations significantly lower than those necessary for unfolding of the disulfide knot domain. Furthermore, in both of these cases, the unfolding of the cystine knot domains is identical in the presence and absence of the prodomains. In contrast, full-length Spätzle isoforms exhibit single, highly cooperative unfolding transitions that can be modeled using a two-state transition; in the

A deep cavity of largely hydrophobic nature is observed along the molecular 2-fold axis that reaches down as far as the intermolecular disulfide bridge (Fig. 9). The extent of this cavity could easily be increased through rearrangement of the charged side chains of Arg14 and Glu45 mentioned above. Interestingly, additional electron density reveals the presence of a glycerol molecule buried deep within the dimer interface, sandwiched between the respective cystine knots of the two monomers. Together with the large Ca displacements observed between the two monomers for residues Asn59-Gln62, this suggests a dynamic behavior of the C106 fold.

**DISCUSSION**

Spätzle has been purified previously in small amounts from Drosophila extracts (1) or has been recombinantly expressed in insect cells (4, 12). Dimerization of Spätzle has been described in vivo (12) and predicted in silico (3). Due to their importance in the insect immune response, Spätzle proteins from the lepidopteran insects Manduca sexta and Bombyx mori (BmSpz) (39) and the mosquito Aedes aegypti (AaeSpz) (40) have also been studied. Injections of periviteline fluid from wild type embryos to spz-deficient embryos (1) and microinjection of spz mRNA or Sf9-expressed proprotein superfamily: in both pro-NGF (16, 38, 41, 42) and pro-BMP-2 (43), the propeptides yield measurable far-UV CD signals in the presence of the (covalently bound) disulfide knot domain. Since this phenomenon is now also observed for the invertebrate Spätzle, it is conceivable that this is a general property of cystine knot proproteins.

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absence of the prodomains, C106 unfolds with limited cooperativity and reduced free energy of unfolding. To the best of our knowledge, this is the first instance in which the cystine knot itself appears to be stabilized by the prodomain.

The crystal structure of C106\textsuperscript{H10} presented here allows a rationalization of these results. The large exposed hydrophobic surface provided by the wings and the long disordered (Trp-containing) loop are generally accepted indicators for the stability in proteins. The proximity of these latter residues with large displacements from NGF residues (in particular within the wings of C106); a strong quenching observed in Spz11.7 and Spz8.19. Based on the deep internal cavity observed in C106, it is tempting to speculate that Spätzle may also play a role in the direct binding of unknown ligands. In mammals, innate immunity to endotoxic lipopolysaccharide appears to be mediated at least in part by complex formation between glycolipid-bound MD-2 and TLR4 (Toll-like receptor 4) (48). It has been shown very recently that Spätzle induces a conformational change in the Toll ectodomain to induce dimerization of the latter (49).

We have shown that the two full-length Spätzle splice variants possess different structural characteristics. In addition to the various isoforms of Spätzle, some eight Toll-like genes (47) have been described in Drosophila. Five Toll and three Spätzle homologues have also been reported in the mosquito, whereby AaeToll5A and AaeSpz1C have been suggested to mediate the immune response to fungal infection (40). Since the prodomain and cystine knot domain of Spätzle remain associated following activation cleavage, it is possible that different splice variants recognize and activate cognate Toll receptors. Alternatively, they might be alternatively processed by the activating proteinases Easter and SPE, providing differentiation between embryogenesis pathways and defense mechanisms.

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to the Toll receptor is proposed to displace the prodomain, thereby leaving this free to perform other biological functions. It is likely that shielding of the extensive hydrophobic surface presented by the wings (in conjunction with residues from the Trp-loop, including Trp\textsuperscript{29}) provides a driving force for both prodomain and Toll receptor binding.

Despite sharing the cystine knot signature, it is clear that members of the superfamily show considerable diversity in the nature and function of the propeptide. Perhaps best characterized is pro-NGF: not only is the propeptide necessary for the correct folding of the cystine knot domain (16), it also plays a role in the p75/sortilin-mediated induction of apoptosis (46) (i.e. the opposite effect of NGF). The propeptides of BMP-2 (43) or vascular epithelial growth factor (44) are not necessary for the in vitro folding of the cystine knot domain, and no explicit functions have been assigned to their propeptides as yet. Transforming growth factor β, on the other hand, is secreted as a complex with its (cleaved) prodomain, the β1 latency-associated peptide (45). Spätzle now provides a novel member of this group, in which the prodomain and cystine knot domain mutually stabilize one another.

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FIGURE 8. Overall structure of Spätzle C106. A and B, orthogonal views of the Spätzle homodimer (A chain in blue, B chain in green), with the cystine disulfide bridges shown as spheres. The order of individual β-strands is denoted by capital letters; note the wings formed by β-strands C', C'', and the NH\textsubscript{2}-terminal portion of strand B from the second monomer. The amino and carboxyl termini are depicted as dark blue and red spheres respectively, whereas the boundaries of the disordered loops (dotted lines) are indicated as balls. C, schematic diagram depicting the secondary structure of mature Spätzle; colors and orientation are as in A. Residues with light backgrounds superimpose with corresponding residues in NGF (compare with F); dark backgrounds denote residues with large displacements from NGF residues (in particular within the wings of C106); a white background indicates residues from the disordered Trp-loop. D–F, corresponding representations of human NGF (25).
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The surface of monomer B is shown after the removal of monomer A; is as in Fig. 8. Colors are according to atom type (green, surface-exposed carbons; white, buried carbons; light blue, polar nitrogen; pink, polar oxygen; dark blue, basic nitrogen; red, acidic oxygen). Knobbed surfaces with *italic* residue numbers indicate atoms in contact with monomer A. The dimer is constructed via a 180° rotation about the vertical axis. Note (i) the hydrophobic nature of the wing; (ii) the hydrophobic cavity that reaches as far as the inter-monomer disulfide bridge (C98), flanked on either side by intermonomeric β-sheet formation; (iii) the extensive burial of hydrophobic surfaces in the neighborhood of the cystine knot; and (iv) the presence of a buried glycerol molecule (orange sticks, with omit map electron density contoured at 3σ).

It is conceivable that xenobiotic-induced conformational rearrangements of Spätzle (isoforms) could initiate diverse Toll-signaling mechanisms in Drosophila immunity.

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