Temperature-induced conformational changes in prosomatostatin-II: implications for processing

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Somatostatin (SRIF) is a 14-residue peptide hormone synthesized in the hypothalamus and pancreatic islets. SRIF-14 and an N-terminally extended form, SRIF-28, are generated by the proteolytic processing of an approx. 102-residue precursor prosomatostatin (proSRIF) at a single set of paired basic residues (Arg-Lys) and at a monobasic (Arg) site respectively. Previous work in our laboratory demonstrated that the propeptide of SRIF mediates intracellular sorting; we suggested that this information resides in the prohormone structure. To identify putative sorting domains we have investigated structural features of recombinant anglerfish proSRIF-II purified from *Escherichia*

coli. Two species of proSRIF-II were obtained: a monomeric form and a disulphide-linked dimer. CD analyses revealed that monomeric proSRIF-II lacks appreciable periodic secondary structure; however, on slow heating (2 °C/min) and cooling, it assumed a predominantly α -helical conformation. When subjected to a second heating-and-cooling cycle, the α -helical conformation was maintained. In contrast, the dimeric form of proSRIF-II was predominantly α -helical and its helicity did not increase in response to heating and recooling. Our results suggest that proSRIF-II might exist in several different folding intermediate states.

INTRODUCTION

Small peptide hormones and neuropeptides are synthesized as larger precursors, which are frequently polypeptides comprising multiple bioactive peptides. During intracellular transport through the secretory pathway, prohormones undergo a series of post-translational modifications, including endoproteolytic cleavage at paired basic amino acids or, less commonly, monobasic residues to generate the bioactive molecule. To identify sorting domains within prohormones, we have been studying prosomatostatin (proSRIF), the precursor to the 14-amino-acid hormone somatostatin (SRIF), which was originally isolated from sheep hypothalamus as a peptide that inhibited growth hormone secretion [1]. In mammals, proSRIFs comprise a highly conserved family of polypeptides that exhibit approx. 96% overall sequence identity in their signal peptides, proregion and the mature hormone. ProSRIFs are among the simplest peptide hormone precursors: the mature hormone is located at the C-terminus and is preceded by a single Arg-Lys pair. Cleavage at this processing site, most probably by the prohormone processing enzyme prohormone convertase 2 (PC2), gives rise to the 14-residue hormone [2]. A second form of SRIF, SRIF-28, which is the tetradecapeptide possessing a 14-residue N-terminal extension, is released by cleavage at a single basic residue, Arg; this might be mediated by an aspartic proteinase related to the *Saccharomyces cerevisiae* enzyme, Yap3 [3,4]. In mammals, SRIF-14 and SRIF-28 result from the tissue-specific processing of a common precursor, whereas in several species of fish separate genes encode two distinct precursors, preproSRIF-I and preproSRIF-II, from which mature hormones SRIF-14 and SRIF-28 respectively are released [5–7].

Evidence from several laboratories including our own [8–11] suggests that information to effect intracellular prohormone sorting and processing resides in the propeptide. A fusion protein comprising the anglerfish SRIF-I propeptide and α -globin was processed correctly when expressed in rat anterior pituitary GH₃ cells to yield ‘mature α -globin’ [11]. Similarly, a chimaera comprising rat proSRIF fused to anglerfish proSRIF-II was also processed in pituitary AtT-20 cells, although there was some aberrant processing [10]. In contrast with proSRIF-I, proSRIF-II is degraded quantitatively when expressed in GH₃ cells [12]; this was surprising because proSRIF-I was accurately processed and efficiently sorted to the regulated secretory pathway [2]. More recently we demonstrated that the propeptide of anglerfish SRIF-I, when fused to proSRIF-II, rescued the latter from intracellular degradation [8]. Rescue occurred only when the propeptide was expressed in *cis* as part of a chimaera rather than in *trans*, and we hypothesized that the propeptide might possess one or more sorting motifs or might function as a ligand for a putative receptor [8]. In addition, recent work with site-directed mutagenesis has suggested that the N-terminal residues (Cys⁸ to Cys²⁰) of pro-opiomelanocortin could function as an intracellular sorting signal [9] that is recognized by a sorting receptor [13]. A chimaera comprising the N-terminal 26 residues of pro-opiomelanocortin fused to chloramphenicol acetyltransferase (CAT) was sorted to the secretory pathway [14]; in contrast, a proSRIF-I–CAT fusion was incompetent for transport and was turned over in the endoplasmic reticulum [15]. Because there is little similarity between different prohormones, the propeptide conformation, rather than a primary amino acid sequence, might be important for intracellular trafficking.

Although three-dimensional models of proinsulin have been

Abbreviations used: DTT, dithiothreitol; ESI, electrospray ionization; MALDI–TOF–MS, matrix-assisted laser desorption ionization–time-of-flight MS; proSRIF, prosomatostatin; SRIF, somatostatin.

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inferred from the crystal structure of mature insulin [16,17], as yet little is known about the tertiary structure of any prohormone. As a first step towards determining the structure of a prohormone, we have expressed proSRIF-II in *Escherichia coli* and purified it to homogeneity. Here we report that the 'native' proSRIF-II isolated from bacteria (conformer 'N') has little periodic secondary structure, suggesting a lack of ordered tertiary structure. Most significantly, proSRIF-II could be induced to undergo a major structural change in response to slow heating and cooling; this resulted in an up to 16-fold increase in α -helical content.

EXPERIMENTAL

Antibodies

Rabbit anti-(SRIF-28) serum, designated R33, was prepared against a synthetic peptide corresponding to anglerfish SRIF-28 [12]. Rabbit antiserum against the propeptide of SRIF-II, designated R60, was generated by constructing a fusion protein of the propeptide and glutathione S-transferase with the use of the pGEX-3x vector (Pharmacia) and expressed in *E. coli*; the fusion protein was purified with a glutathione affinity column [18].

Bacterial expression and purification of anglerfish proSRIF-II

A cDNA encoding proSRIF-II, lacking the signal peptide, was subcloned into the expression vector pET3c and introduced into *E. coli* BL21(DE3)pLysS [19]; proSRIF-II corresponded to approx. 20% of the total soluble protein (see Figure 1). The synthesis and identification of proSRIF-II was determined by Western blot with antibodies specific for the SRIF-II propeptide (R60) and SRIF-28 (R33) respectively. The fidelity of expression was further confirmed by N-terminal sequencing of the first 20 residues of the recombinant protein.

For purification, approx. 30–50 g of frozen *E. coli* were disrupted with a French press. The lysate was centrifuged at 14000 g in a Sorvall SS-34 rotor to remove cell debris and the supernatant was centrifuged for 2.5 h at 150000 g in a Beckman Type 50.2Ti rotor. This post-ribosomal supernatant was adjusted to 40% (w/v) $(\text{NH}_4)_2\text{SO}_4$ and the precipitated proteins were dialysed extensively against approx. 400 vol. of 80 mM KCl/50 mM Tris (pH 7.5)/0.1 mM EDTA (pH 8.0). The dialysate was batch-absorbed to DEAE-cellulose and eluted with 200 mM KCl; the eluate was applied to a column (40 ml) of DEAE-Sepharose CL-6B; proSRIF-II was eluted at approx. 250 mM KCl. This eluate was size-fractionated on a 300 ml Superdex-75 FPLC column and the proSRIF-II peak applied to a 1 ml Mono Q FPLC column; the purified prohormone was eluted at 220 mM KCl (see Figure 1).

HPLC

Purified, recombinant 'native' (conformer 'N') proSRIF-II alone, or after treatment with 50 mM dithiothreitol (DTT) (15 min at 55 °C) was applied to a Vydac reverse-phase C_{18} HPLC column (25 cm $\times$ 4.6 mm) and eluted with the following gradient: 0–7 min, 5% (v/v) CH_3CN ; 7–10 min, 5–12.5% (v/v) CH_3CN ; 10–70 min, 12.5–57.5% (v/v) CH_3CN ; the flow rate was 1 ml/min. All solutions contained 0.1% (v/v) trifluoroacetic acid [2,8]. The proSRIF-II conformers ('N' and 'H') and their tryptic digests were resolved by HPLC under identical conditions.

Tryptic peptide analysis

Purified recombinant 'native' ('N') or the other ('H') conformer of proSRIF-II (100 μg), dissolved in 50 mM NH_4HCO_3 , pH 8.0,

was incubated with 0.4 μg of trypsin for up to 120 min at 37 °C. Aliquots were withdrawn at the end of the respective periods and analysed by reverse-phase HPLC with a C_{18} column (as above) and by matrix-assisted laser desorption/ionization–time-of-flight (MALDI-TOF)-MS.

Aggregation studies

Purified recombinant proSRIF-II (56 μM) was dissolved in buffered solutions of CaCl_2 (pH 5.5, 6.0, 6.5 and 7.0) each containing the following concentrations of free Ca^{2+} : 1 μM , 10 μM , 100 μM , 1 mM and 5 mM Ca^{2+} buffer at pH 5.5, 6.0, 6.5 and 7.0; all buffers contained 1 mM EGTA. After incubation on ice for 10 min, the samples were centrifuged at 12000 g for 5 min each. The pellet and supernatant materials were resolved on a 15% (w/v) polyacrylamide gel containing 0.1% SDS and stained with Coomassie Brilliant Blue.

Structural studies by CD

All CD spectra were recorded with a Jasco Model J-720 spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan) with HPLC-purified recombinant proSRIF-II dissolved in either 50 mM phosphate buffer, pH 7.5, or 80 mM KCl/50 mM Tris/HCl (pH 7.5)/0.1 mM EDTA (pH 8.0) at protein concentrations ranging from 0.4 to 1.5 $\mu\text{g}/\mu\text{l}$, in a water-jacketed quartz cell with a 0.1 mm path length. Measurements were performed at 1 nm bandwidth with 0.125 s response time and 0.5 nm step resolution. Six scans were accumulated for each sample at a scan speed of 50 nm/min. The temperature of the water-jacketed cell was controlled via a programmable thermostat interface. The measured ellipticity data were averaged and converted into mean residue ellipticity, $[\theta]_{\text{m.r.w.}}$, which is defined as $[\theta]_{\text{m.r.w.}} = \theta/10nCl$, where θ is the measured ellipticity in millidegrees, l is the path length of the cell in cm, C is the concentration (mol/l) of the protein, and n is the number of amino acid residues in the protein. The CD data were plotted as mean residue ellipticity $[\theta]_{\text{m.r.w.}}$ (degrees $\cdot \text{cm}^2 \cdot \text{dmol}^{-1}$) against wavelength, in 0.5 nm steps. The protein concentration for calculating the molar ellipticities was determined to be 56 μM by quantitative amino acid analysis of proSRIF-II.

Structural studies by MS

To determine the accurate molecular mass of pure, recombinant 'native' ('N') or the other ('H') conformer of proSRIF-II, and their tryptic fragments, MALDI-MS was performed on a Voyager RP TOF mass spectrometer (Perceptive Biosystem, Framington, MA, U.S.A.). For MALDI-TOF-MS measurement, proSRIF-II or its tryptic peptides dissolved in 50 mM ammonium acetate or sodium acetate buffers was mixed with an equal volume of saturated matrix solution (sinapinic acid for proSRIF-II and α -cyano-4-hydroxycinnamic acid for the tryptic peptides). Water/acetonitrile (1:1, v/v) containing 0.1% (v/v) trifluoroacetic acid was used as the solvent system to prepare the matrix solution. Protein or peptide solution (1 μl) at a concentration of approx. 2.5 mM containing matrix was loaded on the stainless steel target backing and air-dried at room temperature before introduction into the ion source. The accelerating voltage in the ion source was 30 kV. Typically, 20–50 laser shots were accumulated to generate a spectrum. The buffers and salts in the purified protein were removed before analysis by electrospray ionization (ESI) MS by using on-line HPLC with a 1.5 cm $\times$ 0.25 mm C_{18} guard column. All ESI mass spectra were acquired with an API-III triple quadrupole mass spectrometer equipped with an atmospheric pressure ionization (API) source and a SCIEX ion spray

(pneumatically assisted electrospray) interface (PE-SCIEX; Thornhill, Ontario, Canada). The mass scale of the spectrometer was calibrated with poly(propylene glycol). Typically, 10 scans of 10–20 s duration were acquired and added to yield a mass spectrum.

RESULTS

Purification and characterization of proSRIF-II

Our initial goal was to produce sufficient quantities of proSRIF for structural analysis. To this end, human proSRIF and both forms of anglerfish precursor were expressed in *E. coli*; however, only anglerfish proSRIF-II was synthesized abundantly in *E. coli*. ProSRIF-II, lacking the signal peptide, constituted approx. 20% of the soluble protein, facilitating its purification by ion-exchange and gel-filtration chromatography (Figure 1). Seemingly homogeneous recombinant proSRIF-II was resolved into two species by reverse-phase HPLC (Figure 2A), with retention times of 49.7 and 51.8 min (peaks I and II respectively). It was possible that one of these species represented an oligomeric form of the precursor generated as a result of interchain disulphide bond formation. To test this idea, proSRIF-II was treated with the reducing agent DTT. Resolution of this material by HPLC

(Figure 2B) showed only a single peak of proSRIF-II present, with a retention time (49.7 min) corresponding to the first species of prohormone. This result suggested that the second peak of proSRIF-II material (retention time 51.8 min) was a disulphide-bonded oligomer; to confirm this idea we used MS (Figure 3). The first proSRIF-II peak had a molecular mass corresponding to a monomeric form of the prohormone, whereas the second peak was consistent with its being an interchain Cys^{91/102}-Cys^{91/102} disulphide-bonded dimeric form of proSRIF-II; however, for reasons that are unclear, the putative dimer was approx. 16 mass units smaller than expected. ProSRIF-II monomer and dimer were inert to cysteine derivatization with *p*-hydroxy-mercuribenzoate, resulting in no change in their mass, before and after reaction [20] (results not shown). The molecular mass of proSRIF-II (retention time 49.7 min) was determined by ESI-MS and found to be a single species of molecular mass 11 812 Da (Figure 4), which corresponds exactly to the predicted mass of anglerfish proSRIF-II [5]; this conclusively established its identity as the authentic prohormone.

Intermediate folding conformers of proSRIF-II

To determine whether the prohormone possessed secondary structure, we used CD spectroscopy. The CD spectrum of HPLC peak I, 'native' proSRIF-II (conformer 'N'), showed that it had very weak minima at 208 and 222 nm, suggesting minimal α -helical character (Figure 5A). This state was responsive to thermal denaturation and treatment with at least 3 M urea, which resulted in a CD spectrum consistent with a random coil structure, with large minima at approx. 195 nm (results not shown). Remarkably, on slow heating of proSRIF-II to 85 °C (2 °C/min) and cooling to 4 °C, the CD spectra progressively acquired increasing α -helical signature, with pronounced minima at 208 and 222 nm (Figure 5A). Furthermore, this α -helical form of the prohormone, designated the 'H' conformer, was a 'stable' species that retained its α -helical signature when subjected to a second cycle of heating to 85 °C and slow recooling (Figure 5B). Indeed, after two cycles of heating and slow recooling the α -helicity of the 'H' conformer increased slightly compared with proSRIF-II subjected to a single cycle of heating and recooling (Figure 5B). Interestingly, the formation of the peak I 'H' conformer was quite rapid and seemed to be independent of the duration for which proSRIF-II was incubated at 85 °C before recooling (Figure 5A, inset). Thus incubation at 85 °C for 4 min (the shortest time required for the instrument to record the spectra), 10 min or 20 min gave rise to the same CD spectrum when the prohormone was reooled slowly to 4 °C (Figure 5A, inset). In contrast with the peak I form of proSRIF-II, the CD spectrum of peak II was very different from the monomer (Figure 5C). The dimer had a CD spectrum consistent with an α -helical structure, and the degree of α -helicity remained unchanged after heating and recooling.

Aggregation of ProSRIF-II

Numerous studies have demonstrated that polypeptide hormones and members of the granin family of polypeptides, which are acidic glycoproteins present in the secretory granules of all endocrine cells, aggregate under conditions of low pH and high free Ca²⁺ ion concentration [21–25]. Consequently it was of interest to determine whether low pH and/or high Ca²⁺ ion concentrations induced changes in the proSRIF-II CD spectrum that might correlate with aggregation of the prohormone. Purified proSRIF-II was dissolved at pH 6 or 7 in the presence of increasing concentrations of free Ca²⁺ ions from 1 μ M to 5 mM

Figure 1 Purification of recombinant proSRIF-II expressed in *E. coli*

An extract of *E. coli* expressing proSRIF-II (lane 1) was batch-absorbed to DEAE-cellulose. The eluate (lane 2) was applied to a DEAE-Sepharose CL-6B column; the proSRIF-II immunoreactive material was eluted at approx. 250 mM KCl (lane 3). This material was size-fractionated on a 300 ml Superdex-75 FPLC column (lane 4), and the proSRIF-II material was applied to a 1 ml Mono Q FPLC column (lane 5). Each lane contained 12 μ g of protein, which was resolved on an 18% (w/v) polyacrylamide gel containing 0.1% SDS. Lane 6, an aliquot of the material eluted from the Mono Q column (lane 5) was transferred to PVDF membranes, which were blotted with the anti-(SRIF-28) antiserum (R33).

Figure 2 HPLC analysis of purified recombinant proSRIF-II

(A) Purified recombinant proSRIF-II (6 μ g) eluted from the Mono Q column was resolved on a C_{18} reverse-phase HPLC column with a gradient of CH_3CN in 0.1% (v/v) trifluoroacetic acid/water (see the Experimental section). Two peaks of proSRIF-II material, retention times 49.7 and 51.8 min respectively, were observed. (B) Purified recombinant proSRIF-II (6 μ g; approx. 0.5 nmol) treated with excess DTT (50 mM) for 15 min at 55 $^{\circ}C$ (see the Experimental section). After incubation, the reduced proSRIF-II was applied to a C_{18} reverse-phase HPLC column and eluted as in (A).

and the CD spectra were determined at 4 or 22 $^{\circ}C$ (Figure 6). Surprisingly, we observed no change in the spectra at either temperature over a 5000-fold Ca^{2+} concentration range and even under conditions thought to approximate the luminal milieu of the trans-Golgi network (approx. pH 6, 100 μ M Ca^{2+} [26]) or mature secretory granules (pH 7, 5 mM Ca^{2+} [25]); identical spectra were also observed at pH 5.5 and 6.5 (results not shown). Furthermore, in contrast with several polypeptide hormones and the chromogranins [21–25], proSRIF-II did not undergo low-pH or Ca^{2+} -induced aggregation under any of these conditions (Figure 7).

ProSRIF-II conformers are monomeric

Because proSRIF-II has a single disulphide bond between Cys⁹¹ and Cys¹⁰² within the C-terminal mature hormone portion, it could be argued that the change in CD spectra in response to slow heating and cooling represented the formation of multiple interchain disulphide bridges. To test this possibility we used MS to determine the relative sizes of the native proSRIF-II and the 'H' conformer and twice heated and recooled proSRIF-II (Figure 8). The MS data revealed that covalent multimers of proSRIF-II were not generated in response to heating and recooling (Figure 8). In addition the CD spectra were essentially independent of proSRIF-II concentration between 25 and 150 μ M, and the reverse-phase HPLC elution profiles of 'native' (conformer 'N') and the 'heated-cooled' (conformer 'H') forms of proSRIF-II were identical (results not shown). The elution profiles of 'N' and 'H' proSRIF-II conformers on gel-exclusion chromatography were also very similar. The absence of detectable proSRIF-II oligomers by any of these methods confirmed that

Figure 3 MS of purified recombinant proSRIF-II before and after treatment with DTT

(A) Purified recombinant proSRIF-II (6 μ g) eluted from the Mono Q column (Figure 1) was analysed by MALDI-TOF-MS before and after treatment with DTT. Shown are spectra of the proSRIF-II dimer (D^+), 23605 mass units; monomer (M^+), 11812 mass units; and M^{2+} , 5908 mass units. The other three small peaks are unidentified ions. (B) ProSRIF-II (6 μ g) was treated with DTT and the reduced proSRIF-II was analysed by MS as in (A). ProSRIF-II dimer (D^+), 23605 mass units (unreduced remnant after DTT treatment); monomer (M^+), 11812 mass units; and M^{2+} , 5908 mass units.

Figure 4 MS of purified recombinant proSRIF-II

Positive-ion ESI mass spectra of pure recombinant 'native' ('N') proSRIF-II protein (retention time 49.7 min; Figure 2A) dissolved in deionized water. Labels on the peaks indicate the protonation (charge) states of the protein. Inset, molecular mass of pure recombinant proSRIF-II determined by ESI mass spectra. The predicted molecular mass of proSRIF-II is 11809.77 Da (anglerfish proSRIF-II had Val at position 48 rather than Met as in the published sequence [5]).

Figure 6 Effect of Ca^{2+} concentration and pH on the CD spectra of recombinant proSRIF-II

ProSRIF-II (56 μM) was dissolved in buffer containing 1 μM , 10 μM , 100 μM , 1 mM or 5 mM free Ca^{2+} each at pH 5.5, 6.0, 6.5 or 7.0 and the CD spectra were determined at 4 or 22 $^{\circ}\text{C}$. In both panels $[\theta]$ represents $[\theta]_{\text{m.t.w.}}$. Shown are spectra of proSRIF-II (determined at 22 $^{\circ}\text{C}$) at 1 μM , 100 μM , 1 mM and 5 mM Ca^{2+} at pH 6.0 (A) and at pH 7.0 (B); the spectra at pH 5.5 and 6.5 were identical with those shown here. No significant differences in CD spectra were observed at 4 $^{\circ}\text{C}$ compared with 22 $^{\circ}\text{C}$; see Figure 5(A).

heat-induced interchain Cys^{91/102}-Cys^{91/102} disulphide bonds were not formed to any appreciable extent.

Sensitivity of proSRIF-II conformers to trypsin

To probe the structural features of the proSRIF-II 'N' and 'H' conformers further, proSRIF-II was treated with trypsin under limiting conditions. The rationale for these experiments was that if the prohormone had undergone distinct conformational changes in response to heat and recooling, the conformers should exhibit differential sensitivities to protease digestion. Trypsin is predicted to cleave proSRIF-II at 14 sites (Arg⁵, Arg¹⁸, Arg²⁵,

Figure 5 CD spectra of recombinant proSRIF-II

Purified proSRIF-II (56 μM) dissolved in 50 mM phosphate buffer, pH 7.5, was analysed by CD spectroscopy (see the Experimental section). The rate of heating and recooling was 2 $^{\circ}\text{C}/\text{min}$. In all panels $[\theta]$ represents $[\theta]_{\text{m.t.w.}}$: (A) 'Native' ('N') proSRIF-II (HPLC peak I) heated from 4 to 85 $^{\circ}\text{C}$ and re-cooled to 4 $^{\circ}\text{C}$ (conformer 'H'). Inset, 'N' proSRIF-II (HPLC peak I) heated to and held at 85 $^{\circ}\text{C}$ for 4, 10 or 20 min before recooling to 4 $^{\circ}\text{C}$. (B) 'Once heated-cooled' (conformer 'H') proSRIF-II (HPLC peak I) was heated from 4 to 85 $^{\circ}\text{C}$ and cooled to 4 $^{\circ}\text{C}$ a second time. (C) ProSRIF-II (HPLC peak II) heated from 4 to 85 $^{\circ}\text{C}$ and re-cooled to 4 $^{\circ}\text{C}$. Shown are the CD spectra of proSRIF-II after heating to 85 $^{\circ}\text{C}$ and recooling to the indicated temperatures.

Figure 7 ProSRIF-II does not undergo pH- and Ca^{2+} -induced aggregation

ProSRIF-II (56 μM) was dissolved in 1 μM , 10 μM , 100 μM , 1 mM or 5 mM Ca^{2+} buffer at pH 5.5, 6.0, 6.5 or 7.0 each and incubated on ice for 10 min. After incubation, samples were centrifuged at 12 000 g for 5 min and the pellet (P) and supernatant (S) materials were resolved on a 15% (w/v) polyacrylamide gel containing 0.1% SDS which was stained with Coomassie Brilliant Blue.

Figure 8 MS of conformers of recombinant proSRIF-II

(A) 'N' proSRIF-II: 1 μl of 0.66 $\mu\text{g}/\mu\text{l}$ 'native' ('N') proSRIF-II dissolved in 50 mM phosphate buffer, pH 7.5, was used to generate the spectrum (see the Experimental section). (B) 'H' proSRIF-II: 'native' ('N') proSRIF-II was slowly heated once (2 $^{\circ}\text{C}/\text{min}$) from 4 to 85 $^{\circ}\text{C}$ and cooled to 4 $^{\circ}\text{C}$ at the same rate. (C) 'H' proSRIF-II: 'native' ('N') proSRIF-II subjected to two cycles of slow heating from 4 to 85 $^{\circ}\text{C}$ and cooling to 4 $^{\circ}\text{C}$ as indicated in (A) and (B) (see the Experimental section).

Figure 9 Time course of trypsin digestion of recombinant proSRIF-II

(A) 'Native' ('N') proSRIF-II (100 μg) (retention times 49.7 and 51.8 min) dissolved in 50 mM ammonium bicarbonate, pH 8.0, was incubated with 0.4 μg of trypsin at 37 $^{\circ}\text{C}$ for between 0 and 90 min as indicated. At each time point, an aliquot (10 μg) was analysed by reverse-phase HPLC (see the Experimental section). (B) 'Heated-cooled' ('H') proSRIF-II was treated exactly as described in (A).

Arg²⁷, Arg³⁹, Arg⁵⁷, Arg⁶⁹, Arg⁷⁴, Arg⁸⁵, Arg⁸⁷, Lys³⁸, Lys⁸⁸, Lys⁹² and Lys⁹⁷, generating 15 peptide fragments, two of which are single residues, Arg³⁹ and Lys⁸⁸, and two dipeptides, Ala²⁶-Arg²⁷ and Glu⁸⁶-Arg⁸⁷. The 11 other fragments range in size from the tetrapeptide Ala⁸⁹-Lys⁹² to the octadecapeptide Ala⁴⁰-Arg⁵⁷. ProSRIF-II was incubated with a limited concentration of trypsin and, at the indicated times, aliquots from the tryptic digestions were analysed by reverse-phase HPLC (Figure 9) and by MS (Figure 10). The HPLC profiles showed that 'N' proSRIF-II was more sensitive to trypsin than the 'H' conformer (Figure 9A). For instance, full-length 'N' proSRIF-II (retention time 49.7 min) was completely digested by 20 min of treatment with trypsin (Figure 9A); in contrast, approx. 50% of the full-length 'H' conformer remained trypsin-insensitive at this time (Figure 9B). Furthermore, approx. 30% of 'H' proSRIF-II was trypsin-resistant even after 40 min, whereas the 'N' conformer was completely digested. It took 60 min of treatment trypsin to completely digest the full-length 'H' conformer of proSRIF-II trypsin (Figure 9B). In addition, several tryptic peptides from 'N' proSRIF-II appeared at markedly different rates than from 'H' proSRIF-II. For example, the most prominent peptide (retention time 27.5 min) after 60 min of tryptic digestion of 'N'

Figure 10 MS of peptides generated by digestion of recombinant proSRIF-II with trypsin

(A) 'Native' ('N') proSRIF-II was digested with trypsin (1:250, w/w) in 50 mM ammonium bicarbonate, pH 8.0, for 90 min and then analysed by MALDI-TOF-MS. (B) 'Heated-cooled' ('H') proSRIF-II was treated and analysed as above. The bold numerals denote the amino acid positions of the peptide fragments' N-terminus and C-terminus.

proSRIF-II (Figure 9A) was absent from the trypsin-treated 'H' conformer at this time (Figure 9B). Conversely, at 60 min of digestion with trypsin, 'H' proSRIF-II yielded a major peptide (retention time 28.2 min) that was present only at very low levels in the 'N' proSRIF-II digest. Similarly, the peptide eluted at 35.3 min after 90 min of treatment of the 'H' conformer with trypsin (Figure 10B) was less detectable in 'N' proSRIF-II (Figure 9A). These results indicate that proSRIF-II conformers exhibited differential susceptibility to digestion with trypsin, consistent with the idea that they manifest differences in their three-dimensional structures.

Because the complete amino acid sequence of anglerfish proSRIF-II is known [5], MS allowed the identification of those prohormone regions that were resistant to protease. Whereas several tryptic peptides were common to both 'N' and 'H' proSRIF-II after 90 min of tryptic digestion, two peptides, an N-terminal dodecapeptide, Ser²⁸-Arg³⁹ (1361.75 mass units), and a C-terminal tetrapeptide, Ala⁸⁹-Lys⁹² (378.18 mass units), were evident only in the 'N' proSRIF-II digests (Figures 10 and 11).

This result suggests that, in contrast with 'native' ('N') proSRIF-II, residues Arg²⁷, Arg³⁹, Lys⁸⁸ and Lys⁹² in the 'heated-cooled' ('H') proSRIF-II were inaccessible to trypsin and we speculate that as a consequence of heat-induced folding these residues were buried in the 'H' precursor (Figure 11).

DISCUSSION

Many prohormones possess multiple sets of paired basic amino acids, yet only specific subsets of the putative processing sites are cleaved in different cells. In part, cleavage specificity is defined by the expression of different prohormone convertases [27]; however, because there seem to be a limited number of these enzymes, additional factors probably function in defining processing specificity [28,29]. One such 'factor' could be the prohormone structure, which would be expected to fold in such a way that only a particular set of paired basic residues are presented to the processing enzymes.

In an attempt to determine the structure of one of the simplest prohormones, we purified recombinant anglerfish proSRIF-II and demonstrated that as isolated from *E. coli* the 'native' prohormone has little secondary structure. Analysis of the CD spectrum (Figure 5A) estimated that the secondary structure had approx. 43% helical character, approx. 22% β -sheet and approx. 22% random coil. On heating and recooling proSRIF-II underwent a marked increase in its α -helicity (80–90%) and this was maintained after a second cycle of heating and recooling (Figure 5). We hypothesize that proSRIF-II purified from *E. coli*, an environment distinct from the secretory pathway of endocrine cells, was trapped as a kinetic folding 'intermediate' with minimal structure (conformer 'N'). In this model, when proSRIF-II was subjected to the first cycle of heating and cooling, it would overcome an energy barrier to reach a pronounced α -helical conformation, i.e. 'conformer H'. The second heating and cooling cycle drives proSRIF-II to a stable state, which is predicted to be slightly more α -helical than conformer 'H'. In contrast with the CD spectrum of (folded) human proguanylin [30], a 115-residue prohormone precursor to the heat-stable enterotoxin, 15-residue guanylin hormone, the CD spectrum of heated-recooled proSRIF was not indicative of strong β -sheet and β -turn structure. We predict that the 'twice heated-cooled' proSRIF-II might represent a stable, processing-competent configuration.

Earlier studies [31–33] suggested that the secondary structures of mammalian proSRIFs were dominated by alternating helical and turn configurations. The C-terminal hormone portion was predicted to have a β -strand structure preceded by a β -turn at or near the cleavage site. Indeed, the conformation of mature SRIF-14 and its analogues determined by CD and sedimentation equilibrium ultracentrifugation showed that it has an elongated structure with a Cys-S-S-Cys-linked hairpin loop, with four residues in an anti-parallel β -pleated sheet orientation [34,35]. By using CD, Fourier-transform IR spectroscopy of small synthetic

Figure 11 Identification of 'protease-sensitive/resistant domains' within proSRIF-II

Predicted trypsin cleavage sites on proSRIF-II: ↓, actual trypsin cleavage products (90 min incubation) on 'heated-cooled' ('H') proSRIF-II; △, partial cleavage; (△), regions designated A and B are resistant to trypsin digestion in the proSRIF-II 'H' conformer'. The mature 28-residue SRIF-II hormone is shaded.

peptides spanning the dibasic cleavage site of pro-oxytocin/neurophysin and site-directed mutagenesis of human proSRIF, Brakch et al. [36,37] showed that Pro⁻⁵ and the putative β -turn-forming Pro⁸⁴Arg⁸⁵Glu⁸⁶Arg⁸⁷ motif are necessary for efficient prohormone processing *in vitro* and in Neuro2A cells. Several related studies on mutagenized proSRIF [38] and biophysical analyses of peptides corresponding to portions of pro-oxytocin [39] have suggested a role for β -turn/loop motifs at prohormone processing sites. In contrast with these studies, we did not observe changes in CD spectra that were compatible with a β -turn structure. One possible explanation for this discrepancy is that in the earlier studies the CD spectra were obtained with short synthetic peptides, whereas in the present study full-length proSRIF-II was used. Thus it is possible that full-length proSRIF-II could assume one or more conformations different from that of a peptide corresponding to only a small domain of the proregion. It is also possible that peptides corresponding to the C-terminal region of proSRIF-II encompassing the conserved processing site and putative β -turn-forming sequence (Pro⁸⁴Arg⁸⁵Glu⁸⁶Arg⁸⁷Lys⁸⁸) generated the observed CD spectra, whereas the proregion would remain unstructured unless subjected to heating and recooling as demonstrated here. Interestingly, the connecting peptide in proinsulin remains largely unstructured, whereas the overall conformation of insulin in proinsulin closely resembles that of mature insulin [17].

Unlike several polypeptide hormones and chromogranin A or B, proSRIF-II did not aggregate at low pH even in the presence of high concentrations of Ca²⁺ (Figure 7). This was surprising because SRIF is stored in dense core secretory granules in pancreatic islet delta cells [6] and 'milieu-induced aggregation' in the trans-Golgi network has been proposed to be a sorting signal for hormone packaging into immature secretory granules [24]. There are two possible reasons why proSRIF-II failed to aggregate. First, unlike chromogranin A, which is approx. 40% α -helical at pH 5.5 and undergoes a Ca²⁺-induced increase in α -helicity at pH 7.5 [22], proSRIF-II did not exhibit any change in CD spectrum between pH 5.5 and 7.5 at all Ca²⁺ concentrations tested. Secondly, it is possible that as for proinsulin the propeptide actually prevents aggregation [16] and only after proteolytic processing would the mature hormone aggregate [40].

On the basis of the above results, we suggest a model of the proSRIF-II folding pathway that enables the processing site located at the C-terminus of proSRIF-II to be more compact while the N-terminal proregion remains flexible. In this context it is noteworthy that the trypsin digestion of the 'H' conformer suggested that the N-terminal and C-terminal extremities of proSRIF-II had undergone conformational changes after heating and recooling. The N-terminal open conformation might facilitate interaction with processing enzymes, which could then induce desired conformational changes in the prohormone at favourable local pH values and Ca²⁺ concentrations that prevail in processing compartments [26] to enhance their prohormone cleavage. Currently we are testing this idea by analysing the CD spectra of other forms of proSRIF and characterizing their protease-resistant domains.

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