

The mature part of proNGF induces the structure of its pro-peptide

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Abstract Human nerve growth factor (NGF) belongs to the structural family of cystine knot proteins, characterized by a disulfide pattern in which one disulfide bond threads through a ring formed by a pair of two other disulfides connecting two adjacent β -strands. Oxidative folding of NGF revealed that the pro-peptide of NGF stimulates *in vitro* structure formation. In order to learn more about this folding assisting protein fragment, a biophysical analysis of the pro-peptide structure has been performed. While proNGF is a non-covalent homodimer, the isolated pro-peptide is monomeric. No tertiary contacts stabilize the pro-peptide in its isolated form. In contrast, the pro-peptide appears to be structured when bound to the mature part. The results presented here demonstrate that the mature part stabilizes the structure in the pro-peptide region. This is the first report that provides a biophysical analysis of a pro-peptide of the cystine knot protein family.

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1. Introduction

Human nerve growth factor (NGF) belongs to the neurotrophin family, whose members control growth, survival, and differentiation of neuronal cells [1,2]. NGF is a non-covalent homodimer and contains a characteristic structural motif, the cystine knot, denoted by a ring structure formed by two disulfide bridges penetrated by a third disulfide bridge (Fig. 1A and B). The crystal structures of both mouse and human NGF have been reported [3,4].

NGF is expressed *in vivo* as a pre-pro-protein. The 18 amino acids long N-terminal signal sequence targets the pre-pro-protein to the endoplasmic reticulum. The 103 amino acids comprising pro-peptide that does not possess any cystine residues (Fig. 1C) is known to promote correct maturation *in vivo* and guides oxidative refolding of the mature moiety *in vitro* [5–8]. Thus, proNGF joins the group of those proteins, of

which structure formation is significantly aided by their pro-peptides that do not engage in redox reactions (for review, see [9,10]). Pro-sequence assisted folding appears to be a hallmark of the structure formation process of mainly proteases. Structure formation of the pro-peptides themselves is either dependent or independent of their mature moieties.

Besides its verified role *in vitro* as a folding facilitator, the pro-peptide modulates the function of NGF by eliciting pro-apoptotic responses that are likely to be transmitted by both, p75 receptor and sortilin [11,12]. In order to learn more about the biophysical properties of the pro-peptide, its stability and structure were studied in its isolated form and while covalently attached to NGF. Our results suggest that the pro-peptide is stabilized by association with the mature part and that in the absence of the mature moiety the pro-peptide lacks stabilizing tertiary contacts. Thus, the pro-peptide features of NGF described here closely resemble those of the pro-domain of subtilisin BPN^I with respect to structure and stability [13,14].

2. Materials and methods

2.1. Preparation of recombinant human proNGF

Inclusion bodies of proNGF were produced with pET11a in *Escherichia coli* BL21(DE3), and the fully folded protein was obtained as described previously [8].

2.2. Chemical crosslinking

20 μ l pro-peptide (0.3 mg/ml) was incubated with an equal volume of 0.02% (w/v) glutaraldehyde for 30 min at room temperature. The reaction was stopped by addition of 5 μ l of 2 M sodium borohydride in 100 mM NaOH. The protein was precipitated with sodium desoxycholate [15] and analyzed by 15% SDS-PAGE-gel stained with Coomassie Brilliant Blue.

2.3. Analytical ultracentrifugation

For analytical ultracentrifugation, the pro-peptide was analyzed in 20 mM Na-phosphate, pH 7.0, and 1 mM EDTA in a Beckman Optima XL-A centrifuge and a 50Ti rotor. Sedimentation equilibrium measurements (absorption at 230 and 280 nm) were carried out in double sector cells at 25 000 rpm and 20 °C. Data were analyzed with the software provided by Beckman Instruments (Palo Alto, CA).

2.4. Fluorescence measurements

Measurements were carried out on a fluorescence-spectrometer FluoroMax-2 (Jobin-Yvon-Spex). Slit widths for both excitation and emission wavelengths were 5 nm. Experiments were performed in 50 mM Na-phosphate, pH 7.0, and 1 mM EDTA at 20 °C in 1 cm cuvettes. For determination of the denaturation/renaturation transitions,

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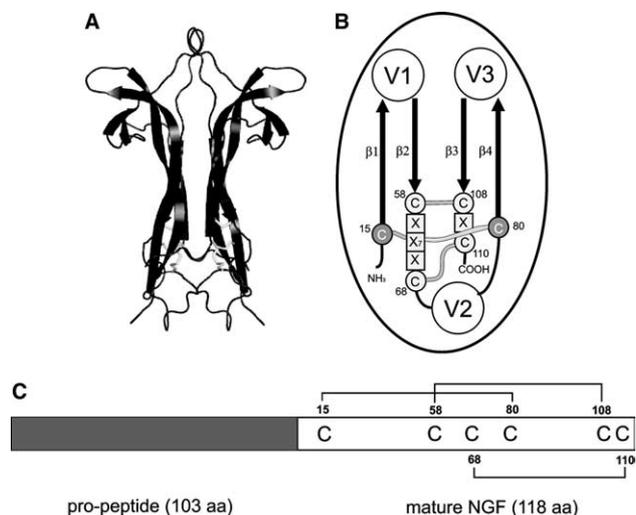


Fig. 1. Schematic presentation of NGF and proNGF. (A) Structure of the NGF dimer [3]. (B) Positions of β -strands and disulfide bridges in a NGF monomer (modified according to [21]). (C) Cysteine connectivities in proNGF.

proteins were incubated at the concentrations provided in the figure legends for at least 6 h. The recorded signals were normalized and linearly extrapolated [16].

2.5. Circular dichroism measurements

Far-UV CD spectra were recorded on an AVIV Model 62 ADS spectropolarimeter from 190 to 260 nm. Measurements were performed in 50 mM Na-phosphate, pH 7.0, at 20 °C. Spectra were buffer-corrected and ellipticities related to the mean residue weight of amino acid residues were calculated according to [17].

2.6. NMR spectroscopy

Proton spectra of 1.5 mM pro-peptide samples were acquired at a BRUKER DRX 500 spectrometer at 25 °C, 512 scans were averaged and a squared cosine window function was applied before Fourier transformation. The residual water resonance in the $^2\text{H}_2\text{O}$ samples, containing 50 mM Na-phosphate, pH 7.0, and 1 mM EDTA, was weakly pre-saturated during the relaxation delay. The concentration of urea was determined by refraction without correction for the isotopic

effect of $^2\text{H}_2\text{O}$. Exchangeable protons of the buffer were removed by dissolving in $^2\text{H}_2\text{O}$ three times and subsequent lyophilization.

3. Results and discussion

3.1. The isolated NGF pro-peptide is monomeric

The NGF pro-peptide was produced with pET11a in *E. coli* BL21(DE3) (Fig. 2A). In contrast to the recombinantly synthesized proNGF [8], the pro-peptide, which does not possess any cysteine residues, remained soluble in the *E. coli* cytosol. Cell lysis and the first purification step on SP-Sepharose were performed in the presence of 8 M urea. After renaturation by dialysis against urea-free buffer, the protein was purified to near homogeneity by hydrophobic interaction chromatography and cation exchange chromatography (Fig. 2A). The protein migrated with an apparent molecular weight of ca. 16 kDa in SDS-PAGE.

We could show that oxidative folding in vitro giving rise to the native homodimer is significantly stimulated by the pro-peptide [7,8]. Thus, the question arises as to whether the pro-peptide exists in isolation in a dimeric form. Analytical ultracentrifugation was performed for the analysis of the oligomeric state of the recombinant pro-peptide. Sedimentation equilibrium analyses yielded an apparent molecular mass of 11.5 kDa, clearly indicating the monomeric state of the pro-peptide (Fig. 2B). Moreover, the correct mass of the pro-peptide (calculated molecular mass: 11.6 kDa) was verified by mass spectrometry (data not shown). The monomeric state of the pro-peptide was confirmed by chemical crosslinking with glutaraldehyde (Fig. 2C). The bands of apparent molecular weights lower than 16 kDa are probably due to intramolecular crosslinking caused by the prevalence of arginines and lysines in the basic protein domain (pI: 11.6).

3.2. The NGF pro-peptide contains only few structural elements

The pro-peptide contains a single tryptophan and four phenylalanine residues. The fluorescence properties of these aromatic amino acids were used to investigate the tertiary

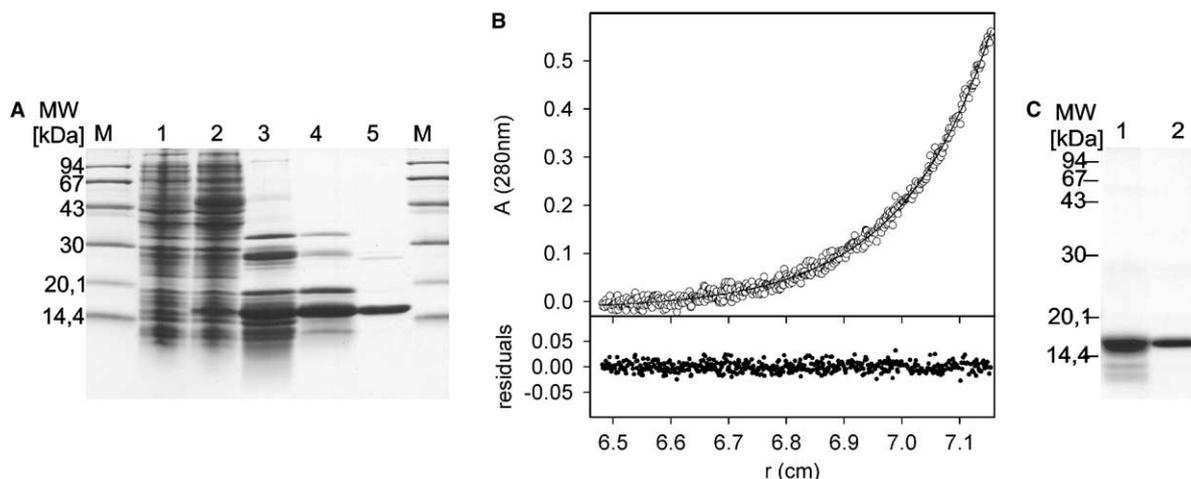


Fig. 2. (A) Isolation of recombinant human NGF pro-peptide from *E. coli* crude extract. 1: crude cell extract before induction, 2: crude cell extract after 3 h induction with 1 mM IPTG, 3: elution sample from the cation exchange chromatography in the presence of 8 M urea at pH 10, 4: sample after hydrophobic interaction chromatography, 5: sample after second cation exchange chromatography step at pH 7.0, M: marker. (B) Sedimentation equilibrium run of the pro-peptide (0.2 mg/ml). (C) Chemical crosslinking of the pro-peptide. 1: NGF pro-peptide after crosslinking with glutaraldehyde, 2: for negative control the protein was treated identically in the absence of crosslinker.

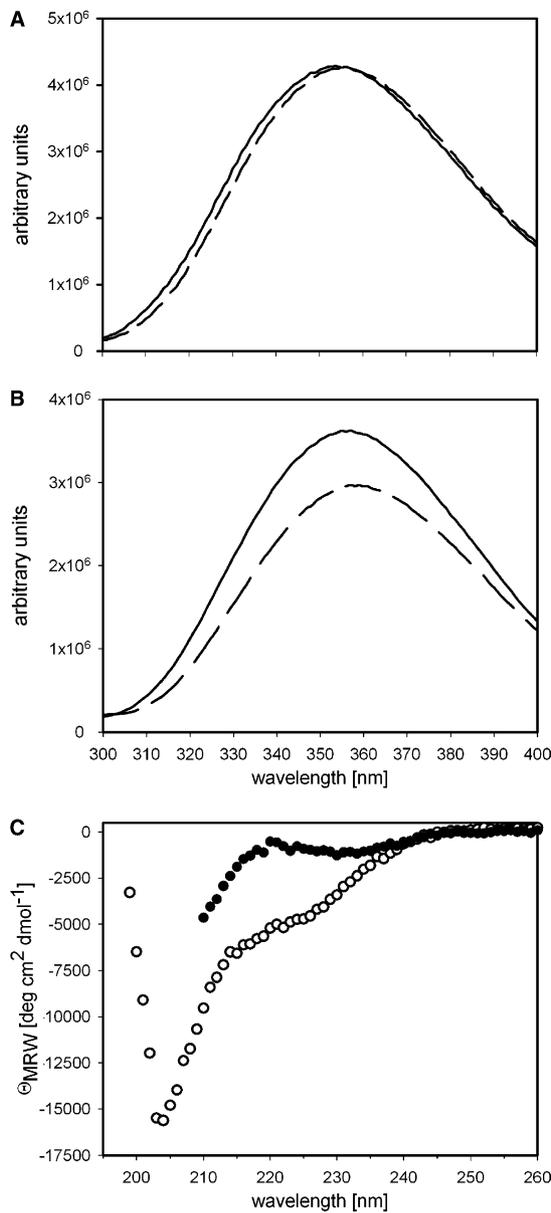


Fig. 3. (A) Fluorescence spectrum of the pro-peptide (50 µg/ml) upon excitation at 280 nm and (B) at 250 nm. Solid line, native pro-peptide; dashed line, pro-peptide denatured in 3.5 M GdmCl. (C) Far-UV CD spectra of the pro-peptide (1.5 mg/ml in 0.2 mm cuvettes). Open circles, native pro-peptide; closed circles, pro-peptide in the presence of 6 M GdmCl.

structure of the pro-peptide using fluorescence spectroscopy. The intrinsic tryptophan fluorescence signals of the native and denatured pro-peptide indicate that the single tryptophan residue is solvent-exposed under native conditions (Fig. 3A). When, however, fluorescence was measured at an excitation wavelength of 250 nm, a clear difference of the spectra of the native and the denatured protein was observed (Fig. 3B). This is due to the excitation of the four phenylalanines that contribute significantly to the overall fluorescence due to an energy transfer from excited phenylalanine to tryptophan. The

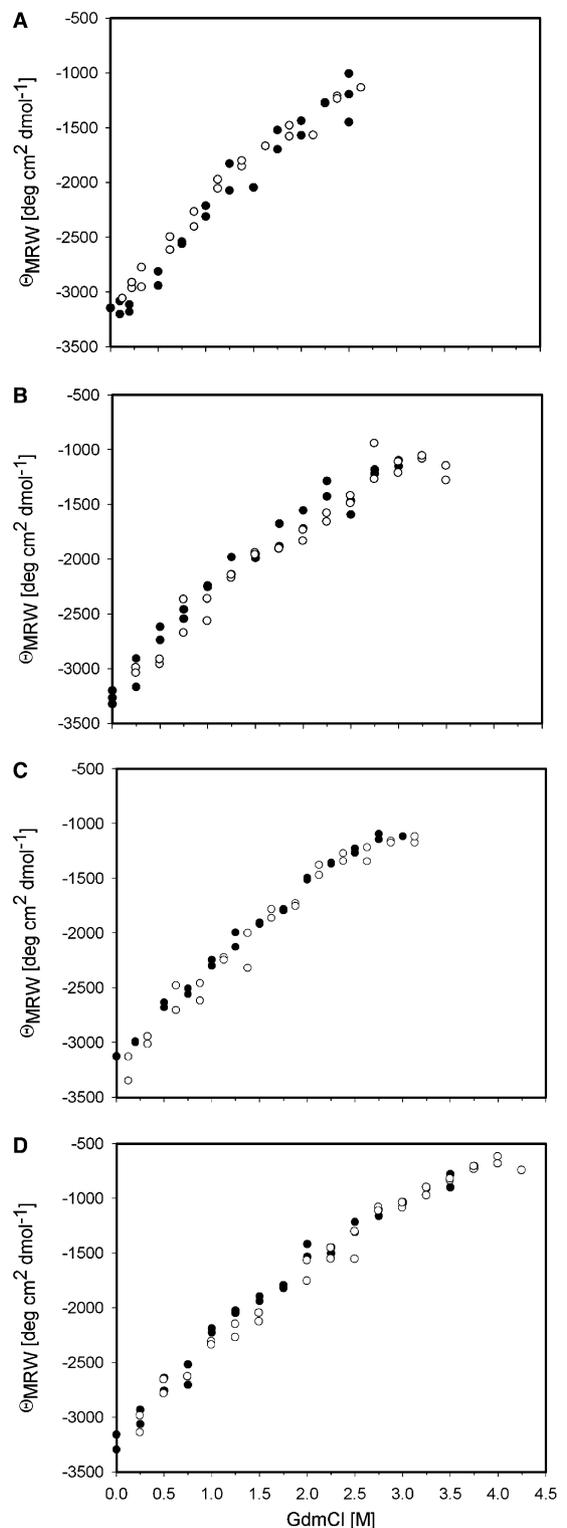


Fig. 4. Denaturation and renaturation transitions of the isolated pro-peptide. Measurements were monitored by far-UV CD spectroscopy. Ellipticities were measured at 220 nm in 1 mm cuvettes. Protein (0.4 mg/ml) was incubated at 20 °C for 6 h in the presence of the indicated GdmCl concentrations. Black circles, denaturation; open circles, renaturation of the pro-peptide. (A) Pro-peptide in 50 mM Na-phosphate, pH 7.0. (B) Pro-peptide as in (A), but +0.25 M (NH₄)₂SO₄, +0.5 M (NH₄)₂SO₄ (C) and +0.75 M (NH₄)₂SO₄ (D).

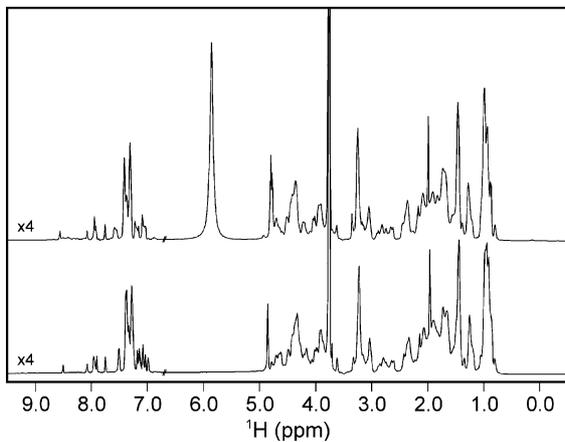


Fig. 5. 1D ^1H NMR spectra of the pro-peptide. The measurements were performed in $^2\text{H}_2\text{O}$ at 0 M urea (bottom) and 4.8 M urea (top). Residual resonances of protonated water and urea are located around 4.8 and 5.9 ppm, respectively. The intense resonance at 3.8 ppm belongs to EDTA. The aromatic region of the spectra between 6.8 and 9.5 ppm has been enlarged by a factor of 4.

higher fluorescence signal of the native protein is due to a more efficient energy transfer than in the denatured form.

In order to further analyze the contribution of secondary structure elements to the overall structure of the pro-peptide,

far-UV CD spectroscopy was performed. The small plateau in the wavelength range 218–223 nm and the minimum at 205 nm indicates a limited amount of β -sheet structure in addition to the prevailing random coil (Fig. 3C). Deconvolution of the spectrum using the program CDNN yielded 24% β -sheet and 34% random coil [18]. However, deviation of the pro-peptide CD spectrum from model spectra for helical, β -sheet or random coil structure suggests that these values should be treated with caution.

3.3. The pro-peptide exhibits only local intrachain contacts in the absence of the mature part

The stability of the pro-peptide was tested by stepwise denaturation with guanidinium-hydrochloride (GdmCl), and structural changes were monitored by far-UV CD spectroscopy. Changes in ellipticity were measured at 220 nm, a wavelength range where the largest difference between the species under native and denaturing conditions was observed (Fig. 3C). The unfolding and refolding changes in ellipticity showed a non-cooperative transition, indicating that the isolated pro-peptide does not possess a cooperatively stabilized structure (Fig. 4A). A similar result was obtained using the energy transfer from phenylalanines to tryptophan as a probe for denaturation (data not shown). Even stabilization by increasing concentrations of $(\text{NH}_4)_2\text{SO}_4$ did not result in cooperative transitions (Fig. 4B–D). $(\text{NH}_4)_2\text{SO}_4$ concentrations above 0.75 M resulted in aggregation of the pro-peptide (data

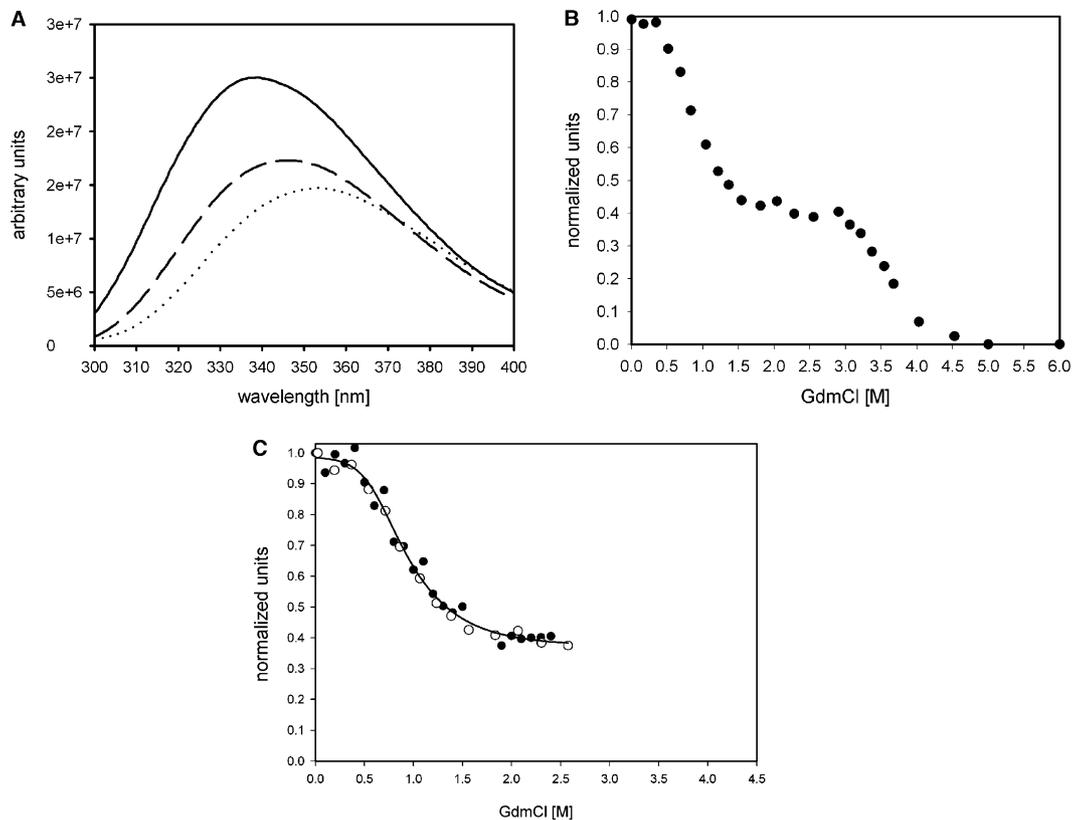


Fig. 6. (A) Fluorescence spectra of proNGF (20 $\mu\text{g}/\text{ml}$) upon excitation at 280 nm. Solid line, proNGF in the absence of GdmCl; dashed line, in the presence of 2.5 M GdmCl; dotted line, in the presence of 6 M GdmCl. (B) Denaturation of proNGF monitored by fluorescence. Protein was incubated at 20 $^\circ\text{C}$ for 24 h in the presence of the indicated GdmCl concentrations. Excitation and emission wavelengths were 280 and 325 nm, respectively. Data were normalized. (C) Transitions of the pro-peptide in proNGF. Black circles, denaturation; open circles, renaturation of the pro-peptide.

not shown) and therefore were not applied for stabilization experiments.

Missing tertiary interactions within the isolated pro-peptide could be confirmed by NMR spectroscopy. Fig. 5 depicts the 1D ^1H spectra of the pro-peptide in 0 and 4.8 M urea, dissolved in $^2\text{H}_2\text{O}$. No high-field shifted aliphatic side chain resonances are observed below 0.7 ppm, which are sensitive measures for the tertiary structure of a protein. The only obvious deviation from random coil chemical shifts [19] shows a histidine $\text{H}^{\text{e}1}$ proton at 8.5 ppm and a H^α proton at 4.9 ppm. Both resonances were only marginally affected by urea, indicating that these deviations are due to local contacts. 2D ^1H NOESY experiments corroborated these findings (data not shown), especially for the H^α proton at 4.9 ppm, which is very close to the residual water resonances at 4.8 ppm in the spectrum at 0 M urea. The very similar overall appearance of the two 1D spectra showed that the pro-peptide is unstructured and the small changes in chemical shifts are caused by a difference in the urea dependence of the individual resonances rather than structural changes upon addition of the denaturant (Fig. 5).

Taking these results together, both the chemical unfolding experiments and the NMR analysis indicate that the isolated pro-peptide lacks structure stabilizing tertiary contacts. Nevertheless, a limited amount of secondary structure appears to be present in the isolated pro-peptide as phenylalanine energy transfer to tryptophan by fluorescence and far-UV CD spectroscopy reveal clear differences between the native and denatured protein.

3.4. Tertiary contacts can only be detected when the pro-peptide is covalently attached to the mature part

For comparison of the structure of the pro-peptide when covalently attached to the mature NGF, fluorescence studies were performed with proNGF. Mature NGF contains three tryptophans, two tyrosines and seven phenylalanines. The crystal structures of mouse and human NGF show that the tryptophans are located close to the dimerization interface [3,4]. The fluorescence spectrum of native proNGF showed a maximum at 337 nm, indicating that the tryptophans are partially shielded from the solvent (Fig. 6A). Unfortunately, no conclusions about the surroundings of the tryptophan residing in the pro-peptide can be made from the spectrum of the native protein, since this signal is overlaid by the signal of the tryptophans in the mature part. However, changes in tryptophan fluorescence were observed upon denaturation of proNGF with low GdmCl concentrations that were not observed in the isolated pro-peptide. These changes are likely to reflect structural transitions of the pro-peptide, since the transition midpoint of the mature part is at a GdmCl concentration of about 3.5 M (see below).

The spectroscopic properties of proNGF allowed structural changes during denaturation to be monitored by fluorescence, so that the thermodynamic stability of the pro-peptide bound to the mature moiety could be explored. Upon chemical unfolding of proNGF with GdmCl, two distinct transitions were observed: one at 0.85 M GdmCl and a second transition at 3.5 M GdmCl (Fig. 6B). The second transition probably reflects the unfolding of the mature part, as the highly homologous murine NGF had been shown to unfold in this concentration range [20]. The small plateau at very low GdmCl concentrations probably represents native proNGF species. Thus, GdmCl concentrations as low as 0.85 M are sufficient to

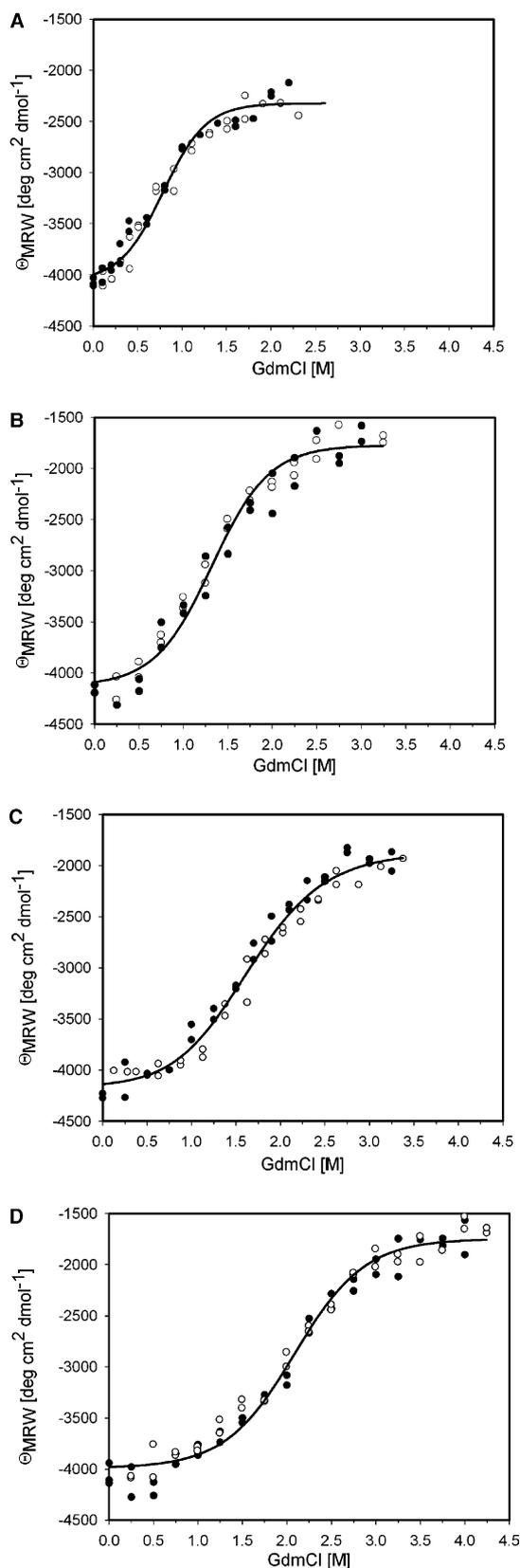


Fig. 7. Denaturation and renaturation of the pro-peptide (0.35 mg/ml) attached to the mature moiety followed by far-UV CD spectroscopy under the same conditions as described in the legend of Fig. 4. (A) in the absence of $(\text{NH}_4)_2\text{SO}_4$, (B) in the presence of 0.25 M, (C) in the presence of 0.5 M and (D) 0.75 M $(\text{NH}_4)_2\text{SO}_4$.

promote dissociation of the pro-peptide from the mature part. Dissociation may be concomitant with the unfolding of the pro-peptide. A ΔG^0 value of -7.8 kJ/mol was calculated from the reversible unfolding/refolding of the pro-peptide (Fig. 6C).

Unfolding was also monitored by far-UV CD spectroscopy. As in the fluorescence measurements, a transition midpoint at 0.9 M GdmCl was observed (Fig. 7A). With increasing $(\text{NH}_4)_2\text{SO}_4$ concentrations, the midpoint of the pro-peptide transition was shifted to higher GdmCl concentrations (Fig. 7B–D). Thus, while the pro-peptide in its isolated form cannot be stabilized by $(\text{NH}_4)_2\text{SO}_4$, stabilization was achieved when the pro-peptide was bound to the mature part, again indicating a significant difference in structure and molecular properties of the isolated and the NGF-bound pro-peptide. Structure stabilization is likely to be brought about via contact of the pro-peptide with the mature part; additional interactions may exist between the pro-peptide moieties themselves in the proNGF dimer.

Structure induction of the pro-peptide by the mature part also raises questions as to the role of the pro-peptide in the oxidative folding process. Clearly, the pro-peptide stimulates oxidative structure formation of the mature part, as proNGF could be renatured with yields of ca. 40%, while with mature NGF refolding yields of only ca. 4% were obtained [8]. These results indicate that, upon folding of mature NGF, the correct disulfide bonding is hardly energetically privileged compared to the multitude of non-native pairings. In contrast, the correctly disulfide-bonded form is the dominant species when proNGF is folded. It is likely that once the cystine knot has formed in a proNGF polypeptide to give rise to a native monomer, the pro-peptide part stabilizes the correct disulfide pattern by associating to this form and adopting a defined tertiary structure. Transient stabilization of correctly disulfide-bonded monomeric species by the pro-peptide is required until dimerization with a second natively folded monomer occurs, that finally results in the stably folded proNGF dimer. Certainly, this hypothesis has to be tested on the experimental level, as, e.g., by a comparison of the thermal unfolding of mature NGF and proNGF under equilibrium conditions in the presence of reducing agents.

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