

Abstract

Engrailed 2 is a transcription factor belonging to the class of homeoproteins. These proteins possess a 60-residue DNA binding globular domain and play an important role in the early stages of development. We expressed and purified a 13.4 kDa fragment of Engrailed 2, which comprises a 54-residue N-terminal extension in addition to the homeodomain region. Almost all backbone and side-chain resonances have been assigned. The weak dispersion in the proton dimension of the $^1$H–$^{15}$N HSQC spectrum indicates the presence of disordered regions that do not belong to the homeodomain. This work is a first step toward the NMR investigation of the structure and dynamics of Engrailed 2 protein that contains a well-structured globular domain and partially disordered regions.

Keywords

Engrailed 2 · Homeodomain · Transcription factor · Disordered proteins · NMR

Biological context

Homeoproteins constitute a large class of transcription factors that are present in a wide range of eukaryotic species, from yeast to humans. They are encoded by homeogenes, characterized by a highly conserved homeobox motif (Gehring et al. 1994) coding for a 60-residue DNA binding domain called homeodomain. Discovered initially in Drosophila (McGinnis et al. 1984), homeogenes have since been found in all metazoan organisms (Gehring et al. 1994). Homeoproteins have been shown in particular to play a fundamental role in embryonic development by modulating the spatio-temporal expression of target genes (Gehring 1987).

Engrailed 2 is a key factor in the development of the encephalon at the mesencephalon-metencephalon junction in the embryo (Joyner 1996). This protein is also expressed in adults in dopaminergic neurons of the mesencephalon. The inhibition of the expression of Engrailed 2 in dopaminergic neurons was shown to lead to apoptosis (Simon et al. 2001). This indicates a major physiologic role played by Engrailed 2 (Alberi et al. 2004; Sonnier et al. 2007). The structures of Engrailed and many other homeodomains have been studied in details by X-ray crystallography or NMR spectroscopy (Fraenkel et al. 1998). On the other hand, little information is available about the structure and dynamics of other parts of homeoproteins.
Chicken Engrailed 2 is a 289-residue protein. We are studying a shorter construct encompassing residues 146–259 that comprises the homeodomain (residues 200–259) and an N-terminal extension. This extension contains several known binding sites for other transcription factors (Pbx, FoxA2) which are implicated in the regulation of transcription (Piper et al. 1999; Foucher et al. 2003). It also comprises a serine-rich region whose post-translation phosphorylation controls the secretion of the protein (Maizel et al. 2002). In this article we report the NMR assignment of the 146–259 fragment of Engrailed 2, in view of further investigations of the three-dimensional structure and dynamics of Engrailed 2 protein.

Methods and experiments

The E. coli BL21RP expression strain was transformed with a pGEX plasmid (Pharmacia) containing a gene coding for sequence 146–259 of chicken Engrailed 2 fused to Glutathion S-Transferase. The recombinant Engrailed 2 protein was expressed and labelled using the Marley protocol (Marley et al. 2001), commonly used for preparing uniformly labelled proteins for NMR. Cells were grown at 37°C in 4 L of LB medium supplemented with 100 μg/mL of ampicillin and chloramphenicol. When the optical density at 600 nm reached 0.7, the cells were harvested by centrifugation at 4,000g for 10 min, and re-suspended in 1 L of M9 minimal medium containing 15NH₄Cl (or 15NH₄H₂Cl and 13C-glucose) as required for 15N (or 15N/13C) labelling. Protein expression was induced by addition of 1 mM isopropyl-b-D-thiogalactoside (IPTG). After 3 h of incubation, the cells were harvested by centrifugation at 4,000g, 4°C for 10 min. The cell pellets were re-suspended in a phosphate buffer (pH 7.4) containing 140 mM NaCl, 1 mM EDTA, 1 μg/mL peptatin, 1 μg/mL leupeptin, 1.5 mM PMSF, 2 mM TCEP, 0.5% (v/v) Triton X-100 and frozen at −80°C. The cell pellets were thawed and lysed by sonication. After centrifugation at 15,000g, 4°C for 45 min, the supernatant was loaded on GSTrap FF columns (GE Healthcare). The GST fusion protein was eluted using a 50 mM Tris–HCl buffer, pH 8, containing 150 mM NaCl, 1 mM EDTA, 1 mM DTT and 30 mM glutathione. The fusion protein was cleaved using PreScission protease (GE Healthcare). Engrailed 2 protein was then loaded on HiTrap Heparin columns (GE Healthcare) and eluted with a phosphate buffer containing 1.5 M NaCl. The collected fractions were concentrated by ultrafiltration using a 3 kDa cutoff membrane (Millipore). Each NMR sample typically contained ~0.6 mM of uniformly labelled protein in 40 mM succinate buffer, pH 6.0, supplemented with anti-proteases (leupeptine, pepstatine, EDTA and AEBSF), 0.1 mM DSS and 0.02% (w/v) NaN₃ in H₂O/D₂O (95/5%). 10 mM TCEP was added to prevent the oxidation of the C175 residue.

Most NMR experiments were recorded at 30°C on a Bruker Avance III spectrometer equipped with a TCI cryoprobe, operating at a 1H frequency of 500 MHz. Five millimetre Shigemi tubes were used for all experiments. Proton chemical shifts were referenced to internal DSS. 15N and 13C resonances were referenced indirectly to DSS. The data were processed with NMRpipe (Delaglio et al. 1995) and analyzed with CCPNmbr Analysis 2.0 (Vranken et al. 2005).

Backbone resonance assignments were obtained using 1H–15N HSQC and a set of 3D spectra HNCACB, CBCA(CO)NH, HNCO, HN(CA)CO and HBHA(CO)NH. Side-chain aliphatic 1H and 13C resonances were assigned mainly using 2D 1H–13C constant time HSQC, 3D 1H–15N TOCSY–HSQC, 3D H(CCO)NH–TOCSY and 3D (H)C(CO)NH–TOCSY experiments (Sattler et al. 1999). To overcome problems of overlap, particularly in the case of resonances corresponding to disordered regions, a 4D HC(CC–TOCSY)(CO)NH with random sampling (Kazimierczuk et al. 2009) was recorded on a Varian 700 MHz spectrometer equipped with a Performa XYZ PFG unit, 3D HBCB(CGCD)HD, 3D HBCB(CGCDCE)HE and 3D 1H–13C NOESY–HSQC experiments were also recorded in D₂O to assign the aromatic resonances.

Assignments and data deposition

The assigned 2D 1H–15N HSQC is shown in Fig. 1. Most backbone NH groups were assigned, with the exception of the N-terminal residue and V173. The absence of correlation for V173 may be due to line broadening. Indeed, all NH resonances of residues in the W169–Y178 segment show lower signal intensities. Moreover, additional weak resonances are observed in the 1H–15N HSQC spectrum corresponding to the aromatic group of W169. These observations suggest some particular dynamic effects in this region that might be due to cis–trans isomerism of the P170 residue. The NH resonances corresponding to homeodomain residues are well dispersed, while those belonging to the N-terminal extension are all in a narrow range between 7.6 and 8.6 ppm (see Fig. 1) which indicates intrinsic disorder in regions outside the homeodomain.

A few side-chain 15N resonances could not be assigned (15N of Pro, 15N of Lys, 15N of Arg). The assignment is complete for all 13C, 13C and 13CO and reaches 98% for 1H. The majority of side-chain resonances have been assigned (91% for 13C, 75% for 15N and 96% for 1H). The chemical shifts have been deposited in the BioMagResBank (http://www.bmrb.wisc.edu) under accession number 17325.
Fig. 1 Assigned 2D $^1$H--$^{15}$N HSQC spectrum of the $^{15}$N-labelled 146–259 fragment of Engrailed 2 protein acquired at 500 MHz. A zoom of the central crowded region of the spectrum is shown in upper-left. Backbone resonances corresponding to residues in the homeodomain (200–259) and in the N-terminal extension (146–199) are marked in black and red, respectively. The side-chain resonances of Trp, Asn and Gln residues are indicated in blue.

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References


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