Supplement: $^1$H, $^{13}$C, and $^{15}$N Resonance Assignments of the Heme-Binding Protein Murine p22HBP

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Biological context

Heme synthesis occurs mainly in erythroid cells (~85%) and hepatocytes although heme is synthesized in virtually all tissues. Δ-amino levulinic acid synthase (ALAS) catalyses the first, and rate-limiting, step in hepatic heme biosynthesis. Hemin, the Fe$^{3+}$ oxidation product of heme, acts as a feed-back inhibitor on ALAS as well as inhibiting transport of ALAS, via an interaction with the 49 amino acid ALAS presequence (Goodfellow et al., 2001), from the cytosol, where it is synthesized, into the mitochondria where it acts. Synthesis of ALAS is also suppressed. As hemes and porphyrins are reactive and poorly soluble under physiological conditions, it is assumed that free heme does not exist and that there is an intracellular heme binding protein or proteins that act as a buffer during induced heme synthesis. p22HBP, a 22 kDa cytoplasmic protein from mouse liver cell extracts, was found to bind heme and was first purified by Taketani et al in 1998. Subsequently in 2002 Blackmon et al determined that p22HBP is a generic tetrapyrrole-binding protein rather than a dedicated heme-binding protein. p22HBP expressed in E. coli is a monomer and has one binding site for heme. The binding properties of p22HBP are unique when compared with those of other heme-binding proteins. The amino acid sequence of p22HBP has 44% homology to SOUL an heme-binding protein expressed in retina and pineal gland. SOUL is reported to be a hexameric protein that binds heme via His42 (Zykla and Reppert 1999; Sato et al., 2004). A recent study, by mass spectrometry, of $^{59}$Fe uptake in developing erythroid cells has identified p22HBP in one of four multiprotein complexes related to heme biosynthesis (Babusiak et al., 2005). As no structural information exists for p22HBP, and sequence analysis has identified no obvious similarity to known protein folds a NMR structure determination of p22HBP was initiated and 2D/3D NMR spectra recorded on $^{15}$N, $^{13}$C and $^2$H labeled protein.

Methods and results

All overexpression and purification steps were carried out at 4°C, except where otherwise stated. A colony of E. coli BL21 (DE3) cells, transformed with the HBP expression plasmid, pNJ2, which contains the gene encoding p22HBP with a N-terminal hexa-His tag under the alkaline phosphatase promoter, was inoculated into 200 ml flasks containing 50 ml of LB with 50 µg/ml ampicillin and was incubated overnight at 37°C with shaking. 20 ml of this culture was inoculated in one liter of LB containing 50 µg/ml ampicillin. The cells were harvested at 0.6-0.8 OD (595nm) by centrifuging at 6000 rpm for 5 minutes. The cells were resuspended in the labeled induction medium (MOPS phosphate limiting) and incubated for 20 hours at 30°C with shaking. The cells were recovered (6000 rpm for 5 minutes) and resuspended in 50 mM PO$_4$ buffer...
pH 8.0 with 300 mM NaCl using an homogenizer. The cells were ruptured using a French Press and the resuspension centrifuged (50000g) for 1 hour. The supernatant was loaded onto a Ni-NTA agarose column equilibrated with standard buffer containing 50 mM PO₄ at pH 8.0 with 300 mM NaCl. The protein was eluted with standard buffer containing 100 mM imidazole and the fractions containing protein were concentrated onto a Superdex 75 column. The collected fractions were concentrated to ca. 1mM using Amicon cell filters. The isotopically labeled NMR samples were prepared using ¹⁵NH₄Cl, ¹³C-glucose and/or D₂O (98%-d) as isotopic sources. All NMR samples were prepared at ~1mM in 50 mM PO₄ buffer at pH 8.0.

NMR spectra were acquired at 30 °C on a Bruker DRX-600 spectrometer equipped with a 5-mm inverse triple-resonance cryoprobe with triple-axis gradient coils. Backbone ¹H, ¹³C, and ¹⁵N resonances were assigned using a combination of automated and manual methods with data from the following experiments: 2D ¹⁵N HSQC, 2D TROSY, 3D trHNCO, trHNCA, trHNCACO, trHNOCACB, ¹⁵N-edited NOESY-HSQC (mixing time, 60 ms), and ¹⁵N-edited TOCSY-HSQC (mixing time, 43.2 ms) spectra. Aliphatic side-chain resonances were assigned manually from 3D HCCH-TOCSY (mixing time 16.3 ms), and aromatic resonance assignments were obtained from a ¹³C-edited NOESY-HSQC (mixing time, 80 ms) centered in the aromatic region. Spectra were processed and analyzed using NMRPipe (Delaglio et al., 1995) and XEASY (Bartels et al., 1995). Chemical shifts were referenced to DSS at 0 ppm (Wishart et al., 1995).

Extent of assignments and data deposition
Excluding the disordered amino terminus (residue 1-17), ~95% of all ¹H, ¹³C and ¹⁵N resonances in the protein have been assigned. The unstructured N-terminus (1-17) and residues 80, 90, 136, 152-3, 157, 164, 177, and 180-1 were unassigned. Chemical shift assignments have been deposited under BioMagResBank accession number 6620 (www.bmrb.wisc.edu).

Figure 1. 2D [¹H,¹⁵N]-TROSY spectrum of murine p22HBP recorded at 600.13 MHz, H resonance frequency, pH 8.0, and 30 °C.

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References