Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Human neuroglobin: crystals and preliminary X-ray diffraction analysis

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Received 7 June 2002
Accepted 17 July 2002

Neuroglobin, a recently discovered member of the haemoglobin superfamily, is primarily expressed in the brain of humans and other vertebrates, where it has been proposed to enhance O_2 supply in response to hypoxia or ischaemia, protecting the neuron from hypoxic injury. Neuroglobin is the first example of a vertebrate haemoglobin in which a hexacoordinate haem geometry has been detected. A triple mutant (replacing three Cys residues) of human neuroglobin (151 amino acids) has been expressed in *Escherichia coli*, purified and crystallized in two crystal forms, the best of which diffracts to 1.95 Å resolution using synchrotron radiation. The crystals belong to space group $P2_1$, with unit-cell parameters a = 39.6, b = 94.9, c = 67.5 Å, β = 94.4°, and contain 2–4 protein molecules per asymmetric unit.

1. Introduction

Two new globins, neuroglobin (NGB) and cytoglobin (CyGB, also known as histoglobin), were recently discovered and included into the vertebrate haemoglobin (Hb) family (Burmester *et al.*, 2000, 2002; Awenius *et al.*, 2001; Trent & Hargrove, 2002). NGB is predominantly expressed in the brain and in other nerve tissues, whereas CyGB is expressed in all tissues. Mammalian NGB and CyGB are composed of 151 and 190 amino acids, respectively. Fish NGB consists of 153 amino acids, whereas fish CyGB has between 174 and 179 amino acids. CyGBs are about 40 residues longer than standard Hbs owing to the presence of N- and C-terminal extensions of about 20 residues each. Although the amino-acid sequences of both NGB and CyGB display some of the structural determinants of the globin fold, they share little amino-acid identity with vertebrate Hbs and myoglobin (Mb) (Burmester *et al.*, 2000, 2002; Awenius *et al.*, 2001; Trent & Hargrove, 2002).

Both NGB and CyGB display hexacoordinated haems, either in their ferrous or ferric forms, having the distal HisE7 as the internal sixth haem Fe ligand. The rate-limiting step in ligand binding to the deoxy hexacoordinated form of NGB and CyGB is represented by the dissociation of the haem distal HisE7 residue; the P_{50} for NGB and CyGB oxygenation is about 133 Pa (Couture *et al.*, 2001; Dewilde *et al.*, 2001; Trent *et al.*, 2001; Trent & Hargrove, 2002).

The biological functions of NGB and CyGB are still unknown. Although expressed at a submicromolar level, NGB is suggested to be involved in providing O_2 to the neuron. Indeed, NGB is induced under hypoxic conditions and protects the neuron from hypoxic injury and stress (oxidative) conditions (Sun *et al.*, 2001; Venis, 2001). Furthermore, NGB is induced by haemin, as are Hb and Mb, but the signal transduction pathway is different (Zhu *et al.*, 2002).

Knowledge of the NGB tertiary structure will be essential in order to understand its function at the molecular level and to shed light on the increasing number of modified globin folds and Hb functionalities currently being discovered (Milani *et al.*, 2001; Pesce *et al.*, 2002; Wittenberg *et al.*, 2002). Here, we report the expression, purification, crystallization and preliminary X-ray diffraction analysis of human NGB (hNGB) as a required step towards determination of the protein tertiary structure.

2. Materials and methods

2.1. Expression, cloning and purification of recombinant NGB

The expression plasmid (hNGB cDNA in pET3a; Dewilde *et al.*, 2001) was transformed into *E. coli* strain BL21(DE3)pLysS. The cells were grown at 298 K in TB medium (1.2% bactotryptone, 2.4% yeast extract, 0.4% glycerol, 72 m*M* potassium phosphate buffer pH 7.5) containing 200 µg ml^{-1} ampicillin, 30 µg ml^{-1} chloramphenicol and 1 m*M* δ-amino-levulinic acid. The culture was induced at A_{550} = 0.8 by the addition of isopropyl-1-thio-D-galactopyranoside to a final concentration of 0.04 m*M* and expression was continued overnight. Purification of the expressed hNGB followed the previously described procedure

(Dewilde *et al.*, 2001), with the exception of a final purification step by semi-preparative isoelectric focusing under native conditions, as described for trematode Hb (Rashid *et al.*, 1997).

Following sequence considerations and feedback from the crystallization experiments on the wild-type protein (see below), two hNGB triple mutants bearing substitutions at residues CysCD5(47), CysD5(56) and CysG19(121) [hNGB residues are identified by their three-letter code, by the topological position they are assigned within the globin fold (Perutz, 1979) and by their sequence number (in parentheses)] were designed and subsequently produced by the megaprimer method (Sarkar & Sommer, 1990). The first mutant bore CysCD5(47)Ser, CysD5(56)Ser and CysG19(121)Ser mutations (hNGB*); the second was designed to bear CysCD5(47)Gly, CysD5(56)Ser and CysG19(121)Ser mutations (hNGB**). Both hNGB* and hNGB** cDNAs were cloned in the pET3a expression vector and expressed as described above.

2.2. Crystallization

Several crystallization experiments were initially conducted on wild-type hNGB (and subsequently on hNGB*), constantly isolating amorphous/insoluble precipitates, even under crystallization conditions held to maintain the Cys residues in reduced form. The precipitation buffers/media explored were identical to those described below. Following expression of the third mutant protein, hNGB** crystals were grown using the hanging-drop vapour-diffusion method. A large number of conditions were screened using Hampton Research Crystal Screen kits I and II, Hampton Research Additive Screen kits and Jena BioScience Screens at 277 and 294 K. Each drop was prepared by mixing 1 µl of protein (at a concentration of 33 mg ml^{-1}) and 1 µl of reservoir solution. The most promising initial conditions were improved by varying protein concentration, precipitant, temperature and pH.

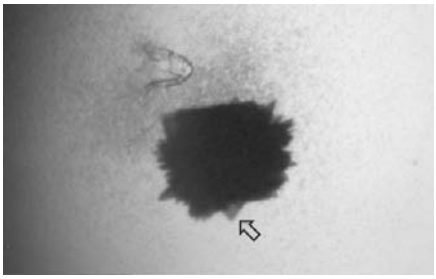

Figure 1
A typical cluster of form II crystals of the recombinant Cys-free human neuroglobin triple mutant [hNGB**, bearing CysCD5(47)Gly, CysD5(56)Ser and CysG19(121)Ser substitutions]. The arrow indicates one single thin plate; the approximate thickness of single plates within the bunch is 5 µm.

2.3. Data collection and processing

A native data set of hNGB** was collected at the ID14-2 beamline (ESRF, Grenoble, France) at 100 K using a MAR CCD detector. All data were processed using *DENZO* and *SCALEPACK* and programs from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994; Otwinowski & Minor, 1997).

3. Results and discussion

Whereas it was not possible to obtain any crystalline precipitate from both wild-type hNGB and from hNGB*, two crystal forms of hNGB** were isolated by improvement of the preliminary crystallization parameters.

3.1. Crystal form I

Initial crystallization conditions were found using condition 40 of Hampton Research Crystal Screen kit I. After optimization, the best hNGB** crystallization condition was: 20% polyethylene glycol (PEG) 4000, 20% 2-propanol, 0.1 *M* sodium citrate pH 5.6 at 277 K. Crystals of rhombic shape and typical dimensions of 0.4 × 0.2 × 0.1 mm grew in about one week. However, the crystals proved very unstable, especially when transferred to the cryosolution for data collection at 100 K; this is likely to be related to the high percentage of volatile 2-propanol. Preliminary diffraction tests were performed in-house using a MAR Research 345 imaging-plate detector coupled to a Rigaku RU-H3R rotating-anode generator (monochromatic Cu $K\alpha$ radiation). The crystals diffracted to 7 Å, with smeared diffraction spots.

3.2. Crystal form II

A second crystal form of hNGB** was isolated starting from condition A6 of the Jena BioScience Screen 6 and then adjusting the growth conditions to 1.4 *M* ammonium sulfate, 3% 2-propanol, 0.05 *M* sodium citrate pH 6.5 at 277 K. In about 3–4 weeks, bunches of extremely thin plates grew, each plate having typical dimensions of 0.3 × 0.3 × 0.005 mm (Fig. 1). The crystals were stored in a stabilizing solution containing 2.4 *M* ammonium sulfate, 3% 2-propanol, 0.05 *M* sodium citrate pH 7.0 at 277 K; they were transferred to the same solution supplemented with 20%(*v*/*v*) glycerol immediately prior to data collection.

Table 1
Data-collection statistics for hNGB**.

Values in parentheses are for the outer resolution shell (1.98–1.95 Å).

Source	ESRF, Grenoble (ID14-2)
Temperature (K)	100
Wavelength (Å)	0.933
Resolution range (Å)	40–1.95
Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 39.6, b = 94.9, c = 67.5, β = 94.4
Mosaicity (°)	0.76
Total reflections	92865
Unique reflections	35871
Completeness (%)	98.5 (97.9)
Redundancy	2.6 (2.5)
$R_{\rm merge}$† (%)	5.5 (22.0)
Average $I/\sigma(I)$	17 (5)

† $R_{\rm merge} = \sum_h \sum_i |I_{hi} - \langle I_h \rangle| / \sum_h \sum_i I_{hi}$.

A high-resolution data set (1.95 Å resolution) was collected using synchrotron radiation (beamline ID14-2, ESRF, Grenoble, France). Inspection of the diffraction pattern and systematic absences allowed the assignment of the hNGB** crystal form II to the monoclinic space group $P2_1$, with unit-cell parameters a = 39.6, b = 94.9, c = 67.5 Å, β = 94.4° (Table 1). Evaluation of the crystal packing parameter (Matthews, 1968) indicated that the lattice can accommodate 2–4 molecules per asymmetric unit ($V_{\rm M}$ in the range 3.8–1.9 Å^3 Da^{-1}), with an estimated solvent content in the 67–35% range.

Owing to the low sequence homology of hNGB** with respect to classical Hbs and because of the likely helix reorientation within the globin fold related to the haem hexacoordination, it was not possible to solve the hNGB** three-dimensional structure using the molecular-replacement method. For this reason, a SAD/MAD experiment based on the haem Fe-atom anomalous scattering is in preparation.

We thank the staff of beamline ID14-2, ESRF, Grenoble (France) for data-collection facilities and support. This work was supported by grants ASI I/R/167/00, CNR PF Biotecnologie and by the EU Project 'Neuroglobin'. The support of Center for Excellence on Molecular Mechanisms of Cell Communication, University of Genova is fully acknowledged.

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