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Expression, purification, crystallization and crystallographic characterization of dimeric and monomeric human neutrophil gelatinase associated lipocalin (NGAL)

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Abstract

Crystals of the monomeric and dimeric forms of human neutrophil gelatinase associated lipocalin have been grown in hanging-drop vapor-diffusion trials using PEG as a precipitating agent with recombinant protein expressed in a baculovirus-based system. Crystals of monomeric NGAL belong to the cubic space group $P432$ with lattice constants $a = b = c = 126.6$ Å; crystals of dimeric NGAL belong to the tetragonal space group $P4_12_12$ (or its enantiomorph $P4_32_12$) with lattice constants $a = b = 54.14$ and $c = 121.56$ Å. Isomorphous crystals of the NGAL dimer can be grown in the presence of ligand: the tripeptide *N*-formyl-Met-Leu-Phe.

1. Introduction

The calycins are a structural superfamily of proteins consisting of the lipocalins, the fatty acid binding proteins (FABP's) and the avidins (reviewed in Flower, North & Attwood, 1993; Flower, 1994, 1995, 1996). Lipocalins and FABP's are diverse families of small proteins which share the ability to bind small, hydrophobic ligands, though the range of ligand specificities is quite diverse. Lipocalins are extracellular while most FABP's are intracellular proteins. Beyond an affinity for small lipophiles, lipocalins have been shown to bind soluble, extracellular macromolecules (Triebel, Bläser, Reinke & Tschesche, 1992; Kjeldsen, Johnsen, Sengeløv & Borregaard, 1993) and specific cell-surface receptors (Bavik, Levy, Hellman, Wernstedt & Eriksson, 1993; Boudejelal, Sivaprasadarao & Findlay, 1993). Originally, lipocalins have been thought of as simple transport molecules. It is now apparent that lipocalins display a diverse set of functions: retinol transport, invertebrate cryptic coloration, olfaction pheromone transport, prostaglandin synthesis, regulation of cellular homeostasis, modulation of immune responses and as carrier proteins involved in the clearance of both endogenous and exogenous compounds. Despite limited sequence similarity, members of these families possess strikingly conserved three-dimensional structures. The lipocalin core structure consists of an eight-stranded antiparallel continuously hydrogen-bonded β-barrel which defines a calyx- or cup-shaped structure with an enclosed ligand binding site. The FABP core structure consists of a ten-stranded orthogonal β-sandwich or barrel which is not continuously hydrogen bonded but with a similar binding site. The strand connectivity in both families is consistently +1, defining the simplest β-barrel topology.

Human neutrophil gelatinase associated lipocalin (NGAL) was originally identified as a component, along with gelatinase, of a disulfide-linked heterodimer secreted by neutrophils (Triebel *et al.*, 1992; Kjeldsen, Johnsen *et al.*, 1993). Gelatinase is a neutral metalloproteinase which has been isolated from neutrophils, macrophages and fibroblasts that degrades a number of the components of the extracellular matrix. NGAL can also be secreted from neutrophils as both a 25 kDa monomer and as a 46 kDa disulfide-linked homodimer in the absence of associated gelatinase (Triebel *et al.*, 1992; Kjeldsen, Johnsen *et al.*, 1993). Unlike other lipocalins, NGAL shows no affinity for retinoic acid, but does bind the tripeptide *N*-formyl-Met-Leu-Phe which is active in bacterial chemotaxis (Pervaiz & Brew, 1987; Sengeløv, Boulay, Kjeldsen & Borregaard, 1994).

NGAL synthesis is highly induced in epithelial cells in both inflammatory and neoplastic colorectal diseases (Nielsen *et al.*, 1996); its expression is restricted to the affected tissue only. Inflammatory bowel diseases are characterized by a pronounced infiltration of a variety of immune system cells (including macrophages, neutrophils, lymphocytes and plasma cells) into the mucosa, particularly around ulcerations in the wall of the bowel. A similar response is also seen in adenocarcinoma of the colon. The mucosa plays a key role in both events (reviewed in Perdue & McKay, 1994). Passage of bacterial products through the colonic mucosa induces expression of a variety of cytokines and mediators of inflammation. Many of these compounds are chemotactic for neutrophils and induce the expression of a number of imunologically active proteins, such as MHC class II molecules, in both colonic epithelial cells and cell lines. Neutrophils in the colonic mucosa do not actively synthesize NGAL; rather, the protein is produced in immature neutrophil precursors in the bone marrow and stored in specific granules for subsequent release (Kjeldsen, Johnsen *et al.*, 1993; Bundgaard, Sengeløv, Borregaard & Kjeldsen, 1994). It has been proposed that NGAL plays a role in mediating these responses by either (1) binding to and transporting compounds into cells in order to stimulate subsequent responses or (2) sequestering compounds that otherwise would stimulate destructive immune responses (Nielsen *et al.*, 1996).

The murine analogue of NGAL, 24p3, was originally identified in a screen for genes overexpressed during a SV40-induced mitotic reaction (Hraba-Renevey, Türler, Kress, Salomon & Weil, 1989). 24p3 expression is also amplified by an autocrine mechanism in response to dexamethasone or retinoic acid (Garay-Rojas, Harper, Hraba-Renevey & Kress, 1996). The synthesis of 24p3 has been shown to be induced in cultured macrophages by lipopolysaccharide (Meheus *et al.*, 1993). The rat analogue of NGAL, *neu*-related lipocalin (NRL) is specifically overexpressed in *neu*-initiated rat mammary carcinomas (Stoesz & Gould, 1995). Therefore, NGAL and its analogues may play a role in neoplastic disease either as a modulator of factors affecting cell proliferation or, through its

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interaction with gelatinase, as a factor in determining the invasiveness and metastatic proclivity of a tumor.

2. Materials and methods

2.1. *Expression and purification*

Recombinant NGAL was expressed as a secreted protein in Sf9 insect cells using the BaculoGold™ baculovirus-based expression system (PharMingen). The coding region of NGAL, excluding the portion encoding the signal peptide (Bundgaard *et al.*, 1994), was amplified by PCR from a human cDNA library with the following primers,

5′-CGCGGATCCCAGGACTCCACCTCAGACCTGATCCC-3′

5′-CGGAATTCTCAGCCGTCGATACACTGGTCGATTGGG-3′.

The PCR product was ligated into the transfer vector pAcGP67 (PharMingen) using the *Bam*HI and *Eco*RI sites in the vector polylinker. Recombinant baculovirus was isolated following the manufacturer's procedure. Sf9 cells (ATCC CRL-1711) were maintained in TC-100 medium (Gibco/BRL) plus 10% fetal calf serum at 300 K in suspension culture. For NGAL expression, cells in logarithmic phase were shifted to serum free SF 900-II medium (Gibco/BRL) at a cell density of $(1.5 \times 10^6$ cells) ml^{-1}. Recombinant baculovirus was added to the cells at an MOI of 1. Medium was typically harvested 72 h post infection and NGAL was purified from infected Sf9 cell supernatants by affinity chromatography. The affinity chromatography matrix was prepared by coupling the murine anti-NGAL monoclonal antibody 211-1 (Kjeldsen, Koch, Arnljots & Borregaard, 1996) to Affigel Hz resin (Bio-Rad) following the manufacturer's protocol. Protein was eluted from the affinity column with 0.2 *M* glycine–HCl (pH = 2.2) followed by immediate neutralization with 2 *M* Tris–HCl (pH = 9.0; see Fig. 1). Initial characterization of the affinity-purified protein by reducing/non-reducing polyacrylamide gel electrophoresis and size-exclusion chromatography indicated that it consisted of a mixture of monomer and disulfide-linked homodimer. In order to resolve this mixture, concentrated NGAL was first incubated overnight in 100 m*M* iodoacetamide (IAA; Sigma) to block any remaining free thiols. The IAA-treated protein was then separated into monomer and dimer species by size-exclusion chromatography (Pharmacia Superdex 200 16/60 column) in 25 m*M* PIPES, 150 m*M* NaCl, 1 m*M* EDTA and 0.02%(*w*/*w*) NaN_3. Isolated monomer and dimer pools were then concentrated to between 18 and 20 mg ml^{-1} (Amicon Centricon ultrafilters) and washed into 25m*M* PIPES, 1 m*M* EDTA and 0.02%(*w*/*w*) NaN_3.

2.2. *Crystallization*

Initial crystallization screens employing a commercially available set of solutions (Hampton Research Crystal Screen) revealed distinct preliminary crystallization conditions for both the monomeric and dimeric forms of NGAL. Subsequent refinement of these conditions yielded the current crystallization protocols. All crystallization trials are conducted by vapor diffusion in a hanging-drop geometry (Hampton Research Q-plates) at a constant temperature of 295 K. Drops consist of equal volumes of concentrated protein solution and well solution (well volume = 1.0 ml). The well solution for growth of crystals of monomeric NGAL consists of 28 to 30%(*w*/*w*) PEG (average M_r = 8 000; Fisher Scientific) plus 10–12% saturated ammonium sulfate (Fisher Scientific); crystals grow within 7–8 d (Fig. 2). The corresponding condition for the dimeric NGAL crystals consists of a well solution of 18 to 22%(*w*/*w*) PEG (average M_r = 8 000) buffered with 50 to 100 m*M* sodium acetate at pH 4.0 to 4.5; crystals appear within 48 h. The bacterial chemotaxis tripeptide used in cocrystallization experiments (*N*-formyl-Met-Leu-Phe) was purchased from Sigma.

Crystals of the complex of NGAL and the ligand *N*-formyl-Met-Leu-Phe can be prepared by cocrystallization or crystal soaks. For cocrystallization, dimeric protein is mixed with tripeptide at an approximate molar ratio of 3:1 (peptide:protein). Stock solutions are prepared by dissolving the peptide in

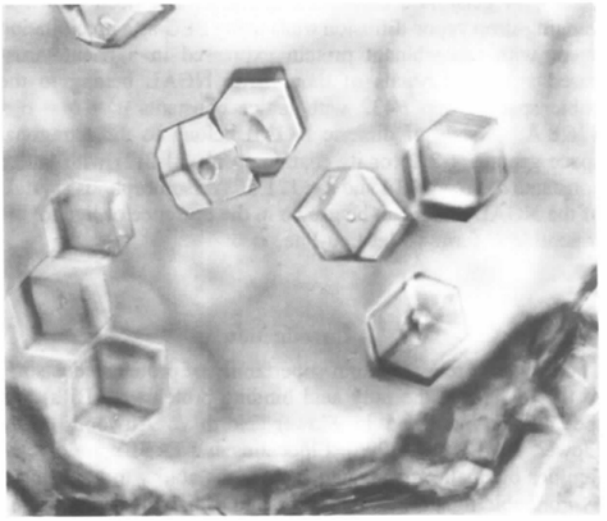

Fig. 1. Crystals of monomeric NGAL grown from PEG/ammonium sulfate. Average crystal dimensions are approximately 350 × 350 × 350 µm.

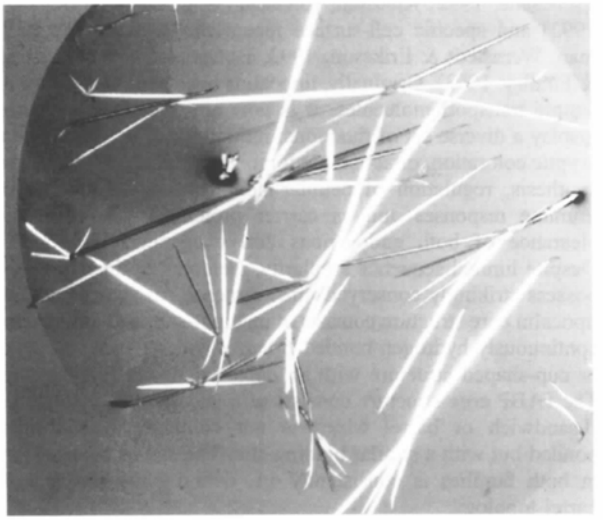

Fig. 2. Crystals of dimeric NGAL, photographed through crossed polarizers, grown from PEG at low pH. Average crystals grow to lengths of between 400 and 700 µm with widths of up to approximately 25 µm.

dimethyl formamide at 20 mg ml^{-1}. Crystallization conditions are identical for the complex. We have not attempted to grow crystals of the peptide complexed with the monomer form of NGAL. The peptide can be soaked into previously grown crystals by transferring the crystals to the appropriate cryo-buffer plus 1 mg ml^{-1} peptide and incubating at 295 K overnight.

2.3. *Crystallography*

For X-ray analysis, crystals were mounted in rayon loops (Woolworth) on magnetic pins (a variation of the methods of Teng, 1990; Rodgers, 1994) and flash-cooled to 103 K (Molecular Structure Corporation Low-Temperature System). Crystals were plunged into cryoprotectant solutions immediately prior to cryopreservation. For the monomeric NGAL crystals, cryo-protectant consists of 30%(*w*/*w*) PEG (average M_r = 8 000), 12% saturated ammonium sulfate and 28%(*v*/*v*) 2-methyl, 2,4-pentanediol (MPD; Fluka); for the dimeric crystals, cryo-protectant consists of 20%(*w*/*w*) PEG (average M_r = 8 000), 50 m*M* sodium acetate and 20%(*v*/*v*) glycerol (Fisher Scientific). Diffraction data were measured with a Rigaku R-AXIS IIc image plate detector mounted on a Rigaku RU-200 generator with a 300 μm focusing cup, a graphite monochromator and a copper anode operating at 100 mA and 50 kV. Space-group assignments, lattice-constant determinations and data reduction were conducted with *DENZO* (Otwinowski, 1991). Data were collected as 1° oscillations, 1 h exposures, at a crystal-to-detector distance of 150 mm.

3. Results and discussion

Crystals of human NGAL were grown from either PEG/ammonium sulfate (monomer) or PEG/low pH (dimer). Attempts to characterize the diffraction behavior of these crystals at room temperature were largely unsuccessful because of their marked sensitivity to radiation damage. Conditions were established for flash-cooling: the cryo-buffer for the monomer crystals included 28%(*v*/*v*) MPD; for the dimer, the cryo-buffer included 20%(*v*/*v*) glycerol. Space groups and lattice constants were determined by processing 1° oscillation diffraction patterns taken from cryopreserved crystals. Crystals of monomeric NGAL belong to the cubic space group *P*432 with lattice constants $a = b = c = 126.6$ Å; crystals of dimeric NGAL belong to the tetragonal space group $P4_12_12$ (or its enantiomorph $P4_32_12$) with lattice constants $a = b = 54.14$ and $c = 121.56$ Å. For the dimer crystals, the unique axis is parallel to the long axis of the needle-like crystals. Based on calculations of solvent content, the monomer crystals have either one (solvent content $\simeq$ 62%) or two (solvent content $\simeq$ 27%) molecules in the asymmetric unit; the asymmetric unit of the dimer crystals contains half a dimer (solvent content $\simeq$ 30%). Cryopreserved monomeric NGAL crystals diffract to a d_{min} of approximately 4.2 Å; dimeric NGAL crystals diffract to a d_{min} of approximately 2.0 Å.

A native diffraction data set has been collected from the dimeric NGAL crystals at 103 K. The crystal-to-detector distance was set at 150 mm which corresponds to a resolution of 2.7 Å at the detector edge. The reduced data set consists of 5 874 unique reflections (36.52–2.50 Å). The data are complete to a resolution of 2.70 Å (96.6% of the unique reflections were recorded from ∞ to 2.70 Å) with a significant amount of data collected to a resolution of 2.50 Å (85.6% of the unique reflections were recorded from ∞ to 2.50 Å, with 23.5% of the unique reflections recorded in the 2.54–2.50 Å resolution shell). The average redundancy is 3.98. The overall R_{symm} [defined as $\sum(|I - \bar{I}|)/\sum \bar{I}$] is 3.9% (calculated with all data from ∞ to 2.50 Å); the R_{symm} for the highest resolution shell (all data from 2.54 to 2.50 Å) is 15.6%. We are also able to collect data from crystals of the complex of dimeric NGAL and its peptide ligand. Crystals of dimeric NGAL plus or minus the *N*-formyl-Met-Leu-Phe ligand behave identically in diffraction experiments. Currently, crystals of the monomer are unusable for data collection because of their poor quality.

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References

Bavik, C. O., Levy, F., Hellman, U., Wernstedt, C. & Eriksson, U. (1993). *J. Biol. Chem.* **268**, 20540–6.
Boudejelal, M., Sivaprasadarao, A. & Findlay, J. B. C. (1993). *Biochem. J.* **317**, 23–27.
Bundgaard, J. R., Sengeløv, H., Borregaard, N. & Kjeldsen, L. (1994). *Biochem. Biophys. Res. Commun.* **202**, 1468–1475.
Flower, D. R. (1994). *FEBS Lett.* **354**, 7–11.
Flower, D. R. (1995). *J. Mol. Recog.* **8**, 185–195.
Flower, D. R. (1996). *Biochem. J.* **318**, 1–14.
Flower, D. R., North, A. C. & Attwood, T. K. (1993). *Protein Sci.* **2**, 753–761.
Garay-Rojas, E., Harper, M., Hraba-Revency, S. & Kress, M. (1996). *Gene*, **170**, 173–180.
Hraba-Renevey, S., Türler, H., Kress, M., Salomon, C. & Weil, R. (1989). *Oncogene*, **4**, 601–608.
Kjeldsen, L., Bainton, D. F., Sengeløv, H. & Borregaard, N. (1993). *Blood*, **83**,799–807.
Kjeldsen, L., Johnsen, A. H., Sengeløv, H. & Borregaard, N. (1993). *J. Biol. Chem.* **268**, 10425–10432.
Kjeldsen, L., Koch, C., Arnljots, K. & Borregaard, N. (1996). *J. Immunol. Methods*, **198**, 155–164.
Meheus, L. A., Fransen, L. M., Raymackers, J. G., Blockx, H. A., Van Beeumen, J. J., Van Bun, S. M. & Van de Voorde, A. (1993). *J. Immunol.* **151**, 1535–1547.
Nielsen, B. S., Borregaard, N., Bundgaard, J. R., Timshel, S., Sehested, M. & Kjeldsen, L. (1996). *Gut*, **38**, 414–420.
Otwinowski, Z. (1991). *DENZO. A Film Processing Program for Macromolecular Crystallography*. Yale University, New Haven, Connecticut, USA.
Perdue, M. H. & McKay, D. M. (1994). *Am. J. Pathol.* **267**, G151–G165.
Pervaiz, S. & Brew, K. (1987). *FASEB J.*, **1**, 209–214.
Rodgers, D. W. (1994). *Structure*, **2**, 1135–1140.
Sengeløv, H, Boulay, F., Kjeldsen, L. & Borregaard, N. (1994). *Biochem. J.* **299**, 473–479.
Stoesz, S. P. & Gould, M. N. (1995). *Oncogene*, **11**, 2233 2241.
Teng, T. Y. (1990) *J. Appl. Cryst.* **23**, 387–391.
Triebel, S., Bläser. J., Reinke, H. & Tschesche, H. (1992). *FEBS Lett.* **3**, 386–388.