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Purification, crystallization and preliminary X-ray diffraction analysis of recombinant human neutrophil-activating peptide 2 (rhNAP-2)

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Abstract

The potent activator and chemoattractant for human neutrophils, neutrophil-activating peptide 2 (NAP-2), has been cloned and expressed in *Escherichia coli*. The protein has been purified to homogeneity (> 98%) by a series of chromatographic techniques, including reversed phase HPLC. The biological activity of recombinant human NAP-2 (rhNAP-2), characterized by the induction of elastase release from human neutrophils, was found to be comparable to natural NAP-2. rhNAP-2 has been crystallized by the hanging drop vapor diffusion method. The crystals belong to space group P222 with unit cell dimensions of a = 30.8 Å, b = 39.5 Å and c = 95.3 Å. A packing density of 3.8 Å^3/Da with a solvent content of approximately 68% is obtained when one molecule per asymmetric unit is assumed. The crystals were shown to diffract to beyond 2.0 Å on a conventional X-ray source. They are stable to X-rays for several days and are thus suitable for high resolution structure determination.

Key words: Neutrophil-activating peptide 2; Chemokine; Cytokine; Crystallization; X-ray diffraction

1. Introduction

Human neutrophils have been shown to exhibit a chemotactic response to neutrophil-activating peptide 1/interleukin 8 (NAP-1/IL-8) [1] and also to the structurally homologous peptides melanoma growth-stimulatory activity (*gro*/MGSA) [2] and neutrophil-activating peptide 2 (NAP-2) [3]. In addition to chemotaxis, NAP-1/IL-8 has been characterized as an inducer of cytosolic free calcium changes, exocytosis and respiratory burst in neutrophils, being indicative of a pro-inflammatory role in vivo. NAP-2 and *gro*/MGSA have been shown to exhibit broadly similar activities [2,4]. These common activities have been explained by the usage of one of the two IL-8 receptors present on human neutrophils by all three peptides, while the other receptor recognizes only NAP-1/IL-8 [5]. Despite their similar effects on neutrophils, NAP-1/IL-8 and NAP-2 differ in their mode of formation and their cellular origin. NAP-1/IL-8 is produced and secreted by mononuclear phagocytes and a wide variety of tissue cells, as a result of transcriptional upregulation following stimulation by lipopolysaccharide, interleukin-1 and tumor necrosis factor.

NAP-2 is produced in a completely different manner, being the proteolytic product of precursor peptides released from platelet α-granules [6], and as such is especially worthy of further study. NAP-2 is the sequential cleavage product of platelet basic protein (PBP), tissue activating peptide III (CTAP-III) and β-thromboglobulin (β-TG)[7]. The carboxytermini of PBP, CTAP-III and β-TG are identical, while the three molecules are shortened by 24, 15, and 11 residues, respectively, at the aminoterminus. In vitro, neutrophil-stimulating activity could be induced by the proteolytic action of human leukocyte cathepsin G and bovine pancreatic chymotrypsin on CTAP-III, pointing to the involvement of serine proteases in vivo [7]. On the basis of its origin, NAP-2 has been proposed to be involved in vascular pathophysiological processes like thrombosis, atherosclerosis, and other inflammatory processes which are accompanied by platelet activation [7]. Inhibition of the interaction of NAP-2 with its receptor(s) by binding of small inhibitor molecules to the ligand or to the membrane receptor presents an attractive approach to chemotherapy of these deseases, and knowledge of the 3D structure of the NAP-2 molecule itself will greatly facilitate the search for such inhibitors.

NAP-2 consists of 70 amino acids (Fig. 1). It has a calculated molecular weight of 7,628 Da and a theoretical isoelectric point of 8.7 [8]. NAP-2 and its precursors contain the four conserved cysteine residues characteristic of the α-chemokine family (CXC), the first two in this case spaced by methionine (Fig. 1). Two characteristic disulfide bonds are formed intramolecularly, linking

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Abbrevations: rhNAP-2, recombinant human neutrophil-activating peptide 2; NAP-1/IL-8, neutrophil-activating peptide 1/interleukin 8; HPLC, high pressure liquid chromatography; GuHCl, guanidinium hydrochloride; MES, morpholino ethane sulfonic acid; FPLC, fast performance liquid chromatography; PEG, polyethylene glycol; AS, ammonium sulfate; aa, amino acid.

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Cys-1 (aa 5) and Cys-3 (aa 31) as well as Cys-2 (aa 7) and Cys-4 (aa 47) [9]. These disulfide bonds were shown to be essential for biological acivity of NAP-1/IL-8 [10]. The NAP-2 sequence contains potential sites for protein kinase C, casein kinase-II phosphorylation (Thr-39), N-myristoylation (Gly-28) and amidation (Asp-42). No N-glycosylation site is contained within the primary sequence. Since chemically synthesized NAP-2 is biologically fully active [8], these modifications do not seem to be important for neutrophil activation. In ultracentrifugation experiments β-TG was identified as a tetramer which dissociates into fragments at low pH [11]. No sedimentation experiments have been performed on NAP-2. The 3-dimensional structure of NAP-1/IL-8 has been well described and the active protein was found to consist of two identical subunits [12–17]. Since the overall homology of NAP-2 and NAP-1/IL-8 is 47%, a similar molecular architecture is expected, including a dimeric quaternary structure for biologically active NAP-2. Moreover, the homology between the two chemokines may assist in solving the structure of NAP-2 by Patterson search methods [18].

2. Materials and methods

All chemicals used in this study were p.a. grade and were used without further purification except PEG 8000 which was re-crystallyzed from isopropyl alcohol/diethyl ether. For polyacrylamide gel electrophoresis commercially available Tris-Glycine gradient gels (4–20% and 10–27%) from NOVEX (San Diego, USA) were used. Staining of the proteins was accomplished either by Coomassie brilliant blue (Bio-Rad, Richmond, USA) or/and by silver [19]. The protein concentration was determined either by measuring the optical density against a well characterized standard of NAP-2 or by using the Pierce assay kit (Pierce, Rockford, USA). Cation exchange FPL-chromatography was performed on a HR5/5 Mono-S column from Pharmacia (Pharmacia, Uppsala, Sweden). For preparative reversed phase HPLC a C-4 column (25 × 100 mm, 15 μm) on a Waters Delta Prep 3000 system (Millipore, Milford, USA) was used. For analytical reversed phase HPLC a C-4 column (Vydac, Hesperia, USA, Cat. 214TP10415) on a Beckman System Gold (Beckman, Fullerton, USA) was used. Capillary electrophoresis was performed on a Beckman P/ACE 2100 system (Beckman, Fullerton, USA). Concentration of the protein was done in Microsep Microconcentrators (Filtron, Northborough, USA). Screening of crystallization conditions was accomplished in Linbro (Flow Laboratories, Virginia, USA) multiwell tissue culture trays. Preliminary X-ray diffraction data were collected on an image plate system (MAR Research, Hamburg, Germany).

3. Results and discussion

Recombinant human NAP-2 (rhNAP-2) used in this study was expressed in *E. coli* as described previously for NAP-1/IL-8 [20]. After breaking the cells by ultrasonication, the protein was extracted from the pellets by 6 M guanidinium-HCl containing 0.1 M Tris-HCl (pH 7.2) and 2 mM EDTA. Centrifugation was followed by dialysis for 24 h of the supernatant against 1 M GuHCl containing 50 mM Tris-HCl (pH 8.2), 2 mM reduced and 0.2 mM oxidized glutathione to obtain the fully reduced form of the protein. After centrifugation, the clear supernatant was dialysed against 5% acetic acid and the gelatinous precipitate was again removed by centrifugation. At this stage, the overall protein concentration was determined to be approximately 4 mg/ml. After buffer exchange to 50 mM MES-NaOH (pH 5.6) containing 5 mM NaN_3 by dialysis, this solution was chromatographed on a Mono-S HR5/5 FPLC column by eluting with a linear gradient (60 min) up to 1 M NaCl (detection at 280 nm). rhNAP-2 elutes with 0.21 M NaCl. The rhNAP-2 containing fractions were pooled, lyophilized and dissolved in water containing 10% acetonitrile and 0.1% trifluoroacetic acid. This solution was then loaded onto a C-4 reversed phase column. A linear gradient (120 min) from 10–90% acetonitrile (containing 0.1% trifluoroacetic acid) was applied. rhNAP-2 eluted at 27% acetonitril (detection at 230 nm). The rhNAP-2 containing fractions (purity > 98%) were pooled and then refolded, under the same conditions as described above, on a Mono-S column.

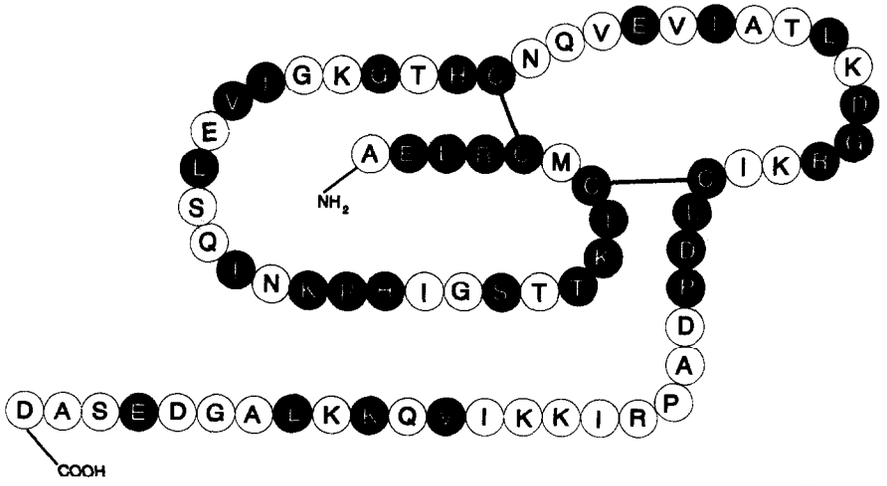

Fig. 1. Sequence of neutrophil-activating peptide 2. Filled circles mark amino acids homologous to NAP-1/IL-8.

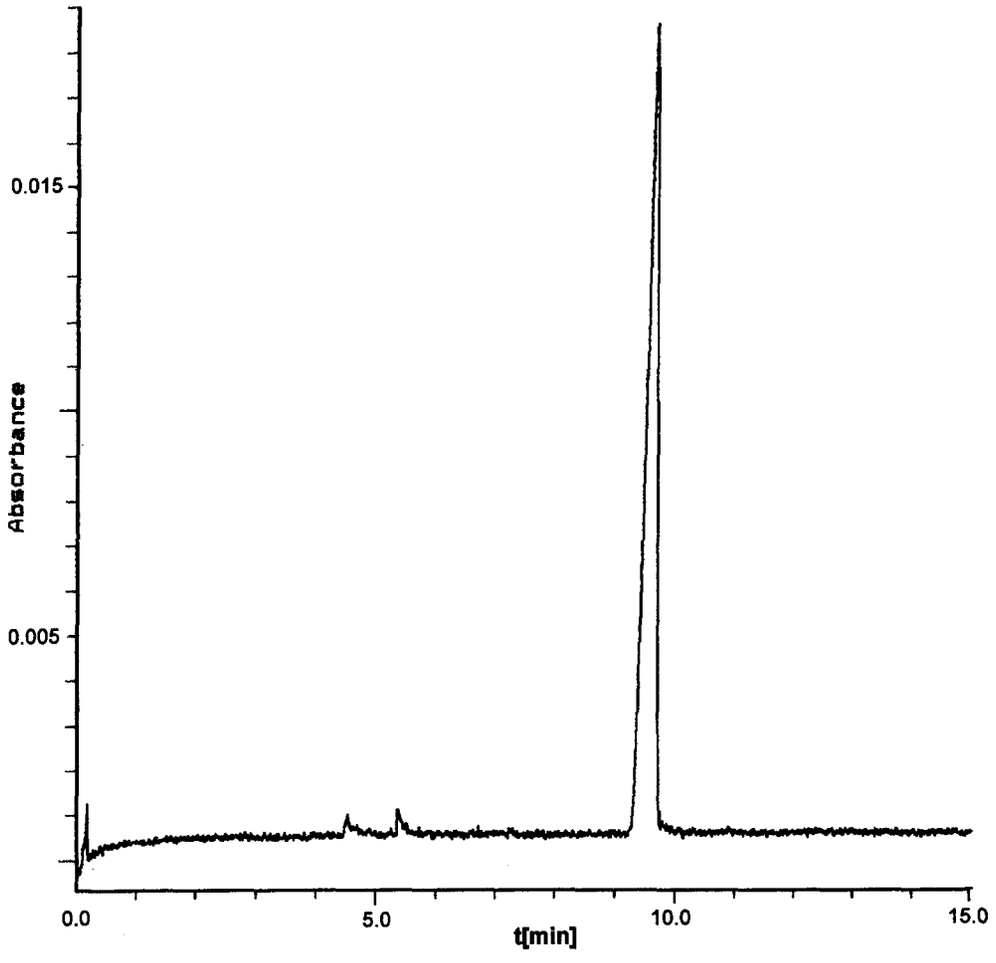

Fig. 2. Capillary electrophoresis of rhNAP-2 (2μg) in 0.1 M Na-borate buffer (pH = 9.5) accomplished at 30°C and 20 kV. The protein was detected at 200 nm.

Every step in the purification protocol was followed by polyacrylamide gel electrophoresis. Commercially purchased 4–20% or 10–27% gradient gels were run and stained by Coomassie blue reagent and/or by silver [19]. After preparative column chromatography the rhNAP-2 containing fractions were analysed by reversed-phase HPLC (C-4) and compared to a NAP-2 standard. The retention time of rhNAP-2 was used to identify and the peak area was used to quantify the protein together with determining its overall amino acid content after total hydrolysis. The final purity was checked by capillary electrophoresis: rhNAP-2, suitable for crystallization experiments, migrated as a single peak (Fig. 2). The biological activity of rhNAP-2 was tested by a neutrophil elastase release assay (Fig. 3) [3]. The recombinant protein was found to be comparable to natural NAP-2, which is itself less active than NAP-1/IL-8. From 50 g wet *E. coli* cells 10 mg purified rhNAP-2 were obtained. The protein solution with a concentration of 2 mg/ml was frozen and could be stored at −20°C for at least 1 month.

Immediately before crystallization experiments the protein solution was concentrated by micro seperation (molecular weight cut-off 3,000 Da) to 15 mg/ml. Diffraction quality crystals of rhNAP-2 were grown by the hanging drop vapor diffusion method using multiwell tissue culture trays [21]. Crystals were obtained in 5 μl drops containing protein at 7.5 mg/ml and PEG 8000 at a concentration of 5–10% w/v. The drops were equilibrated at room temperature either against 70–90% AS (of saturation) or against 30–40% w/v PEG 8000. The buffers contained 5 mM 2-morpholinoethane-sulfonic acid (MES) and 3 mM NaN_3 (pH 6.5). Small crystals of about $0.07 \times 0.07 \times 0.03$ mm size were obtained within 5–7 days. When these rhNAP-2 crystals were allowed to grow for about 2–4 weeks, slightly intercalated parallelepipeds of about $0.3 \times 0.3 \times 0.1$ mm size were obtained which were suitable for X-ray diffraction. The crystals were broken gently to obtain single-crystal fragments.

rhNAP-2 crystals diffract to approximately 2 Å on an image plate system. The space group was determined by precession photographs to be P222 with unit cell dimensions of a = 30.8 Å, b = 39.5 Å, and c = 95.3 Å. The molecular mass weight of the protein (7,268 Da) as well as the volume of the unit cell suggest that the asymmetric

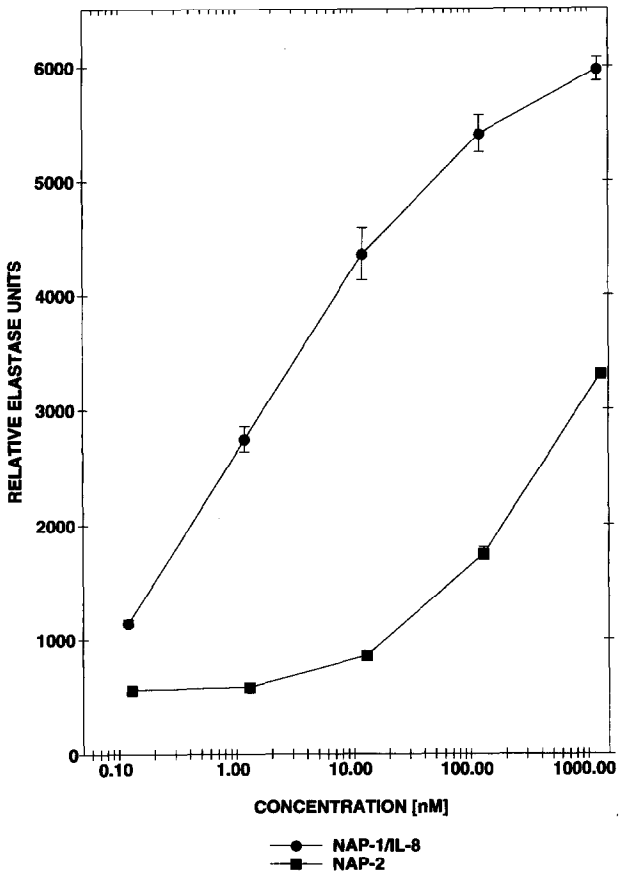

Fig. 3. Concentration-dependent induction of elastase release (arbitrary fluorescence units) from human neutrophils by rhNAP-2 and rhNAP-1/IL-8.

unit contains one monomer of rhNAP-2. According to Matthews [22] a crystal volume per unit of protein molecular weight of 3.8 $Å^3$/Da has been computed. This corresponds to a solvent content of approximately 68%. Since the crystals are stable to long-term X-ray exposure (at least 48 h), they are suitable for high resolution structure determination.

In contrast to NAP-1/IL-8 crystals, which have a trigonal space group when grown from different PEG's and AS (space group $P3_121$), the shorter NAP-2 crystallized in an orthorombic space group. From the series of NAP-1/IL-8 mutants which were crystallized so far [17], an orthorombic space group could only be obtained from the C-terminal truncated fragment 1–66 (instead of 77 aa in the wild type protein). Whether the 50% reduced center to center distance between the two helices of the NAP-1/IL-8 truncation mutant (1–66) will also be seen in NAP-2 awaits clarification by solving the 3D structure.

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