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X-ray crystallographic studies on murine nerve growth factor

N. Q. McDONALD, R. LAPATTO, J. MURRAY-RUST AND T. L. BLUNDELL

Laboratory of Molecular Biology and ICRF Structural Molecular Biology Unit, Dept. of Crystallography, Birkbeck College, Malet St., London, WC1, UK

Summary

The largest and best characterised family of neurotrophic growth factors is that of nerve growth factor (NGF) and its relatives. In order to understand the relation of structure and function, we have undertaken X-ray analyses of murine NGF. The active component β-NGF crystallises as hexagonal bipyramids that give good X-ray diffraction data using a synchrotron to 2.3Å resolution. We have prepared several heavy atom derivatives that are being used in the method of multiple isomorphous replacement to solve the phase problem and determine the three-dimensional structure. We have also prepared crystals of the precursor, 7S NGF, which is a complex of three different subunits of composition $\alpha_2\beta_2\gamma_2$. We have collected X-ray data to 3Å resolution on two crystal forms with related cell dimensions and orthorhombic spacegroups. Detailed analyses of the structures of NGF in these crystal forms, taken together with data on sequence and biological activity, should give clues concerning the role of the precursor complex in storage and assist the identification of the surface region involved in receptor binding.

Introduction

During the development of the vertebrate nervous system, many populations of neurons, including the sympathetic and sensory neurons, depend for their survival on their interactions with target cells. These neuron–target cell interactions are controlled by specific proteins or neurotrophic factors, which are released by target cells in both the peripheral and central nervous system (Thoenen *et al.* 1985 and Levi-Montalcini, 1987). The best characterised neurotrophic factor is nerve growth factor (NGF), first purified from murine submaxillary gland by Cohen (1959). Three decades of intense study on the chemistry and biology of NGF have provided evidence for the trophic role that NGF plays both in the development and throughout the lifetime of the neuron.

When isolated from murine submaxillary gland, the active neurotrophic molecule, β-NGF, is found incorporated in a complex with three different subunits of composition $\alpha_2\beta_2\gamma_2$ (Darling, 1983). This is referred to as the 7S NGF complex, named after its sedimentation coefficient. γ-NGF is an esteropeptidase with a specificity for the C-terminal Arg-Arg sequence of β-NGF and it processes the β-NGF precursor (Edwards *et al.* 1988). The α-subunit is 80 % homologous to the γ-

Abbreviations used: The heavy atom derivatives for NGF are called $NaAuCl_4$ for sodium aurichloride; EMP, for ethyl mercury phosphate; $K_2Pt(NO_2)_4$, for platinum tetranitrate; PCMBS, for p-chloromercuricbenzenesulphonic acid.

Key words: X-ray diffraction, nerve growth factor, structure, neurotrophic, crystallisation.

NGF but lacks esteropeptidase activity. It is clear that both these subunits are related to the kallikrein family of serine proteases (Bothwell *et al.* 1979; Isackson *et al.* 1987).

The β-NGF protomer has a molecular weight of 12 650 M_r, comprises 118 amino acids and associates as a non-covalently bonded dimer (Server and Shooter, 1977). A single asparagine-linked complex oligosaccharide is present in about 2% of murine NGF protomers, and contributes 10% of the total mass of such a protomer (Murphy *et al.* 1989). Each protomer contains three disulphide bridges that give a strongly crosslinked, stable structure analogous to other growth factors (James and Bradshaw, 1978). The biological activity of β-NGF is strongly conformation dependent, since the reduction of the disulphide bonds abolishes NGF activity (Frazier *et al.* 1973). An analysis of the sequences of the NGF family of neurotrophic factors shows five clearly separated and highly conserved regions that may be involved in signal transduction (Fig. 1). The high similarity of sequences indicates similar three-dimensional structures. This is supported by the biological activities and immunological properties of β-NGF from different species (Harper and Theonen, 1980).

β-NGF is a highly basic protein with a pI of 9.3. Chemical modification and site specific mutagenesis have implicated several arginine residues in receptor binding (Bradshaw *et al.* 1976; Ibanez *et al.* 1990). A complementary surface on the NGF receptor must exist and it is likely that multiple ionic interactions are responsible for the high affinity constant observed for β-NGF ($K_d = 10^{-11}$ M). Indeed the recent sequencing of the rat and human NGF receptors (Radeke *et al.* 1987; Johnson *et al.* 1986) showed an overall negative charge distribution of amino acids in their extracellular domains. Other residues such as valine 21

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            ----V1---                                    -----V2----          --
Mouse   NGF SSTHPVFHMGEFSVCDSVSVWV**GDKTTATDIKGKEVTVLAEVNINNSVFRQYFFETKCRA
Human   NGF SSSHPIFHRGEFSVCDSVSVWV**GDKTTATDIKGKEVMVLGEVNINNSVFKQYFFETKCRD
Bovine  NGF SSSHPVFHRGEFSVCDSISVWV**GDKTTATDIKGKEVMVLGEVNINNSVFKQYFFETKCRD
Guinea  NGF SSTHPVFHMGEFSVCDSVSVWV**ADKTTATDIKGKEVTVLAEVNVNNNVFKQYFFETKCRD
Chick   NGF  TAHPVLHRGEFSVCDSVSMWV**GDKTTATDIKGKEVTVLGEVNINNNVFKQYFFETKCRD
Snake   NGF  EDHPVHNLGEHSVCDSVSAWV***TKTTATDIKGNTVTVMENVNLDNKVYKQYFFETKCKN
Pig    BDNF   HSDPARRGELSVCDSISEWVTAADKKTAVDMSGGTVTVLEKVPVSKGQLKQYFYETKCNP
Mouse  NT-3  YAEHKSHRGEYSVCDSESLWVT**DKSSAIDIRGHQVTVLGEIKTGNSPVKQYFYETRCKE

Prediction      TTTTTTTBBBBTT        TTT          BBBBBB    TTTTTBBBB       TT

            -V3---                              ---V4---
Mouse   NGF SNPVESGCRGIDSKHWNSYCTTTHTFVKALTTDEKQ*AAWRFIRIDTACVCVLSRKATRRG
Human   NGF PNPVDSGCRGIDSKHWNSYCTTTHTFVKALTMDGKQ*AAWRFIRIDTACVCVLSRKAVRRA
Bovine  NGF PSPVESGCRGIDSKHWNSYCTTTHTFVKALTTDNKQ*AAWRFIRIDTACVCVLNRKAARRG
Guinea  NGF PNPVDSGCRGIDAKHWNSYCTTTHTFVKALTMDGKQ*AAWRFIRIDTACVCVLSRKTGQRA
Chick   NGF PRPVSSGCRGIDAKHWNSYCTTTHTFVKALTMEGKQ*AAWRFIRIDTACVCVLSRKSGRP
Snake   NGF PNPEPSGCRGIDSSHWNSYCTETDTFIKALTMEGNQ*ASWRFIRIETACVCVITKKKGN
Pig    BDNF MGYTKEGCRGIDKRHWNSQCRTTQSYVRALTMDSKKRIGWRFIRIDTSCVCTLTIKRGR
Mouse  NT-3 ARPVKNGCRGIDDKHWNSQCKTSQTYVRALTSENNKLVGWRWIRIDTSCVCALSRKIGRT

Prediction TTT  TTTTT      TTTTBBBBBBBBBBB          BBBBBBTTTBBBBBBB
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Fig. 1. Sequence alignment of NGF from different species and related neurotrophic factors. A cumulative secondary structure prediction is shown below using the Leeds Prediction Package (Eliopoulos, 1989).

(Ibanez *et al.* 1990) and the tryptophan side chains have also been implicated in receptor binding (Cohen *et al.* 1980). However, recent mutagenesis experiments suggest that the tryptophans are structurally but not functionally important, since their replacement alters but does not abolish NGF activity or receptor binding (Ibanez *et al.* 1990).

A detailed three-dimensional structure of this molecule has so far been elusive despite the availability of X-ray quality crystals for many years (Wlodawer *et al.* 1975). Here we report on recent progress towards defining the active conformation of NGF. We also report on the preliminary characterisation of crystals of the high molecular weight form of NGF in the submaxillary gland, the 7S NGF protein complex.

Purification and crystallisation of β-NGF

With the advent of recombinant DNA techniques, large quantities of relatively scarce proteins such as growth factors can be produced for structural analysis. However, milligram quantities of NGF and EGF (epidermal growth factor) are available from certain tissue sources (Walker, 1982). The adult male submaxillary gland is one such tissue source, and the 7S NGF comprises about 0.2% by weight of this gland (Server and Shooter, 1977).

Purification of the 7S NGF complex to homogeneity by the method of Stach *et al.* (1977) helps protect the β-NGF from proteolytic modification. Proteolytic enzymes in the submaxillary gland can modify the native β-NGF molecule at both the amino and carboxyl termini to give multiple forms of NGF heterodimers (Moore *et al.* 1974). In an effort to reduce the proportion of heterodimers, we have adapted the purification method of Mobley *et al.* (1976) to include an FPLC ion exchange step in place of conventional ion exchangers. After purifying the complex, it is then dissociated by acid pH into its constituent subunits. A Mono-S column gives a crude fractionation of the different β-NGF forms (Fig. 2A) and a higher yield of the final product (typically 3–4 mg from 40 g of glands). The β-NGF is further purified by gel filtration in 2 M acetic acid to remove contaminating γ-NGF and mouse IgG proteins (Fig. 2B).

β-NGF produced in this manner crystallises reproducibly to give well formed hexagonal bipyramids (Fig. 3A) as previously described by Wlodawer *et al.* (1975). This is in contrast to earlier problems with disordered 'bulges' where the two pyramids join (Gunning *et al.* 1982). The crystals diffract to greater than 2.3 Å resolution (1Å = 0.1 nm). A *h0l* precession photograph is shown in Fig. 3B. The crystals belong to the space group $P6_122$ (or its enantiomorph $P6_522$) and have cell dimensions a=b=56.5 Å, c=182.4 Å. A high resolution native dataset has been collected for β-NGF to 2.3 Å using the X-ray synchrotron radiation facilities at the EMBL station at DESY, Hamburg (Table 1).

Multiple isomorphous replacement of β-NGF

After producing well ordered crystals, the remaining obstacle to a crystal

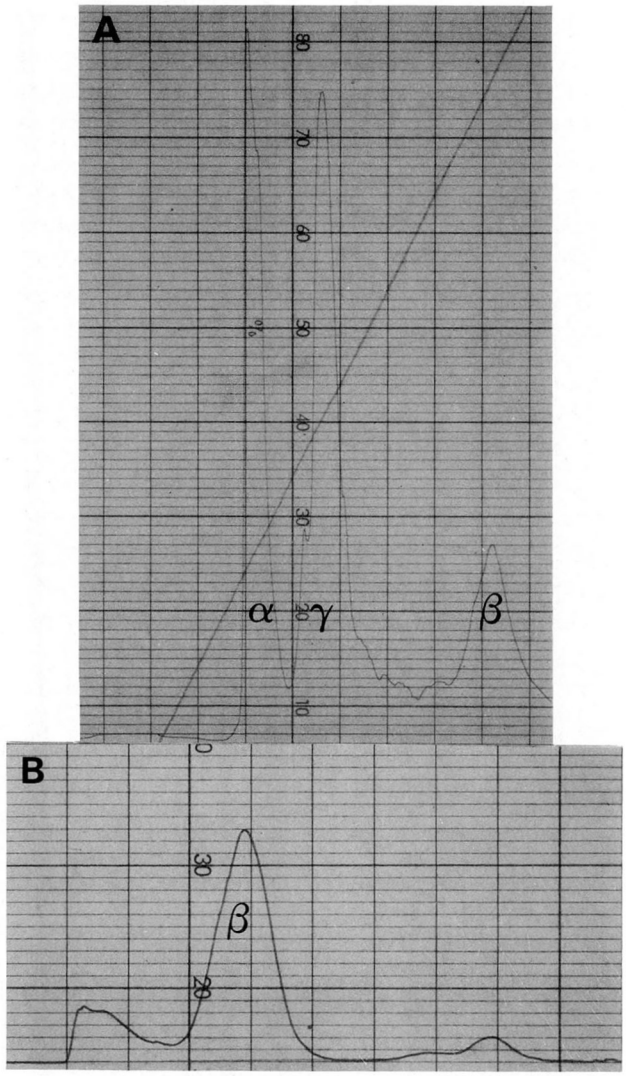

Fig. 2. (A) A 1 ml FPLC Mono S chromatograph of individual subunits from dissociated 7S NGF complex. Buffer conditions are pH 4.0, 50 mM sodium acetate. A flow rate of 1 ml min^{-1}, and a salt gradient of 0–1 M NaCl in 50 ml was used. (B) Gel filtration of β-NGF in 2 M acetic acid using G75 Sephadex.

structure is the solution of the phase problem. Since only the amplitude of each diffracting X-ray wave can be measured, the phase relationship is lost. Therefore a way to evaluate each phase is required. Traditionally new macromolecular structure analyses have used isomorphous replacement with heavy atoms. This is performed by diffusing reactive heavy atom compounds into the native protein crystals. This utilises the large solvent channels present between packed protein molecules in the crystal lattice.

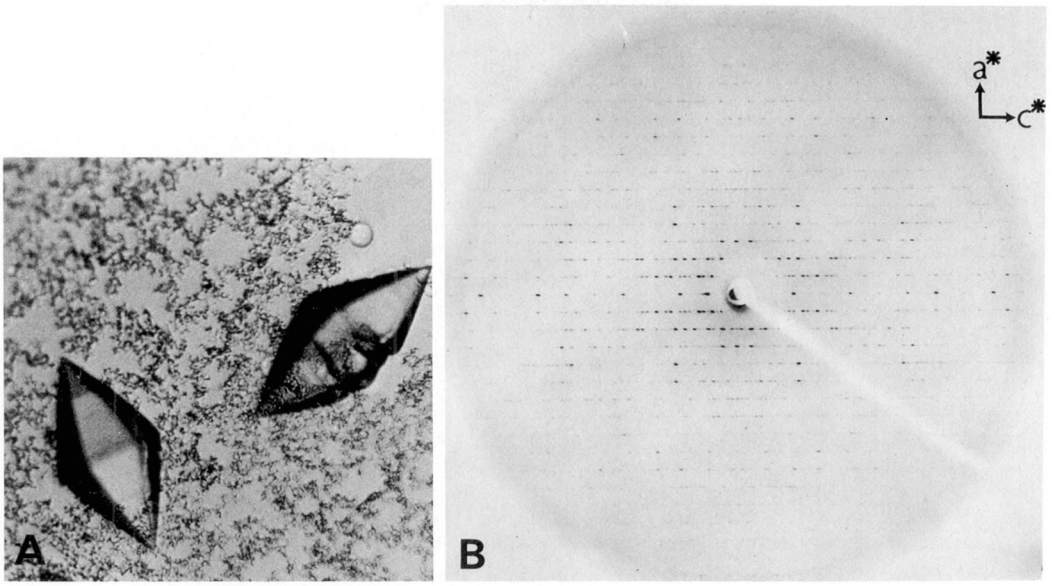

Fig. 3. (A) Hexagonal bipyramid crystals of β-NGF (dimensions 0.5–0.75 mm) grown at pH 6.8 according to Wlodawer *et al.* (1975). (B) A *h0l* precession photograph of a β-NGF crystal, showing systematic absences characteristic of a 6_1 (or 6_5) screw axis along the c axis.

The amino acid sequence of β-NGF indicates three possible types of side chains for heavy atom binding (Angeletti *et al.* 1971). A single methionine and three histidine side chains are potential sites for soft, polarisable heavy atom compounds that form covalent bonds. Also the disulphide bonds are potential interaction sites for mercury and platinum compounds, though these interactions can often give conformational changes (Blundell and Johnson, 1976). As predicted, the best quality heavy atom derivatives have been produced using mercurial and platinum compounds (see Table 1). The harder, less polarisable metals such as the lanthanides often disordered the crystals or changed the cell dimensions (Gunning, 1983).

X-ray data for several heavy atom derivatives have been measured for the β-NGF crystals and are described in Table 1. The quality of the data can be assessed by the internal agreement within the dataset and by the differences found when compared to the native dataset. Previous work at Birkbeck College highlighted the problems of collecting isomorphous heavy atom derivative datasets for β-NGF (Gunning, 1983). Two particular problems were encountered. First, the variation of native crystal cell parameters required that all X-ray data be measured from crystals produced from the same preparation of NGF. Second, even small cell dimension changes during the preparation of heavy atom derivatives resulted in noisy, uninterpretable difference Patterson maps used for locating the heavy

Table 1. *Beta nerve growth factor data processing statistics*

Dataset	Cell		Concentration (mM)	R_{deriv} (%)	R_{merge} (%)	Resolution (Å)	% Data
Native	56.5	182.4	–	–	6.6	2.3	90.3
$SmCl_3$	56.6	182.4	1	14	5.5	3.1	96.5
PCMBS	56.09	183.2	5	19.9	13	3.1	90.8
EMP	56.10	180.32	1	16.4	12	3.3	90.4
$K_2Pt(NO_2)_4$	56.19	181.36	1	16.2	12	3.1	88.2
$NaAuCl_4$	56.10	181.1	5	28.2	11.8	3.3	93.3

$R_{merge}=\sum(|I-\langle I\rangle|)/\sum I$
$R_{deriv}=\sum(|F_{ph}-F_p|)/\sum F_{ph}$.
1 Å=0.1 nm.

atom positions. So a careful control of soaking conditions for heavy atom reagents was necessary.

Single sites in each of the Pt and Hg derivatives have allowed calculation of phases for these derivatives. These phases can be used to locate heavy atom positions for other derivatives, using cross-phased difference Fourier maps (see Blundell and Johnson, 1976) and to check the agreement with the difference Patterson Harker sections. One example is shown in Fig. 4A and 4B, where the phases from the Hg derivative were used with the Pt structure factor amplitudes. A clear single site is evident. The three corresponding Harker vectors from this single site can be seen in the Harker section of the Pt difference Patterson map (Fig. 4B). We have successfully interpreted four of the derivatives listed in Table 1, though the PCMBS and EMP derivatives have sites in common. Therefore the PCMBS dataset was not used. Also the $NaAuCl_4$ dataset showed two heavy atom sites and was included in the phase calculations.

On the basis of the heavy atom positions in Pt, Sm, Au and Hg derivatives described, an electron density map has been calculated to 3.5 Å resolution. It is clear that part of the NGF structure is observable from this map. However, further heavy atom derivatives are currently being screened to improve the MIR phases produced from just three derivatives. We expect these data to allow the solution of the NGF structure in the near future.

X-ray analysis of the 7S NGF protein complex

Varon *et al.* (1967) first reported that NGF exists as a high molecular weight complex. The molecular weight of 7S NGF is 130 000 as estimated from a variety of sedimentation techniques. From the known molecular mass of each subunit and also the relative amounts of each subunit obtained on dissociation, the subunit composition of the complex is two α-NGF subunits of 26 500, one β-NGF dimer of 26 000, two γ-NGF subunits of 26 500 and one or two zinc ions (Pattison and Dunn, 1975).

Three crystal forms of this complex suitable for X-ray analysis have been crystallised using polyethylene glycol as a precipitant (Fig. 5 and McDonald and

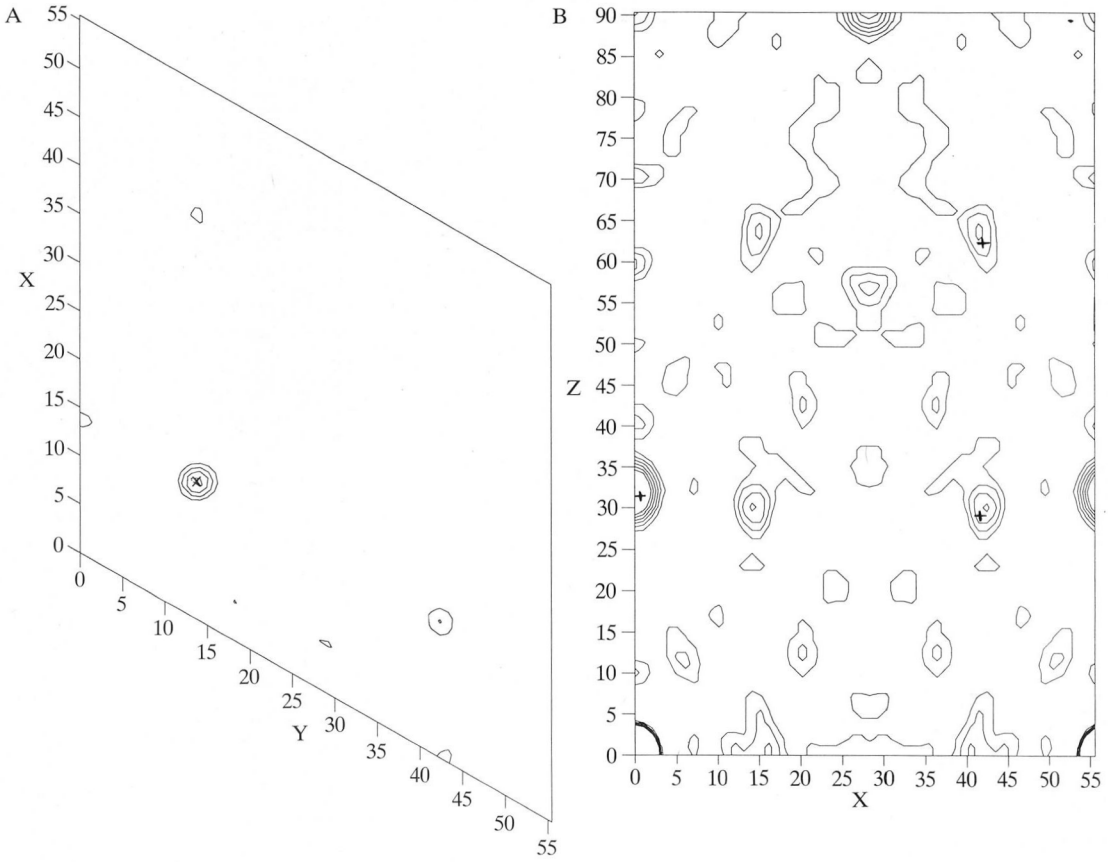

Fig. 4. (A) Cross-phased difference Fourier map section using the phases calculated from the Hg derivative with the Pt isomorphous differences. A single site is located at X and agrees with (B) the three Harker vectors marked X from the difference Patterson map (y=0 section) for the Pt derivative.

Blundell, 1990). Table 2 indicates the cell dimensions and space group for each form. Since the crystal form A_1 is most commonly observed, and has the more convenient cell dimensions for data collection, our X-ray studies have concentrated on this form. The cell dimensions for all forms were estimated from precession photographs, and the space group was derived from the systematic absences from these photos (Fig. 6). The cell parameters shown for form A_1 are the refined cell dimensions used during data collection. A native dataset for form A_1 has been collected to 3.0 Å resolution using synchrotron radiation. Although several high resolution structures are known for the serine protease family (Bode *et al.* 1983), the complexity of the oligomer implies that use of these as search models for a molecular replacement solution of 7S NGF (Rossmann, 1972) may not succeed. Thus, heavy atom trials are in progress so that multiple isomorphous replacement can be used to solve the phase problem.

Electron micrographs using negative staining indicate two features of the

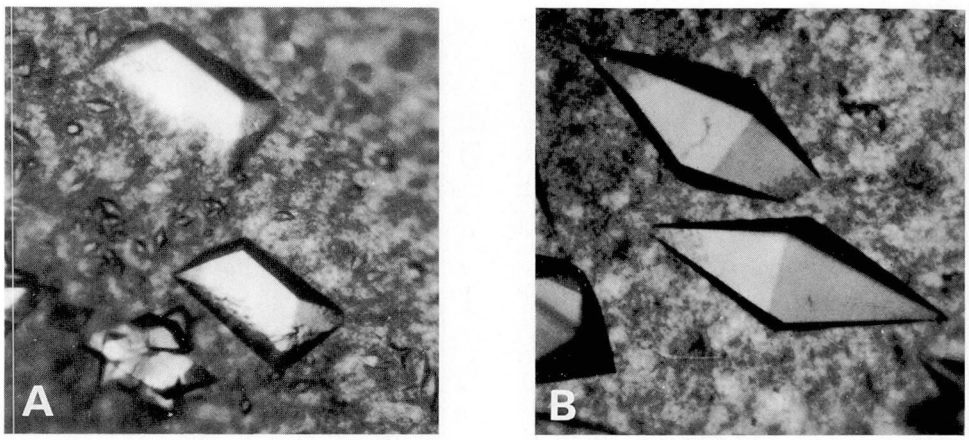

Fig. 5. Crystal forms A_1 (A) and B_1 (B) of 7S NGF grown at pH 4.5 in the presence of zinc according to McDonald and Blundell (1990).

Table 2. *Crystal forms of the 7S nerve growth factor protein complex, crystallized using polyethylene glycol as the precipitant*

Crystal Form	Cell Å			Space Group	V_m(Å^3 Da^{-1})	Z	Resln
Orthorhombic A_2	86.3	91.4	148.5	$P2_12_12_1$	2.3	1 7S complex	3 Å
Orthorhombic A_1	95.4	96.7	146.5	$P2_12_12_1$	2.6	1 7S complex	3 Å
Orthorhombic B_1	93.4	97.8	308.0	$P22_12_1$	2.5	2 7S complexes	3.6 Å

1 Å = 0.1 nm.
V_m = volume of asymmetric unit/molecular mass.
Z = number of molecules per asymmetric unit.

complex (Fig. 7). First, a negatively stained 'hole', surrounded by several unstained protein subunits, can be seen in most of the complex molecules, indicating the subunits are arranged in a symmetric fashion with a closed point group symmetry. Two possibilities exist for the arrangement of the two α and two γ-NGF subunits (which are twice the size of the β-NGF subunits), either with a cyclic or a dihedral point group symmetry. Unfortunately these cannot be distinguished by standard negative stain electron microscopy. Second, the micrographs indicate the globular shape of the complex with an approximate diameter of 80 Å (which comprises two subunit diameters). This is in agreement with approximate dimensions of 35 Å by 30 Å observed for a single serine protease molecule. The size of the 7S complex imposes certain restrictions on the packing of 7S molecules, since four or eight molecules must pack within the unit cell. However, the packing arrangement for the different forms and the relationship between the three crystal forms is not clear at present.

The α and γ-NGF contain primarily β-strand secondary structure motifs, by analogy with other serine protease structures (Thomas *et al.* 1981). The β-NGF is

also predicted to be principally a β type protein from spectroscopic evidence (Williams and Gaber, 1982) and from secondary structure prediction using seven different prediction algorithms (see Fig. 1). In the light of these predictions, the circular dichroism spectra for the whole complex confirm the presence of a large β-sheet contribution in the far UV peptide region (Fig. 8) (see Curtis Johnson, 1988 for review). In addition aromatic side chains from different subunits can become

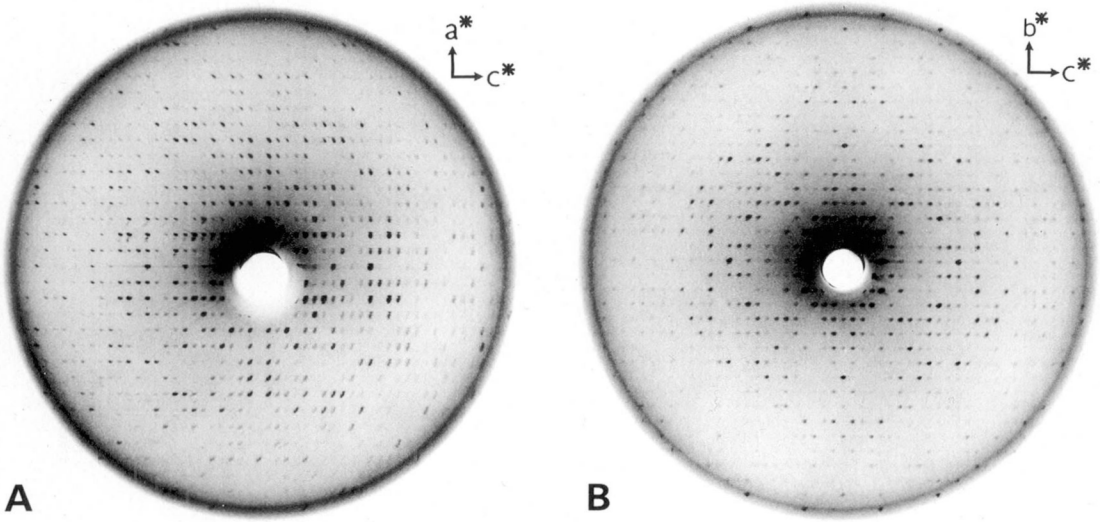

Fig. 6. (A) A *h0l* precession photograph of crystal forms A_2 and (B) an ϕkl photograph of A_1 of 7S NGF. 2_1 screw axes along a and c axes are evident from the systematic absences in these photos.

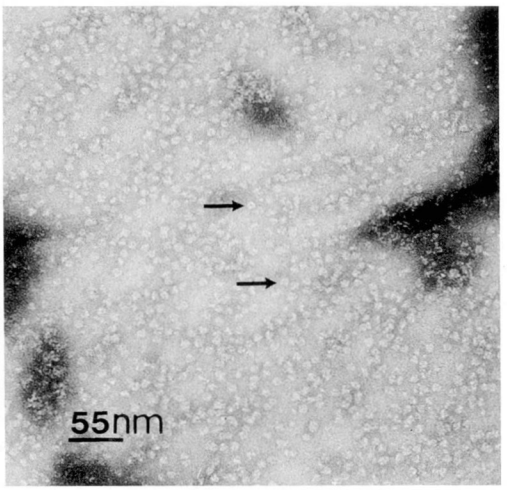

Fig. 7. Electron micrograph of the 7S NGF complex negatively stained with uranyl acetate. The two arrows indicate clear images with a 'hole' and an arrangement of subunits around this 'hole'. Magnification is ×126 500; the bar represents 550 Å (55 nm).

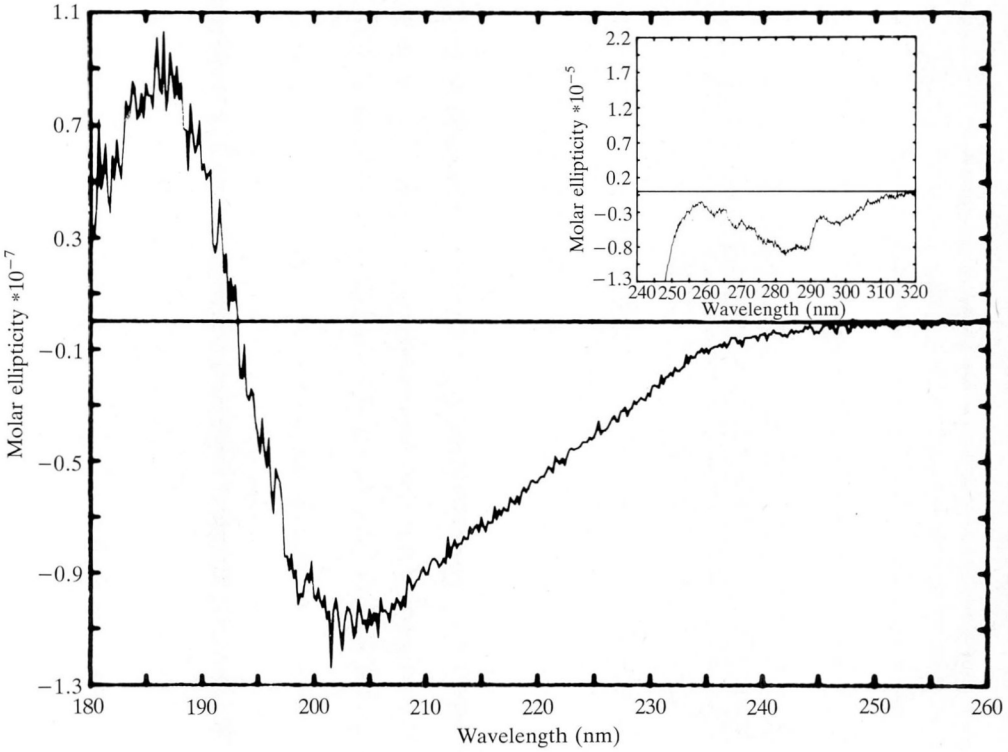

Fig. 8. Far and near (inset) UV circular dichroism spectra of 7S NGF, measured at pH 6.8 at $1\,mg\,ml^{-1}$ concentration in the presence of zinc, showing a β-sheet contribution in the peptide region.

trapped on association in protein complexes (Wood *et al.* 1975). The small CD signals seen in the near UV (inset Fig. 8) are removed on dissociation of the 7S NGF complex (McDonald, unpublished results). This feature of the near UV CD spectra of the associated complex may be a direct consequence of buried aromatic residues between the NGF subunits.

In addition to its high affinity ligand–receptor interaction which is responsible in part for its signalling properties, β-NGF undergoes another important protein–protein interaction with its processing enzmyes α and γ-NGF. The exact physiological role of this complex is not fully understood, though a role in NGF biosynthesis and protection is likely. The serine protease subunits can together competitively inhibit NGF binding to its cell surface receptor and thus inhibit the NGF neurotrophic activity (Woodruff and Neet, 1986; Stach and Shooter, 1980). The three-dimensional structure of such a growth factor-processing enzyme complex should provide information on the receptor binding region of NGF, as well as the manner in which it inhibits the γ-NGF serine protease activity. Thus in parallel with studies on β-NGF, we are pursuing the structure of the 7S NGF complex.

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