Crystallization of Nerve Growth Factor from Mouse Submaxillary Glands

(s-x ray diffraction/vapor diffusion/crystal data)

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ABSTRACT Crystals of the nerve growth factor protein were grown by vapor diffusion from ethanol solution. The crystals are hexagonal, belonging to space group P622 (or its enantiomorph) with a = 56.1 Å, c = 181.4 Å, and V = 494,400 Å³. The unit cell contains six molecules of dimeric protein and thus has one monomer per asymmetric unit. The diffraction pattern extends to at least 2.7 Å, indicating that this crystal form is suitable for structural analysis to near-atomic resolution.

Nerve growth factor (NGF) is a protein that enhances growth and differentiation of sympathetic and embryonic sensory ganglia (1). It was originally isolated by Cohen (2) from snake venom, although its most abundant source was found to be the submaxillary glands of adult male mice (3). Subsequently, Varon et al. (4) showed that NGF activity is associated with a complex protein, 7S NGF. It was also demonstrated that 7S NGF can be dissociated into three different types of subunits, one of which is responsible for the physiological activity of the complex and is active in the absence of the other two subunits (5). This active subunit was named βNGF (5) or 2.5S NGF (6), depending on the method of preparation. The primary sequence of 2.5S NGF has been determined (7, 8). This protein consists of two chains, the A chain, which is 118 residues long, and the B chain, which has 110 residues. The sequence of the B chain is identical to that of the A, except that the first eight amino acids on the NH₂-terminal end are missing. The sequence of βNGF has been independently determined and consists of two A chains (9). Each of the NGF peptide chains contains three disulfide bridges but has no free cysteine residues. Frazier, Angeletti, and Bradshaw (10) noted that in certain regions the amino acid sequence of the NGF chains was homologous to that of proinsulin.

The differences in amino-acid sequence that distinguish the 2.5S NGF from the βNGF dimers result from limited proteolytic cleavage of the NGF chains during isolation. Besides the loss of the NH₂-terminal octapeptide sequences, the COOH-terminal arginine residues are also susceptible to cleavage (8, 11). Loss of one COOH-terminal arginine residue results in the formation of a dimer called βNGF, which has chains with unlike COOH-termini, and loss of both arginine residues produces the dimer, β²NGF, in which both chains have COOH-terminal threonine residues (11). All known procedures of purifying NGF lead to preparations with at least some degree of inhomogeneity at the COOH-termini of the dimer. In βNGF preparations, the major dimer species (90%) retains the arginine residues at both COOH-termini (designated β¹NGF) and the minor species is β²NGF. In 2.5S NGF preparations, however, all three species, β¹, β², and β³NGF, are present, thus reflecting a greater loss of COOH-terminal arginine residues during isolation.

Frazier et al. (12) studied the reactivity of the two tyrosine and three tryptophan residues of 2.5S NGF and found that their topological chemical reactivity is at least consistent with the corresponding reactivity of similarly positioned residues in insulin. It was thus suggested that these two proteins may possess similar conformations. The comparatively low molecular weight of NGF, the knowledge of its sequence, and possible similarity to insulin make crystallographic studies attractive. We report here the crystallization of NGF and some characteristics of the crystals.

RESULTS

Isolation of NGF. The NGF protein was isolated from the submaxillary glands of 60-day-old male Swiss Webster mice (Simonsen; Gilroy, Calif.) by a procedure that combined and simplified the methods for the isolation of 7S and 2.5S NGF (13). The average yield was 5–7 mg of protein from 200 pairs of submaxillary glands. The composition of the NGF protein varied somewhat from preparation to preparation, but a typical preparation contained 73% β¹, 23% β², and 4% β³ NGF. In addition, approximately 35% of the NGF chains were missing the NH₂-terminal octapeptide sequence. Since the extent of proteolytic cleavage fell between that seen in the β and 2.5S NGF preparations and in order to avoid confusion with these two different methods of isolation, the present preparation will be referred to simply as NGF protein. Preparations were stored at 4⁰ in either unbuffered 0.2% acetic acid or 0.05 M Tris buffer, pH 9.0. Each sample was assayed for biological activity by the procedure described by Varon et al. (14). The extent of proteolytic cleavage was determined by the isoelectric focusing method devised by Schenker and Shooter (13).

Crystallization. All experiments leading to crystallization of NGF were conducted at 4⁰. Protein solutions (about 3 mg/ml) were dialyzed against 10 mM sodium phosphate buffer, pH 7.2, and then clarified by centrifugation. Crystals were grown by a modification of the vapor diffusion technique, known as the “hanging-drop” method. The dishes used were Disposal-Tray tissue culture trays with 24 separate wells (Linbro Chemical Co., New Haven, Conn.). Each well was filled with

Abbreviation: NGF, nerve growth factor.

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2 ml of final precipitant. Ten microliters of protein solution were placed with a pasteur pipette on a clean, siliconized cover glass and the well was covered in such a way that the droplet faced the solution. An air-tight seal was assured by coating the rim of each well with silicone grease. This technique allows for the simultaneous checking of up to 24 different crystallizing conditions in one experiment, using only 0.25 ml of protein solution.

Crystals of NGF grow by precipitating the protein with 40-50% ethanol by volume. They reach their final size in 2-3 days. The crystals are hexagonal bipyramids, up to 0.7 mm long and 0.25 mm across (Fig. 1). The appearance of the crystals depends on the heterogeneity of NGF at its COOH-termini. Crystals grown from preparations containing larger proportions of $\beta^4$ and $\beta^4$ NGF have more rounded edges than those grown from preparations containing mostly $\beta^1$ and $\beta^2$ NGF, but are otherwise the same. NGF crystals can be transferred directly into the solution in the bottom of the cell (the precipitant solution containing no protein) without any ill effects. The attempts to seed the droplets of protein solution before the vapor diffusion was begun were unsuccessful, resulting in a large number of imperfect, fused crystals.

**Biochemical Characterization of the Crystals.** Several crystals were dried of mother liquor, dissolved in 400 $\mu$l of 0.2% acetic acid, and extensively dialyzed against the same solution to remove all ethanol and phosphate. Standard bioassay (14) revealed that the protein from dissolved crystals had maximum biological activity at 10 ng/ml, identical to that found with $\beta$NGF. Isoelectric focusing analysis in acrylamide gel (15) of dissolved crystals showed that the proportions of the three NGF species ($\beta^1$, $\beta^2$, and $\beta^4$) were the same as in the starting material, thus revealing that the presence or absence of COOH-terminal arginine is not crucial for crystallization.

**Characterization of the Crystals.** X-ray photographs of NGF crystals were taken in room temperature, using a copper fine-focus tube (40 kV, 30 mA) as a source, on a Nonius precession camera. Crystals diffract to at least 2.7 Å resolution (Fig. 2) and are stable in the x-ray beam for up to 150 hr. The unit cell is hexagonal, with the lattice dimensions $a = b = 56.1$ Å, $c = 181.4$ Å, and $V = 494,400$ Å$^3$. On the basis of the condition $l = 6n$ for the presence of 00l reflections, the space group can be assigned as P6$_2$22 or its enantiomorph P6$_2$22 (to the observed resolution). The unit cell consists of twelve asymmetric units with a volume of 41,200 Å$^3$ each. If each asymmetric unit contained one NGF molecule of molecular weight 26,900, the value of Matthews' parameter $V_M$ (ratio of asymmetric unit volume to molecular weight of protein) would be 1.55 Å$^3$/dalton, while for the situation when each subunit would constitute the asymmetric unit, $V_M$ would be 3.1 Å$^3$/dalton. The first of these numbers is just outside the lower limit of values observed for 116 crystal forms (16), while the second is near the upper limit, but still within the reported range. In order to resolve this ambiguity, we measured the density of NGF crystals. The density measurements were made after crosslinking the crystals with 0.5% glutaraldehyde and transferring them to water. Matthews (17) found that this procedure does not appreciably change the calculated molecular weight of protein. The measurements were done on a xylene-carbon tetrachloride density gradient column and yielded the value of $\rho = 1.17 \pm 0.01$ g/cm$^3$. Crosslinked crystals diffract well, and both the appearance of the diffraction pattern and the unit cell parameters remain unchanged. The molecular weight of the protein in the asymmetric unit was calculated from the expression $M_p = NV(D_e - D_o)/n(1 - \bar{v}_pD_o)$, where $N$ is Avogadro's
number, $V$ the volume of unit cell, $D_u$ is density of crystals, $D_w$ is density of water, $n$ is the number of asymmetric units, and $\rho_p$ is partial specific volume of protein (17). Calculation of $\rho_p$ from the known amino-acid composition of NGF yields the value of $\rho_p = 0.725$, and $M_p$ can be calculated to be 15,300, much closer to the molecular weight of a NGF monomer than dimer. The weight fraction of protein in the unit cell can be calculated as 0.45. In view of these data, we conclude that in this crystal form the asymmetric unit contains a monomer of NGF.

DISCUSSION

Since each unit cell contains six molecules of NGF dimer in twelve asymmetric units, each dimer must occupy crystallographic special position with the monomers related by a 2-fold axis. The diffraction pattern clearly extends to 2.7 Å, and we suspect that this limit is imposed by the small crystal size and relatively low power of the conventional x-ray source, rather than by internal disorder. It may be concluded that the presence or absence of the COOH-terminal arginine (and even NH$_2$-terminal octapeptide) does not appreciably change the conformation of the rest of the molecule, at least to the observed resolution. This result is particularly interesting since the removal of COOH-terminal arginine markedly changes the binding of $\beta$NGF to the $\gamma$ subunit of the 7S complex. It follows that the change in binding properties must be due to facts other than simple conformational change in $\beta$NGF.

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