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REVIEW

Nerve growth factor: Structure/function relationships

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Abstract

Nerve growth factor (NGF), which has a tertiary structure based on a cluster of 3 cystine disulfides and 2 very extended, but distorted β-hairpins, is the prototype of a larger family of neurotrophins. Prior to the availability of cloning techniques, the mouse submandibular gland was the richest source of NGF and provided sufficient material to enable its biochemical characterization. It binds as a dimer to at least 2 cell-surface receptor types expressed in a variety of neuronal and non-neuronal cells. Residues involved in these interactions and in the maintenance of tertiary and quaternary structure have been identified by chemical modification and site-directed mutagenesis, and this information can be related to their location in the 3-dimensional structure. For example, interactions between aromatic residues contribute to the stability of the NGF dimer, and specific surface lysine residues participate in receptor contacts. The conclusion from these studies is that receptor interactions involve broad surface regions, which may be composed of residues from both protomers in the dimer.

Keywords: nerve growth factor; neurotrophins; structure/function relationships

The elucidation of the structure/function relationships of a protein requires a precise knowledge of the 3-dimensional structure and the identification of the residues that participate in biological activity. The latter were traditionally defined by chemical modification, which has now been supplanted, to a large degree, by site-directed mutagenesis, primarily because it offers the possibility of making substitutions anywhere in the molecule. However, despite its limitations, chemical modification does offer 1 major advantage: it is carried out on the intact protein and does not generally require refolding, a problem that has hampered the preparation of many recombinant derivatives. As is often the case, both approaches used in concert may provide much more information than either alone.

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Abbreviations: NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; NT, neurotrophin; *Trk*, tyrosine kinase-containing receptor family for the neurotrophins; LNGFR, low molecular weight NGF receptor (also called p75); NBS, *N*-bromosuccinimide.

Mouse nerve growth factor provides an interesting illustration of this point. The amino acid sequence of the protein was determined more than 20 years ago (Angeletti & Bradshaw, 1971), but the 3-dimensional structure, elucidated by single-crystal X-ray crystallography, has only recently been achieved (McDonald et al., 1991). In the intervening period, many chemical modifications were carried out, with a variety of objectives (see Bradshaw et al., 1977; Server & Shooter, 1977; Bradshaw, 1978; Greene & Shooter 1980; Ebendal, 1992). Results from these modification experiments provided a modest description of the structural features of the molecule that are important for biological activity. In the past few years, this has been extended by site-directed mutagenesis studies (Ibáñez et al., 1990, 1991, 1992, 1993). Interestingly, during the same period, there have also been substantial advances in our understanding of the function of NGF and the nature of its interactions with cell surface receptors (Ebendal, 1992; Meakin & Shooter, 1992; Altin & Bradshaw, 1993; Raffioni et al., 1993). Although we are still short of a complete description of the structure/function relationships, a reexamination and, in some cases, a reinterpretation of the chemical modification data provide some new insights into the molecular features that, along with the newer studies, allow predictions about how this prototypical neurotrophic factor interacts with its receptor.

Properties of NGF

Molecular characterization

The mature form of NGF was first purified to homogeneity from mouse submandibular glands and was designated 2.5S (Bocchini & Angeletti, 1969) (to distinguish it from the higher molecular weight form, 7S) (Varon et al., 1967). Its activity had been identified in tumor tissues (Levi-Montalcini & Hamburger, 1951) and various snake venoms (Cohen, 1959) prior to the identification of the mouse submandibular gland source (Cohen, 1960) and has since been identified in a wide variety of tissues (Barde, 1990). The mouse protein exists (in submandibular tissue) as a complex composed of 3 types of polypeptides, designated α, β, and γ (Varon et al., 1967), and 1-2 g atoms of zinc ion (Pattison & Dunn, 1975). This 7S complex has not been shown to occur in other mouse tissues, or in other species, probably because the α and γ subunits, which are members of a larger glandular kallikrein family (Evans et al., 1987), apparently have a very restricted expression. The α and γ subunits, members of the serine proteinase family, have quite similar amino acid sequences, although only the γ subunit is catalytically active (Thomas et al., 1981; Isackson et al., 1984). The α subunit has undergone several mutations that preclude this function, changes that have been introduced perhaps to allow specific interactions with the β subunit. Based on other serine proteinases of known 3-dimensional structure (such as pancreatic kallikrein) (Chen & Bode, 1983) models have been built (Bax et al., 1993; Blaber et al., 1993) and preliminary structures have been determined directly from X-ray crystallography (B. Bax, J. Murray-Rust, & T.L. Blundell, unpubl. data). Although the nature of the α-β interaction is presently unknown, several lines of evidence suggest that the γ subunit interacts with the carboxyl-terminal sequence of the β subunit through its catalytic center (see Server & Shooter, 1977). It has been further suggested that it cleaves the C-terminal dipeptide sequence from the β subunit and then remains associated as an enzyme product complex (with the carboxyl-terminal arginine of the β subunit occupying the P1 subsite of the γ subunit) (Mobley et al., 1976). Site-directed mutagenesis has shown that additional contacts apparently occur with other residues, particularly in the carboxyl-terminal portion of the γ subunit and, as yet, unidentified residues on the β polypeptide chain (Blaber et al., 1993).

The tightly associated β dimer ($>10^{13}$ M) (Bothwell & Shooter, 1977) is the active principle in all known biological functions of this hormone. (The α and γ subunits actually inhibit β-NGF action and must be dissociated for biological activity to be observed [Server & Shooter, 1977].) The initial molecular characterization of the β subunit of mouse NGF established the subunit organization (Angeletti et al., 1971; Greene et al., 1971) and the amino acid sequence of the protomer (Angeletti & Bradshaw, 1971). The longest isolated polypeptide contained 118 amino acids with 3 intrachain disulfide bonds, but shorter chains, truncated at both termini, were also identified (Bradshaw, 1978). These result principally from the removal of the amino-terminal octapeptide (by another kallikrein present in the submandibular tissue) (Angeletti et al., 1973; Mobley et al., 1976) and the C-terminal arginine residue (by a carboxypeptidase B-like enzyme), which also has the effect of eliminating β-γ interactions (Moore et al., 1974; Silverman & Bradshaw, 1982).

These cleavages have been reported to be without effect on the biological activity of NGF (Mobley et al., 1976), although this has not been systematically tested with defined compositions, and recent studies suggest that some receptor interactions do occur with the N-terminal region (Kahle et al., 1992; Ibáñez et al., 1993). Luo and Neet (1992) have also reported that preparations of recombinant β-NGF prepared in a baculovirus expression system can occur in 2 apparent conformers, one which shows normal biological activity and one which is 30-fold less active. Such preparations contain the carboxyl-terminal dipeptide that is removed from mouse submandibular gland β-NGF preparations. When this dipeptide is subsequently removed, both conformers apparently adopt full biological activity.

The isolation of mouse and human cDNA and genomic clones for NGF established, not surprisingly, that the β-protomer is formed from a larger precursor by limited proteolysis (Scott et al., 1983; Ullrich et al., 1983). In addition to the C-terminal dipeptide removed from the mouse submandibular gland protein (but probably not from any other species of NGF and possibly not from NGF synthesized in any other tissues in the mouse), a substantial amino-terminal pre/pro sequence is also cleaved at a characteristic dibasic site that yields the mature protein. As with most other hormones and growth factors, there is no known function for the material that is excised during processing of the precursor and there is considerable variation in size and sequence of this entity from species to species.

Subsequent cloning experiments have identified additional sequences, making a total of 10 NGF proteins now known (mouse, human, beef, chicken, rat, cobra, African rat, guinea pig, African toad [*Xenopus*], and platyfish [*Xiphophorus maculatus*]). As expected for such a homologous family, there are extensive sequence similarities (Fig. 1), with the higher vertebrates showing a closer similarity to each other and some greater variation occurring in the more distantly related species. As depicted in Figure 1, the regions of sequence similarity and variation are clustered, indicating probable regions of functional or structural importance. It should be noted that, to the extent tested, all species of NGF isolated to date show at least some crossreactivity in biological assays. This clearly emphasizes a retention of 3-dimensional structure (in all regions required for function) as well as a strong conservation of receptor binding sites. The importance of these regions and their potential role in the bioactivity of the molecule was accurately presaged by early sequence comparisons prior to the determination of the 3-dimensional structure (Meier et al., 1986).

Three-dimensional structure

The determination of the 3-dimensional structure of the mature NGF molecule has recently been reported by McDonald et al. (1991). Previous spectroscopic measurements had correctly suggested that the molecule is rich in β-pleated strands (Williams et al., 1982). Indeed, the protomer contains 2 pairs of antiparallel β-pleated strands in an elongated structure. Three loops containing β-hairpin structures are found at one end of the molecule, whereas the opposite end contains a loop structure characterized by reverse turns and all 3 intrachain disulfide bonds. Neither the amino (residues 1-11) nor the carboxyl termini (residues 112-118) were defined in this study. As shown in Figure 2, the 2 protomers are oriented head-to-head in the dimer, with an extensive subunit interface contributed largely by residues located in 2 extended segments of β-pleated structure that make up the body of the molecule. The interface interactions are

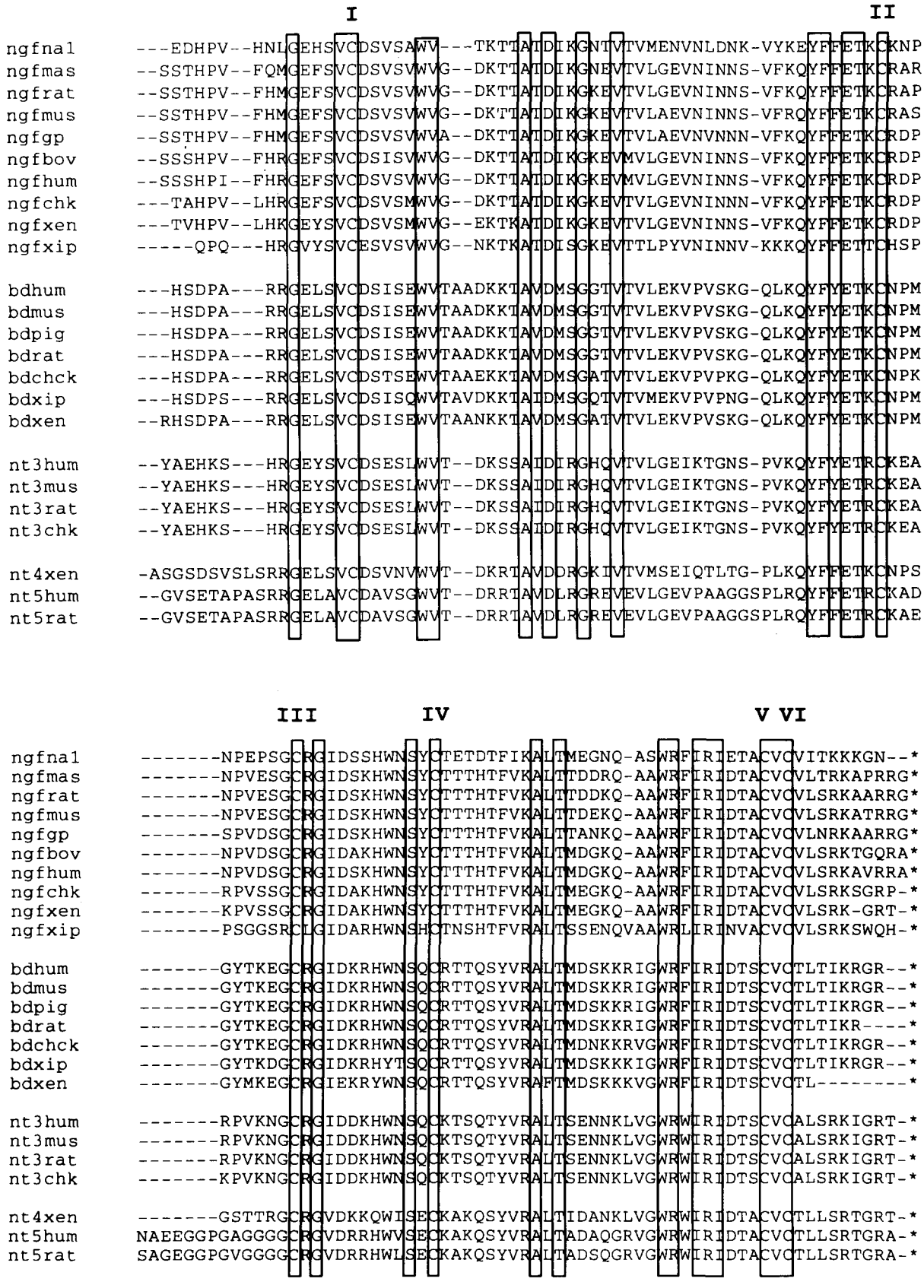

Fig. 1. Sequence alignment of neurotrophins. Data extracted from the OWL database version 23.0 (Bleasby & Wotton, 1990). Residues conserved through all the sequences listed here are boxed; the Cys residues which form the disulfide bond cluster are numbered I–VI. Sequence codes and literature citations for the neurotrophins are provided on the Diskette Appendix.

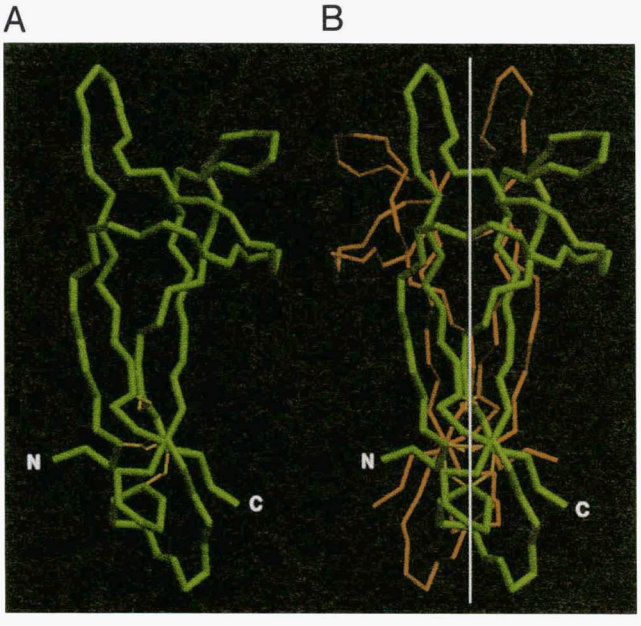

Fig. 2. A: The NGF protomer. **B:** The NGF dimer is formed from 2 parallel protomers which are in contact around an exact (crystallographic) 2-fold axis. The Cα trace of one protomer is shown in green, and the other much thinner in orange; this convention is maintained through the following illustrations. The direction of the 2-fold axis is approximately indicated by the vertical white line.

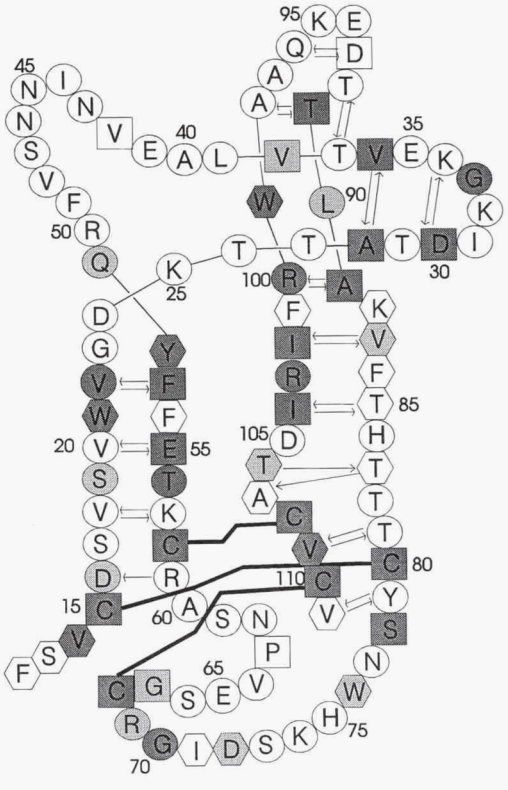

Fig. 3. Schematic of NGF showing location of conserved residues and other residues important to this text. Residues totally conserved on all the neurotrophins of Figure 1 are darkly shaded. Those conserved in all but 1 of the sequences of Figure 1 are shown lightly shaded. Residues in rectangles have buried side chains (accessibility <10%) in the protomer; residues in hexagons are those whose side-chain accessibility changes markedly on dimer formation, although the side chain may not be defined as "buried" using the 10% criterion.

largely hydrophobic and are consistent with the tight noncovalent association that characterizes the β dimer (Bothwell & Shooter, 1977). Dimer formation may be essential for biological activity because no defined monomer that retains biological activity has been prepared. Frazier et al. (1973a) reported the preparation of insolubilized derivatives of NGF, attached to Sepharose beads by cyanogen bromide in the presence of 6 M guanidine-hydrochloride, suggesting the possibility of an active monomeric species; however, the chemical nature of these derivatives did not allow the quaternary structure of the active species to be determined directly. The interface residues, which are indicated schematically in Figure 3, and in stereo projections in Figure 4, involve most of the aromatic residues of the molecule including the 3 tryptophans and 1 of the 2 tyrosines, which have been the targets of extensive modification studies (see below). The remainder of the contacts are provided by aliphatic amino acids. Interestingly, most of these contain β-branched side chains.

As shown in Figure 5, the charged amino acids are relatively evenly distributed throughout the sequence (but not in the 3-dimensional structure; McDonald et al., 1991). None is apparently involved in subunit interactions. Two of the 3 loops found in the upper section of the molecule (in the orientation shown in Figs. 2, 4) are relatively rich in basic residues, although they do contain several acidic residues as well, and site-directed mutagenesis experiments have pinpointed this region as a major contributor to the binding of the low-affinity (p75) receptor. It may contribute to the binding of *Trk*A as well (see below).

Families and superfamilies

NGF is a member of a larger family of proteins, termed neurotrophins, that are characterized by substantial sequence identity (Bradshaw et al., 1993) (Fig. 1). In addition to NGF, 4 other neurotrophin molecules have been identified by either activity or cloning techniques. The first of these homologs to be characterized was brain-derived neurotrophic factor, a molecule that is mainly located in the brain with targets in both the central and peripheral nervous systems (Barde, 1990). Its sequence is more than 50% identical to that of NGF (depending upon species) and is presumed to have an identical disulfide pairing pattern as that determined for mouse NGF (Angeletti et al., 1973). The remaining 3 neurotrophins (NT3, NT4, and NT5) were initially identified from cloning experiments and have been subsequently characterized utilizing recombinant material (Ernfors et al., 1990; Hohn et al., 1990; Maisonpierre et al., 1990; Rosenthal et al., 1990; Berkemeier et al., 1991; Hallböök et al., 1991). All show similarity to NGF and BDNF. It should be noted that NT4 was initially found in toad (*Xenopus*) ovary and may represent only a species variation of NT5, which has been characterized in higher vertebrates (Ip et al., 1992). If this turns out to be the case, the number of unique known neurotrophins in any species is reduced to 4. For this reason, the terminology NT4/5 has been adopted.

A comparison of the amino acid sequences of the neurotrophins gives an alternative view with respect to structural conservation and maintenance of functionality (Fig. 1). Although

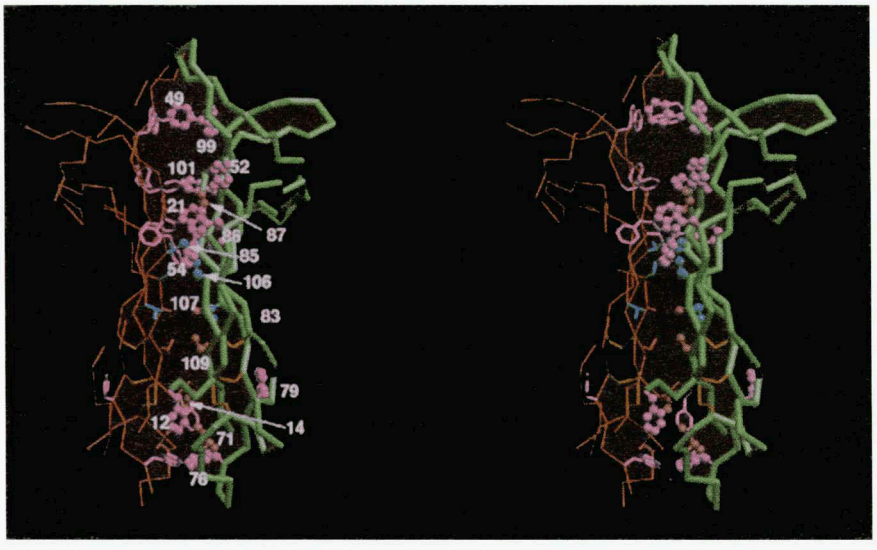

Fig. 4. Stereo view of the dimer interface residues. Aromatic side chains (magenta) intercalate at the dimer interface; other residues buried on dimer formation are shown in cyan (Thr, Ser) and brown (Ala, Leu). In this and the following 2 stereo pictures, side chains belonging to the green main chain are drawn in a ball-and-stick representation and labeled, whereas those of the orange protomer are drawn as wireframe and unlabeled.

all of the molecules display similar *types* of biological activity, crossreaction with the receptors thought to be responsible for biological activity, the *Trk* family, is limited (all of the neurotrophins interact with the p75 receptor with an affinity of 10^{-9} M). Thus, for example, some residues important for the interaction of NGF with *Trk*A must differ from those that characterize BDNF high-affinity receptor interactions with *Trk*B. The residues that are rigorously conserved, on the other hand, might be expected to play primarily a structural role in maintaining the proper conformation of the molecule. It is interesting to note that the residues involved in the putative dimer interface are usually, but not exclusively, conserved in the 4 molecules (Figs. 1, 3); for example, Phe 49 in human NGF is replaced by either leucine or valine in BDNF, NT3, and NT4/5. The degree of conservation of chemical character allows us to assume that the subunit interfaces of these 4 molecules will be largely similar. Importantly in this regard, Radziejewski et al. (1992) have recently demonstrated that recombinant BDNF and NT3 are dimeric.

Recently, the structures of 2 additional growth factors, transforming growth factor-β2 (TGF-β2) and platelet-derived growth factor-BB (PDGF-BB) (Daopin et al., 1992; Oefner et al., 1992; Schlunegger & Grütter, 1992), have been determined, revealing a common structural motif with NGF involving the 3 intrachain disulfides (identically paired in all 3 molecules) and 4 segments of antiparallel β-strands (Murray-Rust et al., 1993). Interestingly, the quaternary structures, although all dimeric, are entirely different in organization and intersubunit bonding.

Functional characterization

The recognition that NGF and, by analogy, other growth factors were basically hormonal in function (Frazier et al., 1972) led directly to the identification of cell-surface receptors on target cells. As with other endocrine systems, these receptors were first characterized with respect to their binding properties using radiolabeled hormones, or tracers, followed by more detailed

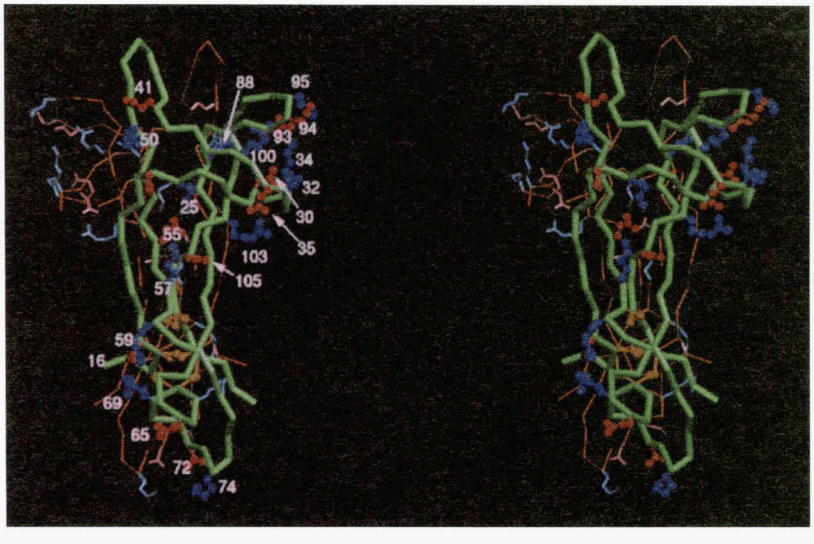

Fig. 5. Stereo view of the location of the charged residues Asp, Glu (red), Arg, Lys (blue) in the NGF dimer.

molecular characterization (see Raffioni et al., 1993). In 1979, Sutter et al. provided a definitive description of NGF binding to responsive neurons, demonstrating the presence of both high- and low-affinity interactions. They differed by about 2 orders of magnitude in their dissociation constants. These binding sites have been subsequently referred to as high (or slow) and low (or fast) to reflect their affinity (or the difference in the dissociation rate of bound ligand). A large body of evidence clearly supports the view that high-affinity binding is required for biological activity (neurite outgrowth) including the ability of NGF to be internalized by receptor-mediated endocytosis (see Meakin & Shooter, 1992). However, determining the molecular components that are responsible for these binding activities has proved to be somewhat more difficult and has not yet been completely resolved (Chao, 1992).

By a combination of isolation, chemical identification (crosslinking), and cloning, it is now clear that there are at least 2 membrane-bound proteins capable of binding NGF specifically. The first of these to be cloned and sequenced was the low-molecular weight NGF receptor, now designated as p75. This molecule has been defined in 3 species (rat, human, and chicken) (Johnson et al., 1986; Radeke et al., 1987; Large et al., 1989) and possesses a molecular weight in the range of 60–70 kDa. (The molecular weight calculated from the amino acid sequence predicted from the cDNA sequence is, in all cases, less than 50 kDa, with the remainder being attributed to both O- and N-linked carbohydrates.) The external domain shows significant relatedness to several lymphocyte proteins and the tumor necrosis factor receptor (see Raffioni et al., 1993). A number of cell lines that contain only this receptor clearly establish that it does not transduce signals that lead to differentiation and neurite proliferation (Meakin & Shooter, 1992); however, recent evidence has linked it to the control of programmed cell death (apoptosis) (Rabizadeh et al., 1993). The second receptor entity, with a molecular weight of 140 kDa, has been defined more recently and corresponds to the protooncogene *Trk*. This molecule was shown to bind NGF specifically (Kaplan et al., 1991; Klein et al., 1991a), and with an affinity of at least 10^{-9} M and is now commonly referred to as *Trk*A. The binding of NGF induces ligand-dependent tyrosine phosphorylation (of both the receptor and internal proteins) and is clearly the predominant (if not sole) signal generator for the biological responses of this factor in responsive neurons.

Two additional receptors, related to *Trk*A and designated *Trk*B and *Trk*C, have been identified as the principal receptors for BDNF and NT3, respectively (Klein et al., 1991b; Lamballe et al., 1991; Squinto et al., 1991). *Trk*B also binds NT3 and NT4/5, making it the least discriminating of the 3 *Trk*-type molecules. *Trk*A has been reported to bind NT3, and NT4/5 (but apparently not BDNF). However, a recent study (Ip et al., 1993) has determined that the "preferred" ligands of *Trk*A, B, and C are NGF, BDNF, and NT4/5, and NT3 and that the other reported interaction, e.g., NT3 to *Trk*A, are unlikely to be physiologically relevant. It should be noted, that although these receptors are clearly distributed through naturally occurring neuronal target cells, the specificities of ligand binding have been, for the most part, determined on recombinant receptors expressed in cultured non-neuronal cells. In fact, it has been pointed out that sensory neurons, which express all 3 *Trk* molecules, bind individual neurotrophins with a much higher degree of specificity than is exhibited in the recombinant experiments (Meakin & Shooter, 1992). The reason for this is presently unknown.

Modifications of NGF

Overview

Many chemical modifications of NGF have been carried out, some of which have led to extensive changes in groups of residues, whereas others have been much more selective. Because these studies were carried out for different reasons, the degree of characterization of the derivatives has been rather variable. Although in some cases this limits the possible interpretations with respect to structure/function relationships, there is a degree of consistency in the findings, particularly as they relate to the now known 3-dimensional structure. In the main, they indicate that the charged residues are not involved in subunit interactions but do play some role in receptor interaction. To the extent that the modification of aromatic residues affects activity, these changes may be related to alterations either in conformation and/or subunit interaction and are not likely to be directly involved in binding with either receptor type.

It should be noted that none of the derivatives or mutant neurotrophins mentioned here have been characterized by structure determination, apart from the recent determination of the structure of bis-des-octa(1–8) mouse NGF (Holland et al., 1994). This confirmed the general topology and flexibility of the molecule and also showed a zinc-binding site involving His 84 and Asp 105.

Charged residues

Lysine

Mouse NGF contains 8 lysine residues, 4 of which are clustered at the "top" of the molecule (when oriented as in Fig. 5). Of these 4, Lys 32 is conserved in all NGFs except platyfish, and Lys 34 in all NGFs except snake and mastomys, but neither is conserved throughout the neurotrophins. Lys 95 is not well conserved, but 88 is lysine or arginine in all neurotrophins. Lys 25, conserved in all neurotrophins except NT-5, where it is arginine, forms a hydrogen bond to the buried Glu 55, which is conserved in all neurotrophins and mutation of either of these residues, which prevented hydrogen bond formation, might be expected to destabilize the structure. The remaining 3 lysines at 57, 74, and 115 are well conserved in the neurotrophin family. Lys 115 is not defined in the crystal structure.

Several procedures leading to extensive modification of lysine residues have produced stable and active derivatives, clearly suggesting that most of the residues of this type are not required for the biological function of the molecule. These include studies in which the NGF β subunit dimer has been crosslinked with dimethylsuberimidate (Stach & Shooter, 1974; Pulliam et al., 1975) and coupled to Sepharose particles through reaction with CNBr (at pH 6.4) (Frazier et al., 1973a). In the crosslinking experiments, greater than half of the lysine residues were substituted, whereas an average of 1 lysine residue was modified in the coupling to the Sepharose beads. (When the reaction was carried out in 6 M guanidine hydrochloride, the number of crosslinks introduced and hence the number of lysines modified were not determined.) Importantly, all of these derivatives were fully

active in appropriate assays using dorsal root sensory (DRS) neurons.

A more complete modification of the lysine residues was carried out by Silverman (1977), who effected the conversion of 93% of the lysine residues to the corresponding homoarginine derivatives by reaction (for 96 h) with 1-guanyl-3-5-dimethylpyrazole. This represents modification of nearly 7.5 of the 8 lysine residues with no significant decrease in the biological activity as measured with the same test (DRS) cells. Similar results were obtained for the reaction of NGF with succinic anhydride, in which extensive conversion of the lysine residues to the corresponding acylated derivatives also did not impair the biological response (Bradshaw et al., 1977). Importantly, these derivatives also retained their dimeric structure.

These results contrast in some degree to the experiments of Levi et al. (1980) and Rosenberg et al. (1986), who found the attachment of rhodamine and biotin derivatives, respectively, to amino groups resulted in modified NGF proteins that showed dramatically decreased binding potential to PC12 cells, an established rat pheochromocytoma cell line used extensively for the study of NGF and other neurotrophic factors because of the reversible differentiation that can be induced (Greene & Tischler, 1976). Interestingly, and importantly in light of subsequent findings, neither group appeared to investigate the biological response, i.e., the ability of the NGF derivative to produce neurites, for these N-modified derivatives.

More recently, a detailed study on the substitution of individual lysine residues by Ibáñez et al. (1992) has established that a subgroup of lysine residues occurring at positions 32, 34, and 95 are apparently involved in interactions with p75. These conclusions were based on experiments in which substitutions were introduced at individual residues and in various combinations. The derivatives were expressed and examined for their receptor binding properties on cells bearing only p75, *Trk*A, or both, and for their ability to induce neurite differentiation in PC12 cells and responsive neurons. Substitution of Lys 32 or Lys 34 with alanine (K32A, K34A) resulted in derivatives that were fully active but showed substantially reduced binding to PC12 cells. A mutant in which both of these lysine residues, as well as Glu 35 (E35A), were converted to alanines yielded a derivative with greater than 99% loss in receptor binding but only a 35% loss in biological activity. These same derivatives showed similar binding profiles with A875 human melanoma cells, which express only p75. However, the 2 single mutations showed little or no decrease in binding to a 3T3 cell expressing recombinant rat *Trk*A receptor protein, whereas the triple mutant showed a loss of about 50% binding to the same cell. The mutation of Lys 95 (K95A), which occurs on a different loop in NGF (than Lys 32 and Lys 34), but is in close juxtaposition in the 3-dimensional space (see Fig. 5), also affects binding to PC12 and A875 cells but shows a considerably reduced loss of binding to the 3T3 cells expressing *Trk*A. A double mutation (K32A/K95A) showed essentially a complete loss of binding to the A875 cells, while retaining some 40% ability to interact with the *Trk*-producing cells. A similar result was found for the mutant (K32A/K34A/E35A/K95A), in which the 3 lysine residues and the glutamic acid residue were all converted to alanine. Importantly, these latter multiple site mutants maintain significant biological activity in responsive cells.

These results clearly indicate that modification of at least 3 of the 8 lysine residues in NGF materially affects its ability to bind to p75 without altering biological activity and provide an explanation for the dramatically decreased binding observed with the rhodamine and biotin derivatives (Levi et al., 1980; Rosenberg et al., 1986), while at the same time supporting the view that the modification of lysine residues has significantly less effect on biological responses, presumably mediated by *Trk*A interactions (Bradshaw et al., 1977). It should be pointed out that the site-directed mutants of NGF have not been rigorously characterized with respect to either conformation or quaternary structure. However, their retention of biological activity strongly argues that neither of these properties are materially altered relative to the native protein. It is also important to stress that these findings provide the strongest evidence to date that p75 interactions are not required to induce the commonly measured neu rotrophic responses in target cells, albeit, they do not eliminate that such interactions occur and that they may have specifying influences that have not been detected in the assays utilized in these experiments (Meakin & Shooter, 1992).

Arginine residues

Mouse NGF contains 7 arginine residues that, as with the lysine residues, are generally found on the surface of the molecule (see Fig. 5). They are generally well preserved in the NGF family (Fig. 1). In the mouse NGF structure, Arg 69 is H bonded to Asp 16, and both of these are conserved in the family except for Leu 69 and Glu 16 in platyfish NGF. Similarly, Arg 100 is totally conserved and forms a buried hydrogen bond to the conserved Thr 91. Modification of these residues is therefore likely to be difficult and to have major structural consequences. Reaction of proteins with cyclohexanedione results in the conversion of arginine residues to the *N*7,*N*8-(1,2-dihydroxycyclohex-1,2-xyline) derivative. The reaction can be reversed by treatment with hydroxylamine, which allows for the detection of irreversibly denatured molecules. The modification of NGF with this reagent indicated that 5 arginines were readily modified at rates comparable to that observed for the modification of free arginine (Silverman, 1977). These derivatives were still largely active in assays using sensory neurons and this modification was fully reversible by treatment with hydroxylamine. However, modification of the last 2 arginine residues was accompanied by a precipitous loss in biological activity that could only be partially reversed, suggesting that 2 of these residues are less than completely available and that their modification probably generally disrupts the integrity of the molecule. The effect of modifying the last 2 arginine residues with this reagent on conformation and quaternary structure was not measured due to the decrease in solubility of the fully modified protein. These studies also did not determine whether inactivation was due to the modification of 2 specific arginine residues, although the kinetic analysis of the course of the reaction was consistent with this view. Examination of the crystal structure (McDonald et al., 1991) suggests that the 2 resistant arginines are at positions 69 and 100. These side chains are involved in internal H bond interactions to Asp 16 and Thr 91, respectively.

Two specific arginines (100 and 103) have been examined by site-directed mutagenesis (Ibáñez et al., 1990). The structural role of Arg 100 has already been described; Arg 103 is conserved in all neurotrophins. Its side-chain hydrogen bonds to the carbonyl of Asp 30, and this may again be important for structural integrity. When Arg 100 was replaced by lysine (R100K), there was no effect either on receptor binding or biological activity,

indicating that this conservative substitution was well-tolerated (despite the H bond to Thr 91). However, when both residues were replaced by glycine (R100G and R103G), receptor binding and biological activity were reduced over 90%. When Arg 100 was replaced by phenylalanine (R100F), the amount of the mutant synthesized was dramatically reduced and could not be rigorously characterized. None of these derivatives were examined with respect to either conformation or quaternary structure. These findings are consistent with the view that arginine residues play a structural role but are not directly involved in receptor interactions.

Histidine

Mouse NGF contains 4 histidine residues, 2 of which are found in the first 8 residues and are removed with the excision of the N-terminal octapeptide (Angeletti et al., 1973). The conformation of this region cannot be assessed because this part of the molecule was not visible in the crystal structure (McDonald et al., 1991). The remaining 2 histidine residues are located at positions 75 and 84 in the mouse protein (Fig. 6). His 75 is conserved in all neurotrophins except *Xenopus* NT (where it is glutamine). In mouse NGF, the side chain of His 75 is hydrogen bonded to the totally conserved Asp 72; this may assist in stabilizing the conformation of this region, but is on the surface of the molecule and mutations should not destabilize the overall fold greatly. His 84 is conserved in all NGFs except snake, but is glutamine in all other neurotrophins. Modification of NGF with diethylpyrocarbonate resulted in the quantitative modification of the histidine residues with a concomitant loss of binding to rabbit superior cervical ganglia (Dunbar et al., 1984). No significant conformational change accompanied the conversion, as judged by fluorescence spectroscopy, suggesting that the quaternary structure was also not altered (although this was not established directly). The modifications were also readily reversed by hydroxylamine, indicating that irreversible denaturation did not accompany the reaction. These results suggest that one or both histidines may, in fact, be more directly involved in binding to either of the receptor molecules. Consistent with this view, His 84 has been shown to be part of the *Trk*A binding region in NGF, as has Gln 84 for binding of BDNF to *Trk*B (Ibáñez et al., 1993).

Carboxyl group modification

Mouse NGF contains 12 side-chain carboxyl groups evenly distributed between aspartic and glutamic acid moieties (Angeletti et al., 1971). There are 3 aspartic acid residues with known structural roles: Asp 16, whose side chain hydrogen bonds to Arg 69; Asp 72, whose side chain hydrogen bonds to His 75; and Asp 30, which is involved in stabilization of the 30–34 loop. One glutamic acid residue is similarly conserved because of its structural role: the side chain of Glu 55 hydrogen bonds to Lys 25. Neither type of group appears to contribute residues directly involved in receptor binding. The coupling of mouse NGF to either rhodamine or biotin derivatives using water-soluble carbodiimides resulted in derivatives that were both biologically active and bound to receptor entities (Levi et al., 1980; Rosenberg et al., 1986). In the rhodamine derivatives, 4–5 of the 12 carboxyl groups were modified, whereas the biotin derivative contained a minimum of 3 substitutions per NGF subunit. Although both derivatives left a substantial number of carboxyl groups unmodified, these studies have not indicated a crucial involvement of any of these residues in receptor binding.

Three carboxyl-containing side chains have been modified by site-directed mutagenesis (Ibáñez et al., 1990, 1992). These are Asp 24, Asp 30, and Glu 35. The substitution of alanine for Asp 24 (D24A) was without effect on either receptor binding or biological activity, although the mutant protein was poorly produced, suggesting that the mutation may have had some effect on stability. Substitution of Asp 30 with alanine (D30A) did not yield a characterizable mutant. However, the substitution of asparagine for this residue (D30N) produced a 75% decrease in both receptor binding and biological activity. The side chain of Asp 30 is involved in the stabilization of the 30–33 loop, which may explain this observation. Substitution of an alanine for Glu 35 (E35A) did not strongly affect either receptor binding or biological activity. However, the derivative was poorly expressed and several high-molecular weight polypeptides were observed suggesting that it may have an effect on the processing of the precursor of the protein.

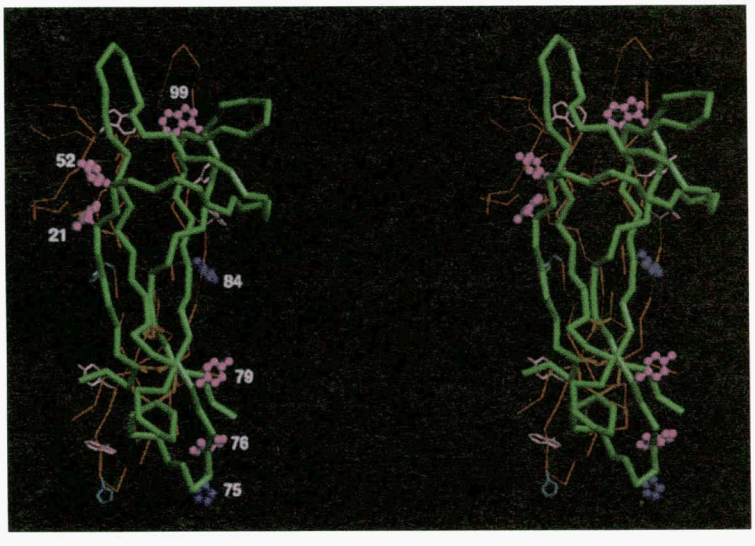

Fig. 6. Stereo view of the location of Tyr, Trp (magenta), and His (blue) residues in the NGF dimer.

Aromatic residues

Tyrosine

Mouse NGF contains 2 tyrosine residues that are well conserved in the 10 NGF proteins sequenced to date (Fig. 1). Tyr 52 is conserved in all the neurotrophins and is adjacent to the dimer interface, where it makes contact with Phe 101 of the other protomer. Tyr 79 is conserved in all the NGFs except platyfish but is glutamine or glutamic acid in the other neurotrophins. In the mouse NGF structure it packs against Val111 from the same protomer, but it could interact with the N-terminus of the second protomer; its environment is not fully determined in the crystal structure.

Frazier et al. (1973b) examined the role of these residues in biological activity by measurement of their physical properties and by chemical modification. Spectrophotometric titrations of native and denatured protein indicated that the average pK_a of the 2 tyrosine residues is about 1 pH unit higher in the native protein than is found in the denatured state (pK_a of 10.75 versus 9.76). These results indicate that the environment of the tyrosines in the native structure is such that their ionization is perturbed. Figure 6 indicates that Tyr 52 is involved in the aromatic packing at the subunit interface, and as mentioned above the environment of Tyr 76 is not completely defined. Adjacent charged residues may also contribute to this perturbation. Modification of the tyrosine residues with tetranitramethane to produce 3-nitrotyrosine also did not affect biological activity. The derivatives in which the tyrosine had been quantitatively converted to this derivative were shown to have no loss in tryptophan. Optical rotatory dispersion indicated some shift in the 220-nm trough, indicating that the fully nitrated derivative had undergone a conformational change, but the quaternary structure of this derivative was not examined. Several iodination studies indicate that the modification of 1 or both tyrosine residues in NGF yielded derivatives with full biological activity and receptor binding characteristics (Herrup & Shooter, 1973; Sutter et al., 1979; Tait et al., 1981). Ibáñez et al. (1990) conservatively substituted Tyr 51 of chick NGF (Tyr 52 in mouse numbering) with phenylalanine (Y51F), producing a derivative that retained full biological activity and receptor binding characteristics and was produced in reasonable yields. From its position in the 3-dimensional structure, such a substitution would be expected to be well tolerated and to have little effect on conformation or subunit interactions.

Tryptophan

Three tryptophan residues are all completely conserved in the neurotrophins, except for a tyrosine substitution in platyfish BDNF, and they are all involved in aromatic stacking at the dimer interface (Fig. 3). There is, however, an additional tryptophan residue in NT3 and NT4/5 at 101 (mouse NGF numbering scheme). In one of the most detailed studies on the effect of modifications on the NGF molecule, Frazier et al. (1973b) studied the properties of the 3 tryptophan residues of mouse NGF. In the first place, they established that these residues could be distinguished by the rate of *N*-bromosuccinimide oxidation, which occurred in a triphasic reaction. The results suggested that 1 tryptophan was completely oxidized before the second was modified, and, in turn, the second was largely derivatized before the third residue was modified. The rate of modification of the first tryptophan was the same as that of tryptophan model compounds. Titration of NGF with *N*-methylnicotinamide chloride, a compound that forms charge-transfer complexes with indole nuclei that are freely available to solvent and that are detectable in the visible spectrum (Deranleau et al., 1969), confirmed the presence of 1 solvent-available tryptophan residue. The charge-transfer titration was obliterated in derivatives in which a total of 1.0 tryptophan had been oxidized. This residue was identified as Trp 21, utilizing chemical modification with dimethyl (2-hydroxy-5-nitrobenzyl)-sulfonium bromide. Similarly, this reagent was also used to determine that Trp 99 was the second residue to be oxidized, thus making Trp 76 the least accessible to *N*-bromosuccinimide oxidation. Thus, oxidation of Trp 21 had no effect on the response of sensory neurons, whereas the oxidation of Trp 99 as well (a derivative in which 2 of the 3 tryptophans had been modified) produced a derivative that had lost all biological and immunological response. The derivative in which all 3 tryptophans had been oxidized was also inactive. Receptor binding experiments were not carried out with these derivatives. However, conformational analysis, as determined by optical rotatory dispersion, indicated that the derivative in which 3 tryptophan residues had been oxidized still maintained substantive native conformation as compared to NGF in 6 M guanidine hydrochloride. Importantly, the derivatives in which 2 tryptophan residues had been oxidized clearly retained their dimeric structure, whereas those with 3 residues of tryptophan oxidized showed sedimentation coefficients consistent with monomeric structures.

All 3 Trp residues are involved in the dimer interface, but they are not buried and have side-chain accessibilities of 42%, 33%, and 31% for Trp 21, Trp 76, and Trp 99, respectively. This is not in order with the Trp 21 > Trp 99 > Trp 76 order of oxidation, but the crystal structure shows that Trp 76 is close to the observed C-terminus (at residue 113) and it is possible that the following C-terminal residues further occlude it. Trp 21 is clearly the most accessible, consistent with the results of the chemical modification and charge-transfer titration experiments. Oxidation of Trp 99 might well be expected to cause distortion in the upper end of the molecule, perhaps causing some parting of the subunits of the dimer as reflected in the change in conformation seen in the optical rotatory dispersion spectra. The introduction of the polar oxindole ring into the Trp 76 side chain clearly is sufficient to disrupt the dimer interface, resulting in the formation of monomers. Nonetheless, these monomers are not grossly denatured. Because the oxidation of Trp 99 (and Trp 76) leads to loss in biological activity, the interaction with *Trk*A may be disrupted by the conformational changes that are induced by these modifications.

Cohen et al. (1980) also examined the tryptophans of mouse NGF and confirmed many of the findings of Frazier et al. (1973b). However, in contrast, they found oxidation of Trp 21 had a significant effect on biological activity, as determined by PC12 cells. A derivative, in which 0.9 tryptophans had been oxidized, had only 1.2% the affinity of the native molecule and a derivative with 2 oxindole residues did not compete with native NGF for receptor binding at all. Drinkwater et al. (1991) also reexamined this question using site-directed mutagenesis, substituting phenylalanine, leucine, and serine for Trp 21 (W21F, W21L, and W21S). W21S was produced at levels that were below detection, but both W21F and W21L were obtained in sufficient quantities for further testing. Interestingly, they found

full biological activity consistent, with the results of Frazier et al. (1973b). However, they also observed substantive decreases in binding to the p75 receptor, which would not have been detected in these earlier experiments. Although conformation and quaternary structure were not examined, it is likely that the changes introduced by these mutations will be reflected in alterations in the subunit interface, which suggest that p75 interactions are sensitive to 1 or both of these parameters.

Ibáñez et al. (1990) reached similar conclusions. Each tryptophan was substituted by phenylalanine and all derivatives showed biological activity and receptor binding, suggesting that replacement of Trp 99 or Trp 76 with phenylalanine, an aromatic residue, is tolerated in the native structure. This also supports the view that the inactivation after NBS oxidation results from the increase of polarity into the hydrophobic area surrounding Trp 99, causing subsequent conformational changes and perhaps a shift in subunit interactions, rather than the modification of receptor interactions per se.

Other modifications

There have been several other modifications of NGF that cast additional light on structure/function relationships. Murphy et al. (1989) have reported the isolation and characterization of a glycosylated form of NGF that occurs in mouse submandibular glands at a level of approximately 2% of the total protein. Characterization of this derivative established that it arises from glycosylation on Asn 45, which is indeed found in a consensus sequence for N-linked glycosylation (Marshall, 1972). When the glycosylated form was isolated to homogeneity and characterized with respect to functional properties, it was found to be active with sensory and sympathetic neurons and in dissociated neuronal cultures. They established that deglycosylation did not occur under the conditions that the bioassays were performed.

Several other point mutations have been introduced, as summarized in Table 1, mainly affecting the region from Ser 19 to Val 36. For the most part, the effects are on stability of the protein and are unlikely to be due to disruption in binding to either receptor (Ibáñez et al., 1990).

Longo et al. (1990) using synthetic peptides to block the activity of NGF, also suggested that the region from residues 28 to 38 is important for receptor interactions. However, shorter peptides derived from this region, including the tetrapeptide Lys-Gly-Lys-Glu and Val-Thr-Val, also showed similar activities. The inability of these same peptides to displace labeled NGF from its receptor raises substantial questions regarding the mechanism of the effect observed. It should be pointed out that these residues, which are also from the same region that was identified in the NGF/BDNF chimera experiments (Ibáñez et al., 1991; Suter et al., 1992), contain some of the same residues that appear to be involved in the interaction with p75 (without affecting *Trk* activities).

Table 1. *Additional modifications of rat βNGF by site-directed mutagenesis (taken from Ibáñez et al., 1992)*

	PC12 cell responses (% of wild type)	
Mutation	Binding	Activity
T26A	100	100
T27A	120	63
T29A	71	74
I31A	30	25
I31M	35	100
I31V	130	100
V36L	33	90

Chimeric neurotrophins

A different type of site-directed mutagenesis, involving the preparation of chimeric proteins in which segments of the homolog, BDNF, have been inserted into the NGF sequence as a means of ascertaining the residues determining the biological specificities of the 2 proteins has been reported from 2 different laboratories. Suter et al. (1992) prepared a series of 12 chimeras in which consecutive sequences of approximately 10 amino acids from BDNF were incorporated into the NGF protein. The expressed proteins were then assayed for their ability to respond in neurite outgrowth and survival assays. Interestingly, all of the chimeras retained NGF-like activity in the bioassays carried out with PC12 cells, embryonic chick dorsal root ganglia and sympathetic ganglia, all of which are normally responsive to NGF. Equally surprising was the fact that most of the chimeras also showed BDNF-like activity on nodose ganglia explants, neurons responsive to BDNF but not to NGF. In spite of being active in nodose ganglia explants, these chimeras were unable to promote the survival of dissociated nodose neurons in culture. Because these results failed to pinpoint either NGF- or BDNF-specific sequences, it was concluded that the overall conformation of the molecule is more important than short "active-site"-like elements.

In a similar approach, Ibáñez et al. (1991) constructed chimeric molecules based on structural analyses. They defined 5 variable regions of the 2 molecules (see Fig. 7) and inserted the corresponding sequences from BDNF into the NGF sequence. The proteins were expressed and assayed in a fashion similar to that used by Suter et al. (1992). Molecules in which 1 or more segments had been replaced were examined. One region, designated Ib, corresponding to residues 29–35, imparted some BDNF-like activity on nodose cells, although it retained NGF-like activity with sympathetic and dorsal root neurons. Substitution of segments III–V also produced a strong BDNF-like response while retaining significant activity against NGF-responsive neurons. Only the substitution of all 5 regions rendered the molecule indistinguishable from BDNF in these neurite outgrowth assays. Importantly, many of the combinations that acquired BDNF-like activity also retained strong NGF-like activity, indicating that hybrid molecules, able to clearly display both specificities, were readily devised.

Ibáñez et al. (1993) used chimeric and mutant molecules to probe for ligand–receptor interactions between NGF, BDNF, and their respective *Trk*A and *Trk*B receptors. The amino- and carboxy-terminal sequences were included in the analysis, as well as a number of point mutants, especially concentrated in variable region II (residues 41–49). The mutants were assayed for receptor binding and receptor activation, measured as tyrosine autophosphorylation, in fibroblasts ectopically expressing *Trk*A and *Trk*B receptors, as well as in biological assays using sympathetic and nodose neurons. The results of this study suggested

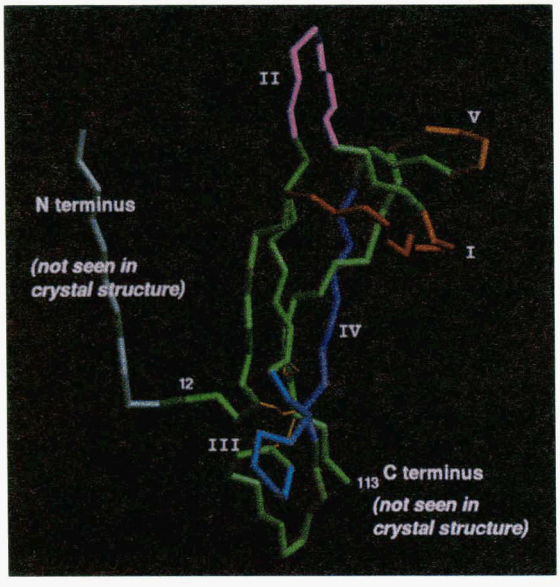

Fig. 7. The sequence variable regions of the neurotrophins mapped onto the 3-dimensional structure. Variable regions I–V are colored to distinguish them from each other and from the rest of the main chain, but otherwise the colors have no special significance. The N-terminus is not seen in the crystal structure but is included (pale blue) in an extended conformation here to indicate its size relative to the rest of the molecule.

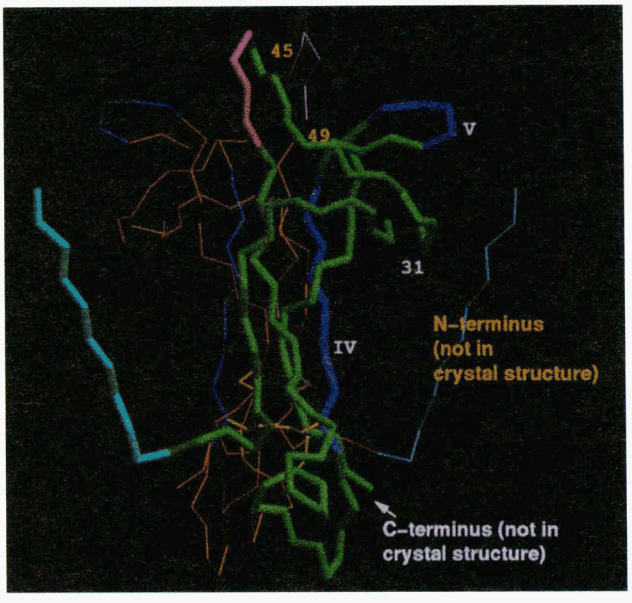

Fig. 8. Location of the *Trk*A binding residues of NGF on the dimer. White lettering refers to the green protomer and yellow lettering to the orange protomer; the proposed binding site involves residues from both protomers. Regions of the main chain whose residues are thought to be involved in *Trk* binding are colored so as to distinguish them from the rest of the main chains. If one looks from the "top" of the dimer molecule along the 2-fold axis, residues 31 to 34 and residues 95 to 97 from the first protomer, and residues 41 to 45 to 49 from the second protomer cluster on a continuous plane stretching across the top of the NGF dimer. Additional contacts may be provided in the middle region of the NGF dimer by residues 79, 81, and 84 from the first protomer, thereby extending the binding surface down to about three-quarters of the molecule. Residues from the N-terminus are known to be important also; although their exact location cannot be determined from this crystal structure, the extended main chain shown here indicates that this part of the molecule is capable of approaching the other *Trk*A binding residues quite closely.

that the interaction of neurotrophins with *Trk* receptors is mediated by multiple, although specific, contacts that cooperate synergistically for receptor binding and activation. In particular, amino acid residues shown to mediate the interaction of NGF with *Trk*A included residues 3–9 from the NH_2-terminus, Ile 31 from variable region I, Glu 41 and Asn 45 from region II, Tyr 79, Thr 81, and His 84 from region IV, and residues 94–98 from region V. In the 3-dimensional structure of NGF, these residues are grouped on 1 side of the NGF dimer, delineating a continuous surface extending approximately parallel to the 2-fold axis of the molecule. The proposed binding surface contains residues from both protomers (Fig. 8). The structural elements mediating the contact of BDNF with the *Trk*B receptor were, in general, localized in variable regions analogous to those involved in the interaction of NGF with *Trk*A.

Based on the functional information obtained with chimeric and mutant molecules, Ibáñez et al. (1993) reported, in the same study, the construction of a chimeric neurotrophin that displayed biochemical and biological properties from 3 different neurotrophins. This molecule, designated pan-neurotrophin-1 or PNT-1, efficiently activated the *Trk*A, B, and C receptors and displayed biological specificities on neurons characteristic of NGF, BDNF, and NT3, demonstrating that the structure/function information obtained can be used for the engineering of molecules with broader or novel biological specificities, with potentially interesting scientific and clinical applications.

Conclusion

NGF was first discovered more than 40 years ago and has been one of the most intensively studied growth factors. The reassessment of chemical modification and site-directed mutagenesis data in the light of the X-ray crystal structure contributes to the emerging picture of the molecular basis of the action of NGF and facilitates the design of further mutants to probe its receptor binding and biological activities. Similar information for the receptors should eventually allow a complete description of the complex(es) that produces NGF responses and aid in the design of therapeutics that are neurotrophin agonists or antagonists.

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