Solution Structure and Internal Motion of a Bioactive Peptide Derived from Nerve Growth Factor

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The conformation and internal dynamics of a bioactive cyclic peptide, N-acetyl-YCTDEKQCY, derived from the C-D loop of β-nerve growth factor (β-NGF) were analyzed by solution NMR spectroscopy. NMR experimental data were used to calculate an ensemble of peptide structures. All of the structures had a β-turn at residues Asp⁴–Gln⁷ but could be divided into two families according to the presence or absence of a hydrogen bond at Gln⁷. Comparison of the calculated structures with the corresponding C-D loops from the x-ray structures of the NGF revealed striking similarity. The orientation of Glu⁶, Lys⁸, and Gln⁷ side chains in the NGF mimetic was very similar to the C-D loop of NGF. These residues are known to participate in interactions with the TrkA receptor. Relaxation measurements of the peptidomimetic α-carbons at ¹³C natural abundance and calculated dynamic parameters suggest that the loop region of peptide is well structured but that residues Thr³, Asp⁴, Glu⁶, and Lys⁸ undergo slow conformational exchange. These results suggest that conformational similarity and possibly peptide dynamics are responsible for the bioactivity of the peptide.

Nerve growth factor (NGF)¹ is a member of the neurotrophin family of polypeptide growth factors. NGF promotes the growth and survival of sympathetic, trigeminal, dorsal root ganglia neurons and cholinergic neurons of the basal forebrain. Two cell surface neurotrophin receptors have been characterized: the low affinity p75 receptor (Kᵢ = 10⁻⁹ M), which is common to all members of the neurotrophin family (1–3), and the intermediate affinity receptors (Kᵢ = 10⁻¹¹ M), TrkA, TrkB, and TrkC, which afford the binding specificity of the different neurotrophins (4–7). TrkA is selective for NGF and has tyrosine kinase enzymatic activity that mediates most of the downstream signaling for this growth factor. Highest affinity binding of NGF (Kᵢ = 10⁻¹₂ M) occurs in cells coexpressing TrkA and p75 (4, 8).

NGF, like other neurotrophins, is a dimer of two identical polypeptides of 118 residues (9). X-ray structures of NGF (10, 11), a brain-derived neurotrophic factor-neurotrophin-3 heterodimer (12), and the 7 S NGF complex (13) have been determined. The neurotrophin protomers have the same overall topology of seven β-strands connected by three disulfide bridges arranged in a cysteine knot motif. Comparison of the primary sequence of different neurotrophins shows clusters of high homology (>50% identity) and high diversity. The hypervariable domains occur in the exposed β-loops (residues 29–35, 43–48, and 92–98), in an exposed reverse turn (residues 59–66), in a solvent-exposed β-strand (residues 79–89), and at both N and C termini. By analogy with findings from other growth factors, the hypervariable domains are believed to be responsible for Trk receptor selectivity (14, 15).

Biological studies have confirmed that these variable regions are involved in NGF receptor binding and specificity (16–19). Either by point mutagenesis or by exchanging blocks of residues between different neurotrophins, amino acids His⁴, Pro⁵, Phe⁷, His⁸, Val⁹, Pro¹⁰, and Gln⁹⁶ have been shown to be important for specific binding of NGF to TrkA (20, 21). Deletion studies have also identified the N terminus (22–24) and C terminus (25–27) as necessary for TrkA binding. NGF binding to p75 is mainly mediated by the positively charged residues Lys⁷², Lys⁷⁴, and Lys⁹⁵ (28) and the reverse-turn residues Asp⁴⁷, Lys⁷⁴, and His⁷⁹ (29).

Since NGF promotes the growth and survival of neurons during development and after neuronal damage, it is a potential therapeutic agent in the treatment of neurodegenerative diseases and nervous system injuries (30, 31). NGF antagonists would be useful in treatment of certain chronic inflammatory or neuropathic pain states mediated by TrkA. Several human malignancies (e.g. melanomas, medulloblastomas, and neuroblastomas) express normal TrkA and are NGF-dependent or -responsive (32–35). Human TrkA-expressing neuroblastoma tumor growth can be arrested in nude mice by TrkA binding ligands, and TrkA ligands can be used to localize TrkA-expressing tumors in vivo (36).²

Small peptides and peptidomimetics overcome many of the drawbacks of proteins as pharmaceutical agents. Compared with proteins such as NGF, peptidomimetics are less antigenic, less subject to proteolytic degradation, and more able to cross blood tissue barriers. Small peptide mimetics of NGF have been produced based on sequences from the β-loops of NGF (37). The β-turn in the loops was induced by the introduction of cysteine residues and intramolecular disulfide cross-linking. These small NGF mimics bind TrkA and antagonize NGF binding and biological activity. A peptide derived from the C-D loop

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† The abbreviations used are: NGF, nerve growth factor; COSY, correlation spectroscopy; TOCSY, total correlation spectroscopy; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy.

² H. U. Saragovi, unpublished results.
of NGF, termed C-(92–96), showed the highest activity, with an affinity for TrkA on the order of 10^{-7} M (37). A linear correlation with the same primary sequence did not show any biological activity, which confirmed the importance of the peptide conformation. In this work, the cyclic NGF β-loop mimic peptide C-(92–96) was the subject of study by high resolution solution NMR with the aim of correlating the structure and dynamics of the peptide with its bioactivity.

**EXPERIMENTAL PROCEDURES**

**Peptide Synthesis and Oxidation—**N-Acetylated peptide N-acetyl-YCTDEKQCY was obtained from BioQuest, Inc. (San Francisco, CA). The peptide was purified on a preparative C18 column using Waters high pressure liquid chromatography (0.1% trifluoroacetic acid and 10–40% acetonitrile gradient in 40 min). The peptide was designed to mimic residues Thr^{92}Asp^{93}Glu^{94}Lys^{95} and Glu^{96} in the primary sequence of NGF and is correspondingly referred to as NAc-C-(92–96). Peptide was oxidized under dilute conditions in 1% ammonium bicarbonate buffer, pH 8.5, overnight and then lyophilized.

**Mass Spectrometry**—Mass spectrometry was used to assess the possible dimerization of the peptide. Purified peptide was dissolved in 10% acetic acid, 20% methanol and analyzed by electrospray ionization mass spectrometry equipped with field ionization quadrupole mass spectrometer (API III MS/MS System, Sciex, Thornhill, Ontario, Canada). The measured mass of the peptide was 1192.3 ± 0.1 Da with no dimers detected.

**NMR Spectroscopy**—For homonuclear one- and two-dimensional experiments, the sample contained 5–10 mM NAc-C-(92–96) in 10 or 100% D_{2}O, pH 5.6, at 275 K. When D_{2}O was used as solvent, the peptide was twice lyophilized and redissolved in D_{2}O. Spectra were acquired at 500-MHz proton frequency on a Bruker DRX500 spectrometer with field pulse gradients. Standard experimental protocols were used for NOESY (38), TOCSY (39), phase-sensitive double-quantum filtered-COSY (40), and COSY-30 spectra. WATERGATE pulse sequence (41) was used for solvent suppression for spectra in H_{2}O. NMR spectra were processed with Silicon Graphics computers using the GIPFEL computer (42). H^1 chemical shifts were referenced to the internal sodium 2,2-dimethyl-2-silapentane-5-sulfonate standard at 0.0 ppm. H^1 chemical shifts were indirectly calibrated using a γC/γH ratio of 0.25144953 (43).

**Determination of Side Chain pK Values**—The pH values of carboxylic acids in the peptide were measured through changes in chemical shifts of amide protons via TOCSY experiments in H_{2}O. Fifteen TOCSY spectra were acquired at the pH 1.1, 6.1, 6.1, and 9.1. The observed chemical shift could be expressed mathematically as δ^n = (δ × 10^{pH} − pK^a) + δ^0(10^{pH} + pK^a + 1), where δ^0 is the chemical shift of the ionized residue and δ is the chemical shift of the un-ionized residue.

**Amide Proton Exchange**—Amide proton exchange kinetics were examined in a mixed sample of reduced and oxidized NAc-C-(92–96). Reduction was carried out by the addition of dithiothreitol and heating. The pH of the fully protonated peptides was then adjusted to 4.6, and Reduction was carried out by the addition of dithiothreitol and heating. The van der Waals energy was represented by a repel function. During the cooling stage, the force constant on the van der Waals repel function was varied from 0.003 to 4 kocal mol^{-1} Å^{-2}. The force constant on the van der Waals repel function was varied from 0.003 to 4 kocal mol^{-1} Å^{-2}. There was no evidence for the presence of cis peptide bonds in the NMR spectra, and all of the peptide bonds were maintained in trans conformation during the structure calculations.

Final acceptance of the structures was based on the following covalent geometry criteria: no bond length violations greater than 0.02 Å, a bond length violation root mean square deviation of less than 0.01 Å, no valence angle violations greater than 3°, an angle violation root mean square deviation of less than 2°, and no improper violations greater than 5°. Criteria for the existence of a hydrogen bond were a donor-acceptor distance of less than 3.2 Å and a donor-proton-acceptor angle of more than 120° (45). Structures were visualized on a Silicon Graphics workstation using the InsightII program (Biosym Technology, San Diego, CA).

**NMR Relaxation Measurements**—All heteronuclear NMR experiments were done at natural ^{1}H abundance with an 18 ms sample in D_{2}O, pH 5.6, 275 K. Relaxation experiments were carried as described by Palmer and colleagues (49). Two-dimensional spectra consisted of 64 and 1024 data points in t1 and t2, respectively, with spectral widths of 5482.5 Hz in ^{1}H and 2500 Hz in ^{13}C dimensions. The number of scans in each t1 increment was 32, with a recovery delay of 2 s. R1 measurements were obtained from 13 experiments with relaxation delays (T) of 0.01, 0.05, 0.1, 0.15, 0.20, 0.30, 0.40, 0.60, 0.80, 1.0, 1.5, 2.0, and 2.5 s. For R2 relaxation measurements, the spin-echo delay between 180° pulses was 5 ms. Eleven experiments were performed with T of 5, 15, 25, 35, 50, 70, 90, 180, 230, 280, and 330 ms. R1 and R2 relaxation rates were calculated from the experimentally measured signal intensities by fitting the equations I(T) = F_0 (1 - (F_0 - exp(-R1 T) - exp(-R2 T)) and I(T) = F_0 exp(-R2 T + T), where F_0 is the intensity that corresponds to 1% magnetization at the beginning of the relaxation period and F_0 is the steady-state intensity. The public domain program, GNUMPLOT (version 3.5), was used to fit the relaxation parameters to the experimental data. In order to estimate the steady-state ^{1}H-^{13}C NOE, two spectra were recorded: one with broad band ^{1}H saturation to obtain the NOE enhancement, the other without ^{1}H saturation. The ^{1}H/^{13}C NOE enhancement, η, was calculated as η = (I_{sat} − I_{unsat})/I_{unsat}, where I_{sat} and I_{unsat} are signal intensities in spectra with and without ^{1}H saturation, respectively. The recovery delay in the heteronuclear NOE experiments was 8 s, with 48 scans acquired for each t1 increment. Four independent pairs of experiments were recorded to reduce uncertainties in the heteronuclear NOE values.

**Diffusion Measurements**—Six independent isotropic self-diffusion experiments were performed at peptide concentrations of 2, 6, 14, and 18 mM, at 275 K, using pulse field gradients (50, 51). Gradient strengths were varied from 0.67 to 63.65 Gcm with a gradient duration of 3.5 ms and diffusion time of 150 μs.

**Estimation of the Overall Rotational Correlation Time**—We calculated the overall rotational correlation time, τ_{r}, from measurements of the translational self-diffusion coefficient (Dtrans). To do this, the correlation time is expressed as τ_{r} = 1/6Drot = ωr KT, where V is the hydrated volume of the molecule, Drot is the rotational diffusion coefficient, η is the solvent viscosity, and k is the Boltzmann constant. Similarly, the translational self-diffusion coefficient is written as Dtrans = kT/6πηr, where r is the radius of the molecule. Assuming a spherical volume (V = 4/3πr^3) and obtaining the radius from Dtrans, we can write τ_{r} = (4πη/3kT)(kT/6πηr^3) = (4πη/3kT)(4/3πr^3). Thus, knowledge of Dtrans and η can be used to calculate τ_{r}, assuming a spherical shape and isotropic rotation. The viscosity, η, of D_{2}O at 275 K is 0.089 cP, and the radius of NGF is 0.098 Å.

**Calculation of Relaxation Parameters**—We used the model free formalism of Lipari and Szabo (54) to approximate the spectral density function as J(ω) = 2/5/5S^2(ω)(I + (ω^2)/(1 - S^2)(ω^2)), where τ_{r} is the overall rotational correlation time, S^2 is the order parameter, τ_{r} = τ_{r} + τ_{r} and τ_{r} is the effective internal correlation time (54, 55). The value of S^2 indicates how restricted the internal motion is. It varies from 1 for completely restrained internal motion to 0 for isotropic
unrestrained internal motion. Values of $\tau_m$ above 100 ps indicate increased motion and usually correlate with large heteronuclear NOEs, small values of $R1$, and decreased order parameters, $S^2$. The longitudinal relaxation rate, $R1$, of a protonated $^{13}$C nucleus is determined primarily by the dipolar interactions with the attached protons, whereas processes such as conformational exchange can contribute to the transverse relaxation rate, $R2$.

Model free parameters were determined from the relaxation data by using the Modelfree version 3.1 program (49). The optimization minimized the function $\chi^2 = \sum \chi^2 = 2(R1 - R1)^2/R1 + (R2 - R2)^2/R2 + (\text{NOE} - \text{NOE} - \text{NOE})/R2$. Quality of the fit was analyzed by comparing the optimal value of $\chi^2$ with the 95 percentile critical value of $\chi^2$ determined from the Monte Carlo simulations. We used the model selection procedure described by Palmer and co-workers (56). We fit $R1$, $R2$, and NOE experimental data for each residue using models with different combinations of $S^2$ alone, of $S^2$ and $\tau_m$ of $S^2$ and $R2\text{exch}$, or of all three parameters: $S^2$, $\tau_m$, and $R2\text{exch}$. In the last run of calculations, the global value of $\tau_m$ was optimized.

### RESULTS

**Sequential Assignment**—We studied the nine-residue peptide, N-acetyl-Tyr$^1$-Cys$^2$-Thr$^3$-Asp$^4$-Glu$^5$-Lys$^6$-Cys$^7$-Tyr$^8$, constrained by formation of an intrachain disulfide between the two cysteines, the sequence between Thr$^3$ and Gln$^7$ corresponds to residues 92–96 (loop C-D) of mouse NGF (37). Resonance assignments were made from inspection of two-dimensional homonuclear spectra using NOESY, TOCSY, and COSY spectra collected in $H_2O$ and $D_2O$ at 275 K (37, 58). The cross-peaks in the spectra were well dispersed, which made the sequential assignment straightforward (Fig. 1). $^{13}$C chemical shifts were determined from a $^{13}$C/$^1$H heteronuclear single-quantum correlation spectroscopy (59) and are listed along with $^1$H assignments in Table I. The spectra of the linear (reduced) peptide showed greatly reduced chemical shift dispersion, suggesting a random coil conformation.

**Aspartic and Glutamic Acid Titration**—In the x-ray structure of NGF by McDonald et al. (10), the C-D loop, which was used to derive NAc-C-(92–96), adopts type I $\beta$-turn conformation. This $\beta$-turn is stabilized by a hydrogen bond between the backbone amide of Glu$^7$ and either the backbone carbonyl or side chain carboxylic acid of Asp$^4$. In order to determine whether the side chain of Asp$^4$ in NAc-C-(92–96) is involved in hydrogen bonding, we measured titration curves for the three carboxyl groups of NAc-C-(92–96) via changes in amide proton chemical shifts. As expected from their proximity to titratable COOH groups, only Asp$^4$, Glu$^5$, and the C-terminal Tyr$^9$ showed significant chemical shift changes as a function of pH (Fig. 2). For all three carboxyls, the calculated $pK_a$ values were very similar in the oxidized and reduced forms, which suggests an absence of hydrogen bonds involving these COOH groups.

**Amide Proton Exchange**—In order to detect the presence of hydrogen bonds in the NAc-C-(92–96) peptide, amide proton exchange kinetics were examined. As a control for primary sequence effects, the $H_2O/D_2O$ exchange rates of both the cyclic (oxidized) and linear (reduced) NAc-C-(92–96) peptide were measured (Table II). The first $D_2O$ exchange spectrum, obtained 8 min after sample transfer to $D_2O$, contained only amide proton peaks of Tyr$^1$ (reduced and oxidized forms), Glu$^5$ (reduced), Gln$^7$ (oxidized), and Tyr$^9$ (reduced and oxidized) (data not shown). We estimate the half-life of amide protons not seen in this spectrum to be less than 3 min. Amide proton half-lives in the linear peptide were also estimated as described by Englander and colleagues (60).

Protection factors were defined as the ratio of the half-lives of the amide proton in the oxidized and reduced peptide (Table II). Analysis of the protection factors allowed us to distinguish slow exchange of amide protons due to peptide conformation from slow exchange due to electrostatic effects from neighboring amino acids. Only the amide proton of Gln$^7$ had a protection factor, $\sim 7$, that was significantly different from 1. This suggests hydrogen bond formation by the amide proton of Gln$^7$. Analysis of amide chemical shifts as a function of temperature confirmed the likely presence of a hydrogen bond for Gln$^7$ (see below). The amide protons of Tyr$^1$ and Tyr$^9$ exchanged slowly in both the reduced and oxidized peptides, which suggests that they exchange slowly due to an intrinsic property of the primary sequence.

Exchange with solvent requires that the amide proton be transiently exposed to solvent (61). If the opening-closing reaction occurs faster than proton exchange, then the equilibrium constant for opening-closing reaction ($K^{\text{open}}$) is equal to the protection factor ($P$). In case of Gln$^7$, the free energy for the opening-closing reaction can be calculated as $\Delta G^{\text{open}} = -RT\ln K^{\text{open}} = -1.07 \pm 0.05$ kcal/mol. Since this value is less than the free energy of the hydrogen bond between an amide hydrogen and carboxyl group, $-3$ kcal/mol, we conclude that Gln$^7$ is weakly hydrogen-bonded and only partially sequestered from exchange (62).

**NMR Restraints for Structure Calculations**—A summary of the short and medium range NOEs, values for $\phi$, $\psi$, and $\chi_1$ coupling constants, and results of amide proton exchange data is illustrated in Fig. 3. $J_{\phi\psi}$, $J_{\phi\chi_1}$, and $J_{\psi\chi_1}$ coupling constants were used to constrain the $\phi$ angles of residues Lys$^6$, Gln$^7$, Cys$^8$, and Tyr$^9$ and the $\chi_1$ angles of residues Glu$^5$, Gln$^7$, Cys$^8$, and Tyr$^9$.

**Converged Structures**—Structures were computed by using a hybrid distance geometry–simulated annealing protocol. No explicit hydrogen bond constraints were used in calculations. Of 200 calculated structures, 195 had no NOE violations greater than 0.5 Å and no dihedral angle violations greater than 5°. From these converged structures, we chose 44 structures with acceptable covalent geometry. This resulted in two distinct families. In both families the loop region was well defined, while Tyr$^1$ and neighboring residues showed significant disorder. The structures in the first family (26 structures) adopt a $\beta$-turn type I conformation with a hydrogen bond between the carboxyl of Asp$^4$ and amide of Gln$^7$. Structures in this family could be further subdivided into two classes based on the conformation of the disulfide bridge and Cys$^8$. Structures in the second family (18 structures) also show a $\beta$-turn between Asp$^4$ and Gln$^7$, but they belong to a less common type of turn, termed $\gamma$-e$\alpha$ in the nomenclature of Wilmot and Thornton (63); there is no Asp$^4$-Gln$^7$ hydrogen bond. Statistics for the NMR structure calculations are summarized in Table III. In both families, the negatively charged carboxyl group of Asp$^4$ is pointed to-
rates for different R2 relaxation rates and NOE enhancements for the methine

ment in conformational exchange, we analyzed the NMR relax-
the peptide’s dynamics, internal motion, and possible involve-

trum.

NOEs were between 0.27 and 0.34 for all residues except the

and terminal carboxyl of Tyr 9 for both reduced and oxidized

B 10.0 ± 3.0 2.42 7.04

Tyr 6 7.93 4.42 3.13 2.86 H2,6 7.05, H5,5 6.79 59.0 39.5

A 8.56 4.75 3.15 2.91 CH 1.22 62.3 69.9 CH 21.7

C 5.44 40.8

D 56.8 32.4 C1 35.1, C1 28.8

E 55.7 29.3 C 33.7

F 55.5 42.2

G 59.0 39.5

H 62.3 69.9 CH 21.7

I 54.4 40.8

J 56.8 32.4 C 35.1, C 28.8

K 55.7 29.3 C 33.7

L 55.5 42.2

M 59.0 39.5

N 62.3 69.9 CH 21.7

O 54.4 40.8

P 56.8 32.4 C 35.1, C 28.8

Q 55.7 29.3 C 33.7

R 55.5 42.2

S 59.0 39.5

T 62.3 69.9 CH 21.7

U 54.4 40.8

V 56.8 32.4 C 35.1, C 28.8

W 55.7 29.3 C 33.7

X 55.5 42.2

Y 59.0 39.5

Z 62.3 69.9 CH 21.7

a 1H chemical shifts were referenced to an internal sodium 2,2-dimethyl-2-silapentane-5-sulfonate standard at 0.00 ppm at 275 K, pH 5.6. 13C chemical shifts were indirectly calibrated using a γC/γH ratio of 0.25144953.

ward Gln7, and its shielding effect on the H2 of Glu5 is not as

profound as in reduced peptide. This may explain the slow

solvent exchange rate of Glu5 amide proton in the linear pep-

peroxide was measured to be (1.05 ± 0.08) × 10−10 cm2/s by pulsed field gradient self-diffusion experiments (50, 51). This gave a correlation time of 1.8 ± 0.4 ns, which correlated well with the average, 1.83 ± 0.06 ns, obtained from the R2/R1 ratio for residues Cys2, Cys8, and Gln7.

Peptide motional parameters were calculated using the model selection procedure described by Palmer and co-workers and the Modelfree version 3.1 program (49, 56). In the calculations, τm was first fixed at 1.83 ns for model selection. The correlation time was then optimized simultaneously for all residues to give 1.81 ± 0.03 ns, and the relaxation parameters for the methine carbons of NAc-C(92–96) were calculated. As

two flanking tyrosines. This is close to the value expected in the

absence of the internal motion. The 2-fold increase in NOE

values for Tyr1 and Tyr9 indicates increased flexibility for these

extracyclic residues and correlates well with their relatively

small R1 values.

As seen in Table IV, the τm of the peptide could not be obtained from the R2/R1 ratios, since a range of values were present. Instead, the correlation time was calculated from the solvent viscosity and Dtrans, assuming a spherical shape and isotropic motion. Dtrans was measured to be (1.05 ± 0.08) × 10−10 cm2/s by pulsed field gradient self-diffusion experiments (50, 51). This gave a correlation time of 1.8 ± 0.4 ns, which correlated well with the average, 1.83 ± 0.06 ns, obtained from the R2/R1 ratio for residues Cys2, Cys8, and Gln7.

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seen in Table IV, the order parameters, $S^2$, for all residues except the two linking tyrosines were high and indicated restricted motion. These $S^2$ values (0.81–0.89) are very similar to those in structured regions of proteins (49, 64–67). The value of overall rotational correlation time $\tau_m$ also indicated restricted motion in the peptide. For the flexible polypeptides, $\tau_m$ is molecular weight-independent and determined by the segmental motions (68). The values of $\tau_m$ for the flexible polypeptides reported in the literature are 0.4–0.6 ns at temperatures of 305–318 K (68–71). These values, corrected to 275 K, give correlation times of 1.2–1.3 ns, which are significantly smaller than the $\tau_m$ of NAc-C-(92–96).

For Tyr1 and Tyr3, the values of the order parameter, $S^2$, were low (0.62 and 0.75), indicative of increased mobility for these extracyclic residues. More surprising were the large conformational exchange terms for residues Thr2 (Cα and Cδ1), Asp4, Glu5, and Lys6. These $R2exch$ values reflect the large $R2$ relaxation rates (and high $R2/R1$ ratios) measured for these residues. The $R2$ rates were concentration- and pH-independent (data not shown) and suggest the presence of the conformational exchange at these residues. No difference was detected between $R2$ rates measured with 1- and 5-ms interpulse delays in the CPMG pulse sequence. For residue Glu2, accurate fitting of the relaxation parameters could be obtained only through a three-parameter model with both $\tau_i$ and $R2exch$ terms.

**DISCUSSION**

The NGF mimetic, NAc-C-(92–96), was designed to reproduce the C-D loop of NGF that is known to interact with TrkA. Four lines of evidence confirmed the formation of $\beta$-turn in the mimetic. 1) There were strong sequential $H^N-H^N(i, i + 1)$ NOEs for residues Glu5, Lys6, and Gln7. 2) There was an NOE crosspeak between $H^\alpha$ of Glu5 and $H^N$ of Gln7 as illustrated in Fig. 1. 3) A number of weak NOEs between side chains of Asp4 and Gln7 were present in the spectra. 4) Solvent H2O/D2O exchange showed that the amide proton of Gln7 was weakly protected against exchange. This protection is probably due to a hydrogen bond between the amide of Gln7 and backbone carbonyl of Asp4.

The structure calculations from NMR distance and dihedral angle constraints generated two families of structures represented in Fig. 4. In both families, a $\beta$-turn is formed by residues Asp4, Glu5, Lys6, and Gln7. The major differences between families occur around residues Asp4 and Glu5. In the more populated family (Fig. 4a), Asp4 makes a hydrogen bond with the amide of Gln7, and Glu5 forms a typical type I $\beta$-turn. In family 2, there is no hydrogen bond acceptor for Gln7, and Glu5 adopts an unusual left-handed $\alpha$-helical conformation to form a yL-\alpha $\beta$-turn. Although Cys7 and Thr8 adopt similar backbone angles in the two families of structures, their orientations relative to the $\beta$-turn differ considerably. Generally, the largest differences between the families occur in the N-terminal half of the peptide (which is also less well defined), while the structures of the C-terminal half are very similar.

Several groups have determined that residues Glu94, Lys95, and Gln96 of NGF are important for high affinity receptor binding and activation (17, 19, 20, 28). In the NAc-C-(92–96) mimetic, these correspond to residues Glu5, Lys6, and Gln7. Since these residues (with the exception of the $\phi$ angle of Glu5) are similar in the two families, the differences observed in the N-terminal portion of the mimetic may not be very important for its bioactivity. In particular, our NMR data suggest that the side chain orientations of the amino acids in the $\beta$-turn are relatively fixed and similar in both families of structures. Analysis of $J_{H_\beta-1H_\gamma}$ coupling constants enabled us to introduce dihedral $\chi_1$ angle restraints on the side chains of Glu5 and Gln7, and NOE's were detected between the side chains of Asp4-Gln7 and Glu5-Lys6. Together, these data restrict the orientation of these side chains to the conformations observed in the two families of structures. The $\beta$-turn formation by residues Asp4–Gln7 and the side chain orientations probably contribute to the ability of peptide to bind the TrkA receptor and antagonize NGF. Further, when linearized, the mimetic acquires a random coil conformation and loses all of its biological activity (37).

Existing crystallographic structures of the NGF show considerable flexibility in the loop regions (10, 11). Interestingly, when we compared the solution NMR structures of NAc-C-(92–96) with the different x-ray structures of C-D loop in NGF, we found very strong similarities. Fig. 5 shows comparisons of the two peptide family structures with C-D loops from NGF. Four different C-D loop structures of $\beta$-NGF are currently available in the Protein Data Bank (Upton, NY). In the structure of McDonald et al. (10), there is one NGF monomer in the asymmetric unit and one C-D loop structure. Residues Asp93–Gln96 form a type I $\beta$-turn with strong structural similarity to the family 1 structures of NAc-C-(92–96). Both the peptide and the NGF $\beta$-turns are stabilized by hydrogen bonds between Glu7 and Asp4. These are present as either a backbone carbonyl-amine hydrogen bond (in NAc-C-(92–96)) or as a bifurcated carbonyl-amine and side chain carboxyl-amine hydrogen bond. We note that the $\beta$-turn type I conformation is the most common type of $\beta$-turn in proteins and that these structures probably represent the lowest energy conformations for the C-D loop.

In the NGF structure reported by Holland et al. (11), three different conformations of the C-D loop were observed (due to the presence of three NGF monomers in the asymmetric unit). While one of the loops adopted a type I $\beta$-turn similar to the McDonald structure, the two other C-D loops presented yL-\alphaR


**Structure and Dynamics of an NGF Mimetic**

**TABLE IV**

| Residue | R1 | R2 | NOE | \(\tau_s\) | \(S^2\) | \(\tau_e\) | R2exch |
|---------|----|----|-----|--------|------|--------|-------|
| Tyr\(^1\) | 3.64 ± 0.05 | 7.18 ± 0.15 | 0.49 ± 0.07 | 1.54 ± 0.07 | 0.62 ± 0.02 | 120 ± 15 |
| Cys\(^2\) | 4.09 ± 0.10 | 9.19 ± 0.16 | 0.31 ± 0.05 | 1.76 ± 0.09 | 0.88 ± 0.01 | 6.3 ± 0.3 |
| Thr\(^3\) | 3.78 ± 0.07 | 14.91 ± 0.29 | 0.32 ± 0.07 | 2.77 ± 0.15 | 0.81 ± 0.02 | 75 ± 23 |
| Asp\(^4\) | 3.89 ± 0.11 | 18.03 ± 0.14 | 0.23 ± 0.03 | 3.08 ± 0.16 | 0.85 ± 0.03 | 9.1 ± 0.3 |
| Glu\(^5\) | 3.95 ± 0.08 | 14.94 ± 0.42 | 0.34 ± 0.04 | 2.71 ± 0.18 | 0.81 ± 0.03 | 6.2 ± 0.5 |
| Lys\(^6\) | 4.13 ± 0.10 | 11.94 ± 0.66 | 0.30 ± 0.03 | 2.19 ± 0.22 | 0.89 ± 0.03 | 2.6 ± 0.7 |
| Gln\(^7\) | 3.88 ± 0.05 | 9.36 ± 0.16 | 0.31 ± 0.04 | 1.85 ± 0.07 | 0.84 ± 0.01 | 0.5 ± 0.2 |
| Cys\(^8\) | 3.98 ± 0.06 | 9.35 ± 0.14 | 0.27 ± 0.05 | 1.84 ± 0.07 | 0.88 ± 0.01 | 85 ± 24 |
| Tyr\(^9\) | 3.76 ± 0.06 | 8.30 ± 0.13 | 0.51 ± 0.10 | 1.73 ± 0.07 | 0.75 ± 0.02 | 5.1 ± 0.7 |
| Thr\(^9\) | 3.92 ± 0.09 | 14.06 ± 0.63 | 0.37 ± 0.06 | 2.31 ± 0.24 | 0.84 ± 0.02 | |

**Fig. 4.** Ensemble of NAc-C-(92–96) structures in family 1 (26 structures) and family 2 (18 structures). The four residues in the \(\beta\)-turn of each family are labeled. In family 1, the carboxyl of Asp\(^4\) is turned into the loop and presents excellent geometry for a hydrogen bond with the amide of Gln\(^7\). Among the structures in family 1, two subclasses can be distinguished based on the conformation of Cys\(^8\) (\(\phi\) angle of 0 or 135°). Experimental coupling constant constraints are responsible for the restricted side chain orientations (\(\chi_1\) of residues Glu\(^5\), Gln\(^7\), Cys\(^8\), and Tyr\(^9\)). Medium range H\(^\alpha\)-H\(^\beta\) NOE constraints also contribute to the restricted side chain orientations of Asp\(^4\) and Tyr\(^9\). Structures were superimposed using the backbone atoms of residues 2 to 8, and only heavy atoms are shown.

**Fig. 5.** Comparison of minimized average structure calculated for each family with the conformation of C-D loop in NGF available from crystallographic data. a, structures in family 1 adopt \(\beta\)-turn type I conformation and correlate with the conformation of C-D loop from McDonald et al. (10). Hydrogen bond distances between Asp\(^4\) (carbonyl or side chain carboxyl) and Asp\(^4\) (side chain carboxyl) are 2.7 Å in NAc-C-(92–96) and 3.45 and 2.75 Å in the NGF structure. b, structures in family 2 adopt a type \(\chi_1\)-ar \(\beta\)-turn and are compared with the structure of subunit A reported by Holland et al. (11). No hydrogen bonding partner of Gln\(^7\) could be identified in the second family of NAc-C-(92–96) structures.

\(\beta\)-turns that are strikingly similar to the second family of NAc-C-(92–96) structures (Fig. 5). In the terminology of Wilmut and Thornton, \(\beta\)-turn types are determined by the dihedral angles \(\phi, \psi\) of the second and the third residues in the turn (63). In the monomer A and B structures of Holland et al. (11), Glu\(^5\) has a strained conformation of approximately \(\phi = 85, \psi = -30\). This is very similar to the \(\chi_1\) conformation of Glu\(^5\) of \(\phi = 50, \psi = +25\) in the peptide structures. While in the Holland structures, a cross-turn side chain carboxyl-amide hydrogen bond was present, in our experiments, we did not detect this additional hydrogen bond, nor was it present in the calculated family of structures.

NMR relaxation data indicate that the NAc-C-(92–96) peptide adopts a well defined conformation that is characterized by high order parameters, \(S^2\), for the loop residues and increased mobility for the extracyclic tyrosine residues. For Tyr\(^1\), this mobility is reflected as disorder in the calculated structures. More unusual relaxation behavior was detected for residues Thr\(^3\), Asp\(^4\), Glu\(^5\), and Lys\(^6\). The methine carbons of these residues exhibited fast R2 relaxation rates, suggesting the presence of slow conformational exchange. As the differences between the calculated structures are largest for these residues, it seems likely that interconversion on a millisecond time scale is occurring between the different families or classes of calculated structures.

In NGF, the C-D loop is flanked by two antiparallel \(\beta\)-strands so that the amino acid side chains are perpendicular to the plane of the sheet and alternate up and down. Thus, residues

\[\text{Thr}^9\text{1}\] and \[\text{Ala}^97\] in NGF have their side chains on opposite sides of the plane formed by the \(\beta\)-sheet. In the NAc-C-(92–96) mimic, these residues were substituted by cysteine residues. Cyclization (oxidation) of the cysteines requires them to twist so that the two side chain sulfur atoms come together. This leads to a rearrangement of the \(\beta\)-sheet in the vicinity of Thr\(^3\) in the NAc-C-(92–96) peptide. This distortion could cause the polymorphism and conformational exchange observed in the NAc-C-(92–96) structures.

This hypothesis is supported by preliminary results from a derivative of NAc-C-(92–96), which contains an additional resi-
ide in the loop (corresponding to Ala97 in the NGF structure). Since the loop now contains an even number of residues, a backbone twist is not be necessary for cyclization. Our preliminary results indicate less conformational exchange and a single family of structures. Surprisingly, this longer peptide, C-(92–97) was somewhat less active in bioactivity tests than C-(92–96) (37). Studies of chimeric NT-3/NGF molecules have found that Ala97 or Ala98 can supplant the requirement of Gly96 for TrkA activation (20). Probably, the small alamine side chains allow NGF to form close contacts with the receptor. In the mimetic peptide, the cysteine following Glu7 has its side chain pointing inward to form the S–S bond. This avoids any unfavorable steric interactions and may mimic Ala97. Although Tyr9 in the peptide has a long side chain compared with Ala98 in NGF, our study of peptide dynamics in solution suggests that the last tyrosine residue is very mobile and, if necessary, could adjust its position upon receptor binding. It is also possible that the decreased activity of C-(92–97) is related to the absence of conformational exchange in the longer mimetic. These arguments point out the utility of NMR relaxation measurements for understanding dynamic factors that contribute to function. Further studies of the conformational changes will help to elucidate the role of each residue in receptor binding and stimulate the rational design of higher affinity mimetics (72).

The goal of this study was to elucidate the structure of NAc-C-(92–96) in solution with the aim of understanding its receptor binding and stimulate the rational design of higher affinity mimetics (72). The goal of this study was to elucidate the structure of NAc-C-(92–96) in solution with the aim of understanding its receptor binding and stimulate the rational design of higher affinity mimetics (72).