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Three-dimensional structure of neurotoxin-1 from *Naja naja oxiana* venom at 1.9 Å resolution

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Neurotoxin-1 from *Naja naja oxiana* venom (NTX-1) has been crystallized by vapor diffusion in sitting drops. The crystals have cell dimensions of $a = 25.2$ Å, $b = 75.6$ Å, $c = 35.9$ Å, and are in space group $P2_12_12_1$. Three-dimensional data to 1.9 Å have been recorded by a Syntex $P2_1$ automatic diffractometer. The atomic structure of the toxin has been determined by molecular replacement using the α-cobratoxin (α-CTX) as the search model. The position of 534 non-hydrogen protein atoms have been determined. The model contains 65 water molecules. Refinement has led to an *R*-factor of 19.3% at 1.9 Å resolution. The secondary and tertiary structures of NTX-1 have been analyzed and a comparison with structure of the α-CTX has been made.

Neurotoxin-1; X-ray structure; *Naja naja oxiana* venom

1. INTRODUCTION

Neurotoxin-1 is one of the components of the venom of the elapid family snakes (cobras, kraits, mambas etc.). The target of neurotoxins is the α-subunit of the nicotinic acetylcholine receptor at the postsynaptic level of the neuromuscular junction, and their function is to prevent binding of acetylcholine and thus block the receptor.

A large number of neurotoxins have been sequenced [1–4]. They can be divided into two groups: 'short' neurotoxins with 60–62 amino acids, and 'long' neurotoxins with 70–74 amino acids. Alignment of amino acid sequences shows a number of conserved or conservatively changed residues and four or five disulfide bridges in the short and long toxins, respectively, in identical positions.

X-ray crystallographic studies of the three-dimensional structures of short erabutoxin b from *Laticauda semifasciata* at 1.4 Å resolution [5,6], of two long toxins: α-cobratoxin from *Naja naja siamensis* at 2.4 Å resolution [7] and α-bungarotoxin from *Bungarus multicinctus* at 2.5 Å resolution [8], showed very high similarity in the overall folding of the polypeptide chains. All of these molecules have comparable structure characteristics, with the polypeptide chain organized in three major loops protruding from a tangle-like part of the molecule stabilized by four disulfide bonds.

The central loop is more prominent than the other two: in the long α-neurotoxins it contains an extra disulfide at its lower tip. Residues Trp^{26}, Asp^{28}, Trp^{30}, Arg^{34} and Gly^{35} [1] are considered to be functionally invariant residues. These residues are responsible for the toxic activity and are located on the central loop [2–4,9,10]. Side chain of these residues are orientated in the same direction from the concave surface of the molecule. The guanidinium group of Arg^{34} located on lower tip of this loop is the only cationic group common to all of the neurotoxins and may represent the counterpart of the quarternary ammonium group of acetylcholine [1].

In this paper we describe the crystallographic study of the structure of neurotoxin-1 from *Naja naja oxiana* venom (NTX-1). This is the first three-dimensional structure of a long α-neurotoxin at high (1.9 Å) resolution. Comparison of the three-dimensional structures of long α-neurotoxins will suggest regions of the molecule that are most important for activity and interaction.

2. MATERIALS AND METHODS

NTX-1 was isolated from *Naja naja oxiana* venom. It was purified and crystallized as described previously [11,12]. The whole data to 1.9 Å resolution was collected from two crystals on a Syntex $P2_1$ diffractometer with an ω-scan.

The 3D structure of NTX-1 was determined by the molecular replacement method with a molecule of α-CTX from *Naja naja siamensis* serving as a starting model at a 2.4 Å resolution [7].

To determine the position of the α-CTX molecule in the unit cell of NTX-1 the programs MERLOT [13] and SEARCH [14] were applied. The structure was refined using the restrained least square minimization procedure of Hendrikson and Konnert [15] incorporating the fast Fourier algorithm [16] as developed by Dodson [17]. Throughout the refinement to the X-ray terms were assigned unit weights relative to one another. All reflections were included, no σ cutoff was applied. The program FRODO [18], running on an Evans and Suther-

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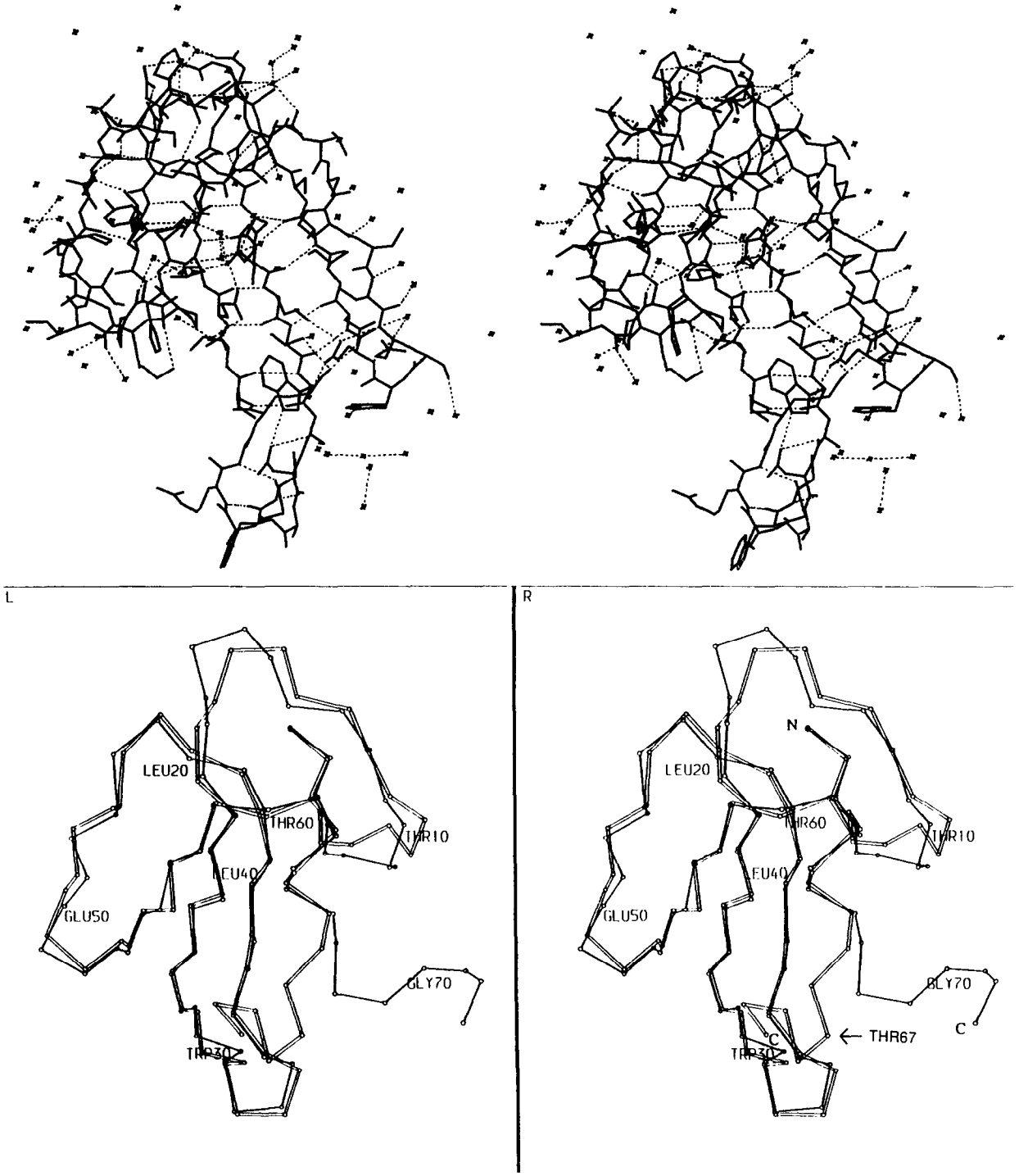


Fig. 1. (Upper panel) Stereo views of the neurotoxin-1 molecule: *, molecules of water; ·····, hydrogen bonds (lower panel). Comparison of a C^{α} backbone tracing of NTX-1 (thin line) and α-CTX (double lined).

land PS 330 interactive graphics unit, was used for all molecular graphics operations. These included inspection of parts of the model with $(3F_o-2F_c)$, $(2F_o-F_c)$ and (F_o-F_c) Fourier syntheses and full 'omit' maps at appropriate steps in the refinement.

3. RESULTS AND DISCUSSION

Crystals (1.5 × 0.4 × 0.2 mm) grew as thin plates over 4 weeks from 20 μl droplets containing 20 mg/ml protein, 0.01 M acetic acid that were plated on glass depression slides and sealed in boxes containing 10 ml of 50% iso-propanol, 5% 2-methyl-2,4-pentadiol, 0.01 M acetic acid, pH 4.7. The space group and cell dimensions were tested on an X-ray diffractometer and were identified as $P2_12_12_1$; a = 25.17 Å, b = 75.58 Å, c = 35.86 Å. Assum-

Table I

Final refinement statistics

	Target σ*	Standard deviations	No. of parameters
Bond distances (Å)			
Bond distances (1–2 neighbors)	0.020	0.014	552
Angle distances (1–3 neighbors)	0.030	0.056	752
Planar distances (1–4 neighbors)	0.040	0.062	201
Planar groups (Å)	0.020	0.018	89
Chiral volumes (Å^3)	0.200	0.227	83
Non-bonded contacts (Å)			
Single-torsion contacts	0.300	0.214	174
Multiple torsion contacts	0.300	0.324	204
Possible hydrogen bonds	0.300	0.216	44
Torsion angles (°)			
Planar (peptide ω)	3.0	3.3	72
Staggered (aliphatic χ)	20.0	25.7	85
Transverse (aromatic χ_2)	20.0	11.5	6

*The weight on each restraint corresponds to $1/\sigma^2$.

ing a molecular weight of 7.8 kDa the packing density was V_m = 2.2 Å^3/D and we had one molecule per asymmetric unit. The final set of the X-ray data contained 4,979 unique reflections in the 38.0–1.9 Å resolution range. The overall R-factor ($\Sigma\sigma/\Sigma|F|$) in this range of resolution was 4.4%.

A total of 175 cycles of least-squares minimization were carried out. The R-factor of 49.0% for the initial model in the resolution range 6.0–3.0 Å fell to 19.3% for the final model NTX-1 containing 534 non-hydrogen atoms of the protein and 65 molecules of water in the resolution range 6.0–1.9 Å. The standard deviations for restrained parameters of the last cycle of refinement are shown in Table I. The overall coordinate error in the atomic positions has commonly been estimated according to Luzzati [19], from which the root mean square error in the atomic coordinates can be estimated as 0.2 Å. All the 4,766 reflections were used in this estimation. For all the atoms in the structure (Fig. 1, upper panel), including water molecules, the mean temperature factor is 15.5 Å^2, for the protein atoms only it is 13.8 Å^2. As a rule the refinement may be assessed from the Ramachandran plot. In this plot for the whole model of NTX-1 only the non-glycine residue with value of conformation angles (ϕ,ψ) outside the allowed region is Ile^9 (−23.0°, 125.0°).

NTX-1 is a flat molecule with an overall dimension of 41 × 36 × 30 Å. The polypeptide chain is held strongly together by four disulfide bridges (Fig. 2) close to each other in the tangle like region of the molecule. The conformation of the disulfide bridges is described in Table II. Three hairpin-type loops and a 'tail' protrude from the tangle in different directions (Fig. 1, lower panel). The sequences for the loops (Fig. 2) are 1–18 (loop 1), 19–43 (loop 2), 44–59 (loop 3), and for the tail is 63–72.

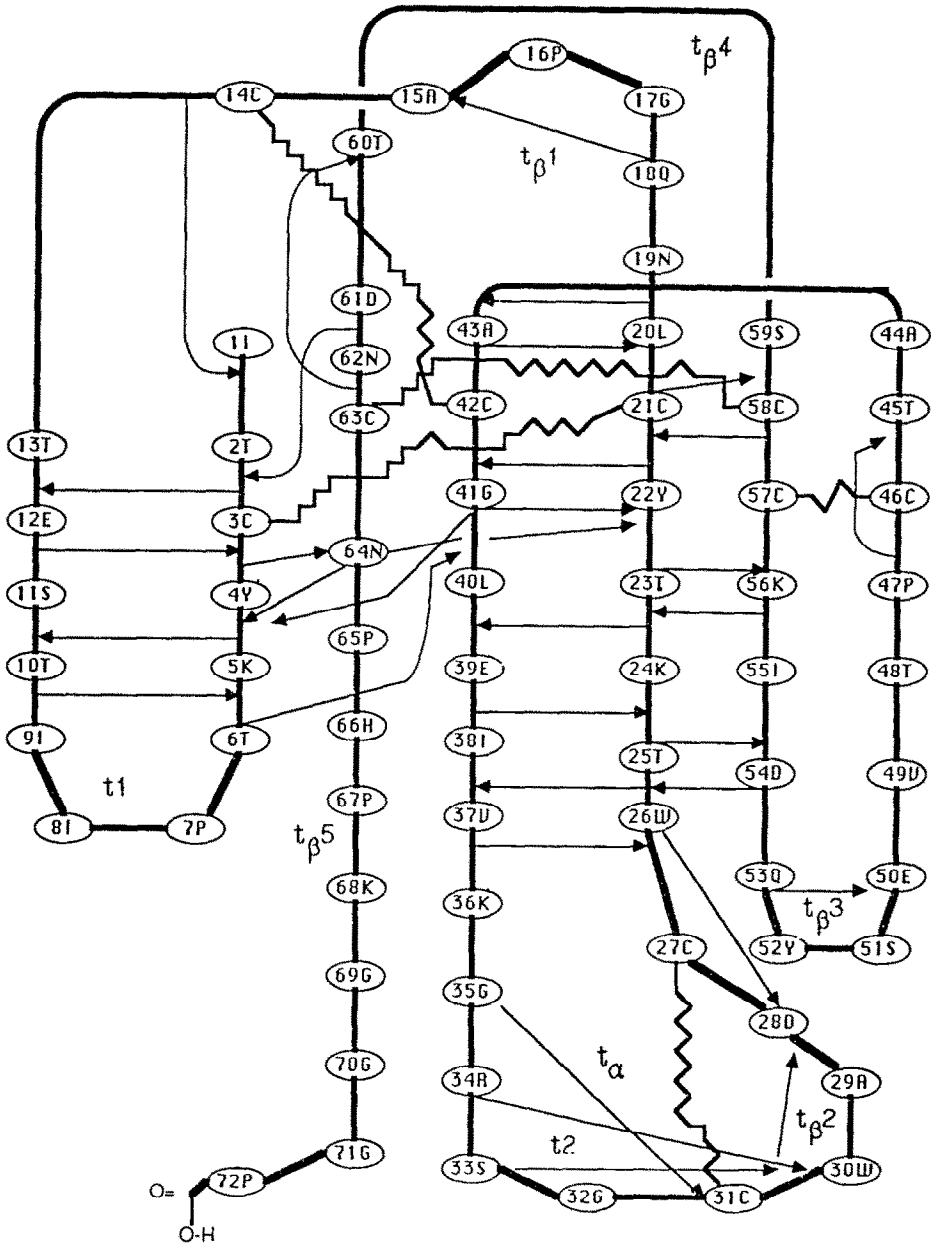
The loops are clamped together in the tangle by four disulfide bonds Cys^3–Cys^{20}, Cys^{14}–Cys^{42}, Cys^{46}–Cys^{56}, Cys^{58}–Cys^{63}; one additional disulfide bond is located at the lower tip of the loop 2, Cys^{27}–Cys^{31}.

Loop 1 is stabilized by an antiparallel β-sheet (βA-, βB-strand, Fig. 3) that is formed by residues 3–5 and 10–12. The central loop 2 is internally stabilized by a β-sheet formation (residues 20–26 (βC) and 37–43

Table II

Torsion angles in the five disulfide bridges

		$Cys_{(i)}$-C_β-S-S-C_β-$Cys_{(j)}$				
		3–21	14–42	27–31	46–57	58–63
χ_1:	N_i–$C_{\alpha i}$–$C_{\beta i}$–S_i (°)	−61	−48	−98	−161	52
χ_2:	$C_{\alpha i}$–$C_{\beta i}$–S_i–S_j (°)	−69	−73	−52	60	92
χ_3:	$C_{\beta i}$–S_i–S_j–$C_{\beta j}$ (°)	−95	−75	−76	91	94
χ_4:	S_i–S_j–$C_{\beta j}$–$C_{\alpha j}$ (°)	−35	−87	−167	125	98
χ_5:	S_j–$C_{\beta j}$–$C_{\alpha j}$–N_j (°)	−62	−75	−138	−78	54
	$C_{\alpha i}$–$C_{\alpha j}$, (Å)	5.62	5.62	5.18	5.95	6.24



Neurotoxin-I from *Naja Naja oxiana*

Fig. 2. Amino acid sequence and intramolecular hydrogen bonding scheme of NTX-1 from *Naja naja oxiana*. Disulfide bridges are indicated by zig-zag lines between cysteines. Hydrogen bonds are indicated by arrows (from donor to acceptor). t_1 and t_2 are turns, $t_{\beta}1$–$t_{\beta}5$ are β-turns, t_{α} is an α-turn.

(βD)), and the β-sheet structure extends to sequence 54–58 (βE) of loop 3 to form a triple-stranded antiparallel β-sheet (Fig. 3).

The central loop contains one additional disulfide bond, Cys^{27}–Cys^{31}, that with a β-turn ((t_{β}, Table III), Fig. 2) type II (residues 28–31), α-helix (residues 31–34 (t_{α})) and disordered turn (residues 30–33) form the bulgy tip of the loop.

The conformation of this part of neurotoxin molecules is of great interest as they contain residues Trp^{30} and Arg^{34} which play an important role in binding with acetylcholine receptor [10].

The tail is tethered to the tangle by the disulfide bridge Cys^{58}–Cys^{63} and by the tightly hydrogen bonded side-chain of Asn^{64} which is in a centrally important

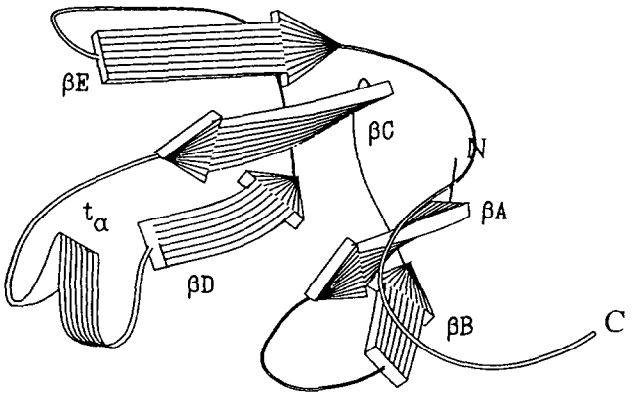


Fig. 3. A ribbon model of the NTX-1 molecule.

position. There are only a few hydrogen bonds that stabilize the conformation of the tail:

Pro^{65} O...H_2O7...OG1 Thr^{23} (2.8 Å, 2.8 Å)

His^{66} O...H_2O...N Pro^{67} (3.2 Å, 3.2 Å)

Pro^{67} O...NZ Lys^{68} (3.2 Å)

The solvent accessibility (Fig. 4) shows that most amino acids are at least partly exposed to the solvent, with a few exceptions, such as amino acids 3, 21, 40, 42 and 63. They are all located in the tangle of the globular NTX-1 structure and consequently are shielded from the solvent.

The non-polar atoms of residues Tyr^{22}, Lys^{24}, Trp^{26}, Val^{37}, Ala^{43}, Cys^{46}, Pro^{47}, Val^{49}, Tyr^{52}, Gln^{53}, Ile^{55} form an elongated hydrophobic core of the neurotoxin molecule.

There are two salt bridges, Lys^5–Glu^{12} and Lys^{36}–Asp^{54}, that additionally stabilize conformation of the polypeptide chain.

The main differences between the structure of NTX-1 and long α-toxins are in the β-sheet and α-helix. So, only the molecule of NTX-1 has an additional antiparallel β-sheet (βA- and βB-strands) and α-helix (t_α-turn).

High homology (about 60%) of amino acid sequences is revealed in the high homology of their 3D structures (Fig. 1, lower panel). The region of 3-chain antiparallel βSh2 β-sheets is the most conservative part of two neurotoxin molecules. The Cys residues of both molecules are located in identical positions: root mean square deviation of the C_α atom coordinates is 0.527 Å. As mentioned above, the conformation of S–S bonds in the NTX-1 molecule corresponds to their conformation in α-CTX. Three S–S bridges are in the left-handed helix conformation and 2 in the right-handed one. This fact confirms the suggestion that the β-structure βSh2 and the 5-disulfur bonds are the most important structural fragments, defining folding of the polypeptide chain in the three-dimensional structure of neurotoxin molecules.

The residues Asp^{64}, Tyr^{22}, Glu^{41} in NTX-1 and the corresponding Asp^{63}, Tyr^{21} and Asp^{40} in α-CTX are structural invariants in these molecules. Asp^{64} (Asp^{63}) (in brackets we show the corresponding residue in the α-CTX molecule) forms hydrogen bonds with Tyr^4 (Phe^4) and Tyr^{22} (Tyr^{21}). It should be pointed out that this residue takes up a unique position. It is the side group, being a donor for the hydrogen bond with Tyr^{22}

Table III

Secondary structure turns

Number turn	Type	Number residue	Distance (Å)	φ2 (°)	ψ2 (°)	φ3 (°)	ψ3 (°)
$t_\beta 1$	II	15...18	5.8	−56	143	99	−26
$t_\beta 2$	I	28...31	4.3	−69	−8	−116	17
$t_\beta 3$	I	50...53	5.2	−57	−40	−86	17
$t_\beta 4$	II	60...63	5.5	−46	140	46	33
$t_\beta 5$	I	67...69	6.4	−79	32	−144	30

Number turn	Number residue	φ2 (°)	ψ2 (°)	φ3 (°)	ψ3 (°)	φ4 (°)	ψ4 (°)
t_2	30...34	−57	−34	−82	−29	−82	−59
t_α	31...35	−82	−29	−82	−59	−86	−14

Number turn	Number residue	φ2 (°)	ψ2 (°)	φ3 (°)	ψ3 (°)	φ4 (°)	ψ4 (°)	φ5 (°)	ψ5 (°)
t_1	5...10	−80	160	−77	−6	−146	97	−30	125

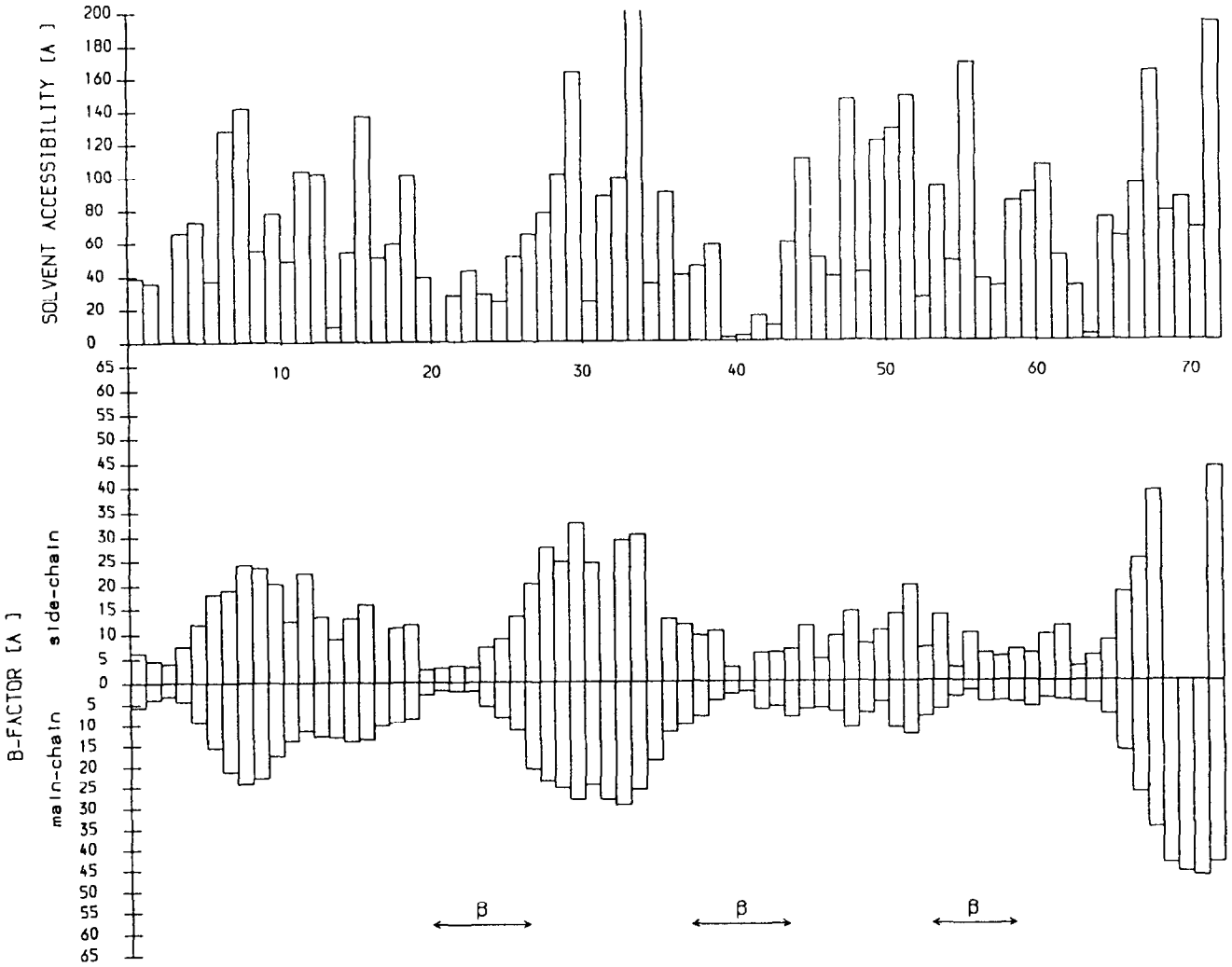


Fig. 4. Solvent accessibility (top) and temperature factor (bottom) for main chain and side chain atoms.

(Tyr[21]) and acceptor in the bond with Tyr[4] (Phe[4]), that takes part in forming the molecule nucleus, attaching to the tail and loops 1 and 2. The hydroxyl group of Tyr[22] (Tyr[21]) forms a hydrogen bond with the side-group of Glu[39] (Asp[38]), which influences the formation of the functionally important region of the polypeptide chain of the NTX-1 molecule.

This structure of neurotoxin-1 is probably the best model of the long toxins currently available for structure based on drug design, modeling acetylcholine receptor as well as for molecular replacement.

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