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Three-dimensional crystal structure of recombinant erabutoxin a at 2.0 Å resolution

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Abstract

Recombinant erabutoxin a (Ea_r) has been crystallized by vapour diffusion in hanging drops. The crystals belong to space group $P2_12_12_1$ with cell dimensions $a = 55.8$ Å, $b = 53.4$ Å, $c = 40.8$ Å. Diffraction data have been recorded on a FAST detector up to 2.0 Å. The atomic crystal structure of Ea_r has been determined by initial refinement of the structure of the isotoxin erabutoxin b (Eb) the crystals of which were grown under identical conditions. The *R*-factor was 23% at 2.0 Å resolution. The secondary and tertiary structures of Ea_r are shown to be identical with that of wild-type Eb, within the experimental error.

Key words: Recombinant snake toxin; Crystal structure

1. Introduction

Curaremimetic toxins are small proteins found in venoms from snakes classified as Elapidae and Hydrophiidae [1]. They block nicotinic acetylcholine receptors from various snake prey with great specificity and high affinity. Their equilibrium dissociation constants frequently range between 1 and $20 \cdot 10^{-11}$ M. With the view to delineate the site by which these toxins recognize their target, the cDNA encoding erabutoxin a (Ea), a curaremimetic toxin from the venom of the sea-snake, *Laticauda semifasciata*, was previously cloned [2], expressed as a hybrid protein fused to protein A from *Staphylococcus aureus* [3], and the recombinant toxin (Ea_r) generated in vitro by treating the hybrid in acidic conditions with cyanogen bromide [4]. Despite this drastic treatment, Ea_r had identical toxicity in mice, similar affinity for AcChoR from *Torpedo marmorata* and superimposable circular dichroism spectra as compared to Ea from snake venom [4]. However, the 3D-structure of Ea_r remained to be elucidated.

Three erabutoxins, a, b and c, are present in the venom of *L. semifasciata* [10]. They all possess 62 amino acids, including four half-cystines, and differ from each other by a single amino acid at position 26 or 51. The crystal structures of these toxins were previously elucidated at different resolutions, starting from crystals grown under various experimental conditions [5,11,12]. All toxins display highly similar polypeptide foldings composed of three adjacent loops rich in β-pleated sheets which emerge from a small globular core where the four disulphides are localized. We therefore investigated whether or not Ea_r adopts the same folding.

Crystals of Ea_r have been obtained using KSCN as a crystallizing agent, according to the procedure already described to crystallize erabutoxin b (Eb) [5]. These crystallization conditions lead to a dimeric form, which was originally reported by Preston et al., for Ec and Eb [6]. X-ray diffraction data were recorded and the 3D structure of Ea_r was solved at 2.0 Å resolution starting from the 3D structure of Eb crystallized under the same conditions [5]. In this paper the 3D structure of Ea_r is compared with that of wild-type erabutoxins and especially with that of Eb.

2. Experimental

2.1. Production and purification of Ea_r

A recombinant expression plasmid encoding a protein A–Ea_r fusion protein was constructed as described by Ducancel et al. [3]. The fused toxin was expressed in the periplasmic space of *E. coli* and purified by a single immuno-affinity step using an IgG Sepharose 6FF (Pharmacia) column. The fused toxin was separated from protein A on a C4 column (Vydac 5μ, 25 × 10 mm) with a trifluoroacetic acid/acetonitrile system. Appropriate mutation in the plasmid had introduced a methionine at position −1 of the Ea_r sequence and a treatment of the hybrid with

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Abbreviations: Ea, Erabutoxin a; Ea_r, recombinant erabutoxin; Eb, erabutoxin b; AcChoR, acetylcholine receptor.

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cyanogen bromide released the unfused Ea_r moiety [4]. The CNBr-treated hybrid was submitted to reverse-phase high pressure liquid chromatography, using a C4 column (Vydac 5μ, 25 × 10 mm) equilibrated with TEAF and eluted with a gradient of acetonitrile. The resulting Ea_r was further chromatographed on a mono S HR5/5 (Pharmacia) column using an ammonium acetate gradient. Ea_r was judged to be homogeneous from both SDS-PAGE and isoelectrofocusing. The amino acid composition of Ea_r was identical to that of Ea from venom.

2.2. Crystallization

Ea_r and Ea were crystallized using the procedure described by Saludjian et al. [5] to crystallize Eb isolated from snake venom [7]. Crystals of Ea_r and Ea were grown at 18°C by vapour diffusion in Linbro tissue culture plates. Four 10 μl hanging drops of a solution of sodium acetate buffer (50 mM, pH 4.5) containing the toxin (2.5 mM) and KSCN (145–160 mM) as crystallizing agent were equilibrated against 290–320 mM KSCN reservoirs, at the same pH. Crystals of Ea_r and Ea grow like prisms in all drops within a few days. They belong to the space group, $P2_12_12_1$, with 2 molecules in the asymmetric unit. Using experimental conditions identical to those described above, we found the same crystal form for Ea and Eb [5]. This crystal form was originally described by Preston et al. [6] for the wild-types Ec and Eb. The unit cell parameters of erabutoxins crystallized with SCN^- are indicated in Table 1.

2.3. Data collection and processing

Data from one single crystal 0.1 × 0.1 × 0.05 mm in size were collected to 2.0 Å reolution on a FAST detector, using a Nonius generator (rotating anode) and Cu Kα radiation selected with a graphite monochromator. The swing angle of the detector was 25°. Data were collected by steps of 0.15°, over 150 s. MADNES software was used to process all the data. After averaging intensities of 12,030 reflections (R_{sym} = 8.9% at 2 Å resolution), a total of 7,708 independent intensities were obtained.

The structure was refined by starting from the Eb three-dimensional structure [5] (6EBX protein Data Bank [8]). The two independent molecules of the asymmetric unit were refined using the XPLOR molecular dynamics program [9]. After several cycles of crystallographic dynamic refinement, with a step-by-step increasing resolution from 3.0 Å to 2.0 Å, a slow-cooling annealing procedure was applied (from 4,000 to 300 K). The *R*-value was reduced to 23% (7,053 $F_{obs} > 2\ \sigma$). Only minor manual adjustments were required for a few side chains. In addition, Pro-48 in both molecules of the asymmetric unit appeared highly disordered, and Gly-20 and Gly-34 in one of the two molecules were poorly defined. The model presently refined includes 1,057 protein atoms, 50 water molecules and one SCN^- ion per asymmetric unit. The thermal factors of water molecules were limited to 60 $Å^2$. The overall rms deviations are 0.007 Å and 1.6° on distances and angles, respectively.

Table 1
Crystal parameters of erabutoxins

	a (Å)	b (Å)	c (Å)	Ref.
Ea_r	55.8 ± 0.1	53.4 ± 0.1	40.8 ± 0.1	This work
Ea	55.9 ± 0.1	53.3 ± 0.1	40.7 ± 0.1	This work
Eb	55.7 ± 0.1	53.3 ± 0.1	40.9 ± 0.1	[5]

3. Results and discussion

As shown in Table 1, the crystal parameters of Ea_r are identical to those of Ea and Eb, grown under identical conditions in the presence of KSCN. As described by Saludjian et al. [5], the recombinant toxin crystallized in a dimeric form. In addition, a preliminary crystal structure of Ea_r (not shown) was similar to those previously reported for the erabutoxins a, b or c isolated from snake venom [5,11,12]. In particular, each molecule of the dimer had five antiparallel β-sheet strands encompassing residues 2–5, 13–17, 24–32, 34–41 and 51–56. Five β-bends were found between residues 7–10 (type II), 18–21 (type II), 31–34 (type I), 47–50 (type II) and 57–60 (type II). The turns 18–21 and 47–50 were close to ideal. Not only did Ea_r and venom erabutoxins have similar secondary structures, but there was excellent agreement regarding their overall three-dimensional architectures. In particular, each molecule had a leaf-shaped structure organized in three adjacent loops emerging from a small globular core. The C-terminal part of the polypeptide chain interacted with residues on the convex side of the toxin. A concave face was predominantly found within the central and third loops.

To get a better insight into the similarity of the crystal structures of recombinant and wild-type erabutoxins, we solved the structure of Ea_r at 2.0 Å and superimposed its backbone with that of Eb [5]. The reason why we selected

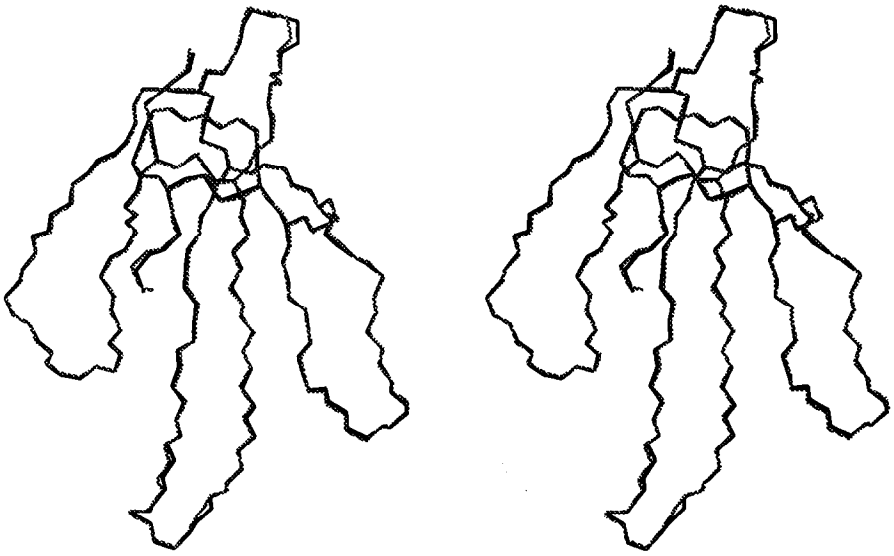

Fig. 1. Superimposition of the backbones of recombinant Ea (—) and wild-type Eb (···). Eb data are from [5] with permission.

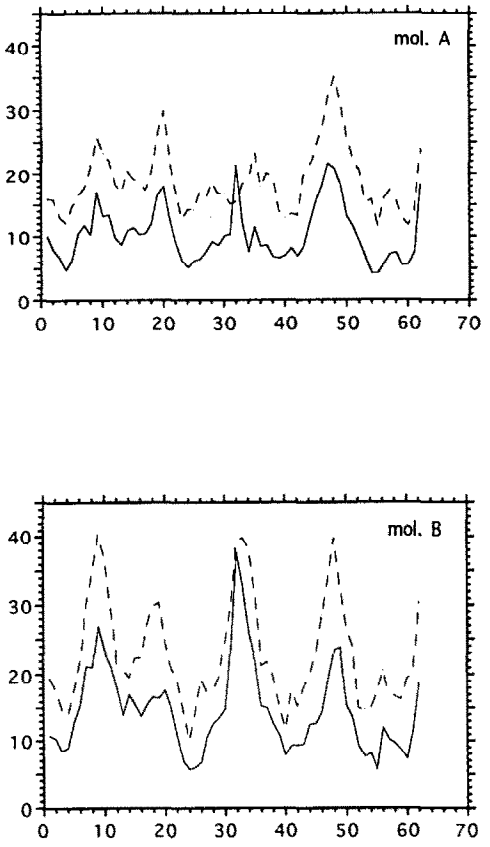

Fig. 2. Averaged thermal factors of main chain atoms of Ear (—) and Eb (– –) of the two molecules, A and B, in the asymmetric unit, vs. amino acid sequences.

the Eb structure for the comparison is twofold. First, the three-dimensional crystal structures of Ea and Eb, two isoforms that differ from each other by a single substitution at position 26, are highly similar to each other [11,12]. Second, the three-dimensional structure of Eb was recently solved from crystals grown under conditions [5] that are identical to those that have been used to produce the Ea_r crystals. As shown in Fig. 1, the foldings of Ea_r and Eb polypeptide chains virtually coincide. The average rms deviation between all α-carbons is 0.21 Å. Furthermore, for both structures, the highest *B*-values are identified in similar regions (Fig. 2) and there is a good fit between the four disulphides of Ea_r and Eb.

In conclusion, the present work demonstrates that the overall crystal structure of recombinant Ea is identical, within the experimental error, to that of Eb determined in the same crystal form [5]. We therefore infer that the 3D structure of the Ea_r backbone is also identical to that of Ea from snake venom. This conclusion is of particular interest for several reasons. First, it fully agrees with our previous findings indicating that the secondary structure of Ea_r and Ea are identical, as judged from circular dichroism spectra [4]. Second, Ea_r was synthesized as a fusion protein in the periplasmic space of *E. coli* [3] and its disulphides have not been submitted to any reduction/reoxidation step. The present data reveal that the disulphides that were formed during the biosynthesis in *E. coli* are paired as they are in the toxin produced in the venom gland of the snake. Third, the data indicate that the drastic treatment to which Ea_r was submitted during its isolation, in particular the cleavage step by CNBr which was carried out under acidic conditions, did not alter the structural properties of the toxin. Finally, Ea_r can now be confidently used to explore the mutation-based effects on the curaremimetic function of Ea, as recently described by Pillet et al. [3].

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