

Crystallization and preliminary X-ray diffraction data of the Fab fragment of a monoclonal antibody against apamin, a bee venom neurotoxin

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Fab fragments of anti-apamin monoclonal antibodies have been purified to homogeneity and crystallized. The crystals belong to the monoclinic space group $P2_1$ with cell dimensions $a=99.0\pm0.3$ Å, $b=137.1\pm0.4$ Å, $c=76.0\pm0.2$ Å and $\beta=92.9\pm0.9^\circ$. They most likely contain four molecules in the asymmetric unit ($V_m=2.39$ Å³/Da). The possibility of the existence of non-crystallographic symmetry is discussed.

Apamin; Monoclonal antibody; Fab purification; X-ray crystallography

1. INTRODUCTION

X-ray crystallography has been extensively used to determine the three-dimensional structures of monoclonal immunoglobulin fragments (Fab) and, more recently, structures of Fab/protein antigen complexes. This has greatly contributed to our understanding of the molecular basis of antibody–antigen recognition (reviews [1,2]). Five such structure determinations have already been reported: three lysozyme/Fab complexes [3–5] and two influenza virus neuraminidase/Fab complexes [6]. The epitopes analysed were all discontinuous, each contained between 15 and 22 residues, and had a buried surface of 650–900 Å². The paratopes appeared to be rather flat surfaces. The structure of one Fab/hapten complex has also been investigated [7]. In this case the paratope appears to be a groove into which the hapten fits. The way a peptide interacts with the paratope of its specific antibody is not well understood. The only relevant reported study is that of a complex between a Fab and

a synthetic peptide analog of the C-helix of myohemerythrin [8].

Apamin, a toxin isolated from bee venom (*Apis mellifera*), is a small polypeptide of 18 amino acids ($M_r=2039$) (review [9]). It specifically blocks a class of Ca^{2+} -dependent K^+ channels [10]. Nuclear magnetic resonance and circular dichroism studies have depicted the compact structure of apamin in solution [11–13]. This type of molecule is of particular interest for studying the immune response to peptides and we have consequently used it as a model antigen. We have found that B and T cell responses to apamin are under the genetic control of the major histocompatibility complex [14,15]. A panel of 20 monoclonal antibodies (mAbs) have been raised against free apamin (mAbs H201) or BSA-coupled apamin (mAbs H196) and their fine epitope specificity has been determined using synthetic apamin analogs [16]. To contribute to the understanding of the nature of the interface established between a peptide and the combining site on a specific antibody, we have undertaken the purification of several anti-apamin mAbs with the goal of crystallizing Fabs and Fab/apamin complexes. In this paper we describe extensive purification, preparation and crystallization trials of the H196-293 mAb-derived Fab fragment (IgG₁,k) which binds apamin with an affinity of 3×10^{-10} M.

2. MATERIALS AND METHODS

Anti-apamin mAb (H196-293) was prepared as described [16]. Ascitic fluid was passed through a protein A-Sepharose column

Abbreviations: BSA, bovine serum albumin; FPLC, fast protein liquid chromatography; MES, 2-morpholinoethanesulfonic acid; HEPES, 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid; PEG, polyethyleneglycol; MPD, 2-methyl-2,4-pentanediol

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(Pharmacia) in 3 M NaCl, 1.5 M glycine, pH 8.9, and the bound IgGs were eluted with 0.1 M sodium citrate, pH 6. Fab fragments were obtained by papain digestion and partial purification on a Sephadex G-100 column according to the general procedure [17]. Isoforms of Fab were separated by FPLC (Mono Q column, Pharmacia) using a linear pH gradient [18]. Optimal separation was obtained by a 20 mM ethanolamine, pH 9.0/20 mM triethanolamine, pH 7.5 gradient, over a period of 180 min. Fractions of each isoform were collected and concentrated by vacuum dialysis to about 10 mg/ml in 5 mM HEPES, pH 6.8. In one case a further increase in purity was obtained using a cation exchange column (Mono S, Pharmacia) and a 20 mM MES, pH 5.8/20 mM HEPES, pH 7.6 gradient. Purity was checked at each step by SDS-polyacrylamide gel electrophoresis and by isoelectrofocusing (IEF; PhastSystem, Pharmacia).

Crystallization experiments were performed by the hanging drop vapor diffusion method at 20°C, initially using the incomplete factorial experimental design described by Carter et al. [19]. Once crystals had grown, crystallization conditions were refined by systematically testing a range of closely related conditions.

3. RESULTS AND DISCUSSION

Analysis of the Fab fraction by IEF showed three major isoforms of pI 8.3, 7.8 and 7.4 which were named I, II and III, respectively. They were purified by FPLC to 90–95% homogeneity. Each isoform was still able to bind to apamin as indicated by the increase in the observed pI of isoforms complexed with apamin. This was due to the highly basic character of apamin (Fig. 1). Crystals were obtained only when PEG 6000 was used as precipitant. Two suitable ranges of conditions were found for isoform II: (i) 15% PEG (w/v), pH 7.4–8.1 with or without 0.1 M NaCl; (ii) 5–10% PEG (w/v), pH 5.4–6.4, with or without either 0.1 M NaCl or 0.2 M ammonium sulfate. Crystals of isoform III grew between 10% and 15% PEG (w/v), pH 5.4–7.4 with 0.1 M NaCl. Isoform I did not crystallize in these experiments.

The best crystals were obtained with isoform II (5 mg/ml) in 13% PEG (w/v), 50 mM sodium phosphate, pH 7.7, and 50 mM NaCl. They grew as thin, roughly rectangular plates ($0.200 \times 0.100 \times 0.020$ mm), that diffracted to at least 3.5 Å resolution. Precession photographs indicated that they belong to the monoclinic space group $P2_1$ with cell dimensions $a = 99.0 \pm 0.3$ Å, $b = 137.1 \pm 0.4$ Å, $c = 76.0 \pm 0.2$ Å and $\beta = 92.9 \pm 0.9^\circ$. Assuming that each asymmetric unit contains four molecules (approximate M_r 50000 per polypeptide molecule) a $V_m = 2.39$ Å³/Da is obtained. This value is within the range generally observed for protein crystals [20]. Data from one of the crystals was collected in a Xentronics/Siemens area detector using a Rigaku RU 200 rotating anode generator as the X-ray source. Data reduction was carried out using the XENGEN package [21]. A total of 7727 independent reflections were obtained from 35926 observations. This constitutes 80% of the theoretically possible reflections at 3.8 Å. The unweighted absolute value R factor on intensities (R3 of XENGEN) is 0.155 at this resolution.

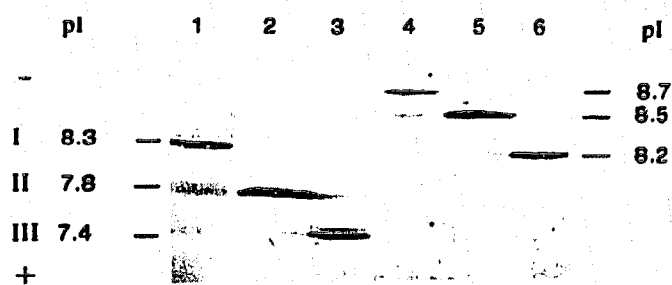


Fig. 1. Comparative electrophoretic migration in an IEF gel of the different isoforms of Fab H196-293 and of their complexes with apamin. Proteins were purified by FPLC (Mono Q column) as described in the text. (Lanes 1–3) Isoforms I, II and III respectively (15 ng each). (Lanes 4–6) Complexes of isoforms I (lane 4), II (lane 5) and III (lane 6) with apamin (ratio 1:1.5) after overnight incubation. The banding pattern was revealed by silver staining.

To explore the possibility that the four Fab molecules in the asymmetric unit are related by non-crystallographic symmetry operations, the self-rotation function was calculated using the program ROTFUN [22,23]. The 180° rotation section of this function shows a strong 222 point symmetry (Fig. 2). The heights of the peaks corresponding to the non-crystallographic dyads (in the periphery of the stereographic projection shown in Fig. 2) are about 60% of that of the origin peak. Based on this observation, the whole data set was re-indexed with all unit cell angles constrained to 90°. The orientation matrices obtained in these calculations systematically showed

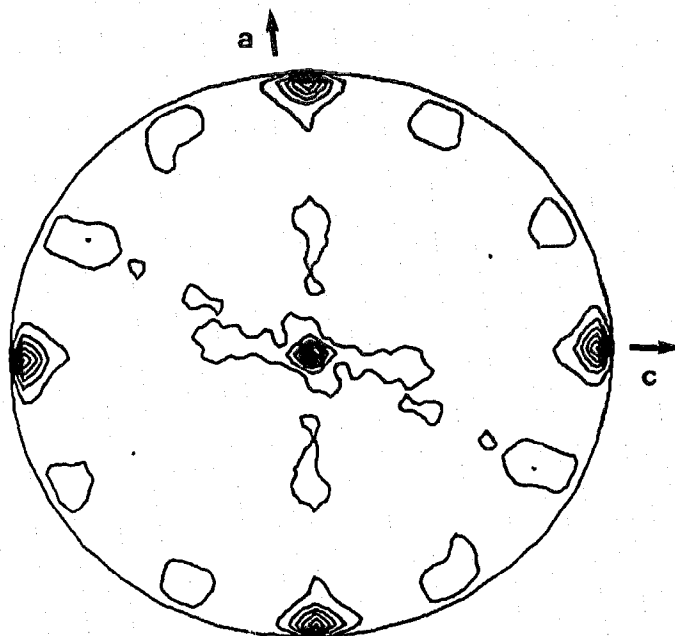


Fig. 2. Stereographic projection (180° rotation section) of the self-rotation function looking down the crystallographic b axis. Contours drawn every two root-mean-square (RMS) deviations of the whole rotation function map.

statistics that were worse than those obtained when using the monoclinic cell parameters described above. This pseudo-orthorhombic symmetry will be of great value in interpreting molecular replacement search functions, as only two essentially independent orientations should be found for the Fab molecule in the unit cell.

Fab/apamin complexes were prepared by mixing Fab and apamin solutions in several molar ratios (from 1:1 to 1:5). They were assayed for crystallization directly or after purification on a Mono Q column. Many different sets of conditions were tried, but no crystals of Fab/apamin complexes were obtained with any of the isoform or mAb used. The complexes induced a phase separation in PEG solutions and amorphous precipitates in MPD, but seemed fairly soluble in saline solutions. Large-scale production of Fab and of their complexes with apamin is proceeding to facilitate the search for crystallization conditions for the complexes.

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