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Crystallization of a carbamatase catalytic antibody Fab fragment and its complex with a transition-state analogue

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Catalytic antibodies showing carbamatase activity have significant potential in antibody-directed prodrug therapy against tumours. The Fab fragment of an IgG1 mouse monoclonal carbamatase catalytic antibody JC1 raised against a transition-state analogue, ethyl *N*-(3,5-dicarboxyphenyl)-*P*-{*N*-[5′-(2″,5″-dioxo-1″-pyrrolidinyl)oxy-1′,5′-dioxopentyl]-4-aminophenylmethyl}phosphonamidate, was obtained by digestion of the whole antibody with papain and was purified by two-step ion-exchange chromatography. Using hanging-drop vapour-diffusion crystallization techniques, three different crystal forms of the Fab fragment were obtained in the presence and absence of the transition-state analogue. All crystals diffract X-rays to between 3.5 and 3.2 Å resolution. The two crystal forms grown in the presence of the transition-state analogue contain up to four or eight copies of the Fab in the asymmetric unit and diffract to 3.5 and 3.2 Å, respectively. The crystal of the Fab alone is most likely to contain only two copies of the Fab in the asymmetric unit and diffracts to beyond 3.5 Å. Determination of the structure will provide insights into the active-site arrangement of this antibody and will help to increase our understanding of the molecular mechanisms by which the immune system can evolve catalytic function.

1. Introduction

Antibodies have been shown to catalyse a broad range of reactions with exquisite control over the reaction pathway (Lerner & Janda, 1995). Based on the natural occurrence of catalytic antibodies, one interesting clinical application of the use of abzymes is to convert prodrugs to active drugs at the desired site of action by harnessing their activity. This is an adaptation of so-called ADEPT (antibody-directed enzyme prodrug therapy) strategies in cancer, where a tumour-specific antibody is coupled to an enzyme that converts a chemotherapeutic agent to its active form at the tumour site (Bagshawe, 1987, 1990; Bagshawe *et al.*, 1988; Senter *et al.*, 1988, 1989). A common problem with ADEPT is that the enzyme is usually bacterial and the immune system raises a response against the enzyme, thus reducing its effectiveness. By using abzymes instead of bacterial enzymes, the problem is potentially resolved. ADAPT (antibody-directed abzyme prodrug therapy) is currently being developed to avoid the problems of ADEPT. Catalytic antibodies capable of activating prodrugs to generate cytotoxic drugs have potential as therapeutic reagents (reviewed in Blackburn *et al.*, 1996). Unfortunately, the catalytic efficiency of the abzymes prepared to date is usually low when compared with their natural enzyme counterparts. An important challenge in abzyme development is the level of understanding of the mechanism of action of these catalysts. The determination of the structural and mechanistic features of abzymes would lead to better manipulation of their properties. Advances in our understanding of abzyme structure and capabilities, together with significant improvements in the methods of generating novel abzymes, are paving the way toward the emergence of abzymes as a significant suite of novel molecular tools.

We previously reported the generation of antibodies that cleave carbamate esters *via* a disfavoured $B_{AC}2$ mechanism and which activate a carbamate prodrug to kill tumour cells *in vitro* (Wentworth *et al.*, 1996, 1997). The monoclonal antibodies (mAbs) were raised against a transition-state analogue ethyl *N*-(3,5-dicarboxyphenyl)-*P*-{*N*-[5′-(2″,5″-dioxo-1″-pyrrolidinyl)oxy-1′,5′-dioxopentyl]-4-aminophenylmethyl}phosphonamidate (TSA). Here, we report the preliminary crystallization of the Fab fragment of one of a panel of catalytic antibodies raised using this TSA. The IgG1 mAb JC1 has an affinity for the TSA of 1×10^{-9} *M* as determined by inhibition ELISA and activates the carbamate prodrug to give ~50% cell kill *in vitro* (Wentworth *et al.*, 1996). We report here the sequencing, isolation, crystallization and preliminary X-ray analysis of the Fab fragment of this antibody and its

complex with the transition-state analogue as the first stage in the structure determination.

2. Materials and methods

2.1. Preparation of JC1 monoclonal antibody

The JC1 IgG1 antibody was generated by conventional monoclonal antibody techniques, essentially as described previously (Wentworth *et al.*, 1996, 1997). Briefly, female Balb/c mice were immunized with TSA linked to keyhole limpet haemocyanin. MAb-secreting cell lines were selected initially on the basis of TSA binding. The catalytic activity of purified mAbs was determined by screening for activation of carbamate prodrug using the Sulforhodamine B (SRB) *in vitro* cell-kill assay (Wentworth *et al.*, 1996). JC1 IgG1 was purified from concentrated tissue-culture supernatant by protein-G chromatography.

2.2. Preparation and purification of Fab fragment

Antibody (1.6 mg ml^{-1} in 20 m*M* Tris–HCl pH 7.5 with 50 m*M* NaCl) was digested with papain at an IgG1:enzyme ratio of 100:1(*w*:*w*). Papain (2× crystallized, lyophilized, Sigma Chemical Co.) was pre-activated with 50 µ*M* cysteine in 2 m*M* EDTA solution. After 30 min activation, the papain solution was added to the protein solution [1:10(*v*:*v*)] and hydrolysis was allowed to continue for about 2 h at room temperature. The reaction was stopped by addition of *N*-ethylmaleimide (Sigma Chemical Co.) to a final concentration of 25 m*M*. After incubation for 20 min at room temperature, 2-mercaptoethanol was added to a concentration of 40 m*M*. The solution was dialysed at 277 K against several volumes of buffer *A* (20 m*M* Tris–HCl pH 8.0, 50 m*M* NaCl). The Fab fragment was separated from the Fc fragment by passing the digest mixture through a column of DEAE-Sepharose Fast Flow (Pharmacia) equilibrated with buffer *A*. The eluate was collected and dialysed against buffer *B* (50 m*M* sodium acetate pH 5.0, 50 m*M* NaCl) and applied to an SP-Toyopearl 650S (Toyo Soda) column equilibrated with buffer *B* for cation-exchange chromatography using an NaCl concentration gradient. The purity of the Fab was checked by both reducing and non-reducing SDS–PAGE. The yield of electrophoretically homogeneous Fab was 20% of the theoretical yield.

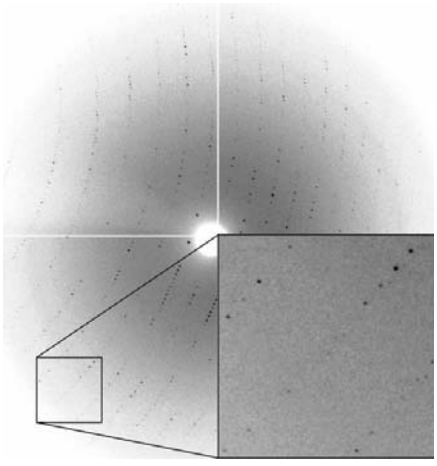

Figure 1
A representative 0.25° oscillation image of data collected on crystals of the form I Fab–TSA complex. The enlarged image on the right is a close-up version of the selected rectangle on the left. The outer edge of the selected square corresponds to 3.2 Å resolution.

Table 1
Crystallization conditions and data-collection statistics.

Values in parentheses are for the highest resolution range.

Complex	Fab–TSA (form I)	Fab–TSA (form II)	Fab (form III)
Precipitant solution	13% PEG 4000, 0.1 *M* sodium citrate pH 5.6, 12% 2-propanol	13% PEG 4000, 0.15 *M* sodium acetate pH 5.0, 12% 2-propanol	20% PEG 3350, 0.2 *M* disodium tartrate
Crystal morphology	Rhombohedral plates	Bricks	Bricks
Crystal size (mm)	0.1 × 0.1 × 0.05	0.6 × 0.2 × 0.2	0.2 × 0.1 × 0.1
Cryoprotection [%(*w*/*v*)]	15% glycerol	15% glycerol	20% glycerol
Oscillation range (°)	0.25	0.5	1.0
Space group	Primitive orthorhombic with at least one twofold screw axis	Primitive tetragonal, 42	$P2_12_12$
Unit-cell parameters			
a (Å)	65.8	185.1	122.8
b (Å)	218.1	185.1	138.2
c (Å)	301.8	139.9	71.3
α (°)	90	90	90
β (°)	90	90	90
γ (°)	90	90	90
No. of molecules in AU	8	4	2
V_M (Å^3 Da^{-1})	2.9	3.2	3.2
Resolution (Å)	15.0–3.2 (3.27–3.2)	15.0–3.5 (3.58–3.5)	15.0–3.5 (3.58–3.5)
R_{merge}	8.7 (29.0)	14.5 (23.5)	12.1 (33.3)
$\langle I/\sigma(I)\rangle$	15.0 (5.0)	8.2 (4.9)	10.4 (3.1)
Completeness	96.8 (97.8)	91.5 (91.8)	98.4 (92.7)
Multiplicity	3.9 (3.8)	3.6 (3.4)	4.4 (3.2)
Mosaicity (°)	0.25	0.9	1.1

2.3. Crystallization and preliminary X-ray analysis of the JC1 Fab in the presence and absence of the TSA

For crystallization, the Fab fragment solution was dialysed into buffer *A*. The protein concentration, estimated by the method of Bradford with IgG as a standard, was adjusted to approximately 10 mg ml^{-1} using a Viva Spin 10 concentrator (Viva Science) by centrifugation at 4000*g*. The complex of the Fab fragment with a transition-state analogue (TSA) was made by incubation of the Fab with the TSA in an approximately 1:5 molar ratio in buffer *A* for 1 h at room temperature. Crystallization of the Fab alone and the Fab–TSA complex was achieved using the hanging-drop vapour-diffusion technique. 1–2 µl of protein solution were mixed with an equal volume of precipitant and equilibrated over the precipitant well at 290 K. Initial crystallization trial conditions for the Fab fragment and the Fab–TSA complex were based on the Crystal Screen crystallization kits (Hampton Research).

In all cases, X-ray diffraction data were collected from flash-frozen crystals in a stream of nitrogen gas at 100 K using an Oxford Cryosystems Cryostream device and glycerol as a cryoprotectant. X-ray diffraction data were collected by the rotation method on a Quantum Q4 CCD detector system at an X-ray wavelength of 0.978 Å on station 14.2 at the CLRC Synchrotron Source, Daresbury (Table 1; Fig. 1). The data sets were processed using the *DENZO*/*SCALEPACK* package (Otwinowski & Minor, 1997) and subsequently handled using *CCP*4 software (Collaborative Computational Project, Number 4, 1994).

2.4. Sequencing of the JC1 variable region

Total RNA was prepared from 10 × 10^6 hybridoma cells using Trizol Reagent and cDNA generated using SuperscriptII with oligo-dT primers according to the manufacturer's instructions (InVitrogen). Heavy-chain (Fd) and light-chain genes were amplified using primers decribed by Huse *et al.* (1989). PCR amplifications were performed using the extensor Hi-fidelity

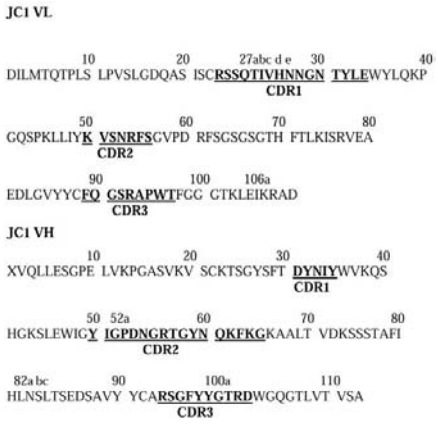

Figure 2
Amino-acid sequences (single-letter codes) of the variable regions of the JC1 light chain (VL) and heavy chain (VH). The CDRs are underlined and in bold. The numbering of the residues is according to Kabat *et al.* (1991).

master mix (Abgene). Cycle conditions for the heavy chain were five cycles of 365 K for 30 s, 318 K for 30 s, 345 K for 45 s and 30 cycles of 365 K for 30 s, 318 K + 0.5 K per cycle for 30 s, 345 K for 45 s, and for the light chain were five cycles of 365 K for 30 s, 325 K for 30 s, 345 K for 45 s and 30 cycles of 365 K for 30 s, 325 K + 0.3 K per cycle for 30 s, 345 K for 45 s.

Products were gel purified using the Nucleotrap kit (Clontech) and then sequenced with the relevant external primers using the Big Dye terminator sequencing method and analysed on an ABI prism 373 sequencer. In addition, internal primers were designed and used to verify the variable-region sequence of the heavy chain (H int-FOR, 5′-AGGCCGCCTTGACTG-TTGAC, and H int-REV, 5′-GGCCAGTG-GATAGACAGATG) and light chain (L int-REV, 5′-TGAGGCACCTCCAGATGTTA). Variable-region genes were assigned using the ImMunGeneTics information system (Lefranc *et al.* (1998) and the Kabat Antibody Sequence Database was accessed as described by Martin (1996).

3. Results and discussion

Initial crystallization-trial conditions for the Fab–TSA complex were based on Crystal Screen 1 crystallization kit (Hampton Research) condition No. 40 (0.1 *M* sodium citrate pH 5.6, 20% 2-propanol and 20% PEG 4000). Subsequent optimization of these crystallization conditions led to the production of two morphologically distinct crystal forms: rhombohedral plates (form I) and bricks (form II). Analysis of the preliminary data sets collected from crystals of form I show that they belong to the primitive crystal system class 22, with unit-cell parameters $a = 65.8$, $b = 218.1$, $c = 301.8$ Å and up to eight copies of the Fab molecule in the asymmetric unit. Analysis of the systematic absences confirms the presence of at least one screw axis (k) in this crystal. Analysis of preliminary data sets collected from crystals of form II show that they belong to the primitive crystal system class 42, with unit-cell parameters $a = 185.1$, $b = 185.1$, $c = 139.9$ Å and up to four copies of the Fab molecule in the asymmetric unit. The h and k axes appear to be pure twofold rotation axes based on the analysis of systematic absences in the diffraction pattern. The symmetry of the fourfold axis is currently unclear. The use of these crystals to solve the structure by molecular replacement is inconvenient owing to the high number of Fab molecules in the asymmetric unit. Therefore, we have opted not to pursue the structure determination of these crystals further and we attempted to crystallize the Fab fragment in the absence of the TSA in order to find a more suitable crystal form for molecular-replacement techniques.

Initial crystallization conditions of the Fab alone were based on PEG/Ion crystallization kit (Hampton Research) condition No. 36 (20% PEG 3350, 0.2 *M* disodium tartrate). Brick-shaped crystals up to 0.2 mm in size appeared within several days (form III). Analysis of a preliminary data set collected to 3.5 Å on these crystals with the auto-indexing routine in *DENZO* (Otwinowski & Minor, 1997) is consistent with a primitive crystal system, class 22. Analysis of systematic absences along all axes show that these crystals belong to space group $P2_12_12$. Taking the subunit molecular weight to be 47.5 kD suggests that the crystals contain two copies of the Fab molecule in the asymmetric unit, with a V_M of 3.2 Å^3 Da^{-1}, which is within the range given by Matthews (1977). Data-collection and processing statistics for all crystals are given in Table 1.

The deduced amino-acid sequence of the JC1 variable regions is shown in Fig. 2. The V_L gene belongs to the $V_\kappa1$ gene subgroup and is joined to $J_\kappa1$. The variable region of the heavy chain is encoded by a gene belonging to the V_H1 subgroup, with a J_H3 joining region and a diversity segment showing homology to D-FL16. Interestingly, the hypervariable loops contain nine basic amino-acid residues and seven tyrosines, some of which may contribute to the catalytic mechanism.

Our efforts are currently being directed towards solving the structure of crystal form III using molecular-replacement techniques. Once this structure is solved, an analysis of the crystals of the Fab–TSA complexes will then be carried out. An analysis of the active-site structure of this antibody will help to increase our understanding of the molecular mechanisms by which the immune system can evolve catalytic function.

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