Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

The preparation and crystallization of Fab fragments of a family of mouse esterolytic catalytic antibodies and their complexes with a transition-state analogue

Tatyana A. Muranova,[a,c] Sergey N. Ruzheinikov,[a] Svetlana E. Sedelnikova,[a] Arthur Moir,[a] Lynda J. Partridge,[a] Hiroyuki Kakinuma,[b] Naoko Takahashi,[b] Kazuko Shimazaki,[b] Jilian Sun,[b] Yoshisuke Nishi[b] and David W. Rice[a]*

[a]Krebs Institute for Biomolecular Research, Department of Molecular Biology and Biotechnology, University of Sheffield, Firth Court, Western Bank, Sheffield S10 2TN, England, [b]Laboratory of Life Science and Biomolecular Engineering, Japan Tobacco Inc., Yokohama, Kanagawa, Japan, and [c]Branch of Institute of Bioorganic Chemistry, Russian Academy of Sciences, 142292 Pushchino, Moscow Region, Russia

Correspondence e-mail: d.rice@sheffield.ac.uk

Received 11 April 2001
Accepted 19 June 2001

The Fab fragments of a family of mouse esterolytic monoclonal antibodies MS6-12, MS6-126 and MS6-164 have been obtained by digestion of whole antibodies with papain, purified and crystallized in a range of different forms either alone or in complex with a transition-state analogue. The crystals diffract X-rays to resolutions between 2.1 and 1.2 Å and are suitable for structural studies. The determination of these structures could be important in understanding the different catalytic power of each of these related catalytic antibodies.

1. Introduction

Antibodies have been shown to catalyze a broad range of reactions with exquisite control over the reaction pathway (Shultz & Lerner, 1986; Lerner *et al.*, 1991; Lerner & Janda, 1995). Catalytic antibodies generally show a high degree of substrate specificity but lack the catalytic efficiency of enzymes. Efforts to engineer antibodies with greater catalytic efficiency require a greater understanding of the molecular features controlling specificity and catalysis and of how such features are elicited through the constraints imposed by the transition-state analogue used in their production. To define the active-site geometries of catalytic antibodies and to enhance the knowledge of the molecular basis of antibody catalysis, structural studies are needed. Recently, there has been an explosion of activity in this area and such structural studies have employed antigen-binding fragments (Fab) rather than whole antibodies because of their ability to form crystals suitable for high-resolution analysis (Haynes *et al.*, 1994; Golinelli-Pimpaneau *et al.*, 1994; Zhou *et al.*, 1994; Charbonnier *et al.*, 1995, 1997; Hsieh-Wilson *et al.*, 1996; Patten *et al.*, 1996; Wedemayer *et al.*, 1997; Buchbinder *et al.*, 1998).

The immune response to a single hapten may produce a range of antibodies generated from the same hybridoma fusion that may be structurally related but exhibit marked differences in their levels of catalytic activity and/or substrate specificity (Patten *et al.*, 1996; Charbonnier *et al.*, 1997; Buchbinder *et al.*, 1998; Angeles *et al.*, 1993; Guo *et al.*, 1995). The structural diversity of antibodies raised to a single hapten makes the catalytic monoclonal antibody pool from a single hybridoma fusion an excellent model system for an experimental and theoretical approach to investigating the structure–function relationships in antibodies. Once a group of isoabzymes (structurally and mechanistically similar catalytic antibodies from the same immunization; Angeles *et al.*, 1993) have been identified, the molecular basis for their differences in catalytic proprties may become apparent either by inspection of their amino-acid sequences or by the determination of their three-dimensional structures and comparative molecular modelling (Charbonnier *et al.*, 1997; Angeles *et al.*, 1993., Guo *et al.*, 1995; Miyashita *et al.*, 1994).

As a contribution towards understanding the catalytic potential of antibodies, we have undertaken an investigation of a number of catalytic esterolytic monoclonal antibodies generated from the same hybridoma fusion with a transition-state analogue *N*-{[2-({[1-(4-carboxybutanoyl)amino]-2-phenylethyl}-hydroxyphosphinyl)oxy]acetyl}-2-phenylethylamine (TSA1; Kakinuma *et al.*, 1999; Takahashi *et al.*, 1999). These antibodies are clonally independent, but the sequences are highly related (unpublished results) and represent the natural mutants of ancestral germlines. Sequence differences are mainly found in the CDR2 and CDR3 in the heavy chains, with the light-chain sequence being almost identical. Whilst some of the antibodies display esterolytic activity to a phenylalanine ester R-12 {[({[(5-dimethylamino-1-naphthalene sulfonyl)-4-aminobutanoyl]-D-phenylalanyl}oxy)-acetyl]-2-phenylethylamine}, others in the series are inactive. Futhermore, there is no correlation between catalytic activity and hapten-binding affinity (Takahashi *et al.*, 1999). In order to understand the molecular basis of this phenomenon, we undertook the investigation of the structural properties of three catalytic esterolytic monoclonal antibodies MS6-12, MS6-126 and MS6-164, which differ by up to sixfold in their esterolytic activity

against a relevant phenylalanine ester R-12 (Kakinuma *et al.*, 1999). We report here the isolation, crystallization and preliminary X-ray analysis of the Fab fragments from these antibodies and of their complexes with the transition-state analogue as the first stage in the structure determination.

2. Materials and methods

2.1. The preparation and purification of the Fab fragments

Mouse monoclonal esterolytic catalytic antibodies MS6-12, MS6-126 and MS6-164 were prepared and purified as previously described (Kakinuma *et al.*, 1999; Takahashi *et al.*, 1999). Antibodies (5 mg ml^{-1} solutions in 75 m*M* sodium phosphate pH 8.0 including 75 m*M* NaCl, 5 m*M* NaN_3, 2 m*M* EDTA) were digested with papain at an IgG:enzyme ratio of 100:1(*w*/*w*). Papain (twice 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 protein solution [1:10(*v*/*v*)] and hydrolysis was allowed to continue for about 3 h in an ice–water bath as described for IgG2b (Guddat *et al.*, 1994). The reaction was stopped by the 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*. After 1 h, the solutions were extensively dialysed at 277 K against several volumes of buffer *A* (20 m*M* Tris–HCl pH 8.0, 50 m*M* NaCl).

The Fab fragments were separated from the Fc fragments by passing the digest mixture through a column of DEAE-Sepharose Fast Flow (Pharmacia) equilibrated with buffer *A*. The non-bound fractions were 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 for cation-exchange chromatography in a NaCl concentration gradient. The purity of the Fabs was checked by both reducing and non-reducing SDS–PAGE. N-terminal sequences of the Fc fragments were determined on an Applied Biosystem gas-phase sequencer, model 475A.

2.2. Crystallization and preliminary X-ray analysis

For crystallization, solutions of pure Fab fragment were 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 VivaSpin 10 concentrator (Viva Science) by centrifugation at 4000*g*. Complexes of the Fab fragments with TSA1 were made by incubation of the hapten with the Fab solution at an approximately 5:1 molar ratio in buffer *A* for 1 h at room temperature.

Crystallization of the Fab fragments and Fab–TSA complexes was achieved using the hanging-drop vapour-diffusion technique. In all but two cases (see below), 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 fragments and the complexes with hapten were based on the Crystal Screen crystallization kits (Hampton Research). The optimized crystallization conditions are given in Table 1.

Table 1
Optimized crystallization conditions for Fab fragments of esterolytic antibodies.

Antibody	MS6-164	MS6-126	MS6-12	
k_{cat}/k_{uncat}	4360	675	1950	
Antibody binding for TSA1 k_d (n*M*)	0.61	0.60	1.83	
Crystal type	Fab–hapten	Fab	Fab	Fab–hapten
Protein concentration (mg ml^{-1})	10	10	1	5.5
Precipitant solution	15–18%(*w*/*v*) PEG 8000, 0.5 *M* Li_2SO_4 pH 3.3†	2.0 *M* $NH_4H_2PO_4$, 0.1 *M* Tris–HCl pH 8.5	50 m*M* sodium acetate pH 5.0, 25 m*M* NaCl‡	22.5%(*w*/*v*) PEG 8000, 0.25 *M* $(NH_4)_2SO_4$, 0.1 *M* sodium cacodylate pH 5.0
Crystal morphology	Prisms	Plates	Prisms	Needles
Crystal size (mm)	0.6 × 0.6 × 0.3	0.5 × 0.3 × 0.1	0.1 × 0.15 × 0.25	0.6 × 0.05 × 0.05
Growing time	48 h	4 months	5 d	48 h
Glycerol cryoprotection [%(*w*/*v*)]	25	25	40	25

† After 24 h pre-equilibration, the pH in the reservoir solution was adjusted to the value of 3.3 by addition of acetic acid (see text for details). ‡ The concentration of NaCl in the well was gradually increased from 0.1 to 0.5 *M* with 0.1 *M* increments every 24 h (see text for details).

For crystallization of the MS6-12 Fab fragment, a drop containing protein at a concentration of 1 mg ml^{-1} at low ionic strength (50 m*M* sodium acetate pH 5.0, 25 m*M* NaCl) was suspended over a reservoir containing 0.1 *M* NaCl. Every 24 h, the NaCl concentration in the reservoir was increased by 0.1 *M*. Crystals first appeared at 0.3 *M* NaCl and achieved maximum dimensions at 0.5 *M* NaCl.

For crystallization of the MS6-164 Fab–hapten complex the protein solution was mixed with an equal volume of 15–18%(*w*/*v*) PEG 8000 in 0.5 *M* Li_2SO_4 and equilibrated against the precipitant solution. After equilibration for 24 h, 15 µl of 20% acetic acid was added to the reservoir solution to adjust the pH to 3.3.

X-ray diffraction data were collected in all cases from flash-frozen crystals in a stream of nitrogen gas at 100 K using an Oxford

Table 2
Data-collection statistics.

Values in parnetheses refer to the highest resolution shell.

Antibody	MS6-164	MS6-126	MS6-12	
Crystal type	Fab–hapten complex	Fab	Fab	Fab–hapten complex
Daresbury synchrotron beamline	14.1	9.6	9.6	14.1
Wavelength (Å)	1.244	0.870	0.870	1.488
Oscillation range (°)	1.0	0.5	0.4	0.5
Space group	*C*2	$P2_1$	$P2_12_12_1$	$P2_12_12_1$
Unit-cell parameters				
a, *b*, *c* (Å)	76.3, 117.1, 114.6	37.0, 138.3, 45.2	56.0, 65.3, 138.6	75.3, 93.8, 142.8
α, β, γ (°)	90, 90.1, 90	90, 106.1, 90	90, 90, 90	90, 90, 90
V_M† (Å^3 Da^{-1})	2.6	2.7	2.7	2.8
No. of molecules in asymmetric unit	1	2	1	2
Resolution (Å)	15.0–2.1 (2.15–2.1)	15.0–1.75 (1.78–1.75)	10–1.22 (1.24–1.22)	10–2.1 (2.15–2.1)
Observed reflections	161896	138196	1177215	214346
Independent reflections	51508	39166	141368	50305
$I/\sigma(I)$	18 (2.0)	31 (4.7)	32 (3.4)	11 (3.0)
R_{merge} (%)	5.2 (30.6)	3.7 (18.3)	3.5 (21.9)	10.0 (32.3)
Completeness (%)	88.5 (47.5)	89.1 (58.4)	93.4 (63.3)	85.0 (49.5)

† V_M (Matthews, 1977) was calculated taking the subunit molecular weight to be 49.0 kDa.

Cryosystems Cryostream device and glycerol as a cryoprotectant (see Table 1 for details). Data collection was performed at the SRS Daresbury Laboratory with a Quantum Q4 CCD detector (Table 2) and the data were processed using the *DENZO*/*SCALEPACK* package (Otwinowski & Minor, 1997).

3. Results and discussion

Practical aspects of Fab investigation involve preparation of a homogenous Fab species following fragmentation by partial digestion with either papain or pepsin (Harlow & Lane, 1988). Sequence variation in the hinge region in distinct subclasses of IgG (IgG1, IgG2a, IgG2b, IgG3; Kabat *et al.*, 1991) results in significant differences in susceptibility to proteolysis and the nature of the cleavage pattern (Parham, 1983; Schreier *et al.*, 1981; Parham, 1986). Conditions for proteolytic hydrolysis of the mouse monoclonal MS6-12, MS6-126 and MS6-164 IgG2a antibodies with papain were first optimized in preliminary experiments. These resulted in the optimum choice of a substrate-to-enzyme ratio of 100:1, with preactivation of papain by cysteine at a twofold to fourfold molar excess. SDS–PAGE analysis of hydrolysates under reducing conditions shows that the heavy chain was completely hydrolysed. The Fab fragments were separated from the Fc fragments by anion-exchange chromatography on DEAE-Sepharose and further purified on an SP-Toyopearl cation-exchange column. The Fab purity was checked by both reducing and non-reducing SDS–PAGE. Under non-reducing conditions Fabs migrated as a single band at approximately 50 kDa, whereas under reducing conditions they appeared as two closely adjacent bands at about 25 kDa. No other bands could be observed. The final yields of electrophoretically homogeneous Fab fragments for different antibodies were between 40 and 50% of the theoretical yield.

The hinge region of the mouse IgG2a antibodies is thought to contain three disulfide cross-links between adjacent heavy chains. N-terminal sequence analysis of the separated Fc fragments revealed only the sequence Cys-Pro-Ala-Pro-, suggesting papain cleaves the peptide bond between Lys241 and Cys242 (numbering according to Kabat *et al.*, 1991) of the heavy chain.

Initial crystallization trial conditions for the Fab fragments and the complexes with hapten produced crystals of the MS6-126 and MS6-12 Fab fragments and crystals of the MS6-164 and MS6-12 Fab–hapten complexes. Refined crystallization conditions are summarized in Table 1.

Initial crystals of the MS6-164 Fab–hapten complex were obtained in Crystal Screen condition 50 [15%(*w*/*v*) PEG 8000, 0.5 *M* Li_2SO_4]. Subsequent optimization showed that pH was very important for crystal formation and the best crystals were grown when the reservoir solution was adjusted to pH 3.3 with dilute acetic acid after 24 h pre-equilibration.

In the absence of hapten, the MS6-12 Fab fragment was found to differ in its solubility from the other Fab fragments of the family, being insoluble at low ionic strength at protein concentrations greater than 2 mg ml^{-1}. On dialysis of a solution of MS6-12 Fab at 3 mg ml^{-1} against a low-salt buffer (50 m*M* sodium acetate pH 5.0, 25 m*M* NaCl), the MS6-12 Fab fragment formed microcrystals. To obtain large crystals, a drop of the above protein solution at 1 mg ml^{-1} was subjected to an increase of the protein concentration in the drop by a stepwise increase of the NaCl concentration in the reservoir from 0.1 to 0.5 *M* over 5 d. The crystals appeared after equilibration of the drop against 0.3 *M* NaCl; further increasing the salt concentration to 0.4 and to 0.5 *M* resulted in the growth of larger crystals (up to 0.1 × 0.15 × 0.25 mm).

Test data were collected at the Daresbury Synchrotron and showed that the various crystals that were obtained diffracted X-rays to between 2.1 and 1.22 Å resolution and are suitable for a full structure determination. Preliminary data sets were therefore collected at the synchrotron and representative oscillation images for all data sets are given in Fig. 1. The data-processing statistics for the different crystals are summarized in Table 2.

Our efforts are currently being directed towards solving the structures of these Fab fragments and their complexes with a transition-state analogue using molecular-replacement techniques. A detailed comparison of these against the activity profile of the antibodies will help to increase our understanding of the molecular mechanisms by which the immune system can evolve catalytic function.

We are grateful to Dr Vladimir Barynin for valuable advice. We thank the support

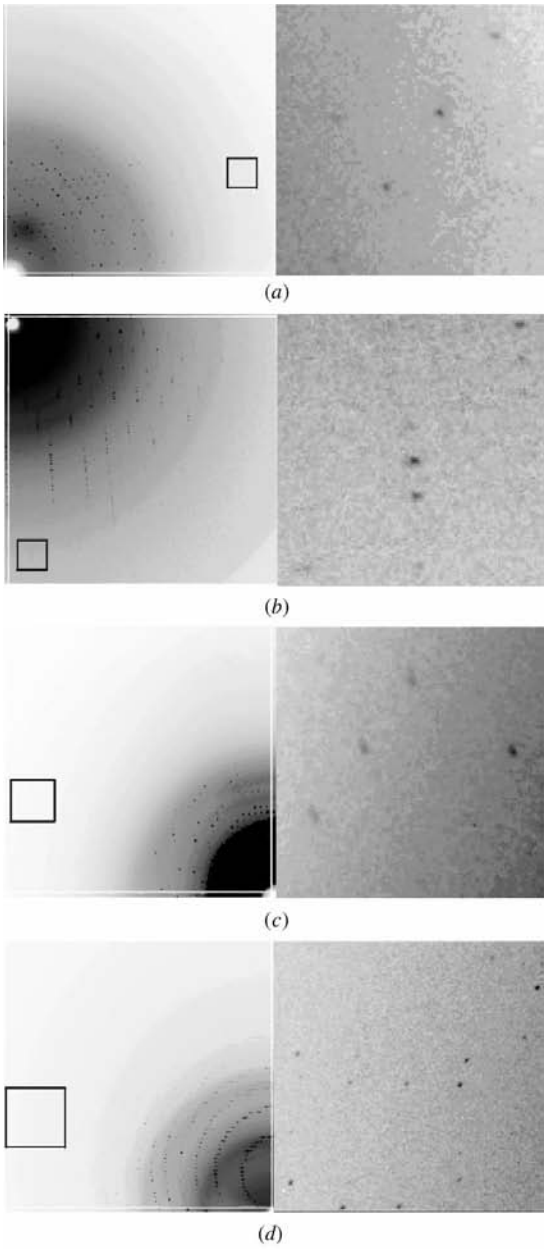

Figure 1
Representative oscillation images for the data collection on the Fab crystals. The enlarged images on the right are close-up versions of the selected rectangle on the left. (*a*) 1° oscillation image of the MS6-164 Fab–hapten complex; the middle of the selected square corresponds to 2.1 Å resolution; (*b*) 0.5° oscillation image of the MS6-126 Fab; the middle of the selected square corresponds to 1.75 Å resolution; (*c*) 0.4° oscillation image of the MS6-12 Fab; the middle of the selected square corresponds to 1.22 Å resolution; (*d*) 0.5° oscillation image of the MS6-12 Fab–hapten complex; the middle of the selected square corresponds to 2.1 Å resolution.

staff at the Synchrotron Radiation Source at the CCLRC Daresbury Laboratory for assistance with station alignment. We are grateful to Dr Masashi Miyano (RIKEN Harima Institute) for valuable advice for Fab preparation. We also thank the support staff at the Laboratory of Life Science and Biomolecular Engineering, Japan Tobacco Inc. for preparing the transition-state analogue TSA1 and the catalytic monoclonal antibodies. This work was supported by the New Energy and Industrial Development Organization, the British Council, BBSRC and the Wellcome Trust. The Krebs Institute is a designated BBSRC Biomolecular Science Centre and a member of the North of England Structural Biology Centre.

References

Angeles, T. S., Smith, R. G., Darsley, M. J., Sugasawara, R., Sanchez, R. I., Kenten, J., Schultz, P. G. & Martin, M. T. (1993). *Biochemistry*, **32**, 12128–12135.

Buchbinder, J. L., Stephenson, R. C., Scanlan, T. S. & Fletterick, R. J. (1998). *J. Mol. Biol.* **282**, 1033–1041.

Charbonnier, J.-B., Carpenter, E., Gigant, B., Golinelli-Pimpaneau, B., Eshhar, Z., Green, B. S. & Knossow, M. (1995). *Proc. Natl Acad. Sci. USA*, **92**, 11721–11725.

Charbonnier, J.-B., Golinelli-Pimpaneau, B., Gigant, B., Tawfik, D. S., Chap, R., Schindler, D. G., Kim, S.-H., Green, B. S., Eshhar, Z. & Knossow, M. (1997). *Science*, **275**, 1140–1142.

Golinelli-Pimpaneau, B., Gigant, B., Bizebard, T., Navaza, J., Saludjian, P., Zemel, R., Tawfik, D. S., Eshhar, Z., Green, B. S. & Knossow, M. (1994). *Structure*, **2**, 175–183.

Guddat, L. W., Shan, L., Anchin, J. M., Linthicum, D. S. & Edmundson, A. B. (1994). *J. Mol. Biol.* **236**, 247–274.

Guo, J., Huang, W., Zhou, G. W., Fletterick, R. J. & Scanlan, T. S. (1995). *Proc. Natl Acad. Sci. USA*, **92**, 1694–1698.

Harlow, E. & Lane, D. (1988). *Antibodies: A Laboratory Manual*, ch. 15, pp. 626–629. Cold Spring Harbor, NY, USA: Cold Spring Harbor Press.

Haynes, M. R., Stura, E. A., Hilvert, D. & Wilson, I. A. (1994). *Science*, **263**, 646–652.

Hsieh-Wilson, L. C., Schultz, P. G. & Steven, R. C. (1996). *Proc. Natl Acad. Sci. USA*, **93**, 5363–5367.

Kabat, E. A., Wu, T. T., Perry, H. M., Gottesman, K. S. & Foeller, C. (1991). *Sequences of Proteins of Immunological Interest*, NIH Publication No. 91-3242, pp. 670–678. Washington, DC: National Institutes of Health.

Kakinuma, H., Shimazaki, K., Takahashi, N., Niihata, S., Aoki, Y., Hamada, K., Matsushita, H. & Nishi, Y. (1999). *Tetrahedron*, **55**, 2559–2572.

Lerner, R. A., Benkovic, S. J. & Schultz, P. B. (1991). *Science*, **252**, 659–667.

Lerner, R. A. & Janda, K. D. (1995). *Exp. Suppl.* **73**, 121–138.

Matthews, B. W. (1977). *X-ray Structure of Proteins*, edited by H. Neurath & R. L. Hill, 3rd. ed., Vol. 3, pp. 468–477. New York: Academic Press.

Miyashita, H., Hara, T., Tanimura, R., Tanaka, F., Kikuchi, M. & Fujii, I. (1994). *Proc. Natl Acad. Sci. USA*, **91**, 6045–6049.

Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.

Parham, P. (1983). *J. Immunol.* **131**, 2895–2902.

Parham, P. (1986). *Cellular Immunology*, 4th ed., edited by D. M. Weir, Vol. 1, ch. 14, pp. 14.1–14.23. California: Blackwell.

Patten, P., Gray, N. S., Yang, P. L., Marks, C. B., Wedemayer, G. J., Boniface, J. J., Stevens, R. C. & Schultz, P. G. (1996). *Science*, **271**, 1086–1091.

Schreier, P. H., Bothwell, A. L. M., Mueller-Hill, B. & Baltimore, D. (1981). *Proc. Natl Acad. Sci. USA*, **78**, 4495–4499.

Shultz, P. B. & Lerner, R. A. (1986). *Science*, **269**, 1835–1842.

Takahashi, N., Kakinuma, H., Hamada, K., Shimazaki, K., Takahashi, K., Niihata, S., Aoki, Y., Matsushita, H. & Nishi, Y. (1999). *Eur. J. Biochem.* **261**, 108–114.

Wedemayer, G. J., Wang, L. H., Patten, P. A., Schultz, P. G. & Stevens, R. C. (1997). *J. Mol. Biol.* **268**, 390–400.

Zhou, G. W., Guo, J., Huang, W., Fletterick, R. J. & Scanlan, T. S. (1994). *Science*, **265**, 1059–1064.