FEBS 17188 FEBS Letters 389 (1996) 195–198

Characterisation, crystallisation and preliminary X-ray diffraction analysis of a Fab fragment of a rat monoclonal antibody with very high affinity for the human muscle acetylcholine receptor

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Received 18 March 1996

Abstract The Fab fragment of a rat monoclonal antibody (no. 192) with very high affinity for the main immunogenic region of the human muscle nicotinic acetylcholine receptor (AChR) has been purified, characterised and crystallised using vapour diffusion techniques. Its K_d for human AChR was determined to be 5×10^{-11} M. Its cross-reactivity pattern suggests that residue α23 of the AChR strongly affects its epitope. Crystals suitable for X-ray analysis, obtained by micro- and macroseeding techniques, belong to the orthorhombic space group $C222_1$ and they diffract to 2.8 Å resolution using synchrotron radiation. The unit cell dimensions are $a=83.4$ Å, $b=110.0$ Å and $c=212.2$ Å and there are two Fab molecules per asymmetric unit.

Key words: Myasthenia gravis; Acetylcholine receptor; Main immunogenic region; Monoclonal antibody; Crystallisation; X-ray crystallography

1. Introduction

The nicotinic acetylcholine receptor (AChR) of mammalian muscle is a ligand-gated cation channel, composed of five homologous subunits in a stoichiometry $\alpha_2\beta\gamma\delta$ or $\alpha_2\beta\varepsilon\delta$ [1]. AChR appears to be the target of an antibody-mediated autoimmune response, which causes the disorder myasthenia gravis (MG). Anti-AChR autoantibodies cause AChR loss and blockage of the function of AChR molecules, resulting in failure of the neuromuscular transmission [2].

The majority of the anti-AChR antibodies in sera of MG patients compete for binding to a region in the extracellular surface of the α-subunit of the AChR, called the main immunogenic region (MIR) [3,4]. Anti-MIR monoclonal antibodies (mAbs) induce experimental MG when injected into rats [4,5] and when added to cell cultures cause AChR loss via antigenic modulation [6,7]. Contrary to the intact antibodies, their Fab fragments, being univalent, do not crosslink the AChR molecules, and thus they do not cause antigenic modulation. In fact it has been shown, in muscle cell cultures, that these Fabs are capable of efficiently protecting the AChR against loss induced by human MG patients' sera [6,7], suggesting that they may be therapeutically useful.

Anti-MIR mAb binding has been localised between amino acid residues 67 and 76 of the α-subunit of Torpedo and human AChR [8]. Peptide analogue studies revealed the antigenic role of each residue within 67–76; the segment 67–71 proved the most critical and sufficient for mAb binding [9]. The approximate location of the MIR on the intact AChR has been recently determined by electron microscopy to be at the extreme synaptic end of the α-subunit of the AChR [10]. Despite thorough investigation of the MIR, little is known about the characteristics of the anti-MIR antibodies. The detailed knowledge of the structure of anti-MIR Fabs is essential in order to understand the molecular details of the interaction between the AChR and anti-MIR antibodies. Based on this knowledge we will be able to construct high affinity humanised mutants of antibody fragments for use as efficient protectors of the AChR against the pathogenic activity of anti-AChR antibodies, in trials for MG treatment.

In this report we present results concerning the anti-MIR Fab 192. The Fab fragment was extensively purified and its affinity for the human muscle AChR was determined. Subsequently the Fab was crystallised and X-ray diffraction data were obtained at 2.8 Å resolution, which is currently used for structure determination.

2. Materials and methods

2.1. Protein purification

The anti-MIR mAb 192 is an IgG2b antibody derived from the fusion of the spleen cells of a Lewis rat immunised by intact human muscle AChR, with the non-secreting mouse myeloma cell line S194/5.XXO.BD.1. The mAb preparation was obtained from hybridoma serum-free supernatants, concentrated 100 times by Amicon ultrafiltration and dialysed against phosphate-buffered saline (PBS, pH 7.4). The concentrate was applied on a 20 ml diethylaminoethyl (DEAE)-Sepharose Fast Flow (Pharmacia), equilibrated with Tris-HCl 20 mM, pH 8.0, and eluted using a continuous salt gradient, 0–1 M NaCl, in the same buffer. The purified mAb was treated for 1 h with mercuripapain (Sigma) at 37°C, at an enzyme/substrate ratio of 1:100 in 150 mM sodium-phosphate buffer, pH 7.0, containing 2 mM EDTA and 10 mM L-cysteine. The reaction was halted by the addition of iodoacetamide to a final concentration of 25 mM.

The Fab was separated from the Fc and traces of remaining intact IgG by running the digest at pH 9.5 (20 mM ethanolamine-HCl) through a 40 ml Q-Sepharose Fast Flow anion exchange column (Pharmacia), using a linear salt gradient 0.0–0.5 M NaCl. The Fab fractions were further purified using a chromatofocusing column (Polybuffer Exchanger 94, Pharmacia) equilibrated with 25 mM ethanolamine pH 9.4 by running a shallow pH gradient 9.0–8.0 using Polybuffer 96-HCl pH 8.0. Finally, the purified Fab was loaded on a gel filtration column (Sephacryl S-200 HR, Pharmacia) to remove the Polybuffer. For all chromatographic steps an FPLC system (Pharmacia) was used.

2.2. AChRs, radioimmunoassays and affinity determination

Extracts of cell culture of the human rhabdomyosarcoma cell line TE671 were used as human muscle AChR source. Rat and mouse

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PII S0014-5793(96)00579-0

skeletal muscle extracts were used as sources of rat and mouse AChR, respectively. Binding of the intact mAb 192 and its proteolytic Fab fragments, as well as of the intact anti-MIR mAbs 198 and 35, to AChRs from various sources was tested by radioimmunoassay using [^{125}I]α-bungarotoxin-labelled AChR [11]. Their binding affinities were calculated by Scatchard plots [12].

2.3. Crystallisation and X-ray data collection

The purified Fab solution was concentrated to 5–15 mg/ml using Amicon Centricon 10 microconcentrator. Protein concentration was determined by the Bradford Coomassie blue protein assay [13]. Crystallisations were carried out using the sitting and hanging drop techniques at 4°C, 16°C and room temperature (20–23°C). 2–5 μl of the protein solution was mixed with an equal volume of reservoir solution containing 10–30% w/v PEG 6000 (Hampton) and 0–300 mM NaCl. All reservoir solutions contained 2mM EDTA and 0.02% (w/v) sodium azide. The pH was screened over a range from 6.0 to 9.5 and the concentration of the protein, PEG 6000 and NaCl was optimised. Also, the influence of various salts on the crystallisation was examined (LiCl, $(NH_4)_2SO_4$, $CaCl_2$, Li_2SO_4, $MgCl_2$, HCOONa) at concentrations of 0.1–0.5 M, but the best crystals were obtained with NaCl.

Diffraction data were recorded from Fab crystals on stations PX 9.5 and 9.6 of the Synchrotron Radiation Source (SRS-CLRC) at Daresbury Laboratory, UK, using a 30 cm diameter MAR Research image plate, and on the EMBL Synchrotron beamline X 11 at DESY, Hamburg. Raw data images were indexed, integrated and corrected for Lorentz and polarisation effects using the program DENZO [14]. All data were scaled and merged using the program SCALEPACK [14].

3. Results and discussion

3.1. Purification

Under the conditions described in Section 2 for the Q-Sepharose fractionation of the mAb proteolysate, the Fab fragments eluted as two adjacent peaks in the flow through and very early in the gradient whereas the intact IgG and the Fc fragment eluted at much higher salt concentrations. The fractions containing the Fab fragment were initially analysed by both reducing and non-reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Under non-reducing SDS-PAGE, all Fab aliquots migrated as a single band at approximately 50 kDa whereas under reducing conditions they appeared as two adjacent bands at 25 kDa.

However, when the same fractions were analysed by analytical isoelectric focusing (IEF) using agarose gel (Pharmacia), each of the two Fab containing peaks was analysed into several bands. This heterogeneity in pI is observed in several cases of Fabs [15,16] and is known to prevent the growth of diffraction quality crystals. Therefore, further purification with the chromatofocusing column was an essential step. Fig. 1 illustrates the chromatofocusing analysis of the first eluted peak from the Q-Sepharose column, into three major peaks. Analytical IEF gels of these peaks showed almost complete separation of the isoforms (Fig. 2). All fractions were tested for binding to the [^{125}I]α-bungarotoxin-labelled AChR. Fractions of the first and second peak in Fig. 1 were found to bind equally well to AChR, whereas the protein of the third peak had a much lower affinity for AChR.

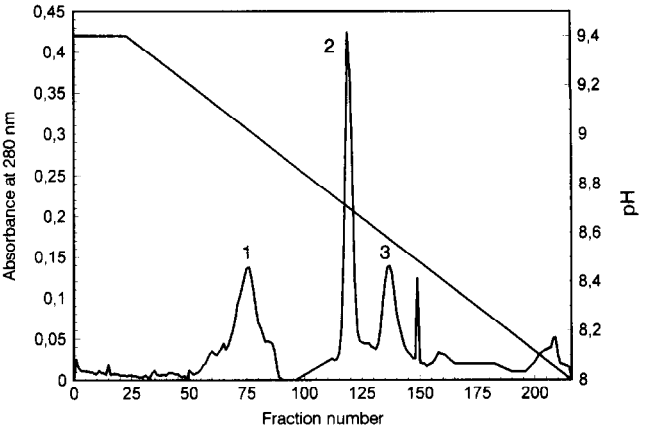

Fig. 1. Schematic presentation of the elution of the various isoforms of Fab 192 on a Polybuffer Exchanger 94 (PBE 94, Pharmacia) column. The column (70 ml) was equilibrated with 25 mM ethanolamine-HCl, pH 9.4 and eluted with Polybuffer 96-HCl, pH 8.0. A linear pH gradient 9.0–8.0 was formed. Fractions (5 ml) were collected at a flow rate of 1.2 ml/min. All peaks contain Fab fragments of different pI.

3.2. Affinity determination

Table 1 summarises the binding affinities of mAb and Fab 192 for AChRs from various species and compares them with those of two extensively studied pathogenic anti-MIR mAbs (nos. 35 and 198). It is apparent that the mAb 192 affinity for the human muscle AChR is about 100-fold higher than the corresponding affinity of mAbs 35 and 198.

The highly reduced binding affinity of mAb 192 for rat, but not for mouse AChR is surprising. Use of AChR hybrids has shown that the epitope for mAb 192 on human α subunit is not affected when the human β, γ and δ subunits are substituted by the, quite different, Torpedo β, γ and δ subunits (Loutrari and Tzartos, unpublished observations). Therefore, the reduced affinity of mAb192 for rat AChR should be due to differences within the α subunit and not to conformational constraints imposed by the other rat subunits. There are only two residues in the extracellular parts of the AChR α subunit (residues α1–209, 268–292 and 428–437) in which the rat α subunit differs from both the human and mouse α subunits [17,18]. These are residues 23 (Glu→Gly) and 195 (Asp, Thr→Asn). α195 is next to Cys192-Cys193 that are involved in the acetylcholine binding, which is remote from the MIR

Table 1
Affinities (K_d in nM) of anti-MIR antibodies for intact AChRs from various sources[a]

Anti-MIR antibody	AChR from:			
	Human muscle	Rat muscle	Mouse muscle	Torpedo electric organ
mAb 192	0.01	646	0.04	∞
Fab 192	0.05	N.T.	N.T	∞
mAb198	21.6	7.4	16.4	1.8
mAb35	>20	42	37	0.6

[a]The K_ds were calculated by Scatchard plots using the results of radioimmunoassays with [^{125}I]α-bungarotoxin-labelled AChRs from cell culture or muscle extracts.
N.T.: not tested.

on the intact AChR [10]. Therefore, we conclude that, on the intact AChR, α23 may be located near the MIR segment α67–76, or that elimination of the negative charge of α23 strongly affects the MIR epitope for mAb 192.

3.3. Crystallisation

The initial crystallisation trials of the protein which was eluted in the second peak in Fig. 1 yielded crystals from drops containing equal volumes of aqueous protein solution (12 mg/ml) and a reservoir solution containing 18% w/v PEG 6000 and 150 mM NaCl, buffered with 100 mM bis-Tris-HCl pH 7.5 at 16°C. Under these conditions crystals were obtained only by the high affinity Fab isoforms eluted from the PBE column (first and second peak in Fig. 1). Nevertheless these crystals were small to medium size rods, all of poor morphology and often badly twinned or fused together. Therefore, micro- and macroseeding techniques [19] were employed in order to obtain larger, single crystals. One of the initial crystal clusters was crushed in 100 μl of stabilising solution (12% w/v PEG 6000), to produce a stock solution of microseeds. These microseeds were diluted before being added to fresh drops. The medium size single rods which were grown from the microseeded drops were transferred after a few days to fresh drops, in order to optimise their size. The macroseeded drops were equilibrated over a reservoir containing a lower concentration of precipitant than the concentration which originally yielded crystals.

Obtaining well ordered crystals of Fab 192, suitable for X-ray diffraction analysis, was found to be critically dependent on an efficient (single band in IEF gel) and fast purification, performed just before crystallisation.

3.4. X-ray crystallographic analysis

Two data sets were collected (3.2 and 2.8 Å) to form the complete data set which is currently used for structural analysis (Table 2). The crystals belong to orthorhombic space group $C222_1$ with unit cell dimensions a=83.4 Å, b=110.0 Å and c=212.2 Å, with two Fab molecules per asymmetric unit and 49.9% of solvent in the crystal. The final data set is 90.4% complete to 4.0 Å and 74.8% complete to 2.8 Å, with an overall R-factor of 8.5%. Attempts to determine the structure by molecular replacement, using the programs AMoRe [20] and X-PLOR [21] and existing Fab structures from the Protein Data Bank as search models, are in progress. The primary structure of the heavy and light variable domains of mAb 192 has been recently determined by cloning and sequencing their cDNA (A. Mamalaki, P. Tsantili, C. Skarimba and S.J. Tzartos, unpublished data).

Table 2
X-ray data collection and processing statistics

	SRS set I	SRS set II
Resolution (Å)	3.2	2.8
Crystal to detector distance	463 mm	465 mm
Exposure time pezr image	100 s	120 s
Wavelength (Å)	1.00	0.87
Number of crystals	1	1
Oscillation range	1.5°	1.0°
N_m	18395	26126
N_u	11295	16163
Overall completeness	67.8%	65.4%
$I/\sigma I$	12.80	16.83
R_{merge} (%)	6.7	7.0
Outer shell (Å)	3.3–3.2	2.9–2.8
Completeness of the outer shell	45.05%	40.10%

N_m: number of measurements recorded; N_u: number of unique reflections; R_{merge}: $\Sigma_I(\Sigma_j|I_{ij}-<I_i>|)/\Sigma_I I_i$, $I_{i,h}$ is the scaled intensity of the jth observation of each unique reflection i and $<I_i>$ is the mean value.

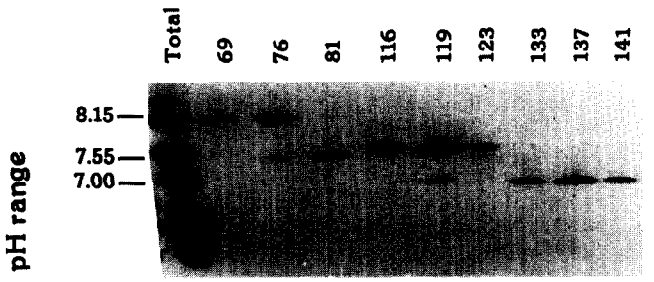

Fig. 2. Native isoelectric focusing agarose gel (pH 3.5–10) of purified Fab molecules eluted from the PBE 94 column. The gel was stained with Coomassie brilliant blue R-250, according to the manufacturer's (Pharmacia) protocol. Lane indications correspond to the eluted fraction numbers (the indication 'total' corresponds to the mAb proteolysate).

The X-ray structure analysis of the Fab 192 should provide insight into the interaction with the human AChR of an antibody that has all the characteristics of an efficient protecting agent of the myasthenic patients' AChR: it binds at the MIR of the human AChR with an unusually high affinity for the receptor (the K_d value is 0.01 nM for the intact mAb and 0.05 nM for the Fab), i.e. it is a unique candidate for future therapeutic applications in myasthenia gravis.

Acknowledgements: Supported by grants from the Association Française contre les Myopathies, the Biomed-1 program of EU (BMH1-CT93-1100) and the HCM program of EU (CHRXCT94-0547). We are grateful to the staff of the Synchrotron Radiation Source, Daresbury Laboratory, UK, and the staff of the EMBL outstation at DESY, for help with the data collection, and Drs A. Papageorgiou and D. Leonidas for help with the data processing. We are especially thankful to Prof. M. Kokkinidis, Ms. D. Kotsifaki, Dr. Y. Papanikolaou and Dr. M. Vlassi (I.M.B.B., Heraclion/Crete) for valuable help with the purification and the crystallisation trials and their general support and encouragement.

References

[1] Changeux, J.-P. (1990) Fidia Res. Found. Neurosci. Award Lect. 4, 21–168.
[2] Lindstrom, J., Shelton, D. and Fungii, Y. (1988) Adv. Immunol. 42, 233–284.
[3] Tzartos, S.J. and Lindstrom, J. (1980) Proc. Natl. Acad. Sci. USA, 77, 755–759.
[4] Tzartos, S.J., Cung, M.T., Demange, P., Loutrari, H., Mamalaki, A., Marraud, M., Papadouli, I., Sakarellos, C. and Tsikaris, V. (1991) Mol. Neurobiol. 5, 1–29.
[5] Loutrari, H., Kokla, A. and Tzartos, S.J. (1992) Eur. J. Immunol. 22, 2449–2452.
[6] Tzartos, S.J., Sophianos, D. and Efthimiadis, A. (1985) J. Immunol. 134, 2343–2349.
[7] Sophianos, D. and Tzartos, S.J. (1989) J. Autoimmun., 2, 777–789.
[8] Tzartos, S.J., Kokla, A., Walgrave, S. and Conti-Tronconi, B. (1988) Proc. Natl. Acad. Sci. USA 85, 2899–2903.
[9] Papadouli, I., Sakarellos, C. and Tzartos, S.J. (1993) Eur. J. Biochem. 211, 227–234.
[10] Beroukhim, R. and Unwin, N. (1995) Neuron 15, 323–331.
[11] Lindstrom, J., Einarson, B. and Tzartos, S.J. (1981) Methods Enzymol. 74, 432–460.
[12] Scatchard, G. (1949) Ann. NY Acad Sci 51, 660–672.

[13] Bradford, M. (1976) Anal. Biochem. 72, 248–254.
[14] Gewirth, D. (1995) The HKL Manual. A Description of the Programs Denzo, XDisplayF, Scalepack. Yale University, New Haven, CT.
[15] Cygler, M., Boodhoo, A., Lee, J. and Anderson, W. (1987) J. Biol. Chem. 262, 643–648.
[16] Vatzaki, E.H., Acharya, K.R., Oikonomakos, N.G. and Tzartos, S.J. (1993) Protein Sci., 2, 1770–1772.
[17] Luther, M.A., Schoepfer, R., Whiting, P., Casey, B., Blatt, Y., Montal, M.S., Montal, M. and Lindstrom, J. (1989). J. Neurosci. 9, 1082–1096.
[18] Witzemann, V., Stein, E., Barg, B., Konno, T., Koenen, M., Kues, W., Criado, M., Hofmann, M. and Sakmann, B. (1990) Eur. J. Biochem. 194, 437–448.
[19] Ducruix, A. and Giege, R. Crystallization of Nucleic Acids and Proteins, A Practical Approach, Oxford University Press.
[20] Navaja, J. (1994) Acta Crystallogr. A50, 157–163.
[21] Brunger, A.T. X-PLOR Version 3.1: System for X-ray Crystallography and NMR. Yale University, New Haven, CT.