Site-Directed Mutagenesis Study of the Antibody 2D7 which Catalyzes a Reaction for Insertion of Cu^{2+} into Mesoporphyrin

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Monoclonal antibody 2D7 generated against a transition-state analog *N*-methyl mesoporphyrin catalyzes a reaction for insertion of a cupric ion into mesoporphyrin. To investigate amino acid residues responsible for the catalytic activity, site-directed mutagenesis of the amino acid residues in the third complementarity determining region of the heavy chain (CDRH3) was performed on the antigen-binding fragment (Fab) of the antibody. Recombinant Fab mutants, in which Arg95 is replaced with Ala (R95A), Asp96 with Asn (D96N) and Met97 with Gly (M97G), were examined in terms of the catalytic efficiency of the reaction (k/K_S) and the dissociation constant for *N*-methyl mesoporphyrin binding (K_d) and these values were compared with those of the wild type. The k/K_S values of the R95A and D96N mutants were 0.96% and 1.0% of that of the wild type, respectively, whereas the M97G mutant had no detectable catalytic activity. The K_d values of the R95A and D96N mutants were 165 and 69 times that of the wild type, respectively, while that of the M97G mutant was similar to that of the wild type. The relationship between the k/K_S and $1/K_d$ values in the wild type and the R95A and D96N mutants suggests that Arg95 and Asp96 are responsible for stabilizing the transition-state in the catalytic reaction. The results of the M97G mutant allow us to propose that Met97 plays an important role in the catalytic activity probably due to a subtle and specific conformation of the antibody.

[**Key words:** catalytic antibody, fluorescence quenching, metalation, porphyrin, site-directed mutagenesis, transition-state stabilization]

Since the first reports from two groups, Tramontano *et al*. (1) and Pollack *et al*. (2), a number of catalytic antibodies have been generated against transition-state analogues as haptens. However, the catalytic activities of the antibodies are usually lower than those of natural enzymes that catalyze the analogous reactions (3, 4), and the molecular mechanisms by which the antibodies perform their catalytic functions largely remain unknown. To gain insight into the mechanisms of the antibody catalysis and to produce an antibody with enhanced catalytic activity, we need to accumulate detailed studies of the individual catalytic antibodies.

Previously, we prepared 14 monoclonal antibodies raised against *N*-methyl mesoporphyrin (*N*-MMP), which was presumed to resemble the transition-state in a reaction for insertion of a metal ion into mesoporphyrin (MP) (5). Five of them accelerate the insertion of Cu^{2+} into MP, but nine of them do not (5, 6). Comparison of the amino acid sequences of the complementarity determining region (CDR) of the five catalytic antibodies with those of the nine non-catalytic ones showed that all of the former conserve a homologous sequence (Arg95-Asp96-X97-Asp101-Tyr102, where X = Met in the four antibodies and X = Gly in the other one) in the third CDR of the heavy chain (CDRH3), and that Asp96 is not found in the latter (6). Furthermore, the CDRH3 was suggested to have a β-turn structure consisting of Arg95-Asp96-X97 according to its canonical structure proposed by Shirai *et al*. (7) and Furukawa *et al*. (8). Consequently, we proposed that the CDRH3 of the catalytic anti-*N*-MMP monoclonal antibodies contains a key residue responsible for the catalytic activity, which is presumably Asp96 (6).

To examine this possibility, in this paper we report the results of site-directed mutagenesis experiments at Asp96 and the residues before and after Asp96 in a catalytic antibody. We have chosen antibody 2D7 as a model for the mutagenesis study of our catalytic antibodies, since the antibody is

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Abbreviations: CDR, complementarity determining region; Fab, antigen-binding fragment; IgG, immunoglobulin G; MP, mesoporphyrin; *N*-MMP, *N*-methyl mesoporphyrin; V_L-C_κ, variable region and constant region of light κ chain; V_H-C_{H1}, variable region and constant region 1 of heavy chain.

TABLE 1. Antibodies catalyzing insertion of Cu^{2+} into MP (6)

Antibody	CDRH3					Subclass	k/K_S[a] $(M^{-1}\cdot min^{-1})$
	95	96	97	101	102		
2B4	R	D	G	D	Y	IgG_{2a}	5.5×10^{-3}
2D7	•[b]	•	M	•	•	IgG_1	1.6×10^{-3}
2H4	•	•	M	•	•	IgG_1	3.5×10^{-4}
1F2	•	•	M	•	•	IgG_1	1.1×10^{-4}
1F9	•	•	M	•	•	IgG_1	4.2×10^{-5}

[a] k/K_S value was obtained at $[Cu^{2+}] = 1.0$ mM.
[b] Conserved amino acid residues are depicted by dots.

the most active among the four antibodies having a homologous conserved sequence, Arg95-Asp96-Met97-Asp101-Tyr102 (6) as shown in Table 1. The three mutants are examined in terms of the catalytic efficiency for the insertion of Cu^{2+} into MP (k/K_S) and the dissociation constant for N-MMP binding (K_d) and these values were compared with those of the wild type. Based on the results, we discuss essential amino acid residues in the catalytic activity of the anti-N-MMP antibodies.

MATERIALS AND METHODS

Materials Tryptone and yeast extract were purchased from Becton Dickinson (Franklin Lakes, NJ, USA). MOPS was purchased from Wako Pure Chemical Industries (Osaka). N-MMP and MP were synthesized and catalytic antibody 2D7 (native IgG) was prepared as described previously (5). The primers were purchased from Invitrogen (Tokyo). All other reagents were of analytical grade.

Construction of Fab expression vector The plasmid pARA6D9 Fab was used as an expression vector, in which the coding regions of the heavy and light chains of the antigen-binding fragment (Fab) of antibody 6D9 are cloned between the *Xho*I and *Spe*I and between the *Sac*I and *Xba*I restriction sites, respectively (9, 10). Total cellular RNA was extracted from the hybridoma cells producing the antibody 2D7 using TRIzol (Invitrogen). cDNA was synthesized from total RNA using a First-Strand cDNA Synthesis Kit (Invitrogen) with oligo-dT primers under the supplier's conditions. For amplification of the heavy chain as a V_H-C_{H1} fragment and the light chain as a V_L-C_κ fragment of the antibody 2D7, PCR was carried out with forward primers (5′-AGGTCCAGCTG***CTCGAG***TCTGG-3′ with the additional *Xho*I site in bold italics for the heavy chain and 5′-CCAGATGT***GAGCTC***GTGATGACCCAGTCTCAA-3′ with the additional *Sac*I site in bold italics for the light chain) and reverse primers (5′-AGGCTT***ACTAGT***ACAATCCCTGGGCACAAT-3′ with the additional *Spe*I site in bold italics for the heavy chain and 5′-GCGCCG***TCTAGA***ATTAACACTCATTCCTGTTGAA-3′ with the additional *Xba*I site in bold italics for the light chain) using *pfx* DNA polymerase (Invitrogen) and a Program Temp Control System PC-800 (Astec, Fukuoka). Amplified DNAs of the heavy and light chains of the antibody 2D7 were ligated into the *Xho*I-*Spe*I and *Sac*I-*Xba*I restriction sites of the expression vector pARA6D9Fab, respectively. The ligation mixture was transformed into *Escherichia coli* strain MC1061 [araD139 Δ(ara-leu)7696 ΔlacX74 galU galK hsr⁻ hsm rpsL]. The clone expressing the Fab of the antibody 2D7 was selected by ELISA for binding to N-MMP as described previously (5) and the resulting clone was designated pARA2D7. Nucleotide sequencing of the coding region of the heavy and light chains in pARA2D7 was carried out with a DNA sequencer, DS-1000L (Shimadzu, Kyoto), by the dideoxy chain termination method.

Site-directed mutagenesis Mutant clones were generated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) according to the supplier's protocol. The sequences of the primers used for site-directed mutagenesis were as follows: R95A (the sense primer: 5′-CTATTACTGTGCAAGAGCGGACATGGACTACTGGGG-3′; the antisense primer: 5′-CCCCAGTAGTCCATGTCCGCTCTTGCACAGTAATAG-3′), D96N (the sense primer: 5′-CTATTACTGTGCAAGAAGGAACATGGACTACTGGGG-3′; the antisense primer: 5′-CCCCAGTAGTCCATGTTCCTTCTTGCACAGTAATAG-3′), and M97G (the sense primer: 5′-GTGCAAGAAGGGACGGGGACTACTGGGGTC-3′; the antisense primer: 5′-GACCCCAGTAGTCCCCGTCCCTTCTTGCAC-3′). All cloning manipulations were confirmed by nucleotide sequencing of the coding region of the heavy chain in the mutated pARA2D7.

Preparation of affinity columns An immobilized N-MMP affinity column was prepared as follows. About 7.0 mg of N-MMP in 20 ml of water was reacted with 310 mg of 1-ethyl-3-dimethylaminopropyl carbodiimide (Peptide Laboratory, Osaka) and 21.7 mg of N-hydroxysuflosuccinimide (Pierce, Rockford, IL, USA) for 30 min at pH 5.0, maintained by the addition of 1.0 M HCl. The reaction mixture was added to 12 ml of EAH-Sepharose 4B (Amersham Bioscience, Tokyo) at pH 8.0 adjusted with 1.0 M NaOH and incubated overnight. The column packed with the resultant N-MMP cross-linked Sepharose was washed repeatedly with 0.1 M sodium acetate buffer (pH 4.0) containing 0.5 M NaCl, 0.1 M sodium carbonate buffer (pH 8.3) containing 0.5 M NaCl, 10 mM sodium phosphate buffer (pH 7.5) containing 0.5 M NaCl, and 50 mM sodium phosphate buffer (pH 6.9) containing 7.0 M guanidine–HCl until free N-MMP was not eluted from the column.

An immobilized anti-mouse $F(ab')_2$ antibody column was prepared as follows. Anti-mouse $F(ab')_2$ antibody (Rockland, Gilbertsville, PA, USA; 1 mg in 2.0 ml of 50 mM sodium borate buffer, pH 8.3) was adsorbed to a Protein-G Sepharose column (bed volume 1.0 ml; Amersham Bioscience). Disuccinimidyl suberate (6.5 mg; Pierce), a bifunctional cross-linker, was dissolved in 1.25 ml of 0.06 M potassium phosphate buffer (pH 7.2) containing 0.09 M NaCl and 40%(v/v) dimethylsulfoxide (DMSO) and the solution was loaded onto the column to couple the adsorbed anti-mouse $F(ab')_2$ antibody with Protein-G in the gel. Then the column was reacted with 0.1 M Tris–HCl (pH 8.0) to chase the remaining cross-linker and washed extensively with 0.1 M glycine–HCl (pH 2.7) and 10 mM sodium phosphate buffer (pH 7.5) containing 0.3 M NaCl.

Expression and purification of recombinant Fabs An overnight culture (5 ml) of the transformant was inoculated into 1 l of superbroth medium (3% tryptone, 2% yeast extract, 1% MOPS, pH 7.0) with 50 µg ampicillin. The cells were cultivated at 30°C with shaking for 120 min, and then expression of recombinant Fab was induced by adding 0.2% arabinose and incubating at 23°C overnight. The culture was separated into supernatant and cell pellet fractions by centrifugation (15,400×g, 30 min, 4°C). The supernatant fraction was concentrated to 20 ml using the Laboscale TFF System equipped with a Pellicon XL device Ultracel-30 PLCTK (Millipore, Tokyo). The cell pellet fraction was washed with 100 ml of 10 mM Tris–HCl (pH 7.5) three times, and then suspended in 100 ml of 30 mM Tris–HCl (pH 7.5) containing 20% sucrose and 1.0 mM EDTA. After incubation for 20 min at room temperature, the cell pellet was harvested by centrifugation (22,200×g, 20 min, 4°C) and resuspended in 100 ml of cold distilled water. After incubation on ice for 60 min, the periplasm fraction was obtained as a supernatant by centrifugation (22,200×g, 20 min, 4°C) and was concentrated to 20 ml using the Laboscale TFF System. The periplasm fraction was mixed with the culture supernatant, and the mixture was treated with Benzonase Nuclease (Novagen, Madison, WI, USA) to digest DNA in the mixture, followed by 20–70% saturated ammonium sulfate precipitation. The precipitates were