Thermostable peroxidase activity with a recombinant antibody L chain–porphyrin Fe(III) complex

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Received 28 August 1995; revised version received 6 October 1995

Abstract In order to engineer a new type of catalytic antibody, we attempt to use a monoclonal antibody L chain as a host protein for a porphyrin. TCPP (meso-tetrakis(4-carboxyphenyl)porphyine) was chemically synthesized and Balb/c mice were immunized using TCPP as a hapten. Two hybridoma cells (03-1, 13-1), that produce monoclonal antibody against TCPP, were obtained. Genes for both H and L chains of monoclonal antibodies were cloned, sequenced and overexpressed using *E. coli* as a host. ELISA and fluorescence quenching method show that the independent antibody L chains from both Mab03-1 and Mab13-1 have specific interaction with TCPP. Furthermore, the recombinant antibody L chain from Mab13-1 exhibits much higher peroxidase activity than TCPP Fe(III) alone. The enzyme activity was detectable with pyrogallol and ABTS (2,2-azinobis-3-ethylbenzthiazolin-6-sulfonic acid) but not with catechol. This new catalytic antibody was extremely thermostable. Optimum temperature of the peroxidase reaction by the complex of 13-1L chain and TCPP Fe(III) was 90°C, while that the TCPP Fe(III) alone was 60°C.

Key words: Catalytic antibody; Peroxidase; Porphyrin; L chain

1. Introduction

Antibodies have been demonstrated to catalyze a wide variety of transformations such as ester hydrolysis [1–3], pericyclic [4], photochemical [5], ligand substitution [6] and redox reactions [7]. These new antibodies were designated as catalytic antibodies or abzymes. We attempt here to engineer a new way to generate catalytic antibody with peroxidase activity. We chose a porphyrin as a hapten based on the fact that active centers of various peroxidases, catalases and cytochromes consist of the porphyrin molecule encapsulated by a protein molecule. In these cases, the protein has the role of a host molecule to enhance the function of porphyrin. Therefore, we planned to use monoclonal antibodies as a kind of host protein to enhance function of porphyrin molecule. It was previously reported that a monoclonal antibody against TCPP (meso-tetrakis(4-carboxyphenyl)porphyine) was raised but enzymatic activity as a catalytic antibody was not characterized [8,9]. Creation of a catalytic antibody using *N*-methylmeso-porphyrin IX, a presumed transition-state analogue for porphyrin metalation as a hapten was also reported [10]. This antibody–porphyrin complex was formed to catalyze the reduction of hydrogen peroxide by several typical chromogenic peroxidase substrates [10]. We synthesized TCPP that exhibits higher substrate specificity than *N*-methylmeso-porphyrin IX as a hapten and monoclonal antibodies were raised. Surprisingly, recombinant antibody L chains were found to have specific interaction with porphyrin. Furthermore, one of the L-chain–porphyrin complex exhibited higher thermostable peroxidase activity than the complex of whole antibody and porphyrin.

2. Materials and methods

2.1. Preparation of monoclonal antibody

TCPP was synthesized as reported previously [9]. In order to raise monoclonal antibody, TCPP was covalently attached to the carrier protein, keyhole limpet hemocyanin (KLH) using water-soluble carbodiimide or 1-(3-dimethylamino)propyl-3-ethyl-carbodiimide. The conjugates were then purified by column chromatography on Sephadex G-50. Balb/c mice were immunized with the KLH conjugate emulsified in complete Freund's adjuvant. Cell fusion was carried out using P3X63-Ag8.653 myeloma as the fusion partner and polyethylene glycol as the fusion reagent. Two hybridoma cells (03-1, 13-1) were screened and propagated in ascites as described previously [9]. Two monoclonal antibodies from hybridoma cells (Mab03-1, Mab13-1) were purified by using protein A column. Specific interaction of Mab03-1 and Mab13-1 with TCPP was examined by ELISA (enzyme-linked immunosorbent assay) [11] and purified monoclonal antibodies were used for further studies.

2.2. Cloning and nucleotide sequencing of antibody genes

Hybridoma cells, 03-1 and 13-1, were cultivated and cellular mRNA was purified by Quick Prep mRNA Purification kit (Pharmacia P-L Biochemicals Inc., Milwaukee, WI). Isolated mRNA was then used to construct the cDNA library using oligo$(dT)_{18}$ primer and reverse transcriptase. Genes for both L and H chains of two monoclonal antibodies (Mab03-1, Mab13-1) were amplified using PCR (polymerase chain reaction) and cloned into Immunozap L and H vectors [12], respectively. Nucleotide sequences of the cloned DNA fragments encoding L and H chains were determined by dideoxy chain termination method [13]. Nucleotide sequences were confirmed by asymmetric PCR followed by direct sequencing [14].

2.3. Expression of antibody genes

Genes for the appropriate L and H chains were subcloned respectively into an expression vector, pET8-c, which has a strong promoter sequence for T7RNA polymerase [15]. Expression of each antibody gene was induced by IPTG (final concentration 0.1 mM). Three hours after the induction, *E. coli* cells were harvested by centrifugation. Cells were then broken by sonication and insoluble fraction was recovered by centrifugation. Insoluble fraction was treated with 2% Triton X-100, 10 mM EDTA (pH 8.0) and sonicated. Inclusion bodies were harvested by centrifugation and washed twice with a buffer (30 mM Tris-HCl, 30 mM NaCl, pH 8.0). The inclusion body fraction was solublized in a solution of 6 M guanidine hydrochloride, 40 mM Tris-HCl, 1 mM dithiothreitol (DTT) pH 8.0. The protein was refolded by dialysis against a refolding buffer (40 mM Tris-HCl, 1 mM DTT). Homogeneity of proteins were confirmed by SDS gel electrophoresis. Binding between porphyrin and antibody proteins was tested by ELISA [11].

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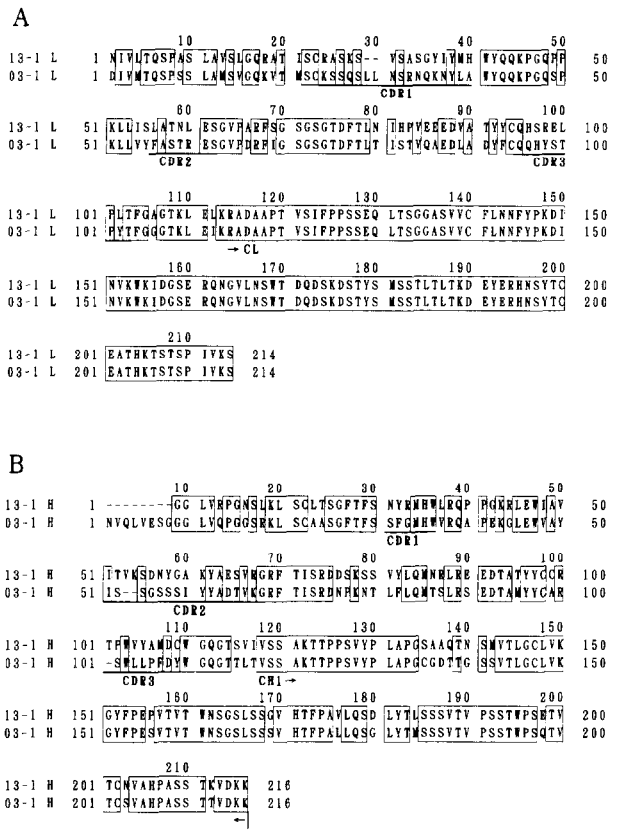


Fig. 1. Amino acid sequences of L chains (Mab03-1, Mab13-1) and H chains (Mab03-1, Mab13-1) derived from nucleotide sequence determined by dideoxy method [13]. The nucleotide sequences were registered by GenBank Accession No. D29667 (H chain of Mab03-1), D29668 (L chain of Mab03-1), D29669 (H chain of Mab13-1) and D29670 (L chain of Mab13-1). Conserved amino acid residues were surrounded by boxes. Three CDRs (complementarity determining region; CDR1–CDR3) were indicated below the amino acid sequence.

2.4. Measurement of dissociation constants

Kinetics of binding was more precisely analyzed by determination of dissociation constants. Fluorescence measurements were performed on a Shimadzu spectrofluorophotometer RF-5020 using wavelength from 300 nm to 450 nm and peak value was detected at wavelength 340 nm. Dissociation constants (K_d) for Mab03-1, Mab13-1 and two L chains were determined from the slope of the Scatchard plot based on the fluorescence quenching of peak values at 340 nm [16,17].

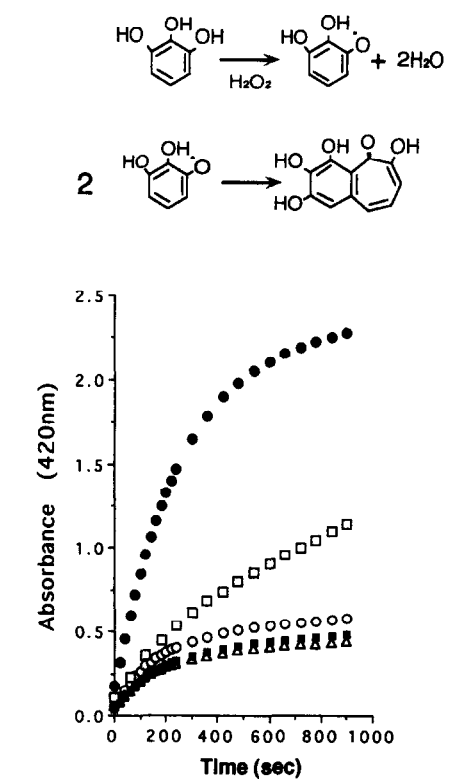
2.5. Assay of peroxidase activity

The reaction for peroxidase activity was performed at 37°C as follows. Reaction mixture containing 5.0×10^{-7} M TCPP Fe(III), 8.0×10^{-7} M antibody protein, 1.2×10^{-3} M substrate, and 5.0×10^{-3} M hydrogen peroxide dissolved into TAB buffer (90 mM Tris-acetate pH 8.0) was prepared and incubated at 37°C. Absorbance of wavelength at 420 nm (pyrogallol) or 414 nm (ABTS) was monitored by Shimadzu UV160 UV-visible recording spectrophotometer. The reaction mixtures without antibody protein and with BSA (bovine serum albumin) at the same concentration were prepared as controls.

3. Results and discussion

3.1. Nucleotide and amino acid sequences of monoclonal antibodies

Nucleotide sequence of the genes for Mab03-1 and Mab13-1 was determined (GenBank Access. No. D29667-D29670) and the deduced amino acid sequences were compared between the



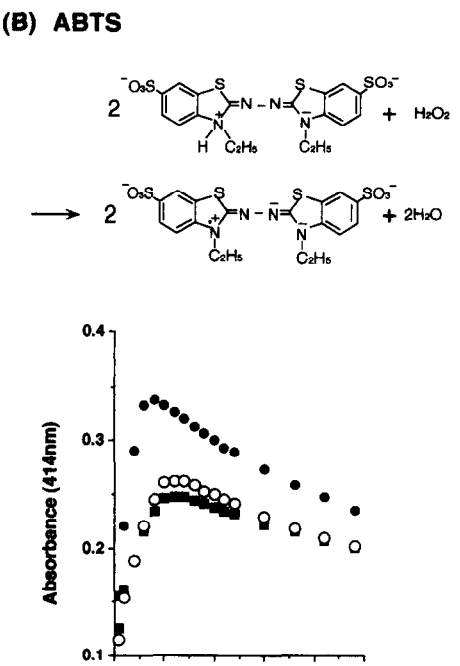


Fig. 2. Reaction scheme of pyrogallol (A) and ABTS (B) by peroxidase and peroxidase reaction by complexes of antibody proteins and TCPP Fe(III). Symbols: ■, △, □, ○ and ● indicate TCPP Fe(III) with none, BSA, Mab03-1, 03-1 L chain, and 13-1 L chain, respectively.

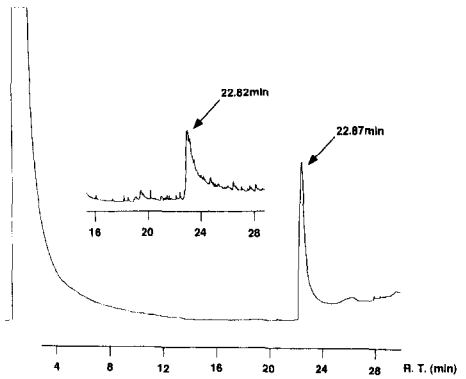


Fig. 3. Detection of purpurogallin by gas chromatography. Reaction mixture with the 13-1 L chain complexed with TCPP Fe(III) was prepared as explained above. Bottom is for control sample of purpurogallin and upper profile is for extractant from the reaction mixture.

two antibodies (Fig. 1). Amino acid sequences of these antibodies were found to be different especially in hyper variable regions (CDR-1, -2 and -3) of H chain.

3.2. Expression and recovery of recombinant antibody proteins

Overexpression of antibody protein was performed using *E. coli* BL21(DE3) as a host and antibody proteins could be recovered as inclusion bodies in insoluble cytoplasmic fraction. H chains from both monoclonal antibodies did not show antigen binding, while specific binding between independent antibody L chain and TCPP was observed for both 03-1 and 13-1 antibodies (data not shown).

3.3. Dissociation constants of antibody proteins

K_d values were measured for antibody proteins by fluorescence quenching as explained in section 2. Specific interaction of independent 03-1 L chain ($K_d = 2.4 \times 10^{-6}$ M) and 13-1 L chain ($K_d = 2.6 \times 10^{-6}$ M) with TCPP was observed. However, K_d values of L chains were higher than those of Mab03-1 ($K_d = 6.4 \times 10^{-8}$ M) and Mab13-1 ($K_d = 1.0 \times 10^{-7}$ M). Most of these antibody proteins had specific interaction even with TCPP Fe(III), while Mab13-1 did not. K_d values of Mab03-1, 03-1L and 13-1L with TCPP Fe(III) are 1.5×10^{-7} M, 1.0×10^{-5} M, and 1.4×10^{-5} M, respectively.

Some naturally occurring active antibodies composed of heavy chain dimers and devoid of L chain have been reported [18]. Recombinant single immunoglobulin variable domains of H chain secreted from *E. coli* had specific binding activities [19]. However, specific antigen binding by L chain is very rare case.

3.4. Assay of peroxidase activity

Peroxidase activity was tested for Mab03-1 and Mab13-1 L chain complexes with TCPP Fe(III) using pyrogallol as a substrate, and compared with those of TCPP Fe(III) alone and Mab03-1–TCPP Fe(III) (Fig. 2A). The L chain of Mab03-1 exhibits slightly higher activity than TCPP Fe(III) but the activity was still lower than that of Mab03-1. Interestingly, 13-1 L chain exhibited much higher peroxidase activity than Mab03-1. This result and the observation that Mab13-1 did not bind to TCPP Fe(III) suggested that H chain of Mab13-1 antibody might interrupt the binding of TCPP Fe(III).

This resultant complex of 13-1 antibody L chain and TCPP Fe(III) exhibited the highest peroxidase activity among all the tested and previously reported catalytic antibodies. Final reaction mixture was tested for detection of purpurogallin, an end product of peroxidase reaction from pyrogallol. Identical profiles of photometric assay was observed (data not shown). Further characterization by gas chromatography confirmed that the L chain complexed with TCPP Fe(III) catalyzes peroxidase reaction from pyrogallol as a catalytic antibody (Fig. 3). Michaelis constants (K_m) and catalytic constants (kcat) were determined by Lineweaver-Burk plot. K_m values were 2.3 mM (H_2O_2) and 4.0 mM (pyrogallol) and k_{cat} value was 667 min^{-1} for both H_2O_2 and pyrogallol. Tentatively, we named this antibody L chain with peroxidase activity 'L-zyme'.

3.5. Specificity of the peroxidase activity

Specificity of the peroxidase activity was also examined using different substrates (ABTS (2,2-azinobis-3-ethylbenz-thiazolin-6-sulfonic acid) and catechol). Significant peroxidase activity for ABTS was observed using the complex of 13-1 L chain and TCPP Fe(III) (Fig. 2B). Yet, no activity was detected when catechol was used as a substrate (data not shown).

3.6. Thermostability of the peroxidase activity

Peroxidase activity of L-zyme was examined at different temperatures and compared with the case of TCPP Fe(III) (Fig. 4). The optimum temperature of the reaction by L-zyme is extremely high (90°C) (Fig. 4) while that by TCPP Fe(III) is only 60°C. Its specific activity at elevated temperature (>80°C) is even higher than that of horse radish peroxidase (data not shown).

We have demonstrated that independent L chains from monoclonal antibodies can recognize and interact with TCPP and TCPP Fe(III). The complex of L chain and TCPP Fe(III) (L-zyme) exhibited much higher peroxidase activity and extreme thermostability than TCPP Fe(III) alone or Mab03-1 with TCPP Fe(III).

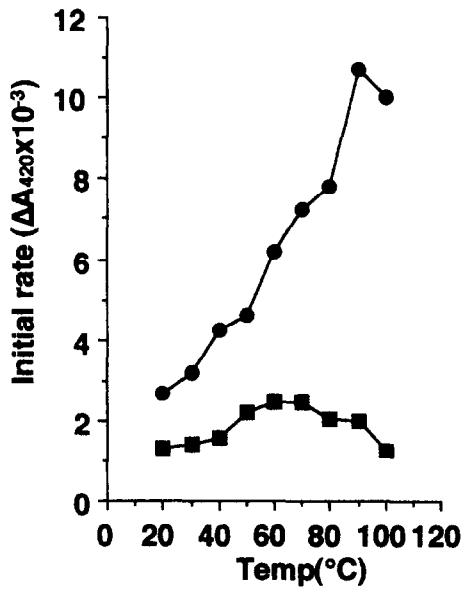


Fig. 4. Peroxidase activity at different temperatures. The activity was tested at different temperatures from 20°C to 100°C as explained in legend of Fig. 2. Initial velocity of catalysis is monitored for 5 s and ΔA_{420}/s was calculated. Symbols: ● and ■ are for the complex of 13-1 L chain and TCPP Fe(III), and TCPP Fe(III) alone, respectively.

We are now determining the molecular structure of this new catalytic antibody by X-ray crystallography and NMR to elucidate structure–function relationship of this new catalytic antibody.

References

[1] Tramontano, A., Janda, K.D. and Lerner, R.A. (1986) Science 234, 1566–1570.
[2] Pollack, S.J., Jacobs, J.W. and Schultz, P.G. (1986) Science 234, 1570–1573.
[3] Jacobs, J.W., Schultz, P.G., Sugasawara, R. and Powell, J. (1987) J. Am. Chem. Soc. 109, 2174–2176.
[4] Braisted, A.C. and Schultz, P.G. (1990) J. Am. Chem. Soc. 112, 7430–7431.
[5] Cochran, A.G., Sugasawara, R. and Shultz, P.G. (1988) J. Am. Chem. Soc. 110, 7888–7890.
[6] Shokat, K.M., Leumann, C.J., Sugasawara, R. and Shultz, P.G. (1989) Nature 338, 269–271.
[7] Janjic, N. and Tramontano, A. (1989) J. Am. Chem. Soc. 111, 9109–9110.
[8] Schwabacher, A.W., Weinhouse, M.I., Auditor, M.M. and Lerner, R.A. (1989) J. Am. Chem Soc. 111, 2344–2346.
[9] Harada, A., Okamoto, K. and Kamachi, M. (1991) Chem. Lett. 953–956.
[10] Cochran, A.G. and Schultz, P.G. (1990) J. Am. Chem. Soc. 112, 9414–9415.
[11] Engvall, E. and Perlman, P. (1971) Immunochemistry 8, 871–879.
[12] Mullinax, R.L., Gross, E.A., Amberg, J.R., Hay, B.N., Hogrefe, H.H., Kubitz, M.M., Greener, A., Alting-Mees, M., Ardourel, D., Short, J.M., Sorge, J.A. and Shopes, B. (1991) Proc. Natl. Acad. Sci. USA 87, 8095–8099.
[13] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[14] Gyllensten, U.B. and Erlich, H.A. (1988) Proc. Natl. Acad. Sci. USA 85, 7652–7656.
[15] Studier,F.W., Rosenberg, A.H., Dunn, J.J. and Dubendorff, J.W. (1990) Methods Enzymol. 185, 60–89.
[16] Janjic, N., Schloeder, D. and Tramontano, A. (1989) J. Am. Chem. Soc. 111, 6374–6377.
[17] Scatchard, G.(1949) Ann. NY Acad. Sci. 51, 660–672.
[18] Harmers-Casterman, C., Atarhouch, T., Muyldermans, S., Robinson, G., Hamers, C., Bajyana Songa, E., Bendahman, N. and Hamers, R. (1993) Nature 363, 446–448.
[19] Ward, E.S., Gussow, D., Griffiths, A.D., Jones, P.T. and Winter, G. (1989) Nature 341, 544–546.