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Studies of high thermostability and peroxidase activity of recombinant antibody L chain-porphyrin Fe(III) complex

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Abstract **A complex of an independent L chain from anti-meso-tetrakis (4-carboxyphenyl) porphyrin (TCPP) monoclonal antibody 13-1 and TCPP Fe(III) was designated as L-zyme and shown to exhibit high peroxidase activity and high optimal reaction temperature (90°C). Heat denaturation study and circular dichroism (CD) spectra analysis suggested that refolded structure of 13-1 L chain exhibited significantly reduced inactivation rate after heat treatment. The secondary structure of 13-1 L chain changed slightly by the encapsulation of TCPP Fe(III) and the complex was found to be less thermostable than the L chain alone. Furthermore, by characterization of truncated forms of the L chain, it was revealed that the hydrophobic region (115–146) and hydrophilic region (147–189) in C_L are important for thermostability and activity, respectively. Tertiary structure of L-zyme was predicted by AbM. Comparison of residues of L-zyme with those in the active centre of known structure of the peroxidase from *Arthromyces ramosus* (ARP) indicated that His38(CDR1), His94(CDR3), Arg96(CDR3) of L-zyme are important residues for peroxidase activity. Moreover, the steric arrangements of these residues in both L-zyme and ARP are similar, respectively. Distance between proximal His and distal His in L-zyme is 9.09 Å, whereas that of ARP is 7.8 Å.**

Key words: Catalytic antibody; Light chain; Thermostability; Peroxidase activity; Porphyrin

1. Introduction

Catalytic antibodies are one of the potential values of monoclonal antibodies (Mabs) and now encompassed with many examples of various chemical reactions catalyzed by antibodies that have been induced to compounds (haptens) that mimic the transition states [1]. In order to create a new catalytic antibody with higher performance, we attempted to use a monoclonal antibody as a host protein for a porphyrin and reported that the independent L chain from anti-porphyrin monoclonal antibody had specific interaction with TCPP, and that L chain-TCPP Fe(III) complex exhibited high peroxidase activity [2]. Accordingly, we named the Mab 13-1 L chain with peroxidase activity L-zyme. Interestingly, optimum temperature of reaction by this L-zyme was very high (90°C). The structures of antibodies in complex with haptens have enabled us to gain insights into the structural basis of immune recognition and mechanisms of catalytic antibodies. We attempted to investigate antibody structure by CD, well known as a major procedure to analyze protein secondary structures. The study of heat denaturation process may reveal a general and theoretical comprehension of the heat denaturation process of antibodies, which will help to engineer more thermostable catalytic antibodies. Furthermore, the field of antibody engineering has made remarkable progress and encouraged the functional investigation of Mabs. Specifically a new potential value of Mabs was revealed by the single chain Fv (sFv) [3], immonotoxin [4] and bispecific antibody [5] as antibody derivates. Elucidation of the mechanism of its high activity and high thermostability will supply valuable information for the improvement of catalytic antibodies. Prediction of tertiary structure is also useful for study of antigen-antibody complex and identification of catalytic residues. The availability of accurate and reliable antibody modelling procedures facilitates the prediction of site directed mutagenesis experiments. Therefore, we predicted tertiary structure model of L-zyme to understand the catalytic mechanism of L-zyme.

2. Materials and methods

2.1. Overexpression of the protein in Escherichia coli

Escherichia coli BL21(DE3) with integration of inducible T7RNA polymerase gene [6] was transformed with the pET-8c plasmid and its derivative plasmids. Expression of antibody proteins were induced by isopropyl β-D-thiogalactopyranoside (IPTG) (final concentration 0.1 mM). 3 h after induction, cells were harvested by centrifugation.

2.2. Recovery of antibody protein

Cells were disrupted by sonication and the insoluble fraction was recovered by centrifugation and precipitate was treated with 2% Triton X-100, 10 mM EDTA (pH 8.0) [2]. 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 was solubilized 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) [2].

2.3. Thermostability test of antibody protein

The solution containing L-zyme was treated at 90°C. Samples taken at appropriate time intervals were chilled quickly with ice water, and the remaining peroxidase activity was measured at 37°C [2].

2.4. CD measurement

The CD spectra were measured on a J-720W automatic spectropolarimeter (Japan Spectroscopic Co., Ltd.). The far CD spectra (200–250 nm) were obtained using solutions containing antibody in 0.1 mM PBB in a cell with an optical path of 10 mm. The mean residue ellipticity (θ) which has the unit of deg cm^2 $dmol^{-1}$, was calculated from molecular weight of antibody protein.

2.5. Measurement of dissociation constants

Kinetics of binding was analyzed by the determination of dissociation constants. Fluorescence quenching method was performed on a Shimadzu spectrofluorophotometer RF5020 using wavelengths from

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300 nm to 450 nm and peak value was detected. Dissociation constants (K_d) for truncated proteins were determined from slope of Scatchard plot based on the fluorescence quenching of peak values [7,8].

2.6. Assay of peroxidase activity

Reaction mixture containing 5.10×10^{-7} M TCPP Fe(III), 8.0×10^{-7} M antibody protein, 1.2×10^{-3} M pyrogallol, 5.0×10^{-3} M hydrogen peroxide, 4% dimethyl sulfoxide (DMSO), and 90 mM Tris-acetate (pH 8.0) was incubated at 37°C. Reactions were monitored at wavelength 420 nm with a Shimadzu UV 160 UV-visible spectrophotometer.

2.7. Modelling of antibody structure

A model of VH-VL complex was predicted by AbM (Oxford Molecular, Inc.) and VL region was constructed by erasing VH region on ProES (Oxford Molecular, Inc.).

3. Results and discussion

3.1. Heat denaturation study of L-zyme

Thermostability of independent L chain from 13-1 Mab and an L chain-porphyrin complex (L-zyme) was tested at 90°C (Fig. 1). It was shown that independent L chain is more thermostable than the complex (L-zyme). During the initial 10 min, rapid heat inactivation was observed in both refolded L chain and its complex (L-zyme). After 10 min, both independent L chain and L-zyme exhibited significantly reduced denaturation rates (Fig. 1). This phenomenon was reproducible. From these results, we speculated that: (1) refolded 13-1 L chain changes its structure by forming complex with TCPP Fe(III), and (2) the structures of these proteins become more rigid and compactly packed by heat treatment at 90°C for about 10 min.

3.2. CD spectra analysis of L-zyme

We further examined above speculations and heat denaturation process by CD. The various states of 13-1 L and complexes were designated as follows. N-state: refolded 13-1 L; N′-state: heat treated N-state; N-complex: N-state complexed with TCPP Fe(III); N′-complex: N′-state complexed with TCPP Fe(III); I-complex: heat treated N-complex. We analyzed these structural conversion processes by thermostability tests and CD spectra analysis. Thermostability tests and CD spectra (Fig. 2a) suggested that the N-state changes to a slightly different conformation (N′-state) by heat treatment. Far UV CD spectra of 13-1 L chain were compared before and after heat treatment (Fig. 2a). Secondary structure content (mainly β-sheet structure) which corresponds to the molecular ellipticity at wavelength 217 nm changed after heat treatment indicating that N-state and N′-state are structurally different. CD spectra also suggested that the N′-state has a thermostable conformation, but N′-state did not change its conformation by interaction with TCPP Fe(III). Refolded recombinant 13-1 L chain (N-state) may change to a rigid and thermostable conformation (N′-state) by heat treatment.

Heat denaturation studies of N-state and N-complex revealed that these two forms have different thermostability. Furthermore, CD spectrum of N-state exhibited clearly different profile from that of N-complex. Indeed molecular ellipticity around 220 nm is significantly reduced (secondary structure contents are increased) by forming complex with TCPP Fe(III). Moreover, peak valley was given at 217 nm for N-state but that for N-complex was at 220 nm. Therefore, it was concluded that N-state and N-complex have different secondary structures. It has become clear that some changes in antibody structure was brought on by interaction with an antigen or a hapten [9]. For the cases when significant changes have been observed, they appear to be of an induced fit nature and resulted in increased contact between the antibody and antigen. It is considered that an increase of secondary structure contributes to stabilize complex between antibody-antigen. Furthermore, CD spectra indicated that N-complex changes to different conformation (I-complex) by heat treatment. On the other hand, comparison of far UV CD spectra (Fig. 2c) suggested that N′-complex and I-complex have almost identical secondary structures. It was also supported by the fact that I-complex and N′-complex exhibited similar values in catalytic constants (N′-complex; $K_{m(H_2O_2)} = 0.98$ mM, $k_{cat} = 147$ min^{-1}, I-complex; $K_{m(H_2O_2)} = 1.04$ mM, $k_{cat} = 150$ min^{-1}). Based on these results, we proposed model for heat denaturation process of L-zyme (Fig. 3).

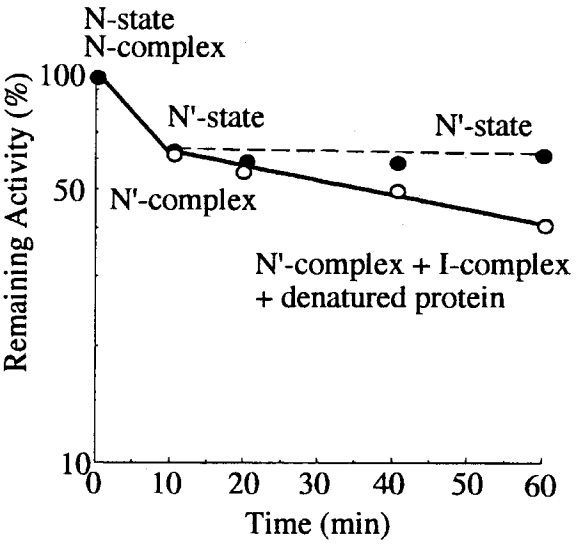

Fig. 1. Thermostability test of 13-1 L and L-zyme. After heat treatment at 90°C, peroxidase activity was measured at 37°C. Symbols: ● and ○ for 13-1 L and 13-1 L+ TCPP Fe(III), respectively.

3.3. Construction of deletion mutants of L chain

We analyzed the hydrophobicity profile of the L-zyme and found that CL region has two distinct regions. One is a region from amino acids 115 to 146, which shows higher hydrophobicity, and the other region is from amino acids 147 to 189, which exhibits higher hydrophilicity. We constructed three different types of truncated L chains to examine role of hydrophobic and hydrophilic regions of the CL region in enzyme activity and thermostability (Fig. 4). Truncated proteins are (1) VL protein which does not contain constant region, (2) Δ31 protein with deletion of 31 amino acids from C terminus (without hydrophilic region) and (3) Δ74 protein with deletion of 74 amino acids from C terminus (without hydrophobic and hydrophilic regions). They were constructed by deletion of corresponding DNA regions by PCR. These truncated proteins were expressed and purified as above-mentioned method. As K_d values of truncated proteins determined by fluorescence quenching method suggested, these proteins have specific interaction with TCPP Fe(III) (13-1 L; $K_d = 1.4\times10^{-5}$ M^{-1}, VL; $K_d = 2.1\times10^{-6}$ M^{-1}, Δ31; $K_d = 1.6\times10^{-5}$ M^{-1}, Δ74;

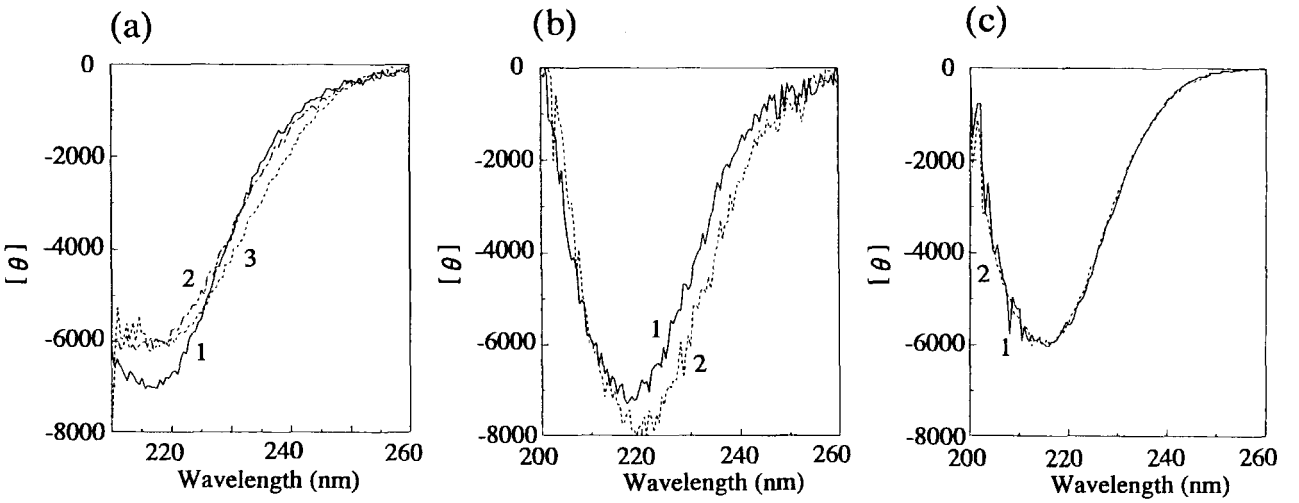

Fig. 2. CD spectra analysis of various states of 13-1 L chain. The mean residue ellipticity (θ) which has the unit of deg cm^2 $dmol^{-1}$, was calculated from molecular weight of antibody protein. a: N-state at 40°C, 1; 90°C, 2; 40°C after heat treatment at 90°C, 3. b: N-state, 1; N-complex, 2. c: N′-complex, 1; I-complex, 2. Far UV CD spectra of (a) were measured between 210 nm and 260 nm, because of strong noise at 90°C.

$K_d = 1.6 \times 10^{-5}$ M^{-1}). Peroxidase activity and optimum temperature for the reaction of the truncated proteins were also examined. The VL protein complexed with TCPP Fe(III) exhibited lower peroxidase activity. Δ31 exhibited high peroxidase activity and relatively high optimal temperature (80°C) for reaction. Δ74 protein exhibited low activity, but the optimum temperature was still high (80°C) (Fig. 5). These results suggested that the hydrophilic region from 147 to 189 has a positive effect on peroxidase activity and the hydrophobic region from 115 to 146 contributes high optimum temperature of L-zyme. Indeed, many hydrophobic amino acid residues like Pro and Ala are found in the 115–146 region and many charged amino acid residues, especially Lys and Asp are found in the 147–189 region. Following points can be concluded: (1) the CL region, especially the hydrophobic region (115–146 region) enhanced thermostability. It is widely recognized that the hydrophobic interaction is important for protein folding and structural stability [11–14]. (2) The hydrophilic region (147–189) may have an influence on oxidation-reduction potential of porphyrin, and resulted in enhancement of the enzyme activity.

3.4. *Prediction of antibody structure and catalytic mechanism of the L-zyme*

Tertiary structure of the Mab 13-1 was predicted by the AbM and then structure of H chain was erased leaving the structures of L chain and porphyrin. AbM implements an algorithm, which combines the advantageous features of a

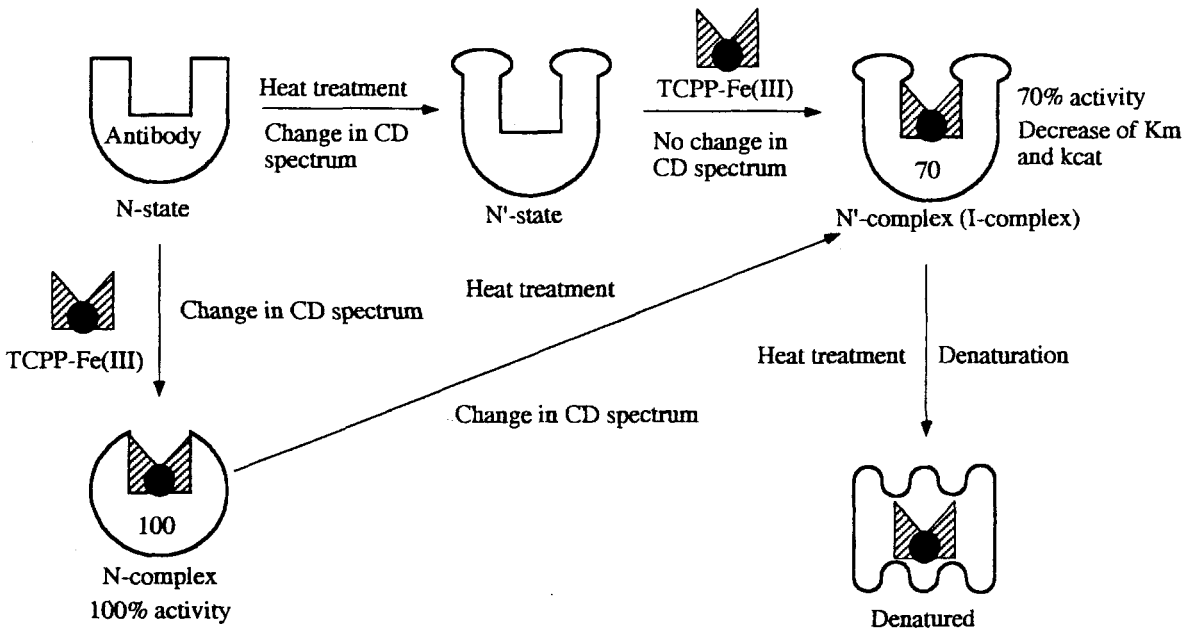

Fig. 3. The model of heat denaturation process of L-zyme. This model was based on thermostability test and CD spectra analysis. N-state, N′-state, N-complex, N′-complex and I-complex were defined in the text.

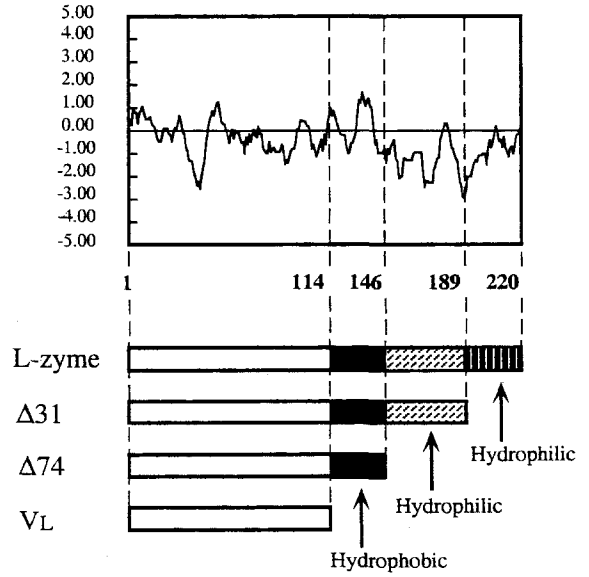

Fig. 4. Hydrophobicity profile of the 13-1 L. Hydrophobicity profile was analyzed by method of Kyte and Doolittle [10]. The amino acid 115–146 region is a hydrophobic region and the 147–189 region is a hydrophilic region.

general protein loop database and ab initio features enabling the user to model antibody combining sites with ease and accuracy [15]. The resultant predicted structure of L chain was compared with the known structure of *Arthromyces ramosus* peroxidase (ARP) [16]. The predicted proximal His38 in CDR1 and distal His94, Arg96 in CDR3 were identified in L-zyme. The steric arrangements of proximal His, and distal His and Arg of L-zyme are similar to those of ARP (Fig. 6). Distance between distal and proximal histidine residues was 7.8 Å for ARP and 9.09 Å for L-zyme, which suggested that

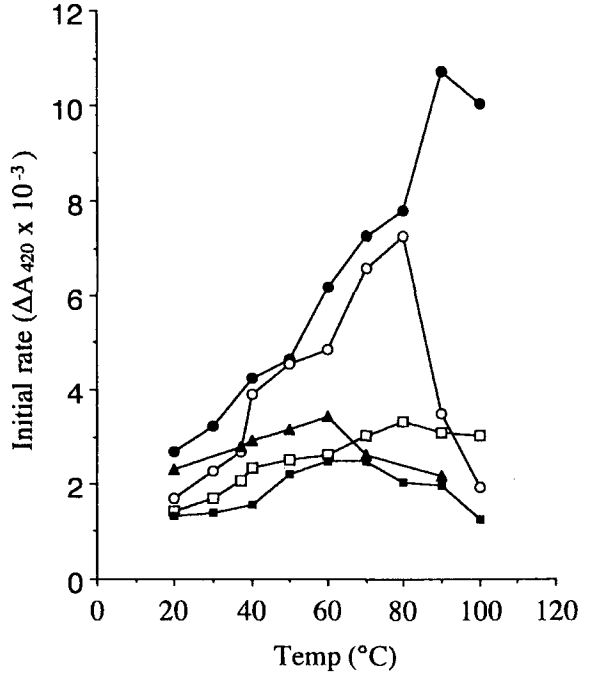

Fig. 5. Peroxidase activity of truncated proteins. Peroxidase activity was assayed at different temperatures. Symbols: ●, ○, □, ▲, ■ indicate L-zyme, Δ31, Δ74, VL, TCPP Fe(III) respectively.

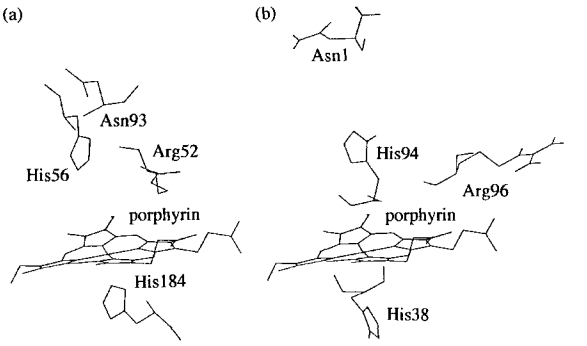

Fig. 6. Comparison of the steric arrangement of amino acids of ARP and L-zyme. a: ARP; b: L-zyme. His38, His96 and Arg96 in L-zyme correspond to catalytic residues (His184, His56 and Arg52) in ARP.

these two enzymes may have similar catalytic mechanisms. It is quite interesting that catalytically important histidine and arginine residues were found in CDR regions of L-zyme. The peroxidase reaction generally consists of the following steps.

$$\text{peroxidase (Fe(III))} + H_2O_2 \rightarrow \text{compound I (Fe(IV)•)} + H_2O$$

$$\text{compound I (Fe(IV)•)} + AH_2 \rightarrow \text{compound II (Fe(IV))} + AH•$$

$$\text{compound II (Fe(IV))} + AH_2 \rightarrow \text{peroxidase (Fe(III))} + AH•$$

$$2AH• \rightarrow A_2H_2 \text{ or } A + AH_2$$

The mechanism of compound I formation, the first step in the above equations, has been proposed for cytochrome *c* peroxidase (CCP) [17]. The mechanism of compound I formation suggested that the distal histidine and arginine concertedly stabilize charge separation and facilitate proton transfer from one oxygen to the other of the peroxide as well as heterolytic of its O-O bond [18]. Similar catalytic mechanism can be applied to the L-zyme.

Recently, it was proposed that β-sheet is more favorable for electron transfer than α-helix [19]. The domains of antibody have an overall topological similarity, characterized by two β-sheets packed closely against each other with disulphide bonds. Therefore, not only catalytic residues but electron pathway between V and C regions may be important for peroxidase activity, which is consistent with our finding that the hydrophilic 147–189 region with charged amino acid residues in constant region enhanced peroxidase activity.

In the future, antibody engineering will have great importance in various fields. This study provides the first example of thermostability about catalytic antibodies and the function of the constant region. We studied the mechanism of heat denaturation, identified the region which contributes to high peroxidase activity and high optimum temperature, and predicted antibody structure and catalytic residues by computer graphics. Mutageneses of predicted proximal and distal His residues, and Arg residue of L-zyme and further elucidation of catalytic mechanism are now in progress.

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