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Peroxidase activity of an antibody–ferric porphyrin complex

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Abstract

The catalytic antibody 2B4 which catalyzes insertion of a cupric ion into porphyrin also combines with ferric porphyrin to form an antibody–ferric porphyrin complex. The antibody has distinct amino acid sequences in complementarity-determining regions compared to other anti-porphyrin antibodies reported. The 2B4-ferric porphyrin complex oxidized *o*-dianisidine and 2,2′-azino-*bis*(3-ethylbenz-thiazoline-6-sulfonic acid) utilizing hydrogen peroxide more efficiently than ferric porphyrin, but did not oxidize pyrogallol nor hydroquinone. The peroxidase reaction of the complex was examined kinetically for *o*-dianisidine, and compared with that of ferric porphyrin. With increasing concentrations of *o*-dianisidine, the reaction rate obtained for ferric porphyrin increased gradually, in contrast, that for the complex increased steeply and then saturated. These results indicated that the interaction of the complex with *o*-dianisidine was much higher than that of ferric porphyrin. At a constant concentration of *o*-dianisidine, the reaction rates obtained for the complex and for ferric porphyrin both showed saturation behavior against hydrogen peroxide concentration. The K_m value for hydrogen peroxide of the complex was similar to that of ferric porphyrin but much larger than that of natural peroxidase, suggesting that the antibody did not have a residue facilitating the binding of hydrogen peroxide as in natural peroxidase. In the reaction of the complex with hydrogen peroxide, active intermediates were not observed. Based on the results, a scheme for the peroxidase reaction by the complex was proposed. It was considered that the enhancement of the peroxidase activity by the antibody was mainly attributed to an increase in the interaction with *o*-dianisidine, and that the substrate specificity of the complex resulted from the difference in the interaction.

Keywords: Abzyme; Catalytic antibody; Porphyrin

1. Introduction

A variety of catalytic antibodies elicited against transition state analogs (TSA) have been produced since the first reports by two groups [1,2]. The catalytic activities of antibodies are usually lower than those of the natural enzymes that catalyze analogous reactions [3–5]. Since cofactors give many proteins chemical functions that might not be possible with proteins alone, the introduction of cofactors into TSA-specific antibodies is a strategy to enhance their catalytic activity and to produce a new type of catalyst.

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Antibodies have been generated that bind specifically to cofactors including flavin [6], metal–trien [7], selenocysteine [8], porphyrin [9–13] and pyridoxal phosphate [14]. Antibodies which combine with ferric porphyrin to form antibody-ferric porphyrin complexes have been prepared by several groups [13,15,16]. The resultant complexes catalyzed the oxidation of substrates utilizing hydrogen peroxide, however, their catalytic mechanisms have not been elucidated.

Previously we prepared a catalytic antibody 2B4 [17], which was raised against *N*-methyl mesoporphyrin (*N*-MMP) as TSA in a similar manner as Cochran and Shultz [11] and showed that it accelerated the insertion of a cupric ion into mesoporphyrin. The antibody readily bound ferric mesoporphyrin (MP-Fe) to form a 1:1 binding complex [18]. In a preliminary experiment, we observed that the complex oxidized *o*-dianisidine in the presence of hydrogen peroxide more efficiently than MP-Fe. In this paper we study the kinetics of the peroxidase reaction by the complex and propose a scheme for the reaction. As well we have determined the primary structures of variable (V) regions of the antibody and compare it with those of the other anti-porphyrin antibodies reported.

2. Experimental

2.1. Materials

MP-Fe was prepared as described previously [18]. The concentration of MP-Fe was determined by the pyridine hemochromogen method [19]. Antibody 2B4 elicited against *N*-MMP was prepared and purified as in the previous report [17]. Protein concentration was determined from the absorbance at 280 nm and calculated assuming an A_{280} of 1% of IgG as 13.7 [20]. The antibody concentration was expressed per binding site assuming a molecular weight of 150 000 (two binding sites per antibody). To prepare the antibody–MP-Fe complex, 0.3 μM and 3 μM of MP-Fe was mixed with 1.5 μM and 6 μM of the antibody, respectively. Since the dissociation constant, K_d, of the complex was 0.064 μM [18], almost all of the added MP-Fe (95% and 98% in the cases of 0.3 μM and 3 μM of MP-Fe, respectively) has been calculated to combine with the antibody to form a complex. So the concentration of the complex was expressed as that of the added MP-Fe. Hydrogen peroxide (30%, v/v) was obtained from Wako. A stock solution of hydrogen peroxide was made up immediately before use and its concentration was estimated using an $\varepsilon_{240\ nm} = 43.6\ M^{-1}\ cm^{-1}$ [21]. In general, no detectable change in concentration occurred for at least 6 h after preparing the stock solution. The buffer used consisted of 0.5% (w/v) Triton X-100, 5% (v/v) dimethyl sulfoxide, and 90 mM Tris–acetate (pH 8.0). All other reagents were of analytical grade.

2.2. Examination of peroxidase activity

Four chromogenic substrates, ABTS (2,2′-azino-*bis*(3-ethylbenz-thiazoline-6-sulfonic acid)) from Sigma, *o*-dianisidine, pyrogallol and hydroquinone from Wako, were used to examine peroxidase activity of the complex. Hydrogen peroxide was added to a cuvette of 0.1 cm light path length containing the substrate and 0.5 μM of the complex or 0.5 μM MP-Fe at 10°C. The concentrations of hydrogen peroxide and the substrates except *o*-dianisidine were 6 mM and 1 mM, respectively. The *o*-dianisidine concentration was 0.5 mM. The peroxidase activity was detected following an increase in absorbance at 500 nm for *o*-dianisidine, 414 nm for ABTS, 420 nm for pyrogallol and a decrease in absorbance at 295 nm for hydroquinone on a Hitachi U-3210 spectrophotometer equipped with HAAKE temperature controllers, F3 and CH.

2.3. Peroxidase activity for o-dianisidine

Hydrogen peroxide was added to a cuvette of 1 cm light path length containing *o*-dianisidine

and 0.3 μM of the complex or 0.3 μM MP-Fe at 10°C. The concentrations of hydrogen peroxide and *o*-dianisidine were in ranges from 1.11 to 33.3 mM and 0.0103 to 0.613 mM, respectively. The initial rates were obtained by measuring an increase in absorbance at 500 nm and expressed as M/min by using $\varepsilon = 6360\ M^{-1}\ cm^{-1}$ for the oxidized *o*-dianisidine under our buffer conditions used. The ε value was determined as follows.

A glucose solution at known concentrations (8.75–35 μM) was oxidized completely by glucose oxidase (from Sigma, 0.67 μM) in the presence of peroxidase (from Life Technologies Oriental, 1.23 μg/ml) and *o*-dianisidine (about 100 μM). The absorbance of the mixtures was measured at 500 nm. From the slope of a plot of the absorbance versus the glucose concentration, the ε value was determined to be 6360 $M^{-1}\ cm^{-1}$.

2.4. Incubation of the complex with hydrogen peroxide

Hydrogen peroxide (14.7 μM) was added to the complex solution (3 μM) or MP-Fe (3 μM) and the mixtures were incubated at 10°C. The absorption of the mixtures was measured immediately and at some time intervals after mixing with hydrogen peroxide in a cell of 1 cm path length at 10°C. At specified time intervals, 30 μ1 aliquots of the mixture of the complex were transferred to cuvettes containing 270 μl of an assay mixture composed of 0.6 mM *o*-dianisidine and 1.15 mM hydrogen peroxide. The peroxidase activity was measured by the increase in an absorbance at 500 nm, and calculated by using $\varepsilon = 6360\ M^{-1}\ cm^{-1}$ for the oxidized *o*-dianisidine.

2.5. Measurements of CD spectra

CD spectra were measured at 10°C with a cell of 1 cm path length using a JASCO J-720 spectropolarimeter. The instrumental scale was set at 290.5 nm using D-camphor-10-sulfonic ammonium as a standard.

2.6. Nucleotide sequence analyses

mRNA was extracted from 10^7 hybridoma cells using QuickPrep Micro mRNA Purification Kit (Pharmacia Biotech) under the supplier's condition. V_H and V_L specific cDNA was synthesized using First-Strand cDNA Synthesis Kit (Pharmacia Biotech) with H3 (5′-GG *AAGCTTA*(T/C)CTCCACCACCAACAGG(A/G)(A/G)CCAGTGGATAGAC-3′ with additional *Hin*dIII site in italics) or L3 (5′-GG *AAGCTT*ACTGGATGGATG-GTGGGAAGATGGA-3′, with an additional *Eco*RI site in italics) primers under the supplier's condition. For cloning of V_H and V_L regions, PCR reactions were performed with H5 (5′-GG-*GAATTC*TCTCTTCCTCCTGTCAG(G/T)AACTGCAGG-3′, with additional *Eco*RI site in italics) and H3 or L5 (5′-GG*GAATTC*TCTTCTGCTCTGGGT(A/G)TCTGGTGC-3′, with an additional *Eco*RI site in italics) and L3 primers using Taq DNA polymerase on a PJ2000 Thermal Cycler (Perkin Elmer). Amplified DNA was digested with *Hin*dIII and *Eco*RI, followed by cloning into the pUC18 vector. The pUC clones were sequenced by the dideoxy chain termination method using a Dye Primer Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase CS + (Perkin Elmer) and ABI 373S DNA Sequencer (Perkin Elmer). For each sequence, five clones were sequenced and aligned to construct consensus sequences.

3. Results

3.1. Examination of peroxidase activity of the antibody-ferric mesoporphyrin complex

Monoclonal antibody 2B4 generated against *N*-MMP as a hapten [17] combined with MP-Fe to form the antibody–MP-Fe complex with a

strong affinity as $K_d = 0.064$ μM [18]. In a preliminary experiment, we observed that the complex oxidized *o*-dianisidine utilizing hydrogen peroxide. To investigate the mechanism of this peroxidase reaction catalyzed by the complex, we first examined the catalytic activity of the complex for typical chromogenic substrates, ABTS, pyrogallol and hydroqinone, as well as *o*-dianisidine. Fig. 1 shows time courses of absorption for the peroxidase reaction catalyzed by the complex and MP-Fe. It is found that the complex exhibited higher activity for *o*-dianisidine and ABTS than MP-Fe but not for pyrogallol nor hydroquinone. These results were

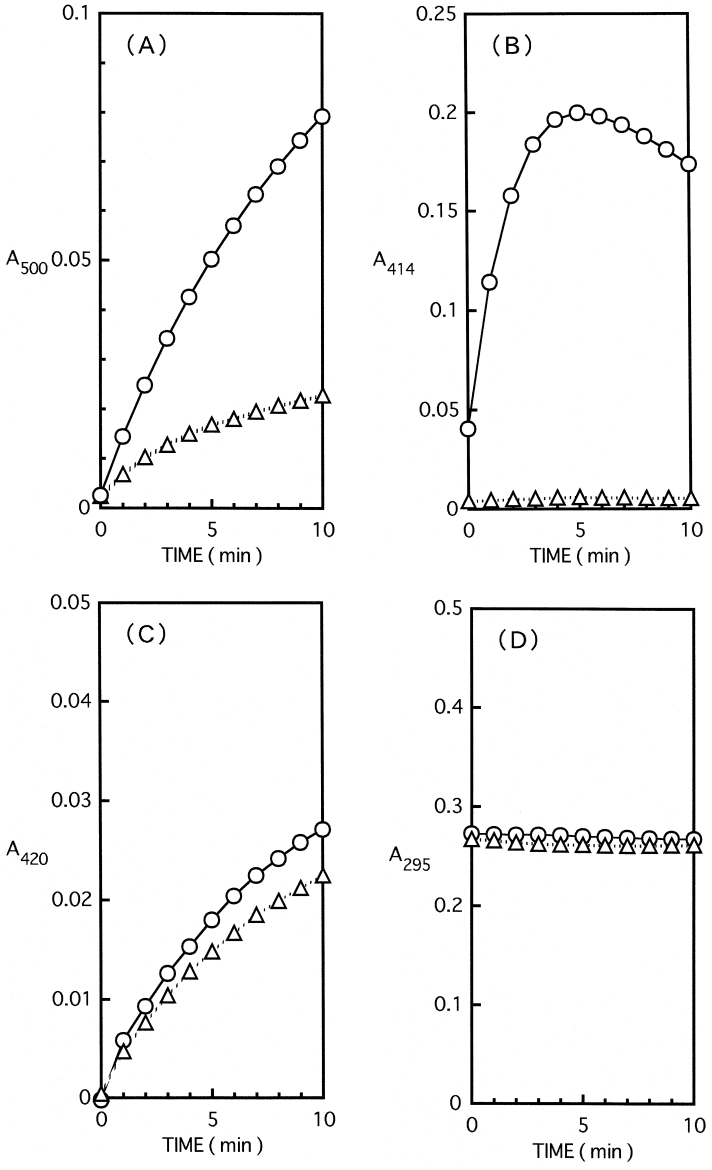

Fig. 1. Oxidation reaction of four substrates by the antibody–MP-Fe Complex (○) and the MP-Fe (△) utilizing hydrogen peroxide. The reaction mixtures contained 0.5 μM antibody–MP-Fe complex or 0.5 μM MP-Fe, 6 mM hydrogen peroxide, (A) 0.5 mM *o*-dianisidine, (B) 1 mM ABTS, (C) 1 mM pyrogallol and (D) 1 mM hydroquinone.

much different from the antibody–MP-Fe complex of Cochran and Schultz [15] which oxidized all four substrates more efficiently than MP-Fe. To elucidate the specificity for substrate and compare with Cochran and Schultz's results, the peroxidase reaction by the complex was investigated kinetically using *o*-dianisidine as substrate.

3.2. Peroxidase activity for o-dianisidine

Fig. 2 represents the initial rate for the complex as a function of *o*-dianisidine concentration and that for MP-Fe. It is clearly shown that the rate for MP-Fe increased gradually with an increase in *o*-dianisidine concentration. In contrast, the rate for the complex increased steeply and was almost constant above 0.3 mM. Further experiments were not performed at higher concentrations of *o*-dianisidine because of its low solubility under our experimental conditions used.

Next, the dependence of the rate on hydrogen peroxide concentration was examined at three concentrations of *o*-dianisidine (Fig. 3). It is found that the rates for the complex and for MP-Fe were depicted by hyperbolic curves against hydrogen peroxide concentration. But

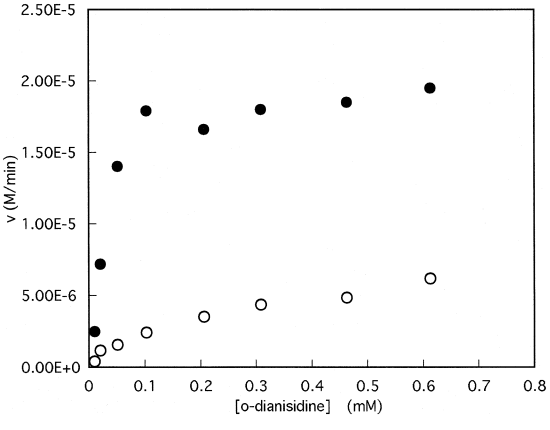

Fig. 2. Relationship between the initial rate and the *o*-dianisidine concentration. The reaction mixture contained 0.3 μM antibody–MP-Fe complex (●) or 0.3 μM MP-Fe (○) in the presence of 8 mM hydrogen peroxide.

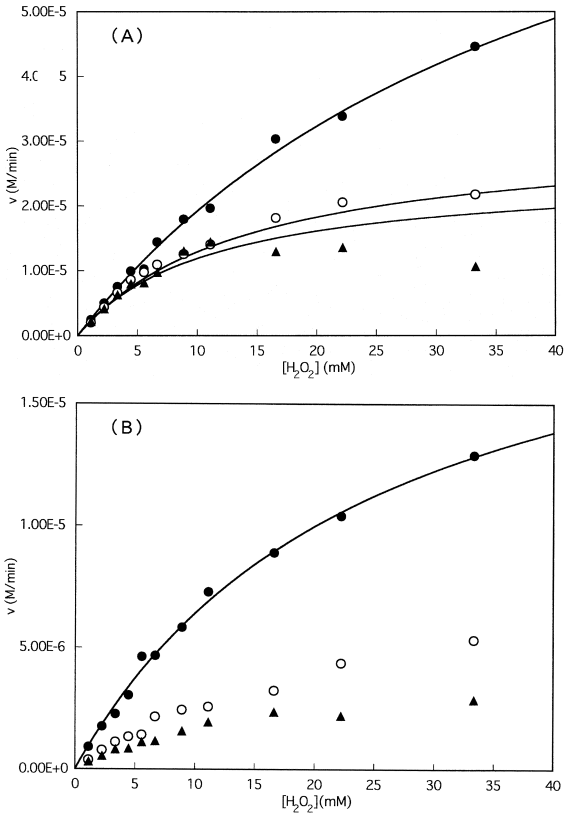

Fig. 3. Relationship between the initial rate and the hydrogen peroxide concentration. The reaction mixture contained 0.5 mM (●), 0.1 mM (○) and 0.05 mM (▲) *o*-dianisidine. (A) 0.3 μM antibody–MP-Fe complex. The lines were calculated values using apparent K_m and k_{cat} values obtained from Fig. 8A. (B) 0.3 μM MP-Fe. The line was calculated values using K_m and k_{cat} values in Table 1.

the complex showed anomalous behaviors at 0.05 mM *o*-dianisidine, that is, the increase in the rates was low with an increase in hydrogen peroxide concentration above 15 mM (Fig. 3A). In these cases, initial rates could not be determined accurately, since time courses of the absorbance increase reached a plateau immediately after starting the reaction (data not shown). The apparent values of the Michaelis constant (K_m) and molecular activity (k_{cat}) for hydrogen peroxide were calculated on the basis of the data at 0.5 mM *o*-dianisidine. These estimates are summarized in Table 1 with other reported values.

Table 1
Kinetic parameters for hydrogen peroxide of the antibody-ferric porphyrin complexes, free ferric porphyrin and natural enzyme HRP

Antibody or protein	Ferric porphyrin	Hapten	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (M^{-1} min^{-1})	Substrate	Temp. (°C)	pH	Ref.
2B4	MP-Fe	*N*-MMP	43 ± 6	330 ± 33	7.7×10^3	*o*-dianisidine (0.5 mM)	10	8	This work
7G12-A10-G1-A12	MP-Fe	*N*-MMP	24	394	1.6×10^4	*o*-dianisidine (0.5 mM)	10	8	[15]
9A5	MP-Fe	*N*-MMP	35	132	3.8×10^3	pyrogallol (0.5%)	20	6	[13]
11D1	MP-Fe	*N*-HMMP[a]	13	86	6.6×10^3	pyrogallol (0.5%)	20	6	[13]
13-1 L chain	TCPP-Fe	TCPP[b]	2.3	667	2.9×10^5	pyrogallol (1.2 mM)	37	8	[16]
HRP	PP-Fe[c]	–	0.0005	306	6.1×10^8	leucomalachite green (15 μM)	25	4	[32]
–	MP-Fe	–	25 ± 3	77 ± 5	3.1×10^3	*o*-dianisidine (0.5 mM)	10	8	this work

[a] *N*-hydroxymethyl mesoporphyrin.
[b] *Meso*-tetrakis (4-carboxyphenyl) porphyrin.
[c] Protoporphyrin.
Each error is the standard deviation.

3.3. Incubation of the complex with hydrogen peroxide

It is known that the catalytic cycle of natural enzyme HRP proceeds via intermediates named as compound I and II [22,23], which each have a distinct optical absorption spectrum [24–26]. To detect any intermediates in the peroxidase reaction by the antibody–MP-Fe complex, we measured the absorption spectrum of the complex after adding some excess equivalents of hydrogen peroxide. When the complex was mixed with hydrogen peroxide by use of a stopped-flow apparatus, no spectral change was observed within 2 ms-1 s (data not shown). After manual mixing with hydrogen peroxide, the spectrum of the complex changed very slowly with a decrease in the peak absorption (Fig. 4A), while such a decrease was not found in MP-Fe (Fig. 4B). If the low absoarbing species observed in Fig. 4A corresponds to an active intermediate as compound I or II, its peroxidase activity will be retained. Then the activity and absorption of the complex were measured at time intervals after mixing with hydrogen peroxide (Fig. 5). The figure revealed that the absorbance decay was concomitant with

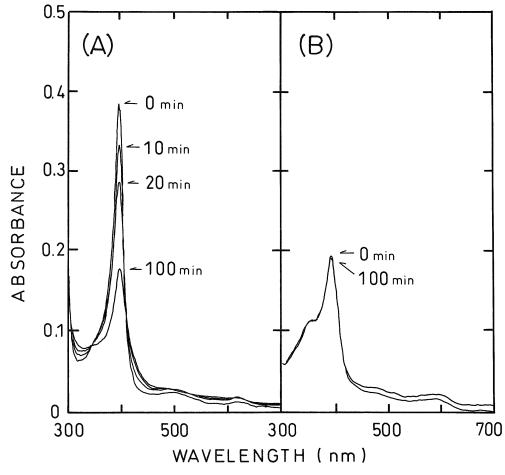

Fig. 4. Absorption spectra of (A) the antibody–MP-Fe complex and (B) MP-Fe at pH 8.0 and 10°C. Traces were made immediately (0 min) and the described times after addition of 14.7 μM hydrogen peroxide. The concentrations of the complex and free MP-Fe were 3 μM.

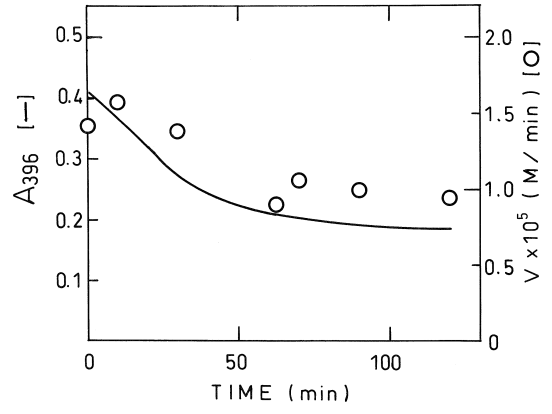

Fig. 5. Time-courses of the absorption at 396 nm (————) and peroxidase activity (○) of the antibody–MP-Fe complex after addition of 14.7 μM hydrogen peroxide at pH 8.0 and 10°C. The concentration of the complex was 3 μM.

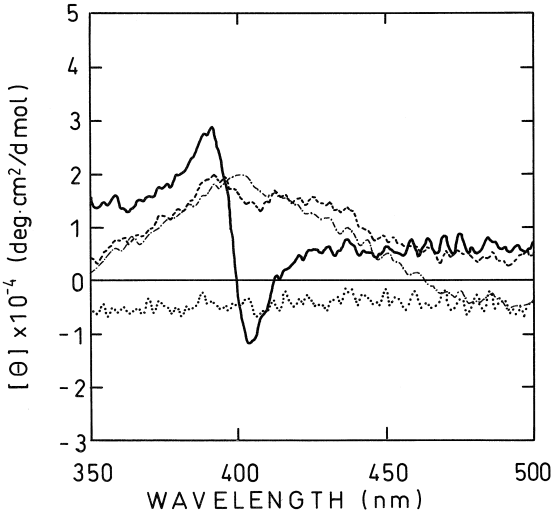

Fig. 6. CD spectra of the antibody–MP-Fe complex and MP-Fe. The spectra of the complex were measured before (————) and at 5–50 min (– – –) 110–155 min (– · –) after addition of 15 μM hydrogen peroxide in the absence of *o*-dianisidine. The spectrum of MP-Fe (· · ·) was measured at 5–50 min after the addition of 15 μM hydrogen peroxide. The concentrations of the complex and MP-Fe were 3 μM.

the activity decline, that is, the low absorbing species observed in Fig. 4A was not an active intermediate.

There is a possibility that MP-Fe was dissociated from the complex upon addition of hydrogen peroxide in the absence of *o*-dianisidine. The dissociation will decrease both the activity and absorption. To clarify this point, the CD spectrum of the complex was measured in the Soret region, since the Soret CD of the complex has been ascribed to the interaction between the antibody and MP-Fe [18]. Fig. 6 shows the CD spectra of the complex before and after addition of hydrogen peroxide compared with the spectrum of MP-Fe. It is found that the complex after the addition had a distinct CD spectrum from not only that before the addition but also MP-Fe, indicating that the complex interacted with MP-Fe even after the addition of hydrogen peroxides. From these results, we concluded that the complex was inactivated by hydrogen peroxide.

3.4. Nucleotide and amino acid sequences of the antibody

For elucidation of the relationship between the structure and function of the antibody, the

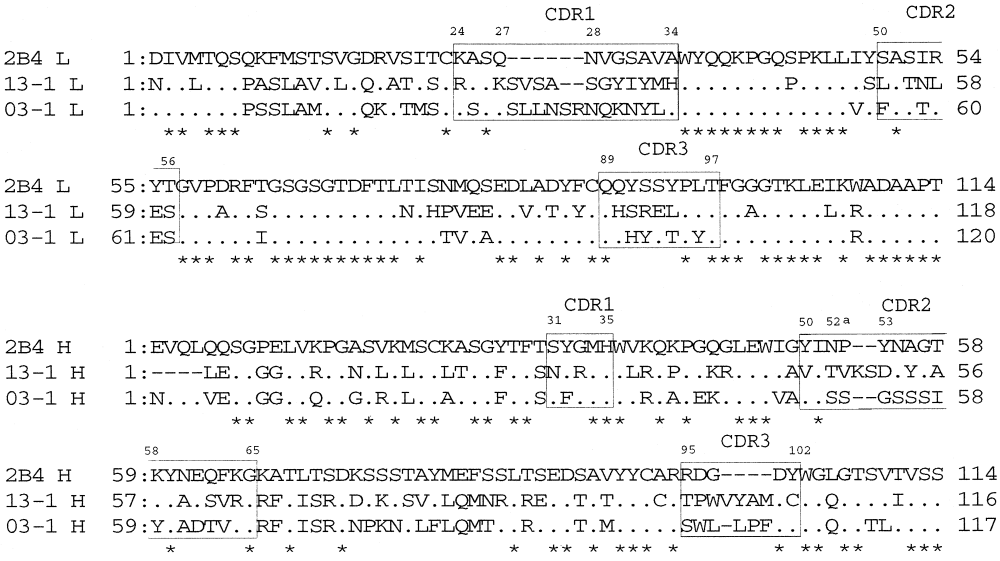

Fig. 7. Homology of amino acid sequences of L and H chains of 2B4 derived from nucleotide sequences with those of *Mab* 03-1 and *Mab* 13-1 [16]. The nucleotide sequences were registered by DDBJ Accession No. D85126 (L chain of 2B4) and D85127 (H chain of 2B4). Conserved amino acid residues were depicted by periods. Asterisks were homology found between three antibodies. Three CDRs (complementarity-determining region; CDR1-CDR3) were surrounded by boxes. The residue numbers above the boxes were indicated according to Kabat et al. [33].

primary structures of V regions were determined. V_L and V_H cDNA were generated from hybridoma mRNA by reverse transcription and the sequences of these cDNA were obtained (DDBJ Access. No. D85126-7) as described in Section 2. The deduced amino acid sequences were shown in Fig. 7.

4. Discussion

Monoclonal antibody 2B4 is generated against *N*-MMP as hapten [17], and was bound to MP-Fe with strong affinity to form the antibody–MP-Fe complex [18]. The complex showed peroxidase activity for *o*-dianisidine and ABTS (Fig. 1). Other anti-porphyrin antibodies were prepared by some groups, and combined with ferric-porphyrin to form the respective complexes which possessed peroxidase activities [13,15,16]. We compared the amino acid sequences in V regions of the antibody 2B4 with those of the other antibodies published [16] (Fig. 7). Particular homology between them were not found in three complementarity-determining regions (CDRs1-3). Recently, Kohda et al. [27] reported that the peroxidase activity of the *Mab* 13-1 L chain depended on three residues in CDRs, as His(L34), His(L90) and Arg(L92) shown in Fig. 7. These residues were not found in CDRs of the 2B4 antibody. Therefore, the antibody 2B4 will have a distinct structure and function from these antibodies.

Kinetic investigations for the peroxidase reaction catalyzed by the antibody 2B4–MP-Fe complex were performed by use of *o*-dianisidine as substrate. The results in Fig. 2 and 3 revealed that the reaction rate for the complex depended on the concentrations of *o*-dianisidine and hydrogen peroxide and exhibited saturation behaviors. In the reaction process of the complex, the ferric iron of MP-Fe in the complex probably reacts with hydrogen peroxide, as well as postulated in that of free ferric porphyrin [28–30], and subsequently may react with *o*-dianisidine to regenerate the ferric state of the complex. In addition, the results in Figs. 4–6 suggested that the complex reacted with hydrogen peroxide to be inactivated. Based on the above considerations, we proposed a scheme for the peroxidase reaction catalyzed by the complex as

$$\mathrm{C(III)} + \mathrm{H_2O_2} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftarrows}} \mathrm{C(III)\cdot H_2O_2} \overset{k_2}{\rightarrow} \mathrm{X} + \mathrm{P_1} \quad (1)$$

$$\mathrm{X} \overset{k_3}{\rightarrow} \mathrm{Y} \quad (2)$$

$$\mathrm{X} + \mathrm{AH_2} \underset{k_{-4}}{\overset{k_{+4}}{\rightleftarrows}} \mathrm{X\cdot AH_2} \overset{k_5}{\rightarrow} \mathrm{C(III)} + \mathrm{P_2} \quad (3)$$

where C(III) is the complex in the ferric state, X the complex in another state, Y the complex in the inactivated state, AH_2 *o*-dianisidine, P products, k the respective rate constants. The k_3 value in Eq. (2) will be negligibly small, since the first-order rate constant of the absorption decay in Fig. 5 was calculated to be about 0.03 min^{-1}. Consequently, the overall reaction rate of the complex, υ, can be expressed by the following equations.

$$\upsilon = k[\mathrm{complex}]/\{1 + K_{\mathrm{m1}}/[\mathrm{H_2O_2}] + K_{\mathrm{m2}}/[o-\mathrm{dianisidine}]\} \quad (4)$$

where

$$k = \{k_2 k_5/(k_2 + k_5)\} \quad (5)$$

$$K_{\mathrm{m1}} = k_5(k_{-1} + k_2)/\{k_{+1}(k_2 + k_5)\} \quad (6)$$

$$K_{\mathrm{m2}} = k_2(k_{-4} + k_5)/\{k_{+4}(k_2 + k_5)\}. \quad (7)$$

Reciprocal of Eq. (4) gives

$$1/\upsilon = \{1 + K_{\mathrm{m1}}/[\mathrm{H_2O_2}] + K_{\mathrm{m2}}/[o-\mathrm{dianisidine}]\}/\{k[\mathrm{complex}]\}. \quad (8)$$

Fig. 8A shows the double reciprocal plots of the data in Fig. 3A according to Eq. (8). In this figure the data at 0.05 mM *o*-dianisidine and hydrogen peroxide concentration above 15 mM were not taken into account, since hydrogen peroxide inhibited the reaction at these concentrations. It is found that the data gave parallel lines, indicating the validity of the above

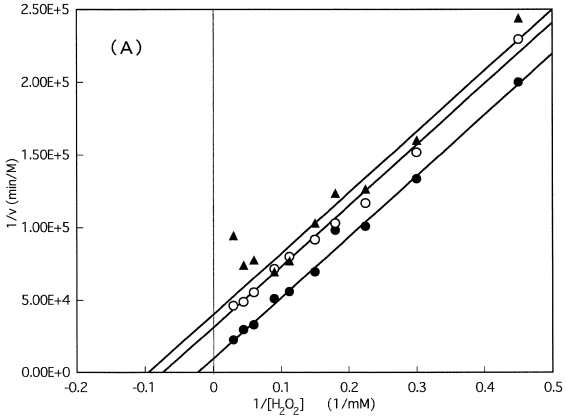

Fig. 8. (A) Double reciprocal plots of the data in Fig. 3A. (B) Plots of intercept value on the ordinate in (A) versus reciprocal of the *o*-dianisidine concentration. (C) Plots of the intercept value on the abscissa in (A) versus reciprocal of the *o*-dianisidine concentration. The *o*-dianisidine concentration of the data was 0.5 mM (●), 0.1 mM (○) and 0.05 mM (▲).

scheme. From the plots of Fig. 8B,C, the k, K_{m1} and K_{m2} values were estimated to be 343 min^{-1}, 45 mM and 0.17 mM, respectively. The values of k and K_{m1} agreed to those of k_{cat} and K_m in Table 1, respectively, which were estimated from the data obtained at 0.5 mM *o*-dianisidine. This agreement indicated that the reaction rate for the complex at 0.5 mM *o*-dianisidine was almost saturated with the *o*-dianisidine concentration. Using the K_m and k_{cat} values obtained from the plots of Fig. 8A, theoretical v values were calculated and shown in Fig. 3A, which explained well the experimental data except those at 0.05 mM *o*-dianisidine and hydrogen peroxide concentration above 15 mM.

It is known that the natural enzyme HRP catalyzes oxidation of various substrates utilizing hydrogen peroxide [31]. The antibody-MP-Fe complex exhibited higher peroxidase activity for *o*-dianisidine and ABTS than MP-Fe but did not for pyrogallol nor hydroquinone, as shown in Fig. 1. The specificity for substrate was not found in the antibody–MP-Fe complex of Cochran and Schultz [15]. From the point of view of the interaction between the antibody and *o*-dianisidine as expressed by Eq. (3), the substrate specificity of the complex can be explained. The molecular size of active substrates as *o*-dianisidine and ABTS is larger than that of inactive ones as pyrogallol and hydroquinone. It is likely that the size difference might relate to the interaction with the complex, as a result, the peroxidase activity depends on substrates. If the specific interaction with a desired substrate is constructed in the antibody, the antibody-ferric porphyrin complex will oxidize the specific substrate more efficiently than free ferric porphyrin.

The list in Table 1 shows that HRP and the antibody-ferric porphyrin complexes, except the 11D1-complex, have similar k_{cat} values in the order of 10^2 min^{-1}. The K_m values of the complexes exist in the range from 2.3 to 43 mM hydrogen peroxide in spite of the difference in hapten [13,15,16], in contrast, HRP has a much smaller K_m value of 0.0005 mM [32]. The strong affinity of HRP with hydrogen peroxide is due to the distal histidine as a ligand to heme iron, since the histidine is postulated to facilitate the formation of compound I [22–26]. The large K_m value of the antibody 2B4–MP-Fe complex

(43 mM) suggested that the complex did not have such a catalytic residue as the distal His in HRP. This view is supported by our previous report [18] in which one side of MP-Fe at the binding site of the antibody was thought to be fully exposed to surrounding solvents like free MP-Fe. To enhance the peroxidase activity of the complex, it will be necessary to introduce a catalytic residue such as the distal His into the binding site of the antibody. Combination with the construction of a specific interaction with a desired substrate into the binding site of the antibody will guide a new type of catalyst. For the challenge, the structure and function of the antibody must be elucidated furthermore.

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