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Chemical modification of a catalytic antibody that accelerates insertion of a metal ion into porphyrin: essential amino acid residues for the catalytic activity

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Abstract

Catalytic antibody 2B generated by immunization with *N*-methyl mesoporphyrin as hapten catalyzes the insertion of a cupric ion into mesoporphyrin. To identify amino acid residues essential for the catalytic activity, we studied effects of various amino acid-reactive reagents on the catalytic activity. The reagents reactive to Arg, Tyr and carboxyl-containing residues inactivated the antibody and mesoporphyrin protected notably the antibody from the inactivation. These results indicated that Arg, Tyr and carboxyl-containing residues are situated in or near the substrate-binding site of the antibody and that some of them would be essential for the catalytic activity. The modified Arg and Tyr residues in the inactivation were quantified in connection with the residual activity. As the result, it was shown that three Arg and one Tyr residues are modified to lead the inactivation. Kinetic analysis indicated that the antibody loses the catalytic activity by modification of one carboxyl-containing residue. In order to find candidates for the modified residues, we performed modeling of the variable domain of the antibody. The model showed that the modified residues are Arg L54, Arg H94, Arg H95, Tyr L91 and Asp H96, and suggested that Arg H95, Tyr L91 and Asp H96 residues would stabilize the transition state of mesoporphyrin in the antibody-mediated reaction.

Keywords: Catalytic antibody; Chemical modification; Porphyrin; Metalation

1. Introduction

Transition state stabilization has been the most important and successful guiding principle for generating catalytic antibodies. Since the first reports of Tramontano et al. [1] and Pollack et al. [2], there have been many reports on catalytic antibodies but the catalytic activities of the antibodies produced by this method are often lower than natural enzymes that catalyze analogous reaction [3,4]. And the mechanism by which these antibodies perform their catalytic functions has been postulated but largely remains unknown. To gain insights into the molecular mechanisms of the antibody catalysis and to produce an antibody with an enhanced catalytic activity, we must accumulate detailed studies of the individual catalytic antibodies.

Previously, we produced catalytic antibodies generated by immunization with *N*-methyl mesoporphyrin (*N*-MMP) as hapten [5], which was designed to mimic a transition-state geometry in insertion of a metal ion into porphyrin [6]. They catalyze insertion of a cupric ion (Cu(II)) into mesoporphyrin (MP) to form Cu(II)-mesoporphyrin (Cu-MP). We have been characterizing the biophysical and kinetic properties of the catalytic antibodies [7–9] and showed that the rate acceleration of the antibodies is attributed to the transition-state stabilization [5]. More recently, efforts have focused

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on the study to know essential amino acid residues for the catalytic activity. Sequence analysis of the catalytic antibodies presented the possibility that a common sequence of $R^{95}D^{96}X^{97}D^{101}Y^{102}$ in CDRH3 would include essential amino acid residues for the catalytic activity [10].

As a general means of studying the functional role of amino acid residue of an enzyme, chemical modification with amino acid-specific reagents of the enzyme has been exploited for many years and the chemistry is well established [11–13]. Therefore, comprehensive chemical modification study may provide a better understanding of essential amino acid residues for the catalytic activity. In this study, we employed the chemical modification of Arg, Tyr, Trp, His, Met, Lys, Asp and Glu residues of the catalytic antibody 2B4, which has the highest levels of metalation activity among our catalytic antibodies obtained, to identify amino acid residues essential for the catalytic activity. These amino acids construct the CDRs of the antibody and are known to be often catalytically important. Coupling with the chemical modification and three-dimensional model of the antibody constructed on the basis of Fv sequence, we propose plausible amino acid residues responsible for the catalytic activity.

2. Experimental

2.1. Materials

Porphyrin derivatives were synthesized as described previously [5]. Monoclonal antibody 2B4 elicited against *N*-MMP was prepared and purified from ascites fluid as in the previous paper [5]. The protein concentration was determined from the absorbance at 280 nm and calculated assuming A_{280} of 1% of IgG as 13.7 [14]. The antibody concentration was expressed per binding-site assuming a molecular weight of 150,000 (two binding sites per antibody). Ammonium chloride (NH_4Cl), 1,3-diaminopropane (DAP), diethylpyrocarbonate (DEPC), glycine ethyl ester (GEE), glycine methyl ester (GME), *p*-nitrobenzenesulfonyl fluoride (NBSF), 3-nitro-L-tyrosine ethyl ester (NTEE), phenylglyoxal (PGO) and tetranitromethane (TNM) were purchased from Sigma–Aldrich Chemical Co., St.Louis, 1-ethyl-3-(3′-dimethylaminopropyl)-carbodiimide (EDC) from Peptide Institute Inc., Japan, *N*-bromosuccinimide (NBS), chloramine-T and 2,4,6-trinitrobenzene sulfonic acid (TNBS) from Wako Pure Chemical Industries Ltd., Japan. All other reagents were of analytical grade.

2.2. Measurement of metalation activity

Catalytic activity for insertion of a cupric ion into MP was determined by measuring an increase in absorbance due to the production of Cu-MP according to the previous method [5]. The reaction mixture (300 μl) contained 1.0 μM antibody, 39 μM MP and 1.0 mM Cu(II) acetate in assay buffer consisting of 0.5% (w/v) Triton X-100, 5% (v/v) dimethyl sulfoxide and 90 mM Tris-acetate (pH 8.0). Time course of the absorbance change was followed at 397 nm and 37 °C. The initial rate was calculated from the time course using the difference absorption coefficient at 397 nm between MP and Cu-MP of 0.318 $\mu M^{-1}\,cm^{-1}$ [5] and was corrected for the background rate. The residual activity in the inactivation experiments was expressed as a relative value of an appropriate control.

2.3. Chemical modification of arginyl residues

A 400 mM PGO solution (37.5 μl) in methanol was added to a 10.26 μM antibody solution (1462.5 μl) in 50 mM HEPES-KOH (pH 8.0) with or without 391 μM MP, and the reaction mixture was incubated at 25 °C in the dark. At appropriate time intervals, aliquots (70 μl) of the reaction mixture were removed and mixed with a 20 mM arginine solution (70 μl) to terminate the reaction, and 60 μl of the solution was assayed for the residual activity. For analysis by Tsou's statistical method, aliquots (200 μl) of the reaction mixture were removed and freed of excess reagent by a gel-filtration through a Sephadex G-25 column (7 mm × 150 mm) equilibrated with 0.1 M Tris-acetate (pH 8.0). The fractions containing the antibody were pooled, and the protein concentration was determined from the absorbance at 280 nm on the assumption of A_{280} of 1% of IgG as 13.7 [14]. The difference spectrum of the modified antibody was obtained with the untreated antibody as the control and the number of modified Arg residues were estimated using a molar absorption coefficient of 11,000 $M^{-1}\,cm^{-1}$ at 250 nm for a diphenylglyoxal derivative [15], and the solution was assayed for the residual activity.

2.4. Chemical modification of tyrosyl residues

A 100 mM NBSF solution (1.5 μl) in 2-propanol was added to a 10 μM antibody solution (148.5 μl) in 0.1 M Tris-acetate (pH 8.0) with or without 391 μM MP, and the reaction mixture was incubated at 25 °C for 15 min. The mixture (70 μl) was added to a 2 mM tyrosine solution (70 μl) to terminate the reaction and 60 μl of the solution was assayed for the residual activity. A 50 mM TNM solution (30 μl) in methanol was added to a 10.2 μM antibody solution (1470 μl) in 0.1 M Tris-acetate (pH 8.0), and the reaction mixture was incubated at 25 °C. At appropriate intervals, aliquots (120 μl) of the reaction mixture were removed and added to a 2 mM tyrosine solution (120 μl) to terminate the reaction, and 60 μl of the mixture was assayed for the residual activity. For analysis by Tsou's statistical method, aliquots (120 μl) of the reaction mixture were removed and added to a 2 mM tyrosine solution (120 μl) to terminate the reaction, and then the buffer was changed to 0.1 M Tris-acetate (pH 8.0) by a gel-filtration through the column described in the Section 2.3. The fractions containing the antibody were pooled, and the protein concentration was determined by the Lowry–Folin method. The number of modified Tyr residues by TNM was estimated

spectrophotometrically using a molar absorption coefficient of 4100 M^{-1} cm^{-1} at 428 nm for 3-nitrotyrosine [16] and the solution was assayed for the residual activity.

2.5. Chemical modification of tryptophanyl residues

A 6 mM NBSF solution (10 μl) in 50 mM sodium-acetate buffer (pH 4.0) was added to a 10.3 μM antibody solution (290 μl) in the same buffer and the reaction mixture was incubated at 25 °C for 10 min. An aliquot (200 μl) of the mixture was passed through the gel filtration column described in the Section 2.3 to remove excessive reagent. The fractions containing the antibody were pooled, the protein concentration was determined by the Lowry–Folin method and the residual activity was measured.

2.6. Chemical modification of lysyl residues

A 10 mM TNB solution (24 μl) in 50 mM HEPES-KOH buffer (pH 7.0) was added to a 10.6 μM antibody solution (376 μl) in the same buffer with or without 391 μM MP, and the reaction mixture was incubated at 25 °C for 90 min in the dark. An aliquot (180 μl) of the reaction mixture was removed and added to a 2 M Lys solution (20 μl) to terminate the reaction, and the whole was passed through the gel filtration column described in the Section 2.3. The fractions containing the modified antibody were pooled, the protein concentration was determined by the Lowry–Folin and the residual activity was measured.

2.7. Chemical modification of histidyl residues

A 100 mM DEPC solution (20 μl) in ethanol was added to a 10.2 μM antibody solution (980 μl) in 0.1 M sodium phosphate buffer (pH 7.0), and the reaction mixture was incubated at 37 °C for 10 min in a cuvette. The change in absorbance at 240 nm with time was recorded, and the number of modified His residues was determined using a molecular absorption coefficient at 240 nm of 3200 M^{-1} cm^{-1} for an *N*-carbethoxyhistidine derivative [13,17]. The reaction mixture (200 μl) was subjected to a gel filtration through the column described in the Section 2.3. The fractions containing the antibody were pooled, the protein concentration was determined from the absorbance at 280 nm on the assumption of A_{280} of 1% of IgG as 13.7 [14], and the solution was assayed for the residual activity. For test of the reactivation, 50 μl of a 7.1 M NH_2OH solution (pH 7.0) was added to the reaction mixture (450 μl) and the whole was incubated at 37 °C for 20 min. Then 200 μl of the reaction mixture was passed through the gel filtration column described in the Section 2.3 to remove the reagent, and the fractions containing the antibody were pooled. The protein concentration was determined from the absorbance at 280 nm on the assumption of A_{280} of 1% of IgG as 13.7 [14], and the residual activity was measured. The number of modified histidine residues was determined spectrophotometrically described above.

2.8. Chemical modification of carboxyl-containing residues

A 10 μM antibody solution was incubated with EDC (20–100 mM) and a nucleophile at 37 °C in the dark. The nucleophile used was NH_4Cl (100 mM), DAP (100 mM), GME (73 mM) and GEE (73 mM). The reaction mixture was maintained at pH 4.8 by adding a small amount of alkaline solution. Aliquots (100 μl) of the mixture were withdrawn at given times and added to the same volume of 2 M sodium acetate buffer (pH 4.0) to quench residual reagent. After dialysis against 0.1 M Tris-acetate (pH 8.0) to remove the reagents, the protein concentration was determined from the absorbance at 280 nm on the assumption of A_{280} of 1% of IgG as 13.7 [14], and the solution was assayed for the residual activity.

A 10 μM antibody solution was incubated with EDC (100 mM) and NTEE (30 mM) at 37 °C in the dark. The reaction mixture was maintained at pH 4.8 by adding a small amount of alkaline solution. At zero and 90 min, the aliquots (500 μl) of the mixture were removed and added to the same volume of 2 M sodium acetate buffer (pH 4.0) to terminate the reaction. The solution was dialyzed extensively against 0.05 M sodium acetate buffer (pH 4.0) and subsequently against 0.1 M Tris-acetate (pH 8.0). The protein concentration of the dialyzate was determined with Advanced Protein Assay Reagent ADV01 (Cytoskelton) and the residual activity was measured. The modified antibody in the dialyzate (500 μl) was precipitated by the addition of 30% (w/v) TCA (250 μl) and the precipitates were collected by centrifugation, washed extensively with chilled acetone, air dried and dissolved in 0.1 M NaOH (400 μl). The number of nitrotyrosyl groups incorporated was determined spectrophotometrically at 430 nm using a molar absorption coefficient of 4600 M^{-1} cm^{-1} [18], and the protein concentration was determined with Advanced Protein Assay Reagent ADV01 after addition of 0.1 M HCl (400 μl).

2.9. Chemical modification of methionyl residues

For chemical modification of Met residues, a 20 mM chloramine-T solution (45 μl) in 0.1 M Tris-acetate (pH 8.0) was added to a 11.8 μM antibody solution (255 μl) in the same buffer, and the reaction mixture was incubated at 25 °C for 20 min. The respective reaction mixtures (200 μl) were passed through the gel filtration column described in the Section 2.3 and assayed for the residual activity.

2.10. Analysis of relationship between chemical modification and inactivation

For determination of number of the amino acid residues essential to the catalytic activity, the inactivation caused by chemical modification was analyzed according to Tsou's statistical method [19]. In the method, the inactivation was classified into following six cases: Case 1, the essential and the non-essential residues are modified at the same rate; Case

2, the essential and the non-essential residues are modified at markedly different rates; Case 3, the essential and the non-essential residues are modified at significantly but not markedly different rates; in the Cases 1–3, the activity is lost completely when all the essential residues are modified; Case 4, some of the activity remains after all the essential residues are modified; Case 5, the protein retains full activity when any one of the essential residues is not modified; Case 6, two kinds of amino acid residues are modified, and both are the essential residues. Our experimental data were applied to the method of Case 4, in which all the modified residues under respective experimental conditions used are classified to three kinds of groups as follows: (1) the first group that reacts most rapidly and does not contain any residues essential for the catalytic activity, (2) the second group that includes the essential residues and reacts appreciably only after the reaction with the first group has almost reached completion, and (3) the third group that reacts most slowly, or not at all, with the reagent. The observed residual activity, a_x, can be expressed by the following equation [19].

$$a_x = \{nx - (n\text{-}p\text{-}s)\}^i \frac{1 - a_T}{p^i} + a_T \quad (1)$$

where a_T is the residual activity of the completely modified species, n a total number of residues corresponding to the modification, s a number of the first group, p a number of the second one, n-p-s a number of the third one and i the number of the essential residues. The overall fraction of unmodified residues, x, is calculated by

$$x = \frac{n - m}{n} \quad (2)$$

where m is the observed number of modified residues.

The residual activities (a_x) were calculated at the number of essential residues (i) by use of Eqs. (1) and (2) on the following assumptions: (1) a total number of modified residues in the antibody (n) is 16 for Arg and 29 for Tyr, (2) the number of the first group of the modifiable Arg or Tyr residues (s) is 0, (3) the number of the second group of the modified residues (p) is 3 for Arg and 2 for Tyr and (4) the fractional residual activity of the modified antibody (a_T) is 0.09 for Arg and 0.17 for Tyr.

2.11. Modeling of the three dimensional structure of the antibody Fv segment

The molecular operating environment (MOE) program (Chemical Computing Group Inc.) was used to perform molecular modeling of the three dimensional Fv structure of the antibody 2B4 under the supplier's conditions. The coordinates for the antibody 7G12 (Protein Data Bank accession code 1NGW) [20] were downloaded from the Protein Data Bank and used as a template for the modeling in Amber89 force field. *N*-MMP was superimposed on the modeled structure in the same coordination as 1NGW.

3. Results

3.1. Effects of amino acid-reactive reagents on the activity of catalytic antibody 2B4

To identify amino acid residues responsible for catalytic activity of the antibody 2B4 which accelerates insertion of Cu(II) into MP to form Cu-MP, we studied effects of following amino acid-reactive reagents on the activity: PGO, NBSF, NBS, TNBS, DEPC, chloramine-T and EDC were used to modify Arg, Tyr, Lys, Trp, His, Met and carboxyl-containing residues, respectively, in the antibody. After the antibody was incubated with and without respective reagents under their typical conditions as shown in Table 1, the remaining activity was measured. The antibody was inactivated by PGO, NBSF, TNBS and EDC, indicating that modification of Arg, Tyr, Lys and carboxyl-containing residues in the antibody led to the loss of the catalytic activity because PGO, NBSF, TNBS and EDC are highly reactive reagents specific to the corresponding amino acid residues in proteins [11–13].

DEPC reacts with His residues in proteins to yield an *N*-carbethoxyhistidyl derivative, followed by an increase in absorbance at 240 nm [13,17]. Number of the modified His residues can be calculated from the absorption increase at 240 nm with a molar absorption coefficient of 3200 M^{-1} cm^{-1} for *N*-carbethoxyhistidine [13,17]. On the other hand, DEPC does not always react specifically with His but also with Tyr, Lys, Cys or carboxyl-containing residues in proteins [13]. Hydroxylamine has been shown to reverse the modified His and Tyr residues, which is much more reactive to the former than the latter, but does not reverse the modified Lys, Cys and carboxyl-containing residues [13]. The inactivation of the antibody by DEPC (Table 1) is ascribable to modification of His residues if the inactivation follows the absorption increase at 240 nm and exposure of the inactivated antibody to hydroxylamine reverses the absorption at 240 nm accompanying regeneration of the activity. Table 2 clearly shows that the hydroxylamine treatment of the inactivated antibody failed to regenerate the activity in spite of the absorption reverse. We therefore concluded that the inactivation by DEPC, as shown in Table 1, is not ascribable to the modification of His residues but to that of other residues.

Table 1 also shows that substrate MP protected notably the antibody from the inactivation by PGO, NBSF and EDC, and that the existence of MP did not affect the inactivation by TNBS. The results indicate that Arg, Tyr and carboxyl-containing residues are located in or near the MP binding-site of the antibody, and that some of them would be catalytically important. We examined in detail the modification of these residues in the antibody given further.

3.2. Modification of arginyl residues in the antibody

Fig. 1A shows that the antibody was inactivated in a time-dependent manner by the arginine-specific modifying

Table 1
Chemical modification of catalytic antibody 2B4

Modified residue	Modification conditions				Residual activity (%)	
	Reagent (mM)	Temperature (°C)	pH	Time (min)	MP (−)[a]	MP (+)[b]
Arg	PGO (10)	25	8.0	240	14	88
Tyr	NBSF (1.0)	25	8.0	15	5	67
Trp	NBS (0.2)	25	4.0	10	89	ND
Lys	TNBS (0.6)	25	7.0	90	17	9
His	DEPC (2.0)	37	7.0	10	31	ND
Met	Chloramine-T (3.0)	25	8.0	20	109	ND
Asp and Glu	EDC (100)	37	4.8	180	4	40

The antibody was reacted with various amino acid-specific reagents in the absence and presence of mesoporphyrin and assayed for resisual activity. Detailes were shown in the Sections 2.3–2.9. ND: the measurement was not conducted, and thus the activity was not determined.
[a] In the absence of mesoporphyrin.
[b] In the presence of 391 μM mesoporphyrin.

Table 2
Modification of catalytic antibody 2B4 by DEPC

Treatement with hydroxylamine[a]	Residual activity (%)	Number of modified His residues[b]
Before	31	5.6
After	10	0.4

The antibody was modified by 2 mM DEPC in 0.1 M sodium phosphate buffer (pH 7.0) containing 2% ethanol at 37 °C for 10 min. Subsequently, the modified antibody was treated with NH_2OH. Details were shown in the Section 2.7.
[a] Incubation of the modified antibody with 0.71 M NH_2OH at pH 7.0 and 37 °C for 20 min.
[b] Number of the modified His residues was determined spectrophotometrically using a molecular absorption coefficient at 240 nm of 3200 M^{-1} cm^{-1} [13,17].

reagent PGO and MP protected the antibody from the inactivation. Fig. 1B shows plots of number of the modified Arg residues versus the remaining activity, in which the number of the modified Arg residues was estimated by measuring absorption change at 250 nm. The figure shows that a partial activity remains even when the modification proceeds, indicating that the modification of the Arg residues related to the inactivation does not result in the complete loss of the activity.

The number of Arg residues essential for the activity was determined on the basis of the data in Fig. 1B according to Tsou's statistical method Case 4 [19]: the residual activities (a_x) were calculated at the number of essential residues (i) of 1, 2 and 3 by use of Eqs. (1) and (2) (see details in Section 2.10). Fig. 1B shows that the theoretical curve in i = 3 explains well the experimental data. Accordingly, we concluded that three Arg residues in the antibody are modified to lead the inactivation.

3.3. Modification of tyrosyl residues in the antibody

NBSF inactivated the antibody as shown in Table 1. However, number of the modified Tyr residues for the inactivation could not be estimated because the modification by NBSF does not yield chromogenic derivatives. We used another reagent, TNM, which specifically reacts with Tyr to produce a nitrated Tyr having an absorption maximum of 428 nm [16]. Incubation of the antibody with TNM resulted in the time-dependent inactivation but the catalytic activity remained after prolonged incubation time (Fig. 2A). The results indicate that the modification of Tyr residue essential for the activity does not result in the complete loss of the

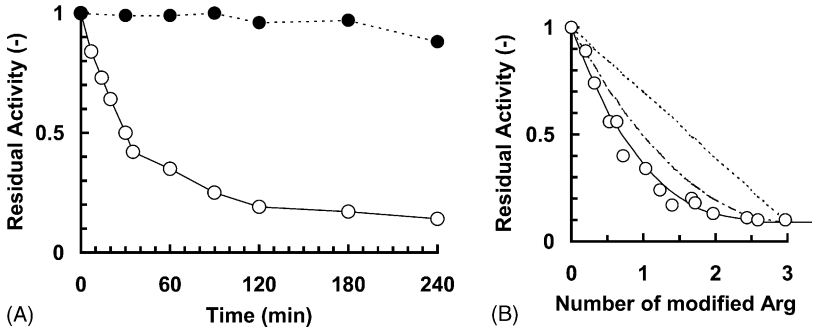

Fig. 1. Modification of Arg residues of the antibody 2B4 by PGO. The antibody (10 μM) was incubated with 10 mM PGO in 50 mM HEPES-KOH (pH 8.0) containing 2.5% methanol at 25 °C in the dark. (A) Time course of the residual activity in the absence (○) or presence (●) of 391 μM MP. (B) Relationship between the number of Arg residues modified and the residual activity. Theoretical curves in i = 1 (····), 2 (– · –) and 3 (—) were calculated according to Tsou's statistical method [19] (details are in the Sections 2.10 and 3.2) for n = 16, s = 0, p = 3 and a_T = 0.09.

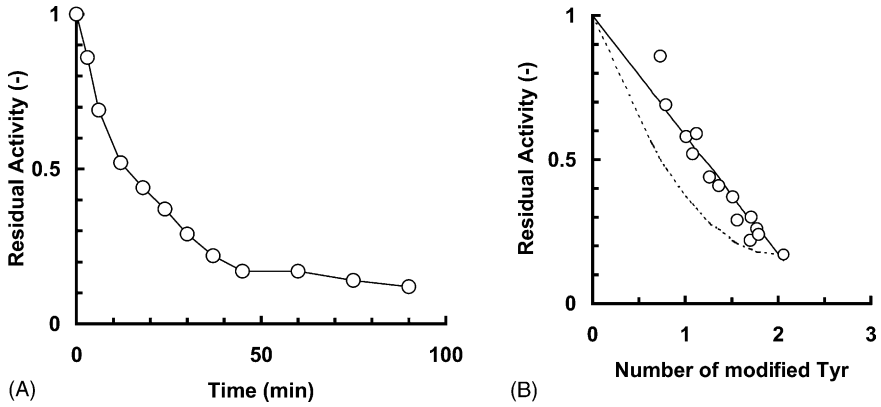

Fig. 2. Modification of Tyr residues of the antibody 2B4 by TNM. The antibody (10 μM) was incubated with TNM (1.0 mM) in 0.1 M Tris-acetate (pH 8.0) containing 2.0% methanol at 25 °C. (A) Time course of the residual activity. (B) Relationship between the number of Tyr residues modified and the residual activity. Theoretical curves in $i = 1$ (—) and 2 (····) were calculated according to Tsou's statistical method [19] (details are in the Sections 2.10 and 3.3) for $n = 29$, $s = 0$, $p = 2$ and $a_T = 0.17$.

activity. It is not clear that MP protects the antibody from the inactivation by TNM since MP reacted with TNM under the experimental conditions used (data not shown). Relationship between the number of the modified Tyr residues and the remaining activity (Fig. 2B) was analyzed according to Tsou's method Case 4 (see Section 2.10). The analysis showed that one Tyr residue is responsible for the inactivation (Fig. 2B).

3.4. Modification of aspartyl and glutamyl residues of the antibody

EDC alkylates carboxyl groups of proteins in the presence of nucleophile [11,21–23]. Fig. 3A shows time courses of the residual activity after the antibody was mixed with 0.1 M EDC and 0.1 M NH_4Cl as a nucleophile in the absence and the presence of MP. The modification resulted in a time-dependent inactivation and MP provided a significant protection of the antibody against this inactivation. However, there is a possibility that carboxyl groups of MP may compete with those of the antibody for EDC. To clarify this possibility, we performed the inactivation experiments in the presence of oxalate that has two carboxyl groups in a molecule as well as MP. As shown in the same figure, the inactivation was not protected by oxalate. This result indicates that carboxyl groups of MP did not compete with those of the antibody for EDC but binding of MP to the antibody protected the antibody from the inactivation, that is, carboxyl-containing residues exist in or near the antigen binding-site of the antibody.

Since neither EDC nor NH_4Cl are chromogenic, number of the modified residues by EDC could not be estimated photometrically. Therefore, we performed the inactivation experiments by use of a chromogenic nucleophile, NTEE [18]: the antibody was treated with 0.1 M EDC in the presence of 30 mM NTEE for 90 min, followed by extensive dialysis, catalytic assay and spectrophotometric determination of nitrotyrosyl groups in the antibody. The modification resulted in the incorporation of 11.3 nitrotyrosyl groups per mole of the antibody with a loss of 98.6 % of its activity. This result suggests that many carboxyl-containing residues were modified by EDC and that the inactivation process cannot be analyzed exactly according to Tsou's statistical method as used in the Sections 3.2 and 3.3. We therefore obtained number of the carboxyl-containing residues for the inactivation kinetically as further.

In a preliminary experiment, the antibody was treated with 0.1 M EDC in the presence of a nucleophile such as: DAP, GME or GEE as well as NH_4Cl. Since semi-logarithmic plots of the residual activity as a function of time were linear in the case of DAP (Fig. 3B), the pseudo first-order rate constants of the inactivation (k) were obtained in the presence of DAP with varying concentrations of EDC. The k value shows a linear dependence on the concentration of EDC with a slope of 1.1 in double logarithmic plots (Fig. 3C). According to the method reported by Hollenberg et al. [24] we concluded that modification of one carboxyl-containing residue by EDC leads to the inactivation in the antibody.

4. Discussion

Comprehensive chemical modification of the catalytic antibody 2B4 indicated that three Arg, one Tyr and one carboxyl-containing residues are situated in or near the substrate-binding site of the antibody, and that some of them are essential for the catalytic activity. In order to find candidates for the residues, we constructed the three-dimensional molecular model of the antibody on the basis of the amino acid sequence in the Fv region. The crystal structure of the antibody 7G12 Fab fragment complex with *N*-MMP [20] (PDB code 1NGW) was selected as a template for the modeling,

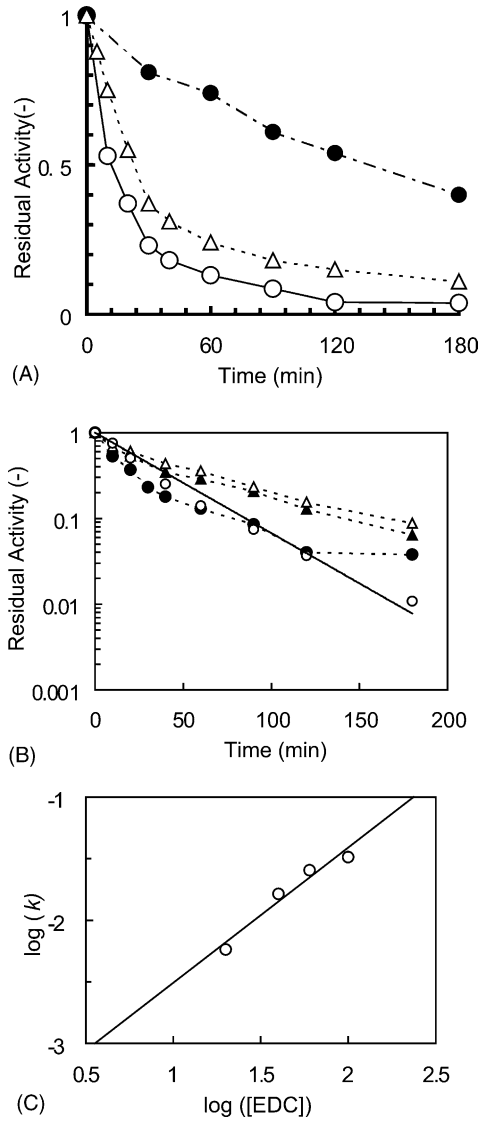

Fig. 3. Modification of carboxyl groups of the antibody 2B4 by EDC at pH 4.8. (A) Time course of the residual activity after the antibody (10 μM) was mixed with EDC (100 mM) and NH_4Cl (100 mM) at 37 °C in the dark; in the absence (◯) or presence (●) of 391 μM MP or in the presence of 391 μM oxalate (△). (B) Semi-logarithmic plots of time course of the residual activity after mixing the antibody (10 μM) with EDC (100 mM) and a nucleophile at 37 °C in the dark. The nucleophile used is 100 mM DAP (◯), 100 mM NH_4Cl (●), 3 mM GME (▲) or 73 mM GEE (△). The straight line represents a linear relationship obtained in the data of DAP. (C) Double logarithmic relationship between the pseudo-first order rate constant, *k*, and EDC concentration. The *k* values were obtained from the time courses of the residual activity after mixing the antibody (10 μM) with varying concentrations of EDC in the presence of DAP (100 mM) at 37 °C. The line represents a linear relationship obtained with slope = 1.1 (γ = 0.95).

because 7G12 and 2B4 antibodies have been raised against *N*-MMP as hapten and accelerate the insertion of Cu(II) into MP and their amino acid sequences of the light and heavy chains in the variable regions are 87.7 and 78.9% homologous, respectively. A possible site of *N*-MMP binding in the model is shown in Fig. 4, which was quite similar to that of the antibody 7G12 [20]. We found that three Arg residues (Arg L54, Arg H94 and Arg H95) are located near the binding site of *N*-MMP (Fig. 4A), indicating that modification of these three residues by PGO brings on a great steric hindrance to the substrate binding and subsequently decreases in the catalytic activity.

As described above, the chemical modification study suggested that one Tyr residue located near the substrate-binding site would be related to the catalytic activity. The model indicates that two Tyr residues (Tyr L91 and Tyr L94) are situated near the binding site of *N*-MMP (Fig. 4A) and that Tyr L91 interacts with a pyrrole ring of *N*-MMP (Fig. 4B). Since Tyr L91 is buried deeper in the binding pocket of *N*-MMP than Tyr L94 as shown in Fig. 4A, the pKa of the hydroxyl group of Tyr L91 must be lowered rather than that of Tyr L94. TNM is subjected to react with a Tyr having a lower pK_a value [25]. We considered, therefore, that Tyr L91 is more reactive to TNM than Tyr L94 and modification of Tyr L91 by TNM loses the catalytic activity in the antibody.

Kinetic analysis of the carboxyl modification indicated that one carboxyl-containing residue would be related to the catalytic activity (Fig. 3C), though the antibody has 65 corresponding residues in the sequence. We found that only two carboxyl-containing residues (Asp H96 and Asp H101) are located in the binding-site of *N*-MMP of the modeled structure (Fig. 4A). Both Asp H96 and Asp H101 are included in CDRH3, which has a characteristic feature in the antibody: its amino acid sequence, $R^{95}D^{96}X^{97}D^{101}Y^{102}$, is conserved in all the catalytic antibodies generated by a single immunization with *N*-MMP as hapten but has not been found in the non-catalytic antibodies generated by the same immunization [10]. Thus we anticipated that there is an amino acid residue responsible for the catalytic activity in the sequence. As shown in Fig. 4B, Asp H96 is in close vicinity to *N*-MMP and the carboxyl group of this residue is oriented toward the center of *N*-MMP, whereas Asp H101 is buried in the binding site of *N*-MMP. Treatment of the antibody with EDC almost completely abolished the catalytic activity, while MP protected partially the antibody from the inactivation (Fig. 3A), suggesting that the modified residue related to the catalytic activity is not buried deep in the binding site of substrate. Consequently, it is suggested that the antibody lost its activity with the modification of Asp H96.

In the modeled structure of the *N*-MMP binding site (Fig. 4B), CDRH3 forms a β turn and the side chain of Arg H95 interacts with adjacent Asp H96 so as to tend the carboxylic side chain of Asp H96 to the center of *N*-MMP, which agrees well with the CDRH3 structure previously predicted [10]. On an opposite side of Arg H95 and Asp H96, the side-chain of Tyr L91 interacts with *N*-MMP in Fig. 4B. These findings lead to the view that the coordination of Arg H95, Asp H96 and Tyr L91 plays a key role in stabilizing the transition state of porphyrin in the insertion of a metal ion into porphyrin.

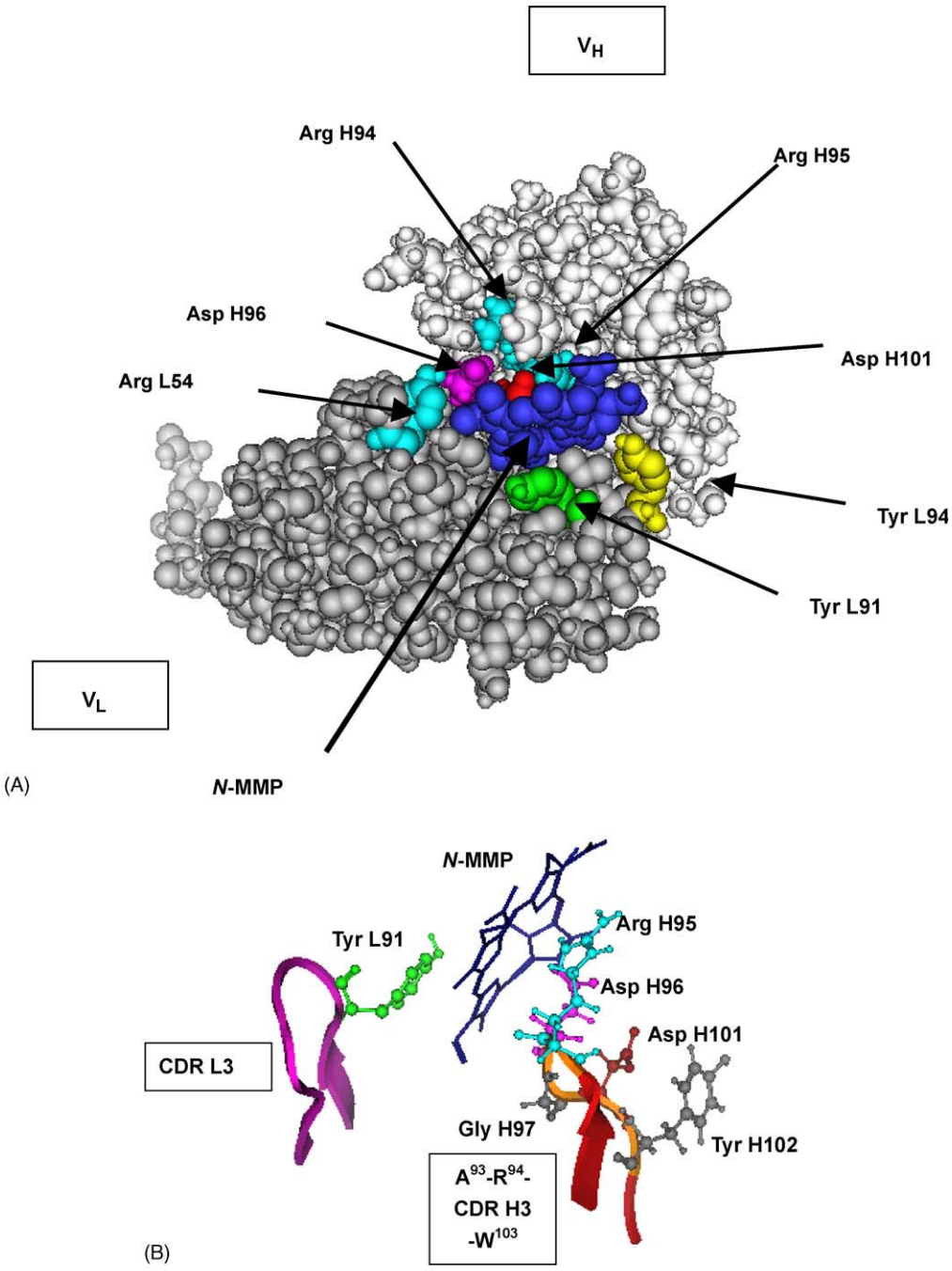

Fig. 4. Modeled structure of the antibody 2B4 Fv fragment with *N*-MMP. The amino acid residues are numbered according to Kabat et al. [26]. (A) Space filling model of the V_H and V_L regions displayed in gray and light gray, respectively, with *N*-MMP (blue). Three Arg residues (L54, H94 and H95) are in light blue, Asp H96 in magenta, Asp H101 in red, Tyr L91 in green and Tyr L94 in yellow. (B) Ribbon model of CDRL3 (magenta) and CDRH3 (orange) structures of the antibody 2B4 Fv fragment with *N*-MMP (blue). Backbone structure adjacent to CDRH3 consisting of Ala H93, Arg H94 and Trp H103 is also displayed with ribbon model in red. Side chains of Arg H95 (light blue), Asp H96 (magenta), Gly H97 (gray), Asp H101 (red), and Tyr H102 (gray) are shown.

5. Conclusions

To identify amino acid residues essential for the catalytic activity of the antibody 2B4, we examined effects of various amino acid reactive reagents on the activity. As a result, it has been shown that modification of three Arg, one Tyr or one carboxyl-containing residues by the reagents inactivates the antibody. Construction of a three-dimensional molecular model of the antibody Fv region demonstrated that candidates for the modified residues are Arg L54, Arg H94, Arg H95, Tyr L91 and Asp H96, and suggested that Arg H95, Tyr L91 and Asp H96 participate in the transition state stabilization in the antibody-mediated reaction.

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