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Immunochemical Features of Complementarity Determining Region (CDR) Peptide in Anti Hemin Monoclonal Antibody

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Abstract: Messenger RNA purified from the anti hemin monoclonal antibody (1D3) secreting hybridoma was amplified by RT-PCR and the nueleotide and amino acid sequences of the antibody were determined. The role of complementarity determining regions (CDRs) in porphyrin recognition and its immunochemical feature of the antibody were investigated by using ELISA, fluorescence measurement and computational calculation of the conformation. All CDR peptides of the heavy chain of the antibody were synthesized and their affinity constants to porphyrins were determined. The value of CDR2 of heavy chain (CDRH2) of 1D3 was 1.5×10^5/M for protoporphyrin and 7×10^7/M for TCPP, respectively, while that of the whole antibody showed to be 1.2×10^7/M for TCPP. Though CDRH2 is a 17 meric peptide, it showed higher affinity than the whole antibody (1D3). Porphyrins can be considered to firmly bind with CDRH2, while CDRH3 is not involved in the antigen binding. CDR-1 may participate in the recognition with a small contribution. By the computational analysis of steric conformation, it was suggested that CDRH1 and CDRH2 co-operatively function in the recognition of porphyrin.

Keywords: anti hemin monoclonal antibody; complementarity determining region; variable region; porphyrin

INTRODUCTION

Many monoclonal antibodies against synthetic porphyrin, for instance TCPP [1], and a partially modified natural type of porphyrin, N-methyl-mesoporphyrin [2], have been produced so far. However, the production of the monoclonal antibody to natural type of porphyrin, hemin, is scarcely reported except our reference in which some interesting immunochemical features of the antibody were described by Uda *et al.* [3].

Molecular analyses of the antigen recognition sites of antibodies have extensively developed [4,5]. The importance of the role of CDRs has especially been pointed out. Many studies with respect to the analysis of the interaction between an antigen and antibody have been made by the method of X-ray crystallography, NMR and so on. These approaches give us much information about the interactions between an antigen and antibody and also lead us closer to an understanding of the recognition sites. These methods are fairly advantageous but a huge amount of data must be accumulated for obtaining a general answer with respect to antigen recogni-

Abbreviations: PP, protoporphyrin; TCPP, meso-tetra (4-carboxy phenyl) porphine; PEG, polyethylene glycol; BSA, bovine serum albumin; PBS, phosphate buffered saline; CDR, complementarity determining region; CDRH1, CDR1 of heavy chain; CDRH2, CDR2 of heavy chain; CDRH3, CDR3 of heavy chain; CDRL1, CDR1 of light chain; CDRL2, CDR2 of light chain; CDRL3, CDR3 of light chain; VH, variable region of heavy chain; VL, variable region of light chain.

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tion. For this purpose, a large molecule antigen such as a protein is not suitable because it is too complicated to analyse and obtain an understanding of the whole recognition phenomena. However, it is important to clarify the immunochemical feature of each CDR segment of the antibody to understand the antigen recognition though the three dimensional conformation may be changed from its original form. In this study, natural- and artificial-type porphyrins were employed as antigens and their interactions with the antibody and its CDR segment peptides were examined.

MATERIALS AND METHODS

cDNA Synthesis, Amplification and Cloning

MRNA was isolated from 5×10^7 hybridoma cells using an mRNA purification kit (Pharmacia, Uppsala, Sweden). For the PCR experiment, a 50 μl reaction mixture containing 10 μg of mRNA, 20 pmol of VH1FOR primer [5′-d(TGAGGAGACG-GTGACCGTGGTCCCTTGGCCCCAG)] or Vκ1FOR primer [5′-d(TGGATGGTGGGAAGATG)], 250 μM of each dNTP, 10 mM dithiothreitol, 100 mM Tris–HCl (pH 8.3), 10 mM $MgCl_2$, and 140 mM KCl was heated at 70°C for 10 min and then cooled to room temperature. Reverse transcriptase (46 units: Toyobo, Osaka, Japan) was added and incubated at 42°C for 1 h.

For amplification using a thermostable DNA polymerase, a 50 μl reaction mixture containing 5 μl of the cDNA RNA hybrid, 10 pmol of the primers VH1FOR or Vκ1FOR and VH1BACK [5′-d(AGGTCCAGCTGCAGCAGTCTGG) or Vκ1BACK [5′-d(GACATTCAGCTGACCCAGTCTCCA)], 250 μM of each dNTP, 67 mM Tris–HCl (pH 8.8), 17 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 200 μg ml^{-1} gelatin, and 1.25 units of thermus aquaticus (Taq) polymerase (Takara, Kyoto, Japan) was overlaid with mineral oil (Sigma, MO, USA) and subjected to 25 rounds of temperature cycling in a DNA thermal cycler. A cycle was 1 min at 95°C (denature), 1 min at 30°C (anneal), and 2 min at 72°C (elongate).

An aliquot of the PCR reaction was analysed by gel electrophoresis and a DNA fragment of 350 base pairs was confirmed. It was excised and submitted to phenol-extraction and then ethanol-precipitation. In the next step, the fragment was ligated to a M13 phage vector (Takara) digested with the restriction enzyme *Sma*I or pGEM-T vector (Promega, WI, USA). In the M13 phage vector, M13mp18 RF was digested with *Sma*I at 37°C for 5 h. The M13 backbone was also treated with calf intestinal phosphatase. The PCR product was reacted with the klenow fragment and extracted with phenol. Then the fragment was also reacted with polynucleotide kinase, extracted with phenol, purified on a 2% low-melting-point agarose gel and ligated to a M13 phage vector. pGEM-T vector was also used because PCR products can be inserted directly. The sequences of the clones containing V gene inserts were determined by Auto Read Sequencing Kit (Pharmacia) using M13 universal and reverse primers.

Nucleotide Sequence Analysis

To determine the nucleotide sequences, the heavy and light chain cDNA fragments were inserted into a M13 or pGEM-T vector (TAKARA). Nucleotide sequencing was carried out using the T7 sequencing kit (Pharmacia) with the M13 universal and reverse primers using automated DNA sequencer (ALF, Pharmacia).

Fluorescence Measurement

Fluometric titrations were carried out on a Spectrofluorometer FP-777 (JASCO, Tokyo, Japan). In the measurements, $\lambda_{Ex} = 332$ nm and $\lambda_{Em} = 630.5$ nm were used for PP-PEG, and $\lambda_{Ex} = 422$ nm and $\lambda_{Em} = 644$ nm for TCPP.

Peptide Synthesis

Peptides used in the experiments were synthesized by the Fmoc solid-phase method using an automated peptide synthesizer (Applied Biosystems 431A, USA). After deprotection of the resultant peptides from the resin, the peptides were purified by reverse-phase high performance liquid chromatography (RP-HPLC, Waters μBONDASPHERE C_{18} column, 17–21% acetonitrile gradient for 20 min at flow rate 2 ml min^{-1}). The purified peptide was submitted to another HPLC analysis under the condition of 10–40% acetonitrile gradient (0.1% TFA) for 30 min at a flow rate of 1 ml min^{-1} using puresil C_{18} column (4.6 × 150 mm; Waters). The HPLC charts for each CDR peptide are presented in Figure 1. The peptide was also identified by ion spray type mass spectrometry (API-III; Perkin-Elmer Sciex, Ontario, Canada). These results are summarized in Table 1 along with the purities.

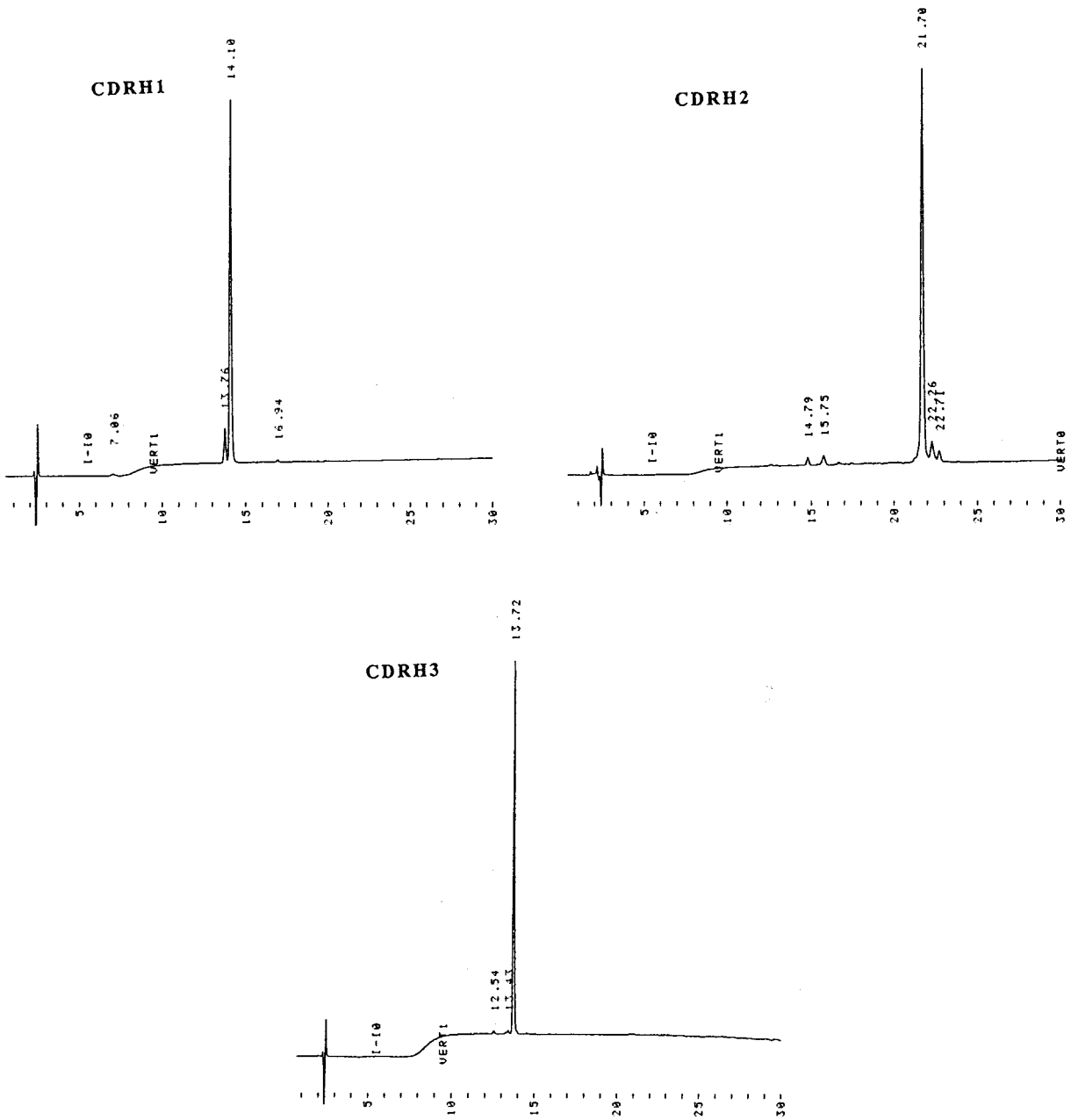

Figure 1 HPLC analysis for CHRH1, CDRH2 and CDRH3.

ELISA

One hundred microlitres of hemin-BSA dissolved in PBS solution (10 μg ml^{-1}) was poured into each well of a 96 immunoplate (Nunc, Denmark) and incubated overnight at 4°C. Blocking was performed using 0.1% gelatin for 30 min at room temperature. After the plate was washed with PBS three times, 50 μl of 1D3 antibody and the same volume of various concentrations of hemin-PEG, PP-PEG or TCPP were simultaneously added, followed by 1 h incubation at room temperature. After washing of the plate, 100 μl of the alkali phosphatase labeled anti mouse Ig antibody (Zymed, USA) was added and then the substrate reaction was carried out for color development. The absorption band at 405 nm was used for measuring the degree of enzymatic reaction by the immuno reader (InterMed NJ-2001).

Table 1 Purity and the Mass for Each CDR Peptide

	Purity (%)	Mass (m/z)
CDRH1	90.9	(+1) 670.0 (670.3)* (+2) 335.6 (335.6)
CDRH2	91.4	(+2) 1002.1 (1002.0) (+3) 668.3 (668.3)
CDRH3	97.9	(+1) 925.4 (925.4)

* ( ), expected mass.

RESULTS

In order to investigate the recognition site for hemin and to clarify its chemical feature in the antibody, ELISAs were performed to evaluate the apparent affinity constants of the 1D3 whole antibody and its heavy and light chain. The purified chains were obtained using two kinds of gel chromatography after the reduction of the 1D3 antibody with dithiothreitol in accordance with the references [6]. Figure 2 shows the results for the immunoreactions of those antibodies with protoporphyrin–polyethylene glycol (PP-PEG). The 1D3 whole antibody displayed the highest affinity being about 2×10^7/M. As the antibody belongs to IgM, the value per ligand becomes 2×10^6/M. That of the heavy chain is roughly estimated to be 10^5/M. With respect to the light chain, the value can not be calculated because the affinity is too small.

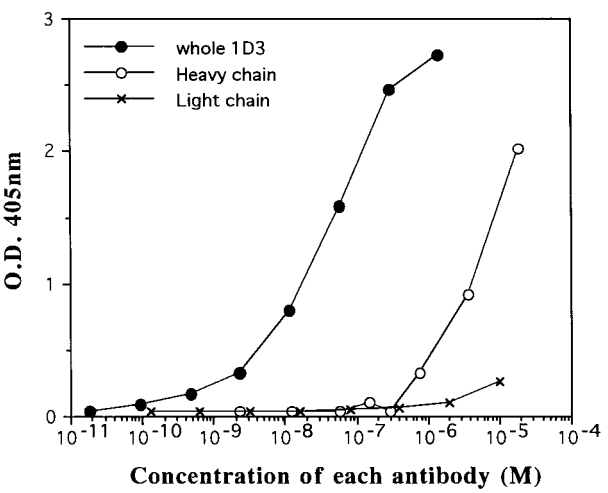

Figure 2 The results of ELISA for 1D3 antibody and its heavy and light chains in the immunoreaction with PP-PEG.

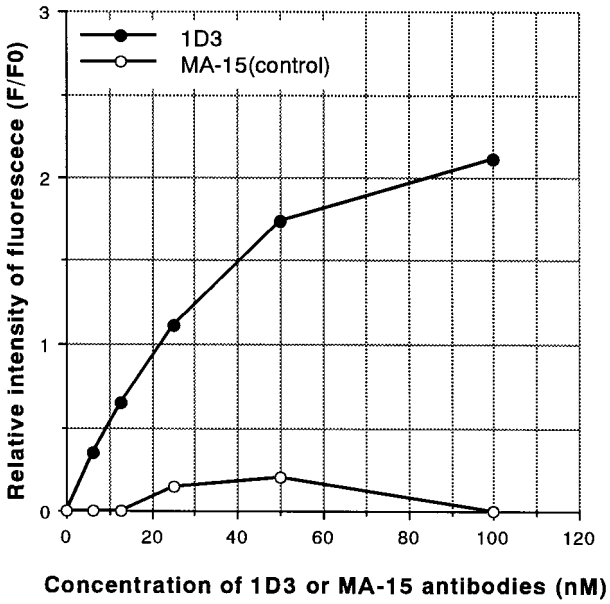

Figure 3 Fluorescence changes of the interaction between antibodies and PP-PEG. F_0: fluorescence intensity of antigen alone. F: fluorescence intensity when antibody was added.

Figure 3 shows the results of the fluorescence changes when 1D3 antibody was reacted with PP-PEG instead of hemin-PEG, as hemin has small fluorescence intensity. The relative fluorescence intensity increased as a function of the concentration of 1D3 antibody. In contrast, another antibody (MA-15 [7]) which was used as a control did not show this change. The affinity constant was calculated from the relative intensity of fluorescence change according to the equation described in explanation of Figure 4. The reciprocal plot $1/(F-F_0)$ versus $1/[C]$ for 1D3 antibody was taken as shown in the figure. A linear relationship was observed. From the slope and intercept of the y axis, the affinity constant per ligand was calculated to be 1.4×10^6 /M. The value is almost the same as the previous value obtained from ELISA.

Messenger RNA was extracted from 5×10^7 hybridoma cells and the nucleotide sequences were determined according to the procedure described in the sections 'cDNA Synthesis, Amplification, and Cloning' and 'Nucleotide Sequence Analysis'. Figure 5 represents the results for the nucleotide and the deduced amino acid sequences of the variable regions of the heavy and light chain of 1D3 antibody. The variable region of heavy chain (VH) subgroup was identified to be II(A) with reference to the data base [8]. The sequences of 1–8 and 103–113 were used for binding of the primers. In 1D3 antibody,

the 1–98 amino acid sequence belongs to the J558 VH family, the DH gene is FL16.1 and JH is the JH4 gene. In the light chain, the Vκ family was III group and the genes were made up of Vκ21 and Jκ2.

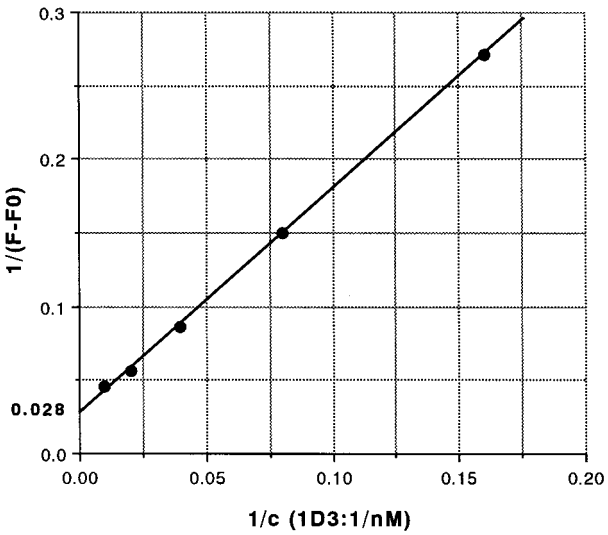

Figure 4 Reciprocal plot of the fluorescence change of 1D3 antibody. $1/(F-F_0)$ was plotted against $1/[A]$, where F is the measured fluorescence of a solution containing the antigen with a given antibody concentration $[A]$ and F_0 is the fluorescence of a solution of the antigen alone. (PP-PEG; 1 μM in PBS.)

$$\mathrm{Ag} + \mathrm{Ab} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \mathrm{Ag \cdot Ab}$$

$$\therefore K = k_{+1}/k_{-1} = [\mathrm{Ag \cdot Ab}]/[\mathrm{Ag}] \cdot [\mathrm{Ab}]$$

where [Ag] is free antigen, [Ab] is free antibody, [Ag · Ab] is antigen–antibody complex.

If $[\mathrm{Agt}] = [\mathrm{Ag}] + [\mathrm{Ag \cdot Ab}]$

where [Agt] is total antigen,

$$\therefore [\mathrm{Ag \cdot Ab}]/[\mathrm{Ag}] = K[\mathrm{Ab}]/\{1 + K[\mathrm{Ab}]\}$$

$$\therefore \{F - F_0\}/\{F_\infty - F_0\} = K \cdot C/\{1 + K \cdot C\}$$

where C is the concentration of antibody.

$$\therefore 1/\{F - F_0\} = 1/(F_\infty - F_0)K \cdot (1/C) + 1/\{F_\infty - F_0\}$$

The relative fluorescence intensity of the antigen bound with antibodies was plotted $1/(F-F_0)$ against $1/[C]$, where F is the measured fluorescence of a solution containing the PP-PEG with a given antibody concentration $[C]$, and F_0 is the fluorescence of a solution of the PP-PEG alone. From the slope and the intercept of y axis of the straight line, the affinity constant was calculated from the bottom equation.

To clarify the role of each CDR segment for antigen recognition, the affinity constant of the antibody and each CDR segment synthesized were examined by the fluorescence measurement.

Figure 6 presents the results of the fluorescence changes of the immunoreaction to TCPP by the whole 1D3 antibody, CDRH1, CDRH2 and CDRH3. The whole 1D3 antibody and the CDRH2 peptide displayed decreases in the relative fluorescence intensity, while those of CDRH1 and CDRH3 slightly increased.

The affinity constants were calculated as represented in Table 2. The value for CDRH2 is a little higher than that for the whole antibody. CDRH1 has a low affinity as expected. In the case of CDRH3, it was impossible to calculate the accurate value because of the very small changes. From the results shown in Figure 6, the value of CDRH3 was estimated to be less than 10^5/M. In general, these facts suggest that CDRH2 plays an important role in the recognition of porphyrins.

Computational calculations were performed for the investigation of the steric conformation of the 1D3 variable region using software AbM (Oxford Molecular Ltd., UK). Plate 1 displays a model of the main amino acid chains. CDRH1 is located in the slightly inner part of the heavy chain, while CDRH2 is on the surface. CDRH3 is difficult to observe because it is hidden by the light chain. The N-terminal of CDRH2 begins at the blue position. The former half is close to the CDRH1 segment. CDRH1 and CDRH2 seem to be closer in distance to each other compared with CDRH2 and CDRH3.

DISCUSSION

The antibody forming system relies very strongly on VH rather than VL, because a similar DNA sequence of VL is frequently used to form antibodies having different specificities [9]. It has also been pointed out that CDRH3 plays an important role in recognizing a large molecule like protein. However, there has been much discussion on the role of CDRH3 for the recognition of or specificity to the antigen. From the reports published so far, we find completely opposite descriptions. Some [10–12] describe the importance of CDRH3. Others [13–15] mention that CDRH3 has little relation with respect to antigen recognition. In these discussions, CDRH3 should be divided into two categories from the point of view of the type of antigen. One is a large molecule like protein and the other is a small molecule like hap-

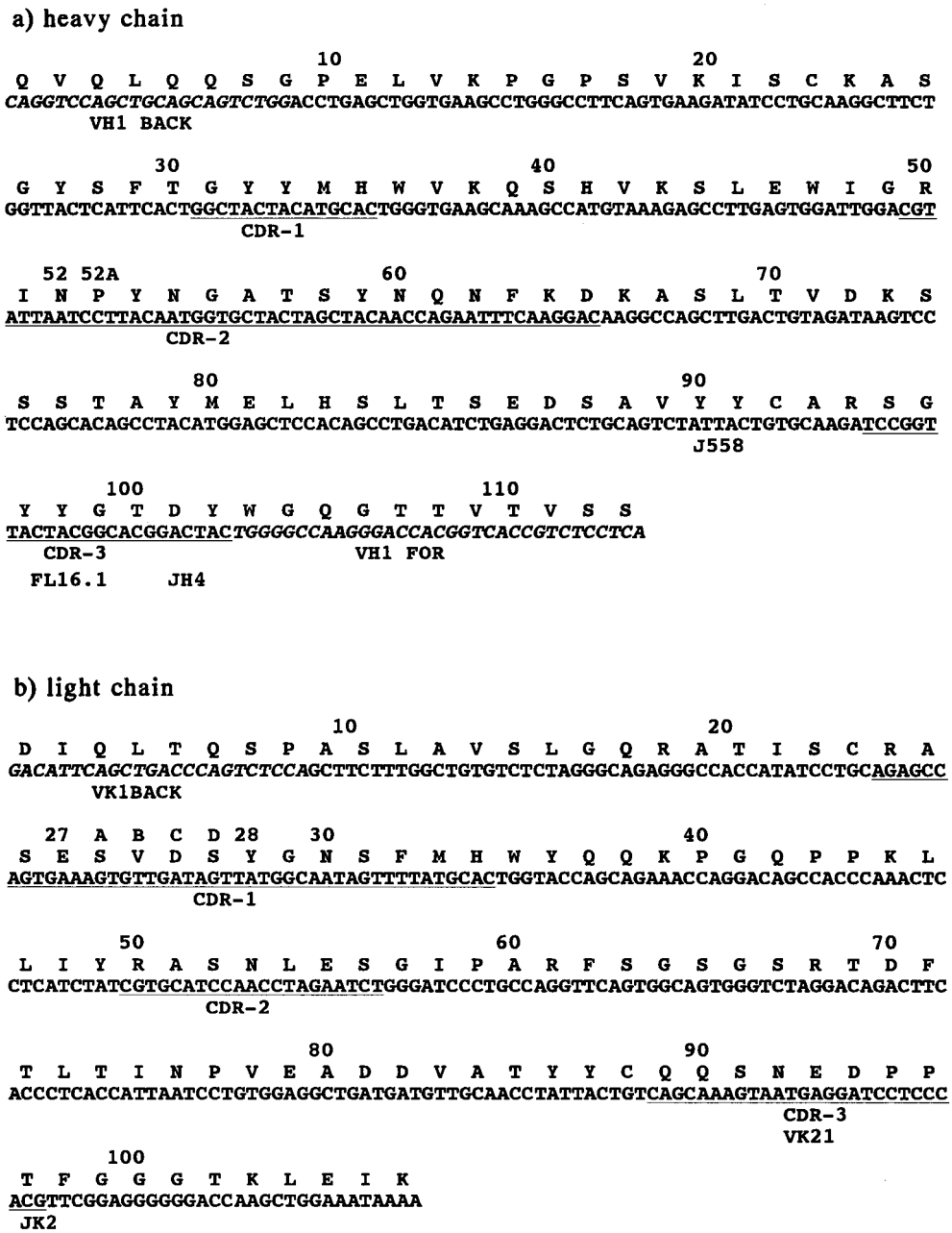

Figure 5 Nucleotide sequence and deduced amino acid sequence of the anti hemin monoclonal antibody (1D3). DDBJ/EMBL/GenBank accession number [heavy chain (D84492); light chain (D84493)]. Complementarity-determining regions are underlined. (a) Heavy chain, (b) light chain.

ten. In this study, we employed the latter case to make it simpler to understand the phenomena. The molecular size of hapten almost corresponds to that of one or several amino acids. On the other hand, there are about 220 amino acids in the Fv segment in the antibody. When the antibody binds a small molecule like porphyrin, it is curious why such a large number of amino acids are needed for the recognition of such a small molecule. It is plausible that some amino acids of the antibody are not necessarily for antigen binding. Or, in some cases, they may disturb the antigen recognition. In the case of a small molecule, it is likely that even CDR does not take part in the antigen recognition. To answer these questions, the chemical features of each CDR should be investigated in detail and their roles must be clarified.

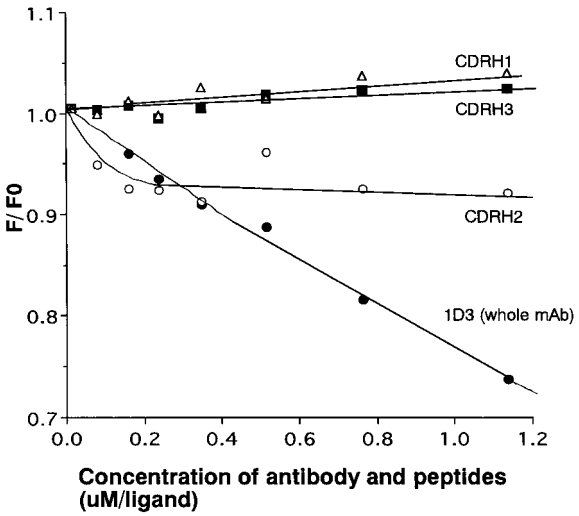

Figure 6 Fluorescence changes of the interaction of 1D3 antibody and the CDRs of the heavy chain to TCPP. TCPP, 0.5 μM.

From the data described previously, it is obvious that the VH domain of 1D3 antibody plays an important role for the binding of a porphyrin molecule rather than the light chain. In the heavy chain, the most important CDR was CDRH2 by considering the results of Figure 6. Surprisingly, the affinity constant of CDRH2 to TCPP was same order or a little larger compared with that of the whole antibody. Some amino acids in the antibody may disturb the antigen binding. When the antigen binds to the recognition site, the affinity constant may increase if there are no conformational constrains in the antibody. Although the strong affinity of CDRH2 with the antigen should be emphasized, it is plausible that the specificity may become low. This point will be clarified in detail in the near future.

From the computational analysis, CDRH1 and the former half of CDRH2 were found to be closely located to each other [Plate 1—The heavy chain is on the right-hand side (the yellow color is the framework of heavy chain), while the light chain is on the left-hand side (the green color is the framework of the light chain). CDRH1 and CDRL1, white; CDRH2 and CDRL2, purple; CDRH3 and CDRL3, red. The conformation of the antibody was analysed by use of a work station (Silicon Graphics Inc., PA, USA). The software was AbM from Oxford Molecular Ltd. (Oxford, UK). Pimms was used as a molecular graphics software (Oxford Molecular Ltd.). It is considered that porphyrins are bound with the former part of CDRH-2 (red color) via hydrophillic interaction and with the latter one via hydrophobic interaction]. As CDRH1 and the former half of CDRH2 are hydrophobic, these can bind the porphyrin ring (pyrrole group) co-operatively via hydrophobic interaction. At this time, electron transfer could occur from protoporphyrin to the antibody. In the case of TCPP, there may be a strong ionic interaction with the latter half of CDRH2, because it is hydrophilic. Then the electron transfer occurs from CDRH2 to TCPP which attracts some electrons. This different flow of electrons will contribute to the different direction of fluorescence change. As ionic interactions are formed in the case of TCPP in addition to the hydrophobic interaction, the affinity constant of TCPP is higher than protoporphyrin.

Table 2 Results of Affinity Measurements

	Affinity constant (/M)
1D3	1.2×10^7
CDRH1	1.3×10^5
CDRH2	6.8×10^7
CDRH3	$< 10^5$

Antigen, TCCP.

Though the separated light chain showed very low affinity, the light chain in the whole antibody may contribute to the recognition of the antigen. As CDRL3 is close to CDRH2 and CDRH1 from Plate 1, CDRL3 may concern with the antigen recognition to a small extent. However, the other two CDRs, CDRL1 and CDRL2 are excluded.

Other investigators [13] have pointed out that CDRH3 does not participate in the hapten binding but relates to the carrier protein. In this study, CDRH3 is not considered to participate in the recognition of the porphyrin molecule from the fluorescence results and the computational analysis. This is ascribed to the following two reasons. One is that the CDRH3 has intrinsically no chemical affinity to bind the hapten. The other will be the location of the CDRH3 in the antibody, which is far from the CDRH2.

Although it has been considered that the intact antibody is best for the antigen binding, there may exist excess amino acids which make it difficult for the antigen to approach its binding site. Therefore, the CDRH2 segment has a possibility to have higher affinity than the native whole antibody.

Some investigators [16,17] have already developed designing of bioactive peptides using the CDR segment of the antibody. For the application to an artificial enzyme, these types of basic studies should be performed extensively. Furthermore, the

specificity must be taken into account along with the binding affinity at the next stage in order to understand the recognition mechanism accurately.

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