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How and Why 41S-2 Antibody Subunits Acquire the Ability to Catalyze Decomposition of the Conserved Sequence of gp41 of HIV-1

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Abstract

It has become well known that antibodies obtained by immunization with the ground state of peptides can display proteolytic activity. Our antibody light chain produced by immunization with the peptide RGPDRPEGIEEEG-GERDRD, a highly conserved sequence in envelope gp41 of HIV-1 showed the ability to cleave this peptide. Moreover, its heavy chain also decomposed the peptide, although this occurred at lower activity levels compared with the light chain, while the whole antibody did not show any catalytic activity. From molecular modeling, the light and heavy chains of the antibody were deduced to possess catalytic triads (Asp, His, and Ser) in their steric conformations, which may be responsible for the observed proteolytic activity.

Index Entries: Catalytic activity of 41S-2 antibody to HIV-1 envelope gp41; catalytic antibody; HIV; gp41; conserved sequence.

Introduction

Recent studies on antibodies have revealed new functional properties that include enzyme-like catalytic activities directed to the antigen. Catalytic antibodies offer great insights in the development of artificial enzymes in chemical and biochemical studies. Further study of such catalysts might result in the development of antibody drugs in medicinal fields. Although most catalytic antibodies have been prepared by the transition-state analog method *(1–3)*, other interesting studies have revealed natural catalytic antibodies with abilities such as hydrolytic scission of peptide bonds by the light chains *(4,5)* and Bence Jones proteins *(6)*, and

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of phosphodiester bonds in DNA by autoantibodies *(7)*. Paul et al. have shown that an antibody raised against vasoactive intestinal peptide (VIP) catalyzes the hydrolysis of VIP *(8)*, and that the active site for the proteolysis reaction is in its light chain. The possibility that antibody light chains have proteolytic activity has also previously been pointed out by Erhan et al. in 1974, based on homology analysis *(9)*. Based on successive works of the antibody gene, Paul has proposed the theory that the catalytic activity of antibody is encoded in the germline *(10)*.

Recently, we found an interesting antibody, the light chain of which decomposed the antigenic peptide of HIV-1 envelope gp41 *(11)*. The sequence of the antigen peptide corresponds to the highly conserved portion of gp41. Because the envelope proteins of HIV mutate frequently, it is significant that a catalytic antibody can target the conserved sequence. In this report, the catalytic triads suggested in our antibody will be discussed from the viewpoint of the catalytic sites based on molecular modeling of the antibody.

Materials and Methods

Preparation and Purification of the Antibody, the Light Chain, and the Heavy Chain

The 41S-2 monoclonal antibody (41S-2 MAb) used in this study was raised against a 19-mer peptide RGPDRPEGIEEEGGERDRD, which is the sequence of a highly conserved region of the envelope protein gp41 of many HIV-1 strains. The peptide was synthesized as in the form of a Cys adduct located at the C terminus. Then, the peptide was coupled with keyhole limpet hemocyanin via the Cys residue. The purified conjugate was used to immunize Balb/C mice three times at an interval of several weeks. Three days after the final booster, hybridomas were prepared by fusion of the spleen cells of the mouse and myeloma cells (SP/2) by HAT selection and screening. After cloning twice, a MAb (MAb)-secreting hybridoma cell line designated 41S-2 [$IgG_{2b}(\kappa)$] was established. An aliquot of the purified MAb dissolved in 2.7 mL of a buffer comprised of 50 m*M* Tris and 0.15 *M* NaCl, pH 8.0, was reduced by 2-mercaptoethanol for 3 h at 15°C. To this solution was added 3 mL of 0.6 *M* iodoacetamide, followed by adjusting the pH to 8 by 1 *M* Tris. Then, the solution was incubated for 15 min at 15°C. The resulting solution was ultrafiltered to 0.5 mL, and it was injected into an HPLC gel-filtration column at a flow rate of 0.2 mL/min of 6 *M* guanidine hydrochloride. The fractions corresponding to the light and heavy chains were collected, and each fraction was diluted with 6 *M* guanidine hydrochloride, followed by dialysis against PBS, replacing the buffer seven times for 3–4 d at 4°C.

Immunoblotting

Blotting analysis to identify HIV proteins was performed by use of the NovaPath Immunoblot Assay kit (for detection of antibody to Human

Immunodeficiency Virus Type-1, Nippon BIO-RAD, Tokyo, Japan). Procedures were mainly carried out according to the manufacturer's protocol, except for the addition of first and second antibodies. As a first antibody, 5.2 μg/mL of whole 41S-2 MAb, 37 μg/mL of its heavy chain (41S-2-H), or 40 μg/mL of its light chain (41S-2-L) was applied. Then, 700 μL of rabbit antimouse Ig(G+A+M) conjugated with alkaline phosphatase [affinity-purified grade $(Fab')_2$; diluted to 1/1000, Zymed, CA, USA] was added as the second antibody, followed by incubation for 30 min at room temperature. HIV-infected human serum was used as the positive control.

Peptide Synthesis

The YP41-1 peptide YPRGPDRPEGIEEEGGERDRD used in the experiments was synthesized by the Fmoc solid-phase method (automated peptide synthesizer, Applied Biosystems 431A, CA, USA). After deprotection of the synthesized peptides from the resin, the peptide was purified by reverse-phase HPLC (RP-HPLC; Waters 490E, Waters μBONDASPHERE C_{18} column; Waters, NY, USA). The peptide was over 99% pure, determined using an ion-spray-type mass spectrometer (API-III, Perkin-Elmer Sciex, Ontario, Canada). In the mass spectrum, $[M+2H]^{2+}$ was 1215.5, $[M+3H]^{3+}$ was 810.7, and $[M+4H]^{4+}$ was 608.2. These *m/z* gave an average mass of 2429.1, which is consistent with the most abundant mass—2429.1—of the YP41-1 peptide.

Reaction Conditions

The catalytic reactions using the light and heavy chain were carried out in 15 m*M* phosphate buffer (pH 6.5) at 25°C. For monitoring the catalytic reaction, 20 μL of the reacting solution was analyzed by HPLC (Water 600S, USA or Jasco, Tokyo, Japan) at room temperature.

Molecular Modeling

The computational analysis of 41S-2 MAb was performed on a workstation (Silicon Graphics Inc. PA, USA). First, AbM software from Oxford Molecular Ltd. (Oxford, UK) was used to build up the three-dimensional molecule of the antibody using the amino-acid sequences of its light and heavy chains. The resulting coordinates were transferred to the software QUANTA 96 (Molecular Simulations Inc., CA, USA) to minimize its CHARM energy using Steepest Descent 400 steps, Conjugate 400 steps, and Adopted Basis Newton–Raphson 800 steps. In the case of the light chain of 41S-2 MAb (41S-2-L) alone, the CHARM energy was minimized again. Then each spatial distance was measured using a ball whose diameter is defined on the display. The data on trypsin were taken from PDB. Discover software (Molecular Simulations Inc., CA, USA) was used for the calculation of steric conformation of YP41-1 peptide.

Results and Discussion

The sequence RGPDRPEGIEEEGGERDRDRS corresponds to amino-acid residues 732–752 of HIV-1 gp160, which is highly conserved and locates in the transmembrane region of gp41 in many HIV-1 strains. It has been established that the sequence DRPEGIEEEGGERDRDRS can play an important role in eliciting a highly neutralizing antibody to HIV-1 *(12)*. Our MAb (41S-2 MAb) *(13)* was found to specifically react with gp160 and gp41 of HIV-1 by immunoblot analysis. The light chain (41S-2-L) isolated from 41S-2 MAb also bound with gp160 and gp41, showing the same specificity as that of the intact 41S-2 MAb. From the previous ELISA experiments *(11)*, it was revealed that the apparent immunoaffinity of the light chain was smaller than that of 41S-2 MAb by about 2,000-fold, but the light chain retained its specificity for gp41 binding.

The Conformation of Antigen Peptide

Figure 1 shows the conformation of YP41-1 deduced by molecular modeling. It is observed that the peptide bends at the position of Pro-Glu-Gly. A similar conformation was also obtained using software Nemesis (version 2.1, Oxford Molecular). This conformation is very consistent with the results of epitope-mapping studies. ELISA results reported previously *(13)* suggest that the affinity of 41S-2 MAb for pentapeptide epitopes decreases in the following order: GIEEE > IEEEG > EGIEE > RGPDR. The sequence RGPDR at the N-terminus position is only moderately recognized by the 41S-2 MAb. The peptide sequence between RGPDR and EGIEEEG was poorly recognized by the 41S-2 MAb. Hence, the sequences RGPDR and EGIEEEG are likely to be positioned so as to create a bend at the intervening sequence. The computer modeling data, therefore, can be seen to confirm the results of epitope mapping.

Immunoblotting Analysis of 41S-2 MAb and its Light and Heavy Chains

Immunoblotting analysis for HIV-1 proteins was performed with 41S-2- MAb and its light chain (41S-2-L) and heavy chain (41S-2-H) (*see* Fig. 2). 41S-2 MAb showed high specificity to gp160 and gp41. 41S-2-L displayed very similar specificity as the 41S-2 MAb. In contrast, 41S-2-H yielded one sharp band corresponding to gp41 and many faint bands between gp41 and gp160. The immunological features of the 41S-2-H are quite different, therefore, from those of 41S-2 MAb and 41S-2-L.

The apparent affinity constants obtained by enzyme-linked immunosorbent assay (ELISA) were 2.3×10^9, 2.5×10^7, and 1.7×10^6 M^{-1} for the intact MAb, 41S-2-H and 41S-2-L, respectively. As expected, the heavy and light chain showed lower binding affinity to the antigen than the intact MAb.

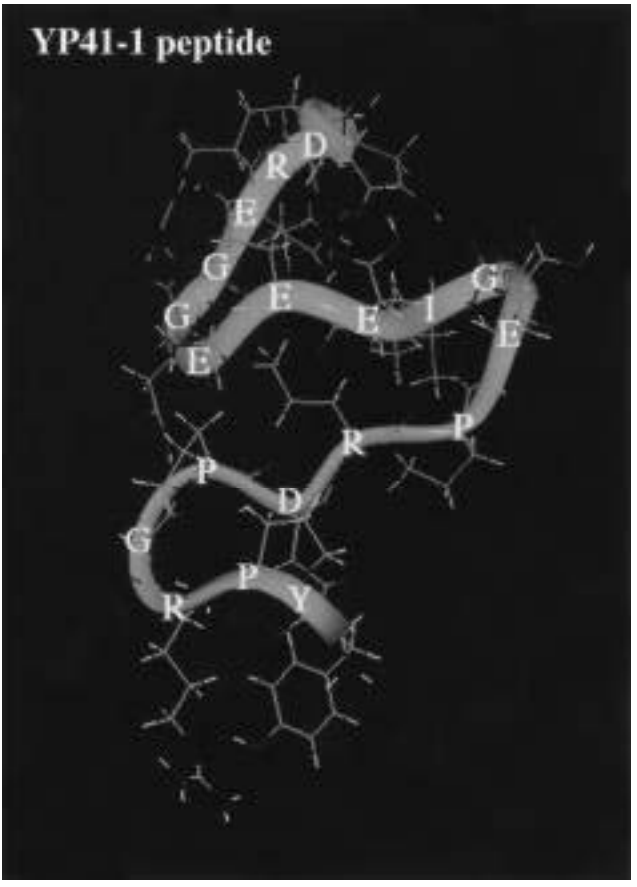

Fig. 1. Conformation of YP41-1 peptide by the molecular modeling.

Catalytic Scission of Antigen Peptide YP41-1 by the Light Chain

The peptide YPRGPDRPEGIEEEGGERDRD (gp160 amino acids number 732–750; YP41-1 peptide) was used as the substrate in this experiment. The degradation reactions of the YP41-1 peptide were carried out at 25°C using 41S-2-L as shown in Fig. 3. The YP41-1 peptide was apparently degraded into amino acids or short peptides within 70 h, as only a few small peaks were observed by HPLC, suggesting near complete consumption of YP41-1 peptide. A control experiment was also carried out using the light chain of MA-15 MAb [$IgG_{2b}(\kappa)$]. MA-15 MAb is a monoclonal antibody to methamphetamine (MA) *(14)*. The light chain of MA-15 MAb [MA-15(L)] was purified in exactly the same way used for the purification of light chain of 41S-2 MAb. The MA-15(L) did not show any catalytic activity to YP41-1 peptide.

An immunoprecipitation experiment was carried out to remove the 41S-2-L from the reaction system by using an antibody-binding matrix

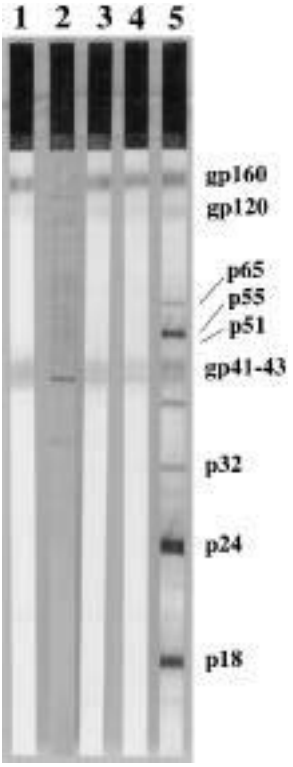

Fig. 2. Immunoblotting analysis for HIV-1 proteins by 41S-2 MAb and its subunits. Lane 1; 41S-2 MAb, Lane 2; 41S-2-H, Lane 3 and 4; 41S-2-L (lot K and lot I, respectively), Lane 5; Positive control (HIV-infected human serum)

(KappaLock-Agarose). When the 41S-2-L was immunoprecipitated, its catalytic activity was largely removed. On the other hand, the nonimmunoprecipitated light chain showed the catalytic activity reproducibly (data not shown). These observations show that the decomposition of the YP41-1 peptide is catalyzed by the light chain 41S-2-L as opposed to contaminants.

Immunological and Catalytic Features of the Heavy Chain

The heavy chain 41S-2-H showed the ability to cleave the YP41-1-peptide, but the reaction occurred only after a long "induction time" as shown in Fig. 4. The degradation started about 100 h after mixing of the 41S-2-H and the YP41-1 peptide. The YP41-1 peptide was completely decomposed at about 140 h. The HPLC analysis showed that the YP41-1 peptide was degraded to amino acids and/or short peptides by the 41S-2-H. As in the case of 41S-2-L, several lots of 41S-2-H independently isolated from 41S-2-MAb showed degradation kinetics similar to those shown in Fig. 4.

The results of the Western-blotting analysis for the purified fractions of the light and heavy chains are presented in Fig. 5. Monomeric and dimeric

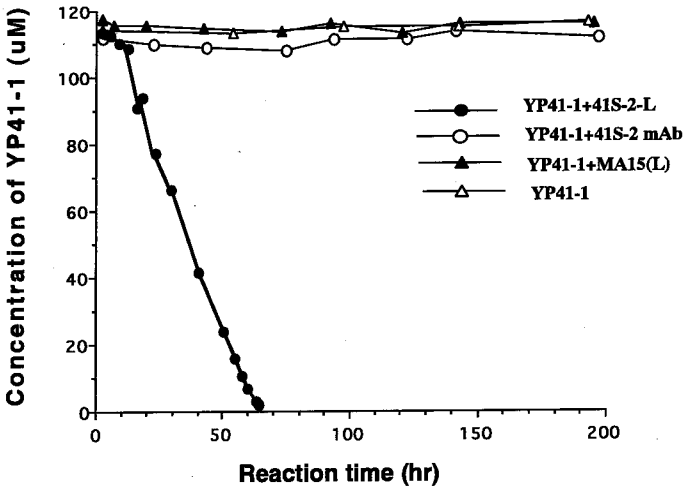

Fig. 3. Time course of the degradation of YP41-1 peptide by the heavy chain (41S-2-L). YP41-1 peptide, 120 μ*M*; each antibody, 0.8 μ*M*; Reactions were carried our in 15 m*M* phosphate buffer at 25°C. Most glassware, plasticware, and buffer solutions used in this experiment were sterilized by heating (180°C, 2 h), autoclaving (121°C, 20 min), or passing through a 0.20-mm sterilized filter prior to the experiment. Manipulations during the experiment were mostly performed in a safety cabinet to avoid contaminations.

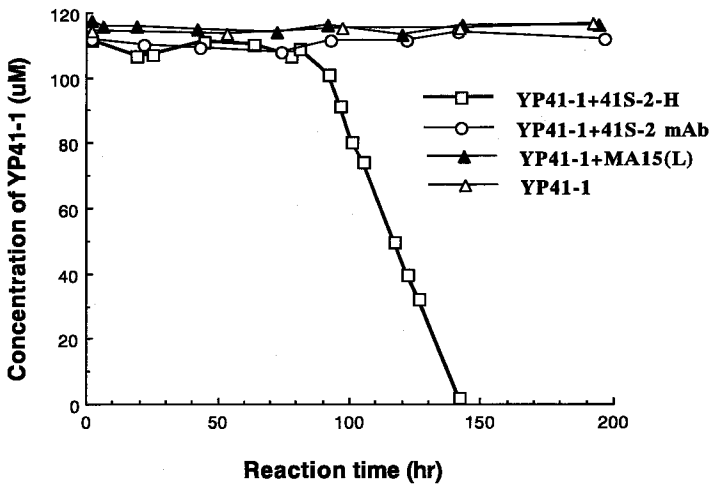

Fig. 4. Time course of the degradation of YP41-1 peptide by the heavy chain (41S-2-H). Reaction conditions were the same as in Fig. 3.

forms of 41S-2-H are evident (*see* Fig. 5). 41S-2-H did not contain any 41S-2-L (Lane 2). Thus, the degradation of the YP41-1 peptide by 41S-2-H is not due to the contamination by 41S-2-L. The degradation of YP41-1 by 41S-2-H obeyed Michaelis–Menten kinetics. Values of K_m and k_{cat} were estimated to be 7.1×10^{-5} *M* and 0.17/min, respectively.

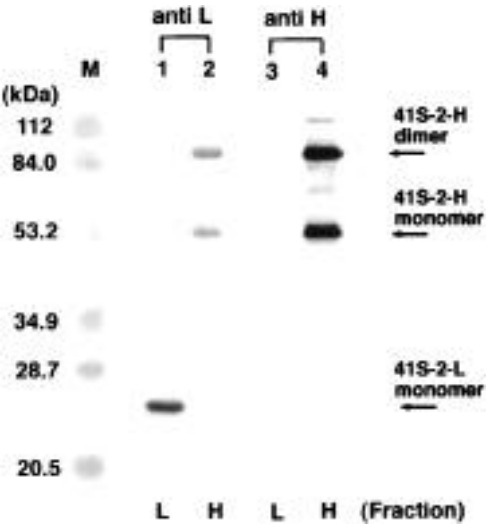

Fig. 5. Immunochemical (Western-blotting) identification of the light chain (41S-2-L) and the heavy chain (41S-2-H). M; Marker, Lane 1; 41S-2-L fraction reacted with antimouse light-chain antibody labeled with alkaline phosphatase. Lane 2; 41S-2-H fraction reacted with antimouse light-chain antibody labeled with alkaline phosphatase. Lane 3; 41S-2-L fraction reacted with antimouse heavy-chain antibody labeled with alkaline phosphatase. Lane 4; 41S-2-H fraction reacted with antimouse heavy-chain antibody labeled with alkaline phosphatase. 41S-2-L was present in a monomeric form (Lane 1). It did not contain any 41S-2-H (Lane 3). 41S-2-H presented as a monomeric and dimeric form (Lane 4) and was not contaminated with any 41S-2-L (Lane 2). The antimouse light-chain antibody displayed low-level cross reactivity to the mouse heavy chain (Lane2).

A comparison of the kinetic constants for the light and heavy chains is summarized in Table 1. The light and heavy chains appear to possess fairly different kinetic features. Regarding the affinity constants of the light chain, the values obtained by ELISA and suggested by cleavage studies ($1/K_m$) are in agreement. However, the affinity constants for the heavy chain obtained by the two methods are quite different. The value obtained by ELISA represents the binding affinity for the antigen recognition site of the antibody. On the other hand, $1/K_m$ value corresponds to the binding affinity for catalytic site. Hence, it is plausible that the recognition site and the catalytic site for the YP41-1 peptide are the same in the case of light chain. On the other hand, in the heavy chain, the affinity constants obtained by ELISA and kinetics are different by three orders of magnitude. This suggests that the antigen recognition site and the active site are located in the different parts of the heavy chain. As the classical antigen-recognition site of the antibody is mostly located in the complementarity-determining regions (CDRs), these data suggest that the catalytic site of the heavy chain may be located outside the CDRs.

Table 1
Some Physical Values for the 41S-2-L and 41S-2-H

		41S-2-L	41S-2-H
Affinity constant (M^{-1})	ELISA	1.7×10^6	2.5×10^7
	Cleavage studies ($1/K_m$)	4.5×10^6	1.4×10^4
kcat (min^{-1})		0.06	0.17
kcatK_m (M^{-1} min^{-1})		2.9×10^5	2.4×10^3

Catalytic Triads in 41S-2 MAb by Molecular Modeling

Figure 6 shows the conformation of 41S-2 MAb deduced by molecular modeling. In the variable region of 41S-2-L (left-hand side in the figure), there are three possible catalytic triads. The first one is comprised of Asp1, His91, and Ser26 or Ser27a (this triad is not displayed). The second is His 53, Ser 56, and Asp 60. The third is His 53, Asp 60, and Ser 63. These two triads are displayed in Fig. 6. The first one is considered to be the same type of catalytic triad reported by Paul et al. *(15)*. In the case of 41S-2-L, the average diameter of the sphere enclosing the three amino-acid residues is about 6 Å for the first catalytic triad. For the second and third, the diameters are about 5 and 4.5 Å, respectively. The third is the smallest among three triads. The location and the spatial distances of the smallest triad are presented as an orange sphere with the diameter 5 Å in Fig. 7. In the case of trypsin, the diameter of the catalytic triad is in the range of 3 and 4 Å. Because of the smaller spatial distances between Ser, His, and Asp in the third triad shown in Fig. 7, we propose that this is the triad constituting the enzymatically active site of 41S-2-L. It is also plausible, however, that more than one catalytic triad (e.g., the second and third triads) contributes to the catalytic activity.

On the other hand, we can see only one catalytic triad on the surface of the heavy chain (*see* Fig. 6), comprised of Asp73, Ser76, and His79. These three amino acids are not contributed by the CDRs of the heavy chain. Like the kinetics and ELISA data described in the preceding section, the computer modeling studies also support the idea that the catalytic triad in the heavy chain is located outside the classical antigen-recognition site.

The catalytic triads in the light and heavy chains are located on the surface of the antibody. However, the big discrepancy is that these sites do not work as catalytic sites in the native antibody. It is likely that the light and heavy chains become flexible in their conformations once they are isolated from he whole antibody. This flexibility allows the three amino acids of the catalytic triad to come closer to each other. Interaction with the antigen might also promote the formation of the catalytic site, generating the ability to hydrolyze the antigen peptide (e.g., *see* Fig. 3). The absence of

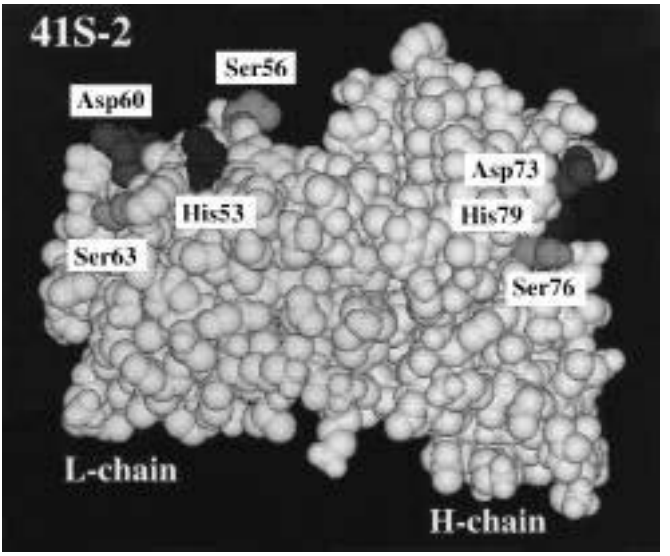

Fig. 6. The locations of catalytic triads in the light and heavy chain of 41S-2- MAb. Catalytic triads (His53, Ser56 Asp60; His53, Asp60, Ser63) in the light chain are presented in the lefthand side of the figure. The triad in the heavy chain (Asp73, Ser76, His79) is displayed in the right-hand side of the figure.

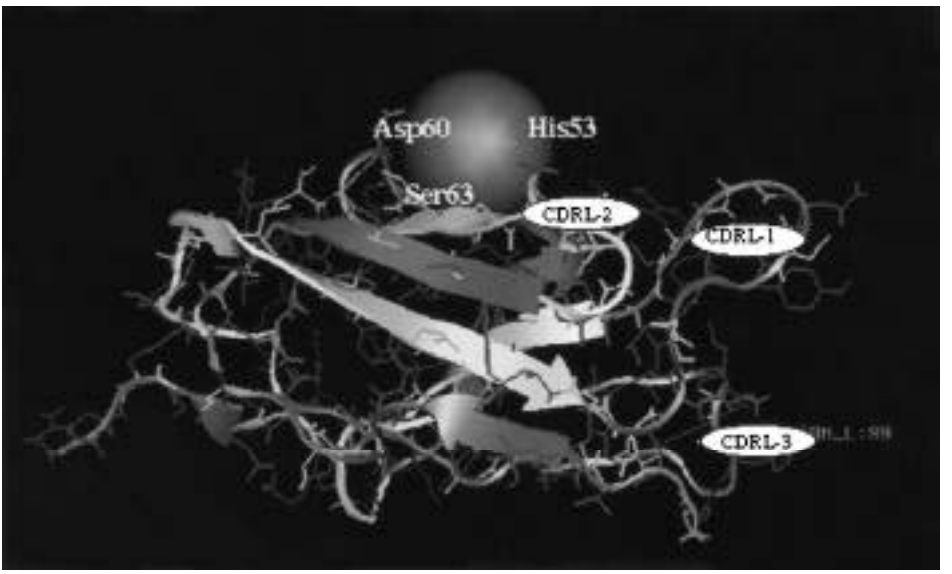

Fig. 7. Location and spatial distance of the smallest catalytic triad for the variable region of 41S-2-L by molecular modeling. The orange ball represents a diameter of 5 Å.

catalytic activity in the whole antibody molecule might be due to the rigid structure of the whole antibody. This holds true even if the whole antibody is denatured and refolded by the same way used to prepare the light and

heavy chains. With respect to the analysis of the active site of light chain against VIP, Paul et al. have reported the importance of the catalytic triad and the conformation and structure of the light chain *(10,15,16)*. Our data are considerably consistent with those data, although the targeted antigens are quite different. The catalytic triads may be encoded in germline—an issue which will be investigated further in near future.

In conclusion, the light and heavy chain appear to be capable of degrading peptide antigens by forming serine protease like catalytic triads.

Acknowledgment

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Discussion

Green: You described one light chain from one MAb. Did you screen a variety of antibodies to choose this antibody? How did you select this from other antibodies that bind to the antigen?

Uda: This was a very lucky case for us. We did not need to screen other MAbs. We obtained only three MAbs. The one I showed here was picked based on specificity for binding to gp41.

Zouali: The antibodies you raised are directed to a peptide derived from the C-terminal part of gp41. However, on your gel, gp41 disappears completely. How do you explain that?

Uda: In the first phase of the reaction, the cleavage is slow and specific, and is limited to the specific peptide against which the antibody is raised. In the later phase of the reaction, we propose that the antibody changes to a different state with reduced specificity, and we see more nonspecific cleavage. Another possibility is that small peptides can be accommodated more easily in the binding site than large proteins. Finally, we cannot exclude that the specific gp41-cleaving site and the small peptide-cleaving site in the light chain are not the same. This is only a speculation at present.

Paul: Two issues need to be considered. First, antibodies are capable of promiscuous cleavage of small peptides. Dr. Sinohara will talk more about that, and we have also published on it. Second, specificity for a large protein combined with cleavage at many bonds in the protein could be explained by promiscuous catalysis within a site that is capable of highly specific initial ground-state binding followed by cleavage at neighboring peptide bonds in the three-dimensional structure. Note that the bonds can be distant from each other in the linear sequence, resulting in a heterogeneous product profile.