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# Effects of substitutions of amino acids on the thermal stability of the Fv fragments of antibodies

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**Abstract** The thermal stability of Fv fragments was examined by circular dichroism (CD) spectrometry and high-performance liquid chromatography. We analyzed three Fv fragments: that of a monoclonal antibody D1.3 and two derivatives of it. After separation of wild-type $V_H$ and $V_L$ fragments, thermal denaturation of each fragment was monitored by CD spectrometry. The results indicated that the dissociation of Fv into $V_H$ and $V_L$ fragments seemed to be coupled with the denaturation of each fragment and that the thermal denaturation of $V_H$ and $V_L$ fragments was prevented when they were associated with one another. The analysis of the three Fv fragments also indicated that, in some cases, differences in amino acids even within the CDRs could have significant effects on the thermal stability of the complex between $V_H$ and $V_L$ fragments.

*Key words:* Artificial antibody; Circular dichroism spectrometry; High-performance liquid chromatography; Phage-display antibody

## 1. Introduction

Phage-display antibody systems seem to hold considerable promise for the preparation of monoclonal antibodies in vitro (for review see [1]). Either a Fab or a single chain Fv fragment is expressed on the surface of a filamentous phage particle, for example fd and M13, in such systems [2,3]. As an alternative, we have proposed another system in which an Fv fragment, namely, a free $V_H$ fragment noncovalently associated with a $V_L$ fragment that is fused with ΔcpIII, is expressed on the phage surface [1,4]. In our system, the stable association between $V_H$ and $V_L$ fragments is essential for the construction of libraries of artificial antibodies. In order to assure the proper association between $V_H$ and $V_L$ fragments for formation of an antigen-binding site, heterogeneous sequences are introduced only in the complementarity-determining regions (CDRs) of $V_H$ and $V_L$ fragments while the sequences of framework regions are kept constant.

Before the development of recombinant DNA technology, it was difficult to prepare an Fv fragment by simple digestion of native antibodies with a particular protease. Therefore, there have been few reports that deal with the stability of Fv fragments [5–7]. After success was achieved in expressing Fv and Fab separately in *Escherichia coli*, it became possible to examine systematically the stability of various Fv fragments [8–10]. In a previous report [11], we described the construction of a library of 512 kinds of Fv fragment, derivatives of a monoclonal antibody, D1.3, that is specific for hen egg-white lysozyme (HEL). In this library, a total of nine of the original amino acids were replaced by closely related amino acids at sites within the CDRs of the heavy (H) chain. More than 80% of the clones in the library have been observed to produce Fv fragments in *E. coli* [11]. However, both the stable expression of $V_H$ and $V_L$ fragments in *E. coli* and the stable association of both fragments to form a proper antigen-binding site are required for preparation of libraries of artificial antibodies. In the present study, we chose three Fv fragments from among these clones and examined the thermal stability of association between $V_H$ and $V_L$ fragments.

## 2. Materials and methods

### 2.1. Fv fragments of antibodies

Fv fragments of antibodies were synthesized in *E. coli* and purified as described previously [11]. Three kinds of Fv fragment were chosen for the present analysis: Wild-O, mutant 2 (#2) and mutant 9 (#9) [11]. The differences in amino acid residues among them are summarized in Table 1.

### 2.2. Recording of CD spectra

Circular dichroism (CD) spectra were recorded with a spectropolarimeter (model J720; Japan Spectroscopic Co. Ltd.). The conditions for the recordings were as follows: 10 μM protein in phosphate-buffered saline (50 mM Na-phosphate, pH 7.0, and 200 mM NaCl; referred to below as phosphate buffer); various temperatures from 30 to 80°C at 5-degree intervals; and a light path of 1 mm. The wavelength ranged from 200 to 250 nm and scans were performed ten times. Mean residual ellipticity [θ] was calculated with the software provided by the manufacturer.

### 2.3. Separation of $V_H$ and $V_L$ fragments

The Wild-O Fv fragment was dissolved in 25 mM MOPS buffer (pH 7.0) that contained 8 M urea and 5 mM NaCl, and the solution was subjected to cation-exchange column chromatography (S sepharose FF; Pharmacia). The $V_H$ fragment did not adsorb to the column, while the $V_L$ fragment was eluted by increasing of the concentration of NaCl in the buffer to 300 mM. The separated $V_H$ and $V_L$ fragments were dialyzed against phosphate buffer and solutions were adjusted to a final concentration of protein of 10 μM. The purity of each sample was examined by SDS-polyacrylamide gel electrophoresis (PAGE).

### 2.4. High-performance liquid chromatography

20 μl of solutions of the separated $V_H$ and $V_L$ fragments were injected separately onto a G2000SW column (Toso Co.) in phosphate buffer. In addition, the $V_H$ and $V_L$ fragments were mixed at a molar ratio of 1:1 at a final concentration of 14 μM each and the mixture was allowed to stand for 5 min at room temperature. This mixture was also injected onto the column. Optical density at 280 nm ($OD_{280}$) of eluates was monitored.

The thermal stability of Fv fragments was also analyzed by HPLC. 10 μM solution of the three kinds of Fv fragment in phosphate buffer were incubated at various temperatures from 30 to 55°C for 1 h, and then each was injected onto a G3000SW connected in tandem with a

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*Abbreviations:* CD, circular dichroism; CDR, complementarity-determining region; HEL, hen egg-white lysozyme; PAGE, polyacrylamide gel electrophoresis; OD, optical density; HPLC, high-performance liquid chromatography.

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G2000SW column (Toso Co.). During chromatography, the columns were kept at the temperature that corresponded to the incubation temperature of the specific sample. $OD_{280}$ was monitored. The G3000SW and G2000SW columns are for gel permeation and the separation performance does not alter between 30 and 55°C.

## 3. Results

### 3.1. Analysis of the thermal stability of Fv fragments by CD spectrometry

The CD spectra of three Fv fragments, Wild-O and two of its derivatives, #2 and #11 [11], were recorded at various temperatures, as shown in Fig. 1A. Since a CD spectrum is considered to reflect primarily the secondary structure of a polypeptide, such as its $\beta$-sheet and $\alpha$-helical contents, our analysis should have provided evidence of the thermal denaturation of the $V_H$ and $V_L$ fragments rather than direct evidence of the dissociation of Fv fragments into $V_H$ and $V_L$ fragments. In the case of the Wild-O fragment, below 50°C, the CD spectra at 218 nm were all alike. Between 55 and 65°C, the spectrum changed but, above 70°C, the spectra were again similar to each other. A 50% change in circular dichroism was observed at 62°C (Fig. 1B). In the case of the other two mutant Fv fragments, a 50% change was observed at 52°C for #2 and at 50°C for #11 (Fig. 1B).

We separated $V_H$ and $V_L$ fragments by denaturation of Wild-O with 8 M urea, with subsequent cation-exchange chromatography, and the separated fragments were renatured by dialysis against phosphate buffer. As shown in Fig. 2A, the $V_H$ and $V_L$ fragments were well separated without contamination. Each fragment and the mixture were analyzed by HPLC. The analysis by HPLC indicated that half of the separated $V_L$ fragment appeared to form a complex (Fig. 2B). By contrast, most of the separated $V_H$ fragments existed in a monomeric form. When $V_H$ and $V_L$ fragments were mixed at a molar ratio of 1:1, monomer forms of $V_H$ and $V_L$ fragments disappeared and a new peak that corresponded to an Fv fragment appeared. The position of the complex formed by the $V_L$ fragment was slightly shifted towards the position of a larger complex. Although the molecular composition of this large complex is not known, the results indicate that the majority of the $V_H$ and $V_L$ fragments were effectively renatured during the process of preparation of these fragments. The CD spectra of the separated $V_H$ and $V_L$ fragments were recorded at various temperatures and the results are shown in Fig. 2C. About 50% of $V_H$ and $V_L$ fragments, respectively, were denatured at 43°C when they were present alone in solution. Thus, the dissociation of Fv into $V_H$ and $V_L$ fragments seemed to be coupled with the denaturation of each fragments and the thermal denaturation of $V_H$ and $V_L$ fragment was prevented when they were associated with one another. Although the thermal denaturation of the $V_H$ and $V_L$ fragments of mutant forms of Fv was not examined, the temperature dependency of the values of $[\theta]$ of Fv fragments observed in the analysis by the CD spectrometry should reflect the degree of dissociation of Fv fragments into $V_H$ and $V_L$ fragments.

### 3.2. Analysis of the stability of Fv fragments by HPLC

The wild-type Fv fragment was analyzed by HPLC over a range of temperature from 30° to 55°C at 5-degree intervals,

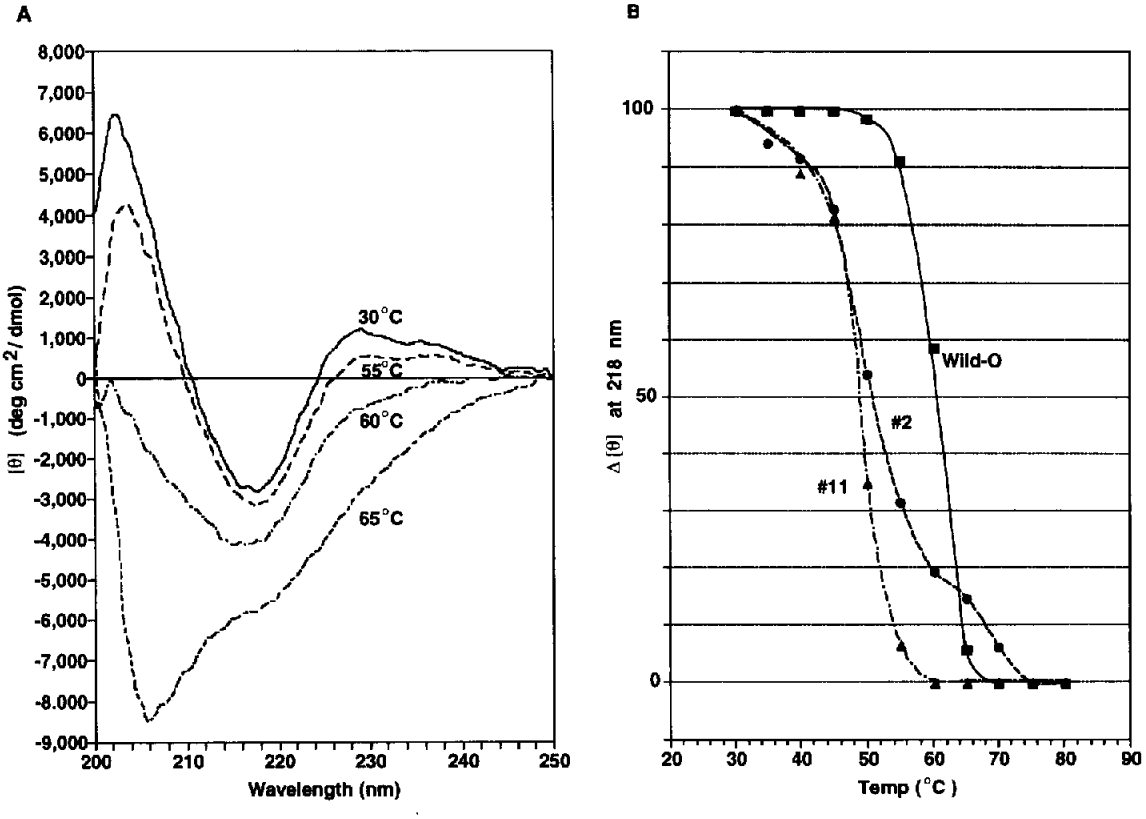


Fig. 1. Thermal stability of three Fv fragments, analyzed by CD spectrometry. (A) The mean residual ellipticity $[\theta]$ was measured over the range from 200 to 250 nm at various temperatures. Only the data for the Wild-O sample are shown. (B) The extent of changes in the value of $[\theta]$ at 218 nm were calculated and plotted as follows: the value at 30°C was taken as 100% and the value at 80°C as 0%.

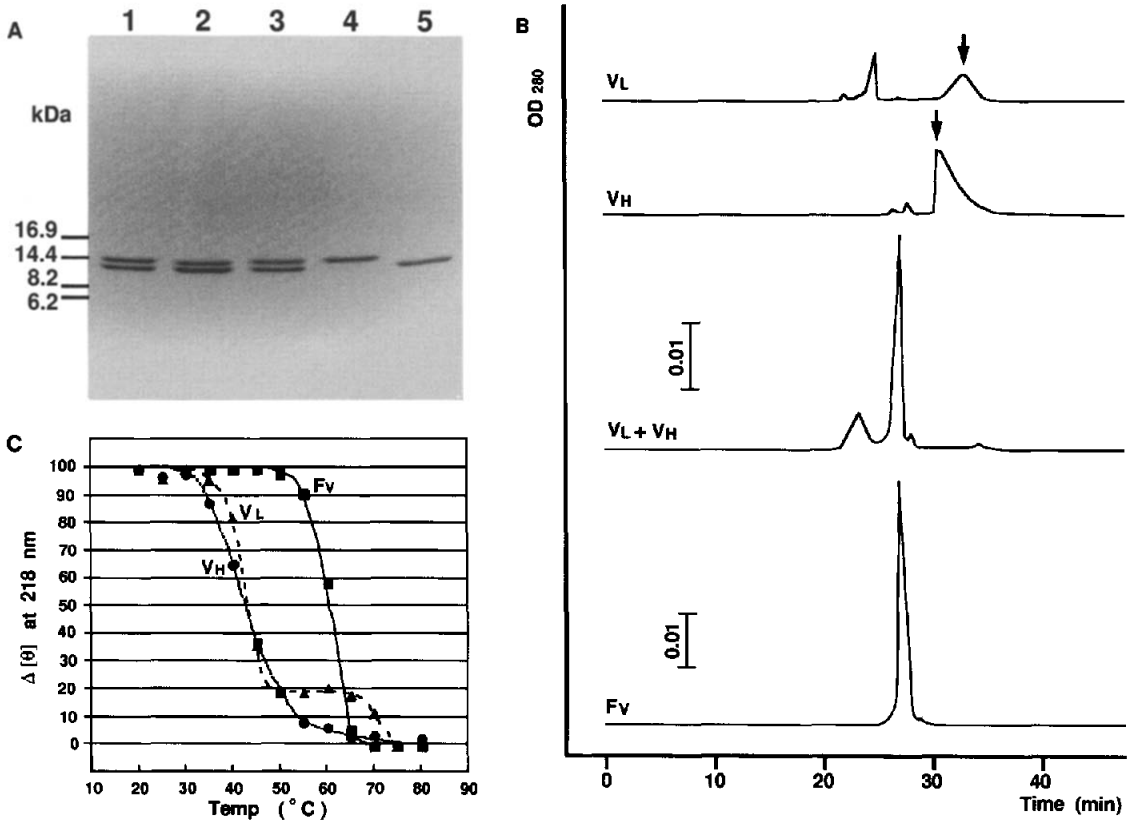


Fig. 2. Analysis of Wild-O and the separated $V_H$ and $V_L$ fragments derived from it. (A) SDS-PAGE. Lane 1, Wild-O; lane 2, #2; lane 3, #11; lane 4, $V_H$; lane 5, $V_L$. (B) Analysis by HPLC. The separated $V_H$ and $V_L$ fragments of Wild-O, a mixture of the $V_L$ and $V_H$ fragments, and Wild-O were subjected to HPLC. $OD_{280}$ was monitored. The positions corresponding to $V_L$ and $V_H$ monomers are indicated by arrows. (C) Thermal stability of the separated $V_H$ and $V_L$ fragments of Wild-O as well as that of Wild-O was analyzed by CD spectrometry. The data obtained were calculated in the same way as the data in Fig. 1B.

as shown in Fig. 3A. A single peak that corresponded to its intact Fv fragment was observed from 30 to 40°C. Above 40°C, the height of this peak decreased and tailing was observed. At 55°C, the peak was completely absent. At 48°C, 50% of the sample was observed at the position of the Fv fragment (Fig. 3B). The two mutant Fv fragments, #2 and #11, were also analyzed by HPLC. About 50% of the sample remained at 42°C in the case of #2 and at 36°C in the case of #11. Since, in the case of analysis by HPLC, the concentration of the sample should have been dynamically changed when the sample was moving through the gel, it is difficult to estimate the equilibrium constant between the Fv fragment and its separated fragments. However, the data should reflect the strength of the association between $V_H$ and $V_L$ fragments, and the results from the analysis by HPLC are consistent with those obtained by CD spectrometry.

## 4. Discussion

In the present study, the CD spectra of three Fv fragments and the separated $V_H$ and $V_L$ fragments were monitored at various temperatures. The temperatures associated with 50% changes in circular dichroism were quite different between separated $V_H$ and $V_L$ fragments and the Fv fragment. When the $V_H$ and $V_L$ fragments were alone in their respective solutions, denaturation occurred at a temperature about 20 degrees lower than that of the denaturation when they were associated with one another. Therefore, it appears that the association between $V_H$ and $V_L$ fragments increases the stability of the individual fragments. From 50 to 62°C, at which the 50% changes in the circular dichroism were observed for the three Fv fragments, the majority of the dissociated fragments should be denatured. Thus, the changes in the CD spectra of the three Fv fragments corresponded closely to degree of dissociation.

The analysis of three Fv fragments by HPLC indicated that dissociation of Fv fragments into $V_H$ and $V_L$ fragments occurred at temperatures 10–15°C lower than that deduced from the CD spectra. Such differences can mainly be ascribed to differences in concentrations since the degree of the dissociation should directly depend on their concentrations. In the analysis of CD spectra, the concentration of each fragment was fixed at 10 μM. In the analysis by HPLC, 5 μl of a 10 μM sample were injected onto the column, but the concentration

Table 1
Differences in amino acids among the heavy chains of the Fv fragments analyzed

| | | CDR I | | | CDR II | | | | CDR III |
|---|---|---|---|---|---|---|---|---|---|
| | 27 | 30 | 32 | 46 | 52 | 53 | 54 | 56 | 96 |
| Wild-O | F | T | Y | E | W | G | D | N | R |
| #2 | N | T | F | Q | W | G | N | D | W |
| #11 | N | S | F | Q | R | S | N | N | R |

The details of the amino acid sequences of these three Fv fragments are described in a previous paper [11].

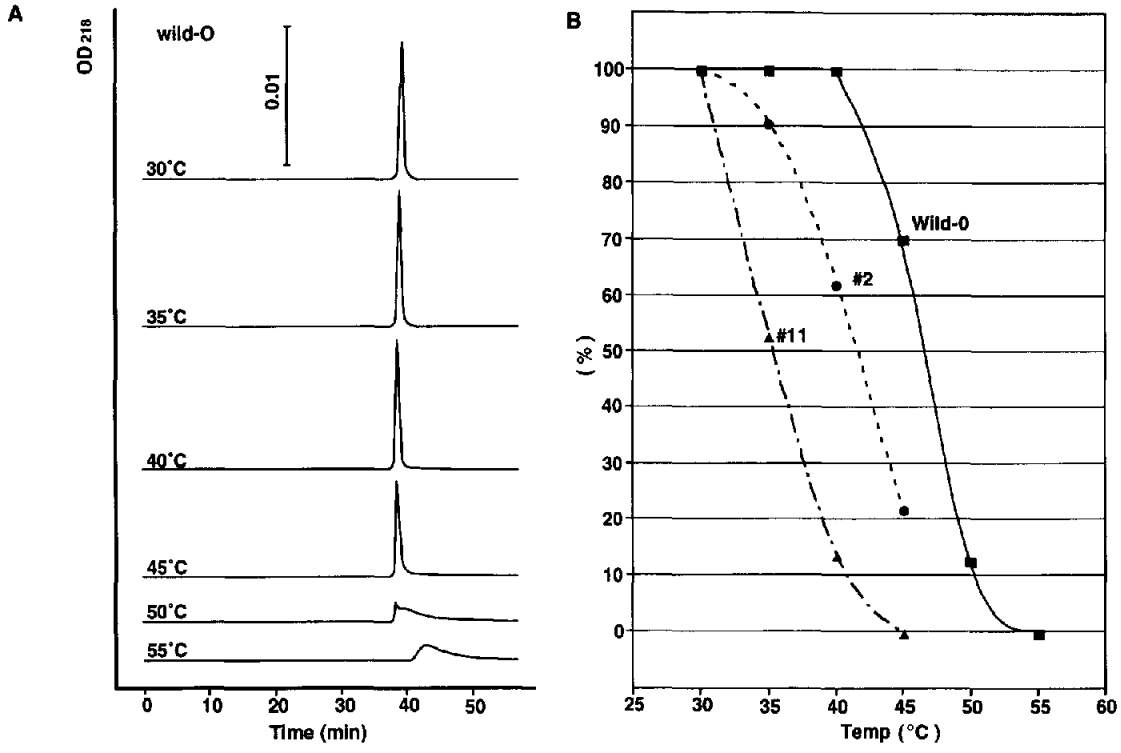


Fig. 3. Thermal stability of three Fv fragments, analyzed by HPLC. The Fv fragment was subjected to HPLC at various temperatures and $OD_{280}$ was monitored. Only the results for Wild-O are shown. (B) The relative values of $OD_{280}$ at the position of Fv were compared and plotted.

of the sample must have been reduced close to twenty- to thirty-fold during passage through the column. A slight difference was observed as for #2 between the results in Fig. 1B and those in Fig. 3B. The results from the HPLC analysis should reflect the dissociation constant at respective temperature since Fv, $V_H$ and $V_L$ fragments should not be denatured in the temperature range from 30 to 40°C as shown in Fig. 2C. On the other hand, in the CD measurements, the dissociation seemed to be coupled with the denaturation. Therefore, presence of a hypothetical intermediate structure may exist in some temperature range. A non-sigmoidal profile of #2 observed in Fig. 1B might reflect such situations. In any case, we can conclude that differences in amino acids even in the CDRs have effects on the thermal stability of the complex between $V_H$ and $V_L$ fragments in some cases. Foote and Winter [12] reported an example where substitutions of amino acids in framework regions affected the conformation of the hypervariable loops.

We have been constructing libraries of artificial antibodies in which an Fv fragment, namely a free $V_H$ fragment noncovalently associated with a $V_L$ fragment that is fused with $\Delta$CpIII, is expressed on the surface of phage [1]. The sequences of the CDRs of $V_H$ and $V_L$ fragments are highly divergent while the sequences of framework regions are kept constant. At least two conditions must be fulfilled in our system: stable expression of both $V_H$ and $V_L$-$\Delta$cpIII fused fragments in *E. coli*; and stable association of $V_H$ and $V_L$ fragments to form an antigen-binding site. Our present analysis indicates that the introduction of different amino acids into the CDRs can have a considerable effect on the strength of the association between $V_H$ and $V_L$ fragments in some cases. Our present genetic construct is based on the D1.3-derived Fv. Below 30°C, the three Fv fragments analyzed in this study were retained as stable Fv complexes. Therefore, we do not anticipate major problems in many cases. Ideally, however, we should search for combinations of $V_H$ and $V_L$ fragments that can generate a stronger complex than Fv of D1.3.

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