Comparative thermodynamic analyses of the Fv, Fab* and Fab fragments of anti-dansyl mouse monoclonal antibody

Nobuhisa Shimba[a], Hidetaka Torigoe[b], Hideo Takahashi[a,**], Katsuyoshi Masuda[a,**], Ichio Shimada[a,*], Yoji Arata[a,**], Akinori Sarai[b]

[a]*Faculty of Pharmaceutical Sciences, University of Tokyo, Hongo, Tokyo 113, Japan*
[b]*Gene Bank, Tsukuba Life Science Center, The Institute of Physical and Chemical Research (RIKEN), Tsukuba, Ibaraki 305, Japan*

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Abstract In order to investigate the role of the constant domains on the antigen-binding property of the variable domains, we have carried out a comparative thermodynamic study of the anti-dansyl Fv, Fab* and Fab fragments that possess the identical amino acid sequence of the variable domains. The thermodynamic analyses have shown that binding constants, enthalpy changes and entropy changes are similar for the three antigen-binding fragments, whereas the thermal stability of Fab is much higher than that of Fv and Fab*. We have concluded that (i) the variable domains of the three antigen-binding fragments possess identical intrinsic capability for antigen binding and (ii) the two constant domains serve to improve the stability of the variable domains.

Key words: Fv; Fab*; Fab; Isothermal titration calorimetry (ITC); Differential scanning calorimetry (DSC)

1. Introduction

Immunoglobulin G (IgG) consists of two identical heavy chains and two identical light chains. The heavy chains are composed of four homologous units, V_H, C_H1, C_H2 and C_H3 domains, whereas the light chains are divided into two homologous units, V_L and C_L domains. Papain digestion of the whole antibody gives Fab and Fc fragments. Antigen recognition is carried out by the Fab fragment, which consists of V_H, C_H1, V_L and C_L domains. Fv and Fab* fragments are other types of antigen-binding fragments. The Fv fragment, which is the heterodimer of V_H and V_L domains, is a smallest antigen binding fragment. An antigen binding fragment composed of V_H, V_L and C_L domains is referred to as the Fab* fragment.

In previous papers, we have shown that anti-dansyl Fv, Fab* and Fab can be prepared by the limited enzymatic digestion of the IgG antibodies that originate from the identical anti-dansyl switch variant cells [1–3]. We have recently reported a multinuclear NMR study of the comparison of the static and dynamic structures for the anti-dansyl Fv and Fab [4]. It has been indicated that the hydrogen–deuterium (H–D) exchange rates for some of the residues in the V_L domain of Fab are not as fast as those of Fv. On the basis of the results of this experiment, we have concluded that the fluctuation of the variable domains of Fab is more restricted than that of Fv.

It is therefore of interest to compare the binding properties and thermal stability of the three antigen-binding fragments. It has been demonstrated that calorimetry can provide thermodynamic information on the antigen–antibody interaction and thermal stability of the proteins involved [5–7]. We have used the calorimetric method for the comparison of the thermodynamic property of Fv with those of Fab and Fab*.

In the present study, we determined the changes associated with antigen binding of free energy, enthalpy, entropy and heat capacity for Fv, Fab* and Fab. We have also examined the stability of the antigen binding fragments by measuring the denaturing process for these fragments. On the basis of the results of the thermodynamic studies, the antigen binding mechanism for Fv, Fab* and Fab and the role of the constant domains will be discussed.

2. Materials and methods

2.1. Materials

Clostripain, papain and ε-dansyl-L-lysine (DNS-Lys) were purchased from Sigma Chemical Co. Anti-dansyl Fv and Fab* were prepared by the clostripain digestion of the short-chain IgG2a, in which the entire C_H1 domain is deleted. Anti-dansyl Fab was prepared by the papain digestion of the IgG2a, and the disulfide bond between C_L and C_H1 in the obtained Fab was reduced and alkylated. Methods for the preparation were described previously in detail [1,2].

2.2. Isothermal titration calorimetry (ITC)

The enthalpy changes (ΔH) and the binding constants (K_a) were determined by using an isothermal titration calorimetry (Microcal Inc.; OMEGA). The solutions of Fv, Fab* and Fab were extensively dialyzed against 10 mM phosphate buffer, 200 mM sodium chloride, pH 6.0. DNS-Lys was then dissolved in the same dialysis buffer. During a period of 8 s, 5 μl of the DNS-Lys solution was added to the calorimeter cell that contained the Fv, Fab* or Fab solution. The heat for each injection was determined using the Microcal Origin software supplied by the manufacturer and was subtracted by the heat of dilution of DNS-Lys. Then, we plotted the observed values of heat as a function of the total ligand injected into the cell. In order to determine ΔH and K_a, the obtained plots were fitted by non-linear least-squares analysis.

2.3. Determination of thermodynamic parameters

Free energy change (ΔG), entropy change (ΔS) and heat capacity change (ΔCp) were calculated by the equations: $\Delta G = -RT\ln K_a = \Delta H - T\Delta S$ and $\Delta Cp = \delta\Delta H/\delta T$ where R is the gas constant and T the absolute temperature. In the antigen–antibody reaction, the magnitude of the free energy change depends upon the concentration units chosen

*Corresponding author. Fax: (81) (3) 3815-6540.

***Present addresses:* H.T., School of Pharmaceutical Sciences, Kitasato University, Shirokane, Minatoku, Tokyo 108, Japan; K.M., Faculty of Pharmacy, Meijo University, Tempakuku, Nagoya 468, Japan; Y.A., Water Research Institute, Sengen 2-1-6, Tsukuba, Ibaraki 305, Japan.

Abbreviations: C_H1, C_H2, C_H3, constant domains of the heavy chain; C_L, constant domain of the light chain; DNS-Lys, ε-dansyl-L-lysine; DSC, differential scanning calorimetry; Fv, Fab*, Fab, antigen binding fragment; ΔCp, heat capacity change; ΔGu, unitary free energy change; ITC, isothermal titration calorimetry; NMR, nuclear magnetic resonance; ΔSu, unitary entropy change; V_H, the variable domain of the heavy chain; V_L, the variable domain of the light chain.

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for the standard state. In order to obtain unitary entropy change (ΔSu) and unitary free energy change (ΔGu), which are independent of the concentration units chosen for the standard state, we used the following equations [8]: $\Delta Su = \Delta S - R\ln X_m = \Delta S + 7.98$ (cal·mol^{-1}·K^{-1}) and $\Delta Gu = \Delta G - 7.98 \times 10^{-3}T$ (kcal·mol^{-1}) where X_m is the mole fraction of a molecule at a concentration of 1.0 M. In this case, since the concentration of water is about 55.6 M, X_m is equal to 1/55.6, and therefore the cratic contribution to entropy is −7.98 cal·mol^{-1}·K^{-1} [9].

2.4. Differential scanning calorimetry (DSC)

Thermal denaturation experiments were carried out on a Microcal MC-2 or MCS calorimeter interfaced with a microcomputer. The sample solutions for DSC were prepared by extensive dialysis against the experimental buffer. The sample solutions and the reference dialysis buffer were degassed with a vacuum pump at room temperature, and they were immediately loaded into the respective cells. The obtained DSC curves were subtracted by the instrumental baseline, which was measured with both cells filled with degassed dialysis buffer.

3. Results and discussion

Fig. 1 shows a typical result of an ITC experiment for the association between Fv and DNS-Lys. As shown in Fig. 1a, a large negative heat pulse is observed upon addition of DNS-Lys to Fv. The area of small negative peaks observed at the last several injections is equivalent to the heat of dilution observed upon injection of DNS-Lys to the buffer. Integrated areas for the peaks plotted against the molar ratio are shown in Fig. 1b. The K_a and ΔH values obtained at 30°C were 1.8×10^7 M^{-1} and −18.8 kcal·mol^{-1}, respectively. ΔGu and ΔSu calculated from K_a and ΔH were −12.5 kcal·mol^{-1} and −20.9 cal·mol^{-1}·K^{-1}, respectively. It should be noted that large negative enthalpy and entropy changes were observed. The same analyses were performed for the binding of DNS-Lys to Fab* and Fab.

In Fig. 2, the values of ΔGu, ΔH and $-T\Delta$Su for Fv, Fab* and Fab are plotted against temperature. The plots of ΔH vs. temperature are linear, indicating that ΔCp is constant in the temperature range used in the present experiments. For each antigen-binding fragment, ΔGu exhibits a small temperature dependence, which results from enthalpy–entropy compensation with the opposite signs for the slopes of ΔH and $-T\Delta$S vs.

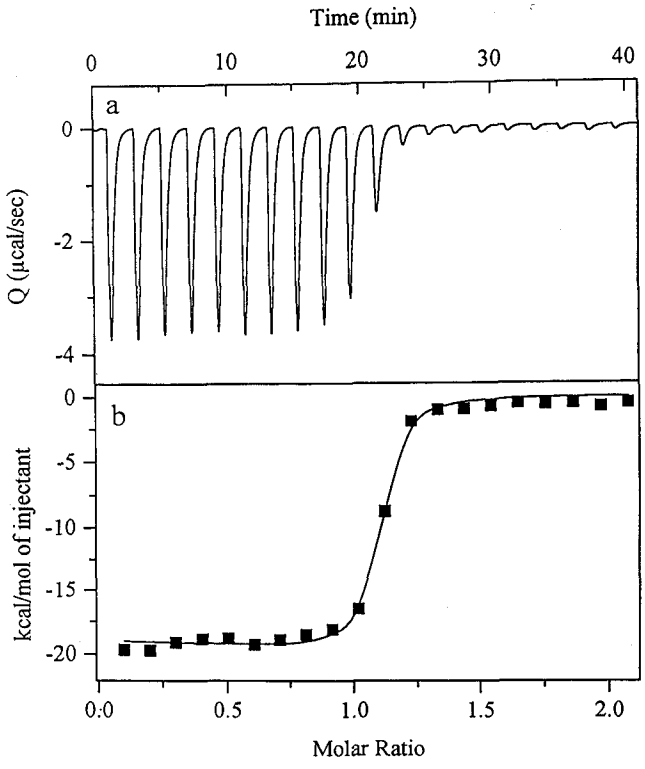

Fig. 1. Isothermal titration calorimetric profiles of Fv–DNS-Lys association. (a) Incremental heat liberation upon titration of DNS-Lys solution into Fv solution at 30°C. Twenty 5 μl injections of DNS-Lys (1.1 mM in 10 mM sodium phosphate, 200 mM NaCl, pH 6.0) were made into 41 μM Fv. Injections occurred over 8 s at 2 min intervals. (b) Integrated areas for the above peaks plotted against the molar ratio ([DNS-Lys]/[Fv fragment]).

Table 1
Thermodynamic parameters[a] for the binding of DNS-Lys to Fv, Fab* and Fab

	Temperature (°C)	K_a[a] (M^{-1})	ΔH[a] (kcal·mol^{-1})	ΔGu[a] (kcal·mol^{-1})	ΔSu[a] (cal·mol^{-1}·K^{-1})	ΔCp[b] (cal·mol^{-1}·K^{-1})
Fv	25	$2.1 \pm 0.1 \times 10^7$	−18.7 ± 0.1	−12.4 ± 0.0	−21.1 ± 0.4	−123 ± 13
	30	$1.6 \pm 0.4 \times 10^7$	−19.3 ± 0.5	−12.4 ± 0.2	−22.8 ± 2.0	
	35	$1.5 \pm 0.2 \times 10^7$	−20.5 ± 0.3	−12.5 ± 0.1	−25.7 ± 1.3	
	40	$1.2 \pm 0.1 \times 10^7$	−20.8 ± 0.2	−12.6 ± 0.1	−26.1 ± 0.8	
	45	$5.3 \pm 0.9 \times 10^6$	−21.0 ± 0.4	−12.3 ± 0.1	−27.4 ± 1.2	
	50	$4.3 \pm 0.6 \times 10^6$	−21.9 ± 0.1	−12.4 ± 0.1	−29.4 ± 0.1	
Fab*	25	$1.7 \pm 0.4 \times 10^7$	−18.5 ± 0.6	−12.2 ± 0.1	−20.9 ± 1.6	−151 ± 15
	30	$1.8 \pm 0.6 \times 10^7$	−18.5 ± 0.2	−12.4 ± 0.2	−20.0 ± 0.3	
	35	$1.1 \pm 0.5 \times 10^7$	−19.7 ± 0.2	−12.3 ± 0.3	−24.0 ± 0.3	
	40	$6.0 \pm 0.1 \times 10^6$	−20.1 ± 0.1	−12.2 ± 0.0	−25.3 ± 0.3	
	45	$4.1 \pm 0.6 \times 10^6$	−21.3 ± 0.5	−12.1 ± 0.1	−28.6 ± 1.9	
	50	$3.2 \pm 0.1 \times 10^6$	−22.0 ± 0.1	−12.2 ± 0.0	−30.4 ± 0.3	
Fab	25	$2.4 \pm 0.4 \times 10^7$	−18.1 ± 0.1	−12.4 ± 0.1	−19.0 ± 0.6	−150 ± 15
	30	$1.8 \pm 0.2 \times 10^7$	−19.1 ± 0.3	−12.5 ± 0.1	−21.9 ± 1.0	
	35	$1.2 \pm 0.4 \times 10^7$	−19.1 ± 0.3	−12.4 ± 0.2	−21.9 ± 0.7	
	40	$1.1 \pm 0.2 \times 10^7$	−20.4 ± 0.1	−12.6 ± 0.1	−25.0 ± 0.1	
	45	$5.0 \pm 1.5 \times 10^6$	−21.4 ± 0.2	−12.2 ± 0.2	−28.9 ± 1.1	
	50	$2.7 \pm 0.9 \times 10^6$	−21.7 ± 0.1	−12.1 ± 0.2	−29.9 ± 0.8	

[a] $K_a\Delta$H, ΔGu and ΔSu with standard deviations are the means of two or more measurements.
[b] ΔCp is obtained from the slope of ΔH vs. temperature given in Figure 2.

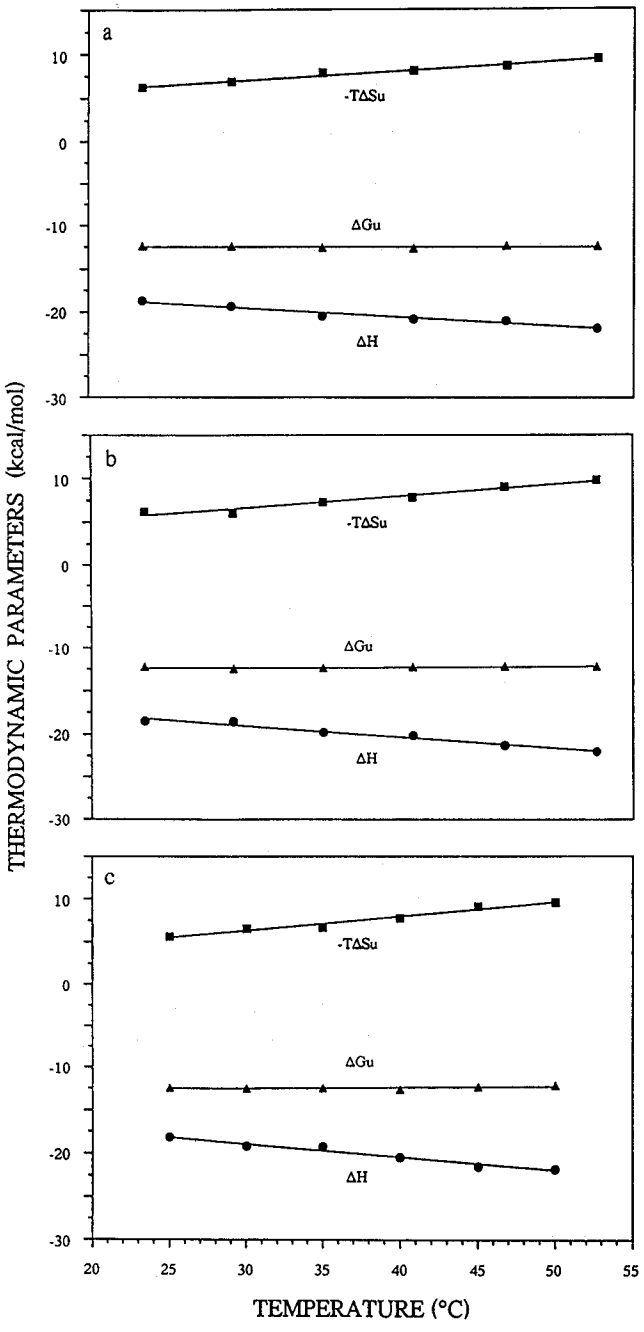

Fig. 2. Thermodynamic parameters (●, ΔH; ▲, ΔGu; ■, −TΔSu) plotted against temperature for the binding of DNS-Lys to (a) Fv, (b) Fab* and (c) Fab.

temperature [10–14]. The thermodynamic parameters obtained are summarized in Table 1.

We have shown that the chemical shifts observed in the $^{1}H,^{15}N$ HSQC spectra for most of the aromatic residues of Fv are quite similar to those for the variable regions of Fab [4]. This indicates that the overall structures of the variable regions of Fv and Fab are virtually identical. As shown in Table 1, no significant differences in magnitude were detected among Fv, Fab* and Fab for all the thermodynamic parameters. These results suggest that the presence of the constant domains has little effect on the binding of DNS-Lys. The thermodynamic results obtained in the present study are consistent with the conclusion drawn on the basis of the NMR data.

We have observed large negative enthalpy changes upon binding of DNS-Lys to Fv, Fab* and Fab. A previous NMR study of Fv performed in the presence of DNS-Lys showed that the aromatic ring of Tyr^{96} and Tyr^{104} at both ends of the third loop of the complementarity-determining region in the heavy chain (H3) are located within 5 Å of the dansyl ring [1,15]. Furthermore, we have found by using deuterated Fv analogues that the side chain of Val^{2} in the N-terminal of the heavy chain and the aromatic ring of Phe^{27} in the first loop of the complementarity-determining region in the heavy chain (H1) are in close contact with DNS-Lys (manuscript in preparation). We have suggested that the side chains of these residues in V_H contribute to the binding of DNS-Lys through van der Waals interactions. It is therefore quite likely that the observed large negative enthalpy change is induced by the van der Waals interaction. We therefore conclude that the van der Waals interactions involving the above four residues are the driving force for the binding of DNS-Lys to Fv, Fab* and Fab.

Our experimental results of transferred nuclear Overhauser effects have suggested that both the dansyl ring and the side chain of DNS-Lys are tightly bound to the antibody combining site [16]. There is an empirical relation between the hydrophobic component of $|\Delta Cp|$ and the amount of accessible surface area of non-polar atoms buried upon the protein folding or the association of molecules [17]. If we assume that the surface of DNS-Lys is entirely buried, the upper limit to the hydrophobic component of $|\Delta Cp|$ can be estimated. With the extended conformation, the non-polar surface area of DNS-Lys is estimated at 280 $Å^2$, which gives a maximum of $|\Delta Cp|$, 92 ± 25 $cal \cdot mol^{-1} \cdot K^{-1}$. However, this value is smaller than the observed $|\Delta Cp|$. Thus, the present $|\Delta Cp|$ result suggests that some additional factors, such as the changes in the vibrational frequencies and conformational changes of molecules [18], may contribute to the antigen–antibody binding.

The hydrophobic effect, which drives the association of nonpolar surfaces of molecules by excluding water from the interface, would contribute to a positive entropy change. In con-

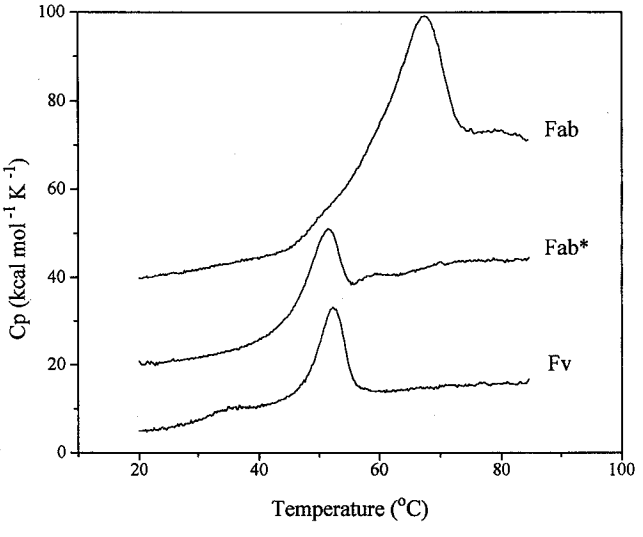

Fig. 3. Thermal denaturation DSC curves of Fv, Fab* and Fab in 50 mM acetate buffer, pH 4.5. The scanning rate is 45 K/h. The concentration of each sample is 20 μM.

trast, we actually observed negative entropy changes upon binding of DNS-Lys to Fv, Fab* and Fab. This means that some other factors counteract the hydrophobic effect and make a larger contribution to the negative direction. Such an effect can be caused by the reduction in the translational and rotational degrees of freedom upon antigen–antibody binding [19,20]. Conformational change of the flexible regions of the protein molecules may also be induced by the ligand binding, which can contribute to the negative entropy change.

NMR analysis of Fv has shown that the spin–spin relaxation times of the backbone amide nitrogens in the H3 loop change significantly upon addition of DNS-Lys, suggesting that the binding of DNS-Lys restricts the flexibility of the H3 region that contains four tyrosine residues [21]. Furthermore, the freezing of the side chains such as the aromatic ring of the tyrosine residue induced by the ligand binding can make a significant contribution to the negative entropy change [22].

In the previous NMR experiments performed on Fv and Fab, it has been indicated that the existence of the constant domains affects the H–D exchange rates of the main chain amide protons in the variable domains [4]. On the basis of the NMR results, we have shown that the H–D exchange rates for the residues of Fab located in the V_L domain (His^{34}, Trp^{35}, Tyr^{36}, Tyr^{49}, Phe^{71}, Tyr^{86}, Phe^{87}, His^{93}), are not as fast as those of Fv [4]. Moreover, we have observed that the H–D exchange rates for aromatic residues of the variable regions of Fv and Fab* are similar to each other (data not shown).

In order to obtain information about the contribution of the constant domains to the overall stability of the antigen-binding fragments, thermal stability of Fv, Fab* and Fab were examined (Fig. 3). In the absence of DNS-Lys, melting temperatures (T_m) for Fv, Fab* and Fab were 52.3, 51.4 and 67.4°C, respectively. T_m for Fv is lower than that for Fab by 15.1°C. In contrast, T_m for Fab* is comparable to that for Fv. It should be noted that in Fab used in the present study, the disulfide bond between C_L and C_H1 was reduced and alkylated. Therefore, we suggest that the increase in the thermal stability observed for Fab is not due to the disulfide bond. This result is consistent with the H–D exchange data [4], indicating that (i) the pair of the constant domains (C_H1 and C_L) is necessary for the stabilization of the variable domains and (ii) the single constant domain (C_L) is not quite sufficient for the stability of the variable domains.

In the present study, we have indicated that (i) the three antigen-binding fragments possess sufficient ability for antigen binding and (ii) the two constant domains serve to improve the thermal stability of the variable domains. We conclude that the formation of the quaternary structure comprising the two variable and two constant domains renders the antigen-binding fragment stable with the full binding ability expressed.

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