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Pronounced effect of hapten binding on thermal stability of an anti-(4-hydroxy-3-nitrophenyl)acetyl antibody possessing a glycine residue at position 95 of the heavy chain

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ABSTRACT

Immune response to T-cell-dependent antigens is highly dynamic; several B-cell clones responsible for antibody production appear alternately during immunization. It was previously shown that at least two-types of antibodies are secreted after immunization with (4-hydroxy-3-nitrophenyl)acetyl (NP); one has Tyr and another has Gly at position 95 of the heavy chain (referred to as Tyr95- and Gly95-type). The former appeared at an early stage, while the latter appeared at a late stage, i.e., after secondary immunization, although Fv domains of these antibodies were encoded by same genes of variable heavy and light chains. We examined whether any biophysical properties of antigen-combing sites relate to this shift in B-cell clones by preparing single-chain Fv (scFv). Thermodynamic and kinetic parameters of the interaction of scFv with various haptens are in accordance with those of intact antibodies, indicating that scFvs are appropriate models for the study on structure and function of antibodies. Next, we measured thermal stability of scFvs using differential scanning calorimetry and found that the apparent melting temperature of free Tyr95-type was 64–66 °C,while that of Gly95-type was 47–48 °C, indicating that the latter was highly unstable. However, Gly95-type greatly gained thermal stability because of hapten binding. We discussed the relationship between thermal stability resulted by hapten binding and dynamism of antibody response during immunization.

1. Introduction

Assembly of variable (*V*), diversity (*D*), and joining (*J*) gene segments generates antibody diversity (Tonegawa, 1983). In addition, immunization with T-cell-dependent antigens induced somatic hypermutation in the V regions of antibodies resulting in an increase in binding affinity, referred to as affinity maturation (Eisen and Siskind, 1964; Milstein and Rada, 1995; Azuma, 1998). Primary antibodies produced at an early stage of immunization have a low affinity, while those produced at a later stage have a higher affinity. Elucidation of structural and physical properties of antibodies that appeared during the process of affinity maturation can also help us to understand the molecular recognition mechanism of protein, and also inform the basis of rational protein design, for example, by suggesting ways to increase the affinity and specificity of antibodies *in vitro* (Wedemayer et al., 1997; Yin et al., 2001; Adhikary et al., 2015). For this purpose, studies using various hapten systems, such as (4-hydroxy-3-nitrophenyl)acetyl (NP), phosphorylcholine, and 2-phenyl oxazolone, have been carried out. These studies revealed that dynamic changes in B-cell clones are responsible for antibody production during immunization (Bothwell et al., 1981; Gearhart et al., 1981; Kaartinnen et al., 1983; French et al., 1989).

Structural properties of anti-NP antibodies during affinity maturation have been well analyzed (Cumano and Rajewsky, 1985; Allen et al., 1988; Azuma, 1998; Furukawa et al., 1999; Murakami et al., 2010). The antigen binding affinity of germline-type antibody is as low as 10^5–10^6 M^{-1}, and that of affinity-matured antibody sometimes reached to 10^9 M^{-1}. Rajewsky's group reported that the mutation from Trp to Leu at position 33 of the heavy chain (W33L mutation) resulted in an increase in the affinity by approximately 10-fold (Allen et al., 1988). We used the Kabat amino acid numbering system throughout this study (Kabat et al., 1991). Azuma's group reported that anti-NP antibodies could be classified into at least two groups, Tyr95- and Gly95-type, based on the amino acid

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residue at position 95 of the heavy chain, which corresponds to the junction of VH and D segments (Furukawa et al., 1999; Murakami et al., 2010). They showed that W33L mutation resulted in an increase in affinity of Tyr95-type, while it brought about a decrease in affinity of Gly95-type that employed the other mutations to gain higher affinity (Murakami et al., 2010), indicating that affinity maturation of Tyr95- and Gly95-type anti-NP antibodies proceeds through different pathways (strategies).

In addition to the different strategies employed, the ceiling affinities attained by affinity maturation were different between Tyr95- and Gly95-type. Namely, association constants (K_a) of germline anti-NP antibodies irrespective of amino acids at position 95 were shown to be in the range of 10^5–10^6 M^{-1}, while the ceiling affinity attained after maturation was $\sim10^7$ M^{-1} for Tyr-95 and $\sim10^9$ M^{-1} for Gly95-type (Furukawa et al., 1999). These two types of antibodies are produced at different stages of immunization; Tyr95-type appeared at an early stage of immunization, while Gly95-type appeared after secondary immunization (Tashiro et al., 2015). The origin of such a difference in appearing time points between Tyr95- and Gly95-type antibodies has not been understood yet. Recently, it was reported that somatic hypermutation affects both antibody stability and binding affinity and that B-cell clones are selected on the basis of affinity and stability (Sun et al., 2013; Wang et al., 2013). To address how the amino acid residues at position 95 as well as those arisen from somatic hypermutation affect the stability, we used a single-chain Fv (scFv) antibody and analyzed its structural, physical, and functional properties (Sato et al., 2016). One of the advantages to use scFv is that it consists of minimum unit of antibody domain and provides information on structure and function without perturbation of other domains. However, since VH and VL domains are linked with a linker peptide in scFv, it is necessary to verify if the linker peptide provided little effect on the structure and function.

In the present study, we selected two representative anti-NP antibodies, N1G9 (Tyr95-type) and C6 (Gly95-type) and prepared scFvs, N1G9-scFv and C6-scFv, respectively. We carried out detailed kinetic and thermodynamic analyses of the interaction between scFvs and hapten using surface plasmon resonance (SPR) biosensor and isothermal titration calorimetry (ITC). Essentially, no significant differences were observed in the profiles of hapten binding between scFvs and parent antibodies, suggesting that a linker peptide had little effect on structure and function, allowing us to use scFvs instead of parent antibodies. Then, we measured the thermodynamic stability using differential scanning calorimetry (DSC), whose parameters were compared with those obtained by ITC measurements. We attempted to explain the prevalence of Tyr95-type in primary response and appearance of Gly95-type after secondary immunization in terms of affinity and stabilizing effect of haptens.

2. Materials and methods

2.1. Expression and purification of scFv

Gene encoding scFv (-VL-$(G_4S)_3$ linker-VH-) of N1G9 was amplified by PCR, similar to that of C6, as described previously (Sato et al., 2016). The mutant plasmids of W33L were constructed by site-directed mutagenesis. The plasmid was transformed into *E. coli* BL21 (DE3) codon plus, and the transformed cells were cultured in LB medium at 37 °C. After addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), cells were cultured for a further 12 h at 22 °C. Because the protein was expressed in the insoluble fraction, it was solubilized using 8 M urea, and refolded by stepwise dilution of urea from 8 M to 0 M, via 4, 2, 1 M, as similar to the reported procedure (Sato et al., 2016). As additives, 0.4 M L-arginine and 0.7 mM glutathione oxidized form or a combination of 0.7 mM oxidized and 1.4 mM glutathione reduced forms were added at the 1 M urea step. The refolded protein was purified using an antigen column, in which NP conjugated to BSA was immobilized on the resin (NHS-activated sepharose 4; GE Healthcare). The concentration of scFv was spectrophotometrically determined using molar absorption coefficient of 5.94×10^4 M^{-1} cm^{-1} for N1G9-scFv, 5.39×10^4 M^{-1} cm^{-1} for N1G9-W33L-scFv, 5.64×10^4 M^{-1} cm^{-1} for C6-scFv, and 5.09×10^4 M^{-1} cm^{-1} for C6-W33L-scFv at 280 nm.

2.2. Antigen preparation

NP-coupled ε-aminocaproic acid (NP-Cap) was used for ITC and DSC measurements. NP-coupled bovine serum albumin (BSA) at a molar ratio of 8.2:1 ($NP_{8.2}$-BSA), and (4-hydroxy-3,5-dinitrophenyl)acetyl (NNP)-coupled BSA at a molar ratio of 6.4:1 ($NNP_{6.4}$-BSA) were used for SPR experiments. These conjugated molecules were synthesized, as reported previously (Oda and Azuma, 2000). The concentrations of NP and NNP were determined using molar absorption coefficients of 4.23×10^3 M^{-1} cm^{-1} at 430 nm and 7.12×10^3 M^{-1} cm^{-1} at 445 nm, respectively (Oda and Azuma, 2016).

2.3. Circular dichroism (CD) experiments

Far UV CD spectra were measured in 40 mM potassium phosphate buffer (pH 7.3) at 20 °C on a Jasco J-820 spectropolarimeter. The scFv concentration was 0.02 mg/ml. CD spectra were obtained under the following condition: optical path length of 1 cm, scan rate of 20 nm/min, response time of 1 s, and bandwidth of 1 nm.

The melting curves were recorded for scFv in the absence or presence of NP (40 μM), in temperature mode at 218 nm, from 20 to 80 °C with a heating rate of 1.0 °C/min. The analysis of the transition curve to determine the transition temperature (T_m) was performed on the basis of two-state transition model, as described previously (Inaba et al., 2013).

2.4. SPR experiments

Antigen-antibody interactions were analyzed using SPR biosensor (Biacore T200, GE Healthcare). Antigens, $NP_{8.2}$-BSA and $NNP_{6.4}$-BSA were immobilized on the dextran surface of a CM5 sensor chip (GE Healthcare) with amine coupling method. The scFv was diluted at eleven concentrations (0.78–800 nM) in PBS containing 0.005% Tween 20 (running buffer) and the association of each solution was recorded at rate of 20 μl/min for 180 s. The dissociation of the complexes was measured by injecting running buffer alone for 180 s, followed by injecting of 3 M Gdn-HCl containing 1 M acetate for 45 s as regeneration. The experiments were performed at 25 °C and collected data were analyzed using the BIAevaluation 3.1 software. Obtained curves were fitted to a 1:1 binding model to determine the kinetic rate constants, association rate constant (k_{on}) and dissociation rate constant (k_{off}). The K_a was calculated from the two rate constants.

2.5. ITC experiments

ITC measurements were carried out using an iTC200 calorimeter (Malvern). NP-Cap solution (100 μM) was titrated into the scFv solution (10 μM) using a 40 μl syringe. Both solutions were prepared in PBS. Each titration consisted of a preliminary 1 μl injection followed by 19 subsequent 2 μl additions at 25 °C. The heat for each injection was subtracted from the heat of dilution of the injectant, which was measured by injecting the antigen solution into buffer. Each corrected heat value was analyzed on Origin software supplied by the manufacturer.