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Improvement of antibody affinity by introduction of basic amino acid residues into the framework region

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ABSTRACT

Antibodies are widely used not only as therapeutic agents but also as research tools and diagnostic agents, and extensive efforts have been made to generate antibodies that have higher affinity. It was recently reported that introduction of charged residues into the framework region of an antibody improved its affinity; however, the underlying molecular mechanism has not been elucidated. In this study, we used kinetic and thermodynamic analyses of the antibody–antigen interaction to investigate the molecular mechanism by which an antibody with introduced charged residues recognizes its antigen with higher affinity. The introduction of basic amino acid residues resulted in improvement of the affinity whereas the introduction of acidic residues weakened the interaction. For two mutant antigen-binding fragments (Fabs) with improved affinity (named K5- and R5-mutants), the balance between the association rate constant k_{on} and the dissociation rate constant k_{off} was distinct despite each mutant having the same number of charged residues. Moreover, thermodynamic analysis of the interactions in the transition state revealed a difference between the K5- and R5-mutants in terms of enthalpic energy change following formation of the encounter complex with the antigen. These results suggest that the affinity of the K5- and R5-mutants is improved by distinct mechanisms. Although the mutations destabilize the Fab and necessitate further studies, our strategy is expected to become a versatile and simple means to improve the affinity of antibodies to their antigens.

Introduction

Antibodies are widely used not only as therapeutic agents but also as research tools and diagnostic agents due to their high specificity and affinity towards their antigens [1]. Antibodies acquire their affinity and specificity towards a variety of target antigens by changing the composition of amino acid residues in the six hyper-variable regions known as complementarity-determining regions (CDRs)[2]. Although CDRs comprise only a small number of amino acid residues, antibodies can precisely recognize numerous types of antigen [3]. As the high affinity of an antibody towards its antigen is a critical factor for therapeutic applications such as molecularly targeted anti-cancer drugs [4–7], extensive efforts have been made to generate higher-affinity antibodies, mainly through a directed evolutionary approach [8–12].

Recently it was reported that the affinity of an antibody towards its antigen was improved by introducing charged amino acid residues into the framework region of the antibody [13]; however, the molecular mechanism by which the modified antibody recognized its antigen with higher affinity remained to be elucidated. Here, we investigated the molecular mechanism by which introduction of charged amino acid residues affects an antibody's recognition of the antigen. Although the affinity was improved by introducing basic amino acid residues (either arginine or lysine), the thermodynamic parameters of the antibody–antigen interaction in the transition state were significantly different. Our results suggested that the introduction of basic residues into the framework region of antibodies improved the affinity by distinct mechanisms. A more detailed characterization of the interaction between antigens and antibodies with charged residues would contribute to the development of a versatile strategy to improve the affinity of antibodies.

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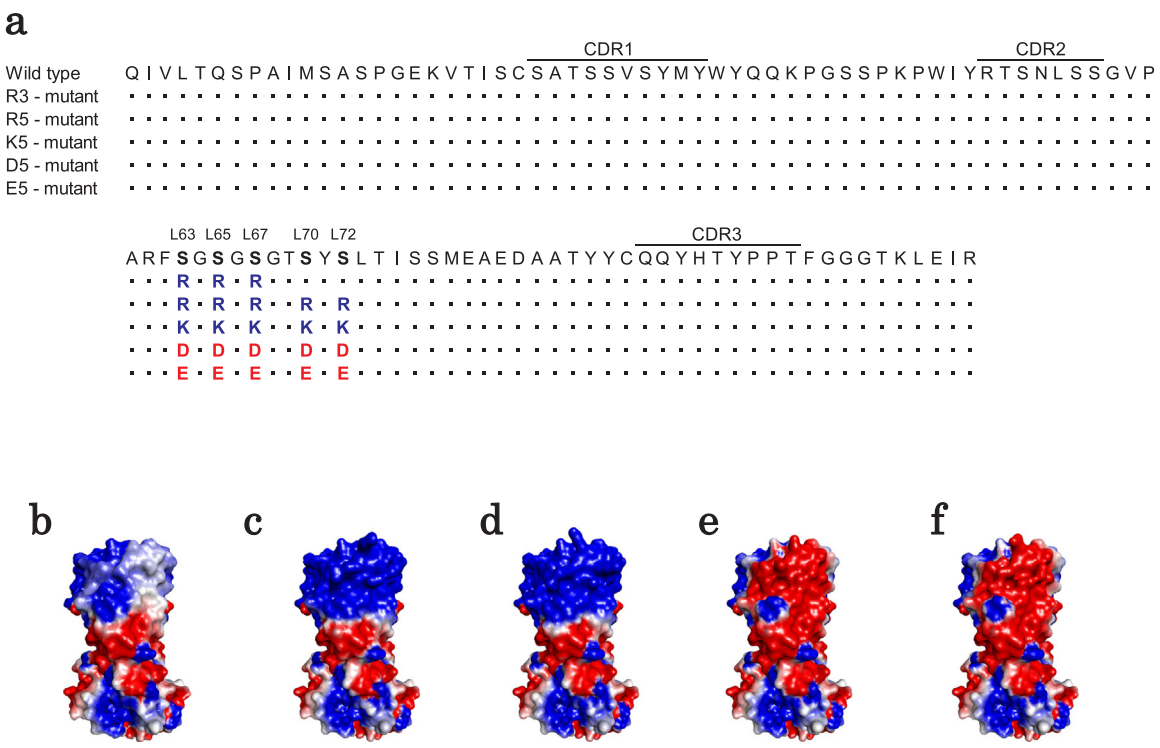

Fig. 1. Light chain variable region (VL) amino acid sequences of mutants and surface representations of Fab mutants. (**a**) VL amino acid sequence of each mutant. L63, L65, L67, L70, and L72 were selected as mutation points. All points were included in framework region 3. The mutants were named R3-mutant, R5-mutant, K5-mutant, D5-mutant, and E5-mutant. (**b–f**) The electrostatic potentials around the VL binding site of wild type (b), R5-mutant (c), K5-mutant (d), D5-mutant (e), and E5-mutant (f) depicted by using Discovery Studio (ver. 4.5; BIOVIA) with contours drawn at 2 kT per electron at 0.018 mM NaCl (blue for positive and red for negative) by using only full charges. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

Materials and methods

Genetic engineering and protein expression and purification

We used an anti-insulin Fab, an antigen binding fragment of antibodies, as a model. The mouse monoclonal antibody against human insulin was developed by conventional hybridoma technology from Sysmex Corporation [14]. All mutants in framework region 3 (FR3) were generated by site-directed mutagenesis using a KOD -Plus- Mutagenesis Kit (Toyobo) in accordance with the manufacturer's protocol.

Antibodies were expressed using the pcDNA 3.4 TOPO expression vector (Life Technologies) and Expi293TM expression system (Life Technologies). Cell culture supernatant was filtered through a 0.8-μm-pore-size filter (ADVANTEC) and antibodies in the filtered supernatant were added to protein A resin (GE Healthcare). After washing with phosphate-buffered saline (PBS), antibodies were eluted with 0.1 M glycine-HCl (pH 2.7), neutralized with 100 mM Tris-HCl (pH 8.0), and dialyzed against PBS.

Fabs were prepared by using Mouse IgG1 Fab and F(ab′)2 Preparation Kits (Pierce). Eluted Fabs were further purified with size-exclusion chromatography using Superdex 200 Increase 10/300GL (GE Healthcare) in PBS.

Binding and thermodynamic analysis of the interaction between Fabs and insulin using surface plasmon resonance (SPR)

To obtain kinetic and thermodynamic parameters, SPR experiments were performed using a BIAcoreT200 system (GE Healthcare). Insulin (Funakoshi) was immobilized on research-grade CM5 sensor chips (GE Healthcare). The amount of Insulin immobilized for kinetic analysis were determined according to the manufacture's protocol. Each purified Fab was dialyzed against HBS-EP buffer (10 mM HEPES [pH 7.4], 150 mM NaCl, 3.4 mM EDTA, 0.05% surfactant P20) and injected over the immobilized insulin at a flow rate of 50 μl/min. The data were normalized by subtracting the response from a blank cell in which bovine serum albumin (BSA) alone was immobilized. BIA evaluation software version 2.0.2.(GE Healthcare) was used to analyze the data. Kinetic parameters were calculated by a global fitting analysis with the assumptions of the 1:1 Langmuir binding model.

Thermodynamic analyses of each Fab were performed at five temperature points (283.15 K, 288.15 K, 293.15 K, 298.15 K, and 303.15 K). The standard state Gibbs energy change upon binding was obtained from Eq. (1):

$$\Delta G = R\,T \ln K_d \quad (1)$$

where K_d is the dissociation constant, expressed in units of $mol \cdot l^{-1}$, R is the gas constant, and T is the absolute temperature. The ΔG values of each data set were plotted against the temperatures, and were fitted with the nonlinear van't Hoff equation (Eq. (2)),

$$\Delta G = \Delta H - T\Delta S + \Delta Cp(T - 293.15) - \Delta Cp\ T \ln(T/293.15) \quad (2)$$

where ΔH and ΔS are the binding enthalpy change and entropy change at 293.15 K, respectively, and ΔCp is the heat capacity change, which is assumed to be temperature independent.

The activation energy parameters were obtained from the temperature dependence of the association rate constant following the Eyring approximation:

$$\ln(k_{on})/T) = -(\Delta H^{\ddagger}/RT) + (\Delta S^{\ddagger}/R) + \ln(k_B/h)$$

where k_{on} is the association rate constant, $\Delta H^{\ddagger}$ is the activation enthalpy, R is the gas constant, T is the absolute temperature, $\Delta S^{\ddagger}$ is the activation entropy, k_B is the Boltzmann constant, and h is the Planck constant.