Heat denaturation of the antibody, a multi-domain protein

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Abstract

The antibody is one of the most well-studied multi-domain proteins because of its abundance and physiological importance. In this article, we describe the effect of the complex, multi-domain structure of the antibody on its denaturation by heat. Natural antibodies are composed of 6 to 70 immunoglobulin fold domains, and are irreversibly denatured at high temperatures. Although the separated single immunoglobulin fold domain can be refolded after heat denaturation, denaturation of pairs of such domains is irreversible. Each antibody subclass exhibits a distinct heat tolerance, and IgE is especially known to be heat-labile. IgE starts unfolding at a lower temperature compared to other antibodies, because of the low stability of its CH3 domain. Each immunoglobulin domain starts unfolding at different temperatures. For instance, the CH3 domain of IgG unfolds at a higher temperature than its CH2 domain. Thus, the antibody has a mixture of folded and unfolded structures at a certain temperature. Co-existence of these folded and unfolded domains in a single polypeptide chain may increase the tendency to aggregate which causes the inactivation of the antibody.

Keywords Antibody · Heat denaturation · Aggregation · Protein stability · Single-domain antibody

Introduction

Mammalian antibodies usually consist of multiple immunoglobulin fold domains; for instance, IgE and IgG have 14 and 12 domains, respectively (Fig. 1). Although VH and VL domains are sufficient to bind antigens, there are several artificial and smaller antibody formats, such as Fab and single-chain Fv that possess four and two domains, respectively. The camelid heavy-chain antibody that naturally lacks the light-chain and CH1 domain is half the size of an IgG. It consists of six domains and a separated VH domain, known as VHH, which can bind to an antigen with a single domain. In addition to the

complexity of the overall structure and the number of domains, the differences in intra- and inter-domain interactions, which is determined by the amino acid sequences, give a distinct physical character to each antibody format. In this article, we compare the heat denaturation in several antibody formats and discuss the mechanism of heat denaturation of the antibody at the domain level.

Heat-induced denaturation of IgG

IgG denaturation becomes significantly irreversible at temperatures higher than 65 °C (Mainer et al. 1999; Indyk et al. 2008). IgG almost completely loses its antigen-binding activity after heat treatment for several minutes at 90 °C (Augener and Grey 1970; van der Linden et al. 1999; Ladenson et al. 2006; Akazawa-Ogawa et al. 2014). Other classes of antibodies, such as IgA and IgM, are more sensitive to heat treatment than IgG. At 72 °C, IgG, IgA, and IgM in bovine milk are denatured by more than 75% after incubating for 25, 5, and 1.5 min, respectively (Mainer et al. 1997).

A series of heat-induced denaturation experiments have been carried out, mainly by using differential scanning calorimetry (DSC) to elucidate the mechanism of IgG denaturation (Vermeer and Norde 2000; Vermeer et al. 2000; Garber and

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Fig. 1 The domain structure of IgG, IgE, and heavy-chain antibody. Heavy and light chains are shown in white and black, respectively. The ovals indicate the domain composing the antibody and the names of domains are shown inside. The Fv, VHH, Fab, and Fc regions are

indicated by thick, thin, dotted, and broken lines, respectively. The Fv with an artificial polypeptide linker between VH and VL domains is known as single-chain Fv

Demarest 2007; Brader et al. 2015). Since heat-induced denaturation of IgG is irreversible in most cases, it is impossible to precisely analyze DSC data. However, the data allow us to compare the relative stability of domains in a single IgG, and those in different IgGs when the experimental condition is identical. Two or three distinct heat absorption peaks are observed in the DSC experiment with an increase in temperature, and each peak corresponds to a cooperative structural unit. After the analysis of isolated Fab and Fc regions from IgG by papain digestion, it was observed that Fab was less stable than Fc, and that heat denaturation of isolated Fc was irreversible (Augener and Grey 1970; Vermeer et al. 2000). Isolated Fab was also irreversibly denatured by heat (Deutscher et al. 1996). Later, it was found that Fc is not a cooperative unit, and that CH2 and CH3 were denatured independently. CH2 is the least stable cooperative structural unit in IgG (Feige et al. 2004; Chen et al. 2016). The interaction between carbohydrate chains and proteins enhances the stability of CH2 (Ghirlando et al. 1999; Voynov et al. 2009; Zheng et al. 2011). On the other hand, CH3 is the most stable structural unit (Demarest et al. 2004). Thus, the DSC experiment involving IgG shows three heat absorption peaks and the order of peak formation is usually CH2, Fab, and CH3, from low to high temperature. However, the peaks sometimes overlap and the order of the peaks may change depending on the stability of Fab and the subclass of IgG (Ito and Tsumoto 2013; Brader et al. 2015).

The heat-induced denaturation of isolated single CL, CH2, and CH3 domains is reversible at a neutral pH (Hagihara et al. 2002; Demarest et al. 2004; Feige et al. 2004). Thus, these isolated domains are considered to be highly heat resistant (i.e. doesn't aggregate). The heat denaturations of VH and VL are generally irreversible, although there is a naturally occurring VH domain that is heat-resistant and that refolds after heat denaturation (Jespersen et al. 2004b). Mutation and acidic pH dramatically improve the heat resistance of VL and VL (Martsev et al. 2000; Jespersen et al. 2004a; Hagihara et al. 2005). The

isolated CH1 domain of murine IgG1 is disordered; it can form a structure only with the CL domain (Feige et al. 2009).

Recently, our group compared the heat resistance of murine IgG, Fab, single-chain Fv, and llama VHH (Akazawa-Ogawa et al. 2014). Each protein was subjected to repetitive 5-min incubation cycles at 90 °C and 20 °C. Additionally, the effect of continuous incubation at 90 °C was examined. With an increase in the number of cycles or incubation time at 90 °C, the residual antigen-binding activity at 20 °C decreased. After five cycles of incubation at 90 °C and 20 °C, and a corresponding total incubation of 25 min at 90 °C, the residual activity of the three IgGs that were examined were less than 10% of untreated proteins. Although the Fab domains of those IgGs were slightly more stable than those of IgG, less than 10% of residual activity was observed in these Fabs after ten cycles of heat treatment. The single-chain Fv exhibited higher heat resistance than IgG and Fab, where approximately half the activity was observed after one cycle of heat treatment. Forty cycles or 200 min of continuous incubation at 90 °C were required to inactivate a single-chain Fv exhibiting less than 10% of the original activity. The heat-induced inactivation of single-chain Fv was concentration-dependent and, thus, caused by aggregation of proteins, as expected, in other larger antibody formats. VHH exhibited significantly greater resistance towards repetitive and continuous heat treatments than any other antibody format. Even after 40 cycles of heat treatment corresponding to 200 min of incubation at 90 °C, approximately half of the original antigen-binding activity was retained. We found that inactivation of VHH under a long period of incubation at a high temperature is not caused by aggregation, but by chemical modification of asparagine and cystine (Akazawa-Ogawa et al. 2014, 2016).

Heat-induced irreversible denaturation of IgG has been utilized to prevent nonspecific polymerase chain reaction (PCR) (Kellogg et al. 1994; Sharkey et al. 1994; Mizuguchi et al. 1999). At the very first step of PCR, the temperature is increased from room temperature to DNA denaturing

temperature around 95 °C. In this process, mismatched PCR templates remain and cause a nonspecific PCR product. Monoclonal antibody neutralizing thermostable DNA polymerase activity keeps inhibiting the PCR at low temperature where the mismatched PCR template is formed. At DNA denaturing temperature, the antibody is irreversibly denatured and does not disturb the amplification of DNA after the first ramp of the temperature. The antibodies against a number of DNA polymerases have been produced and commercialized.

The mechanism enabling lower stability in IgE than that in IgG

IgE has been known to be more heat-labile than other antibodies (Ishizaka et al. 1967). The incubation of IgE at 56 °C for a few hours significantly reduces anaphylactic activity, triggered by the binding of Fc of IgE to the Fc ϵ receptor, but does not have a notable effect on the antigen-binding activity in the Fab region (Prouvost-Danon et al. 1977; Binaghi and Demeulemester 1983; Ishizaka et al. 1986). Under the same experimental conditions, anaphylactic IgG (IgG2a) did not lose its activity (Bloch et al. 1968), indicating the difference in heat resistance between the Fc domains of IgE and IgG. After DSC scanning, carried out from 10 to 90 °C, both the Fc domains of IgE and IgG were irreversibly denatured (Demarest et al. 2006).

The Fc of IgE is composed of one pair each of three heavy chain domains, CH2, CH3, and CH4. The Fc ϵ receptor binds to the homodimeric interface of CH3 domains; CH2 and CH4 do not directly participate in the antibody–receptor interaction (Holdom et al. 2011). The Fc of IgE starts unfolding at a temperature approximately 15 °C lower than that of the Fc of IgG in the DSC experiment at a neutral pH (Demarest et al. 2006). The first phase of heat absorption in the Fc of IgE corresponds to the unfolding of CH3 and CH4, and the second phase corresponds to the unfolding of CH2. CH3 and CH4 are cooperatively unfolded as a single structural unit. Although the isolated CH3–CH4 construct is active and has a well-defined structure (Wurzburger et al. 2000; Hunt et al. 2005), isolated single-domain CH3 produced by *E. coli* exhibited weak receptor-binding activity and was partially unfolded (Henry et al. 2000). These findings indicate that the interaction between CH3 and CH4 is indispensable for maintaining the structure of CH3. The low heat resistance of anaphylactic IgE can be explained by the intrinsically low stability of the structural unit composed of CH3 and CH4.

Conclusions

Isolated domains in antibodies, such as VHH, CL, CH2, and CH3 in IgG, can be refolded after heat denaturation and are, thus, considered to be highly heat-resistant.

Single-domain VHH has a low tendency to aggregate when denatured by heat. Additionally, the heat denaturation in homodimeric CH3 of IgG is reversible. On the other hand, the hetero-pairing of two different domains apparently reduces its heat resistance, as revealed by the inactivation of Fc after heat denaturation. Thus, the heat resistance of an antibody format might be restricted by formation-related interactions between two or more different domains. The intrinsic stability of each domain in the antibody format is different; thus, some domains are folded, while others are unfolded at a certain temperature. Fc of IgE is inactivated at 56 °C, whereas IgG remains active at that temperature. CH3 and CH4 of IgE unfold at a lower temperature than that of CH3 of IgG, although the CH2s of both antibody subtypes start unfolding at a similar temperature. At 56 °C, the structure of Fc of IgE is a mixture of folded and unfolded parts, while the Fc of IgG is completely folded. The inactivation of an antibody format with more than two domains is often caused by aggregation. These suggest that the co-existence of folded and unfolded domains in a single polypeptide chain increases the tendency to aggregate and causes the inactivation of the antibody format.

Compliance with ethical standards

Conflict of interest Yoko Akazawa-Ogawa declares that she has no conflict of interest. Hidenori Nagai declares that he has no conflict of interest. Yoshihisa Hagihara declares that he has no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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