Special Issue
"Protein-Ligand Interactions"

Vol. 13, pp. 135–138 (2016)
doi: 10.2142/biophysico.13.0_135

Biophysics and Physicobiology

https://www.jstage.jst.go.jp/browse/biophysico/

Regular Article

Effects of substrate conformational strain on binding kinetics of catalytic antibodies

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Received January 7, 2016; accepted January 18, 2016

We analyzed the correlation between the conformational strain and the binding kinetics in antigen-antibody interactions. The catalytic antibodies 6D9, 9C10, and 7C8 catalyze the hydrolysis of a nonbioactive chloramphenicol monoester derivative to generate a bioactive chloramphenicol. The crystal structure of 6D9 complexed with a transition-state analog (TSA) suggests that 6D9 binds the substrate to change the conformation of the ester moiety to a thermodynamically unstable twisted conformation, enabling the substrate to reach the transition state during catalysis. The present binding kinetic analysis showed that the association rate for 6D9 binding to the substrate was much lower than that to TSA, whereas those for 9C10 and 7C8 binding were similar to those to TSA. Considering that 7C8 binds to the substrate with little conformational change in the substrate, the slow association rate observed in 6D9 could be attributed to the conformational strain in the substrate.

Key words: antigen, conformational change, surface plasmon resonance, transition-state analogue

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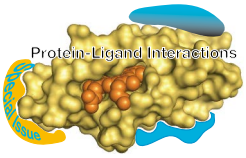
An antibody can specifically recognize an antigen with a different size and shape by changing the residues in the variable region, and the binding is typical for ligand-protein interactions. Antibodies generated by immunizing an animal with a putative transition-state analog (TSA) of the chemical reaction are expected to possess antigen-combining sites to be both geometrically and electronically complementary to the transition state of the reaction and to successfully catalyze the chemical reaction [1,2]. These catalytic antibodies have attracted wide attention as possible tailor-made catalysts for chemical transformations, and have been used in biotechnology and in clinical applications [3]. The catalytic antibodies 6D9, 9C10, and 7C8 were induced by immunization with a phosphonate TSA **3**, designed on the basis of the stabilization of transition state **4**; these antibodies catalyzed the hydrolysis of a nonbioactive chloramphenicol monoester derivative **1** to generate a bioactive chloramphenicol **2** (Fig. 1). A previous study showed that the catalytic antibodies could similarly catalyze the water-soluble substrate derivative, whose carboxyl group is conjugated to Lys, via a peptide bond (sub-Lys **5**) [4]. Among the three catalytic antibodies, 6D9 and 9C10 stabilize the transition state to catalyze the hydrolysis reaction strictly according to the following theoretical relationship; the ratio of the dissociation constants for the transition-state analog, K_{TSA}, and the corresponding sub-



◀ ***Significance*** ▶

The binding kinetics of catalytic antibodies, 6D9, 9C10, and 7C8, with substrate and transition-state analogue were determined and correlated with structural and thermodynamic information. The efficient catalytic antibody, 6D9, binds the substrate, changing the conformation to an unstable twisted conformation with enthalpic strain. The present analysis showed that the association rate for 6D9 binding to the substrate was much lower than that to transition-state analogue, whereas those for 9C10 and 7C8 binding were similar to those to transition-state analogue. The correlation of conformational strain with binding kinetics would widely apply for other protein-ligand interactions.

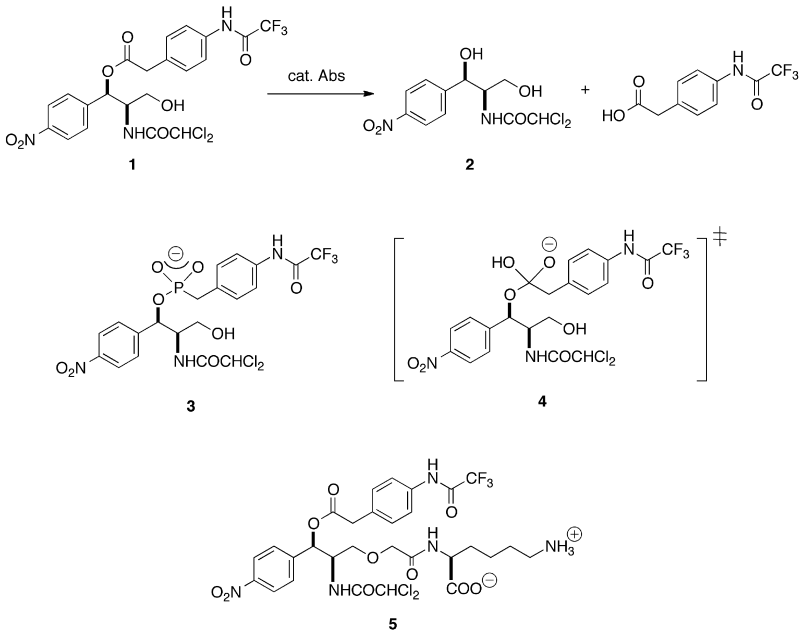


Figure 1 Chemical transformation resulting from antibody-catalyzed prodrug activation, and chemical formulae of the compounds used in this study. Catalytic antibodies, 6D9, 9C10, and 7C8, were raised against chloramphenicol phosphonate **3**, designed on the basis of stabilization of transition state **4**, and catalyzed the hydrolysis of chloramphenicol ester **1** to generate chloramphenicol **2** and the acid product. Sub-Lys (**5**) and TSA-BSA were used for the SPR measurements.

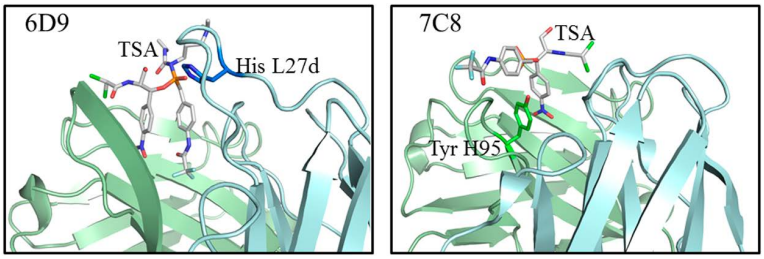


Figure 2 Crystal structures of 6D9 and 7C8 complexed with TSA, based on the crystallographic coordinates (PDB codes, 1HYX and 1CT8). The heavy and light chains are indicated in light green and light cyan, respectively, and the side-chains of His L27d in 6D9 and Tyr H95 in 7C8 are indicated as stick models. TSAs are indicated as stick models. Figures were generated with Pymol [13].

strate, K_S, must be equal to the rate enhancement, k_{cat}/k_{uncat}; for 6D9, k_{cat}/k_{uncat} = 895, K_S/K_{TSA} = 900, and for 9C10, k_{cat}/k_{uncat} = 56, K_S/K_{TSA} = 60 [5]. This suggests that binding in the antibody-TSA complex is essentially identical to that in the transition state of the antibody-catalyzed reaction. In contrast, the k_{cat}/k_{uncat} value for 7C8, namely 707, was considerably different from its K_S/K_{TSA} value, 12; this indicates that 7C8 catalyzes the reaction by a different mechanism. The effects of pH and hydroxylamine on the catalytic activity of 6D9 and 7C8 indicate that the rate determining steps of each antibody-catalyzed reaction are OH^- attack and nucleophilic attack by deprotonated TyrH95, respectively [6].

The crystal structures of TSA complexed with 6D9 Fab and 7C8 Fab were reported previously [6,7]. One of the most prominent differences between the two structures is that in 6D9, the two aromatic rings of TSA are stacked and buried deep in the antigen-combining site, whereas in 7C8, only the *p*-nitrobenzyl group is buried and the trifluoroacetyl group of TSA is exposed to the solvent (Fig. 2). Structural and thermodynamic analyses showed that 6D9 binds the substrate, changing the conformation of the ester moiety to a thermodynamically unstable twisted conformation with enthalpic strain [4,7]. In contrast, 7C8 binds the substrate with much lesser constraint on the ester bond [6].

In this study, we analyzed the substrate binding to the antibodies 6D9, 9C10, and 7C8 using a surface plasmon resonance (SPR) biosensor, and we evaluated the effects of the substrate-bound conformation on binding kinetics. In addition, the effects of the antigen-antibody complex stability on the binding kinetics were evaluated in comparison with the binding kinetics of TSA.

Table 1 Kinetic parameters for interactions between catalytic antibodies, 6D9 Fab, 9C10 Fab, and 7C8 Fab, and immobilized antigens, sub-Lys and TSA-BSA, on the sensor chip

antibody	antigen	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_a (M^{-1})	$K_{a,ITC}$ (M^{-1})[a]
6D9 Fab	sub-Lys	6.7×10^3	2.4×10^{-1}	2.8×10^4	6.0×10^4
6D9 Fab	TSA-BSA	6.8×10^5	2.0×10^{-3}	3.5×10^8	1.3×10^8
9C10 Fab	sub-Lys	1.6×10^6	1.0×10^{-2}	1.6×10^8	1.2×10^8
9C10 Fab	TSA-BSA	8.1×10^5	1.1×10^{-3}	7.6×10^8	9.5×10^8
7C8 Fab	sub-Lys	7.1×10^5	2.0×10^{-2}	3.6×10^7	n.d.[b]
7C8 Fab	TSA-BSA	8.3×10^5	1.9×10^{-3}	4.3×10^8	6.3×10^8

[a] The K_a values determined using isothermal titration calorimetry were reported previously [4,14].
[b] not determined.

Materials and Methods

Antigen and antibody preparation

Antigens, sub-Lys and TSA conjugated to bovine serum albumin (TSA-BSA) (Fig. 1), and Fabs of catalytic antibodies, 6D9, 9C10, and 7C8, were prepared as reported previously [4,8].

SPR measurement

The Biacore biosensor system, Biacore 2000 (GE Healthcare Bioscience, Uppsala, Sweden), was used to measure real-time antigen-antibody interactions. Antigens, sub-Lys and TSA-BSA, were covalently linked to the sensor chip, CM5, and antibodies at various concentrations in a 10 mM phosphate buffer (pH 6.0), containing 0.14 M NaCl and 0.005% Tween20, were applied over the sensor chip at a rate of 20 µl/min during a period of 3 min. The surface was regenerated with one 15 µl injection of a solution of 3 M guanidine hydrochloride containing 1 M acetic acid. All experiments were performed at 25°C.

The sensorgrams for antigen-antibody interactions were examined by first adjusting for background changes reflected by the bulk refractive indices, and then by anlysis using the BIAevaluation 3.2 software. In this program, a global fitting method was used to determine the association and dissociation rate constants, k_{on} and k_{off}, using a model of 1:1 Langmuir binding. The kinetic values for 6D9 Fab binding to sub-Lys and for others were determined by a global fitting method for Fab concentrations of 400–3,200 nM and 3.1–25 nM, respectively. The equilibrium association constant, K_a, was calculated from the two rate constants using the equation, $K_a = k_{on}/k_{off}$.

Results and Discussion

In order to immobilize antigens on the sensor chip, CM5, via amine-coupling, sub-Lys **5** and TSA-BSA were used (Fig. 1). When an intact antibody was used as the analyte, the apparent binding affinity toward the immobilized antigen was higher than that of monovalent binding; this is because an intact antibody can use two antigen-combining sites simultaneously [9]. Therefore, in order to analyze the monovalent binding, Fabs of catalytic antibodies were used as analytes. Because the catalytic antibodies hydrolyze chloramphenicol monoesters efficiently at high pH, the Fab binding to antigens was examined at pH 6.0 under conditions of negligible catalysis. As reported previously, the binding affinity toward sub-Lys at pH 6.0 is similar to the K_m value determined at pH 8.0, suggesting that the mode of substrate binding at pH 6.0 is essentially identical to that at pH 8.0 [4]. SPR experiments for Fab binding to sub-Lys, along with that to TSA-BSA, were carried out at pH 6.0, and the binding kinetics were determined (Table 1). The K_a values calculated from the binding kinetic parameters are comparable to those determined using isothermal titration calorimetry, which indicates that the SPR experiments can be performed without any artificial effects from antigen immobilization.

It should be noted that the k_{on} value for the 6D9 Fab binding to the immobilized sub-Lys was significantly lower than those of other interactions analyzed (Table 1). The crystal structure shows that the two aromatic rings of TSA are buried deep in the antigen-combining site, suggesting that the conformation of the ester moiety of the substrate is changed to a thermodynamically unstable twisted conformation upon 6D9 binding [7]. In contrast, upon 7C8 binding, only the *p*-nitrobenzyl group is buried in the 7C8 antigen-combining site and the trifluoroacetyl group is exposed to the solvent (Fig. 2), suggesting that the substrate binds to 7C8 with little conformational change [6]. Considering these observations, the slower association rate can be attributed to the conformational change in the antigen or the population of the native format determinant [10]. This relation can be used to predict the substrate strain or the conformational change for the antigen-antibody complex, which have not been determined thus far. Because the k_{on} value for the 9C10 Fab binding to the immobilized sub-Lys is similar to that to the immobilized TSA-BSA (Table 1), the bound conformation of substrate in the antigen-combining site of 9C10 may be perturbed only slightly.

The k_{off} value for the 6D9 Fab binding to the immobilized sub-Lys is approximately two orders higher than that to the immobilized TSA-BSA, and the k_{off} values for the 9C10 Fab and 7C8 Fab binding to the immobilized sub-Lys are approximately one order higher than those to the immobilized TSA-

BSA. The dissociation rate is likely largely affected by the specific interactions between molecules, such as hydrogen bonds and van der Waals contacts [11]. The antibodies 6D9, 9C10, and 7C8 bind the phosphonyl oxygen of TSA by establishing one hydrogen bond with it and stabilize the TSA relative to the substrate [6,7]. The corresponding residue of 6D9 and 9C10 is His L27d in the CDR1 of the light chain, and that of 7C8 is Tyr H95 in the CDR3 of the heavy chain (Fig. 2) (the amino acid sequences are numbered according to the consensus nomenclature of Kabat *et al*. [12]). The loss of these specific hydrogen bonds in the Fab binding to the substrate should mainly contribute to the faster dissociation rates. In addition, the conformational strain in sub-Lys in the bound state would contribute to the difference in k_{off} between sub-Lys and TSA-BSA, which is larger for the 6D9 Fab binding than the 9C10 Fab and 7C8 Fab binding.

The TSA used in this study is a true analog of the actual transition state in 6D9 and 9C10-catalyzed hydrolysis; 7C8, however, uses a different catalytic mechanism [5,6]. In the catalysis by 7C8, nucleophilic attack by Tyr H95 would be the major cause. Because 7C8 could efficiently hydrolyze the substrate in the stable Z form, similar to the bound form of TSA observed in the crystal structure complexed with 7C8 Fab [6], the k_{on} value for the 7C8 Fab binding to the immobilized sub-Lys was similar to that of the immobilized TSA-BSA (Table 1). The hydrolysis reaction by 6D9 and 9C10 is accelerated by the differential affinity of the transition state relative to the substrate. The antibody stabilizes the transition-state binding; in other words, it destabilizes the substrate binding. For the more efficient catalytic antibody 6D9, relative to 9C10, the degree of transition-state stabilization and substrate destabilization must be larger; this is supported by the present binding kinetics results (Table 1). Our thermodynamic analysis showed that enthalpic strain leads to the destabilization of antibody-substrate complexes [4]. In this study, we showed that this enthalpic and conformational strain in a substrate could be detected by using the antibody binding kinetics.

Conclusion

We analyzed the interactions of catalytic antibodies, 6D9, 9C10, and 7C8, with substrate and TSA using an SPR biosensor. Among them, 6D9 binds the substrate, changing the conformation of the ester moiety to a thermodynamically unstable twisted conformation. In this study, we found that this conformational strain results in slow association and fast dissociation rates, the latter of which includes the contribution from the loss of specific hydrogen bonds, in comparison with the binding to TSA. The correlation of the conformational strain with binding kinetics will apply widely to other ligand-protein interactions.

Acknowledgments

The authors thank Kayo N. Suzuki for technical support.

Conflicts of Interest

The authors declare no competing interests exist.

Author Contributions

M. O. and I. F. directed the project. T. T. synthesized the antigens and purified Fabs of catalytic antibodies. M. O. carried out the SPR measurements and wrote the manuscript.

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