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# KINETIC ANALYSIS OF RECOMBINANT ANTIBODY–ANTIGEN INTERACTIONS: RELATION BETWEEN STRUCTURAL DOMAINS AND ANTIGEN BINDING

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**The relation between domain structures of recombinant monoclonal antibody fragments and their reaction kinetics was studied for the first time using a novel biosensor based on surface plasmon resonance technology. The association and dissociation rate constants of Fab, Fv and single domain (VH fragment) anti-lysozyme antibodies were determined and compared to the intact monoclonal antibody. Fab and Fv fragments showed similar reaction kinetics and had affinity constants of $6 \times 10^9$ $M^{-1}$ and $25 \times 10^9$ $M^{-1}$, respectively. The single domain antibody had significantly different reaction kinetics compared to the fragments consisting of paired heavy and light chain domains. The VH domain had both a higher dissociation and a lower association rate constant, which resulted in an affinity constant approximately 250 times lower than the Fab fragment. This rapid evaluation of antibody reaction kinetics should prove to be an important selection parameter when comparing antibody fragments for their utility in therapeutic or other applications.**

Antibody association rate constants were recently suggested to be an important factor in the antigen-dependent mutational "fine-tuning" of the structural antibody repertoire[1]. This indicates a biological significance of kinetic parameters in the antibody-antigen interactions during an immune response, which would also have implications when constructing designer antibodies using bacterial expression systems[2]. Since these recombinant antibody fragments are potentially important tools for *in vivo* diagnosis and therapy of human disease[2], we have undertaken kinetic measurements on the interactions between recombinant fragments (Fab, Fv and single domain antibody (VH)) and the relevant antigen.

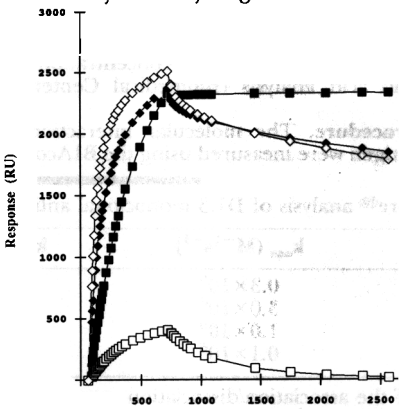
In this study we have determined the association/dissociation rate constants and affinity constants for the intact anti-hen egg lysozyme D1.3 monoclonal antibody, and its recombinant fragments[3], using a novel and very rapid biosensor technology[4,5]. The association and dissociation rate constants differed significantly between antibody fragments consisting of paired VH and VL domains (Fv and Fab) as compared with single VH domain fragments. This indicates an important role of the light chain variable domain when constructing recombinant antibody fragments for therapeutic applications.

## RESULTS AND DISCUSSION

The three-dimensional structure of the Fab D1.3 monoclonal antibody bound to its antigen lysozyme was recently determined by x-ray diffraction at 2.5-Å resolution[6]. Twelve lysozyme residues made contact with the antibody combining site, involving six VL residues and seven VH residues. Thus, recombinant fragments consisting of paired domains, such as Fab and Fv, should be able to bind to lysozyme in a way similar to the intact D1.3 monoclonal antibody, but the single domain VH antibody fragment would have fewer contact points with the antigen.

To investigate the effect of antibody domain structure on the binding kinetics of the antigen interaction, we studied the reaction of the relevant antibody fragments with immobilized lysozyme using a biosensor based on surface plasmon resonance. The relative responses of the intact monoclonal D1.3 antibody and its antibody fragments were plotted against time as shown on the overlaid plot in Figure 1. From the slopes of these curves we can draw several conclusions regarding the association and dissociation rate constants for the binding of lysozyme to each of the molecules. The Fab and Fv fragments bind significantly more quickly compared to the VH single domain fragment. The recombinant Fab fragment associated with the highest rate followed by Fv, intact antibody and VH. The intact D1.3 monoclonal antibody had an association rate constant ($k_{ass}$) of $0.3 \times 10^6$ $M^{-1}s^{-1}$, whereas the Fab, Fv and VH fragments had association rate constants of $3.0 \times 10^6$, $1.0 \times 10^6$ and $0.1 \times 10^6$ $M^{-1}$ $s^{-1}$, respectively. The high association rate constant of the Fab fragment, versus native antibody, is most probably due to a higher diffusion rate constant of the fragments. The intact D1.3 antibody dissociated at a rate which was too slow to be measured by the biosensor. From previous measurements of dissociation rate constants ($k_{diss}$) of antibodies using this biosensor, we have been able to

**FIGURE 1** The binding of lysozyme to intact D1.3 monoclonal antibody (■) and its recombinant Fab (◇), Fv (◆), and VH (□) fragments, as illustrated by the relative response, as a function of time. The antibody/antibody fragment concentration was 11 nM.



measure constants as low as $1 \times 10^{-6}$ $s^{-1}$ (data not shown), indicating an apparent affinity constant ($K_a$) for the intact D1.3 molecule of at least around $10^{11}$ $M^{-1}$. The recombinant Fab and Fv fragments seem, from the slopes shown in Figure 1, to have approximately the same dissociation rate constants. However, the single domain antibody (VH) differs significantly from the other fragments in that the dissociation and association rate constants were in the range $10^{-3}$ $s^{-1}$ and $10^5$ $M^{-1}s^{-1}$, respectively. This is approximately one order of magnitude higher/lower as compared with fragments consisting of paired VH and VL domains. The dissociation could also be divided into two different phases, since it seemed that the dissociation was initiated at a somewhat higher rate ($k'_{diss1}$) which then slowed to a lower rate ($k'_{diss2}$). The reason for this behavior of the antigen-antibody interaction in the biosensor is presently not fully understood[4], although steric hindrance and/or modification of some antigenic epitopes during the immobilization procedure might be involved. Furthermore, it was recently shown that the valency of the antibody correlated to the extent of biphasic dissociation, i.e. IgM had a more pronounced biphasic dissociation as compared to IgG[4]. Consequently, using the monovalent antibody fragments in the present study, the rapid initial dissociation ($k'_{diss1}$) was very brief and of little importance for the kinetic evaluation. The affinity constants of the intact antibody and the recombinant fragments were calculated, using the $k_{ass}$ and $k_{diss}$ values (Table 1), the latter were derived without taking the minor biphasic dissociation into consideration.

The biosensor seemed to give higher affinity constant values compared to homogeneous assays, such as the fluorescence quenching technique[3], previously used to evaluate the anti-lysozyme antibody. This is due to the differences in kinetic parameters that are obtained by solid-phase or liquid-phase based systems, as previously reported by Nygren and coworkers[7,8]. Thus, both dissociation and association rate constants of antibodies bound to surface-immobilized antigen are normally significantly lower than constants determined in solution[7,8]. This is in agreement with the biosensor determined $k_{ass}/k_{diss}$ for VH which was much lower than previously reported using the liquid-phase fluorescence quenching technique[3]. Furthermore, discrepancies between test methods might also arise from molecular weight dependent diffusion limitations, especially in solid-phase based systems[8].

In summary, we have investigated the relation of domain structure of recombinant antibody fragments and kinetic binding constants. The rapid evaluation of these kinetic parameters should prove valuable in the design of recombinant antibody fragments for therapeutic, imaging or other uses.

## EXPERIMENTAL PROTOCOL

**Antibody and antibody fragments.** The intact D1.3 (IgG1, κ) monoclonal antibody was derived from a mouse hybridoma, previously described by Mariuzza and coworkers[9], and the different recombinant antibody fragments were expressed in *E. coli*, as recently described[3,10]. The protein concentration was determined by amino acid analysis (Biomedical Center, Uppsala, Sweden).

**Analytical procedure.** The molecular interactions between antibody and antigen were measured using the BIAcore™ system (Pharmacia Biosensor AB, Uppsala, Sweden), as described by the manufacturer. Briefly, the BIAcore™ system is built of a processing and a computer unit. The processing unit consists of an integrated fluidic cartridge, a sensorchip and a detector. The interaction between biomolecules takes place on the sensor chip, which consists of an optically flat glass slide covered with a thin layer of gold on one side. A hydrophilic dextran matrix is covalently bound to the gold film. Antibody or antigen is coupled to the matrix and allowed to react with its counterpart. The amount of protein bound to the gold surface is analyzed by measuring the surface plasmon resonance (SPR). A change of 0.1 degree corresponds to 1 ng bound protein/$mm^2$ or 1000 RU (RU = resonance unit, which is an instrument specific unit). The SPR-signals are illustrated as a sensorgram, where resonance units (RU) are plotted against time[4,5]. Immobilization of ligand in the BIAcore™ system was performed in 10 mM HEPES complete buffer, pH 7.4 (containing 3.4 mM Titriplex III, 0.15 M sodium chloride, and 0.05% surfactant P20 (Pharmacia Biosensor AB)) while maintaining a continuous flow rate of 5 μl/min. About 1000 RU of hen egg lysozyme (Sigma Chemical Co., St. Louis, MO.) was immobilized on the carboxymethylated dextran surface of a CM5 sensorchip, as described by the manufacturer (Pharmacia Biosensor AB), using 25 μl of a lysozyme solution (45 μg/ml in 10 mM sodium acetate buffer, pH 4.5). The specific interactions were then carried out at a continuous flow rate of HEPES complete buffer, pH 7.4, of 3 μl/min. The D1.3 antibody and the recombinant fragments were diluted in HEPES complete buffer, pH 7.4, at the concentrations 2.75, 5.5, 11.0, and 22.0 nM, run in duplicate, and 33 μl of each sample was injected, giving a total contact time between antibody/recombinant fragment and antigen of 11 minutes. The dissociation between antibody/recombinant fragments and antigen was followed for 30 minutes and the dissociation rate constants were calculated as recently described[4]. A mouse monoclonal antibody with the same isotype was used as a control antibody. The surface was finally regenerated with 9 μl, 100 mM HCl, at a flow rate of 3 μl/min. All solutions used in the BIAcore™ were prepared from ultrapure water and filtered through a 0.2 μm filter. The kinetic constants were determined as described[4,5], using computer software supplied by the manufacturer.

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**References**

1. Foote, J. and Milstein, C. 1991. Kinetic maturation of an immune response. Nature **352**:530–532.
2. Winter, G. and Milstein, C. 1991. Man made antibodies. Nature **349**:293–299.
3. Ward, E. S., Güssow, D., Griffith, A. D., Jones, P. T. and Winter, G. 1989. Binding activities of a repertoire of single immunoglobulin variable domains secreted from *Escherichia coli*. Nature **341**:544–546.
4. Malmborg, A-C., Michaëlsson, A., Ohlin, M., Jansson, B. and Borrebaeck, C. A. K. 1992. Real time analysis of antibody-antigen reaction kinetics. Scand. J. Immunol. *In press.*
5. Karlsson, R., Michaëlsson, A. and Mattsson, L. 1991. Kinetic analysis of monoclonal antibody-antigen interactions with a new biosensor based analytical system. J. Immunol. Methods **145**:229–240.
6. Fischmann, T. O., Bentley, G. A., Bhat, T. N., Boulot, G., Mariuzza, R. A., Phillips, S. E. V., Tello, D. and Poljak, R. J. 1991. Crystallographic refinement of the three-dimensional structure of the FabD1.3-lysozyme complex at 2.5-Å resolution. J. Biol. Chem. **266**:12915–12920.
7. Nygren, H., Czerkinsky, C. and Stenberg, M. 1985. Dissociation of antibodies to surface-immobilized antigen. J. Immunol. Methods **85**:87–95.
8. Nygren, H., Werthen, M. and Stenberg, M. 1987. Kinetics of antibody binding to solid-phase-immobilized antigen. Effect of diffusion rate limitation and steric interaction. J. Immunol. Methods **101**:63–71.
9. Mariuzza, R. A., Jankovic, D. L. J., Boulot, G., Amit, A. G., Saludjian, P., Le Guern, A., Mazie, J. C. and Poljak, R. J. 1983. Preliminary crystallographic study of the complex between the Fab fragment of a monoclonal anti-lysozyme antibody and its antigen. J. Mol. Biol. **170**:1055–1058.
10. Ward, E. S. 1991. Expression and purification of antibody fragments using *Escherichia coli* as a host, p. 121–137. *In:* Antibody Engineering. A Practical Guide. Borrebaeck, C. A. K. (Ed.). W. H. Freeman and Company, New York.

**TABLE 1** BIA core™ analysis of D1.3 monoclonal anti-lysozyme antibody and corresponding recombinant antibody fragments.

| Antibody | $k_{ass}$ ($M^{-1}s^{-1}$) | $k'_{diss1}$ ($s^{-1}$) | $k'_{diss2}$ ($s^{-1}$) | $k_{diss}$ ($s^{-1}$) | $K_a$ ($M^{-1}$) |
|---|---|---|---|---|---|
| | | | | $<10^{-6}$ | |
| | | | | $1.2\times10^{-4}$ | |
| | | | | $1.7\times10^{-4}$ | |
| | | | | $10\times10^{-4}$ | |

Calculations of the association/dissociation rate constants and the affinity constants are based on duplicate and triplicate runs for intact D1.3 antibody and for each fragment. The affinity constants ($K_a$) were calculated using $k_{ass}/k_{diss}$, without considering the biphasic dissociation ($k'_{diss1}$ and $k'_{diss2}$)[4].