Probing Changes in Antibody Binding-Site Flexibility with Affinity Maturation using Fluorescence Spectroscopy

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Abstract

The vertebrate immune system defends the body against a host of pathogens by various humoral and cell-mediated responses. The binding of antibody to antigen stimulates the removal of the pathogen by phagocytic white blood cells. During the course of humoral response, the average affinity of the antibodies produced increases by the process of affinity maturation. It is hypothesized that affinity maturation reduces binding site flexibility, making hapten-binding more thermodynamically favorable. Steady-state and time-resolved fluorescence spectroscopy were used to investigate the evolution of binding affinity of antibodies obtained at different stages of affinity maturation. Frequency-modulated fluorescence lifetime studies of Fab fragments derived from four monoclonal antibodies elicited against β-diketone hapten indicate that antibody binding sites rigidify with affinity maturation, as evidenced by a narrowing in the half-width lifetime distribution. Though the binding sites do appear to rigidify with affinity maturation, they do not become perfectly rigid. Fluorescence anisotropy measurements will be performed on each Fab system to confirm the results.

Introduction

It has been proposed that affinity maturation leads to the production of antibodies with structurally rigid binding sites that are preconformed to the antigen. Conformationally rigid binding sites have higher binding affinities because entropic cost is reduced compared to conformationally flexible binding sites. The purpose of the current work was to examine this hypothesis using fluorescence spectroscopy. Here we present the results from the study of a panel of antibodies raised against β-diketone hapten I (Figure 1) by the Richard Goldsby laboratory at Amherst College and obtained at different stages of the immune response. The β-diketone hapten I was synthesized and purified in the laboratory of David Hansen at Amherst College.

The panel includes a primary antibody A3.1.1, secondary antibody 2C26.1, tertiary antibody A3.2.2 and commercially available mature antibody 38C2. Pauyo and Mohan in the O'Hara group previously used fluorescence to probe binding site flexibility using the intact monoclonal antibodies in this panel. Table 1 shows the binding constants obtained for each antibody. The Kds indicate that the secondary antibodies bound the hapten better than primary antibody, which bound better than the tertiary antibody.

Antibodies were purified from Ascites fluid using Pierce ImmunoPure® (G) IgG Purification Kit according to the manufacturer's protocol. Fab fragments were prepared using Pierce ImmunoPure® Fab Preparation Kit according to the manufacturer's protocol. Pure antibodies and Fab fragments were suspended in phosphate buffered saline at pH 7.2. Steady state excitation and emission spectra of all systems were taken using ISS K2 Multifrequency Phase Fluorometer and the Perkin-Elmer LS50 B Luminescence Spectrometer. ISS K2 Multifrequency Phase Fluorometer was used to perform the frequency-modulation lifetime measurements. ISS Vinci software was used to fit data to an appropriate lifetime model.

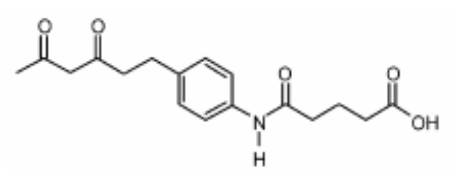

Figure 1 β-diketone Hapten I

Results and Discussion

The fluorescence of the amino acid tryptophan was essential for this study (Figure 2). The quenching of fluorescence intensity was used to determine whether binding was occurring. Quenching that exceeded that of the negative control case was considered to be due to binding. Primary antibody A3.1.1, secondary antibody 2C26.1, tertiary antibody A3.2.2 and the positive control mature antibody 38C2 all bind β-diketone Hapten I because fluorescence intensity is significantly quenched (Table 2). Figures 3 and 4 show differences in fluorescence quenching when hapten was added to a solution of intact A3.3.3 versus A3.2.2 Fab.

Fluorescence lifetimes typically decreased upon addition of hapten (Figure 5, Table 2). A significant decrease was considered to be due to binding. Fwl for the intact antibodies and the Fabs changed when hapten was added (Figure 6). However, changes in Fab Fwl were more informative about the behavior of antibody binding site as framework tryptophan fluorescence was eliminated. The Fwl changed more when hapten was added to immature antibodies than when hapten was added to the mature antibodies. This indicates that the immature antibodies were undergoing more conformational change in order to bind, and are therefore more flexible than the mature antibodies.

Figure 2. Cartoon image of the Fab fragment for 38C2 derived from pdb coordinate 1AXT. The heavy chain is colored pink and the light chain white. The binding site for the hapten is at the top of the image. The nine tryptophan in the Fab are shown spacefilled and colored CPK. Five are on the heavy chain and four on the light chain. Five Trp are in the variable domain and are close to the binding site and four Trp are in the constant domain and are further from the binding site.

Table 1 Dissociation constants as determined for hapten

Antibody	K_d hapten Trp quenching
38C2 mature	9.03 ± 3.16 nM[a]
3.22 tertiary	25.4 ± 6.8 μM
2C26.1 secondary	0.023 ± 0.006 μM
A3.1.1 primary	5.46 ± 2.57 μM
Control IgG[b]	7.26 ± 5.25 μM

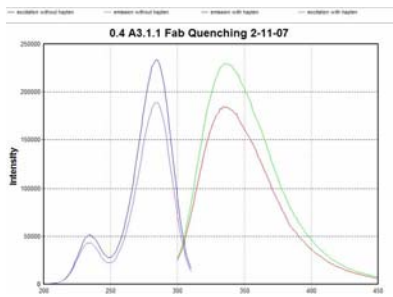

Figure 3 Excitation and Emission Scans of a solution of A3.1.1 Fab Fragments before and after Hapten I was bound, showing quenching.

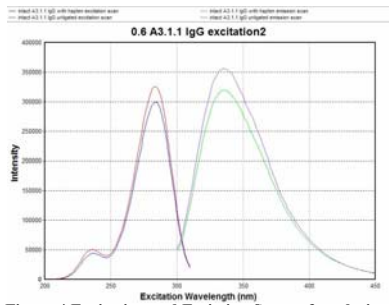

Figure 4 Excitation and Emission Scans of a solution of intact A3.1.1 before and after Hapten I was bound, showing quenching.

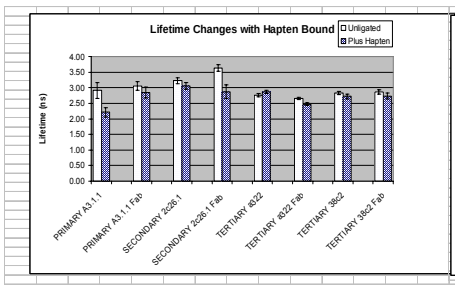

Figure 5 Lifetme changes with hapten bound

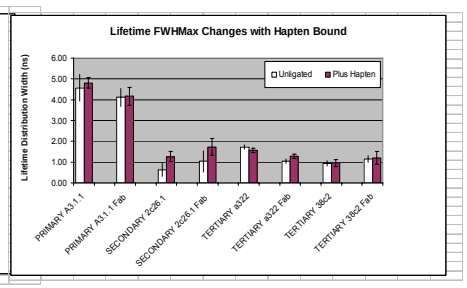

Figure 6 Changes in Fwhmax with hapten bound.

Table 2. Raw Lifetime and Intensity quenching data

System	tau(ns)	tau(ns) "+ hapten"	% quench	fw_L(ns) free	fw_L(ns) "+ hapten"
Primary A3.1.1	2.910(0.250)*	2.210(0.150)	0.759	4.577(0.650)	4.810(0.250)
Primary A3.1.1 Fab	3.057(0.140)	2.847(0.170)	0.931	4.110(0.430)	4.163(0.420)
Secondary 2c26.1	3.223(0.090)	3.06(0.100)	0.949	0.622(0.320)	1.265(0.260)
Secondary 2c26.1 Fab	3.637(0.100)	2.870(0.220)	0.789	1.040(0.510)	1.730(0.410)
Tertiary a322	2.755(0.050)	2.870(0.050)	1.042	1.730(0.100)	1.570(0.100)
Tertiary a322 Fab	2.655(0.040)	2.470(0.050)	0.930	1.051(0.100)	1.275(0.100)
Tertiary 38c2	2.835(0.050)	2.72(0.08)	0.959	0.941(0.120)	0.953(0.160)
Tertiary 38c2 Fab	2.857(0.070)	2.733(0.100)	0.957	1.143(0.160)	1.204(0.320)
nonspecific IgG	3.153(0.143)	3.300(0.184)	1.047	1.867(0.278)	1.660(0.346)
Nonspecific Fab	3.043(0.147)	2.965(0.160)	0.974	1.840(0.274)	1.890(0.287)
Free Tryptophan	2.590(0.022)	2.625(0.030)	1.014	d	d

*Uncertainties in parentheses

Time-resolved fluorescence anisotropy will be conducted on these samples in order to obtain more information about how binding site flexibility changes with affinity maturation. The Fab samples should be particularly useful for this purpose, since they contain the binding site without the remainder of the molecule which can rotate about the hinge region.

Conclusion

Fluorescence spectroscopy appears to be an appropriate tool for probing antibody binding-site flexibility. To probe binding-site flexibility with fluorescence spectroscopy, Fab fragments are more useful than intact antibodies. Antibody binding-site flexibility appears to decrease over the course of affinity maturation, though a perfectly rigid antibody does not form.

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