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PROBING A CATALYTIC ANTIBODY ACTIVE SITE USING SPIN-LABELLED HAPTENS

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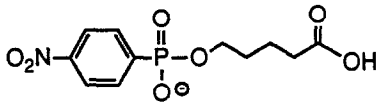
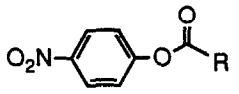
Abstract: Spin-labelled hapten analogues have been used to probe the structure of the active site of the nitrophenylphosphonate specific catalytic antibody 48G7. The results of the ESR experiments suggest that substrates are held rigidly in a cleft with the labile ester or carbonate bond exposed to solvent.

The spin labelling technique is a well established tool for probing the structure of proteins, particularly the topography of enzyme active sites.[1] Nitroxide spin labels have also been used to probe the combining site structure of antibodies, including the dinitrophenyl specific antibody MOPC315.[2-4] By incorporating nitroxide spin labels onto haptens, these ligands can be used as a molecular "dip stick" to probe the depth and width of the combining site. We now report the use of this technique to probe the active site of the hydrolytic catalytic antibody 48G7.[5] Antibody 48G7 was generated[5] against the nitrophenylphosphonate transition state analogue **1**[6] and catalyzes the hydrolysis of a variety of nitrophenylcarbonates and esters with rate accelerations of 10^3 to 10^4 over the uncatalyzed reaction.[5]



1



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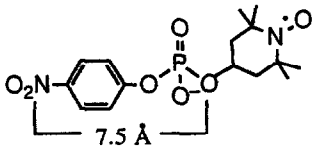

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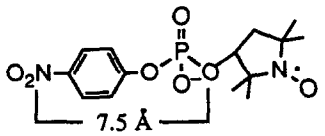

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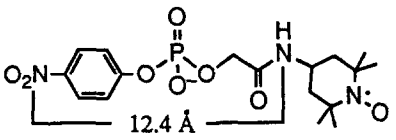

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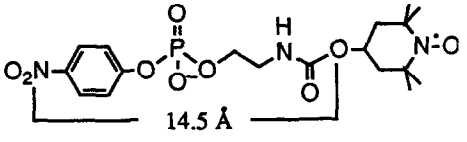
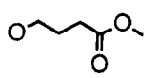
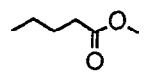


6

Methylnitrophenylphosphonate inhibits the antibody-catalyzed reaction with a K_I of 560 nM. That the antibody-catalyzed reaction is first order in hydroxide between pH 6.4 and 9 and that an acyl antibody intermediate could not be detected[5] suggests that hydroxide ion from solution may be the nucleophilic species. The antibody may simply function as a "tailored solvent" for the transition state.

Table 1. Kinetic parameters of antibody 48G7 in 10mM Tris•HCl, 50mM NaCl, pH 8.2[7]

Substrate	k_{cat} (min^{-1})	K_M (μM)	Rate acceleration[a]
2a: R = OMe	7.4 ± 0.4	430 ± 70	7,300
2b: R = 	26.2 ± 2.4	360 ± 40	40,000
2c: R = Me	3.8 ± 0.4	1.02 ± 0.12	1,600
2d: R = 	22.0 ± 2.6	300 ± 25	15,700

[a] relative to the hydroxide ion catalyzed reaction

In order to begin to characterize the nature of the combining site, spin-labelled haptens of varying dimensions were synthesized by condensation of the corresponding alcohols with 4-nitrophenylphosphorodichloridate [compounds were purified by ion exchange chromatography on DEAE sephadex A-25, followed by chromatography on Dowex 50 (Na^+ form)].[5] The ESR spectra of spin labels **3** - **6**, recorded in the presence of the Fab fragments, are shown below (solid line) along with the theoretical spectra (dashed line) calculated with a Brownian rotational diffusion model.[8,9] The calculated rotational correlation times, τ_1 and τ_2, where τ_1 corresponds to rotation about a molecular axis parallel to the N-O bond of the nitroxide, and τ_2 corresponds to rotational reorientation about the two axes perpendicular to the N-O bond, are listed in Table 2 (note that the values of τ were determined from a model and hence are not absolute numbers; however, the relative values with respect to each spin-label are accurate and generalizations regarding the environment of the spin-labels are valid).

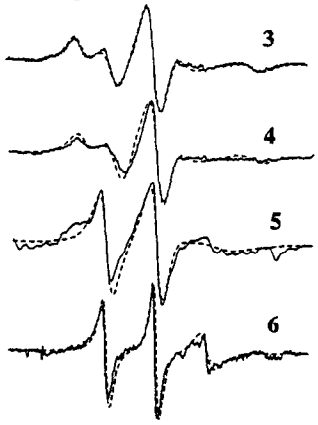


Table 2. Rotational correlation times, τ, for antibody-bound spin-labelled haptens **3-6**.[10]

Spin-Label	Length (Å)	τ_1	τ_2
3	8.75	15.0 ns	15.0 ns
4	8.75	7.0 ns	15.0 ns
5	12.4	1.6 ns	15.0 ns
6	14.5	0.9 ns	2.0 ns

Interestingly, the best fit between simulation and experiment for spin label **3** was obtained by assuming $\tau_2 = \tau_1 = 15$ ns, the same value of τ calculated for rotation of the entire Fab fragment from the Stokes-Einstein equation. These results reveal that the nitroxide ring of spin label **3** is rigidly held in the combining site of antibody 48G7, indicating that the lateral dimensions of the subsite of the antibody that accommodates the alkyl substituent of substrate **2** are approximately 6x9 Å based on the dimensions of the spin label.[11] Using a "dip stick" approach similar to that described by Hsia and Piette,[3] the depth of each combining site was also estimated using spin labels **4 - 6**. The spectra could not be simulated by assuming isotropic reorientation; however, reasonable fits were obtained for the case of anisotropic rotation with faster rotation about the axis parallel to the N-O bond. The simulations were generated by systematically varying the label's two rotational correlation times to minimize the least-squares difference between the calculated and experimental spectra. The calculated rotational correlation times obtained from the ESR spectra of the Fab-ligand complexes of spin labels **3-6**, in which the distance between the oxygens on the nitro group of the spin-labels and the heteroatom at the C-4 carbon of the nitroxide ring varies from 8.8 Å to 14.5 Å,[12] exhibited a sharp decrease in τ_1 between **4** and **5** and τ_2 between **5** and **6** (Table 2). From these observations the depth of each combining site can be approximated at 10-11 Å, extending about 3-4 Å beyond the phosphorus atom.

The results of the ESR experiments suggest that the substrate is held rigidly in a cleft in the variable region of the antibody, as is the case with many small haptens,[13] and that the carbonyl group is accessible to solvent. There appears to be a subsite for the alkyl substituent of substrate **2** that can accommodate a range of substituent sizes. The increasing values of k_{cat} with increasing size of the substrate alkyl substituent also suggests that the subsite interacts favorably with these substituents in the transition state. The mechanism of 48G7 may be very similar for that of the phosphorylcholine specific catalytic antibody, S107, in which OH^- from solvent attacks the antibody bound substrate and the resulting transition state is stabilized by a positively-charged amino acid side chain.[14] Chemical modification experiments to determine the presence of such a residue were ambiguous.[5]

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7. Protein molarity was determined by absorbance at 280 nm by using $E_{1\ cm}^{0.1\%} = 1.37$ and a molecular weight of 150,000 for IgG. Reactions were initiated by adding 10 μL of a stock substrate solution (THF) to the antibody in 0.5 mL of reaction buffer. Kinetic constants were determined by the method of initial rates. The value of $k_{uncat}[OH^-]$ was determined by extrapolation of the rate of the uncatalyzed reaction to zero buffer concentration.
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9. The dissociation constants of **3** and **4** were determined to be approximately 4.3 μM and 6.4 μM, respectively, from ESR titration experiments.
10. Spectra were recorded on an IBM ER200D-SRC spectrometer operating at a microwave frequency of 9.45 GHz. Spectra were recorded on 100 μL samples of 20 μM Fab solutions in 50 mM Tris•HCl buffer, pH 7.3 at a constant temperature of 23 °C.
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12. These distances were calculated by first performing energy minimizations on each spin-label using MacroModel[a] software which utilizes Allinger's MM2 program.[b] Each structure was minimized to a root-mean square of less than 2.0 kJ/Å-mol. Distances between both nitro oxygens and the heteroatom at C-4 of the nitroxide ring were determined and the average of these two measurements used. [a]Still, W.C.; Mohamadi, F.; Richards, N.G.J.; Guida, W.C.; Lipton, M.; Liskamp, R.; Chang, G.; Hendrickson, T.; De Gunst, F.; Hasel, W. *MacroModel V2.5*, Department of Chemistry, Columbia University, New York, New York, 10027. [b]Allinger, N.L. *J. Am. Chem. Soc.* **1977**, *99*, 8127.
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