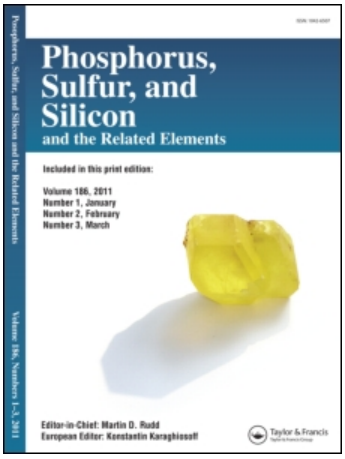

Phosphorus, Sulfur, and Silicon and the Related Elements
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713618290

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To cite this Article Fujii, Ikuo , Tanaka, Fujie , Miyashita, Hideaki , Tanimura, Ryuji and Kinoshita, Keiko(1996) 'Transition State Analysis within a Panel of Catalytic Antibodies Generated Against a Phosphonate Transition State Analog', Phosphorus, Sulfur, and Silicon and the Related Elements, 109: 1, 253 — 256
To link to this Article: DOI: 10.1080/10426509608545138
URL: http://dx.doi.org/10.1080/10426509608545138

Phosphorus, Sulfur, and Silicon, 1996, Vols. 109-110, pp. 253-256
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TRANSITION STATE ANALYSIS WITHIN A PANEL OF CATALYTIC ANTIBODIES GENERATED AGAINST A PHOSPHONATE TRANSITION STATE ANALOG

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Abstract The biochemical properties within a panel of six hydrolytic catalytic antibodies elicited against a phosphonate transition state analog were investigated. Although the individual values for *k*cat, *K*m, and *K*TSA (the affinity for the transition state analog) of the six antibodies differed substantially, the transition state analysis (*k*cat /*k*uncat versus *K*S/*K*TSA) displayed a linear relationship (slope = 0.99) with the four antibodies 6D9, 8D11, 4D5, and 9C10, which have homologous primary amino acid sequences, providing evidence that all of the differential binding energy of the transition state vs the ground state is available for the rate enhancement. This also suggested that these four antibodies catalyze the hydrolysis by variations of the same basic mechanism of transition state stabilization. The analysis of the substrate specificity suggested that the catalytic antibodies with highly homologous primary amino acid sequences possess homogeneous binding modes to the substrate or hapten.

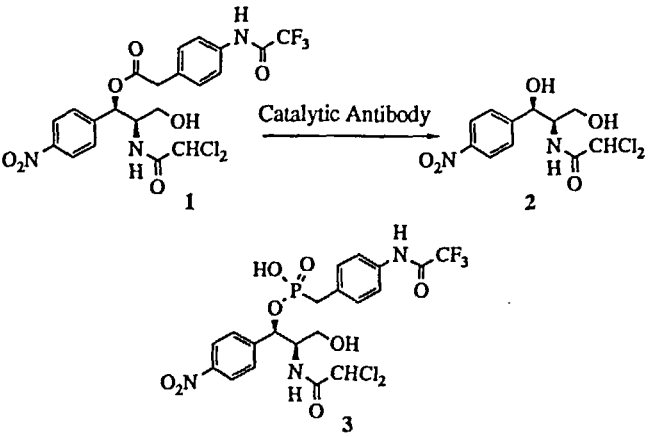
Key Words: Catalytic antibody, Transition state analog, Antibody diversity, Ester hydrolysis, Antigen-combining site.

INTRODUCTION

The diversity of the immune response, which can provide a panel of catalytic antibodies with varying degrees of catalytic activity and substrate specificity by immunization with a single hapten, raises the question concerning the extent to which a rationally designed hapten dictates the paratopes for catalytic function in the antigen-combining site. The study of the correlation between the antigen-combining-site structures and the chemical properties within a panel of catalytic antibodies elicited against a single hapten can potentially provide a more global understanding of the molecular mechanisms by which catalytic antibodies are generated in immune responses.

Previously, we have reported prodrug activation via catalytic antibodies that catalyze the hydrolysis of the nonbioactive chloramphenicol monoester derivative **1** to generate chloramphenicol **2**.[1] Immunization with a KLH conjugate of the transition state analog **3**, designed on the basis of the transition state stabilization concept, yielded 12 immunoglobulin G (Ig G) proteins binding to the hapten **3**, six of which were found

to catalyze the hydrolysis with varying degrees of activity. Despite the antibody diversity, the catalytic antibodies, 6D9, 8D11, 4B5, 9C10, and 3G6, share significant structural identity to one another and have 89-95% and 74-84% sequence homology in the complete VL and VH regions, respectively.[2] An exception is antibody 7C8, which was found to be catalytic, but its structure was different from the other five catalytic antibodies. In this work, we report the detailed biochemical properties of the six catalytic antibodies, to enhance our understanding of the active site structure and function relationship assignments.



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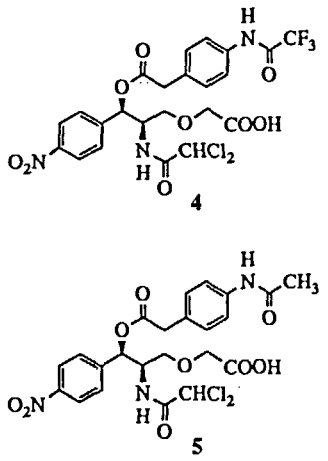
CATALYTIC AND BINDING ASSAYS

To survey the catalytic activity within the six catalytic antibodies, the kinetic parameters of the antibody-catalyzed hydrolysis with substrate **4** were determined at 25 °C in 10% DMSO/50 mM Tris (pH 8.0). The first-order rate constants per antigen-combining site (*k*cat) and Michaelis constants (*K*m) in the six catalytic antibodies were in the range of 0.008-0.145 min^{-1} and 2.5-60 μM, respectively.

According to transition state theory, under ideal conditions, one can predict the rate enhancement of an antibody-catalyzed reaction from the ratio of the affinity for the substrate relative to the affinity for the transition state. Since we have generated monoclonal antibodies against the putative transition state analog, with the expectation that the antibodies may be catalytic by virtue of the theoretical relationship between the affinity for the transition state and the catalytic efficiency, the ratio of the affinity (*K*S) for the substrate **4** relative to the affinity (*K*TSA) for the transition state analog **3** within the six catalytic antibodies was determined by competitive inhibition enzyme immunoassay (CIEIA) and was analyzed on the basis of transition state theory. Figure 1 shows plots of *k*cat/*k*uncat versus *K*S/*K*TSA for the six catalytic antibodies. Although

the catalytic antibodies possess varying values of *K*m, *k*cat, *K*s, and *K*TSA, the transition state analysis displays a linear relationship among antibodies 6D9, 8D11, 4B5, and 9C10, with high homologous amino acid sequences. The slope of the straight line is 0.99. This suggests that the entire differential binding energy of the four catalytic antibodies to the transition state vs the ground state might be available for rate enhancement. On the other hand, the plots for antibodies 7C8 and 3G6 deviate from the linear relationship, suggesting that factors other than transition state stabilization, such as a functioning acid or base or nucleophilic catalyst, are involved in the catalysis.



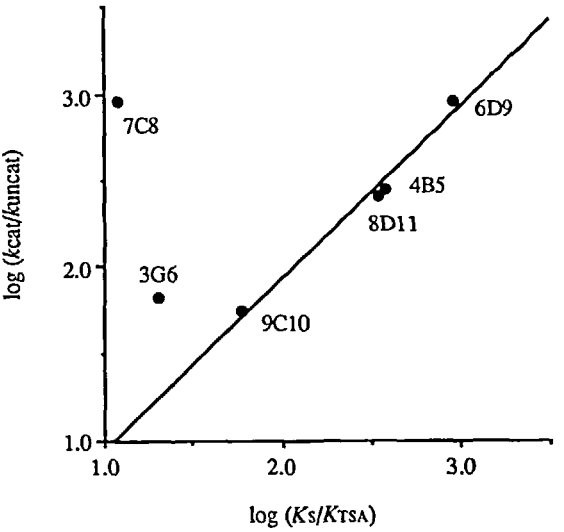


Figure 1. Plot of log (*K*s/*K*TSA) versus log (*k*cat/*k*uncat) for catalytic antibodies generated against phosphonate transition state analog 3. A linear relationship was obtained for antibodies 6D9, 4B5, 8D11, and 9C10, suggesting that the antigen-combining sites act as catalysts by stabilizing the transition state; slope = 0.99, $R^2 = 0.98$.

SUBSTRATE SPECIFICITIES

The substrate specificity of the antibodies was examined using substrate **4** and its derivative **5**, in which the trifluoroacetyl group of **4** was converted to an acetyl group. If the combining-site structures and the binding modes to the hapten and the substrates of these catalytic antibodies are related each other, the antibodies should display a homologous substrate specificity.

The relative velocities for substrates **4** and **5** in the antibody-catalyzed hydrolysis with the six catalytic antibodies were compared under the conditions of 5 μM of antibody and 200 μM of substrate in 10 % DMSO/50 mM Tris (pH 8.0). Antibodies 6D9, 8D11, 4B5, 9C10, and 3G6, with highly homologous amino acid sequences,

displayed reduced catalytic activities for **5**, with rates of 3.5-14 times lower than those for **4**. On the other hand, antibody 7C8, with an amino acid sequence different from those of the other five antibodies, was found to catalyze the hydrolysis of substrate **4** and **5** with the same rate enhancement.

CHEMICAL MODIFICATIONS

Chemical modification of the antibodies showed that the hydrolytic activity for substrate **4** was reduced by tyrosine-, and especially histidine-specific reagents. When the six catalytic antibodies were treated with diethyl pyrocarbonate (DEPC) to modify any histidine residues, antibodies 6D9, 8D11, 4B5, and 9C10 completely lost the hydrolytic activity. Antibody 3G6 had a 40% reduction in activity under the same conditions. On the other hand, antibody 7C8 resisted chemical modification with DEPC under the same conditions and retained the same activity as that before the modification. Nitration of tyrosine residues by tetranitromethane reduced the activity of the antibodies by 60-75%, with the exception of antibody 3G6. In the case of antibody 3G6, treatment with tetranitromethane completely abolished the hydrolytic activity.

His (L27d) in the CDR 1 of the light chain is conserved in the catalytic antibodies 6D9, 8D11, 4B5, 9C10, while antibody 3G6 has a tyrosine residue at the corresponding position. We suspected, therefore, that the His at position L27d is a catalytic amino acid residue participating in transition state stabilization in the antibody-catalyzed reactions.

CONCLUSION

It is noteworthy that the majority of these catalytic antibodies, generated against a single transition state analog, display high homology in the biochemical and structural properties and catalyze the reaction with the same mechanism expected from designing the transition state analog. These findings emphasize the critical importance of hapten affinity to transition state stabilization and of chemically designing haptens that closely resemble the true transition state for the generation of catalytic antibodies.[3]

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