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Investigation of an Antibody-Ligase. Evidence for Strain-Induced Catalysis

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Abstract—Catalytic antibody 16G3, a peptide ligase with extended substrate scope has been characterized mechanistically exploiting a set of systematically designed perspective substrates **6**–**9**, two of which, thioesters **8** and **9** act instead as inhibitors. Taken together the structure/activity relationships suggest a catalytic mechanism dependent on induction of strain, programmed via specific structural deviations between the hapten and the substrates. General mechanistic implications for de novo induced catalysis are presented.

Several years ago we initiated a program on catalytic antibodies directed towards de novo induction of peptide-ligase activity from an immunoglobulin scaffold. Subsequently, we disclosed the preparation of phosphonate hapten **1** (Fig. 1), designed as a transition-state analogue/high-energy intermediate for peptide bond formation.[1]

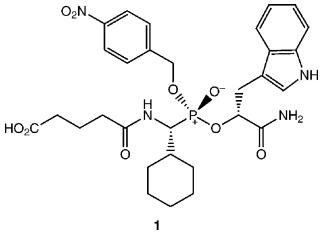
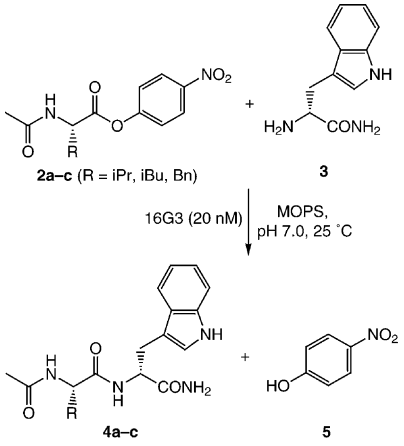


Figure 1. Phosphonate hapten designed for induction of peptide-ligase activity.

Immunization with phosphonate **1** resulted in the identification of monoclonal antibody 16G3 (MAb16G3), which indeed promoted peptide coupling between amine **3** and *several p*-nitrophenyl (*p*-NP) esters of amino acids **2a**–**c** (Scheme 1); rate enhancements of up to 2×10^4 over the uncatalyzed reaction were observed.[2] Importantly,



Scheme 1. Dipeptide formation, catalyzed by MAb16G3.

the reactivity profile of the catalytic antibody correlated well with the hapten architecture. Thus, the observed initial rates increased as the size of the ester α-sidechain matched more closely that of the cyclohexyl group of the hapten, and the hapten itself acted as a potent inhibitor of MAb16G3.[3] Furthermore, the catalysis was found to be selective for an α-amino group over a β-amino group,[4] which is consistent with the hapten structure and is of practical importance for potential utility of the abzyme-ligase in effecting coupling between other unprotected substrates. Finally, no catalysis was detected for esters other than *p*-NP.

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We, therefore, associated both the scope and the low level of product inhibition[2] of the antibody-ligase with structural deviations in the substrates with respect to the hapten. Specifically, the antibody appeared to rely on both *residual* recognition of the *p*-nitrophenyl leaving group by the *p*-nitrobenzyl-sculpted pocket,[5] and *generic* recognition of the hydrophobic residues (i.e., Val, Leu, and Phe) by a cyclohexyl-generated cavity, thereby ensuring a critical balance between substrate recognition, transition state accommodation, and product desorption. Pleasingly, the synthetic potential of MAb16G3 was subsequently extended to include tri- and tetrapeptide formation[4] and the *cyclization* of appropriately functionalized hexapeptide substrates.[6,7]

Having achieved the first antibody-ligase for peptide bond formation,[8] we next sought to decipher the mechanism responsible for the catalytic activity. Two complementary approaches were explored: (1) molecular modeling using the antibody structural database as a guide, and (2) an empirical analysis of the 'structure–reactivity' relationships.

The modeling effort by Roberts at the Scripps Research Institute disclosed earlier,[6] relied on structural conservation of the antibody variable domain,[9] to generate a MAb16G3 recognition model, illustrated schematically in Figure 2. The homology model suggested that

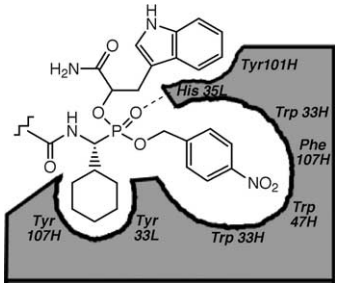

Figure 2. Schematic illustration of the hapten–MAb16G3 complex. The key contact residues, within 5 Å of the hapten are listed.

the *p*-nitroaryl group extends into a well-defined, deep pocket, featuring three tryptophan and one phenylalanine residues. The deeply embedded leaving group places the P=O bond of the phosphonate well inside the complementary determining regions (CDR), permitting H-bond formation with the imidazole ring of His35L. Closer to the surface, a shallow, auxiliary cleft, produced primarily by two tyrosines (Tyr107H and Tyr33L), is well-suited to accommodate the bulky hydrophobic cyclohexyl group. The indole ring, on the other hand, was found to make only superficial contact with the CDR loops through Tyr101H.

The static snapshot, produced via the modeling study, provided important, yet only limited insight into the mechanism of the abzyme-catalyzed aminolysis. We sought, therefore, to generate kinetic and molecular recognition probes to pinpoint the driving force, for the catalytic activity. To address these issues we chose to 'graft' individual structural elements of the hapten onto perspective acyl donor substrates **6–9**, modified as monoglutaramides for enhanced solubility (Fig. 3). The L-Phe derivative **6**, previously demonstrated to be a substrate of the abzyme,[5] was included to be a convenient control of antibody catalysis. As a probe for side chain influence on substrate recognition, the L-cyclohexylglycine (L-Chg) derivative **7** was designed. An attempt was also made to analyze the influence of the leaving group structure on both catalysis and binding. Towards this end, *p*-nitrobenzyl (*p*-NBn) thioesters emerged as intriguing mechanistic probes, in view of both their close structural resemblance to the *p*-nitrobenzyloxy group and their comparable activation level to that of *p*-NP esters [p*K*a(*p*-nitrophenol) = 7.2,[10] p*K*a(*p*-nitrobenzyl mercaptan) = 8.50[11]]. Thus, thioesters **8** and **9** were designed to expand our systematic investigation of substrate binding and activation.

Active ester **7** was prepared from Boc-L-Chg-OH in three steps, similar to the protocol reported for **6**.[4] Two *p*-NP esters **6** and **7** were subjected to a steady-state kinetic analysis, measuring initial rates via integration of HPLC traces of reaction aliquots.[12] Surprisingly, *both* the affinity and the turnover rate of **7** were noticeably compromised by the benzyl-to-cyclohexyl transformation (Table 1), despite the presence of the cyclohexyl substituent, also incorporated in the hapten. This unexpected result is discussed later.

Next, thioesters **8** and **9** were prepared in a similar fashion by condensation of the respective Boc-amino acids with *p*-nitrobenzyl mercaptan,[13] followed by Boc removal and monoglutaramide installation. Remarkably, the thioesters were not recognized as substrates of the MAb16G3, as the rates of their acyl transfer were

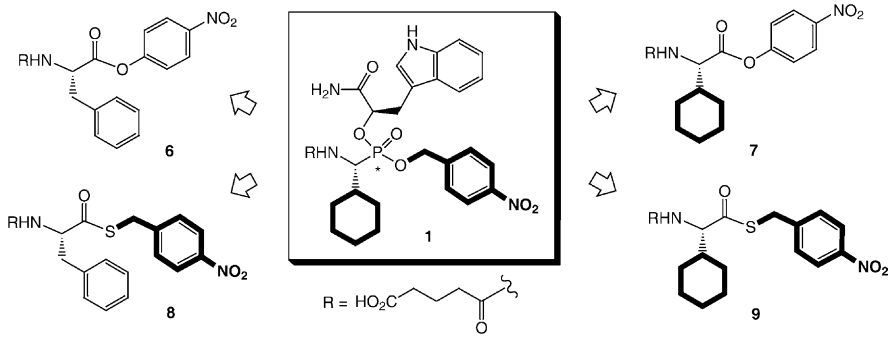

Figure 3. Perspective substrates **6–9** for the systematic analysis of 'structure–reactivity' relationships with MAb16G3. The structural elements 'grafted' directly from the hapten **1** are highlighted.

Table 1. Kinetic characterization of substrates **6** and **7**[a]

Kinetic parameter[b]	Substrate **6**	Substrate **7**
K_M(D), μM	100	300
k_{cat}, min^{-1}	0.6	0.1
V_{max}, μM/min	6	1
k_{uncat}, $M^{-1}\cdot min^{-1}$	0.04	0.05
Substrate specificity: k_{cat}/K_m(D), $M^{-1}\cdot min^{-1}$	6×10^3	3×10^2
Rate acceleration:[c] $k_{cat}/K_m(A)/k_{uncat}$	2×10^4	2×10^3
Effective molarity, M	15	2

[a]The kinetic study was carried out at 25 °C in 5:95 DMSO/0.1 M MOPS (pH 7.0) with the following component concentrations: 1 mM esters (**6** and **7**), 2 mM **3**, and 20 μM MAb16G3.
[b]'D' and 'A' refer to acyl *d*onor and *a*cceptor, respectively.
[c]For weaker substrate **3**: $K_M = 900$ μM (ref [4]).

identical to those of the background reactions. Notwithstanding the lack of catalysis, a recognition profile could be inferred from the inhibitory activities, observed when the substrates **3** and **6** were subjected to MAb16G3 in the presence of the thioesters. For example, at an equimolar (1 mM) concentration of the individual thioesters and substrate **6**, only 6% MAb16G3 catalytic activity could be detected in the presence of **9**, whereas **8** resulted in rate reduction to 18%. Since other related molecules (e.g., *p*-nitrophenyl anilides, *o*-nitrophenyl esters[2]) did not exhibit detectable inhibition, we concluded that both *p*-NP esters and *p*-NBn thioesters compete for the same active site, and that their SARs can be rationalized via a common binding model.[14]

Significantly, the more potent inhibitor **9**, the best substrate **6** and hapten **1** share a common structural feature, depicted schematically in Figure 4. Specifically, the distance comprised of five covalent bonds between the two non-flexible hydrophobic groups [i.e., *p*-nitrophenyl and side chain ring (cyclohexyl or phenyl)] is conserved in these molecules. Thus, the reactivity and inhibition profiles can be interpreted collectively, albeit simplistically, in terms of a rigid distance requirement between two well-defined hydrophobic subsites elicited by the hapten. The 5-bond requirement is consistent with the distance of 7–9 Å between the approximate centers of the two hydrophobic subsites observed in the Roberts homology model. Note that the 'two-subsite recognition' cartoon provides only comparative profiling of the substrate and inhibitor families, while disregarding a potential misalignment of the hydrogen-bonding network and/or other catalytically relevant recognition elements that are involved in accommodating the substrates over the inhibitors. This issue will be addressed below.

Important mechanistic implications can be ascertained from the fact that the thioesters were not recognized as substrates by MAb16G3. We offer the following scenario to rationalize the contrasting behavior of the active esters, possessing similar, on the basis of the p*K*a's, activation levels. When the abzyme pocket, generated immunogenically in response to the *p*-nitrobenzyl group, is challenged with a *p*-nitrophenyl ester, retention of recognition would require some realignment of the substrate, antibody, or both to compensate for this change. Stabilization of the transition state by MAb16G3 could be, therefore, directly programmed in the destabilization of the ground state of the complex. Without this structural deviation, on the other hand, catalytic aminolysis is energetically forbidding, as evidenced by the inhibitory *p*-nitrobenzyl thioesters. The latter are expected to bind the antibody in a manner similar to that of a hapten, in an unstrained and, therefore, *unproductive* conformation.

The structural origin of catalytic activity can be potentially deduced from the fact that both *tetrahedral* *p*-nitrobenzyl phosphonate and *trigonal* *p*-nitrobenzyl thioesters inhibit aminolysis of *p*-nitrophenyl esters, implying that the common structural features are responsible for the active site recognition. This in turn suggests that the principle driving force for MAb16G3 catalysis is different from the well-understood stabilization of a tetrahedral transition-state $\mathbf{A}^{\ddagger}$[15] (Fig. 5), where distortion of the trigonal substrate geometry and electrostatic complementarity are paramount. Instead, we propose that the catalytic activity was programmed by the methylene unit of the *p*-nitrobenzyl group, a common structural feature in both types of inhibitors. As a topological 'spacer' the methylene group fits the role of a strain inducer, encoding, for example, for elongation of the scissile bond in an early, trigonal-like transition state $\mathbf{B}^{\ddagger}$,[16] more likely to operate with *p*-nitrophenyl esters.[17] Alternatively, the flexible methylene group may dissipate leaving group distortion, required for catalysis, rendering the *p*-nitrobenzyl thioesters inactive. Both scenarios are consistent with the argument presented earlier by Tawfik et al. to rationalize catalytic activity of *p*-nitrophenyl hydrolytic antibodies, elicited by a *p*-nitrobenzyl phosphonate hapten.[5] Our observations are also consistent with the

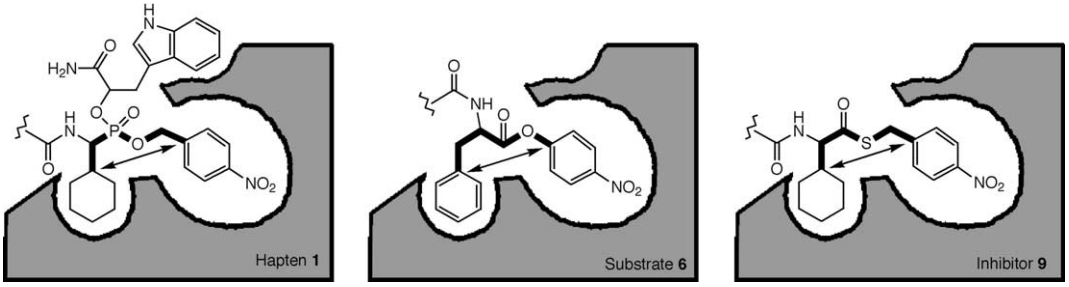

Figure 4. Schematic illustration of the recognition of the hapten, substrates and inhibitor by MAb16G3. The model accounts for better accommodation of both substrate **6** over substrate **7** (not shown) and inhibitor **9** over inhibitor **8** (not shown). The preferred distance of five covalent bonds between the two hydrophobic groups, found also in the hapten **1**, is highlighted.

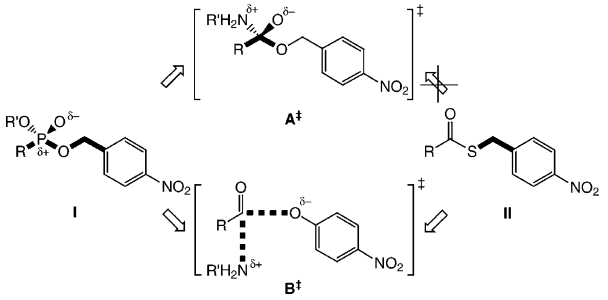

Figure 5. Transition state analogue **I**, inhibitor **II**, and the respective transition states: tetrahedral-like, late $\mathbf{A}^{\ddagger\ddagger}$ and trigonal-like, early $\mathbf{B}^{\ddagger}$. Both limiting transition states were fortuitously programmed in the design of the phosphonate hapten **I**, yet inhibition with **II** points to $\mathbf{B}^{\ddagger}$, as the rate-limiting transition state in the MAb16G3-catalyzed aminolysis of *p*-nitrophenyl esters.

classical 'strain induction' hypothesis,[18] invoked to rationalize catalytic efficiency of enzymes. In retrospect, it was the fortuitous choice of the hapten/substrate pair, which happened to program for the two limiting transition states at once, that enabled us to encounter a catalytic mode, which would not have been revealed otherwise, and which was not envisioned at the design stage. Combining the strain induction with site-specific recruitment of mechanistically relevant residues is being attempted with our next generation haptens and will be reported in due course.

Acknowledgements

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References and Notes

1. Smith, A. B. I.; Taylor, C. M.; Benkovic, S. J.; Hirschmann, R. *Tetrahedron Lett*. **1994**, *35*, 6853.
2. *p*-Nitrobenzyl esters were not viable substrates, with catalysis being either too ineffectual to detect reliably or absent altogether, suggesting that more activated acyl donors were required. Conveniently chromogenic *p*-nitrobenzyl esters were chosen because of their residual structural similarity to the hapten and, at the same time, higher reactivity than *p*-nitrobenzyl derivatives: Hirschmann, R.; Taylor, C. M.; Benkovic, P. A.; Taylor, S. D.; Yager, K. M.; Sprengler, P. A.; Smith, A. B., III; Benkovic, S. J. *Science* **1994**, *265*, 234.
3. The peptide bond formation was completely inhibited by addition of an amount of hapten **1** equal to twice the antibody concentration.
4. Smithrud, D. B.; Benkovic, P. A.; Benkovic, S. J.; Taylor, C. M.; Yager, K. M.; Witherington, J.; Phillips, B. W.; Sprelenger, P. A.; Smith, A. B., III; Hirschmann, R. *J. Am. Chem. Soc*. **1997**, *119*, 278.
5. Tawfik et al. subsequently reported *p*-nitrophenyl *hydrolytic* antibodies elicited by a *p*-nitrobenzyl phosphonate hapten: Tawfik, D. S.; Lindner, A. B.; Chap, R.; Eshhar, Z.; Green, B. S. *Eur. J. Biochem*. **1997**, *244*, 619.
6. Smithrud, D. B.; Benkovic, P. A.; Benkovic, S. J.; Roberts, V.; Liu, J.; Neagu, I.; Iwama, S.; Phillips, B.; Smith, A. B., III; Hirschmann, R. *Proc. Natl. Acad. Sci. U.S.A*. **2000**, *97*, 1953.
7. In contrast, enzyme-derived ligase, sublitigase, is incapable of cyclizing linear peptide precursors, containing less than 12 amino acids: Jackson, D. Y.; Burnier, J. P.; Wells, J. A. *J. Am. Chem. Soc*. **1995**, *117*, 819.
8. Schultz et al. subsequently disclosed generation of an abzyme-ligase: Jacobsen, J. R.; Schultz, P. S. *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 5888.
9. Roberts, V. A.; Stewart, J.; Benkovic, S. J.; Getzoff, E. D. *J. Mol. Biol*. **1994**, *235*, 1098.
10. Kortum, G.; Vogel, W.; Andrussow, K. *Dissociation Constants of Organic Acids in Aqueous Solution*; Plenum Press: New York, 1961.
11. Brembilla, A.; Roizard, D.; Schoenleber, J.; Lochon, P. *Can. J. Chem*. **1984**, *62*, 2330.
12. Both the desired and diasteriomeric dipeptide standards were prepared for the kinetic assay.
13. Ho, P. T.; Ngu, K. Y. *J. Org. Chem*. **1993**, *58*, 2313.
14. The residual activity data was consistent with reversible inhibition.
15. (a) Pauling, L. *American Scientist* **1948**, *36*, 51. (b) Wolfenden, R. *Acc. Chem. Res*. **1972**, *5*, 10. (c) Lienhard, G. E. *Science* **1973**, *180*, 149.
16. Satchell, D. P. N.; Secemski, I. I. *J. Chem. Soc. B* **1969**, 130.
17. Menger, F. M.; Smith, J. H. *J. Am. Chem. Soc*. **1971**, *94*, 3824.
18. Haldane, J. B. S. *Enzymes*; Longmans, Green and Co: London, 1930.