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A Single Antibody Catalyzes Multiple Chemical Transformations upon Replacement of the Functionalized Small Nonprotein Components

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Enzymatic reactions enlist amino acid residues and cofactors during the catalytic process. In designed biocatalysts such as catalytic antibodies (abzymes),[1] stereoelectronic complementarity between the antibody and hapten has been used to elicit catalytic amino acid residues in the antigen-combining site. These amino acids are involved in transition-state stabilization,[2] approximation,[3] general acid/base reactions,[4] covalent-bond catalysis,[5] and cofactor binding.[6] The introduction of functionalized small nonprotein components acting as "chemical teeth" into antibodies would broaden their catalytic versatility. Here, we demonstrate a single antibody catalyzing multiple chemical transformations by the generation of an antigen-combining site that functions as an apoprotein for binding functionalized components. We immunized mice with a hapten designed to induce both a substrate- and functionalized component-binding site and isolated two antibodies, which catalyze an acyl-transfer reaction by using an alcohol component. Replacement of this component with acidic and amino components enabled the antibodies to catalyze β-elimination, decarboxylation, and aldol reactions with large rate accelerations. These results demonstrate a new strategy for generating catalytic antibodies, namely, by controlling the reactivity and mechanism of the antibody using designed small molecules. This approach promises to both broaden the scope of catalytic antibodies and push back the boundaries of protein-based catalysis.

Phosphonate diester hapten **1** was designed to induce a substrate binding site and a functionalized component binding site into an antigen-combining site (Figure 1). Given the inherent antigenicity of *p*-nitrophenyl and *N*-acetylphenyl groups, immunization with hapten **1** would elicit both substrate- and functionalized component-binding sites simultaneously in the antigen-binding site. Hapten **1** was synthesized in nine steps from commercially available triethyl phosphate and ethyl 6-bromohexanoate. Although some phosphonate diesters are labile and have been used as haptens for reactive immunization,[5] hapten **1** showed a half-life ($t_{1/2}$) of 200 h in PBS (pH 7.4, 37 °C) and thus was deemed sufficiently resistant to the many chemical entities encountered in vivo during immunization. The hapten was conjugated to keyhole limpet heamocyanin (KLH) and was used to immunize five Balb/c mice, and standard hybridoma technology produced 50 IgGs specific for the hapten.[7]

First, we examined the acyl transfer reactions of *p*-nitrophenyl ester **2** with alcohol **3** by the hapten-binding antibodies. Out of 50 monoclonal antibodies, 22 were found to catalyze this acyl transfer reaction. The two most active antibodies, 25E2 and 27C1, showed a tight binding affinity to hapten **1** (25E2: K_d = 27 nM; 27C1: K_d = 8.7 nM) and catalyzed the reaction according to saturation kinetics (25E2: k_{cat} = 0.044 min^{-1}; k_{cat}/k_{uncat} = 198 M; K_m for ester **2** = 9.2 mM at 150 mM alcohol **3**; K_m for **3** = 88 mM at 10 mM **2**; 27C1: k_{cat} = 0.024 min^{-1}; k_{cat}/k_{uncat} = 109 M; K_m for **2** = 2.7 mM at 150 mM **3**; K_m for **3** = 86 mM at 10 mM **2**) (Table S1, Figures S1 and S2). Therefore, we examined whether these antibodies could accept a variety of functionalized components into the antigen-combining site. It was found that with nucleophilic amine **4**, 25E2 and 27C1 catalyzed acyl-transfer reactions with rate accelerations of 14 000-fold and 55 000-fold, respectively (Table S2, Figures S3 and S4). The antibody-catalyzed reactions were competitively inhibited by addition of hapten **1** (25E2: K_i = 110 nM; 27C1: K_i = 313 nM) (Figures S5 and S6) and followed a completely random, sequential kinetic mechanism that proceeds via a ternary antibody−substrate complex (the binding of one ligand has no effect on the other).[8] Furthermore, interestingly, replacement of **4** with thiol **5** resulted in significant enhancement of hydrolysis of *p*-nitrophenyl ester **2** via acyl-transfer reactions (Table S3, Figures S7 and S8). In the absence of **5**, neither antibody enhanced the rate of cleavage of **2**, showing that acyl transfer to **5** is essential for the antibody-catalyzed hydrolysis of ester **2**. These acyl-transfer reactions demonstrated that both the substrate and functionalized small molecule binding sites correctly orient their respective guest molecules, leading to productive substrate binding and catalysis. Having shown that the binding site of the functionalized component is versatile and can accept a variety of molecules, we next introduced a wider selection of functionalized small nonprotein components and examined other chemical transformations.

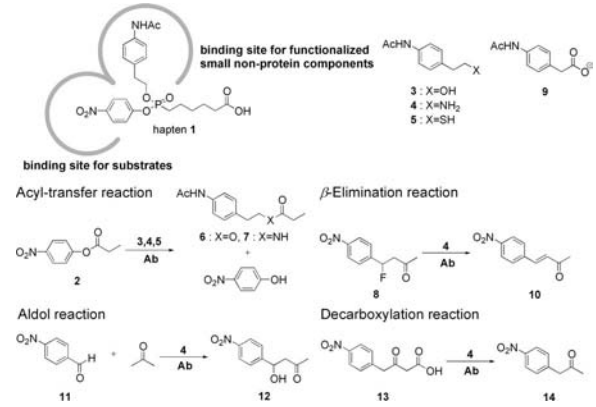

Figure 1. Structure of hapten and antibody-catalyzed chemical transformations.

We targeted reactions involving proton abstraction from a carbon center; these reactions utilize the carboxylate side chains of glutamic or aspartic acids in enzymes. When phenyl acetic acid derivative **9** was used as a functionalized component, antibody 25E2 catalyzed β-elimination of β-haloketone **8** to exclusively yield the trans isomer of enone **10**. The antibody-catalyzed reaction proceeded in the manner of random, sequential binding[8] and was competitively inhibited by addition of hapten **1** or removal of functionalized molecule **9**. Catalysis by 25E2 compared to the noncatalyzed reaction was remarkably efficient, showing a rate enhancement [(k_{cat}/K_m **8**)/k_{uncat}] of 2.4 × 10^5; k_{cat} (per binding site) = 0.89 min^{-1}, K_m

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Table 1. Kinetic Parameters for the Antibody-Catalyzed Aldol Reaction in the Presence of Amine **4**

	K_m				
	11 (mM)	4 (mM)	V_{max} ($\mu M \cdot min^{-1}$)	k_{cat} (min^{-1} per binding sites)	k_{cat}/K_m(**11**)/k_{uncat} k_{cat}/K_m(**4**)/k_{uncat}
25E2	1.1	186	1.43×10^2	28.6	4.4×10^4 251
27C1	0.958	67	2.58×10^2	25.8	4.4×10^4 629

In the aldol reaction with **11**, acetone, and amine **4**, kinetics were measured at 25°C in 50 mM Tris-HCl, pH 8.0, 5% acetone and 5% DMSO. The V_{max}, k_{cat}, and K_m were calculated from initial rates using a random, rapid equilibrium mechanism. The bimolecular noncatalyzed rate constant ($k_{uncat} = 6.13 \times 10^{-3}$ mM^{-1} min^{-1}) for the aldol reaction was determined by the method of initial rates with the fixed acetone concentration (5%) under otherwise identical conditions.

(for **8**) = 864 μM, K_m (for **9**) = 122 μM, pH 6.0 (Figure S9). Interestingly, antibody 27C1 could not catalyze the β-elimination reaction. This lack of catalytic activity could be due to differences in the electrostatic microenvironment of the antigen-combining sites in antibodies 25E2 and 27C1.

Both 25E2 and 27C1 were found to also catalyze the aldol reaction, which is arguably the most basic C−C bond forming reaction. Class I aldolases[9] and catalytic antibodies 38C2 and 33F12[10] utilize the ϵ-amino group of a lysine residue in the active site to form a Schiff base with the substrate which functions as an aldol donor. Since the antibody-combining sites of 25E2 and 27C1 can be equipped with amino component **4**, the antibodies should exhibit aldolase activity. To test this, the ability of the antibodies in the presence of amine **4** to catalyze the addition of acetone to *p*-nitrobenzaldehyde **11** was measured. The assay monitored the production of β-hydroxy ketone **12** and showed that both antibodies catalyzed the aldol reaction with large rate accelerations [25E2: (k_{cat}/K_m **11**)/$k_{uncat} = 4.4 \times 10^4$; 27C1: ($k_{cat}/K_m$ **11**)/$k_{uncat} = 4.4 \times 10^4$], as shown in Table 1 (Figures S10 and S11). As with the other antibody-catalyzed reactions described above, the aldol reaction was competitively inhibited by addition of hapten **1** or removal of amino component **4**. In the aldol reaction, the direct precursor of enamine is the imminium ion; the presence of the imminium ion was established by isolation of the reduction product following $NaBH_4$ treatment (Figure S12). Examination of enamine formation with acetone and amine **4** in the presence of $NaBH_4$ showed that antibodies 25E2 and 27C1 catalyzed the formation of the isopropylation product, providing evidence for an enamine mechanism for the antibody-catalyzed reaction. This enamine formation was also inhibited by addition of hapten **1**, showing that the enamine was forming in the antigen-combining site. The antibody-catalyzed aldol reaction gave no enantiomeric excess of product **12**, probably due to the small size of acetone for the molecular recognition. Therefore, the reaction with more bulky aldol donors would improve enantioselectivity. Finally, we examined whether antibodies 25E2 and 27C1 could decarboxylate β-keto acids, since Class I aldolase and aldolase antibodies can catalyze decarboxylation via the enamine mechanism.[11] Antibodies 25E2 and 27C1 were found to catalyze the decarboxylation of *p*-nitrophenyl β-keto acid **13** in the presence of amino component **4** (data not shown). In summary, antibodies 25E2 and 27C1, elicited by immunization with hapten **1**, have a substrate and functionalized small nonprotein component binding site. By simply exchanging the functionalized components, these antibodies can catalyze a wide range of chemical transformations including acyl-transfer, β-elimination, aldol reaction, and decarboxylation. The catalytic activities are comparable or superior to those of other "specialized" antibodies catalyzing the same reactions (Table S4). It has been reported that an antibody possesses substrate versatility to hydrolyze a variety of substrates (enol ethers, acetals, and epoxides) with general acid catalysis,[12] whereas antibodies 25E2 and 27C1 show catalytic versatility. This is the first example of a single antibody catalyzing multiple chemical transformations via versatile catalytic mechanisms. Our results demonstrate that replacement of functionalized small molecules can regulate the type of chemical transformation catalyzed by antibodies. In other words, a functional group in the active site can be easily replaced with other functional groups to change the chemical transformations. This is essentially a chemical version of site-directed mutagenesis used in protein science; for example, the replacement of alcohol **3** with acid **9**, as demonstrated in the antibody-catalyzed β-elimination, is equal to a mutagenesis of serine with aspartic acid. So, we have named this approach "site-directed chemical mutation". Using this approach, a variety of functional groups can be introduced into the active site, thereby providing new insights into biomolecular catalysis. In this work, the diversity of the immune system was used for the generation of tailor-made binding sites for functionalized small nonprotein components. Our results demonstrate that antibodies incorporating newly devised components acting as "chemical teeth" will enlarge the scope and broaden the reaction boundaries of catalytic antibodies.

Supporting Information Available: Synthetic procedures and spectral data for all compounds and kinetic assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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