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A Modified Bait and Switch Strategy for the Generation of Esterolytic Abzymes Using Concerted Catalytic Mechanisms

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Abstract

Esterolytic abzymes were generated via immunization with a haptenic diphosphonate mounted with two distinct traps actuated by the "bait and switch" and "transition-state mimic" concepts. One monoclonal antibody, 12F12, was characterized in detail, and showed significant rate enhancement and unique substrate specificity. Experimental data support that the antibody catalyzes the reactions with a concerted catalytic mechanism.

Keywords: antibodies; catalysis; ester hydrolysis; amino acids and derivatives

The construction of a system capable of controlling the acyl transfer processes is one of the most challenging themes in chemistry, because such an event should open the door to unlimited possibilities in organic synthetic [1], biological [2], and medicinal [3] applications. Catalytic antibody technology has been implemented as a reliable method for generating specific catalysts of acyl transfer processes, and the last decade witnessed its explosive development [4]. However, the catalytic activity of antibodies is thus far insufficient for use in practical applications, with few exceptions [5]. The next step in the catalytic antibody field is to find the principles for improvement of the catalytic activity [6].

Conventional catalytic antibodies display weak activities as compared with natural enzymes, because the antibodies have been designed to use only a single catalytic mechanism; for example, transition-state stabilization [7]. Therefore, we reasoned that an approach toward increasing the catalytic efficiency of antibodies is to generate an antigen-combining site, in which several catalytic mechanisms work in concert, as in natural enzymes. Herein, as a strategy to elicit hydrolytic antibodies with concerted catalytic mechanisms, we report a novel class of haptens, mounted with two distinct traps actuated by the "bait and switch" and "transition-state mimic" concepts [8]. Thus, we designed a hapten carrying two negatively charged phosphonate groups (Figure 1). In the hapten, the central phosphonate moiety, which mimics a high-energy transition state derived from the attack of a hydroxyl anion during hydrolysis, was designed to elicit an "oxyanion hole" in the antigen-combining site [9]. In addition, according to the "bait and switch" concept, the terminal phosphonate group was expected to attract a positively charged amino acid residue [10] in the S_1' subsite of the abzymes, which acts as general acid/base catalysis in the formation and/or decomposition of the tetrahedral intermediate. The elicited antibodies would catalyze the reactions by using a combination of the catalytic mechanisms of transition-state stabilization and general acid/base catalysis.

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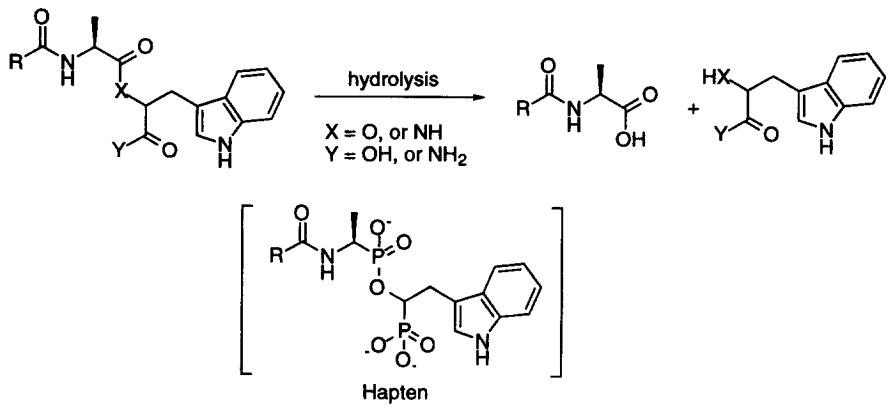
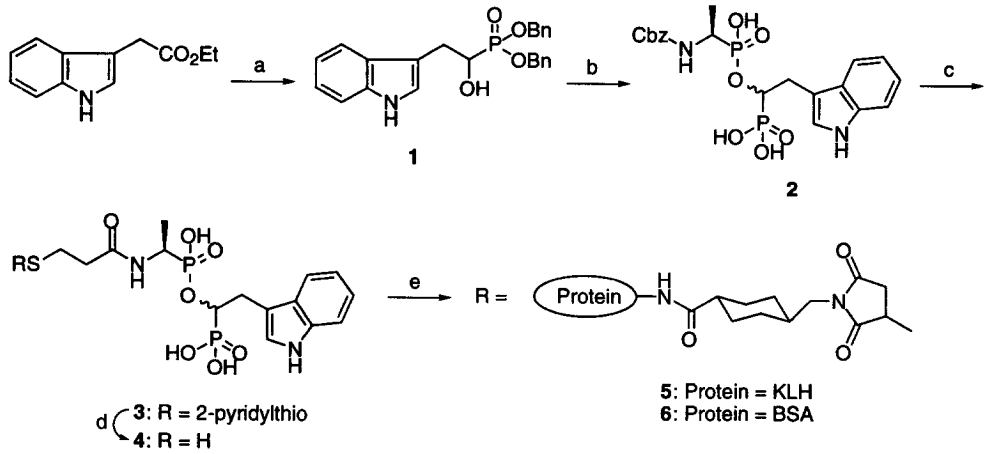
Figure 1



Scheme 1[a]



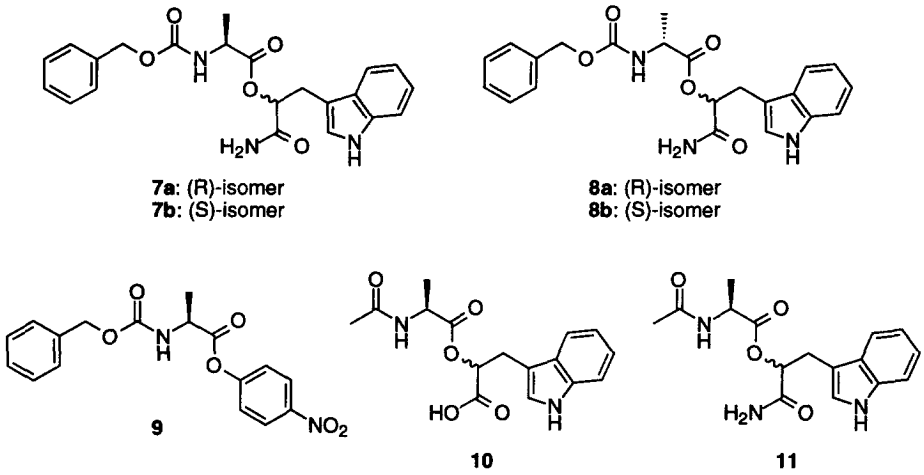
[a]Reagents and conditions: (a) (1) DIBAL-H, toluene-DME, -78°C, (2) dibenzyl phosphite, NaH, DMF-THF, 68%; (b) *N*-Cbz-(*R*)-(1-aminoethyl)phosphonic acid, DCC, pyridine, 56% (1:1 mixture of the diastereomers); (c) (1) H_2, $Pd(OH)_2$, MeOH, (2) *N*-succinimidyl 3-(2-pyridyldithio)propionate, 0.2 M phosphate buffer (pH 7.95), 75%; (d) dithiothreitol, 50 mM phosphate buffer (pH 7.95); (e) maleimide activated KLH or maleimide activated BSA, 83 mM phosphate buffer, 0.1 M EDTA, 0.9 M NaCl (pH 7.2) (17 haptens/KLH).

Hapten **2** was synthesized as a roughly equimolar mixture of two diastereomers via the condensation of *N*-Cbz-(*R*)-(1-aminoethyl)phosphonic acid[1] and racemic alcohol **1** [11],[2] and was coupled via its amino terminus to the carrier proteins, keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA) (Scheme 1). Balb/c mice were immunized with the KLH-conjugate **5**, and monoclonal antibodies were generated by standard protocols [12]. Hybridoma supernatants were screened for antibodies with binding affinities to the BSA-conjugate **6** by ELISA. Of the 930 hybridomas generated, nine clones were finally

[1] (*R*)-(-)-(1-aminoethyl)phosphonic acid was selected in relation to the L-configuration of naturally occuring amino acids, and was purchased from Aldrich Co.

[2] **1** was prepared in 68% yield via the condensation of indole-3-acetaldehyde, prepared *in situ* by DIBAL-H reduction of ethyl indole-3-acetate, and sodium dibenzyl phosphite.

Figure 2



established.[3] Screening for the hydrolytic activity was examined by using the 1:1 diastereomixture of substrate **7** (Figure 2). The reaction was followed by monitoring the production of indolelactic acid (280 nm) via high performance liquid chromatography (HPLC). As a result, three antibodies, 2H3, 12F12, and 17H6, were found to catalyze the hydrolysis. The most potent antibody, 12F12, was characterized in detail.

Since the hapten for immunization possessed the racemic α-carbon of the terminal phosphonate, the enantioselectivity of the elicited catalytic antibody was determined by using the chiral substrates, **7a** and **7b**.[4] The reactions were examined with 5.0 μM of 12F12 and 2.0 mM of the substrate in 10% DMSO/50 mM BisTris buffer (pH 7.5) at 25°C. As a result, it was found that antibody 12F12 was highly specific against the (*R*)-isomer to catalyze the hydrolysis of **7a**.[5] The antibody-catalyzed reaction of **7a** proceeded in a manner consistent with the Michaelis-Menten kinetics [15], and a Lineweaver-Burk plot of the steady-state data afforded a k_{cat} of 0.36 min^{-1} and a K_m of 3.3 mM. The rate acceleration (k_{cat}/k_{uncat}) was a 5 x 10^3-fold over that of the uncatalyzed reaction.[6] The reaction was competitively inhibited by hapten **2**: a Dixon analysis with hapten **2** afforded a K_i of 4.4 μM. In addition, the antibody-catalyzed reaction displayed strict substrate specificity for the stereochemistry of both P_1 and P_1' residues. Thus, of the D-alanine containing counterparts, neither **8a** nor **8b** was the substrate. Even the activated ester **9** was not hydrolyzed. Together with the competitive inhibition by the hapten, the strict substrate specificity that was programmed with the hapten used for immunization, demonstrated that the catalysis took place in the antibody combining site.

The observed catalytic activity of 12F12 was relatively high, as compared with those of the other catalytic antibodies hydrolyzing unactivated alkyl ester substrates [3b, 14]. This led us to examine the possibility that 12F12 uses a combination of catalytic mechanisms. To examine whether the terminal phosphonate of the hapten induced a positively charged catalytic residue, we prepared charged and non-charged substrates, **10** and **11** (Figure 2). In the antibody-catalyzed reaction, the negatively charged carboxylate group in **10** was expected to make an electrostatic interaction with the elicited catalytic residue to destroy the function. In a comparison of the antibody-catalyzed reactions between **10** and **11**, interestingly, the existence of the

[3] We attribute the low yield to the resemblance of the hapten to naturally abundant amino acids and small peptides.
[4] Chiral indole lactamide was prepared starting from resolved methyl indolelactate [13] by aminolysis (NH_3-MeOH, sealed tube, 80 °C). Its optical purity was confirmed after esterification with both enantiomers of *N*-Cbz-alanine by using HPLC.
[5] Compound **7b** was not a substrate. Similar stereospecificity in hydrolytic abzymes was reported by Schultz *et al.* [14].
[6] The background hydrolysis (k_{uncat}) of **7a** was measured to be 7.25 x 10^{-5} min^{-1} at pH 7.5.

negatively charged group in the substrate resulted in a 5-fold decrease in the rate enhancement.[7] This result suggests the crucial role of an amino acid residue in the S_1' subsite for the catalysis. The remaining activity of 12F12 against the charged substrate **10** seems to be due to transition-state stabilization by a catalytic residue in the S_1 subsite, which was programmed with the central phosphonate group in the hapten. Finally, the pH dependence of the hydrolysis was examined in the region from pH 6.0 to 9.0 (pH 6.0 to 7.5 in BisTris buffer, pH 8.0 to 9.0 in Tris buffer) at 25°C. In the pH region, the plot of pH vs log k_{cat} provided a single acid limb at pH 7.6.[8] Given a concerted mechanism with two catalytic residues, the pKa values are 7.6 and over 9.0. Although it is difficult to completely understand the details of catalytic mechanisms from only kinetic data, these observation suggest that the two phosphonates in the hapten induced at least two different catalytic residues, which work in concert in the antibody-catalyzed reaction. We are now performing site-directed mutagenesis and x-ray structural analyses, which will provide more detailed information on the catalytic mechanisms.

In this work, we demonstrated a modified bait and switch strategy for the generation of antibody catalysts with multiple catalytic functions. Although the abzyme 12F12 is still at the primordial level, as compared with natural hydrolases, the induced catalytic functionalities should provide a reliable basis for evolving catalytic efficiency by applying protein engineering methods [16].

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[11] Analytical data for compound **3** (a mixture of two diastereoisomers): ^{1}H NMR (500 MHz, D_2O): δ 8.44 (d, J = 4.0 Hz, 0.5H), 8.25 (d, J = 4.0 Hz, 0.5H), 7.82 (m, 0.5H), 7.80 (m, 0.5H), 7.64 (d, J = 7.0 Hz, 0.5H), 7.61 (d, J = 7.0 Hz, 0.5H), 7.36 (d, J = 7.0 Hz, 0.5H), 7.33 (d, J = 7.0 Hz, 0.5H), 7.23 (d, J = 5.9 Hz, 1H), 7.20-7.04 (m, 3.5H), 7.02 (t, J = 7.0 Hz, 0.5H), 4.87 (m, 0.5H), 4.75 (m, 0.5H), 4.25-4.02 (m, 1H), 3.47-3.38 (br m, 1H), 3.30-3.20 (m, 1H), 3.10-2.78 (m, 2H), 2.54-2.38 (m, 1H), 2.20-1.90 (m, 1H), 1.27-1.10 (m, 3H); HR-(-)FABMS calcd for (M-H) $C_{20}H_{24}N_3O_7P_2S_2$ 544.0531, found 544.0532.

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[7] The rate enhancement (**10**: V_{cat}/V_{uncat}= 3.0, **11**: V_{cat}/V_{uncat}= 15.1) was observed in the reaction using 10 μM antibody 12F12 and 2.0 mM substrate in 10% DMSO/50 mM Tris buffer (pH 8.0) at 25 °C.

[8] Unfortunately, no bell-shaped profile was observed in the pH region. In general, it is difficult to follow antibody-catalyzed reactions over pH 9.0, due to the instability of antibodies at high pH.