Antibody Catalyzed Cleavage of an Amide Bond Using an External Nucleophilic Cofactor

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The antibody-mediated hydrolysis of amides constitutes an important step toward the development of biocatalysts for the sequence-specific cleavage of peptides.[1] Previous efforts toward this end, including our own, used the selective binding of transition-state analogs,[2,3a] proximity effects supplied by a metal cofactor,[3b] intramolecular rearrangements,[3c,d] and complementary acid−base catalysis[4] similar to natural hydrolytic enzymes. As these efforts did not seem to provide a reliable strategy to generate amide-hydrolyzing antibodies, we decided to explore a different mode of catalysis, namely nucleophilic catalysis. This mechanism is well understood for serine and cysteine proteases.[5] Ideally, the nucleophile of the hydrolytic reaction is programmed into the antibody binding site in the form of an amino acid side chain residue. In practice, it is not a simple task to generate such a precisely placed residue even with prior knowledge of the binding site geometry.[6] On the other hand, an auxiliary nucleophile can be tightly bound in an appropriately created pocket of the antibody binding site, and it should prove equally effective compared with an internal nucleophilic residue. In this present study, phenol was chosen as the auxiliary nucleophile since it is a readily water-soluble compound, incorporating a phenyl ring that can take advantage of hydrophobic binding interactions to optimally place it in the antibody binding pocket. Furthermore, it is a good nucleophile that would generate a water-labile phenyl ester **7** through the reaction with the target amide **1**. The phenol-assisted cleavage of propionyl *p*-nitroanilide **1** is schematically shown in Figure 1.

Recently, we reported the generation of three antibody catalysts, 6-17, 3-49 and 14-10, raised against the haptens **O1**, **O2**, and both, respectively (Figure 2).[7] In a preliminary screen, these antibodies were shown to accelerate the cleavage of an amide bond in the form of an *intra*molecular N → O acyl transfer reaction of substrate **2** (Figure 3). Here, we report that these three antibodies also catalyze the *inter*molecular N → O acyl transfer reaction, the cleavage of propionyl *p*-nitroanilide **1** using phenol as an external nucleophilic cofactor.

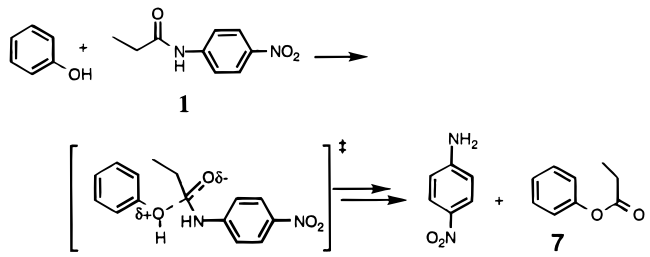

Figure 1.

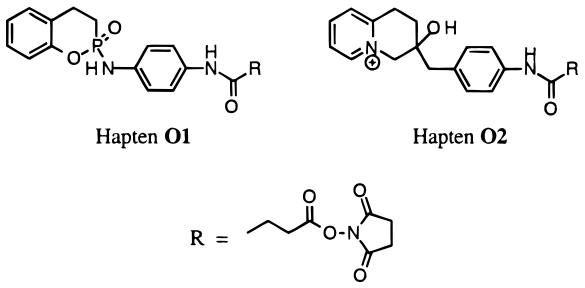

Figure 2.

The design features of haptens **O1** and **O2** have been already discussed in detail.[7] In short, both haptens were designed to program specific antibody binding pockets for the transition state of the amide cleavage and the phenol cofactor. Furthermore, the hapten designs sought to generate acidic and/or basic catalytic residues in the antibody binding sites, via charge complementarity. These residues would then augment the nucleophilic catalysis provided by the phenol. The two haptens were used separately (homologous immunization) to immunize Balb/C mice, as well as in sequence (heterologous immunization).[8] The details of the immunization protocol have been reported,[7] and the monoclonal antibodies were generated according to standard procedures.[9]

The three antibody catalysts were screened for the acceleration of the cleavage of propionyl *p*-nitroanilide **1** at varying concentrations of phenol and at different pH values. All three were found to catalyze this reaction, and the largest rate accelerations were observed at pH 8.0 and 10-fold excess of phenol to propionyl *p*-nitroanilide **1**. The Michaelis−Menten parameters were determined for the three antibody catalysts by varying either the phenol or the propionyl *p*-nitroanilide concentration and holding the other one in excess. These values are listed in Table 1. As previously observed for the *intra*molecular N → O transfer reaction, the heterologously generated antibody 14−10 was again found to be an almost 7-fold more efficient catalyst than the two homologously generated antibodies 6-17 and 3-49. More importantly, the catalytic activity of the (heterologous) antibody 14-10 was competitively inhibited by both haptens, while 6-17 (elicited against **O1**) was only inhibited by **O1** and 3-49 (elicited against **O2**) was only inhibited by **O2**.

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Table 1

catalytic antibody	haptens	immunization	app k_{cat}[a] (10^{-5}/min^{-1})	K_m^{phenol} (μM)	$K_m^{substrate\ 1}$ (μM)
6-17	**O1**/**O1**/**O1**	homologous	2.1	78	310
3-49	**O2**/**O2**/**O2**	homologous	1.3	14	77
14-10	**O1**/**O1**/**O2**	heterologous	13.3	136	370

[a] The k_{cat} values were measured by holding the phenol concentration in excess (pseudo-first-order rate constants).

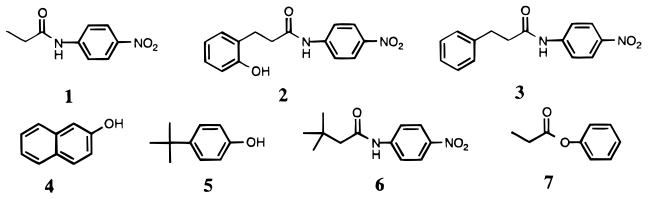

Figure 3.

The participation of phenol in antibody catalysis was clearly established with a number of simple experiments. First and foremost, in the absence of phenol, none of the three antibodies showed any rate acceleration of the cleavage of amide **1**. Furthermore, neither 2-naphthol (**4**) nor 4-*tert*-butylphenol (**5**) was accepted as nucleophilic cofactors for catalysis by any of the three antibodies (Figure 3). These results indicate the existence of a specific binding pocket for the phenol auxiliary nucleophile. Similarly, these catalysts were also specific for anilide **1**. *tert*-Butylacetyl *p*-nitroanilide **6** was not accepted as a substrate, indicating that a dedicated and specific binding pocket also exists for **1**. Together with the observation that substrate **3** lacking the nucleophilic hydroxyl group was not subject to antibody catalysis, these results confirm that the participation of phenol is essential for the antibody-catalyzed cleavage of amide **1**. The product of the reaction, phenyl propionate **7**, was shown not to inhibit the antibody-catalyzed cleavage of **1**, even at relatively high concentrations (5 mM **7** and 0.5 mM **1**). Furthermore, we found that none of the three antibodies displayed any acceleration of the rate of hydrolysis of **7**. Thus, it can be stated that the product **7** readily diffuses out of the antibody binding pocket upon its formation. Finally, the comparatively fast uncatalyzed rate of hydrolysis of product **7** regenerates the phenol cofactor, thus completing the catalytic cycle.

The background rate of the hydrolysis of amide **1** was found to be 2.1×10^{-7} min^{-1} at pH 8.0. Interestingly, this rate does not vary with a change in the concentration of phenol in aqueous solution, indicating that phenol does not participate in the uncatalyzed hydrolysis of amide **1**. Therefore, the k_{cat} values in Table 1 cannot be directly compared with this background hydrolysis rate. Rather, the antibody-catalyzed reaction appears to proceed by a pathway that is kinetically "disfavored" in aqueous solution.[10,11] An analysis of K_m values shows that the K_m^{phenol} values of all three antibodies are smaller than the respective $K_m^{substrate\ 1}$ values (Table 1). This may be explained by the consideration of the hapten designs. In both cases, the part of the hapten that corresponds to phenol is positioned furthest from the linker site of the haptens. Therefore, it seems likely that phenol is bound in a deep, hydrophobic pocket in the antibody binding sites while the binding pocket for amide **1** is more solvent accessible. It is also interesting to note that the antibody-catalyzed bimolecular reaction between **1** and phenol is only about 2 orders of magnitude slower than the antibody-catalyzed lactonization of **2**.[7,12]

Given that the product phenyl ester **7** appears to freely diffuse out of the binding pocket (i.e. no product inhibition is observed), and its rate of hydrolysis in the buffer employed ($k = 2.0 \times 10^{-4}$ min^{-1} at pH 8.0) is 3 orders of magnitudes faster than the buffer-catalyzed rate of hydrolysis of amide **1** at this pH, it can be said that all three antibodies have, in fact, achieved the cleavage of the amide bond in substrate **1**. This represents a formal, two step hydrolysis of an amide bond where the initial transfer of the acyl group to phenol is followed by the relatively facile uncatalyzed hydrolysis of the formed phenol ester **7**. It should be pointed out that this is the first time that a comparatively weak phenolic nucleophile is turned into a powerful cofactor by a catalytic antibody to effect the cleavage of an amide bond. Previously, a tyrosine residue within an antibody combining site was implicated to assist the hydrolysis of ester bonds.[13] This concept should prove generally applicable to other hydrolytic reactions of biological significance such as glycolysis or phosphodiester hydrolysis.[14] Our future efforts will seek to improve on this approach for amide cleavage by using different external nucleophiles that are more potent without sacrificing the lability of the subsequently formed acyl intermediates.

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(11) Mechanistically, it is possible that phenol can attack the amide bond in the presence of competing water (even though phenol is a rather poor nucleophile). However, the breakdown of the formed tetrahedral intermediate would most likely favor the expulsion of phenol due to the huge pK_a difference (greater than 6 units) beween the phenol and nitroaniline. The antibodies are capable of rerouting this process in favor of nitroanilide expulsion.

(12) There could be steric repulsion between the *o*-hydrogen of phenol and the β-hydrogens of propionamide **1** since the hapten structure represents this as a carbon−carbon bond. As a result, phenol and amide **1** might not assume an optimal orientation within the antibody in terms of the phenol attack angle, phenol deprotonation, and anilide protonation. However, it seems that antibodies do provide some flexibility in accommodating different substrates.

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