N → O Acyl-Transfer Reaction Catalyzed by Antibodies

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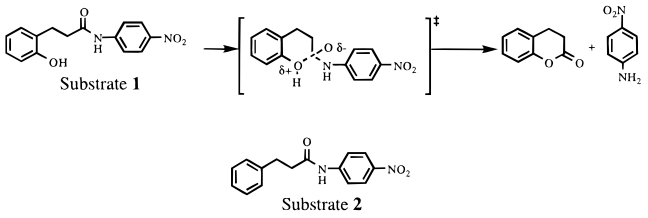
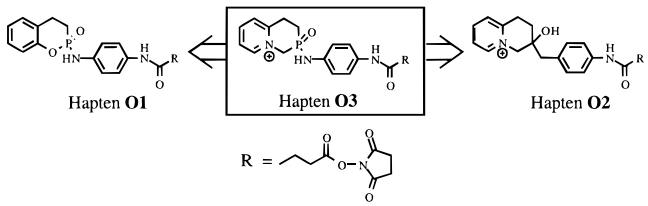
The generation of amidase-like antibodies has been and still is an extremely challenging task that bears high therapeutic potential.[1] An earlier study showed some promising results,[2] but only modest progress has been accomplished thus far.[3] Our recent work directed primarily toward this end was based on the hydrolytic mechanism accepted for aspartic protease catalysis.[4] We have also devised a new strategy of heterologous immunization where the two charged functional groups of a zwitterionic hapten are divided into two structurally similar but differently functionalized haptens which are then used to immunize animals in sequence. In addition to the facilitation of hapten synthesis, we have found that this method generated more efficient antibodies than a homologous immunization with only one of these haptens.[5] Thus considerable rate accelerations for ester hydrolysis were achieved, but amide hydrolysis met with little success, indicating the need for a new approach to this problem.

Serine proteases are another class of proteolytic enzymes which utilize an optimally placed internal serine residue in their active site to achieve the nucleophilic attack at the amide bond.[6] The following step is the acyl transfer from the amide to the internal serine residue.[7] Unfortunately, neither improved hapten design nor site directed mutagenesis[8] has consistently generated an optimally placed nucleophile in the antibody combining site. In order to study this crucial acyl transfer reaction, we decided to incorporate the nucleophile into the substrate.[9] Herein we report the successful antibody mediated catalysis of a N → O transfer reaction of substrate **1** as shown in Scheme 1 and emphasize the importance of the role played by the phenolic nucleophile.[10] This transfer reaction formally represents the hydrolysis of an amide bond. It should be noted that antibodies

Scheme 1



Scheme 2



capable of catalyzing this reaction could bind (externally supplied) phenol molecules which would then act like a cofactor in the amidolysis of properly chosen substrates.

Complementary haptens **O1** and **O2** were designed by sorting out the functional groups of a hypothetical hapten **O3** which is expected to generate acidic and/or basic residues in the antibody combining sites (Scheme 2). Hapten **O2**, bearing a pyridinium salt, was intended to induce a basic antibody residue to accept the proton from the attacking phenolic hydroxyl group.[11] Furthermore, this protonated basic residue could thereafter deliver its proton to the amine functionality of the tetrahedral intermediate, facilitating its departure. The tertiary hydroxyl group in **O2** serves as an isosteric replacement for the tetrahedral intermediate.[11] Phosphonamidate hapten **O1** was designed to generate in the catalytic site an acidic residue that would stabilize the developing oxyanion of the transition state. The arrangement of the phenol and the phosphonamidate in a six-membered cyclic structure is intended to ensure the correct orientation of the hydroxyl group in close proximity to the amide carbonyl group during the process of catalysis. The synthesis of the haptens and substrates will be reported elsewhere.

Balb/c mice were immunized according to two different protocols. In the homologous immunization protocol, mice were immunized three times at 2-week intervals with either **O1** or **O2** conjugated to KLH. In the heterologous immunization protocol, mice were immunized twice with the same hapten (**O1** or **O2)** followed by a final boost with the complementary hapten. Three days after hyperimmunization, mice were sacrificed and monoclonal antibodies were prepared according to standard protocols.[12] The amide hydrolysis of **1** was carried out with or without antibodies at 25 °C and at the appropriate pH's (three component buffer: 12.5 mM Mes, 12.5 mM Hepes, 25 mM diethanolamine). The reaction was followed by HPLC, monitoring *p*-nitroaniline release at 382 nm.

The background rate of hydrolysis of amide **1** at pH 6.8 was measured to be 4.7×10^{-7} min^{-1}, and that of substrate **2** lacking the phenolic hydroxyl of substrate **1** was found to be almost identical (k for **2** $= 4.8 \times 10^{-7}$ min^{-1} at pH 6.8).[13] These data show that if the phenol participates in the hydrolysis of substrate

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amide **1**, the rate of this lactonization reaction should not exceed that k observed for the hydrolysis of substrate amide **2**.[10] This result sets the upper limit for the uncatalyzed lactonization, effecting the N → O acyl transfer reaction.

Out of 39 monoclonal antibodies generated against hapten **O1** two were found to be catalytic. The more efficient catalyst 6−17 was analyzed further. It obeyed Michaelis−Menten kinetics with $k_{cat} = 1.8 \times 10^{-3}$ min^{-1} and $K_m = 0.86$ mM at pH 6.8. It was inhibited competitively by hapten **O1** with $K_i = 36$ μM. As expected, no inhibition was seen with hapten **O2** ($K_i > 10$ mM).[14] This antibody displayed a pH optimum at 8.0 where $k_{cat} = 2.7 \times 10^{-3}$ min^{-1} and $K_m = 0.98$ mM.[15]

One out of 20 monoclonal antibodies generated by hapten **O2** was found to be a catalyst. The catalytic efficiency of antibody 3−49 increased with increasing hydroxide concentration and its Michaelis−Menten parameters were determined at pH 9.0 ($k_{cat} = 4.2 \times 10^{-3}$ min^{-1}, $K_m = 0.77$ mM). It was also shown to be competitively inhibited by its respective hapten **O2** ($K_i = 380$ μM), while no inhibition was observed with hapten **O1** ($K_i > 10$ mM at pH 7).[14,15]

Antibodies were also generated heterologously by immunizing twice with **O1** followed by a final boost with **O2** (the reversed order, **O2** followed by **O1**, did not yield crossreactive antibodies).[16] All of the 15 antibodies displayed similar binding affinities to both haptens as determined by ELISA. Three of them were found to be catalytic and the most efficient catalyst 14−10 was characterized by Michaelis−Menten kinetics (at pH 6.8 $k_{cat} = 9.3 \times 10^{-3}$ min^{-1} and $K_m = 1.40$ mM). In accord with its binding affinity to both haptens, the catalytic activity of this antibody was competitively inhibited by both **O1** ($K_i = 51$ μM) and **O2** ($K_i = 790$ μM). The pH optimum of this antibody was determined to be pH 8.0.[17] At this pH, $k_{cat} = 1.5 \times 10^{-2}$ min^{-1} and $K_m = 1.27$ mM. As we observed in our previous studies with esterolytic antibodies,[5] antibody 14−10 generated by heterologous immunization ($k_{cat}/K_m = 11.8$ M^{-1} min^{-1}) proved to be a more efficient catalyst (approximately five-fold in this instance) than antibody 6−17 ($k_{cat}/K_m = 2.7$ M^{-1} min^{-1}), the best antibody generated by homologous immunization.

The evidence for the intramolecular participation of the phenol of **1** emerged from the studies with substrate **2**. While this substrate that lacks the phenolic hydroxyl group was shown to have an identical background rate of hydrolysis as compared with **1**, none of the three antibody catalysts, 6−17, 3−49, 14−10, accepted **2** as a substrate in the catalytic reaction. This shows that the phenol moiety is required for antibody catalysis. Furthermore, significantly faster rates observed for antibody catalyzed amide hydrolysis of **1** as compared with those for **1** and **2** without an antibody strongly indicate that the antibodies catalyze the nucleophilic attack of the phenol at the carbonyl group and subsequent breakdown of the tetrahedral intermediate.[18]

We have generated antibodies that are capable of efficiently catalyzing the acyl transfer from an amide to an internal nucleophile. Efforts are now underway to achieve the hydrolysis of a substrate in which the amide is not covalently linked to a nucleophile. It can be envisioned that with a nucleophile provided externally like a cofactor, nucleophilic catalysis could be accomplished without the laborious engineering of similar residues into a pre-existing antibody binding pocket.

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(13) This background rate is in accordance with a previously determined background rate for a similar *p*-nitroanilide substrate, see ref 2.

(14) 10 mM is the solubility limit of the haptens in water.

(15) The K_i at pH 8.0 and higher could not be determined due to the instability of hapten **O1** at this pH.

(16) We have observed a similar phenomenon in our previous studies, see ref 5b.

(17) Preliminary results showed that antibody 14−10 displays a bell shaped pH−rate profile suggesting two ionizable binding site residues that participate in catalysis. However, further investigations will be carried out to support this hypothesis and will be the subject of a full account of our work.

(18) There is a remote possibility that the hydroxyl group is only needed for binding, and that catalysis occurs by some other mechanism.