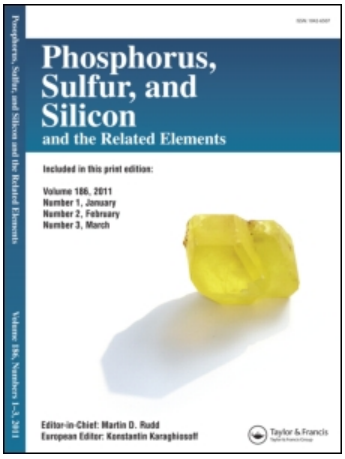

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Antibody Catalysis of Phosphodiester Hydrolysis: a Survey of Hapten Strategies

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Transition state analog and bait-and-switch hapten strategies for the elicitation of antibodies with phosphodiesterase actiivity are discussed.

Keywords: Catalytic antibody; hapten; transition state analog; bait-and-switch; ribonuclease inhibitor; rhenium

INTRODUCTION

The phosphodiester linkage forms the backbone of DNA and RNA and therefore its hydrolysis represents a reaction of fundamental importance in living systems. In our contribution to the intense efforts towards the development of novel phosphodiesterases for use in biochemistry and medicine[1,2], we have elected to explore the diversity of the murine immune system[3,4] to generate phosphodiesterase antibodies.

Transition State Analog (TSA) Approach

The naturally occurring enzyme RNase (ribonuclease) A catalyzes the hydrolysis of the P-O$^{5'}$ bond of RNA in two steps: attack on phosphorus by the 2'-hydroxyl to form a 2'-3'-cyclic phosphate, followed by hydrolysis of this intermediate to yield a 3'-phosphate monoester. It is generally accepted that both the ring closure and opening steps proceed *via* a similar pentacoordinate negatively-charged transition state (TS) **1** (see Figure 1). Classically this TS has been assumed to be trigonal bipyramidal (TBP)[5] though several studies have implicated a distorted TBP/square pyramidal (SP) geometry[6].

The challenge in designing mimics of TS **1** for use as haptens reduces to representing its geometry and charge characteristics in a stable molecule. The suitability of vanadate esters, established by their excellent inhibition of RNases[7], is thwarted by their tendency to undergo rapid ligand exchange and disproportionation reactions rendering them unsuitable for immunizations[8]. Therefore we turned to oxotechnetium(v) and

oxorhenium(V) complexes which we have shown to be excellent inhibitors of ribonuclease (RNase) U_2 (EC 3.1.27.4) activity ($K_i < 90\ \mu M$)[9-11].

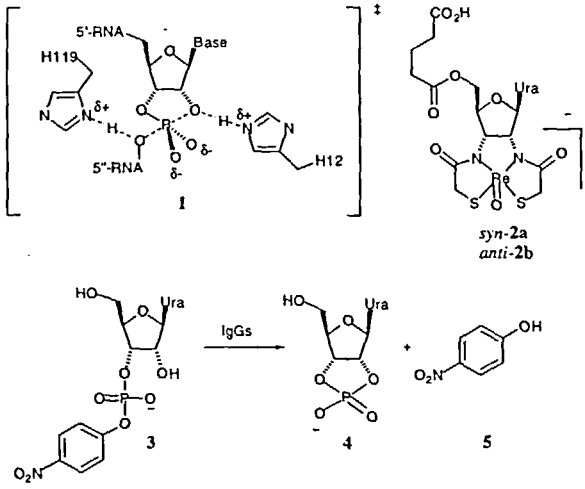

FIGURE 1 Oxorhenium(v) complexes **2a-b**, analogs of the putative TS **1** of RNase A mediated hydrolysis of RNA, were utilized as haptens for the elicitation of antibodies that catalyze the cyclization cleavage reaction of phosphodiester **3** to uridine 2',3'-cyclic phosphate **4** and *p*-nitrophenol **5**.

Keyhole limpet hemocyanin (KLH) conjugates of the *syn* and *anti* diastereomeric oxorhenium(v) complexes **2a-b**, were used to immunize BALB/c mice leading to the production of 50 hapten-specific monoclonal antibodies (25 each for **2a** and **2b**). From this library, 3 antibodies (all elicited to the *anti*-**2b** hapten) were found to catalyze the unassisted hydrolysis of uridine 3'-(4-nitrophenyl)phosphate **3** to the cyclic phosphate **4** and *p*-nitrophenol **5**. The most active antibody, 2G12, obeys Michaelis-Menten kinetics ($K_m = 240\ \mu M$, $k_{cat} = 1.53 \times 10^{-3}\ s^{-1}$ and $k_{cat}/k_{uncat} = 312$)[12]. The hapten **2b**, was found to be a tight-binding inhibitor of 2G12 activity ($IC_{50} = 6\ \mu M$, $K_i < 0.4\ \mu M$)[13]. Interestingly, both the *anti*-**2a** and *syn*-**2b** oxorhenium(v) diastereomers have indistinguishable K_i values suggesting that antibody 2G12 recognition is primarily based around the rhenium core while possessing broad tolerance for the apical oxygen ligand. Antigen binding fragments (Fabs) of 2G12 have been prepared and possess indistinguishable kinetic parameters from the parent antibody. This is the first example of an antibody being generated *de novo* as a phosphodiesterase catalyst and serves as an important benchmark for all future work in this field.

Bait-And-Switch Approach

The bait and switch paradigm is a well established strategy for the elicitation of antibody catalysts. Significant success has been realized by the use of positively charged haptens to yield negatively charged catalytic residues in antibody combining sites possessing general base properties[14-16]. With the importance of general base catalysis (supplied by His12) utilized by RNase A as precedent (see Figure 1), the applicability of this bait-and-switch approach to the generation of phosphodiesterase antibodies was investigated using the quaternary ammonium hapten **6** (see Figure 2).

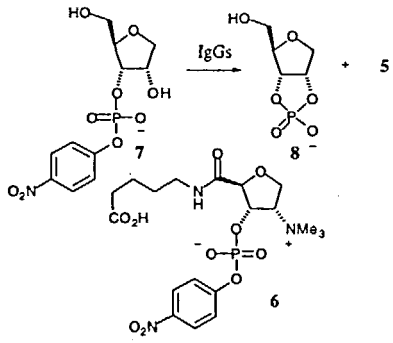

FIGURE 2 Quaternary ammonium hapten **6** elicited antibodies that catalyze the cyclization cleavage of phosphodiester **7**.

Following immunization protocols with the KLH-6 conjugate and monoclonal antibody production, 35 hapten-specific hybridoma cell lines were isolated. Two antibodies from this library were found to catalyze the cyclization cleavage of substrate **7** to the cyclic phosphate **8** and *p*-nitrophenol **5**. The most active clone, MATT.F-1, was studied in depth at pH 7.49 (Hepes, 20 mM). The reaction progress curves remain linear when followed for more than one turnover, suggestive of no product inhibition and the kinetic profile of MATT.F-1 obeys classical Michaelis-Menten kinetics (K_m = 104 ± 11 μM, k_{cat} = 0.44 min^{-1}, k_{cat}/k_{uncat} = 1.65 x 10^3)[17].

When studying any catalyst for processes where natural enzymes catalyze the same reaction it is essential to exclude the possibility of enzyme contamination. In addition to the inherent stability of substrate **7** to naturally occurring phosphodiesterases, several experiments have also been performed which help rule out the potential contribution of enzymatic contamination. First, hapten **6** was found to be a stoichiometric inhibitor of MATT.F-1. Second, antibody activity was destroyed upon boiling for 3 min, mitigating against contamination by thermostable RNases. Finally, MATT.F-1 preparations purified by precipitation, affinity (Protein-G column) and ion-exchange chromatography from different batches of ascites retain the same catalytic activity.

The catalytic proficiency of a biocatalyst, defined by division of its second order specificity constant (k_{cat}/K_m) with the background rate (k_{bkgd}) of substrate hydrolysis has been implemented by Wolfenden[18] to enable a comparison of different biocatalysts catalyzing the hydrolysis of different substrates. MATT.F-1 has a proficiency of 1.6 x 10^7 M^{-1}, which is 10 fold higher than that of the phosphodiesterase antibody 2G12 (1.3 x 10^6 M^{-1})[10], elicited by the TSA hapten **2b** *vide supra* and is in fact only 3 orders of magnitude lower than the proficiency of the naturally occurring enzyme RNase A for its unnatural substrate **3** (1.1 x 10^{10} M^{-1}).

In this report we have shown the powerful utility of both TSA and bait and switch hapten paradigms for the generation of catalytic antibodies. By immunization with either an oxorhenium(v) complex **2b** or a quaternary ammonium molecule **6** phosphodiesterase antibodies with inherently unique catalytic mechanisms and substrate specificities but catalyzing the hydrolysis of the same phosphodiester bond have been generated, highlighting the remarkable nature of these programmable biocatalysts.

Acknowledgments

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