A Pentacoordinate Oxorhenium(V) Metallochelate Elicits Antibody Catalysts for Phosphodiester Cleavage

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The cleavage of phosphodiester bonds, such as those found in DNA and RNA, is a reaction of key importance in living systems. Consequently there are intense efforts to develop novel phosphodiesterases for use in biochemistry and medicine.[1] Our approach to this problem has been to exploit the diversity of the vertebrate immune system[2] to generate antibodies with phosphodiesterase activity, focusing initially on antibody catalysis of RNA cleavage.[3] The most-studied natural ribonuclease is RNase A which catalyzes the hydrolysis of the P−O$^{5'}$ bond of RNA in two steps: attack on phosphorus by the 2′-hydroxyl group to form a 2′, 3′-cyclic phosphodiester intermediate, followed by hydrolysis of this intermediate to give a 3′-phosphomonoester (Scheme 1).[4]

While there is currently a lively debate on the precise mechanism of this enzyme,[5] it is generally accepted that both the ring closure and ring opening steps involve a similar pentacoordinate negatively-charged transition state (TS) (Figure 1). Classically this TS has been assumed to be trigonal bipyramidal (TBP)[6] though several studies have suggested more of a distorted TBP/square pyramidal (SP) geometry.[7] The challenge in designing mimics of this TS for use as haptens reduces to representing its geometry and charge characteristics in a stable molecule. Vanadates would be promising candidates and have been shown to be potent inhibitors of several phosphoryl transfer enzymes including RNase A.[8] However, vanadium alkoxides undergo rapid ligand exchange in aqueous solution, making them unsuitable for immunization.[9] Recently we have shown that oxorhenium(V) (oxoRe(V)) and oxotechnetium(V) adenosine complexes are good inhibitors of the purine-specific ribonuclease, RNase U_2.[10] These metallochelates possess a water-stable negatively-charged pentacoordinate structure, most likely existing in a distorted TBP/SP geometry[11] (Figure 1). Therefore we reasoned that oxoRe(V) uridine complexes **1a** and **1b** could be mimics of the TS for either the first or second step in RNA hydrolysis and thus be promising haptens for the generation of catalytic antibodies for this reaction.

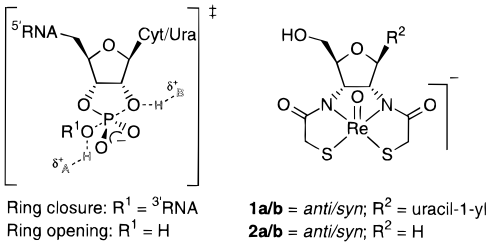
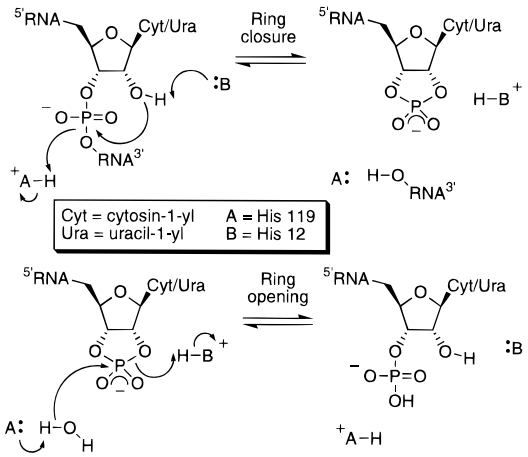


Figure 1. Comparison of the putative transition state for RNase A-catalyzed RNA cleavage and the oxorhenium(V) transition-state analogues (**1a**, **1b**).

Scheme 1



Hapten **1** was synthesized as a pair of diastereomers in ten steps starting from 1-β-D-arabinofuranosyluracil.[12] The *anti* and *syn* epimers (**1a** and **1b**) were separated by preparative reverse-phase HPLC and coupled to keyhole limpet hemocyanin (KLH) through the 5′-hydroxyl group of the ribose ring using glutaric anhydride. Mice were immunized with the hapten-KLH conjugates, and 50 hapten-specific hybridoma cell lines (25 each for **1a** and **1b**) were isolated using standard hybridoma technology.[13] Monoclonal antibodies (IgG) were purified to homogeneity from tissue culture and ascites fluid.[14] All 50 antibodies were then screened for their ability to catalyze the cleavage of uridine 3′-(*p*-nitrophenyl phosphate) (UpOC$_6$H$_4$-*p*-NO$_2$)[15] (Scheme 2).

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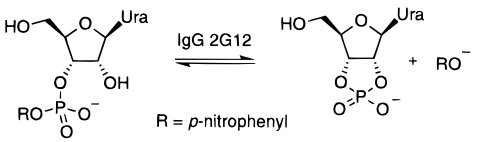
S0002-7863(97)00107-8 CCC: $14.00

Scheme 2



Three antibodies (all raised to the *anti* hapten, **1a**) were found to catalyze the reaction. These antibodies all lost activity upon boiling for 3 min, mitigating against contamination with thermostable RNases. Moreover, their activities were unaffected by the addition of 1 mM EDTA excluding the possibility of contamination by a metal-dependent phosphodiesterase.

The most active antibody (2G12) was studied in more detail. The greatest catalytic rate enhancement by this antibody was observed at pH 6.0, yielding a k_{cat} of $1.53 \pm 0.09 \times 10^{-3}$ s^{-1} and a K_M of 240 ± 30 μM. The reaction was typically followed for several turnovers with no indication of product inhibition. Base-catalyzed cleavage of $UpOC_6H_4$-*p*-NO_2 was found to be first-order with respect to hydroxide ion. A small linear dependence on buffer concentration was measured, while the antibody-catalyzed reaction was independent of buffer effects.[16] Extrapolating to zero buffer concentration yielded $k_{uncat} = 4.9 \times 10^{-6}$ s^{-1} and $k_{cat}/k_{uncat} = 312$.[17] The catalytic proficiency of an enzyme can be expressed as $[(k_{cat}/K_M)/k_{uncat}]$[18] which for Ab 2G12 is 1.3×10^6 M^{-1}. This compares to a value of 1.1×10^{10} M^{-1} for RNase A at pH 6.0 using the same substrate.[5d]

When studying any artificial catalyst where natural enzymes catalyze the same reaction, it is essential to exclude the possibility of enzyme contamination. This is especially important for the cleavage of phosphodiesters. Several experiments have been performed that together unequivocally rule out enzyme contamination, the most important of which are hapten inhibition, retention of specific activity by Fab fragments, and protein G immunoprecipitation, *vide infra*.

The hapten (**1a**) was found to be a tight-binding inhibitor of Ab 2G12. An IC_{50} value of 6 μM was measured by varying the inhibitor concentration in reactions containing 5 μM antibody (10 μM active sites) and 400 μM substrate. Assuming a competitive mode of inhibition this gives an upper limit to the K_i of 0.4 μM.[19] Interestingly both the *anti* (**1a**) and *syn* (**1b**) epimers of the hapten were found to give indistinguishable IC_{50} values. The oxoRe(V) complexes of 2,5-anhydro-3,4-diamino-3,4-dideoxy-D-ribitol (**2a** and **2b**), lacking a base at the 1′ position of the sugar, were also investigated as inhibitors[20] and found to behave in a similar way to **1a** and **1b**. This suggests that the antibody primarily recognizes the Re core common to both classes of compounds but does not markedly distinguish between the two configurations of the Re oxygen. The apparent lack of importance of the base for inhibition in the antibody contrasts with experiments on RNase U_2[10c] where oxoRe(V) complexes of 9-(2′,3′-diamino-2′,3′-dideoxy-β-D-ribofuranosyl)-adenine were found to be much more potent inhibitors of the enzyme than **2a** and **2b**. However, between *anti* and *syn* diastereomers there was only a small (6-fold) difference in K_i.[10c] The lack of strong diastereoselectivity in the antibody and enzyme may reflect the fact that the negative charge of the Re complex is delocalized over the diamide dimercaptide ligand system reducing the impact of the Re oxygen configuration in molecular recognition.

Antigen-binding fragments (Fab) of 2G12 were prepared and showed indistinguishable kinetic properties to the parent antibody, while the constant region fragments (Fc) exhibited no catalysis. This further supports the notion that catalysis takes place in the antibody binding site.

We have performed an experiment analogous to immunoprecipitation[21] but using immobilized protein G as the precipitant. By treating identical solutions of IgG 2G12 with varying amounts of immobilized protein G and filtering the samples through 0.22 μm spin-filters, it was possible to selectively remove varying amounts of antibody from the solutions. Analysis of the protein G-treated filtrates showed that catalytic activity was linearly proportional to the remaining protein concentration providing perhaps the most direct evidence that catalysis is due to the antibody. This is a relatively simple experiment to perform and offers a general and powerful control for all antibody-catalyzed reactions where the possibility of a contaminating enzyme exists.

In this communication we have reported the isolation of antibodies that cleave a phosphodiester bond. This finding further supports our hypothesis that oxoRe(V) complexes can be effective transition-state analogues for phosphoryl transfer reactions. Work is underway to isolate more efficient phosphodiesterase antibodies by improving on the original hapten design. Two ways that this might be achieved are by minimizing the size of the Re ligand system and/or by incorporating a leaving group mimic into the ligand design.[3]

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Supporting Information Available: Buffer effect on the cyclization of $UpOC_6H_4$-*p*-NO_2, Michaelis−Menten analysis of Ab 2G12, details of the protein G immunoprecipitation, and procedures for antibody preparation and purification (5 pages). See any current masthead page for ordering and Internet access instructions.

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(16) Reaction conditions were 10 μM Ab active sites, 20 mM 4-morpholineethanesulfonic acid (MES) pH 6.0. The second-order rate constant for buffer catalysis is 2.04×10^{-4} s^{-1} M^{-1}. Thus the ratio of the k_{cat}/K_M for Ab 2G12 to the second-order rate constant for buffer catalysis is 3.1×10^4.

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