Stereoselective Antibody-Catalyzed Oxime Formation

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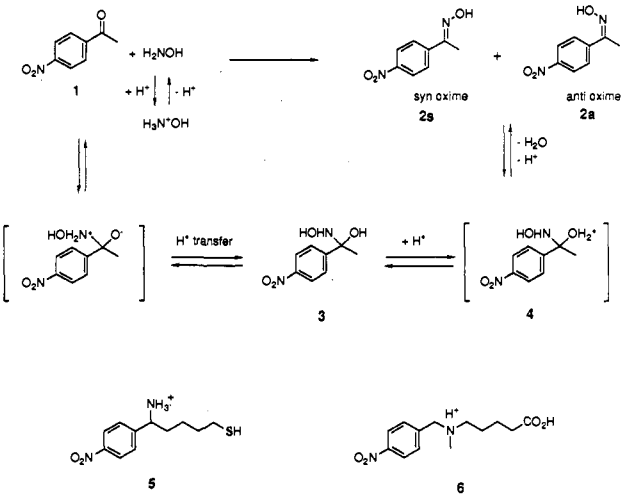
The addition of hydroxylamine to carbonyls to yield oximes is not only one of the best understood examples of a nonenzymatic addition–elimination reaction[1] but also an important reaction in synthesis.[2] Except in the case of symmetric ketones, two isomeric oximes are generated, syn and anti, which have different physical properties and biological activities.[3] Chemical methods for the synthesis of oximes usually give a mixture of the two geometrical isomers which must be separated by chromatography or recrystallization. Recently, it has been shown that antibodies can catalyze the formation of products that are disfavored or difficult to obtain by chemical methods.[4] Consequently, antibody catalysis may provide an approach to controlling the stereochemistry of this useful condensation reaction.

Oxime formation proceeds through the formation of a tetrahedral intermediate followed by an acid-catalyzed dehydration step,[1] which at neutral pH is rate determining. This mechanism suggests that antibodies specific for haptens **5** and **6** might catalyze oxime formation from 4′-nitroacetophenone and hydroxylamine. Both haptens contain a positively-charged ammonium ion which mimics the transition state involved in breakdown of the protonated tetrahedral intermediate (Scheme 1). The protein carrier–hapten conjugation scheme should ensure accessibility of hydroxylamine to the antibody active site, and the asymmetry of the combining site might be expected to lead to selectivity in formation of the syn/anti products. Because the reaction product is sp^2 hybridized, product inhibition should be minimized.

Thirty-eight monoclonal antibodies (mAb) specific for hapten **5** were screened for oxime formation using a spectrophotometric assay.[5] Thirty-six antibodies were found to accelerate the reaction rate compared to the uncatalyzed reaction. Reverse-phase high-performance liquid chromatography (HPLC) analysis indicated that only the starting ketone and various ratios of two oxime isomers were present.[6] Addition of 40 μM free hapten **5** to the antibody-catalyzed reactions led to complete inhibition of catalysis. Interestingly, of the 36 antibodies specific for **5**, mAb 20A2F6 showed the highest activity. This antibody also was found to have the highest activity in another reaction for which these antibodies were previously assayed, the β-elimination of water from 4-(4′-nitrophenyl)-4-hydroxy-2-butanone.[7] Antibody 43D4-3D12, which is specific for hapten **6** and was earlier shown to catalyze the β-elimination of hydrogen fluoride from 4-(4′-nitrophenyl)-4-fluoro-2-butanone,[8] also catalyzed oxime formation.

Scheme 1



The reactions catalyzed by antibodies 20A2F6 and 43D4-3D12 follow Michaelis–Menten kinetics. Apparent values of k_{cat} and K_m for the conversion of *p*-nitroacetophenone to the syn and anti products at a fixed concentration of hydroxylamine (20 mM) were determined: antibody 20A2F6, $K_{m\ app}$ = 2.7 ± 0.2 mM, $k_{cat\ app}$ = 11.0 ± 0.6 min^{-1}, $k_{bg\ app}$ = 6.3 × 10^{-4} min^{-1}, pH 7.25; antibody 43D4-3D12, $K_{m\ app}$ = 0.94 ± 0.15 mM, $k_{cat\ app}$ = 6.7 ± 0.7 min^{-1}, $k_{bg\ app}$ = 2.3 × 10^{-3} min^{-1}, pH 6.5. The rate accelerations, $k_{cat\ app}/k_{bg\ app}$, are 1.7 × 10^4 (20A2F6, pH 7.25) and 2.9 × 10^3 (43D4-3D12, pH 6.5), where $k_{bg\ app}$ is $k_1k_{H^+}[H^+][NH_2OH]/(k_{-1} + k_{H^+}[H^+])$.[1] No significant product inhibition was observed for either antibody-catalyzed reaction. Moreover, it was shown that 20A2F6 does not catalyze 3′-nitroacetophenone oxime formation.

The ratio of the two oxime isomers was determined by reverse-phase HPLC analysis.[5] The background reaction gave a 3:1 syn:anti isomer ratio under the reaction conditions. The syn/anti ratio of the condensation reaction catalyzed by antibody 20A2F6 was 9.0, and that of antibody 43D4-3D12 was reversed (anti:syn = 9.0).[9] Thus, while chemical methods always yielded the syn

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(5) Velocities were determined spectrophotometrically by measuring the initial absorbance increase at 310 nm. Because the syn and anti isomers have different extinction coefficients at this wavelength, an apparent extinction coefficient, $\Delta\epsilon_{app}$, was determined by analysis of the syn:anti product ratio using reverse-phase HPLC, namely,

$$\Delta\epsilon_{app} = \Delta\epsilon_{310\ nm}(\mathbf{2s–1})\left(\frac{\mathbf{2s}}{\mathbf{2s}+\mathbf{2a}}\right) + \Delta\epsilon_{310\ nm}(\mathbf{2a–1})\left(\frac{\mathbf{2a}}{\mathbf{2s}+\mathbf{2a}}\right)$$

$\Delta\epsilon_{310\ nm}$(**2s–1**) = 8370 M^{-1} cm^{-1}, and $\Delta\epsilon_{310\ nm}$(**2a–1**) = 2280 M^{-1} cm^{-1}. The reaction was initiated by adding 10 μL of a 50 mM solution of **1** in CH_3CN and 10 μL of a 1 M aqueous solution of hydroxylamine hydrochloride, pH 7.0, to 480 μL of the antibody solution (10 mM phosphate, 100 mM NaCl, pH 7.0) preincubated at 25 °C. The background rate was measured in the absence of antibody under otherwise identical conditions. The assay solution was applied to a Microsorb 5-μm C18 column (25 cm × 4.6 mm) equilibrated with 45% CH_3CN in aqueous 10 mM triethanolammonium acetate buffer, pH 7.2. The peaks were eluted under isocratic conditions and monitored at 278 nm, where **2s** and **2a** have the same UV absorbance. Retention times for **1**, **2s**, and **2a** were $t_{r(1)}$ = 8.1 min, $t_{r(2s)}$ = 7.5 min, and $t_{r(2a)}$ = 5.6 min, respectively. Both syn and anti oximes were found to be stable at pH 7, 25 °C, in the presence or in the absence of the antibodies by reverse-phase HPLC analysis.

(6) Authentic samples of the syn and anti oximes were prepared chemically. Condensation of 4′-nitroacetophenone and hydroxylamine in ethanol/water in the presence of sodium acetate as base gave almost exclusively the syn isomer. The syn oxime was isomerized by UV light to the anti isomer, which is more polar by TLC analysis. They were separated from each other in high purity by a combination of silica gel flash column chromatography (R_f(**2s**) = 0.58, R_f(**2a**) = 0.29, 30 v/v % EtOAc in hexanes) and recrystallization (**2s** was recrystallized from EtOAc, mp 172–174 °C; **2a** from ether, mp 123–124 °C). The structure of the anti oxime was confirmed by X-ray analysis.

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(9) The antibody-catalyzed product distribution is corrected for the background reaction.

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isomer as the major reaction product, both the syn and anti isomers could be produced as the major reaction product using antibody catalysis.

The pH dependence of the 20A2F6-catalyzed reaction was studied in the presence of 20 mM hydroxylamine.[10] The pH profile of $k_{cat\ app}$ for antibody 20A2F6 is bell-shaped and shifted approximately 2 pH units from that of the uncatalyzed reaction (Figure 1). The $K_{m\ app}$ increases as the reaction pH is raised. These results are consistent with an active site group with pK_a of approximately 7.5 that is either acting as a general acid or stabilizing the protonated transition state. The drop in rate on the acid side of the curve may represent a change in the pK_a of bound hydroxylamine. A catalytic group has yet to be identified, but glutamate and aspartate are not likely because treatment of the antibody with diazoacetamide[11] (DAA) failed to inactivate 20A2F6. In contrast 43D4-3D12 loses its catalytic activity upon treatment with diazoacetamide, consistent with the fact that 43D4-3D12 was previously shown to have an active site glutamate[8] which likely functions as a general acid in catalyzing oxime formation.

The high number of antibodies specific for hapten **5** that catalyze oxime formation suggests that ammonium ions are a good mimic for the tetrahedral positively-charged transition state of this reaction. It should be possible to design new haptens that include stereochemical considerations in their design in order to obtain even higher stereoselectivity.

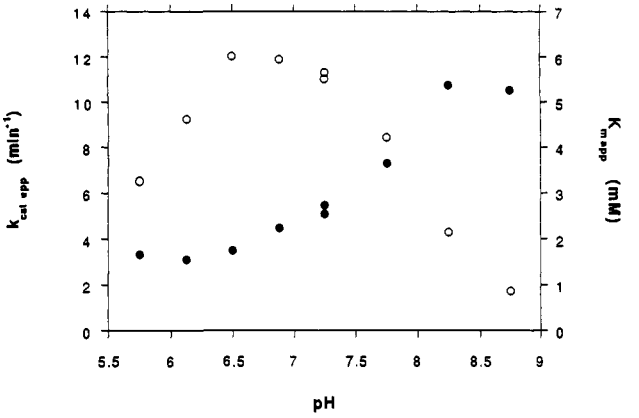

Figure 1. The pH profile of $k_{cat\ app}$ (○) and $K_{m\ app}$ (●) for oxime formation catalyzed by antibody 20A2F6 at a fixed concentration of hydroxylamine (20 mM) at 25 °C.

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(10) The buffers used were 20 mM [bis(2-hydroxyethyl)imino]tris-(hydroxymethyl)methane, 20 mM hydroxylamine hydrochloride, 100 mM NaCl for the pH range 5.75–7.25, and 20 mM *N*-(2-hydroxyethyl)piperazine-*N*′-3-(propanesulfonic acid), 20 mM hydroxylamine hydrochloride, 100 mM NaCl for the pH range 7.25–8.75. The concentration of 20A2F6 was 1.9 μM as determined by absorbance at 280 nm using ε (1 cm, 0.1%) = 1.37 and a molecular weight of 150 000 for IgG. The reaction was initiated by adding 20 μL of a stock solution of **1** in CH_3CN to 480 μL of the antibody solution preincubated at 25 °C.

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