Antibody-catalysed glycosyl transfer reactions from *in vitro* immunization

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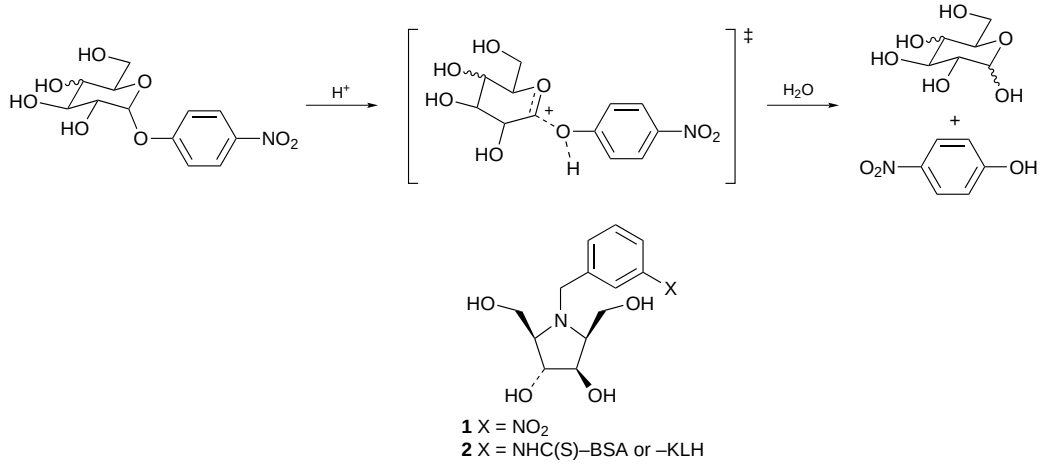
***In vitro* immunization using a 5-membered ring iminocyclitol afforded antibodies that catalyse the hydrolysis of *p*-nitrophenyl glucopyranoside and *p*-nitrophenyl galactopyranoside with rate enhancements (k_{cat}/k_{uncat}) of greater than 10^4.**

The generation and characterization of antibodies that catalyse glycosyl transfer reactions should provide insights into the mechanisms of naturally occuring glycosidases, and may lead to the development of glycosidases with novel specificities.[1–3] Previous efforts to generate antibodies with glycosidase activity made use of cyclic acetals as model substrates and antibodies generated against charged and conformationally restricted analogues of the oxocarbonium ion intermediate.[1–3] Rate accelerations of the order of 10^2–10^3-fold were reported for these catalytic antibodies. More recently, an antibody that catalyses the hydrolysis of *p*-nitrophenyl galactopyranoside with a rate enhancement (k_{cat}/k_{uncat}) of 7×10^4 was isolated by screening a library of antibodies specific for a five-membered ring iminocyclitol using a suicide substrate.[4] We have independently used a similar positively-charged twist boat hapten to directly generate an antibody that catalyses the cleavage of *p*-nitrophenyl-β-D-glucopyranoside **3** with a rate enhancement (k_{cat}/k_{uncat}) of *ca.* 2×10^4. Antibodies were produced by *in vitro* immunization with free hapten, suggesting this might be a general method for generating catalytic antibodies, especially for unstable or toxic immunogens.[5]

The pyranoside ring is known to have a half-chair or twist-boat conformation in the transition state for glycosidic bond cleavage.[6] Another distinctive feature of the transition state is the development of partial positive charge at the anomeric position. Based on these structural and electronic characteristics, hapten **1** was used as a transition state analogue[7] to elicit antibodies that catalyse the cleavage of the β-glucosidic bond of *p*-nitrophenyl-β-D-glucopyranoside (Scheme 1). Hapten **1** was synthesized from 5-keto-D-fructose and benzhydrylamine in three steps.† The $NaBH_4$ dependent reductive amination of 5-keto-D-fructose with benzhydrylamine[8] afforded, after recrystallization, the indicated diastereoisomer (Scheme 1) of the corresponding *N*-benzhydryl azasugar. Removal of the protecting group by catalytic hydrogenation, and alkylation of the resulting secondary amine with 3-nitrobenzyl bromide, yielded hapten **1**. The nitro group was then reduced with H_2 using Lindlar catalyst, converted to the isothiocyanate and coupled to the carrier proteins keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA).[9] Sixteen hybridomas antibody specific for hapten **1** were generated by standard methods[10] and ascites from each cell line were generated. Antibodies were purified using Protein-A affinity chromatography[11] followed by ion-exchange chromatography using a mono S column,‡ and were tested using the following substrates: *p*-nitrophenyl-α- and -β-D-glucopyranosides, *p*-nitrophenyl-α- and -β-D- galactopyranosides and *p*-nitrophenyl-α- and β-mannopyranosides. HPLC analysis of the reaction products indicated that none of the antibodies showed any hydrolytic activity under a range of buffer conditions.‡

However, encouraged by recent progress in immunization methods, we decided to generate antibodies by *in vitro* immunization, wherein carrier free hapten is mixed directly with spleen cells in culture.[11] Using this method, fourteen hybridoma cell lines secreting antibodies specific for hapten **1** were identified by enzyme-linked immunosorbent assay and ascites were generated. The ascites were purified and assayed as described above. Two antibodies were found to be catalytic: Ab24 and AB21 catalysed the hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside **3** and *p*-nitrophenyl-β-D-galactopyranoside **4**, respectively. These antibodies displayed saturation kinetics and their Michaelis constants were determined in 10 mM 2-(4-mor-



1 X = NO_2
2 X = NHC(S)–BSA or –KLH

Scheme 1

pholino)ethanesulfonic acid (MES), 100 mM NaCl, 0.02% NaN_3 buffer, pH 4.5, at 37 °C; rate constants for the uncatalysed reaction were determined under the same conditions.‡ Antibody Ab21 catalysed the hydrolysis of *p*-nitrophenyl-β-D-galactopyranoside **4** (and not **3**) with a k_{cat} of 0.035 h^{-1} and K_m of 310 μM (k_{cat}/k_{uncat} = 2.5 × 10^4); antibody Ab24 catalysed the hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside **3** (and not **4**) with a k_{cat} of 0.02 h^{-1} and K_m of 160 μM (k_{cat}/k_{uncat} = 2.2 × 10^4). Antibodies purified from different preparations of ascites showed the same specific activities. Moreover, the catalytic activity could be stoichiometrically titrated by absorbing antibodies on increasing amounts of protein A agarose and analysing the activity of the filtrate *versus* the amount of bound antibody. The isolation of two antibodies, one specific for substrate **3** and the other for substrate **4**, also argues against enzymic contamination.

The antibodies were inhibited by hapten **1** with a K_i value of 60 μM for the Ab21-catalysed hydrolysis of glucopyranoside **3**, and a K_i value of 380 μM for the Ab24-catalysed hydrolysis of galactopyranoside **4**.[12] For both antibodies the rate acceleration does not correlate with the relative affinities of the antibody for substrate and hapten **1**. This behaviour suggests that a structural feature of the hapten (*i.e.* an amino or hydroxy group) results in the presence of a catalytic group, such as aspartic or glutamic acid, in the antibody combining site which can function as a general acid in catalysis but does not contribute substantially to the overall binding energy for the hapten. Indeed, diazoacetamide treatment of antibody Ab21 led to a 97% reduction in catalytic activity; 60% of the catalytic activity was retained when modification was carried out in the presence of 2 mM hapten **1**.[13] The relatively low affinities of the antibodies for hapten **1** resulting from the *in vitro* immunization likely reflect the absence of affinity maturation that normally occurs during an immune response.[14] In addition, antibodies generated against carbohydrates typically show relatively low affinities. It is of interest that both β-glucosidase and β-galacosidase activities were generated using hapten **1**. The five-membered ring in hapten **1** is flattened, mimicking a boat or twist-boat conformation of the pyranoside ring in the transition state. Either hapten **1** has some conformational flexibility or its geometry must map onto the critical recognition elements for both substrates and/or transition states.

Finally, Lerner[4] reported that direct immunization of a related hapten afforded catalytic antibodies with only 100-fold rate enhancements; we isolated no catalytic antibodies from direct immunization. This suggests that *in vitro* immunization, like antibody phage display methods, may complement conventional approaches for producing catalytic antibodies.

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Footnotes and References

† 5-Keto-D-fructose was synthesized by incubating D-fructose with *Gluconobactor cerinus* (ATCC catalogue number IFO 3267). Hapten **1** was purified on silica gel using CH_2Cl_2–MeOH (9 : 1 v/v) as eluent (R_f = 0.37); δ_H (300 MHz, $CDCl_3$) 8.1 (s, 1 H), 8.0 (d, 1 H, *J* 3), 7.6 (d, 1 H, *J* 8), 7.45 (dd, 1 H, *J* 8, 3), 4.0–3.8 (m, 4 H), 3.6–3.3 (m, 8 H including 4H from 4 OHs), 3.1 (m, 1 H), 2.7 (m, 1 H); δ_C (75 MHz, [$^2H_6$]DMSO) 147.6 (3′ phenyl), 143.4 (1′ phenyl), 135.3 (6′ phenyl), 129.3 (4′ phenyl), 122.9 (2′ phenyl), 121.6 (4′ phenyl), 77.0, 76.1 (C3 and C4), 72.6 (C benzyl), 67.4, 62.0 (C2 and C5), 60.3, 57.4 (C1 and C6); *m/z* 299 (MH^+).

‡ Antibodies were purified using a SP-Separose® (HiLoad™ 16/10) column (Pharmacia). For the mobile phase, buffer A (50 mM MES, pH 5.5) and buffer B (50 mM MES, 1.0 M NaCl, pH 5.5) were co-eluted with a linear gradient of 0–50% B over 40 min and a flow rate of 2 ml min^{-1}. Assays were carried out by mixing 90 μl of antibody (2–3 mg ml^{-1}) and 10 μl of substrate (20 mM), each in reaction buffer, and then incubating at 37 °C for 3 days. The reaction was analysed by HPLC using a C18 microsorb column (4.6 mm × 15 cm, 5 μm), eluting with 50% aqueous MeOH at 0.8 ml min^{-1} and monitoring a 315 nm. The rate was determined when less than 1% of the substrate was converted to product. The data were fitted directly to the Michaelis–Menten equation, $v = (V_{max}[S])/([S] + K_m)$, using the nonlinear curve fitting program from the Kaleidagraph software suite. The second order rate constants for the H^+, OH^- and AcOH catalysed reactions and the psuedo-first order rate constant for the reaction in water are: k_{H^+} = 1.1 × 10^{-3} M^{-1} h^{-1}, k_{OH^-} = 0.29 M^{-1} h^{-1}, k_{AcOH} = 1.2 × 10^{-6} M^{-1} h^{-1}, and k'_{H_2O} = 8.0 × 10^{-7} h^{-1} for substrate **3**; k_{H^+} = 3.8 × 10^{-3} M^{-1} h^{-1}, k_{OH^-} = 0.58 M^{-1} h^{-1}, k_{AcOH} = 2.0 × 10^{-6} M^{-1} h^{-1} and k'_{H_2O} = 1.6 × 10^{-6} h^{-1} for substrate **4**.

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