Reactive Immunization Strategy Generates Antibodies with High Catalytic Proficiencies

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Acyl-transfer processes are ubiquitous in both biochemical metabolism and organic chemistry and as such have remained a major focus for antibody catalysis since the first reports of these programmable proteins appeared in the mid-1980s.[1] The numerous antigens utilized to elicit catalysts for these reactions can be broadly categorized as being either transition-state analogues (TSA) or bait and switch haptens.[2] To expand the scope and improve the catalytic abilities of antibodies we are directing efforts toward new strategies for immunogen design. Recently we reported a method termed reactive immunization (RI), which utilizes a labile phosphonate diester hapten which can either hydrolyze at physiological pH or trap a nucleophile at the B-cell level of the immune response.[3] We have reasoned that the combination of these events, which incorporate chemical reactivity and transition state stabilization into antibody selection, may encompass a better overall strategy for catalyst generation.[3] In the present study we combine the known stereoselectivity of antibodies[4] with the new RI approach to generate catalysts for a kinetic resolution of a racemic mixture of *p*-methylsulfonylphenyl esters of naproxen **1** (Scheme 1).[5] Chemical synthesis of **1** leads to a racemic mixture[6] and consequently there is a considerable effort being devoted to improve the methods for its asymmetric synthesis and resolution.[7]

The hapten chosen for immunization, phosphonate diester **3**, hydrolyzes under physiological conditions with expulsion of a *p*-methylsulfonylphenol leaving group (pK_a 7.8).[8] By immunizing with a racemate, it was expected that antibody catalysts with differing enantioselectivities would be generated and that during screening, antibodies with the required (+)-(*S*)-selectivity could be highlighted.[4a,e]

Following synthesis and coupling of hapten **3** to carrier proteins,[9] 129 Gix$^+$ mice were immunized with a keyhole limpet haemocyanin (KLH)-**3** conjugate. Monoclonal antibodies were produced by standard techniques[10] and purified from hybridoma supernatants as described previously.[11] Of 20 monoclonal antibodies that bound to a bovine serum albumin (BSA)-**3** conjugate, 12 catalyzed the hydrolysis of the substrate *rac*-**2**.[12] The kinetic parameters and enantioselectivities of the five most active antibody catalysts were studied in detail (Table 1). In all cases the catalysis was competitively inhibited by both phosphonates **4** and **5**.[13] This cross-reactivity supports the notion that catalysis occurs in the antibody combining site and that the RI strategy involves antibody recognition of structural components along the hydrolysis coordinate of the phosphodiester hapten. A defining precept of RI is the installation of chemical reactivity within the antibody binding-site to the hapten. This was determined kinetically for antibody 15G12, which catalyzes the hydrolysis of the hapten analogue **4** (K_m = 232 μM, $k_{cat} = 2.1 \times 10^{-3}$ min^{-1}). Interestingly, no phosphonylation of 15G12 was detected during this process, and in contrast to our previous report,[3] no acyl−antibody intermediate was detected, suggesting either that acylation is rate-limiting or that the chemical reactivity engendered by RI, in this case, is not covalent in origin.

The most active members of the panel have enhancement ratios, k_{cat}/k_{uncat}, of greater than 10^5, highlighting the catalytic power imparted by RI. Antibodies 5A9 and 6C7 have high substrate specificity, which coupled with their high turnover numbers, k_{cat}, results in reaction specificity constant values, $k_{cat}/K_m > 10^5$ M^{-1} min^{-1}. Wolfenden[14a] has defined the catalytic proficiency of a biocatalyst by division of its second-order specificity constant with the background rate of substrate hydrolysis. By this rationale, the three most active monoclonals, 5A9, 6C7, and 15G12 have proficiency constants of 4.66, 2.75, and 2.20×10^9 M^{-1}, respectively.[14b] These values are among the highest achieved by catalytic antibodies for any acyl transfer process, whether elicited by transition state analogue, bait and switch, or heterologous immunization approaches,[15] and are comparable with a number of lipases and esterases (10^9−10^{12} M^{-1}),[16a] including A-esterase (EC 3.1.1.2).[16b]

At present, industrial production of (*S*)-naproxen **1** involves diastereomeric crystallization of a racemic acid mixture.[6] Several reports of enzymatic resolution of naproxen alkyl esters

(1) (a) Tramontano, A.; Janda, K. D.; Lerner, R. A. *Science* **1986**, *234*, 1566−1570. (b) Pollack, S. J.; Jacobs, J. W.; Schultz, P. G. *Science* **1986**, *234*, 1570−1573.

(2) (a) Janda, K. D.; Shevlin, C. G.; Lo, C.-H. L. In *Comprehensive Supramolecular Chemistry*; Yakito, M., Ed.; Pergamon: London, 1996; Vol. 4, pp 43−78. (b) Lavey, B. J.; Janda, K. D. *ACS. Symp. Ser.* **1995**, *604*, 123−137.

(3) Wirsching, P.; Ashley, J. A.; Lo, C.-H. L.; Janda, K. D.; Lerner, R. A. *Science* **1995**, *270*, 1775−1782.

(4) For leading papers on antibody stereoselectivity, see: (a) Janda, K. D.; Benkovic, S. J.; Lerner, R. A. *Science* **1989**, *244*, 437−440. (d) Shoji, I.; Weinhouse, W. I.; Janda, K. D.; Lerner, R. A.; Danishefsky, S. J. *J. Am. Chem. Soc.* **1990**, *113*, 7763−7764. (e) Fujii, I.; Lerner, R. A.; Janda, K. D. *J. Am. Chem. Soc.* **1991**, *113*, 8258−8529. (f) Pollack, S. J.; Hsuin, P.; Schultz, P. G. *J. Am. Chem. Soc.* **1992**, *114*, 2257−2258.

(5) Profens are a widely prescribed class of non-steroidal anti-inflammatory drugs (NSAIDs) for the treatment of rheumatoid arthritis [Payan, D. G.; Shearn, M. A. In *Basic and Clinical Pharmacology*; Katzung, B. G., Ed.; Appleton and Lange: London, 1989; pp 431−432]. Naproxen **1**, a prominent member of this drug class, exhibits both stereoselective activity and disposition [(a) Wechter, W. J.; Loughhead, R. J.; Reischer, G. J.; VanGiessen, G. J.; Kaiser, D. G. *Biochem. Biophys. Res. Commun.* **1974**, *61*, 833−835. (b) Roszowski, A. P.; Rooks, W. H., II; Tomolonis, A. J.; Miller, L. M. *J. Pharmacol. Exp. Ther.* **1971**, *179*, 114−117].

(6) Harrison, I. T.; Lewis, B.; Nelson, P.; Rooks, W.; Roszowski, A.; Tomolonis, A.; Fried, J. H. *J. Med. Chem.* **1970**, *13*, 203−207.

(7) (a) Gu, Q.-Ming; Chen, C.-S.; Sih, C. J. *Tetrahedron Lett.* **1986**, *27*, 1763−1766. (b) Hernáiz, M. J.; Sánchez-Montero, J. M.; Sinisterra, J. V. *Tetrahedron Lett.* **1994**, *50*, 10749−10760. (c) Manimaran, T.; Stahly, G. P. *Tetrahedron: Assymetry* **1993**, *4*, 1949−1954. (d) Pirkle, W. H.; Welch, C. J.; Lamm, B. *J. Org. Chem.* **1992**, *5*, 3854−3860. (e) Pirkle, W. H.; Liu, Y. *J. Org. Chem.* **1994**, *59*, 6911−6916.

(8) The phosphonate diester **3** hydrolyzes to its monoester with a half-life, $t_{1/2}$, of 2 d in phosphate-buffered saline (PBS) (200 mM, pH 7.4) at 310 K as determined by HPLC.

(9) The synthesis and spectroscopic data for hapten **3** is reported in the Supporting Information. Protein conjugates were prepared by the sulfo-NHS ester method in PBS (10% DMF).

(10) (a) Köhler, G.; Howe, S. C.; Milstein, C. *Eur. J. Immunol.* **1976**, *6*, 292−295. (b) Köhler, G.; Milstein, C. *Ibid.* **1976**, *6*, 511−519.

(11) Janda, K. D.; Schloeder, D.; Benkovic, S. J.; Lerner, R. A. *Science* **1988**, *241*, 1188−1191.

(12) BSA an "off the shelf protein" which has been reported to accelerate medium-sensitive reactions (Hollfelder, F.; Kirby, A. J.; Tawfik, D. S. *Nature* **1996**, *383*, 60−63. Kikuchi, K.; Thorn, S. N.; Hilvert, D. *J. Am. Chem. Soc.* **1996**, *118*, 8184−8185) does not catalyze the hydrolysis of *rac*-**2**.

(13) For assay conditions and K_i data see Supporting Information.

(14) (a) Radzicka, A. R.; Wolfenden, R. A. *Science* **1995**, *267*, 90−93. (b) Kinetic investigation of the antibodies in Table 1 revealed neither accumulation of detectable acylated intermediate nor pronounced curving of the progress curves during antibody-catalyzed hydrolysis of **2**, both of which lends support to this analysis [Koshland, D. E., Jr. *Bio. Rev. Camb. Philos.* **1953**, *28*, 416−418. Wolfenden, R. A. *Annu. Rev. Biophys. Bioeng.* **1976**, *5*, 271−306].

(15) Data taken from the following key reviews: Thomas, N. R. *Appl. Biochem. Biotechnol.* **1994**, *47*, 345−372 and references cited therein. Stewart, J. D.; Benkovic, S. J. *Nature* **1995**, *375*, 388−391 and references cited therein.

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Scheme 1. Phosphonate Diester **3**, Inhibitors **4** and **5**, and Racemic Esters **2** Utilized in a Reactive Immunization Approach for the Antibody-Catalyzed Kinetic Resolution of Esters of Naproxen **1**

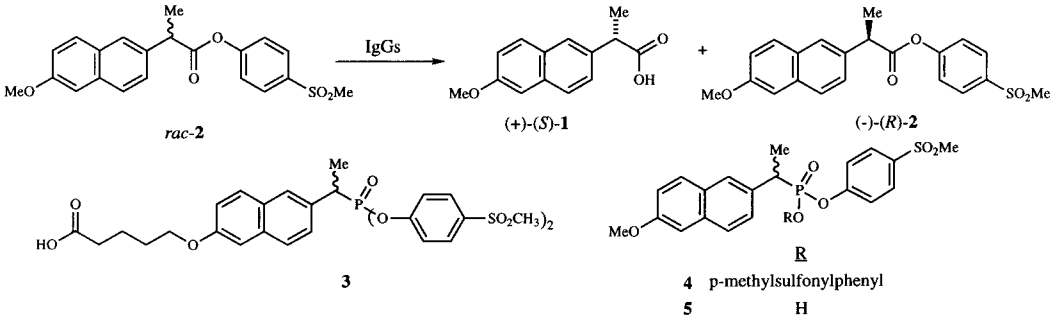

Table 1. Parameters for Antibody Catalysis of (+)-(*S*)-**2** and (−)-(*R*)-**2** Hydrolysis[a]

antibody	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} min^{-1})	E[b]
		(+)-(*S*)-**2** Hydrolysis		
15G12	28.1	300.8	9.34×10^4	29.0
7H9	3.3	576.0	5.73×10^3	30.5
5A9	2.3	11.5	2.00×10^5	22.5
7E1	1.6	206.6	7.74×10^3	56.9
6C7	0.2	1.6	1.25×10^5	123.4
		(−)-(*R*)-**2** Hydrolysis		
5A9	0.49	55.4	8.80×10^3	
15G12	0.23	69.9	3.29×10^3	
7E1	0.17	1228.5	1.38×10^2	
7H9	0.14	735.7	1.90×10^2	
6C7	0.06	58.0	1.03×10^2	

[a] Kinetic assays were performed in aqueous buffer (100 mM bicine, pH 8.0) with 5% DMF and 1% Tween 80 to enhance substrate solubility. The reaction was followed by monitoring generation of either (+)-(*S*)-**1** or (−)-(*R*)-**1** by reversed-phase HPLC. The assay was started by addition of the substrate in DMF, either (+)-(*S*)-**2** or (−)-(*R*)-**2** (1−300 μM), to a mixture of the antibody (0.1−1.75 μM) in the aqueous buffer system. Kinetic parameters were calculated using nonlinear regression analysis of the raw data with the Grafit computer program. The uncatalyzed rate, $k_{uncat} = 4.24 \times 10^{-5}$ min^{-1}, of (+)-(*S*)-**2** and (−)-(*R*)-**2** is the observed rate of hydrolysis in the aqueous buffer system (100 mM bicine, pH 8.0) used for the antibody kinetics. [b] E is the ratio of the second-order specificity constants k_{cat}/K_m of the two enantiomers.[19]

state observed ee's of >98% of **1** for up to 39% conversion.[7a,b17] All five monoclonal antibodies in Table 1 possess the correct (+)-(*S*)-**2** selectivity, although this was not programmed by racemic hapten **3**, as discussed *vide supra*. The most stereoselective clone, 5A9, produces (+)-(*S*)-naproxen acid **1** with a 90% ee after 26% conversion.[18] All the antibodies have E values of >20 which should yield >90% ee for this nondynamic kinetic resolution for up to 35% conversion.[19] This analysis assumes that neither product inhibition nor nonproductive binding of substrate enantiomers contributes during the resolution. While studying the antibody-catalyzed hydrolysis of *rac*-**2**, it became clear that the poorer substrate enantiomer, (−)-(*R*)-**2**, binds more tightly and inhibits the antibody-catalyzed hydrolysis of the (+)-(*S*)-**2** enantiomer for 6C7 ($K_{i\ app}$[(−)-(*R*)-**2**] = 1 μM) and 7E1 ($K_{i\ app}$[(−)-(*R*)-**2**] = 37 μM), thus contributing to the observed reduction of ee's. It is reasoned that, for future studies, this unwanted recognition of the *R* enantiomer can be minimized by utilizing a homochiral hapten.

In conclusion, these experiments have highlighted RI as a strategy for generation of a panel of antibody catalysts with proficiency quotients among the highest yet observed for acyl-transfer processes. One clone, 5A9, couples high catalytic activity with a 90% ee for production of the (+)-(*S*)-naproxen acid **1**. Perhaps one of the most exciting features of this work is its applicability. The programmable nature, specificity, and power of catalysts produced by RI mean that reactions thought previously to be out of the realms of catalytic antibodies may now be a step closer.

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Supporting Information Available: Spectroscopic data and synthetic information for hapten **3**, details of antibody production and purification, full kinetic assay protocols and K_i values for inhibitors **4** and **5** (9 pages). See any current masthead for ordering and Internet access instructions.

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(16) (a) Data taken from: Schomburg, D.; Salzmann, M. In *Enzyme Handbook*; Springer-Verlag: Berlin, 1991; Vol. 3 and references cited therein. (b) A-esterase is an aryl esterase which hydrolyzes substituted phenyl esters of acetic acid [Florkin, M.; Stotz, E. H. In *Comprehensive Biochemistry, Vol. 13: Enzyme Nomenclature*; Elsevier: Amsterdam, 1965; p 126]. Its natural substrate is unknown, but *p*-nitrophenylacetate, one of its best substrates, is hydrolyzed with a proficiency quotient of 10^9-10^{10} M^{-1} [Bosmann, H. B. *Biochim. Biophys. Acta* **1972**, *276*, 180−191].

(17) (a) Mutsaers, J. H. G. M.; Kooreman, H. J. *Recl. Trav. Chim. Pays-Bas* **1991**, *110*, 185−188.

(18) For ee determination the assay conditions were as described above (footnote, *a*, Table 1) with the following exceptions. Racemic ester *rac*-**2** was the substrate (300 μM), and the enantiomers of the naproxen acid **1** were resolved by chiral HPLC [Phenomenex reversed-phase column with an isocratic NH_4OAc (30 mM, pH 4.5) in methanol mobile phase].

(19) (a) Chen, C.-S.; Wu, S.-H.; Girdaukas, G.; Sih, C. J. *J. Am. Chem. Soc.* **1987**, *109*, 2812−2817.