anti-Sulfonamide antibodies catalyse the hydrolysis of a heterocyclic amide

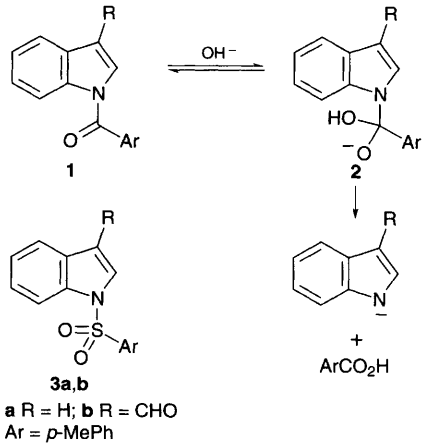
Fabio Benedetti,*[a] Federico Berti,[a] Alfonso Colombatti,[b] Cynthia Ebert,[c] Paolo Linda[c] and Federico Tonizzo[a]

[a] *Dipartimento di Scienze Chimiche, Università di Trieste, via Giorgieri 1. 34127 Trieste, Italy*
[b] *Dipartimento di Scienze e Tecnologie Biomediche, Università di Udine, Via Gervasutta 48, 33100 Udine, Italy*
[c] *Dipartimento di Scienze Farmaceutiche, Università di Trieste, p. le Europa 1, 34127 Trieste, Italy*

The hydrolysis of *N*-*p*-toluoylindole and its 3-formyl derivative, at pH 8.0, are accelerated by a factor of nearly 10^3 by an antibody obtained against a KLH conjugate of *N*-*p*-toluensulfonylindole.

Hydrolysis of the amide bond is a major target of research in catalytic antibodies. This approach could be of use in the design of catalysts for sequence specific peptide hydrolysis; however, amide hydrolysing antibodies are still rare in spite of many efforts. So far, the only successful approach to amidase-like antibodies has been based on the use of phosphinate or phosphonate esters[1] and amides[2] as haptenic transition state analogues (TSA). In contrast, esterase-like antibodies have been obtained against several types of TSA,[3] including well known enzyme inhibitors such as tetrahedral phosphorus compounds,[4] α-fluoro ketones[5] and amino alcohols.[6] Thus the design of new haptens capable of producing antibodies with amidase activity is still a challenge. Here we report that the hydrolysis of a heterocyclic amide is accelerated by monoclonal antibodies obtained against a sulfonamide hapten

As a model reaction we chose the hydrolysis of a heterocyclic amide **1**, Scheme 1. It has been shown that the base catalysed hydrolysis of closely related aroyl pyrroles proceeds *via* rate determining decomposition of the tetrahedral intermediate, from which the leaving group departs as an anion.[7,8] This step could be either spontaneous[7] or catalysed by OH^-[8] and it has been calculated[7] that the transition state for the spontaneous decomposition of the intermediate, which appears to be the likely process, is reagent-like and thus similar to the tetrahedral intermediate itself. This transition state is mimicked, in our approach, by the sulfonamide analogue **3**, Scheme 1; TSA **3** and intermediate **2** adopt a similar conformation and an RMS overlay of the PM3 minimized molecules gives an error of only 0.73 Å.† Unlike the phosphorus derivatives previously used as TSAs sulfonamides lack a net charge; however the S–O bonds are strongly polarized and a partial charge of −0.82 is localized by PM3 on the oxygen atoms of **3**.

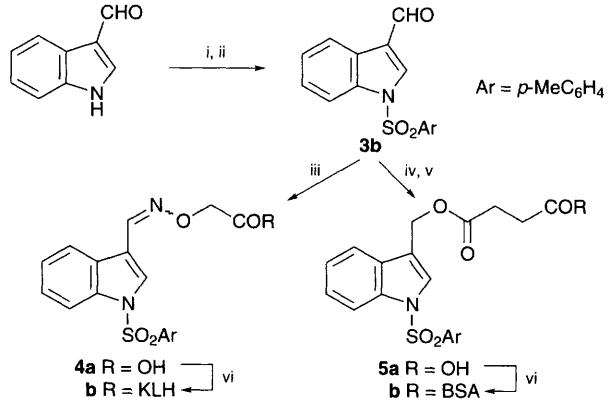


Scheme 1

Haptens **4a** and **5a** were synthesized from commercially available 3-formyl indole, as outlined in Scheme 2, and then conjugated with the carrier proteins keyhole limpet hemocyanin (KLH) and bovine serum albumine (BSA), respectively. Balb/c mice were then immunized with the KLH conjugate **4b** and monoclonal antibodies, obtained by standard methods,[9] were screened by ELISA for binding to the BSA conjugate **5b**. Seven antibodies were thus selected that were specific for the indole sulfonamide epitope. In a preliminary kinetic test two out of seven antibodies induced a marked acceleration in the hydrolysis of the amide **1b** at pH 8. Antibody 312D6, an IgG2a which showed the higher acceleration, was chosen for a more detailed study.

Amide **1a** was incubated at 25 °C and pH 8.0 (10 mmol dm^{-3} TRIS buffer) in H_2O containing 10% dioxane with the antibody (2 μmol dm^{-3}), purified from ascite fluid by ammonium sulfate precipitation and protein G affinity chromatography.‡ The reaction was followed by HPLC, (C_{18}; H_2O:MeCN:TFA 1.2:1.8:0.01), monitoring the appearance of indole with a fluorescence detector (at 270 nm excitation and 343 nm emission) and 3-hydroxymethylindole as internal standard. Initial rates were measured, within 5% conversion, with substrate concentrations of 0.033–1.0 mmol dm^{-3}, and corrected for the background reaction. Hydrolysis of **1a**, catalysed by 312D6, follows saturation kinetics and the kinetic parameters were obtained from the Lineweaver–Burk plot shown in Fig. 1. The values thus obtained for the apparent pseudo-first order rate constant k_{cat} and for the Michaelis constant K_m are reported in Table 1, together with the apparent rate constant k_0 for the background reaction, at the same pH. The results give a k_{cat}/k_0 ratio equal to 1500 per antibody molecule or 750 per catalytic site.

Antibody 312D6 also accelerates the hydrolysis of amide **1b** with k_{cat}/k_0 = 780 (390 per site), Table 1. The background reaction for this substrate, activated by the 3-formyl group, is



Scheme 2 *Reagents and conditions*: i, NaH, THF, 25 °C; ii, $ArSO_2Cl$, reflux, 81%; iii, *O*-carboxymethyl hydroxylamine hydrochloride, NaOH, EtOH–H_2O, reflux, 87%; iv, $NaBH_4$, MeOH–H_2O; v, succinic anhydride, pyridine, reflux, 73%; vi, KLH or BSA, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC), pH 4.5

approximately 5000-fold faster than the uncatalysed hydrolysis of **1a** at the same pH. Also with this substrate the catalysed reaction follows Michaelis–Menten kinetics and k_{cat} and K_m (Table 1) were obtained in the usual way, Fig. 1. It is remarkable that k_{cat}/k_0 ratios vary by only a factor of two on going from amide **1a** to **1b**, in spite of the substantial reactivity difference between the two substrates.

The 312D6-catalysed hydrolysis of both **1a** and **1b** was completely inhibited by the addition of transition state analogue **3a** or **3b** in a concentration twice that of the antibody. This clearly demonstrates that catalysis is taking place in the antibody combining site and is not due to interactions between the substrate and the antibody's surface. In a further experiment, antibody 312D6 (7.5 μmol dm^{-3}) was incubated with a 15 fold excess of amide **1b** until the UV absorption of the solution was constant and corresponding to the end point of the reaction; a second batch of substrate **1b** (15 fold excess) was added and the reaction was again followed spectrophotometrically to show that the rate of the catalysed reaction had not changed. The antibody thus exhibits multiple turnover and, at these concentrations, is not inhibited by the products.

For an antibody accelerating a reaction by transition state complementarity, it can be derived that $K_m/K_i \cong k_{cat}/k_0$.[10] Binding constants K_i for the complexes between 312D6 and haptens **3a** and **3b** were measured by a competition ELISA[11] and were 1.0 and 1.3 μmol dm^{-3}, respectively; a similar value (0.86 μmol dm^{-3}) was obtained, by a direct assay, for the binding constant of the BSA conjugate **5b**. Since this method is likely to overestimate the binding constant, then K_i obtained this way should be taken as an upper limit. K_m/K_i ratios for the 312D6 catalysed hydrolysis of amides **1a** and **1b** are somewhat lower than the corresponding k_{cat}/k_0 ratios, Table 1; considering, however, the approximation on K_i, it appears that a substantial fraction of hapten complementarity is reflected into catalytic activity.

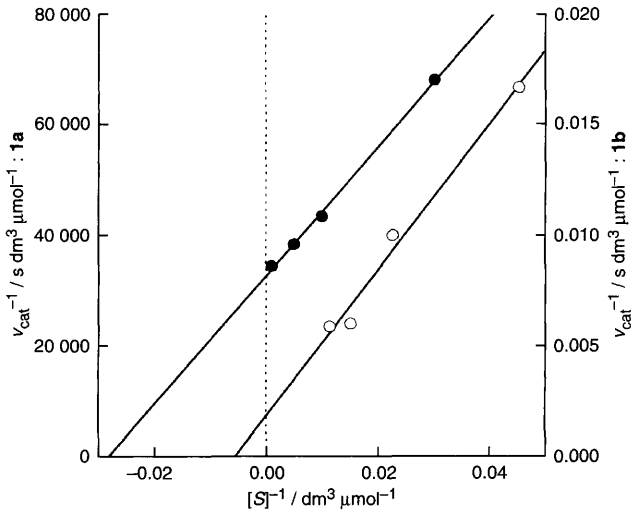


Fig. 1 Lineweaver–Burk plot for the 312D6-catalysed hydrolysis of amides **1a** (●; left axis) and **1b** (○; right axis) 10% aq. dioxane, pH = 8.0, T = 25 °C, [antibody] = 2 μmol dm^{-3}, [TRIS] = 10 mmol dm^{-3} [NaCl] = 0.1 mol dm^{-3}

Table 1 Kinetic parameters for the 312D6-catalysed hydrolysis of amides **1a** and **1b**[a]

Amide **1**	k_0[b]/s^{-1}	k_{cat}[b]/s^{-1}	K_m/mol dm^{-3}	K_m/K_i	k_{cat}/k_0
a	1.01×10^{-8}	7.5×10^{-6}	3.57×10^{-5}	36	750
b	5.24×10^{-5}	2.1×10^{-2}	1.5×10^{-4}	150	400

[a] Conditions: 2 μmol dm^{-3} antibody, 10% aq. dioxane, 10 mmol dm^{-3} TRIS buffer, 0.1 mol dm^{-3} NaCl, pH 8.0, 25 °C. [b] Observed pseudo-first order rate constant at pH 8.0.

The immunological work was carried out at the Centro di Riferimento Oncologico (CRO - Aviano) and we are grateful to Mrs Maria Teresa Mucignat for the preparation of monoclonal antibodies. This work was supported by the CNR (Progetto Finalizzato Biotecnologie e Biostrumentazione) by the European Network on Antibody Catalysis, Human Capital and Mobility Programme, and by Glaxo Italia (Postdoctoral fellowship to F. Berti).

Footnotes

† PM3 calculations were performed with the HYPERCHEM program (Hypercube, Inc.). Geometry optimization was carried out by the conjugate gradient method with a final gradient norm smaller than 0.01 kcal Å^{-1} (1 cal = 4.184 J).

‡ Purity (>95%) was assessed by SDS-polyacrylamide gel electrophoresis.

References

1 M. T. Martin, T. S. Angeles, R. Sugasawara, N. I. Aman, A. D. Napper, M. J. Darsley, R. I. Sanchez, P. Booth and R. C. Titmas, *J. Am. Chem. Soc.*, 1994, **116**, 6508; L. J. Liotta, P. A. Benkovic, G. P. Miller and S. J. Benkovic, *J. Am. Chem. Soc.*, 1993, **115**, 350; G. Gallacher, M. Searcey, C. Jackson and K. Brocklehurst, *Biochem. J.*, 1992, **284**, 675.

2 K. D. Janda, D. Schloeder, S. J. Benkovic and R. A. Lerner, *Science*, 1988, **241**, 1188.

3 Reviews: R.A. Lerner, S. J. Benkovic and P. G. Schultz, *Science*, 1991, **252**, 659; U. K. Pandit, *Recl. Trav. Chim. Pays-Bas*, 1993, **112**, 431.

4 J. Guo, W. Huang and T. S. Schanlan, *J. Am. Chem. Soc.*, 1994, **116**, 6062; D. A. Campbell, B. Gong, L. M. Kochersperger, S. Yonkovich, M. A. Gallop and P. G. Schultz, *J. Am. Chem. Soc.*, 1994, **116**, 2165; K. Ohkubo, Y. Urata, K. Seri, H. Ishida, T. Sagawa, T. Nakashima and Y. Imagawa, *J. Mol. Catal.*, 1994, **90**, 355; B. H. Wilmore and B. L. Iverson, *J. Am. Chem. Soc.*, 1994, **116**, 2181.

5 T. Kitazume, T. Tsukamoto and K. Yoshimura, *J. Chem. Soc., Chem. Commun.*, 1994, 1356.

6 H. Suga, O. Ersoy, T. Tsumuraya, J. Lee, A. J. Sinskey and S. Masamune, *J. Am. Chem. Soc.*, 1994, **116**, 487; H. Suga, O. Ersoy, S. F. Williams, T. Tsumuraya, M. N. Margolies, A. J. Sinskey and S. Masamune, *J. Am. Chem. Soc.*, 1994, **116**, 6025.

7 R. S. Brown, A. J. Bennet, H. Slebocka-Tilk and A. Jodhan, *J. Am. Chem. Soc.*, 1992, **114**, 3092.

8 A. Cipiciani, P. Linda and G. Savelli, *J. Heterocycl. Chem.*, 1979, **16**, 673; F. M. Menger and J. A. Donohue, *J. Am. Chem. Soc.*, 1973, **95** 432.

9 E. Harlow and D. Lane, *Antibodies, a Laboratory Manual*, Cold Spring Harbor Laboratory, New York, 1988; pp. 139–243.

10 S. J. Benkovic, *Annu. Rev. Biochem.*, 1992, **61**, 29; J. D. Stewart and S. J. Benkovic, *Nature*, 1995, **375**, 388.

11 B. Friguet, A. F. Chaffotte, C. Djaudi-Ohaniance and M. E. Goldberg, *Immunol. Methods*, 1985, **77**, 305.

Received, 6th February 1996; Com. 6/00864J