Antibody Catalysis of $B_{Ac}2$ Aryl Carbamate Ester Hydrolysis: A Highly Disfavored Chemical Process

Paul Wentworth, Jr., Anita Datta, Simon Smith, Ann Marshall, Lynda J. Partridge, and G. Michael Blackburn*

Departments of Chemistry and Molecular Biology The Krebs Institute for Biomolecular Research University of Sheffield, Sheffield, S3 7HF, United Kingdom

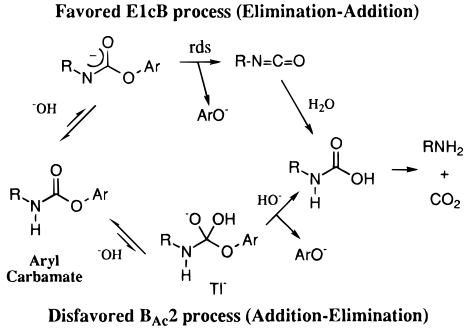
Received May 15, 1996

The recent application of antibody catalysis to the area of disfavored chemistry is one of the most significant contributions to have emerged from this rapidly expanding subject. Notable achievements to date include the reversal of kinetic control in reactions such as ring-formations,[1] Diels−Alder cycloadditions,[2] cationic cyclizations,[3] and eliminations.[4] Herein we describe and characterize an antibody which effectively reroutes aryl carbamate ester hydrolysis through the highly disfavored $B_{Ac}2$ pathway.

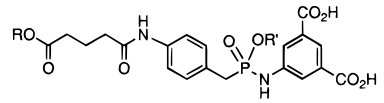
Aryl carbamate ester hydrolysis is known to occur dominantly *via* an E1cB mechanism, while the $B_{Ac}2$ pathway involving a tetrahedral intermediate (Scheme 1) operates effectively only for carbamate esters lacking an ionizable N−H group.[5] The difference in the relative rates of these two alternative mechanistic processes has been studied extensively by Hammett $\sigma-\rho$ relationships,[6] inverse deuterium isotope effects,[5] trapping of reactive intermediates,[7] and the use of *N*,*N*-disubstituted aryl carbamate esters.[8] Hammett correlations of the exponent of the rate constant for hydroxide promoted E1cB hydrolysis with the parameter σ for *N*-monosubstituted aryl carbamates[6a,7b] have given ρ values of +2.87 and +3.16. By contrast, the linear free energy analysis for *N*,*N*-disubstituted aryl carbamate ester[5d] and aryl ester[8] hydrolyses (both of which proceed *via* a $B_{Ac}2$ mechanism) give ρ values of +1.24 and *ca.* +1.0, respectively.

In the present study we have synthesized phosphonamidates **1a** and **1b** as haptens for the production of monoclonal antibodies capable of hydrolyzing the *p*-nitrophenyl *N*-aryl carbamate **2a** (Figure 1). While **2a** hydrolyzes rapidly[9] *via* the E1cB mechanism, the rate of its $B_{Ac}2$ hydrolysis is immeasurably small but can be estimated from the rate of hydrolysis[10] of its *N*-Me derivative **3a**. The ratio of these rates is *ca.* 10^8, a value entirely consistent with previous comparisons of the E1cB and $B_{Ac}2$ rates of hydrolysis of substituted carbamates.[5c,d]

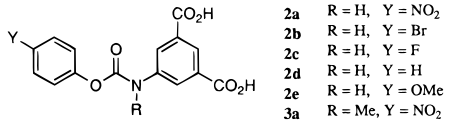
Scheme 1. Duality of Mechanism for Carbamate Ester Hydrolysis[a]



[a] Mechanistic studies have shown that the rate of alkaline hydrolysis *via* the $B_{Ac}2$ process is up to 10^8 times slower than for the E1cB process (dependent on substitution in the aryl ring).[5,6]



1a R' = Et, R = succinimidyl
1b R' = H, R = succinimidyl
1c R' = H, R = H



2a R = H, Y = NO_2
2b R = H, Y = Br
2c R = H, Y = F
2d R = H, Y = H
2e R = H, Y = OMe
3a R = Me, Y = NO_2

Figure 1. Phosphonamidate haptens **1a** and **1b** were used for the generation of antibodies for the hydrolysis of the carbamate substrate **2a**. Modified hapten **1c** was used for inhibition studies. The carbamates **2b**−**e** were used as a range of substrates to support a Hammett $\sigma-\rho$ correlation (see Table 1). The rate of hydrolysis of the *N*-methyl carbamate **3a** was used to estimate a k_{app} for the $B_{Ac}2$ hydolysis of **2a**.

Haptens **1a** and **1b** have three key features. First, in common with previous strategies for the elicitation of antibodies that catalyze $B_{Ac}2$ hydrolysis reactions,[11] the inclusion of a tetrahedral phosphoryl core was considered essential. Second, the phenolic oxygen of the substrate was replaced with a benzylic methylene moiety in the haptens **1a** and **1b** to minimize recognition of phenolate anion character known to be a prime feature of the E1cB transition state.[6a] Third, the glutaric acid linker was appended at the para-position of the benzylphosphonate group in both haptens with the expectation of eliciting an antibody with little or no recognition of this feature in its substrates as supported by previous work in the catalytic antibody field, including X-ray crystal structure analysis.[12] Such an antibody could then be used in a Hammett correlation of reactivity for a series of *p*-substituted-phenyl carbamate esters. This analysis, a focal postulate of our design, allows for a mechanistic differentiation of the catalytic antibodies, because it is clear that the E1cB process is much more dependent on the leaving group ability than the disfavored $B_{Ac}2$ process.

Haptens **1a** and **1b** were synthesized[13] and conjugated to the carrier protein keyhole limpet haemocyanin (KLH) for im-

* Author to whom correspondence should be addressed.

(1) Janda, K. D.; Shevlin, C. G.; Lerner, R. A. *Science* **1993**, *259*, 490−493.

(2) Gouverneur, V. E.; Houk, K. N.; De Pascual-Teresa, B.; Beno, B.; Janda, K. D.; Lerner, R. A. *Science* **1993**, *262*, 204−208.

(3) Li, T. Y.; Janda, K. D.; Ashley, J. A.; Lerner, R. A. *Science* **1994**, *264*, 1289−1293.

(4) Cravatt, B. F.; Ashley, J. A.; Janda, K. D.; Boger, D. L.; Lerner, R. A. *J. Am. Chem. Soc.* **1994**, *116*, 6013−6014.

(5) (a) Bender, M. L.; Homer, R. B. *J. Org. Chem.* **1965**, *30*, 3975−3978. (b) Bruice, T. C.; Holmquist, B. *J. Am. Chem. Soc.* **1968**, *90*, 7136−7138. (c) Christenson, I. *Acta. Chem. Scand.* **1964**, *18*, 904−922. (d) Dittert, L. W. Ph.D. Thesis, WI, 1961. (e) Holmquist, B.; Bruice, T. C. *J. Am. Chem. Soc.* **1969**, *91*, 2993−3002. (f) Pratt, R. F.; Bruice, T. C. *J. Am. Chem. Soc.* **1970**, *92*, 5956−5964.

(6) (a) Williams, A. *J. Chem. Soc., Perkin Trans. II* **1972**, 808−812. (b) Williams, A.; Douglas, K. T. *J. Chem. Soc., Perkin Trans. II* **1972**, 1455−1459. (c) Williams, A.; Douglas, K. T. *J. Chem. Soc., Perkin Trans. II* **1972**, 2112−2115.

(7) (a) Hegarty, A. F.; Frost, L. N. *J. Chem. Soc., Chem. Commun.* **1972**, 500−501. (b) Hegarty, A. F.; Frost, L. N. *J. Chem. Soc., Perkin Trans. II* **1973**, 1719−1728. (c) Hegarty, A. F.; Frost, L. N.; Coy, J. H. *J. Org. Chem.* **1974**, *39*, 1089−1093.

(8) (a) Bruice, T. C.; Mayahi, M. F. *J. Am. Chem. Soc.* **1960**, *82*, 3067−3071. (b) Kirsch, J. F.; Clewell, W.; Simon, A. *J. Org. Chem.* **1968**, *33*, 127−132.

(9) Datta, A. Ph.D. Thesis (Sheffield, 1996).

(10) In a typical run, the carbamate ester **3a** (20 μL of a stock solution in DMSO) was added to sodium hydroxide (pH range 12 to 14, $I = 1.0$) to a final volume of 500 μL in a quartz cuvette. The UV kinetic assay was performed in a thermostatted cuvette rack (288 K) at 405 nm in a Philips PU 8720 UV/vis spectrophotometer.

(11) (a) Janda, K. D.; Schloeder, D.; Benkovic, S. J.; Lerner, R. A. *Science* **1988**, *241*, 1188−1191. (b) Tramontano, A.; Ammann, A. A.; Lerner, R. A. *J. Am. Chem. Soc.* **1988**, *110*, 2282−2286. (c) Lerner, R. A.; Tramontano, A. *TIBS* **1987**, *12*, 427−430. (d) Stewart, J. D.; Benkovic, S. J. *Chem. Soc. Rev.* **1993**, *22*, 213−219. (e) Stewart, J. D.; Benkovic, S. J. *Nature* **1995**, *375*, 388−391.

(12) Haynes, M. R.; Stura, E. A.; Hilvert, D.; Wilson, I. A. *Science* **1994**, *263*, 646−652.

S0002-7863(96)01625-3 CCC: $14.00

Table 1. Kinetic Parameters for Spontaneous and DF8-D5 Catalyzed Hydrolysis of the Substituted Aryl Carbamates **2a**−**e**

substrate group **Y**	k_{cat}, min^{-1}	K_m[a], mM	k_{cat}/K_m, $mM^{-1}\ min^{-1}$	k_{uncat}[b], min^{-1}	k_{cat}/k_{un}
NO_2 (**2a**)	18	120	0.15	6.0×10^{-2}	3.0×10^3
Br (**2b**)	6	80	0.075	6.0×10^{-4}	1.0×10^4
F (**2c**)	7.2	41	0.17	1.8×10^{-4}	4.0×10^4
H (**2d**)				3.0×10^{-5}	
MeO (**2e**)	4.9	58	0.08	4.2×10^{-6}	1.2×10^6

[a] The kinetic measurements were all carried out at 14 °C and pH 6.5 (50 mM MES). [b] The uncatalyzed rates were determined by multiplication of the second-order rate constant (see Supporting Information) by $1 \times 10^{-7.5}$ (the hydroxide ion concentration at pH 6.5).

munization purposes. Fifty monoclonal antibodies specific for **1a** and **1b** were tested for catalysis. The rate of hydrolysis of **2a** was assayed in 2-(*N*-morpholino)ethanesulfonic acid (MES, pH 6.5, $I = 1.0$) at 288 K, with 4% dimethyl sulfoxide (DMSO) both in the presence and absence of antibody.[14] The most active of these, DF8-D5,[15] raised against hapten **1a**, effects hydrolysis of **2a** with Michaelis−Menten kinetics and with K_m 120 μM and k_{cat} 18 min^{-1}. This catalytic activity is stoichiometrically inhibited by hapten **1c**.

Catalytic and spontaneous rate constants (k_{cat}, k_{uncat}) for a series of *p*-substituted-phenyl *N*-aryl carbamates **2a**−**e** were then determined and plotted against σ/σ^- of the phenol leaving group to determine the Hammett ρ value for the antibody catalyzed reaction (Table 1 and Figure 2).[16] The difference in the slopes of the hydroxide ($\rho = +2.68$) and DF8-D5 ($\rho = +0.53$) catalyzed hydrolyses of the carbamates (**2a**−**e**) clearly establishes a major mechanistic difference between the antibody catalyzed and spontaneous processes: the value of ρ for the hydroxide mediated hydrolysis is typical for an E1cB mechanism, while that for the DF8-D5 process is characteristic of a $B_{Ac}2$ mechanism.

As antibodies are discovered that catalyze ever more difficult processes, it becomes interesting to inquire whether there is a limiting binding energy that can be supplied in an antibody binding site to generate catalysis. Several workers have

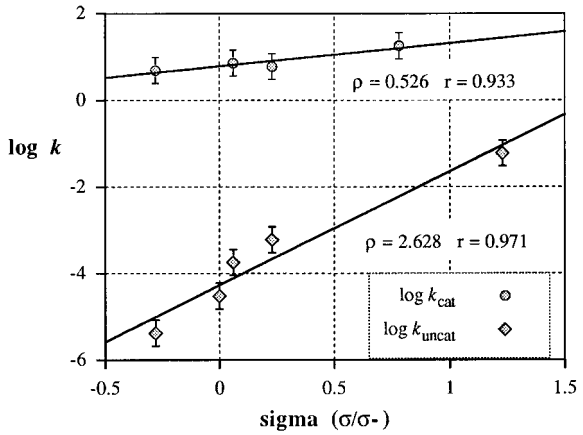


Figure 2. Hammett $\sigma-\rho$ correlation for the hydroxide catalyzed and DF8-D5 catalyzed hydrolyses of the carbamate series **2a**−**e** at pH 6.5 (50 mM MES, $I = 1.0$) and 288 K.

estimated that up to 20 kcal mol^{-1} of binding energy may be available.[17] The most energetically demanding disfavored process hitherto promoted by an antibody is that of a *syn*-elimination,[4] disfavored by 5 kcal mol^{-1}. The present data[18] estimates that for hydrolysis of the aryl carbamate **2a** there is a free energy difference between the spontaneous E1cB and $B_{Ac}2$ transition states of some 13 kcal mol^{-1}. Thus, the generation of a monoclonal antibody capable of catalyzing the highly disfavored $B_{Ac}2$ mechanism of carbamate hydrolysis is a major advancement in the catalytic antibody field.

The application of another antibody raised against hapten (**1a**) for cleavage of a carbamate prodrug to effect human tumor cell kill *ex vivo* has been reported elsewhere.[19]

Acknowledgment. The authors would like to thank Mr. Andrew Bassett (Sheffield Hybridomas) for expert assistance in monoclonal antibody production, Professor Kim. D. Janda (Scripps Research Institute) for advice concerning antibody purification, Zeneca Pharmaceuticals plc for financial support (A.M.) and for a CASE studentship (P.W.), and the BBSRC and EPSRC for research studentships (P.W. and A.D.).

Supporting Information Available: Experimental details including full analytical data for the haptens **1a**−**c** and the carbamates **2a**−**e** and **3a**, full details of antibody production and purification protocols, and kinetic data (15 pages). See any current masthead page for information and Internet access instructions.

JA961625T

(13) The synthetic route with analytical details for all intermediates in the synthesis of haptens **1a**−**c** are supplied in the supporting information.

(14) In a typical experiment, the substrate **2a** (20 μL in DMSO) was added to a solution of antibody (0.6 μM) in MES (pH 6.5, 50 mM, $I = 1$, final volume 500 μL)) thermostatically controlled at 288 K. The progress of the reaction was followed by monitoring the release of *p*-nitrophenol at 340 nm (ϵ 8124).

(15) DF8-D5 was purified by protein G chromatography and Mono Q ion exchange fplc and characterized as being of IgG_1 serotype. Its catalytic activity was identified with the dominant (>95%) protein component eluting from the Mono Q column. The slower running, minor protein component (<5%) was identified as a physical complex of IgG and serum albumin.

(16) The Hammett σ^- value (+1.27) was used for the *p*-NO_2 group in the plot of the rate constants for the hydroxide reaction in line with practice elsewhere for the background E1cB process. For reactions where inductive effects predominate ($B_{Ac}2$), then the standard σ value of +0.78 is employed.

(17) (a) Schultz, P. G.; Lerner, R. A. *Acc. Chem. Res.* **1993**, *26*, 391−395. (b) Benkovic, S. J. *Annu. Rev. Biochem.* **1992**, *51*, 29−54.

(18) The value of 13 kcal mol^{-1} for the difference in the free energies of the E1cB and $B_{Ac}2$ transition states is calculated from the ratio of the second-order rate constants for the hydrolysis of **2a** and **3a** of 1×10^8.

(19) Wentworth, P.; Datta, A.; Blakey, D.; Boyle, T.; Partridge, L. J.; Blackburn, G. M. *Proc. Natl. Acad. Sci. U.S.A.*, **1996**, *93*, 799−803.