Evidence that the mechanism of antibody-catalysed hydrolysis of arylcarbamates can be determined by the structure of the immunogen used to elicit the catalytic antibody

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A kinetically homogeneous anti-phosphate catalytic antibody preparation was shown to catalyse the hydrolysis of a series of *O*-aryl *N*-methyl carbamates containing various substituents in the 4-position of the *O*-phenyl group. The specific nature of the antibody catalysis was demonstrated by the adherence of these reactions to the Michaelis–Menten equation, the complete inhibition by a hapten analogue, and the failure of the antibody to catalyse the hydrolysis of the 2-nitrophenyl analogue of the 4-nitrophenylcarbamate substrate. Hammett σ – ρ analysis suggests that both the non-catalysed and antibody-catalysed reactions proceed by mechanisms in which development of the aryloxyanion of the leaving group is well advanced in the transition state of the rate-determining step. This is probably the E1cB (elimination–addition) mechanism for the non-catalysed reaction, but for the antibody-catalysed reaction might be either E1cB or B_{Ac}2 (addition–elimination),

in which the elimination of the aryloxy group from the tetrahedral intermediate has become rate-determining. This result provides evidence of the dominance of recognition of phenolate ion character in the phosphate hapten in the elicitation process, and is discussed in connection with data from the literature that suggest a B_{Ac}2 mechanism, with rate-determining formation of the tetrahedral intermediate for the hydrolysis of carbamate substrates catalysed by an antibody elicited by a phosphoramidate hapten in which phenolate anion character is minimized. The present paper contributes to the growing awareness that small differences in the structure of haptens can produce large differences in catalytic characteristics.

Key words: antibody, catalytic antibody, hydrolysis, mechanism.

INTRODUCTION

Among the best potentially successful enzyme mimics are catalytic antibodies, proteins whose catalytic properties rely on aspects of transition-state stabilization [1] as proposed by Jencks [2]. The design of stable analogues of transition states [3,4] as haptens involves consideration of conformational, stereochemical and electronic characteristics of the reaction to be catalysed. At its simplest, the hapten should resemble the postulated transition state rather than the ground state of the substrate [5,6]. A typical example is the successful use of a stable phosphate analogue of the postulated anionic tetrahedral transition state of the B_{Ac}2 (addition–elimination) mechanism of the reaction of a carbonate ester substrate with an hydroxide ion as the haptenic determinant of the immunogen [7]. One approach to understanding how binding energy can be exploited in catalysis is to investigate how small, relatively subtle, changes in the structures of transition-state analogues affect the kinetic characteristics of the resulting antibodies as catalysts for particular reactions. Some of our recent work aimed at contributing to this objective involved catalytic antibodies elicited by using two closely related immunogens (a phosphate and a phosphonate), differing only in the flexibility of the atomic framework around the structural motifs of the haptens analogous to the reaction centres of the corresponding carbonate ester **1** and carboxylic ester **2** substrates (Figure 1) [8,9]. The small change in the structure of the hapten, i.e. removal of one of the phosphate oxygen atoms of **3** in the phosphonate **4** resulted in a catalytic antibody preparation with enhanced catalytic characteristics [8] and increased substrate selectivity [9].

The aim of the present study was to investigate whether a small key structural change in a hapten might cause the reaction catalysed by the resulting antibody to proceed by a different mechanism. Aryl carbamates are useful for this type of investigation because their hydrolysis can proceed potentially by two alternative mechanisms. One of these is an B_{Ac}2 mechanism involving (usually) the rate-determining formation of a tetrahedral intermediate (Figure 2a). The other is an E1cB (elimination–addition) mechanism involving the rate-determining expulsion of the aryloxyanion from the anionic intermediate formed by abstraction by an hydroxide ion of the N–H proton to produce an isocyanate (R–N=C=O) intermediate (Figure 2b). This is subsequently hydrated across the C=N bond to produce R–NH–CO₂H, which is common to both mechanisms, prior to formation of RNH₂ and CO₂.

The work reported in the present paper was stimulated by the Hammett σ – ρ analysis [10] of the hydrolysis of a set of substituted arylcarbamates **7** catalysed by an antibody elicited by a phosphoramidate hapten **8** reported by Wentworth et al. [11]. These authors demonstrated a major mechanistic difference between the hydrolysis catalysed by their anti-phosphoramidate antibody (where for k_{cat} $\rho = 0.53$) and the non-catalysed hydroxide ion reaction where for $k_{\text{non-cat}}$ ρ was shown to be much larger (+2.68 in the text on p. 2316, and shown as +2.628 in their Figure 2). The value of ρ for the hydroxide ion reaction is typical for an E1cB mechanism (e.g. $\rho = +2.87$ [12]; $\rho = 3.16$ [13]), whereas that for their antibody-catalysed reaction is characteristic of a B_{Ac}2 mechanism with the attack of an hydroxide ion rate-determining (see [11] and references cited therein). Wentworth

Abbreviations used: B_{Ac}2, addition–elimination; E1cB, elimination–addition; PCA, polyclonal catalytic antibody; PCA 271-100, PCA preparation from the antiserum of sheep 271 in week 100 of the immunization programme.

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Figure 1 Structures of haptens and substrates

1 and 2 are the carbonate and carboxylic esters designed as the original substrates for catalytic antibodies generated by the phosphate hapten 3, and the phosphonate hapten, 4 respectively; 5a–5e are the *O*-aryl *N*-methyl carbamates used in the Hammett σ - ρ analysis as substrates for PCA 271-100 elicited by the phosphate hapten 3; 5f is the 2-nitrophenyl isomer of the 4-nitrophenyl *N*-methyl carbamate substrate 5a; 6 is the phosphate analogue used as an inhibitor for PCA 271-100; 7 is the series of carbamate substrates [11] of a catalytic antibody elicited by the phosphonamidate hapten 8; 9 is the truncated 4-nitrophenyl carbonate shown to be a specific substrate for our anti-phosphate catalytic antibody preparations elicited by hapten 3.

Figure 2 Transition states of the alkaline hydrolysis of *O*-aryl carbamates proceeding by (a) the $B_{AC}2$ mechanism with rate-determining formation of a tetrahedral intermediate and (b) the $E1cB$ mechanism

In mechanism (a), a rate-determining attack of an hydroxyl ion at the carbonyl carbon produces a tetrahedral species from which an aryloxyanion is eliminated; in mechanism (b), rapid abstraction of the N-H proton by an hydroxyl ion produces the anion shown, from which rate-determining expulsion of an aryloxyanion produces the isocyanate intermediate $R-N=C=O$. This intermediate is subsequently hydrated across the $C=N$ bond to produce the intermediate $R-NH-CO_2H$, common to both mechanisms, which undergoes decarboxylation to RNH_2 and CO_2 .

et al. [11] suggested that, in the context of attempting to divert the mechanism from $ElcB$ to $B_{AC}2$ for the antibody-catalysed reaction, the key design feature was the replacement of the phenolic oxygen of the substrate 7 by a benzylic methylene moiety in the hapten 8. This was an attempt to minimize recognition of the phenolate anion character of the $ElcB$ transition state (see Figure 2b). The hypothesis that the change in mechanism might depend on the presence or absence of the relevant oxygen atom in the hapten was investigated in the present work using an antibody preparation elicited by the phosphate hapten 3 in which the phenolic oxygen of the arylcarbamate substrates 5a–5e is retained. Hammett σ - ρ analysis for the hydrolysis of the arylcarbamates showed that the values of ρ for both the non-catalysed hydroxide ion reaction and the reaction catalysed by the anti-phosphate antibody are similar to each other and typical of mechanisms in which development of the leaving aryloxy group is well advanced

in the transition state of the rate-determining step. One possibility is that the E1cB mechanism of the non-catalysed hydroxide ion is maintained in the anti-phosphate antibody-catalysed reaction. Another is that the mechanism involves a tetrahedral intermediate ($B_{Ac}2$) but the rate-determining step has changed to elimination of the aryloxy ion. In either event, this result contrasts with that observed for the anti-phosphonamidate antibody used by Wentworth et al. [11]. The present study supports the hypothesis of a key role for the presence or absence of the phenolic oxygen in the hapten and contributes to the growing awareness [8,9] that small but key changes in hapten structure can control antibody-catalysed mechanisms.

MATERIALS AND METHODS

Materials

Materials used in the production and characterization of the PCA (polyclonal catalytic antibody) preparation PCA 271-100 (PCA elicited from the antiserum of sheep 271 in week 100 of the immunization programme), the substrates **5a–5f** and the hapten-analogous inhibitor **6**, other than those described below, were purchased or synthesized as described previously [7,14,15]. Chemicals, including all of the phenyl chloroformates used as starting materials for the synthesis of the *N*-methyl carbamate substrates, were from Sigma–Aldrich. Chromatographic materials were obtained from Pharmacia. The non-antibody products were characterized by 360 MHz ^1H NMR spectroscopy and high-resolution MS.

Purification of PCA 271-100

Anhydrous sodium sulphate (360 mg) was added to the relevant sheep antiserum (2 ml). The resulting suspension was vortex-mixed for 30 min at room temperature and then centrifuged at 3000 *g* at 25 °C for 10 min. The supernatant was discarded and the pellet was suspended in 2 ml of 18 % (w/v) sodium sulphate solution and vortex-mixed for 10 min. This wash procedure was repeated once more and the pellet was then redissolved in 2 ml of distilled water. This solution was applied to a Protein G Sepharose 4 Fast Flow chromatography column (5 ml) and washed with 0.05 M sodium phosphate buffer (pH 8.0) until all of the non-IgG protein (monitored at 280 nm) had been removed. The IgG adsorbed on to the column was eluted with 0.1 M glycine buffer (pH 2.6) and quenched by collecting in tubes containing 0.2 ml of 0.1 M Tris buffer (pH 8). The collected fractions of IgG, each of 2 ml, were then passed through PD10 columns using the sodium phosphate buffer as eluent and three 1.2 ml fractions were collected. The IgG concentration of each fraction was determined by measuring the absorbance at 280 nm ($\epsilon_{280} = 2 \times 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$). The 1.2 ml IgG fractions were stored at –20 °C. A typical purification would give 12 fractions each containing 1.2 ml and varying in IgG concentration from 5 to 50 μM .

Synthetic routes

The general route to the substrates **5a–5f** is exemplified by the procedure detailed below for 4-nitrophenyl *N*-methyl carbamate **5a**. To a stirred mixture of methylamine (2.48 ml) and triethylamine (502 mg, 4.96 mmol, 691 μl) in anhydrous acetonitrile (10 ml) at 0 °C, protected from atmospheric moisture by a drying tube, 4-nitrophenyl chloroformate (1 g, 4.96 mmol) was added portion-wise and the mixture was left to stir at room temperature for 2 h during which time a precipitate formed. After removal of the acetonitrile by evaporation, the residue was partitioned

between ethyl acetate (40 ml) and water (30 ml). After separation, the organic layer was washed with cold water ($2 \times 30 \text{ ml}$) and dried over anhydrous magnesium sulphate. The latter was removed by filtration and the filtrate was then evaporated. The crude product was recrystallized from chloroform/diethyl ether to give white, needle shaped crystals of 4-nitrophenyl *N*-methyl carbamate **5a**, melting point 142–146 °C (633 mg, 65 %). ^1H NMR (360 MHz, C_2HCl_3) δ (p.p.m.): 8.25 (2 H, d, *J* 8.7 Hz, H_A of ABq), 7.32 (2 H, d, *J* 8.7 Hz, H_B of ABq), 5.08 (1 H, broad s, NH), 2.94 (3 H, d, *J* 4.36 Hz, NCH_3); IR ν (cm^{-1}) 3337, 1719, 1545, 1525, 1510 and 1489; *m/z* 196.1. The phosphate inhibitor **6** was synthesized from 4-nitrophenylphosphoro-dichloridate and phenol using the procedure described in [7].

Kinetic studies

The general method is first described for 4-nitrophenyl *N*-methyl carbamate **5a**. The release of 4-nitrophenolate from 50–350 μM **5a** was monitored in 50 mM sodium phosphate buffer (pH 8.0) containing 4 % (v/v) acetonitrile at 25 °C by recording the increase in A_{400} , and quantified using $\epsilon_{400} = 1.65 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$. In some cases, reaction mixtures also contained 2–4 μM PCA 271-100 or non-specific IgG from a non-immunized sheep. Initial rates (v) were determined from slopes of linear progress curves. The initial rates for the reactions carried out in the absence of IgG or in the presence of IgG prepared from non-immunized sheep increased linearly with an increase in $[S]_0$ and were closely similar at a given value of $[S]_0$. These initial rates were subtracted from those obtained in the presence of PCA 271-100 to provide the rates of the PCA-catalysed reaction. After demonstration of adherence of the corrected v versus $[S]_0$ data to the Michaelis–Menten equation by the linearity of an $[S]_0/v$ versus $[S]_0$ plot, the provisional values of the parameters V and K_m thus obtained were refined by fitting the Michaelis–Menten equation to the v , $[S]_0$ data pairs by weighted non-linear regression using the Sigmaplot 8.0 program. Values of the catalytic rate constant (k_{cat}) were calculated from $k_{\text{cat}} = 10 V/2[\text{IgG}] = 5 V/[\text{IgG}]$ (10 % of the IgG catalytic and two potential active centres per molecule) to provide lower limits for this parameter [16–18]. $[\text{IgG}]$ was determined from A_{280} and $\epsilon_{280} = 2.0 \times 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$. For the other carbamates, analogous procedures were followed using the following wavelengths (nm) with absorption coefficients ($\text{M}^{-1} \cdot \text{cm}^{-1}$) in parenthesis: for 4-bromo-**5b**, 278 (1310); for 4-fluoro-**5c**, 280 (2180); for the unsubstituted carbamate **5d** at pH 9.0, 280 (750); for 4-methoxy-**5e** at pH 9.0, 318 (128) and for 2-nitro-**5f**, 416 (4103). In the case of the reactions of phenyl *N*-methyl carbamate **5d** and 4-methoxyphenyl *N*-methyl carbamate **5e** more accurate data were obtained by also performing the reactions at pH 9.0 (50 mM borate buffer) and converting the k_{cat} values into those corresponding to the reaction at pH 8.0. Inhibition by the hapten-analogue phosphate **6** was investigated using the procedure described above for substrate **5a** but with equimolar amounts of phosphate **6** incorporated into the reaction solution.

RESULTS AND DISCUSSION

Choice of substrates for the present Hammett σ – ρ analysis

The choice of the particular series of mono-*O*-aryl *N*-methyl carbamates **5a–5e** was based on (i) the fact that an analogous carbonate, methyl 4-nitrophenyl carbonate **9**, which is devoid of the additional recognition features in **1**, is a specific substrate for catalytic antibodies generated using the hapten **3** [9], and (ii) the ease of synthesis of suitable 4-substituted phenyl methyl carbamates from commercially available starting materials.

Figure 3 Demonstration of the adherence to the Michaelis–Menten equation of the hydrolysis of *O*-4-nitrophenyl *N*-methyl carbamate **5a** catalysed by PCA271-100 at pH 8.0 and 25 °C

The continuous line corresponds to the Michaelis–Menten equation with $V = (5.69 \pm 0.36) \times 10^{-8} \text{ Ms}^{-1}$ (best fit value; mean $\pm$ S.E.M.) and $K_m = 118 \pm 19 \mu\text{M}$. For further details see the text.

Kinetic characteristics

As expected, the hydroxide ion (non-catalysed) hydrolysis of the carbamate substrates **5a–5f** is first-order in the substrate, with linearity in the plots of v versus $[S]_0$ demonstrated to at least $500 \mu\text{M}$ (results not shown). By contrast, the hydrolysis of **5a–5d** (i.e. the substrates with 4-NO₂, 4-Br, 4-F and 4-H) catalysed by PCA 271-100 was shown to obey the Michaelis–Menten equation with K_m values in the range $30\text{--}183 \mu\text{M}$. A typical hyperbolic saturation curve is shown in Figure 3 and values of $k_{\text{cat}}/k_{\text{non-cat}}$ are shown in Table 1. It is important to emphasize that (i) polyclonal IgG preparations investigated to-date in different laboratories have not deviated from single-phase (Michaelis–Menten) saturation kinetics (e.g. see references cited in [19]), (ii) polyclonal preparations are free from contamination by enzymes, as shown in our laboratories by the use of isomeric substrates [7,20] and in other laboratories by the observation of catalysis of reactions for which there are no known enzymes [14,21], and (iii) the generation of PCAs by immunization with a transition-state analogue [7,22–24] is a good way to sample the entirety of the immune response to the haptenic moiety of an immunogen. Catalysis of the hydrolysis of the substrate **5e** (4-CH₃O) was too weak to be quantified reliably when using the concentration of PCA 271-100

available. Evidence that the catalysis occurs in the active centres elicited by the 4-nitrophenyl phosphate haptenic determinant of **4** is provided by (i) the abolition of the catalysed hydrolysis of **5a** by the hapten analogue **6** when present at a concentration equimolar with substrate **5a**, and (ii) the lack of catalysis of the hydrolysis of **5f**, the 2-nitrophenyl isomer of the 4-nitrophenyl methyl carbamate substrate **5a**. The 2-nitrophenyl isomer **5f** is not a substrate for PCA 271-100 because antibody specificity is sensitive to structural changes, particularly at sites distant from the site of conjugation in the immunogen, as discussed in [7] where the diagnostic use of isomeric substrates was introduced.

The ways in which $k_{\text{non-cat}}$ (for the hydroxide ion reaction) and k_{cat} , k_{cat}/K_m and the proficiency constant, $k_{\text{cat}}/(k_{\text{non-cat}} \cdot K_m)$ for the antibody-catalysed reaction, together with values of $k_{\text{cat}}/k_{\text{non-cat}}$, vary with the substituent in the 4-position of the phenyl ring of the substrates **5a–5d** are shown in Table 1. Whereas k_{cat} (and k_{cat}/K_m) decrease with a decrease in the electron-attracting power of the substituent that replaces the 4-H atom, as indicated by the σ or σ^- value, the proficiency constant is relatively insensitive to the nature of the substituent. The proficiency constant, introduced by Radzicka and Wolfenden [25], is related to the specificity constant, k_{cat}/K_m , with the intrinsic reactivity of the substrate taken into account. The electronic effect of the substituents in the leaving group may be quantified by use of the Hammett equation, eqn (1), e.g. see [10], in which k is the rate constant for the reaction of a member of the substrate series with a given substituent, k_0 is the rate constant for the reaction of the parent compound with H at the substituent site, σ is the substituent constant and ρ is the reaction constant

$$\log k = \rho\sigma + \log k_0 \quad (1)$$

The substituent constant, σ , represents the ability of the group to attract or donate electrons. Its values are given by the difference between the $\text{p}K_a$ values of substituted benzoic acids and benzoic acid itself. Positive values of σ indicate greater electron attraction than H and negative values indicate weaker electron attraction (i.e. electron donation). In the present study, common practice has been followed in using the Hammett σ^- value ($+1.27$ rather than $+0.78$) for the 4-nitro group for reactions with relatively large values of ρ [11,12]. The value of ρ , the reaction constant, is a measure of the susceptibility of the rate constant for a given mechanism of a given reaction type to the influence of substituents. Reactions with positive ρ values are aided by electron withdrawal from the benzene ring and vice versa for reactions with negative ρ values.

In the present study, both mechanisms (Figures 2a and 2b) predict that the reaction would be aided by electron withdrawal from the aromatic ring. The sign of ρ therefore should be positive for both cases and not able to distinguish between them. The mechanism shown in Figure 2(b) [ElcB], however, would be

Table 1 Kinetic parameters for the non-catalysed (hydroxide ion) and PCA 271-100-catalysed hydrolysis of the substituted arylcarbamates **5a–5d** at pH 8.0 and 25 °C

The Hammett σ^- value ($+1.27$) rather than the standard σ value of $+0.78$ is generally used for the 4-nitro group in reactions such as the hydroxide ion reaction (E1cB) characterized by large values of ρ , (>2) [11,12]; values of k_{cat} were calculated from $k_{\text{cat}} = 5V/[\text{IgG}]$ (see the text and [16–18]). The proficiency constant [25], $k_{\text{cat}}/(k_{\text{non-cat}} \cdot K_m)$, is related to the specificity constant, k_{cat}/K_m , with the intrinsic reactivity of the substrate taken into account.

Substituent	Substituent constant	$k_{\text{non-cat}}$ (s^{-1})	k_{cat} (s^{-1})	$k_{\text{cat}}/k_{\text{non-cat}}$	k_{cat}/K_m ($\text{M}^{-1} \cdot \text{s}^{-1}$)	Proficiency constant ($\times 10^{-6} \text{ M}^{-1}$)
4-NO ₂	$\sigma^- = 1.27, \sigma = 0.78$	1.3×10^{-3}	1.5×10^{-1}	114	1.2×10^3	0.9
4-Br	$\sigma = 0.23$	1.4×10^{-5}	7×10^{-4}	49	23	1.6
4-F	$\sigma = 0.06$	1.7×10^{-6}	3×10^{-4}	178	3.3	2.0
4-H	$\sigma = 0.00$	1.7×10^{-6}	9.1×10^{-5}	53	0.5	0.3

Figure 4 Hammett σ - ρ correlation for the reactions of the carbamate substrates **5** with an hydroxide ion at pH 8.0 and 25 °C

(a) Catalysed by PCA271-100 and (b) non-catalysed. The substituent constant is σ^- for the 4-nitrophenyl carbamate **5a** [11,12] and σ for the other substrates. The slopes of the regression lines provide the values of ρ for (a) 2.36 ± 0.20 and for (b) 2.25 ± 0.22 , both of which are characteristic of mechanisms in which development of the aryloxyanion of the leaving group is well advanced in the transition state of the rate-determining step; for the non-catalysed reaction it was possible to obtain a value of $k_{\text{non-cat}}$ also for the 4-methoxycarbamate and its inclusion in the regression gives $\rho + 2.55 \pm 0.20$. For the consequences of using σ rather than σ^- for the 4-nitrocarbamate, see the text.

expected to be more sensitive to electron withdrawal from the aromatic ring of the aryloxy leaving group and thus the value of ρ should be greater for this mechanism than for the mechanism shown in Figure 2(a). This expectation is supported by values of ρ reported in the literature (+2.87, 2.68 and 3.16) for the reactions of the hydroxide ion with *N*-monosubstituted arylcarbamates [11–13], which proceed via the E1cB mechanism (Figure 2b), and for the reactions with an *N,N*-di-substituted arylcarbamate ($\rho = +1.24$, see [11]) and an arylester ($\rho = \text{approx.} +1.0$, [15,26]). The last two necessarily proceed via the B_{Ac}2 mechanism (Figure 2a) because of the absence of the N-H group required to produce the anionic intermediate shown in Figure 2(b).

Hammett plots for the reactions of the carbamate substrate **5a–5d** are shown in Figure 4. The values of ρ for the non-catalysed reactions of the substrates with an hydroxide ion (+2.25; Figure 4b), and for k_{cat} for the PCA 271-100-catalysed reactions (+2.36; Figure 4a) are closely similar to each other and >2.0 . This compels the view that these phosphate-elicited antibody-catalysed reactions, like the non-catalysed reactions, proceed by a mechanism in which development of the aryloxyanion of the leaving group is well advanced in the transition state of the rate-determining step. It is necessary to point out that the values of ρ for these Hammett plots depend on the value of the substituent constant used for the 4-nitrocarbamate substrate. Use of σ however, results in values of ρ ($+3.82 \pm 0.25$ and $+3.97 \pm 0.28$ for the non-catalysed and antibody-catalysed reactions respectively) that are even larger than those when σ^- is used ($+2.25 \pm 0.27$ and $+2.36 \pm 0.20$). The conclusion reached in the present study about the well advanced development of the aryloxyanion in the transition state of the rate-determining step, therefore, is not affected despite the uncertainties about the choice of the substituent constant. The E1cB mechanism has been established convincingly for the reactions of an hydroxide ion with *N*-monosubstituted arylcarbamates by deuterium isotope effects,

trapping of reactive intermediates and the use of *N,N*-disubstituted arylcarbamates in addition to Hammett relationships with values of $\rho > 2$. In the present paper, in the case of reactions catalysed by PCA 271-100 there are two mechanisms that involve advanced development of the aryloxyanion in the transition state. One is the E1cB mechanism and another is the B_{Ac}2 mechanism, with the breakdown of the tetrahedral intermediate being rate-determining. In either case the result for the anti-phosphate antibody PCA 271-100 contrasts markedly with that for the anti-phosphonamide antibody investigated by Wentworth et al. [11]. This difference supports the hypothesis of a key role for the presence or absence of the phenolic oxygen atom of the hapten [11] and contributes to the growing awareness [8,9] that small but key changes in hapten structure can control antibody-catalysed mechanisms.

REFERENCES

- Pauling, L. (1946) Molecular architecture and biological reactions. *Chem. Eng. News*, **24**, 1375–1377
- Jencks, W. P. (1969) *Catalysis in Chemistry and Enzymology*, pp. 282–320, McGraw-Hill, New York
- Resmini, M., Ostler, E. L., Brocklehurst, K. and Gallacher, G. (2004) in *Catalytic Antibodies*, (Keinan, E., ed.), pp. 468–488, UCH-Wiley
- Hilvert, D. (2000) Critical analysis of antibody catalysis. *Ann. Rev. Biochem.* **69**, 751–793
- Mader, M. M. and Bartlett, P. A. (1997) Binding energy and catalysis: the implications for transition-state analogs and catalytic antibodies. *Chem. Rev.* **97**, 1281–1301
- Wolfenden, R. and Radzicka, A. (1991) Transition state analogues. *Curr. Opin. Struct. Biol.* **1**, 780–787
- Gallacher, G., Jackson, C. S., Searcey, M., Badman, G. T., Goel, R., Topham, C. M., Mellor, G. W. and Brocklehurst, K. (1991) A polyclonal antibody preparation with Michaelian catalytic properties. *Biochem. J.* **279**, 871–881
- Gul, S., Sonkaria, S., Pinitglang, S., Florez-Alvarez, J., Hussain, S., Thomas, E. W., Ostler, E. L., Gallacher, G., Resmini, M. and Brocklehurst, K. (2003) Improvement in hydrolytic antibody activity by change in haptenic structure from phosphate to phosphonate with retention of a common leaving-group determinant: evidence for the 'flexibility' hypothesis. *Biochem. J.* **376**, 813–821
- Sonkaria, S., Boucher, G., Florez-Alvarez, J., Said, B., Hussain, S., Ostler, E. L., Gul, S., Thomas, E. W., Resmini, M., Gallacher, G. and Brocklehurst, K. (2004) Evidence for 'lock and key' character in an antiphosphonate hydrolytic antibody catalytic site augmented by non-reaction centre recognition: variation in substrate selectivity between an anti-phosphonate antibody, an anti-phosphate antibody and two hydrolytic enzymes. *Biochem. J.* **381**, 125–310
- Johnson, C. D. (1973) *The Hammett Equation*, Cambridge University Press, U.K.
- Wentworth, P., Datta, A., Smith, S., Marshall, A., Partridge, L. J. and Blackburn, G. M. (1997) Antibody catalysis of B_{Ac}2 aryl carbamate ester hydrolysis: a highly disfavoured chemical process. *J. Am. Chem. Soc.* **119**, 2315–2316
- Williams, A. (1972) Alkaline hydrolysis of substituted phenyl *N*-phenylcarbamates, Structure-reactivity relationships consistent with an E1cB mechanism. *J. Chem. Soc. Perkin Trans.* **2**, 808–812
- Hegarty, A. F. and Frost, L. N. (1973) Elimination-addition mechanism for the hydrolysis of carbamates, Trapping of an isocyanate intermediate by an *o*-amino-group. *J. Chem. Soc. Perkin Trans.* **2**, 1719–1728
- Stephens, D. B. and Iverson, B. L. (1993) Polyclonal catalytic antibodies. *Biochem. Biophys. Res. Commun.* **115**, 623–628
- Bruice, T. C. and Mayahi, M. F. (1960) The influence of the leaving tendency of the phenoxy group on the ammonolysis and hydrolysis of substituted phenyl acetates. *J. Am. Chem. Soc.* **82**, 3067–3071
- Resmini, M., Gul, S., Carter, S., Sonkaria, S., Topham, C. M., Gallacher, G. and Brocklehurst, K. (2000) A general kinetic approach to investigation of active-site availability in macromolecular catalysis. *Biochem. J.* **346**, 117–125
- Brocklehurst, K., Resmini, M. and Topham, C. M. (2001) Kinetic and titration methods for determination of active site contents of enzyme and catalytic antibody preparations. *Methods* **24**, 153–167
- Topham, C. M., Gul, S., Resmini, M., Sonkaria, S., Gallacher, G. and Brocklehurst, K. (2000) The kinetic basis of a general method for the investigation of active site content of enzymes and catalytic antibodies: first order behaviour under single turnover and cycling conditions. *J. Theor. Biol.* **204**, 239–256

- 19 Odenbaugh, A. L., Helms, E. D. and Iverson, B. L. (2000) An investigation of antibody acyl hydrolysis catalysis using a large set of related haptens. *Bioorg. Med. Chem.* **8**, 413–426
- 20 Ostler, E. L., Resmini, M., Boucher, G., Romanov, N., Brocklehurst, K. and Gallacher, G. (2002) Polyclonal antibody catalysed hydrolysis of a β -lactam. *Chem. Commun.* 226–227
- 21 Hu, Y. J., Ji, Y. Y., Wu, Y. L., Yang, B. H. and Yeh, M. (1997) Polyclonal catalytic antibody for hetero-cycloaddition of hepta-1,3-diene with ethyl glyoxylate an approach to the synthesis of 2-nonulosonic acid analogs. *Biorg. Med. Chem. Lett.* **7**, 1601–1606
- 22 Gallacher, G., Jackson, C. S., Searcey, M., Goel, R., Mellor, G. W., Smith, C. Z. and Brocklehurst, K. (1993) Catalytic antibody activity elicited by active immunisation. *Eur. J. Biochem.* **214**, 197–207
- 23 Resmini, M., Vigna, R., Simms, C., Barber, J., Hagi-Pavli, E. P., Watts, A., Verma, C., Gallacher, G. and Brocklehurst, K. (1997) Characterization of the hydrolytic activity of a polyclonal catalytic antibody preparation by pH-dependence and chemical modification studies: evidence for Tyr and Arg side chains as hydrogen bond donors. *Biochem. J.* **326**, 279–287
- 24 Ostler, E. L., Resmini, M., Brocklehurst, K. and Gallacher, G. (2002) Polyclonal catalytic antibodies. *J. Immunol. Methods* **269**, 111–124
- 25 Radzicka, A. and Wolfenden, R. (1995) A proficient enzyme. *Science* **267**, 90–93
- 26 Kirsch, J. F., Clewell, W. and Simon, A. (1968) Multiple structure reactivity correlations. Alkaline hydrolyses of acyl and aryl substituted benzoates. *J. Org. Chem.* **33**, 127–132

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