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

Sheraz GUL*[1], Sanjiv SONKARIA*, Surapong PINITGLANG*[2], José FLOREZ-ALVAREZ*, Syeed HUSSAIN*, Emrys W. THOMAS†, Elizabeth L. OSTLER‡, Gerard GALLACHER‡, Marina RESMINI§[3] and Keith BROCKLEHURST*[3]

*Laboratory of Structural and Mechanistic Enzymology, School of Biological Sciences, Queen Mary, University of London, Mile End Road, London E1 4NS, U.K., †Department of Biological Sciences, University of Salford, The Crescent, Salford M5 4JW, U.K., ‡School of Pharmacy and Biomolecular Sciences, University of Brighton, Cockcroft Building, Lewes Road, Moulsecoomb, Brighton BN2 4GJ, U.K., and §Department of Chemistry, Queen Mary, University of London, Mile End Road, London E1 4NS, U.K.

To investigate the hypothesis that decreased hapten flexibility may lead to increased catalytic antibody activity, we used two closely related immunogens differing only in the flexibility of the atomic framework around the structural motif of the haptens, analogous to the reaction centre of the corresponding substrates. Identical leaving-group determinants in the haptens and identical leaving groups in the substrates removed the ambiguity inherent in some data reported in the literature. Anti-phosphate and anti-phosphonate kinetically homogeneous polyclonal catalytic antibody preparations were compared by using carbonate and ester substrates respectively, each containing a 4-nitrophenolate leaving group. Synthetic routes to a new phosphonate hapten and new ester substrate were developed. The kinetic advantage of the more rigid anti-phosphonate/ester system was demonstrated at pH 8.0 by a 13-fold advantage in $k_{cat}/k_{non\text{-}cat}$ and a 100-fold advantage in the proficiency constant, $k_{cat}/k_{non\text{-}cat} \cdot K_m$. Despite these differences, the pH-dependences of the kinetic and binding characteristics and the results of chemical modification studies suggest closely similar catalytic mechanisms. The possible origin of the kinetic advantage of the more rigid hapten/substrate system is discussed.

Key words: flexibility of hapten, hydrolytic catalytic antibody, kinetic characterization, leaving-group determinant, phosphonate synthesis.

INTRODUCTION

The successful production of polyclonal catalytic antibodies by immunization with a transition-state analogue was reported in the early 1990s (e.g. [1–3]). This made possible systematic study of the relationship between haptenic structure and antibody recognition and catalytic activity (see [4] and the first 21 references cited therein). The development of the polyclonal catalytic antibody field (reviewed recently in [5]) followed earlier successes reported with monoclonal catalytic antibodies (initially reported in [6,7]; reviewed in e.g. [8,9]). The generation of polyclonal catalytic antibodies remains the best way to sample the entirety of the immune response to an immunogen. In addition, the investigation of antibody catalysis using polyclonal IgG is much faster and less expensive than analogous studies using monoclonal antibodies.

Two early anxieties were that (i) the polyclonal preparations might be catalytically heterogeneous and thus give rise to complex kinetics, and (ii) catalytic activity might derive from contamination by enzymes. Both anxieties have been shown to be without foundation. Thus, as far as the first is concerned, catalysis by all of the polyclonal IgG preparations investigated to date in different laboratories has not deviated from single-site (Michaelis–Menten) saturation kinetics (e.g [2–4,10–18]). Given the heterogeneous nature of the polyclonal antisera, this observation may be regarded as counter-intuitive. Nevertheless, it may be that polyclonal immune responses towards small haptens are less heterogeneous than is commonly supposed. The catalytic IgG present in a polyclonal preparation is a subset of the binding IgG, which itself is a subset of the total IgG and, in our experience, the content of catalytic IgG is very small. Recent work in these laboratories [16,19,20] using a combination of steady-state and single-turnover kinetics demonstrated that the upper limit of the catalytic IgG content of our sheep polyclonal preparations is at most 10 % of total IgG, and may be < 1 %. The upper limit determined kinetically is in good agreement with that (12 %) determined by Stephens and Iverson [13] for some rabbit polyclonal antibodies using hapten inhibition experiments. The observed functional homogeneity could be due to different antibodies interacting with the hapten in closely similar binding modes; the alternative explanation would need to involve the postulate that the catalytically active IgG is essentially monoclonal in nature [21].

With regard to the second anxiety, the evidence summarized below compels the view that catalytic activity in the polyclonal preparations discussed is not due to contaminant enzymes. The approach that we introduced in 1991 [2] using a pair of isomeric substrates makes use of the exquisite specificity of antibodies, particularly for regions of the substrate remote from those analogous to the site of attachment of the hapten to the carrier protein. We demonstrated that although, as expected, the IgG produced from sheep by using the 4-nitrophenyl phosphate immunogen (**1a**; Figure 1) catalysed the hydrolysis of the 4-nitrophenyl

Abbreviations used: EDC, 1-(dimethylaminopropyl)-3-ethylcarbodi-imide; KLH, keyhole-limpet haemocyanin; NSS, normal sheep serum; PCA, polyclonal catalytic antibody preparation; PCA 271-22 (etc.), PCA isolated from the antiserum of sheep no. 271 in week 22 of the immunization programme (etc.).

[1] Present address: Assay Methodology Development, GlaxoSmithKline, New Frontiers Science Park, Third Avenue, Harlow, Essex CM19 5AW, U.K.
[2] Present address: Department of Food Science, University of Thai Chamber of Commerce, Vibhavadee-Rangsit Road, Bangkok 10320, Thailand.
[3] To whom correspondence should be addressed (e-mail m.resmini@qmul.ac.uk or kb1@qmul.ac.uk).

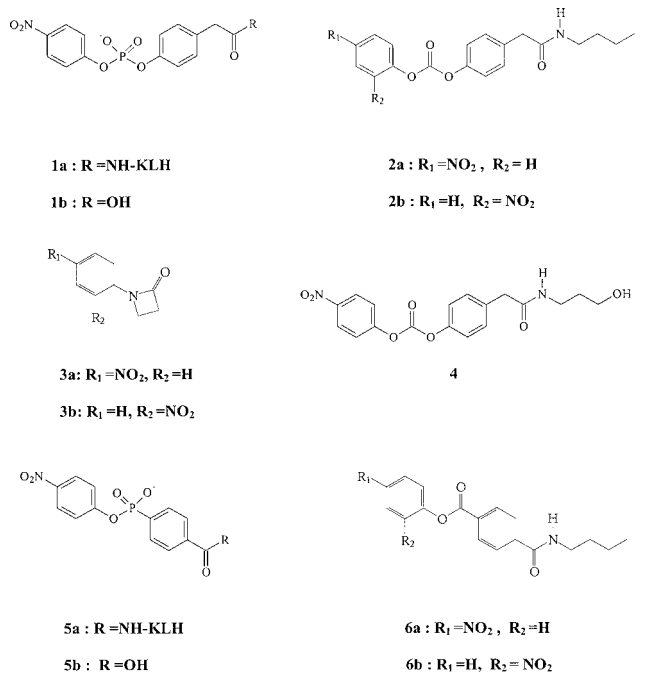


Figure 1 Structures of immunogens, haptens and substrates

1a is the 4-nitrophenyl phosphate immunogen used to elicit PCA 271-22, and **1b** is the associated hapten 4-nitrophenyl 4′-(carboxymethyl) phenyl hydrogen phosphate; **2a** is the 4-nitrophenyl carbonate substrate, 4-nitrophenyl 4′-(3-aza-2-oxoheptyl) phenyl carbonate, and **2b** is its 2-nitrophenyl isomer; **3a** is the 4-nitrophenyl β-lactam substrate *N*-(4-nitrophenyl)-azetidine-2-one, and **3b** is its 2-nitrophenyl isomer; **4** is the more soluble 4-nitrophenyl carbonate substrate, 4-nitrophenyl 4′-(3-aza-7-hydroxy-2-oxoheptyl) phenyl carbonate, used to demonstrate kinetic homogeneity in PCA 271-22 up to > 300 μM and for the other kinetic studies in the present work; **5a** is the 4-nitrophenyl phosphonate immunogen used to elicit PCA 2649-16, and **5b** is the associated hapten; **6a** is the 4-nitrophenyl ester substrate, 1-(butylamido)-4′-(4-nitrophenoxycarbonyl)benzene carboxylic ester used in kinetic studies with PCA 2649-16, and **6b** is its 2-nitrophenyl isomer used to establish the lack of an enzyme contaminant in PCA 2649-16.

carbonate ester substrate (**2a**), it failed to catalyse the hydrolysis of the 2-nitrophenyl isomer (**2b**). In contrast, two different carboxylesterases failed to discriminate substantially between the two substrates, as did whole serum from a non-immunized sheep or from a sheep immunized with an immunogen structurally unrelated to the substrate (a derivative of 3-*O*-methylnoradrenaline). This approach was also used more recently [18] to demonstrate that hydrolysis of the 4-nitrophenyl β-lactam (**3a**) catalysed by one of our anti-(4-nitrophenyl phosphate) IgG preparations is antibody-mediated, by showing that the 2-nitrophenyl β-lactam (**3b**) is not a substrate. The evidence provided by using isomeric substrates is complemented by the fact that catalysis by polyclonal antibody preparations of reactions for which there are no known enzymes has been demonstrated. Examples include the hydrolysis of a trityl ether [13] and a Diels–Alder cycloaddition [22].

Hapten design is now a priority in the catalytic antibody field and, as indicated above, its investigation is facilitated by the use of polyclonal antibodies. Recent work by Iverson's group has begun to address the important question of the relationship between haptenic structure and the resulting success or otherwise in eliciting antibody catalysts, given a high-affinity immune response. A paper [4] describes an investigation of the effects of small modifications in haptens on the resultant catalytic antibody activity. The hydrolysis of six isomeric benzyl ester or benzyl carbonate substrates was not catalysed by polyclonal antibodies generated by six congruent benzyloxy phosphorus transition-state analogues. Some other structurally related benzyloxy phosphorus haptens did generate antibodies that catalysed the hydrolysis of congruent benzyl esters, but the activity was poor by comparison with that generated by the phenyl equivalents. The results led to the hypothesis that flexibility in the region of the hapten remote from the linker might be responsible for the decreased effectiveness of the resulting catalytic antibodies. Unfortunately the data presented in [4] do not allow the flexibility hypothesis to be distinguished from the alternative possibility that the differences between the successful and less successful haptenic systems might be due to differences in leaving-group ability of the respective phenylcarbonate and benzylcarbonate cognate substrates of the resulting antibodies.

Investigation of this potentially important feature of hapten design requires kinetic characterization of catalytic antibodies elicited from closely related haptens differing only in the rigidity of the atomic framework around the reaction centre analogue, but with identical leaving-group determinants. Similarly, characterization requires cognate substrates closely related to the haptens, in particular with identical leaving groups. In the present paper we provide evidence for the flexibility hypothesis. This involves comparing the kinetic characteristics of a PCA (polyclonal catalytic antibody) preparation (PCA 271-22), elicited by using a 4-nitrophenyl phosphate immunogen (**1a**; Figure 1), towards a cognate 4-nitrophenyl carbonate substrate (**4**) with those of PCA 2649-16, elicited by using the less flexible 4-nitrophenyl phosphonate immunogen (**5a**), towards its cognate 4-nitrophenyl ester substrate (**6a**). These two immunogens and the two cognate substrates contain the 4-nitrophenyl moiety, and thus the leaving group is common to both substrates. The larger values of $k_{cat}/k_{non\text{-}cat}$ (by a factor of 13) and $k_{cat}/k_{non\text{-}cat} \cdot K_m$ (by a factor of 100) for the anti-phosphonate antibody/carboxylic ester system demonstrates the greater effectiveness of the less flexible phosphonate haptenic structure in eliciting catalytic activity. Despite these differences, the pH-dependence characteristics of k_{cat} and k_{cat}/K_m, and the results of group-selective chemical modification studies, suggest similar features in the mechanism of the two PCA preparations. In both cases, rate enhancement appears to involve assistance to nucleophilic attack of hydroxide ion by hydrogen-bond donation to the substrate from the side chains of tyrosine and arginine residues. The possible origin of the kinetic advantage of the more rigid hapten/substrate system is discussed.

MATERIALS AND METHODS

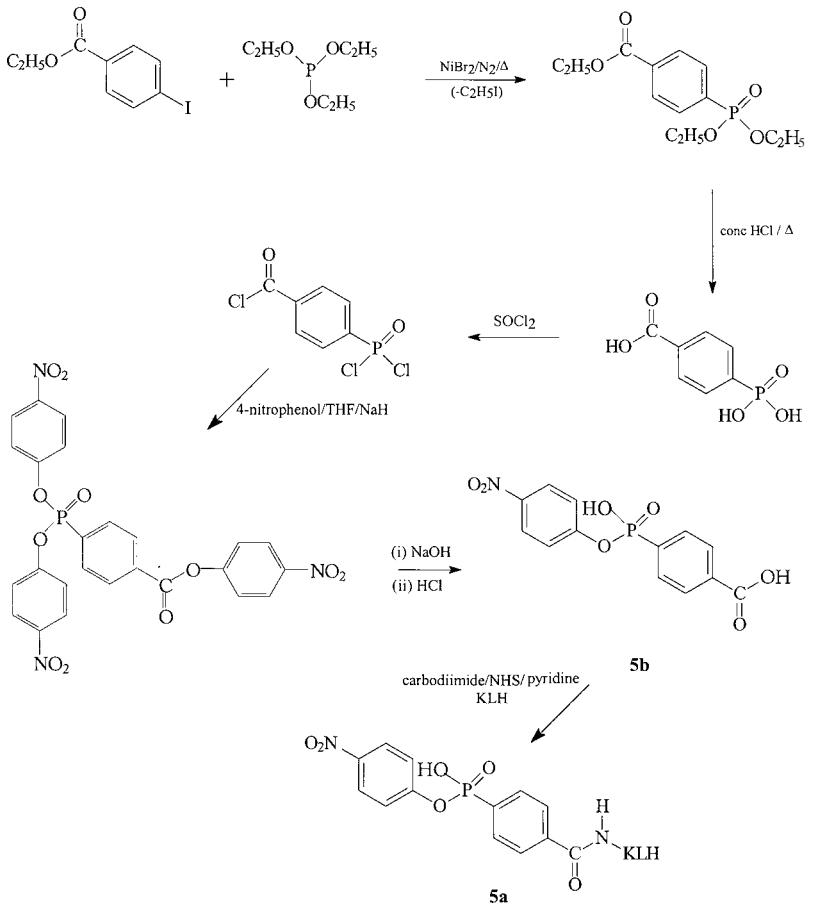
Materials

Materials used in the production and characterization of antibodies other than those described below were purchased or synthesized as described previously [2,12,16]. Unless otherwise stated, chemicals were from Sigma-Aldrich Co. (Poole, Dorset, U.K.).

New syntheses

Phosphonate immunogen **5a** (Scheme 1)

Ethyl-4-iodobenzoate (28 g, 101 mmol) was added to triethyl phosphite (20 ml, 117 mmol) in the presence of anhydrous nickel bromide (1.2 g, 5.5 mmol). The mixture was refluxed at 180 °C under N_2 for 30 min. After cooling, dichloromethane (CH_2Cl_2; 250 ml) was added to the solution, which was then washed twice with 5 % (v/v) HCl and once with water, and dried with Na_2SO_4; after its removal, the filtrate was concentrated *in vacuo*, to give the



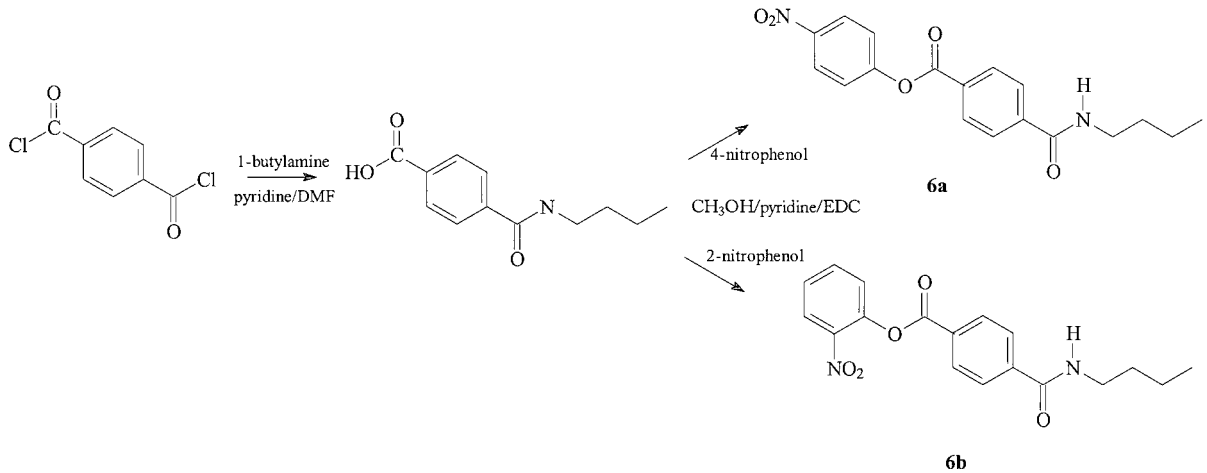
Scheme 1 Synthesis of the phosphonate immunogen 5a

A five-step synthesis produced the phosphonate hapten **5b**, which was coupled to the carrier protein KLH to provide the immunogen **5a**. THF, tetrahydrofuran; NHS, *N*-hydroxysuccinimide.

crude triester. Hydrolysis of this was achieved by heating under reflux with stirring in conc. HCl (100 ml) for 12 h. After cooling on ice, the crude product was isolated by filtration and dried *in vacuo* over P_2O_5 to give 13.5 g of 4-carboxyphenylphosphonic acid (6.7 mmol, 67 % yield, m.p. > 300 °C). The crude diacid (1 g, 5 mmol) was suspended in $SOCl_2$ (1.5 ml, 20 mmol) with stirring and under N_2. *N*,*N*-Dimethylformamide (100 μl) was added as catalyst, and the mixture was kept at 55 °C for 12 h. After cooling, excess $SOCl_2$ was removed *in vacuo*, and the residue was distilled under reduced pressure (0.5 mmHg) using Kügelrohr distillation equipment. The trichloride product was collected as an oil (140–150 °C at 0.5 mmHg), which solidified to a cream-coloured solid. This was converted into the tri-4-nitrophenyl ester as follows. To 4-nitrophenol (2.78 g, 20 mmol) in dry tetrahydrofuran (50 ml) was added NaH (0.48 g, 20 mmol) with stirring at 20 °C. Stirring was continued until evolution of gas (H_2) ceased. A solution of the trichloride (13.5 g, 5 mmol) in dry tetrahydrofuran (10 ml) was added gradually with vigorous stirring, and stirring at 20 °C was continued for 12 h. The reaction mixture was poured into ice-cold water (200 ml), and the resulting precipitate was isolated by filtration, washed with ice-cold water and air-dried to give approx. 1.2 g of crude product. This was recrystallized from hot acetonitrile to give the pure triester ($\nu_{C=O} = 1730\ cm^{-1}$, m.p. 195–196 °C). The triester (1.12 g, 2 mmol) was stirred vigorously in NaOH (0.1 M, 200 ml) for 48 h at room temperature. After removal of unreacted triester by filtration, the filtrate was acidified at pH 4.0 with 0.5 M HCl and evaporated to dryness *in vacuo* at 30 °C. 4-Nitrophenol was removed from the residue by trituration and extraction with dichloromethane several times. Further trituration of the resulting solid with ethanol/methanol (5:1, v/v) extracted the crude monoester hapten (**5b**, Scheme 1). This was precipitated from the alcoholic extract by treatment with excess ethyl acetate and isolated by filtration to give a white solid that was recrystallized from methanol/ethyl acetate (m.p. 280 °C, with decomposition). The results of both potentiometric titration and nitrophenolate release (0.1 M NaOH, 100 °C, 30 min; spectral analysis at 400 nm) were in accordance with those predicted for **5b**. ^{1}H-NMR (DMSO; 250 MHz): δ 8.10 (2 H, d, *J* 8.5 Hz, *H*ArNO$_2$), 7.85 (2 H, d, *J* 8 Hz, *H*ArCO$_2$H), 7.70 (2 H, d, *J* 8.5 Hz, *H*ArNO$_2$), 7.30 (2 H, d, *J* 8 Hz, *H*ArCH$_2$CO$_2$H); ^{31}P-NMR (DMSO; 250 MHz): δ 6.0. Carbodi-imide/*N*-hydroxysuccinimide coupling of the phosphonate hapten **5b** to KLH (keyhole-limpet haemocyanin), as described for the phosphate hapten in [2], provided the phosphonate immunogen **5a**.

4-(1-Butylamido)benzoic acid, the common intermediate used in the synthesis of isomeric ester substrates **6a** and **6b** of Scheme 2

Terephthaloyl chloride (5 g, 24.6 mmol) was dissolved in dry dimethylformamide (10 ml), and dry pyridine (3.5 ml, 3.42 g, 43.3 mmol) and butylamine (2.4 ml, 1.8 g, 24 mmol) were added.



Scheme 2 Synthesis of the 4-nitrophenyl (6a) and 2-nitrophenyl (6b) aryl ester substrates via the common intermediate 4-(1-butylamido) benzoic acid

DMF, dimethylformamide.

The resulting exothermic reaction gave rise to a pale yellow slurry, which was stirred for 17 h at room temperature. NaOH (1 M, 200 ml) was then added to the mixure and stirring was continued for further 20 min, followed by extraction with ethyl acetate (2 × 200 ml). Subsequently the aqueous layer was acidified to pH 1 with HCl (2 M, 100 ml). The required product was then extracted with ethyl acetate (3 × 300 ml) and the combined organic extracts were dried with $MgSO_4$. After filtration, evaporation gave a cream solid. This was recrystallized from boiling ethyl acetate (125 ml), with insoluble material (terephthalic acid) being removed by hot filtration, to give a white crystalline powder (1.21 g, 5.47 mmol, 22 %, m.p. 225–226 °C). TLC (dichloromethane/methanol, 5:1, v/v): $R_F = 0.4$. ^{1}H-NMR (360 MHz, [$^2H_6$]DMSO): δ 8.08 (2 H, d, J 8.9 Hz, *Ar*C<u>H</u>CCOOH), 7.88 (2 H, d, J 8.8 Hz, *Ar*C<u>H</u>CCONH), 3.37 (2 H, t, J 7.1 Hz NH<u>CH$_2$</u>), 1.60 (2 H, m, NH<u>CH$_2$</u>CH$_2$), 1.40 (2 H, m, <u>CH$_2$</u>CH$_3$), 0.97 (3 H, t, J 7.1 Hz, CH$_2$<u>CH$_3$</u>). MS: $m/z = 221.7$ (M^+), 179, 178 $(M - Bu)^+$, 150, 149, 121.

1-(Butylamido)-4′-(4-nitrophenoxycarbonyl)-benzene (substrate **6a**)

4-(1-Butylamido)benzoic acid (150 mg, 0.68 mmol) was suspended in dry acetonitrile (9 ml) containing dry pyridine (57 μl, 58 mg, 0.73 mmol) and 4-nitrophenol (97 mg, 0.7 mmol). EDC [1-(dimethylaminopropyl)-3-ethylcarbodi-imide; 268 mg, 1.4 mmol] was added portionwise over 30 min and the solution was stirred for 5 h. The mixture was diluted with ethyl acetate (100 ml) and extracted with HCl (0.5 M, 2 × 50 ml) and then with saturated $NaHCO_3$ (2 × 50 ml). The organic layer was dried over $MgSO_4$ and, after filtration, evaporation provided a pale yellow solid (180 mg, 5.26 mmol, 77 %). This was recyrstallized from ethyl acetate (10 ml), washed with diethyl ether and dried to give a white solid (95 mg, 2.77 mmol, 40 %, m.p. 138–139 °C). ^{1}H-NMR (360 MHz, C^{2}HCl$_3$): δ 8.34 (2 H, d, J 8.8 Hz, ArC<u>H</u>CCOOAr), 8.25 (2 H, d, J 8.0 Hz, ArC<u>H</u>CNO$_2$), 8.0 (2 H, d, J 7.1 Hz C<u>H</u>CO), 7.44 (2 H, d, J 8.8 Hz, ArC<u>H</u>CCONH), 6.32 (1 H, s, <u>NH</u>), 3.50 (2 H, t, J 7.1 Hz, NHCH$_2$), 1.64 (2 H, m, NHCH$_2$<u>CH$_2$</u>), 1.46 (2 H, m, <u>CH$_2$</u>CH$_3$), 0.98 (3 H, t, J 7.1 Hz, CH$_2$<u>CH$_3$</u>); ^{13}C-NMR (C^{2}HCl$_3$): δ 166.3, 163.5 (C=O), 155.4 (ArCNO$_2$), 130.8, 130.5, 127.3, 125.4, 125.3, 122.6 (Ar), 40.0 (NH<u>C</u>H$_2$), 31.6 (NHCH$_2$<u>C</u>H$_2$), 20.1 (<u>C</u>H$_2$CH$_3$), 13.8 (CH$_2$<u>C</u>H$_3$). MS: $m/z = 343$, 342 (M^+).

1-(Butylamido)-4′-(2-nitrophenoxycarbonyl)benzene (**6b**), the 2-nitro analogue of **6a**

4-(1-Butylamido)benzoic acid (75 mg, 0.32 mmol) was suspended in dry acetonitrile (4.5 ml) containing dry pyridine (29 μl, 28 mg, 0.37 mmol) and 2-nitrophenol (48.5 mg, 0.35 mmol). EDC (132 mg, 2.8 mmol) was added in three portions over 3 h and the solution was stirred for a further 2 h. The mixture was diluted with ethyl acetate (50 ml), extracted quickly with HCl (0.5 M, 2 × 25 ml), and then saturated with aqueous $NaHCO_3$ (2 × 25 ml). The organic layer was dried over $MgSO_4$ and, after filtration, evaporation provided a pale cream solid (176 mg). This was recyrstallized from ethyl acetate (10 ml), washed with diethyl ether and dried to give a white solid (52 mg, 0.15 mmol, 45 %, m.p. 147–148 °C). Elemental analysis: C 62.97 %, H 5.36 %, N 8.01 % ($C_{18}H_{18}N_2O_5$ requires C 63.16 %, H 5.26 %, N 8.19 %). ^{1}H-NMR (360 MHz, C^{2}HCl$_3$): δ 8.26 (2 H, d, J 8.8 Hz, ArC<u>H</u>CCOOAr), 8.17 (1 H, d, J 8.0 Hz, ArC<u>H</u>CNO$_2$), 7.90 (2 H, d, J 8.0 Hz, ArC<u>H</u>CCONH), 7.73 (1 H, t, J 8.0 Hz, ArC<u>H</u>CHCNO$_2$), 7.48 (1 H, t, J 8.0 Hz, ArC<u>H</u>CHCO), 7.42 (1 H, d, J 8.0 Hz, ArC<u>H</u>CO), 6.19 (1 H, s, N<u>H</u>), 3.50 (2 H, t, J 7.0 Hz, NHCH$_2$), 1.64 (2 H, m, NHCH$_2$C<u>H$_2$</u>), 1.46 (2 H, m, C<u>H$_2$</u>CH$_3$), 0.98 (3 H, t, J 8.0 Hz, CH$_2$C<u>H$_3$</u>).

Procedures for the production, purification and non-kinetic characterization of the anti-phosphate and anti-phosphonate polyclonal catalytic antibody preparations

The following procedures were carried out as described in [16]: preparation of the affinity column for use in the determination of the content of non-antigen-binding antibodies, preparation of the BSA–hapten conjugates, production of the antisera containing anti-phosphate or anti-phosphonate antibodies, and isolation of immunoglobulin fractions from the antisera of sheep 271 and 2649. The antibody dilution curves for PCA 271-22 (isolated from the antiserum of sheep 271 in week 22 of the immunization programme) were constructed using data obtained as described in [12], and those for PCA 2649-16 (designated in an analogous manner: sheep 2649, week 16) were constructed using an analogous procedure, with the phosphate hapten **1b** replaced by the phosphonate hapten **5b**.

Chemical modification

Chemical modification reactions were targeted at the side chains of tyrosine (using tetranitromethane), lysine (using methyl acetimidate), histidine (using diethyl pyrocarbonate), cysteine (using methyl 4-pyridyl disulphide) and arginine (using phenylglyoxal) residues of PCA 271-22 and PCA 2649-16. These procedures, and the evaluation of their effects on both binding and catalytic characterization, were carried out as described for another anti-phosphate antibody preparation, PCA 270-29 [12], using the cognate substrate for each antibody preparation. In addition to the evaluation of loss of binding and catalytic activity, evidence of chemical modification was sought by spectral analysis as follows (see [12]): (i) for reactions of tetranitromethane, at 428 nm ($\varepsilon_{428} = 4.1 \times 10^3\ M^{-1} \cdot cm^{-1}$); (ii) for reactions of diethylpyrocarbonate, at 242 nm ($\varepsilon_{242} = 3.2 \times 10^3\ M^{-1} \cdot cm^{-1}$); and (iii) for reactions with methyl 4-pyridyl disulphide, at 342 nm ($\varepsilon_{342} = 1.98 \times 10^4\ M^{-1} \cdot cm^{-1}$).

Kinetics

Kinetic studies of the hydrolysis of the 4-nitrophenyl carbonate substrate **2a** catalysed by PCA 271-22, and of the 4-nitrophenyl ester substrate **6a** catalysed by PCA 2649-16, were performed at various pH values in the range approx. 7–10 in potassium phosphate buffers containing 10 % (v/v) acetonitrile at 25 °C and I 0.3. Other buffers such as Tris, borate and carbonate were not used because of their accelerating effects on background (non-IgG-catalysed) rates and specific buffer effects on the IgG itself (including effects on binding detected using the ELISA [12]). Because of these complications, phosphate buffers were used over the whole pH range (approx. 7–10), despite their low buffering efficiency above pH 8.5. The pH of the reaction mixtures was monitored throughout the reactions, and no pH changes were detected. This is not surprising, in view of the small extent of conversion of substrate into product, with consequent low uptake of hydroxide ion, during measurement of initial rates over a short time scale. The ionic strength was maintained by the phosphate salts to avoid the inhibitory effect of Cl^- that had been noted for the catalytic activity of PCA 270-29 when NaCl was used in the preparation of the buffers [12]. It is important to note that, although phosphate might have been anticipated to be a potential inhibitor of anti-phosphate antibodies, there is no evidence for this, at least under the conditions used in our laboratories. Thus increasing the ionic strength from 0.15 to 0.90 by use of phosphate salts resulted in no change in $\tilde{k}_{cat}$ and a small increase in $\tilde{k}_{cat}/\tilde{K}_m$ (pH-independent values of k_{cat} and k_{cat}/K_m respectively [12]).

The IgG concentration in the 1 ml reaction mixture was 1.2 μM in the case of PCA 271-22 and 0.5 μM in the case of PCA 2649-16. The reactions were initiated by addition of various volumes of solutions of substrate in pure anhydrous acetonitrile. The release of 4-nitrophenolate from the substrate was monitored at 410 nm using a Cary 1 spectrophotometer over 2–5 min. Initial rates (v_i^{obs}) were calculated in units of $M \cdot s^{-1}$ from absorbance–time data by using the relationship $\varepsilon_{410} = 16\,963/(1 + [H^+]/K_a)\ M^{-1} \cdot cm^{-1}$, where $K_a = 10^{-7.15}$ M. These values were corrected for background hydrolysis determined in the absence of catalytic antibody under otherwise identical experimental conditions. The rates of reactions at pH 8.0 of the carbonate substrate **2a** were corrected by subtracting the rates of reaction with 1.2 μM IgG from NSS (normal sheep serum) calculated from $k_{(aq+NSS)} = 2.47 \times 10^{-4}\ s^{-1}$; those of the reactions of the ester substrate **6a** were corrected by subtracting the rates of the aqueous reaction ($k_{aq} = k_{non\text{-}cat} = 3.15 \times 10^{-5}\ s^{-1}$), since these were not enhanced by the addition of 0.5 μM IgG from NSS. For the reactions at other pH values, the appropriate background rates were determined separately at each substrate concentration. Rates thus corrected are designated v_i. The pH of each reaction mixture was measured 5 min after initiation of the reaction. The reaction was then allowed to go to completion, and the resulting A_{410} value was used to calculate the initial value of the substrate concentration ($[S]_0$) in each kinetic run. Upper limits of the active-site contents of the antibody preparations were determined by the method involving a combination of pre-steady-state and steady-state kinetics described previously [16,19,20]. As with other catalytic antibody preparations evaluated in these laboratories, the upper limits were found to represent approx. 10 % of the total IgG content.

Parameter evaluation

The adherence of each set of corrected initial rate (v_i) against $[S]_0$ data to the Michaelis–Menten equation was first checked by observation of an intersecting pattern of lines in a direct linear plot [23] and the linearity of an $[S]_0/v_i$ against $[S]_0$ plot [24]. Values of the parameters V_{max} and K_m were then determined by using the weighted non-linear regression program in SIGMAPLOT 5.0 (Jandel Scientific) using a Research Machines Pentium III PC/500 MHz. An error structure of constant relative error was assumed, and weighting factors were inversely proportional to v_i^2. Values of the catalytic rate constant (k_{cat}) were calculated from $k_{cat} = 10V_{max}/2[IgG] = 5V_{max}/[IgG]$ (assuming 10 % of the IgG is catalytic and two potential active centres per molecule).

Characterization of the pH-dependence of k_{cat} and k_{cat}/K_m was carried out by using the multi-tasking application program SKETCHER [25] written in ANSI C running under RISCOS on an Acorn Archimedes microcomputer, and by regression of $1/k_{cat}$ and k_{cat}/K_m on $[H^+]$, since both k_{cat} and k_{cat}/K_m increase with an increase in pH along a single ionization curve [26]. SKETCHER permits rapid estimation of characterizing parameters in the generic set of equations for various models by means of interactive manipulation of calculated curves. For simple models, as in the present paper, values of the parameters thus obtained are useful as provisional estimates for analysis by weighted non-linear regression. For more complex models with closely spaced K_a values, some determined separately by independent experiments [27], SKETCHER facilitates evaluation of the unknown parameters.

RESULTS AND DISCUSSION

Design and synthesis of the immunogens and cognate substrates

The objective of the work here reported was to investigate the flexibility hypothesis referred to in the Introduction. The approach involved comparison of the kinetic characteristics of catalytic antibodies elicited by using closely related immunogens differing only in the rigidity of the atomic framework around the structural motif of the hapten analogous to the reaction centre of the corresponding substrate. The inclusion of identical leaving-group determinants in the haptens and identical leaving groups in the corresponding substrates was essential in order to remove the ambiguity inherent in the systems discussed in [4].

In the present work, as one example of this approach, we compared the kinetic characteristics of the two catalytic antibody preparations, PCA 271-22 and PCA 2649-16 (see the Introduction), making use of the phosphate immunogen **1a**/carbonate substrate **4** and phosphonate immunogen **5a**/ester substrate **6a** systems respectively. The 4-nitrophenyl group, which provides a chromogenic leaving group in the substrates and a strongly

antigenic group in the corresponding immunogens, is common to all four components of the two systems. The other common features, i.e. the additional phenyl ring and the amide group, were introduced to provide opportunities for recognition of the substrates by the antibodies at hydrophobic and hydrogen-binding sites. All of these features were introduced into the phosphate/carbonate systems discussed previously [2]. The major respect in which the phosphonate **5a**/ester **6a** system differs from the phosphate **1a**/carbonate **4** system is that, in **5a** and **6a**, the phenyl ring of the non-leaving-group moiety in **6a** and its analogue in **5a** are bonded directly to the carbonyl carbon (in **6a**) or to the phosphorus atom (in **5a**) rather than via an oxygen bridge. This difference provides the decrease in degrees of freedom needed to investigate the flexibility hypothesis. The two single bonds of the oxygen bridge in **1a** and **4** are replaced by one single bond in **5a** and in **6a**, which is part of the conjugated system and thus will restrict the rotation of the phenyl ring in the latter immunogen and substrate.

The syntheses of the phosphate immunogen **1a** and the carbonate substrates **2a**, **2b** and **4** relating in the present work to PCA 271-22 have been reported previously [2,12] in connection with work on other anti-phosphate PCA preparations. The syntheses of the phosphonate immunogen **5a** and the ester substrates **6a** and **6b** that relate to PCA 2649-16 are outlined below, summarized in Schemes 1 and 2 respectively and detailed in the Materials and methods section.

The route to the 4-nitrophenyl phosphonate immunogen **5a** makes use of the strategy for the synthesis of diethyl arylphosphonates reported by Grabiak et al. [28]. Thus, in the present case, elimination of ethyl iodide in the reaction of ethyl 4-iodobenzoate with triethyl phosphite catalysed by $NiBr_2$ gave a triethyl ester, hydrolysis of which provided 4-carboxyphenyl phosphonic acid (Scheme 1). Treatment of this with thionyl chloride gave the trichloride, which was used to produce the corresponding tri-4-nitrophenyl ester. Alkaline hydrolysis of this triester gave the 4-nitrophenyl arylphosphonate hapten **5b**, which was carbodi-imide-coupled to the carrier protein KLH to provide the 4-nitrophenyl phosphonate immunogen **5a**. The successful production of **5b** relies on the fact that one of the phosphonate diester groups is considerably more base-labile than the other.

Synthesis of the 4-nitrophenyl (**6a**) and 2-nitrophenyl (**6b**) aryl ester substrates (Scheme 2) involved reaction of terephthaloyl chloride with 1-butylamine to produce 4-(1-butylamido)benzoic acid. The carboxy group of this intermediate was then esterified with 4-nitrophenol or 2-nitrophenol.

Catalytic antibody preparations

Anti-phosphate PCA preparations have been isolated in these laboratories from three sheep (270, 271 and 272) at various times during the immunization programme. Although some characterization of PCA preparations from all three sheep has been reported [2,3,12], most of the detailed kinetic studies have used PCA 270 preparations.

In the present work, the little-investigated anti-phosphate antibody preparation PCA 271-22 and the newly elicited anti-phosphonate antibody preparation PCA 2649-16 were investigated kinetically. Use of the two pairs of isomeric substrates (**2a** and **2b** for PCA 271-22, and **6a** and **6b** for PCA 2649-16) provided strong evidence for the absence of enzyme contaminants, as has been found for other PCA preparations [2,18]. As is the case for all other PCA preparations investigated in these laboratories, catalysis by PCA 271-22 and PCA 2649-16 was found to obey single-site saturation kinetics (Figure 2). There is no evidence, therefore, for kinetic heterogeneity in these preparations.

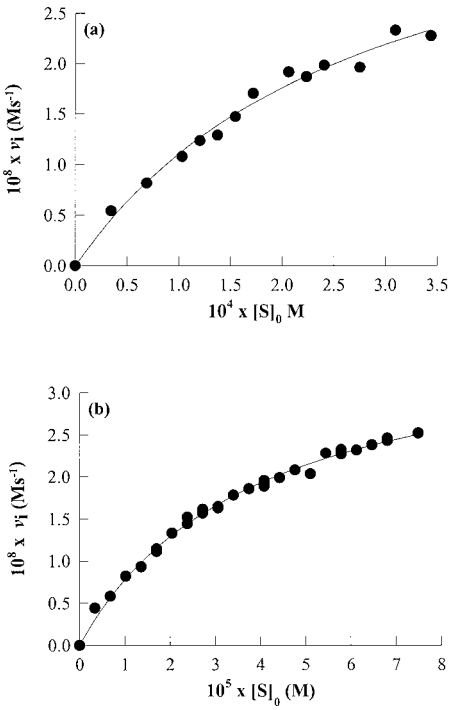


Figure 2 Demonstration of the adherence to the Michaelis–Menten equation of the hydrolysis at 25 °C in 0.1 M potassium phosphate buffer (*I* 0.3) containing 10% (v/v) acetonitrile of (a) the carbonate substrate 4 catalysed by PCA 271-22 (1.2 μM IgG), and (b) the ester substrate 6a catalysed by PCA 2649-16 (0.5 μM IgG)

The points are experimental and the continuous lines correspond to $v_i = V_{max}[S]_0/(K_m + [S]_0)$ with for (**a**) $V_{max} = (4.32 \pm 0.38) \times 10^{-8}$ M · s^{-1} (best-fit values; mean ± S.E.M.) and $K_m = 290 \pm 44$ μM, and for (**b**) $V_{max} = (3.82 \pm 0.08) \times 10^{-8}$ M · s^{-1} and $K_m = 39 \pm 2$ μM. The data in (**a**) were obtained by correcting the observed initial rates (v_i^{obs}) for the background reaction by using the first-order rate constant for the reaction of **4** with 1.2 μM NSS IgG (2.47×10^{-4} s^{-1}); the data in (**b**) were obtained from values of v_i^{obs} by subtracting background rates of aqueous hydrolysis, calculated by using $k_{non\text{-}cat} = 3.15 \times 10^{-5}$ s^{-1}, which were not affected by inclusion of 0.5 μM NSS IgG.

Evidence for the flexibility hypothesis

By maintaining the 4-nitrophenolate leaving group in both the carbonate substrate (**4**) and the ester substrate (**6a**), and the corresponding 4-nitrophenyl group in both immunogens (**1a** and **5a**), it was possible to isolate the effects on the kinetic characteristics of PCA 271-22 and PCA 2649-16 of changing the flexibility around the reaction centre of the substrate and its determinant in the immunogen. The values of V_{max} and K_m for the catalyses at pH 8.0 (Figure 2) and other kinetic parameters are collected in Table 1. As pointed out in the Materials and methods section and in the footnote to Table 1, values of k_{cat} were calculated by assuming that 10% of IgG is catalytic and possesses two potential active centres per molecule. The values of $k_{cat}/k_{non\text{-}cat}$, i.e. values of k_{cat} (see the Materials and methods section) corrected for the differences in the intrinsic reactivity of the substrate towards aqueous hydrolysis, were 9×10^2 for the anti-phosphate antibody and 1.2×10^4 for the anti-phosphonate antibody. Thus there is a kinetic advantage in favour of the latter of 13-fold ($1.2 \times 10^4/9 \times 10^2$) in terms of k_{cat}, and a 100-fold advantage ($3.1 \times 10^8/3.1 \times 10^6$) in terms of the proficiency constant, $k_{cat}/k_{non\text{-}cat} \cdot K_m$. The proficiency constant, introduced by Radzicka and Wolfenden [29], is related to the well known specificity constant, k_{cat}/K_m, with account taken of the intrinsic reactivity of the substrate. It measures the ability of an

Table 1 Kinetic parameters at 25 °C and pH 8.0 of the hydrolysis of (i) the carbonate substrate 4 catalysed by the anti-phosphate catalytic antibody preparation PCA 271-22, and (ii) the ester substrate 6a catalysed by the anti-phosphonate catalytic antibody preparation PCA 2649-16

The reactions were carried out in potassium phosphate buffer, pH 8.0, *I* 0.3, containing 10 % (v/v) acetonitrile; for further details, see the text. $[(k_{cat}/k_{non\text{-}cat})_{2649\text{-}16/\mathbf{6a}}]/[(k_{cat}/k_{non\text{-}cat})_{271\text{-}22/\mathbf{4}}] = 13$; $[(k_{cat}/k_{non\text{-}cat} \cdot K_m)_{2649\text{-}16/\mathbf{6a}}]/[(k_{cat}/k_{non\text{-}cat} \cdot K_m)_{271\text{-}22/\mathbf{4}}] = 100$.

Antibody	[IgG] (μM)	Substrate	K_m (μM)	V_{max} ($M \cdot s^{-1}$)	$5V_{max}/[IgG] = k_{cat}$ (s^{-1})*	$k_{non\text{-}cat}$ (s^{-1})†	$k_{cat}/k_{non\text{-}cat}$	$k_{cat}/k_{non\text{-}cat} \cdot K_m$ (M^{-1})
271-22	1.2	**4**	290 ± 44	$(4.32 \pm 0.38) \times 10^{-8}$	(0.18 ± 0.02)	$(2.0 \pm 0.1) \times 10^{-4}$	9.0×10^2	3.1×10^6
2649-16	0.5	**6a**	39 ± 2	$(3.82 \pm 0.08) \times 10^{-8}$	(0.38 ± 0.08)	$(3.15 \pm 0.1) \times 10^{-5}$	1.2×10^4	3.1×10^8

* Values of k_{cat} were calculated from $k_{cat} = 10V_{max}/2[IgG]$, i.e. by assuming 10 % of the IgG to be catalytic and to possess two potential active centres per molecule.
† Values of $k_{non\text{-}cat}$ are those of the first-order rate constants for aqueous hydrolysis at pH 8.0.

enzyme or enzyme-like catalyst to lower the activation energy of a reaction. Expressed in units of M^{-1}, it represents the lower limit of the affinity of the catalyst for the perturbed substrate in the transition state.

These kinetic data compel the view that decreasing the flexibility from that of the phosphate/carbonate system (**1a/4**) to that of the phosphonate/ester system (**5a/6a**) does indeed result in substantial enhancement of the catalytic effectiveness of the catalytic antibody. A haptenic structure that has fewer degrees of freedom around the reaction centre determinant would be expected to elicit antibodies with fewer relevant conformational variants associated with the reaction-centre binding site. If at least one variant has better complementarity to the reaction transition state, the catalytic effectiveness of the antibody preparation will be improved. The fact that the polyclonal preparations investigated exhibit kinetic homogeneity suggests that if a given preparation contains multiple conformational variants, the variations that affect catalytic activity are below detectable limits.

Common features of the structures and mechanisms of PCA 271-22 and PCA 2649-16

The kinetic advantage of the anti-phosphonate antibody preparation PCA 2649-16 raised the question of whether its active-centre characteristics might be qualitatively different from those of the anti-phosphate antibody preparation PCA 271-22. To investigate this possibility, we used approaches involving pH-dependent kinetics [26,30] coupled with binding studies and group-selective chemical modification. These have been used previously to characterize another anti-phosphate antibody preparation, PCA 270-29 [12].

The results from these experiments lead to the conclusion that, as was found for PCA 270-29, the catalytic activity of both PCA 271-22 and PCA 2649-16 requires contributions from the side chains of tyrosine and arginine residues. By contrast, there is no evidence for the involvement of side chains of lysine, histidine or cysteine residues. The evidence from the kinetic and chemical modification studies and from studies on the pH-dependence of binding suggests that, in all three cases, catalysis might involve assistance to the reaction of the substrate with hydroxide ion by hydrogen-bond donation at the reaction centre by tyrosine and arginine side chains. This combination of hydrogen-bond donors is a feature common to a number of other catalytic antibodies [9]. High-pK_a acidic side chains may be essential for the catalytic effectiveness of catalytic antibodies that utilize hydroxide ions as the attacking nucleophile. The postulated antibody mechanism differs substantially from those of hydrolysis catalysed by hydrolytic enzymes such as pig liver serine esterase and α-chymotrypsin. As is well known, particularly for α-chymotrypsin, the enzyme mechanisms involve general base catalysis by a histidine side chain of attack initially by a serine hydroxy group and subsequently by an undissociated water molecule. The results of the present studies suggest the absence of gross differences in mechanism between the anti-phosphonate antibody preparation PCA 2649-16 and the anti-phosphate antibody preparation PCA 271-22. The kinetic advantage, therefore, may be due to more subtle differences involving the closer complementarity of the active centre of the anti-phosphonate antibody to key features of the reaction transition state within a common gross mechanism involving reaction of HO^- assisted by hydrogen-bond donation by the antibody.

Figure 3 shows that, for both PCA 271-22 and PCA 2649-16, k_{cat} and k_{cat}/K_m each increase with an increase in pH across pK_a approx. 9. These results, taken together with those of the chemical modification studies, which provide evidence for essential tyrosine side chains (but not for lysine, histidine or cysteine side chains), suggest a key role for a relatively unperturbed phenolic hydroxy group in the common catalytic mechanism. In addition to its suggested role in binding (see below), the pH-dependence of k_{cat} suggests a role in catalysis. As is well known (e.g. see [26]), kinetic analysis alone can define proton stoichiometries, but is only rarely able to define the proton locations in the transition state of a pH-dependent reaction. Thus the sigmoidal curves in Figure 3 are consistent with two types of mechanism. The mechanism that relates more obviously to the observed pH-dependence characteristics is that of the reaction of the carbonate or ester substrate (S) with a water molecule, assisted by the ionized form of the postulated Tyr side chain, Ab-Tyr-O^-, acting as either a general base or a nucleophile. The other involves reaction of the substrate with HO^- assisted by hydrogen-bond donation from the undissociated form of the Tyr side chain, Ab-Tyr-OH. The ambiguity arises from the rapid equilibration of Ab-Tyr-OH and Ab-Tyr-O^- and the resulting kinetic equivalence illustrated by eqns (1) and (2), in which k_1 and k_2 are pH-independent values of k_{cat} or k_{cat}/K_m:

$$\text{Rate} = \tilde{k}_1[\text{Ab-Tyr-OH}][HO^-][S] \quad (1)$$

$$\text{Rate} = \tilde{k}_2[\text{Ab-Tyr-O}^-][H_2O][S] \quad (2)$$

Evidence in favour of eqn (1), i.e. reaction of HO^- assisted by Ab-Tyr-OH, was provided in the case of PCA 270-29 from the ELISA studies on both native and nitrated antibody preparations described in [12], and analogous results were obtained in the present work for both PCA 270-29 and PCA 2649-16. Data from the ELISA demonstrated that (a) binding to native antibodies is stronger at pH 6 than at pH 9, and (b) binding to tetranitromethane-nitrated antibodies is retained at pH 6, but abolished at pH 9. The latter result is consistent with the expected decrease in the pK_a value of the nitrophenolic hydroxy group of the postulated Tyr side chain from approx. 9 to approx. 7. Thus, in the native antibodies (Ab-Tyr-OH; p$K_a \sim 9$), approx. 50 % of the undissociated Tyr hydroxy group should exist at pH 9, with consequent retention of

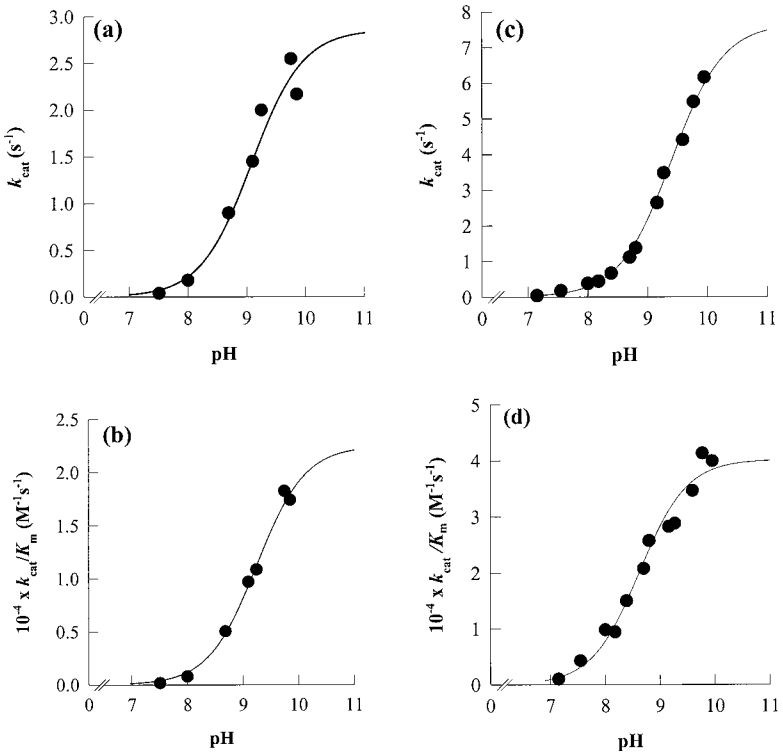


Figure 3 pH-dependence of (a) k_{cat} and (b) k_{cat}/K_m for the hydrolysis of the carbonate substrate 4 catalysed by PCA 271-22, and (c) k_{cat} and (d) k_{cat}/K_m for the hydrolysis of the ester substrate 6a catalysed by PCA 2649-16

The reactions were carried out at 25 °C in potassium phosphate buffers (see the text), *I* 0.3, containing 10 % (v/v) acetonitrile. The points are experimental and the continuous lines are theoretical for the single-ionization equation $k = \tilde{k}/(1 + [H^+]/K_a)$, where $k = k_{cat}$ or k_{cat}/K_m and $\tilde{k} = \tilde{k}_{cat}$ or $\tilde{k}_{cat}/\tilde{K}_m$ (pH-independent kinetic parameters), with the following values of the parameters: (**a**) $pK_a = 9.08 \pm 0.04$, $k_{cat} = 2.86 \pm 0.6\ s^{-1}$; (**b**) $pK_a = 9.25 \pm 0.05$, $\tilde{k}_{cat}/\tilde{K}_m = (2.25 \pm 0.01) \times 10^4\ M^{-1} \cdot s^{-1}$; (**c**) $pK_a = 9.40 \pm 0.02$, $\tilde{k}_{cat} = 7.65 \pm 0.04\ s^{-1}$; (**d**) $pK_a = 8.62 \pm 0.04$, $\tilde{k}_{cat}/\tilde{K}_m = (4.02 \pm 0.08) \times 10^4\ M^{-1} \cdot s^{-1}$.

some binding ability. By contrast, in the nitrated antibodies [Ab-Tyr-(NO_2)-OH; $pK_a \sim 7$], the hydroxy group should be almost fully ionized at pH 9, with consequent essentially complete loss of binding ability.

It is the combination of the pH-dependent kinetic data, the pH-dependent binding data and structural considerations that suggests that it is unlikely that there is substantial conformational change between binding and nucleophilic attack at the carbonyl centre of the substrate. In the absence of a substantial conformational change, a Tyr side chain that provides hydrogen-bond donation to the carbonyl oxygen atom of the substrate could not provide nucleophilic or general-base catalysis in addition. Nucleophilic attack by either Tyr-O^- or water assisted by Tyr-O^-, both of which would occur by rear-side attack at carbonyl carbon, would require the essential Tyr side chain to be in a different location to that in which Tyr-OH could donate a hydrogen bond to the carbonyl oxygen atom. Geometric considerations, therefore, do not support a mechanism in which a Tyr side chain is required in the undissociated state for binding and in the ionized state for catalysis. This suggests that a mechanism involving nucleophilic attack by HO^- (i.e. eqn 1) is the more probable. Possible additional binding interactions within the antibody–substrate complex involving Arg side chains were discussed in [12], and apply also to the present work.

Concluding comments

Characterization of two catalytic antibody preparations elicited by closely similar phosphate and phosphonate immunogens containing identical leaving group determinants has provided evidence both for the kinetic superiority of the latter and for a common gross mechanism probably involving nucleophilic attack by hydroxide ion with assistance by hydrogen-bond donation to the reaction centre from undissociated Tyr-OH groups, and possibly also from Arg side chains. The only major difference perceived in the structures of the haptenic components of the two immunogens is the greater flexibility of the less effective phosphate hapten. Thus, in the more rigid system, it may be concluded that binding energy is utilized more effectively in stabilizing the transition state. Kinetic studies on catalytic antibodies elicited by a wide range of carefully designed, related immunogens should help to establish patterns of reactivity leading to general concepts as an aid to effective rational hapten design.

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