Characterization of the hydrolytic activity of a polyclonal catalytic antibody preparation by pH-dependence and chemical modification studies: evidence for the involvement of Tyr and Arg side chains as hydrogen-bond donors

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The hydrolyses of 4-nitrophenyl 4′-(3-aza-2-oxoheptyl)phenyl carbonate and of a new, more soluble, substrate, 4-nitrophenyl 4′-(3-aza-7-hydroxy-2-oxoheptyl)phenyl carbonate, each catalysed by a polyclonal antibody preparation elicited in a sheep by use of an analogous phosphate immunogen, were shown to adhere closely to the Michaelis–Menten equation, in accordance with the growing awareness that polyclonal catalytic antibodies may be much less heterogeneous than had been supposed. The particular value of studies on polyclonal catalytic antibodies is discussed briefly. Both the k_{cat} and k_{cat}/K_m values were shown to increase with increase in pH across a pK_a of approx. 9. Group-selective chemical modification studies established that the side chains of tyrosine and arginine residues are essential for catalytic activity, and provided no evidence for the involvement of side chains of lysine, histidine or cysteine residues. The combination of evidence from the kinetic and chemical modification studies and from studies on the pH-dependence of binding suggests that catalysis involves assistance to the reaction of the substrate with hydroxide ions by hydrogen-bond donation at the reaction centre by tyrosine and arginine side chains. This combination of hydrogen-bond donors appears to be a feature common to a number of other hydrolytic catalytic antibodies. High-pK_a acidic side chains may be essential for the effectiveness of catalytic antibodies that utilize hydroxide ions.

INTRODUCTION

Following the first demonstration of antibody-mediated catalysis in 1986 [1,2], the catalytic antibody field has developed rapidly in a number of directions. Most investigations have been concerned with monoclonal catalytic antibodies, and a principal objective of the early work was the demonstration of the wide range of chemical reactions for which antibody catalysts could be produced [3,4]. Currently, particular attention is being given to catalytic mechanism [5], and papers reporting kinetic, protein chemical and structural studies are beginning to appear [6–11].

Another aspect of the field is concerned with polyclonal catalytic antibodies (PCAs). The finding (reviewed in [12]) that, despite early discouraging results [13,14], substantial catalytic activities can be generated reproducibly in polyclonal preparations [15–20] suggests valuable opportunities for studies and applications complementary to those involving monoclonal catalytic antibodies. The particular value of the former, pointed out in [16] and emphasized by others [12,20,21], includes: (i) the relative simplicity of producing polyclonal as against monoclonal antibodies, (ii) their potential value as catalysts for technological applications, (iii) the rapid and cost-effective application in evaluating a range of haptens for production of catalytic activity, and (iv) their potential value in the development of novel therapies when produced by active immunization [18,19]. Because polyclonal IgG necessarily represents the entirety of the immune response, the relative immunogenic capabilities of a series of haptens may be assessed more effectively than by studies on a small selection of isolated monoclonal antibodies [20].

Although a potential problem in the evaluation of the catalytic activity of polyclonal IgG is that heterogeneity might result in complex kinetic behaviour, this has not, so far, proved to be the case. We have reported the kinetic characteristics of catalytically active polyclonal IgG preparations purified from sheep serum [15–18]. IgG from sheep immunized with the 4-nitrophenyl phosphate–keyhole-limpet haemocyanin (KLH) conjugate [compound (Ia) in Figure 1] was shown to catalyse the hydrolysis of the analogous 4-nitrophenyl carbonate [Figure 1, compound (II)] at pH 8.0. It is an important observation that the catalysis obeyed the Michaelis–Menten equation (at least in the range of substrate concentrations studied, i.e. ~ 0.2–$17\ \mu$M). This provides no evidence for functional heterogeneity in the various IgG preparations. As is well known, catalysis by mixtures of enzymes with significantly different characteristics deviates from adherence to the Michaelis–Menten equation, such that plots of [S]/v against [S] (where v is initial velocity) are markedly curved concave downwards [22,23]. The kinetic homogeneity of polyclonal antibodies is somewhat surprising in view of current perceptions of clonal selection. It is important to emphasize, therefore, that close adherence to the Michaelis–Menten equation has been demonstrated using IgG preparations isolated from the antisera of three different sheep (nos. 270–272) collected at different times in the immunization programme. Thus single-site saturation behaviour has been observed for the following PCA preparations (see [15–18]): PCA270-22 (a preparation isolated from the antiserum of sheep 270 in week 22 of the immunization programme), PCA270-n (where $n = 26$ and 29 weeks), PCA271-n (where $n = 22$, 29, 34, 38, 42, 92 and 100 weeks) and PCA272-

Abbreviations used: DEP, diethyl pyrocarbonate; KLH, keyhole-limpet haemocyanin; PCA, polyclonal catalytic antibody; PCA270-29 (etc.), PCA isolated from the antiserum of sheep no. 270 in week 29 of the immunization programme (etc.); TNM, tetranitromethane.

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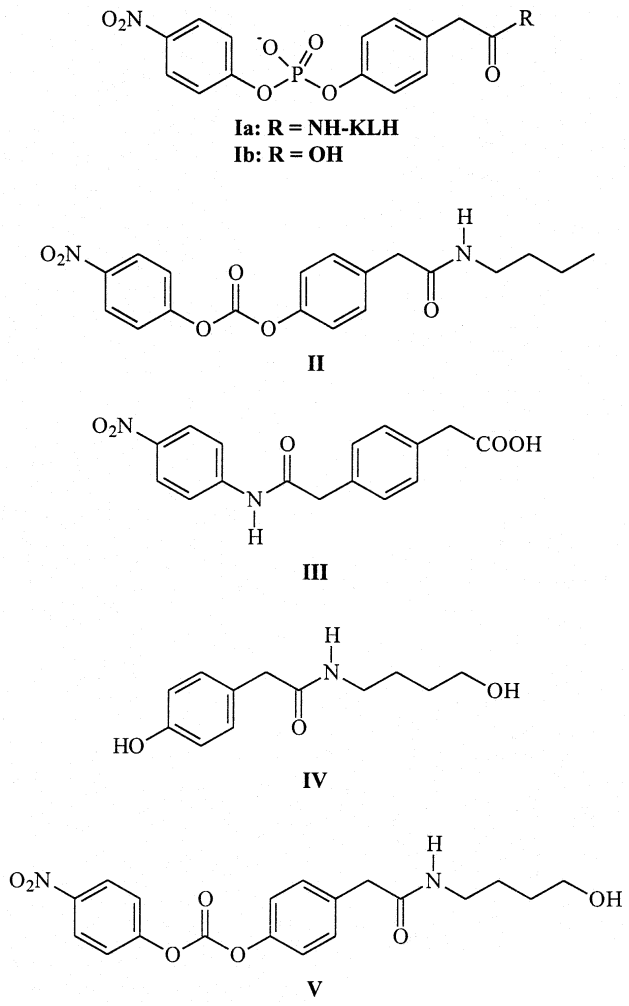

Figure 1 Structures of compounds used in the present study

(Ia) is the phosphate immunogen used to elicit PCA270-29; (Ib) is the hapten 4-nitrophenyl 4′-(carboxymethyl)phenyl hydrogen phosphate; (II) is the carbonate substrate 4-nitrophenyl 4′-(3-aza-2-oxoheptyl)phenyl carbonate used in the pH-dependence studies; (III) is the anilide ligand 4-carboxymethyl-*N*-(4′-nitrophenyl)phenylacetamide used in the ELISA; (IV) is 4-(3-aza-7-hydroxy-2-oxoheptyl)phenol, the precursor of (V); and (V) is 4-nitrophenyl 4′-(3-aza-7-hydroxy-2-oxoheptyl)phenyl carbonate, the more soluble substrate used to demonstrate kinetic homogeneity in PCA270-29 at substrate concentrations up to at least 85 μM.

n (where $n = 22$, 29 and 42 weeks). In the present work, further evidence for the kinetic homogeneity of PCA270-29 is presented, both for the carbonate substrate [Figure 1, compound (II)] and for a new, more soluble, carbonate substrate, 4-nitrophenyl 4′-(3-aza-7-hydroxy-2-oxoheptyl)phenyl carbonate [Figure 1, compound (V)], which permits single-site saturation behaviour to be established with substantially higher substrate concentrations. Our finding that polyclonal catalytic IgG exhibits functional (kinetic) homogeneity, which permits valid kinetic characterization in terms of a simple one-site saturation model, is supported by other demonstrations of analogous functional homogeneity by kinetic [20] and time-resolved luminescence [24] studies. 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. One explanation for the observed functional homogeneity is that different, genetically unrelated, antibodies could interact with the hapten in closely similar binding modes. As Shreder et al. [24] have pointed out, the alternative explanation for functional homogeneity would need to involve the postulate that the catalytic IgG is essentially monoclonal in nature.

In the present work, we have further characterized the catalytic activity of the polyclonal antibody preparation designated PCA270-29 [18] (the PCA preparation isolated from the antiserum of sheep 270, in week 29 of the immunization programme) by determining the pH-dependence characteristics of k_{cat} and k_{cat}/K_m, and of binding by using an ELISA and by group-selective chemical modification studies. The evidence from these studies leads to the hypothesis that rate enhancement involves assistance to nucleophilic attack of hydroxide ion by hydrogen-bond donation to the substrate from Tyr and Arg side chains.

MATERIALS AND METHODS

Materials

Imject® BSA and ImmunoPure® rabbit anti-sheep IgG (H + L) were from Pierce and Warriner Ltd. (Chester, U.K.). Protein G–Sepharose (4 Fast Flow) and Sephadex G-25 were from Pharmacia Biotech (Uppsala, Sweden), and KLH was from Calbiochem-Boehring (San Diego, CA, U.S.A.). Microtitre plates (flat bottomed; Immunoplate Maxisorp F96) were from Nunc. Solvents (analytical grade) and aluminium-backed silica gel 60 TLC plates (Merck 5554) and silica gel 60 for flash column chromatography (Merck particle size 0.040–0.063 nm) were from Merck Ltd. (Lutterworth, Leics., U.K.). All other chemicals were from Sigma–Aldrich Co. (Poole, Dorset, U.K.). 4-Isopropyl phenol was recrystallized from aq. 10% (v/v) methanol to a constant melting point (58–60 °C).

Syntheses

Immunogen (Ia) and carbonate substrate (II)

Preparation of the phosphate immunogen (Ia) and the synthesis of 4-nitrophenyl 4′-(carboxymethyl)phenyl hydrogen phosphate (Ib), which is coupled to KLH to produce the immunogen, and of the carbonate substrate 4-nitrophenyl 4′-(3-aza-2-oxoheptyl) phenyl carbonate (II), were carried out as described previously [16].

Anilide ligand (III)

Synthesis of the anilide ligand, 4-carboxymethyl-*N*-(4′-nitrophenyl)phenylacetanilide (III), for use in the ELISA was carried out as follows. Pyridine (166 μl; 2.06 mmol) was added to a stirred suspension of 1,4-phenylenediacetic acid (200 mg; 1.03 mmol) in acetonitrile (4 ml) at 0 °C. Pivaloyl chloride (253 μl; 2.06 mmol) was then added and the resulting mixture was stirred and allowed to warm to room temperature (approx. 22 °C). Stirring was continued for 1 h at room temperature until TLC monitoring (developing solvent dichloromethane/methanol, 6:1, v/v) indicated that formation of the mixed dianhydride was complete (1,4-phenylenediacetic acid has an R_F of 0.21 and the mixed dianhydride has an R_F of 0.86 in this solvent system). 4-Nitroaniline (142 mg; 1.03 mmol) and pyridine (83 μl; 1.03 mmol) were added to the reaction mixture and stirring was continued at room temperature for 18 h. The mixture was cooled to 0 °C, saturated aqueous $NaHCO_3$ (20 ml) was added and the resulting solution was stirred for 30 min at 0 °C. The acetonitrile was removed *in vacuo* and the remaining aqueous

solution was extracted with diethyl ether (2 × 35 ml). The aqueous layer was adjusted to pH 7.5 by adding 2.0 M HCl (10 ml) followed by sodium phosphate buffer solution (pH 7.5; 1.0 M; 20 ml). The aqueous solution was extracted with ethyl acetate (5 × 60 ml). The ethyl acetate extracts were combined, dried and concentrated *in vacuo* to give the crude product (R_F 0.36 in the above solvent system) as a white solid (230 mg; 70% yield). A portion (100 mg) of the crude product was purified by trituration with ethyl acetate to give the pure 4-carboxymethyl-*N*-(4′-nitrophenyl)phenylacetanilide as a white solid [85 mg; 85% yield; m.p. > 260 °C (decomposed)].

The compound had the following properties: (KBr) IR: ν (cm^{-1}) 3341 (NH), 3272 (broad, OH), 1696 (carbonyl, carboxylic acid) and 1669 (carbonyl, anilide); MS (fast-atom bombardment): $m/2z$ 157 (M^{2+}); ^{1}H-NMR (250 MHz): δ ($^2H_6$-DMSO) 3.53 (2 H, s, CH_2CO_2), 3.69 (2 H, s, CH_2CONH), 7.20 (2 H, d, J 8.3 Hz, H_A of ABq, HAr), 7.27 (2 H, d, J 8.3 Hz, H_B of ABq, HAr), 7.84 (2 H, d, J 8.2Hz, H_A of ABq, $HArNO_2$), 8.21 (2 H, d, J 8.2 Hz, H_B of ABq, $HArNO_2$).

BSA–anilide (III) conjugate

The anilide (III) (3 mg) was dissolved in acetonitrile (0.5 ml) and this solution was added to BSA (20 mg) in sodium phosphate buffer (6 ml; 50 mM; pH 7.4). 1-Ethyl-3-(3-dimethylaminopropyl)carbodi-imide hydrochloride (30 mg) was added and the mixture was left to react at room temperature for 4 h. The solution was dialysed overnight against the phosphate buffer with two changes. Determination of the number of mol of ligand bonded per mol of BSA (hapten density) was performed as follows. A small aliquot of conjugate solution (0.5 ml) was added to 3 M NaOH (0.5 ml) and left to react overnight. The sample was clarified by centrifugation and the A_{410} of the supernatant was measured to determine the 4-nitroaniline released (ϵ_{410} 8800 $M^{-1} \cdot cm^{-1}$ [17]). The hapten density for the conjugate was calculated as mol of 4-nitroaniline released/mol of BSA.

4-(3-Aza-7-hydroxy-2-oxoheptyl)phenol (IV) [precursor of the more soluble substrate (V)]

To a solution of 4-hydroxyphenylacetic acid (1.0 g; 6.6 mmol) and *N*-hydroxysuccinimide (1.5 g; 13 mmol) in acetonitrile (5 ml) was added 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide hydrochloride (1.26 g; 6.6 mmol) portionwise over 20 min. The mixture was stirred at room temperature for 1 h, at which time TLC monitoring (developing solvent dichloromethane/methanol, 6:1, v/v) showed that formation of the activated ester was incomplete (4-hydroxyphenylacetic acid has an R_F of 0.37 and the activated ester has an R_F of 0.66). A second equivalent (1.26 g; 6.6 mmol) of the carbodi-imide was added, portionwise, over 40 min and stirring was continued at room temperature. After 20 min, TLC monitoring indicated that formation of the activated ester was complete. The resulting solution was added dropwise over 1 h to a stirred solution of 4-amino-1-butanol (3 ml; 33 mmol) in water (5 ml) at room temperature, and the mixture was stirred for 18 h at room temperature. The acetonitrile was then removed *in vacuo* and the remaining mixture was basified with 0.5 M aqueous sodium hydrogen carbonate (10 ml) and extracted with diethyl ether (2 × 25 ml). The aqueous solution was then extracted with ethyl acetate (8 × 25 ml). The ethyl acetate extracts were combined, dried and concentrated *in vacuo* to give the crude product as a yellow oil (R_F 0.36; developing solvent dichloromethane/methanol, 7:1, v/v). Purification by flash column chromatography (eluting solvent dichloromethane/methanol, 20:1, v/v, then dichloromethane/methanol, 15:1, v/v, then dichloromethane/methanol, 11:1, v/v) gave the pure 4-(3-aza-7-hydroxy-2-oxoheptyl)phenol as a white solid (641 mg; 43% yield). Recrystallization from acetonitrile gave white crystals (518 mg; 35% yield), with an m.p. of 117–118 °C.

Fourier-transform IR: (KBr) ν (cm^{-1}) 3330, 3245, 3044, 2925, 2883, 1641, 1600, 1548, 1240; ^{1}H-NMR (250 MHz): δ ($^2H_6$-DMSO) 1.38 (4 H, m, -CH_2CH_2-), 3.02 (2 H, m, -CH_2O), 3.23 (2 H, t, -$NHCH_2$-), 3.34 (2 H, s, -$PhCH_2CO$-), 4.33 (1 H, s, -OH-), 6.65 (2 H, d, J 8.7 Hz, H_A of ABq, HAr), 7.03 (2 H, d, J 8.7 Hz, H_B of ABq, HAr), 7.83 (1 H, s, -NH), 9.15 (1 H, s, broad, ArOH).

4-Nitrophenyl 4′-(3-aza-7-hydroxy-2-oxoheptyl)phenyl carbonate (V) (the more soluble carbonate substrate)

To a stirred solution of 4-(3-aza-7-hydroxy-2-oxoheptyl)phenol (IV) (200 mg; 0.9 mmol) and triethylamine (126 μl; 0.9 mmol) in acetonitrile (6 ml) at 60 °C was added a solution of 4-nitrophenylchloroformate (180 mg; 0.9 mmol) in acetonitrile (500 μl) dropwise over 6 min. Stirring was continued for 1 h at 60 °C. Volatiles were then removed *in vacuo* and the residue was suspended in ethyl acetate (40 ml) and washed with a mixture of water and ice (2 × 5 ml). The organic phase was dried and concentrated *in vacuo* to give the crude product as an off-white solid (282 mg; 81% yield) (R_F 0.17; developing solvent dichloromethane/methanol, 20:1, v/v). Purification by flash chromatography (eluting solvent dichloromethane/methanol, 30:1, v/v, then dichloromethane/methanol, 20:1, v/v, then dichloromethane/methanol, 15:1, v/v) gave the pure 4-nitrophenyl 4′-(3-aza-7-hydroxy-2-oxoheptyl)phenyl carbonate as a white solid (147 mg; 42% yield). Recrystallization from ethyl acetate gave white needles (107 mg; 31% yield; m.p. 121–122 °C).

Fourier-transform IR: (KBr) ν (cm^{-1}) 3411, 3130, 2951, 2869, 1780, 1647, 1535, 1508, 1491, 1385, 1203; ^{1}H-NMR (250 MHz): δ ($^2H_3$-CH_3CN) 1.48 (4 H, m, -CH_2CH_2-), 2.56 (1 H, t, -OH-) 3.13 (2 H, m, -CH_2O), 3.46 (2 H, m, -$NHCH_2$-), 3.46 (2 H, s, -$PhCH_2CO$-), 6.55 (1 H, s, -NH), 7.27 (2 H, d, J 9 Hz, H_A of ABq, HAr), 7.37 (2 H, d, J 9 Hz, H_B of ABq, HAr), 7.56 (2 H, d, J 10.25 Hz, H_A of ABq, $HArNO_2$), 8.33 (2 H, d, J 10.25 Hz, H_B of ABq, $HArNO_2$)

Production and purification of antibodies

The polyclonal antibody preparation PCA270-29 was obtained from sheep no. 270 immunized with the KLH–(Ia) conjugate. The IgG was isolated by salt fractionation using Na_2SO_4 and purified by affinity chromatography on a Protein G–Sepharose 4 Fast Flow column (5 ml), as described previously [16].

ELISA procedure

Microtitre plates were coated with the anilide (III)–BSA conjugate (hapten density 8 mol/mol; 3 μg/ml; 100 μl/well in 50 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl; 4 °C, overnight). After extensive washing with the sodium phosphate buffer/NaCl solution also containing 0.05% (w/w) Tween 20, the plates were blocked with BSA solution (0.25%, w/w, in phosphate buffer/NaCl/Tween 20; 200 μl/well; 1 h at 37 °C). The polyclonal antibody preparation (PCA270-29) was diluted with the appropriate buffer, i.e. the sodium phosphate buffer, pH 7.4, containing 150 mM NaCl, or 10 mM 2-(*N*-cyclohexylamino)ethanesulphonic acid (Ches), pH 9.2, or 10 mM Mes, pH 6.0, and incubated at 37 °C for 1 h (100 μl per well). The plates were then washed with the phosphate buffer/NaCl/Tween 20 solution, the secondary antibody (rabbit anti-sheep

IgG conjugated to peroxidase; 1 μg/ml; 100 μl/well) was added and the plates were kept at room temperature for 1 h. After washing with the phosphate buffer/NaCl/Tween 20 solution, the peroxidase substrate [2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid)] in 0.05 M sodium phosphate/citrate buffer, pH 4.5; 1 mg/ml; containing 0.1 % (w/w) H_2O_2] was added, and the A_{405} [25] was recorded by using a Titertek Reader (Multiskan Plus MKII) connected to an Epson LX850 printer.

Kinetics of the hydrolysis of the carbonate substrates (II) and (V)

All experiments were performed at various pH values in sodium phosphate buffer containing 0.7 % (v/v) acetonitrile at 25 °C and constant ionic strength (I 0.15 or 0.9). The use of other buffers such as Tris, borate and carbonate was abandoned because of their accelerating effects on the background (non-IgG-catalysed) rates and because of specific buffer effects on the IgG itself (including effects on binding detected using the ELISA). Because of these complications, it was decided to use phosphate buffers over the whole of the pH range (approx. 8–10), despite the low buffering efficiency above pH 8.5. The pH of the reaction mixtures was monitored throughout the reaction, and no pH change was 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 the 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^- on the catalytic activity of PCA270-29 that was noted when NaCl was used in the preparation of the buffers.

Stock solutions of the substrate [(II) or (V)] at different concentrations were prepared in acetonitrile. A solution of PCA270-29 (24 μl of a 12.7 μM stock in phosphate buffer, pH 8.0) was added to phosphate buffer of a particular pH value (969 μl). The reaction was initiated by the addition of aliquots of stock solutions of substrate and acetonitrile to give a total volume of organic solvent of 7 μl. This resulted in 1 ml reaction mixtures containing 0.3 μM PCA270-29. The release of 4-nitrophenolate from the substrate was monitored at 410 nm using a Cary 1 spectrophotometer over a period of 1 min. Initial rates were determined from the linear increase in A_{410} and calculated in $M \cdot s^{-1}$ by using the relationship $\epsilon_{410} = 16963/(1+[H^+]/K_a)$ $M^{-1} \cdot cm^{-1}$, where $K_a = 10^{-7.15}$ M. These values were corrected for background (uncatalysed) hydrolysis determined in the absence of the antibody under otherwise identical experimental conditions. The rates of uncatalysed reactions have been shown previously [16] to be closely similar to those determined in the presence of IgG from non-immunized sheep. Values of the first-order rate constants for the background aqueous (uncatalysed) hydrolysis of substrates (II) and (V) at pH 9.33, I 0.9 and 25 °C were determined as 1.38×10^{-3} s^{-1} and 8.97×10^{-4} s^{-1} respectively. Thus the initial rates of background hydrolysis were 3 % and 11 % respectively of the initial rates corrected for background hydrolysis at the lowest and highest values of the concentration of substrate (II) used to determine the Michaelis parameters in Figure 2(a); the corresponding values for substrate (V) (Figure 2c) were 4.5 % and 20 % respectively. The pH of the 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 concentration of the substrate in each kinetic run.

Analysis of kinetic data

The adherence of the corrected initial rate (v_i)-against-[S] data to the Michaelis–Menten equation was first checked by observation of an intersecting pattern of lines in a direct linear plot [26] and the linearity of an [S]/v against [S] plot [22,23]. Values of the parameters V_{max} and K_m were then determined by using the weighted non-linear regression program of SIGMAPLOT 4.1 (Jandel Scientific) using a Tandon MCS 486/33 computer. An error structure of constant relative error was assumed and weighting factors were inversely proportional to v^2. Values of the apparent catalytic rate constant (k_{cat}) were calculated from $k_{cat} = V_{max}/2[IgG]$, i.e. by assuming all of the IgG to be catalytic (but see the Results and discussion section) and to possess two active centres per molecule.

Characterization of the pH-dependences of k_{cat} and k_{cat}/K_m was carried out by using the multitasking application program SKETCHER written in ANSI C running under RISCOS on an Acorn Archimedes microcomputer [27,28], and by regression of $1/k_{cat}$ or K_m/k_{cat} on $[H^+]$, since both k_{cat} and k_{cat}/K_m increase with the increase in pH along a single ionization curve [29].

Determination of the pK_a value of 4-isopropylphenol

This was determined from the pH-dependence of A_{293} in aqueous buffers in the pH range 8.5–11.3 at I 0.9 and I 0.15.

Computer modelling

The substrate (II) was modelled using QUANTA (MSI, Burlington, MA, U.S.A.) and CHARMM [30,31] to provide a structure for the schematic diagram (see Figure 4). Electron density was assigned to the atoms using standard CHARMM parameter sets [32]. Non-bonded interactions were truncated using a shifted potential [30] with a 14 Å cut-off [33]. Electrostatic interactions between atoms separated by three covalent bonds were scaled to 0.5 of their value. Minimizations were carried out using the steepest descent and adopted-basis Newton–Raphson algorithms [30]. QUANTA and IDRAW were used for visualization and graphics (see [34] for details of the computational techniques).

Group-selective chemical modification of PCA270-29

Chemical modification reactions targeted at the side chains of tyrosine, lysine, histidine, cysteine and arginine residues (see [35,36]) in PCA270-29 were carried out using 2–500-fold excesses of modifying reagent over the IgG concentration. Typical experiments are described below. For each reaction, control experiments were carried out in the absence of the modifying reagent under otherwise identical conditions to check that any loss of binding ability or catalytic activity was due to the chemical modification and not to denaturation of the protein promoted by the reaction conditions. All of the modified antibody preparations were evaluated for changes in binding characteristics by using the ELISA, and for changes in catalytic activity towards the carbonate substrate (II) at pH 9 (the latter after gel filtration through Sephadex G-25). In addition to the evaluation of loss of binding and catalytic activity, evidence of chemical modification was sought by spectral analysis as follows: (i) for reaction of tyrosine side chains with tetranitromethane (TNM), ΔA_{428} (ϵ_{428} 4.1×10^3 $M^{-1} \cdot cm^{-1}$ [36]); (ii) for reaction of histidine side chains with diethyl pyrocarbonate (DEP), ΔA_{242} (ϵ_{242} 3.2×10^3 $M^{-1} \cdot cm^{-1}$ [36]); (iii) for reaction of cysteine side chains with methyl 4-pyridyl disulphide (a gift from Dr. Surapong Pinitglang, Laboratory of Structural and Mechanistic Enzymology, Department of Biochemistry, Queen Mary and Westfield College, University of London), ΔA_{324} (ϵ_{324} 1.98×10^4 $M^{-1} \cdot cm^{-1}$ [37]; and see [38,39] for descriptions of 2- and 4-pyridyl disulphides as thiol titrants).

Tyrosine side chains

A freshly prepared solution of TNM in acetonitrile (20 μl; 105 mM) was added to PCA270-29 (400 μl; 12.7 μM) at 4 °C. The reaction mixture was kept at 4 °C for 90 min, then at room temperature (approx. 22 °C) for 90 min, and was then quenched by addition of 2-mercaptoethanol (20 μl) and diluted to a total volume of 1 ml with 0.1 M sodium phosphate buffer, pH 8.2. Changes in binding characteristics were investigated by performing the ELISA at pH 6 and pH 9 (pH values of the buffers used to dilute the modified IgG preparation immediately prior to incubation on the plate). To ascertain whether the modification of tyrosine residues was protected by the hapten (Ib), an analogous experiment was carried out in which compound (Ib), used as an inhibitor (0.36 mg; 400 mol/mol of IgG), was added to the solution of the antibody at 4 °C before the addition of TNM.

Lysine side chains

A sample of the stock solution of PCA270-29 (0.5 ml; 12.7 μM) was added to 0.1 M sodium borate buffer, pH 9.6 (0.5 ml). The pH of the solution was readjusted to 9.5 if necessary. To this solution was added an aliquot (3.5 μl) of a solution of methylacetamidate hydrochloride (1 M in 1 M NaOH). The mixture (pH 9.5) was kept at room temperature for 1 h and was then dialysed overnight against 1 litre of 50 mM sodium phosphate buffer, pH 8.2.

Histidine side chains

A solution of PCA270-29 (0.5 ml; 12.7 μM) was added to sodium acetate buffer (0.5 ml; 0.1 M, pH 5.0). A solution of DEP (20 μl; 30 mM) was added and the reaction mixture was kept at room temperature for 90 min prior to analysis.

Cysteine side chains

A sample of the stock solution of PCA270-29 (0.3 ml; 12.7 μM) was treated with methyl 4-pyridyl disulphide (1.51 μmol; approx. 400 mol/mol of IgG in 0.5 ml of sodium phosphate buffer, pH 8.0). Thiol groups would be expected to react rapidly with this type of reagent under these conditions [38].

Arginine side chains

Experiments were performed using stock solutions of phenylglyoxal at different concentrations. A solution of PCA270-29 (100 μl; 12.7 μM) was added to sodium phosphate buffer, pH 9.1, I 0.9 (100 μl). An aliquot (10 μl) of phenylglyoxal solution (5, 10, 20 or 50 mM) was added, and the reaction mixture was kept in the dark for 3 h at room temperature before analysis.

RESULTS AND DISCUSSION

Single-site saturation behaviour exhibited by PCA270-29

Many of the papers on catalytic antibodies in which kinetic experiments are reported and discussed do not show the v–[S] data, which makes it difficult to assess how closely such catalysed reactions obey single-site saturation kinetics. Figure 2 demonstrates the close adherence to the Michaelis–Menten equation of the hydrolyses of the carbonate ester substrates (II) and (V) catalysed by PCA270-29 (0.3 μM) at pH 9.33 (I 0.9). This serves to emphasize the point made previously [16,18] for a number of PCA preparations produced by using the immunogen (Ia), that there is no evidence of functional heterogeneity in these preparations. Use of the more soluble substrate (V) permitted demonstration of kinetic homogeneity up to [S] $\simeq$ 85 μM.

The value of V_{max} for the data in Figure 2(a) (5.7×10^{-7} $M^{-1} \cdot s^{-1}$) gives an apparent k_{cat} value of 0.95 s^{-1} for the catalysed hydrolysis of substrate (II), if the assumption is made that the concentration of functional antibody active centres is given by 2[IgG]. If the more reasonable assumption that only a small fraction of the IgG is catalytically active is made, the value of k_{cat} becomes correspondingly larger (e.g. 19 s^{-1} if only 5% of the IgG is active). The value of K_m for the data in Figure 2 is 9.9 μM and, with the k_{cat} assumed to be 19 s^{-1}, the value of k_{cat}/K_m [$0.95/(9.9 \times 10^{-6}) = 9.6 \times 10^4$ $M^{-1} \cdot s^{-1}$ using the initial assumption] becomes 1.9×10^6 $M^{-1} \cdot s^{-1}$. Analogous values of the parameters for the catalysed hydrolysis of substrate (V) (Figure 2c) are: $k_{cat} = 0.77$ s^{-1} (15.5 s^{-1} if only 5% of IgG is active), K_m = 14 μM and, with k_{cat} assumed to be 15.5 s^{-1}, $k_{cat}/K_m = 1.1 \times 10^6$ $M^{-1} \cdot s^{-1}$.

pH-dependence of k_{cat}/K_m and k_{cat} for hydrolysis of the carbonate substrate (II) catalysed by PCA270-29

The status of the proton as the least sterically demanding general perturbant of protein structure, and the importance of the protonation state of specific side chains in enzyme (and, by extension, catalytic antibody) function, combine to make properly designed and carefully interpreted pH-dependent kinetic studies one of the most useful approaches to probing catalytic function [29,40]. The principal experimental objectives of such studies are the identification and determination of the characteristics (pK_a values approximating to those of individual ionizing groups, and rate constants characteristic of the reaction of specific ionic forms of the reactants) of the kinetically influential ionizations.

Figure 3 shows the pH-dependence, in the pH range ~ 8–10, of k_{cat}/K_m and of k_{cat} at I 0.9 (Figures 3a and 3b) and I 0.15 (Figures 3c and 3d). The values of the characterizing parameters (kinetically influential pK_a values and pH-independent rate constants, $\tilde{k}_{cat}/\tilde{K}_m$ and $\tilde{k}_{cat}$) are collected in Table 1. Ratios of the values of the overall second-order rate constants for the reaction of antibody and substrate (II) to form product ($\tilde{k}_{cat}/\tilde{K}_m$) assuming only 5% of the IgG to be catalytically active (Table 1), and those of the second-order rate constants for the reaction of hydroxide ion and substrate (II) ($k_{OH^-} = 57$ $M^{-1} \cdot s^{-1}$ at I 0.9 and 60 $M^{-1} \cdot s^{-1}$ at I 0.15) provide values for the rate enhancement factor as $\tilde{k}_{cat}/\tilde{K}_m \cdot k_{OH^-} = 2.2 \times 10^3$ at I 0.9 and 1.3×10^3 at I 0.15. All four curves in Figure 3 are of simple sigmoidal form, with k_{cat} and k_{cat}/K_m increasing with increase in pH and with a pK_a value of approx. 9 in each case.

The pK_a value (~ 9) of the kinetically influential ionization suggests the possibility of the involvement in catalysis by PCA270-29 of the side chain of either a tyrosine or a lysine residue. Group-selective chemical modification, described below, provides evidence in favour of the former and against the latter. In an attempt to provide additional evidence for this conclusion from the chemical modification studies, the kinetic experiments originally carried out at I 0.9 (Figures 3a and 3b) were repeated at I 0.15 (Figures 3c and 3d). The pK_a of a cationic acid (Lys-N^+H_3) should be insensitive to changes in ionic strength, whereas that of an uncharged acid (Tyr-OH) should increase with a decrease in ionic strength, due to the decreased stabilization of the anionic conjugate base (Tyr-O^-). A decrease in I from 0.9 to 0.15 resulted in only a small increase in the pK_a value for the pH-dependence of k_{cat}/K_m (8.85 to 8.95), and no change in that of k_{cat} (9.20). This magnitude of increase, however, is similar to that

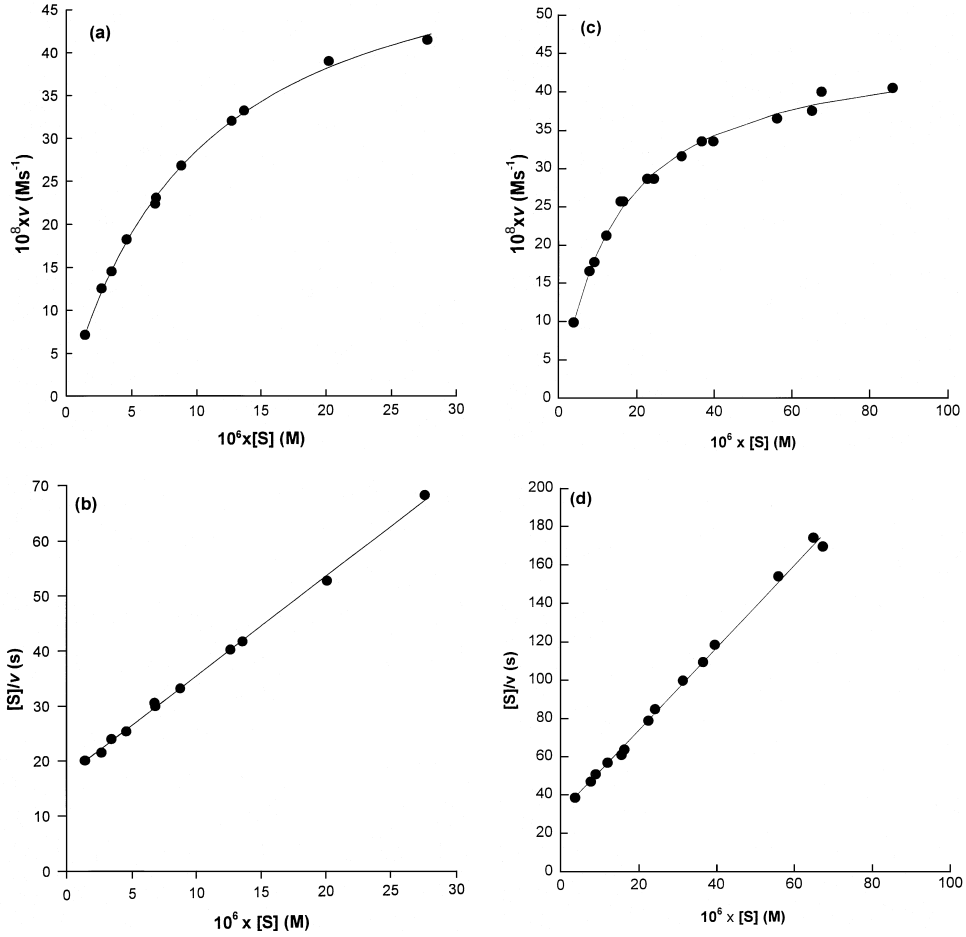

Figure 2 Demonstration of adherence to the Michaelis–Menten equation of the hydrolysis of (a, b) the carbonate substrate (II) and (c, d) the carbonate substrate (V) catalysed by PCA270-29

Catalysis was performed with 0.3 μM PCA270-29 at pH 9.33, I 0.9 and 25 °C. In (**a**) and (**c**), the points are experimental and the continuous lines correspond to $v = V_{max}[S]/(K_m + [S])$, with (**a**) $V_{max} = (5.66 \pm 0.09) \times 10^{-7}$ M^{-1}·s^{-1} (best fit value ± S.E.) and $K_m = 9.87 \pm 0.33$ μM, and (**c**) $V_{max} = (4.64 \pm 0.07) \times 10^{-7}$ M^{-1}·s^{-1} and $K_m = 14.03 \pm 0.64$ μM. These best-fit values of V_{max} and K_m were obtained by fitting the v-against-[S] data to the hyperbolic form of the Michaelis–Menten equation by weighted non-linear regression analysis, as described in the Materials and methods section. The linearity of the plots of [S]/v against [S] in (**b**) and (**d**) [as well as the good fit of the data points to the hyperbolic curves in (**a**) and (**c**)] demonstrates the adherence of the catalysed reaction to the Michaelis–Menten equation. This type of plot is particularly effective in demonstrating any deviation from Michaelis–Menten kinetics that might exist, due for example to the presence of mixtures of catalysts with different characteristics, which would produce downward concavity in the plot [23].

found for the change in pK_a of a model compound, 4-isopropyl phenol (pK_a 9.83 at I 0.9 to pK_a 10.0 at I 0.15). It was not possible to use an ionic strength substantially greater than 0.9 because of the low solubilities of the buffer salts under such conditions. Also, it was not possible to decrease the ionic strength to values lower than 0.15 due to the low buffering capacity above pH 8.5. The fact that the pK_a value for the pH-dependence of k_{cat} is somewhat higher than that for the pH-dependence of k_{cat}/K_m is consistent with the postulated tyrosine side chains being in a more anion-destabilizing environment in the antibody–substrate complex (k_{cat}) than in the free antibody molecule (k_{cat}/K_m) [29,40]. This could be a more hydrophobic environment provided either by direct shielding by the bound substrate or by a conformational change in the protein promoted by substrate binding.

As is well known, kinetic analysis alone is only rarely able to define the protonation state of the reactive molecular species. In the present case, the sigmoidal curves in Figure 3 are consistent with both the reaction of substrate (II) with water assisted by the ionized form of the postulated Tyr side chain (by a general-base or nucleophilic mechanism) and the reaction of (II) with HO^- assisted by hydrogen bonding with the undissociated form of the Tyr side chain. Thus both eqns. (1) and (2) below could describe the rate of catalysis when $[S] \ll K_m$ (reflected in the pH-dependence of k_{cat}/K_m), and an analogous pair of equations involving antibody-bound complexes could describe the rate when $[S] \gg K_m$ (reflected in the pH-dependence of k_{cat}).

$$\text{Rate} = (\tilde{k}'_{cat}/\tilde{K}'_m)\cdot[(II)][\text{Ab–Tyr-O}^-]\,[H_2O] \quad (1)$$

$$\text{Rate} = (\tilde{k}''_{cat}/\tilde{K}''_m)\cdot[(II)][\text{Ab–Tyr-OH}]\,[HO^-] \quad (2)$$

Eqns. (1) and (2) are kinetically equivalent, the pH-independent rate constants being related through the equilibrium constant, eqn. (3):

$$K = [\text{Tyr-O}^-][H_2O]/[\text{Tyr-OH}][HO^-] \quad (3)$$

Evidence in favour of eqn. (2), i.e. reaction of HO^- assisted by Tyr-OH, from the results of studies using the ELISA with both native and nitrated antibody preparations is described below.

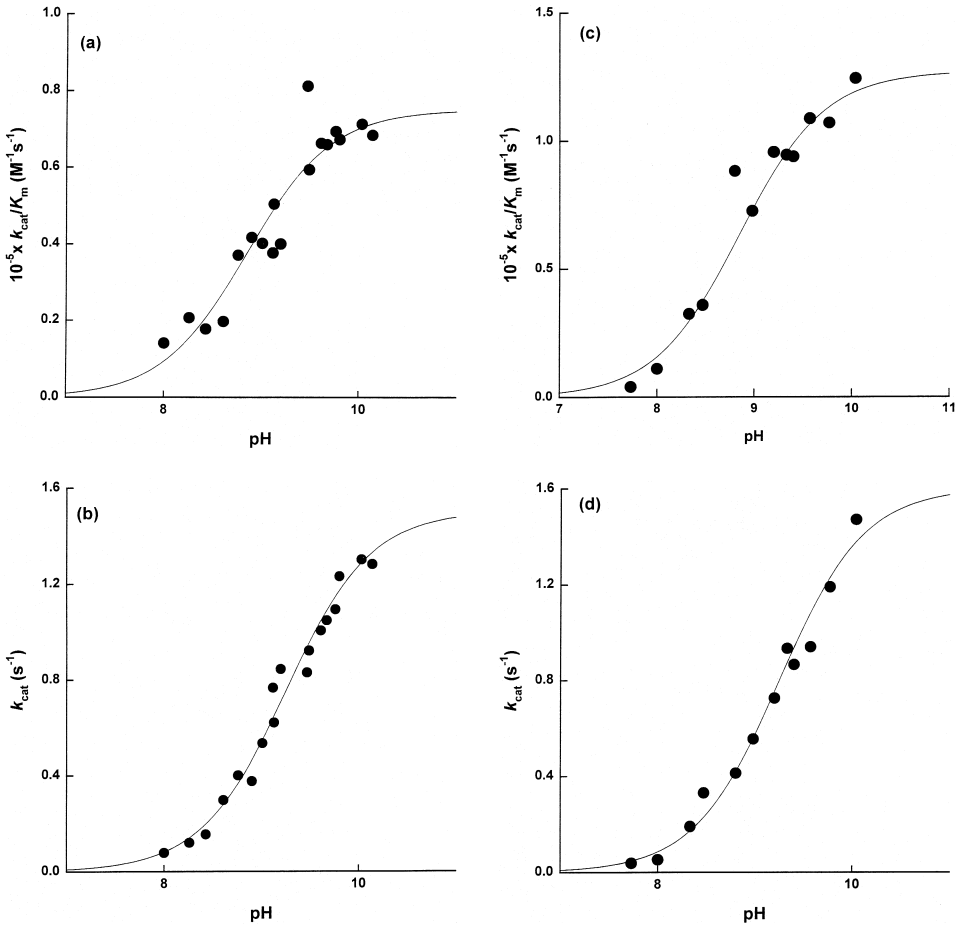

Figure 3 pH-dependence of (a, c) k_{cat}/K_m and (b, d) k_{cat} for the hydrolysis of the carbonate substrate (II) catalysed by PCA270-29

Catalysis was performed at 25 °C and at I 0.15 (**a**, **b**) or I 0.9 (**c**, **d**). The points are experimental and the continuous lines are theoretical for the single-ionization equation $k = \tilde{k}/(1 + [H^+]/K_a)$, where $k = k_{cat}/K_m$ or k_{cat} and $\tilde{k} = \tilde{k}_{cat}/\tilde{K}_m$ or $\tilde{k}_{cat}$ (the pH-independent kinetic parameters), with the following values of the parameters: (**a**) $pK_a = 8.95$, $\tilde{k}_{cat}/\tilde{K}_m = 7.7 \times 10^4$ $M^{-1} \cdot s^{-1}$; (**b**) $pK_a = 9.20$, $\tilde{k}_{cat} = 1.48$ s^{-1}; (**c**) $pK_a = 8.85$, $\tilde{k}_{cat}/\tilde{K}_m = 1.25 \times 10^5$ $M^{-1} \cdot s^{-1}$; (**d**) $pK_a = 9.20$; $\tilde{k}_{cat} = 1.48$ s^{-1}.

Table 1 Characteristics of the hydrolysis of the carbonate substrate (II) catalysed by PCA270-29 at 25 °C

The pK_a values of 4-isopropyl phenol (a model compound relating to a Tyr side chain) in aqueous buffers were determined by spectral analysis at 293 nm to be 10.0 at I 0.15 ($\tilde{\epsilon}_{293} = 2118$ $M^{-1} \cdot cm^{-1}$) and 9.95 at I 0.90 ($\tilde{\epsilon}_{293} = 2300$ $M^{-1} \cdot cm^{-1}$). The values given in parentheses were calculated by assuming that only 5% of the IgG is catalytically active (see the text).

	pH-dependence of k_{cat}/K_m		pH-dependence of k_{cat}	
I	pK_a	$10^{-5} \times (\tilde{k}_{cat}/\tilde{K}_m)$ ($M^{-1} \cdot s^{-1}$)	pK_a	$\tilde{k}_{cat}$ (s^{-1})
0.15	8.95	0.77 (15.4)	9.20	1.48 (29.6)
0.90	8.85	1.25 (25)	9.20	1.48 (29.6)

Studies on the binding of carbonate substrate (II) to PCA270-29 using the ELISA

Investigation of the binding interaction of substrate (II) and PCA270-29 was carried out by using the ELISA described in the Materials and methods section. This uses the BSA–anilide ligand substrate (III) conjugate, which is an analogue of the carbonate substrate (II), rather than the BSA–hapten (Ib) conjugate to facilitate the detection of even small changes in binding characteristics. The detection of such changes is much less sensitive when the latter conjugate is used because of the very high affinity of PCA270-29 for the hapten (Ib). Use of the ELISA (with the former conjugate) demonstrated binding of the anilide (III) to be stronger at pH 6 than at pH 9. This result supports the hypothesis that the side chain associated with the kinetically influential ionization of $pK_a \sim 9$ may be required for activity in its undissociated form, as represented by eqn. (2). Additional evidence for this conclusion was provided by analogous experiments described below using PCA270-29 nitrated by reaction with TNM.

Group-selective chemical modification of PCA270-29

Chemical modification was targeted at Tyr, Lys and Cys side chains, in view of the kinetically influential ionization observed at $pK_a \sim 9$, at the side chain of Arg because this has been implicated in the functioning of some other antibody preparations (see, e.g., [7,9,35,41]), and at that of His because of the well known involvement of the imidazole/imidazolium side chain in

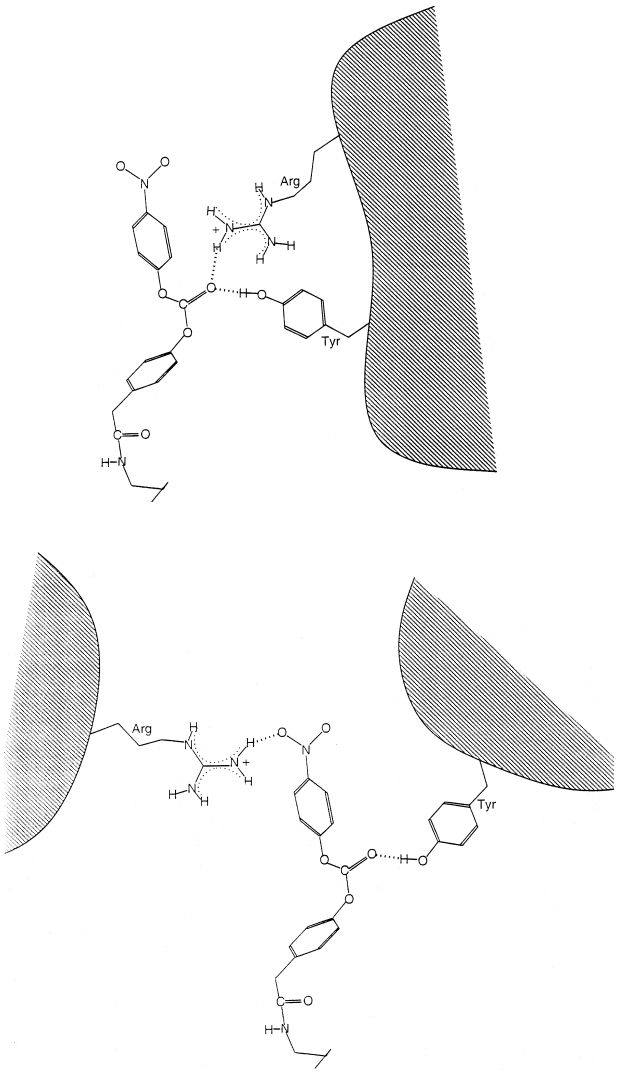

Figure 4 Schematic diagram illustrating possible roles for the side chains of an Arg residue and a Tyr residue implicated in binding of the carbonate substrate (II) by PCA270-29 and in catalysing its hydrolysis

Substrate (II) is shown in an optimized extended conformation generated by the RIS random sampling protocol of QUANTA. In the upper panel, both side chains are shown in interactions analogous to those in the oxyanion holes of serine proteinases and cysteine proteinases, In the lower panel, the Tyr-OH, which contributes to both the binding of (II) and the kinetics of the reaction within the adsorptive complex, is shown as an oxyanion hole contributor, and the Arg side chain is shown binding to the nitro group of (II) (see the text); in this scheme an additional contributor to the postulated oxyanion hole, e.g. a second Tyr-OH, is consistent with the results of the chemical modification studies.

enzyme catalysis and reports of its involvement in antibody-mediated catalysis (see, e.g., [7,9–11,41]). Modification of Lys side chains by methyl acetimidate, and of His side chains (up to ~ 10 per IgG molecule) by DEP resulted in negligible decreases in catalytic activity and binding. Incubation with methyl 4-pyridyl disulphide did not result in thiol group modification and, as would be predicted from this, had no effect on either catalytic activity or binding. In contrast with these results, modification of Arg side chains by reaction with phenylglyoxal resulted in loss of binding and a decrease in catalytic activity by more than 90%.

The most extensive series of modification experiments involved the phenolic side chains of Tyr residues. Nitration by reaction with TNM resulted in the loss of more than 95% of the catalytic activity towards substrate (II) measured at pH 9. The strongly binding hapten (Ib) provided protection against the modification, in accordance with the suggestion that Tyr residues exist in the binding sites of PCA270-29. Comparison of the binding characteristics of PCA270-29 with those of its nitrated derivative showed that, whereas binding ability at pH 6 was retained in both cases, at pH 9 it was abolished (rather than decreased) only in the case of the nitrated antibody. This result is consistent with the expected decrease in the pK_a value of the nitrophenolic hydroxy group of the postulated active-centre Tyr side chain from ~ 9 to ~ 7. The particular importance of the results of these experiments is that they define the functional ionization state of the essential Tyr side chain as that of the undissociated phenol. Thus in the native antibody (Tyr-OH; $pK_a \sim 9$), approx. 50% of the undissociated Tyr hydroxy group should exist at pH 9, with consequent retention of some binding ability. In contrast, in the nitrated antibody (O_2N-Tyr-OH; $pK_a \sim 7$) the hydroxy group should be almost fully ionized at pH ~ 9, with consequent essentially complete loss of binding ability. Identification of the form of the Tyr side chain essential for binding as the undissociated phenol provides support for eqn. (2) (assistance by Tyr-OH to attack of HO^-) as the simplest interpretation of the pH-dependent kinetic data shown in Figure 3.

Concluding comments

The convincing demonstration of adherence to the Michaelis–Menten equation of the hydrolysis of the carbonate substrates (II) and (V) catalysed by PCA270-29 here reported lends further support to the developing view that PCAs may be functionally much more homogeneous than had been supposed initially. The combination of studies on the pH-dependence of both binding and kinetics and group-selective chemical modification studies suggests that catalysis depends on assistance to the reaction of HO^- with substrate (II) provided by the proton-rich form of at least one Tyr residue and by at least one Arg residue of PCA270-29. Two rational roles for these residues are illustrated schematically in Figure 4. In the upper panel of Figure 4, one of the hydrogen-bond donors (the alkyl guanidinium cation of an Arg residue) is shown hydrogen-bonded to the nitro group of substrate (II), and the Tyr-OH group is shown hydrogen-bonded to the carbonyl oxygen atom of the carbonate reaction centre. In the lower panel, both hydrogen-bond donors are shown associated with the carbonyl oxygen atom of the substrate in a manner similar to the well known interactions found in the oxyanion holes of serine proteinases and cysteine proteinases. The former would be in accordance with the concept that binding interactions between antibody and ligand that include those remote from the site of attachment of the hapten to the carrier protein can be particularly effective.

It is important to emphasize that, as is well known, kinetic analysis alone normally defines proton stoichiometries in transition states, and not their location. In the present work it is the combination of the evidence from the pH-dependence of binding assessed by the ELISA and that from the pH-dependence of the k_{cat}/K_m and k_{cat} values, together with structural considerations, that suggests a mechanism involving nucleophilic attack by HO^- assisted by hydrogen bonding provided by an undissociated Tyr-OH side chain. The ELISA data define the ionization state of the tyrosine side chain for binding. The pH-dependent kinetic data alone are ambiguous, and are in accordance both with hydrogen-bond donation by Tyr-OH and with Tyr-O^- acting as a nucleophile or general base. On the assumption that a substantial

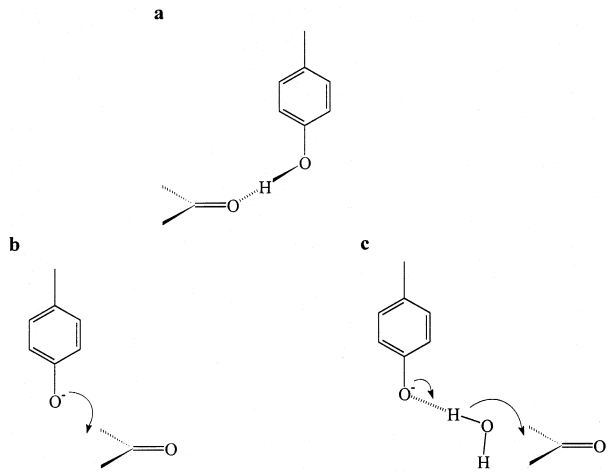

Figure 5 Illustration of the different dispositions of a tyrosine side chain with respect to the carbonyl group of a substrate needed to effect various mechanisms

The mechanisms shown are: (**a**) hydrogen bonding to carbonyl oxygen, (**b**) nucleophilic attack by the phenolate anion at carbonyl carbon, and (**c**) general-base catalysis by the phenolate anion of nucleophilic attack by water at carbonyl carbon. The difference between the geometric disposition in (**a**) and those in (**b**) and (**c**) suggests that a mechanism in which binding by the undissociated tyrosine side chain, as in (**a**), could be followed by catalysis using the dissociated ionization state, as in (**b**) or (**c**), is improbable.

conformational change between binding and nucleophilic attack at carbonyl carbon does not take place, a tyrosine side chain providing hydrogen bonding in an oxyanion hole motif could not provide nucleophilic or general-base catalysis as well. This is illustrated in Figure 5. Nucleophilic attack either by Tyr-O^- (Figure 5b) or by water assisted by Tyr-O^- (Figure 5c), both of which occur by rear-side attack on carbonyl carbon, would require the essential tyrosine side chain to be in a different location to that in which Tyr-OH could supply a hydrogen bond to the carbonyl oxygen atom (Figure 5a). Geometric considerations, therefore, suggest as unlikely a mechanism in which a tyrosine side chain is required in the undissociated state for binding and in the dissociated state for catalysis. In this circumstance the attacking nucleophile is defined as hydroxide ion rather than either a water molecule or the phenolate anion of the essential tyrosine residue.

If antibodies that catalyse reactions of substrates with hydroxide ion require assistance from hydrogen-bond donors, these would be expected to have high pK_a values. This would ensure the coexistence of significant concentrations of both nucleophile and hydrogen-bond donors. This situation might explain the high intrinsic catalytic effectiveness of PCA270-29.

We thank the Biotechnology and Biological Sciences Research Council for support, including an Earmarked Studentship for C.S. and a Quota Studentship for A.B.W.; the European Network for Antibody Catalysis for support, including a Postdoctoral Research Assistantship for M.R.; the University of Brighton for a Studentship for N.J.B.; Therapeutic Antibodies Inc. for support, including a Studentship for E.P.H.-P.; and the Wellcome Trust for a Fellowship for C.V.

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Received 19 June 1996/17 March 1997; accepted 7 April 1997