Evidence for ‘lock and key’ character in an anti-phosphonate hydrolytic antibody catalytic site augmented by non-reaction centre recognition: variation in substrate selectivity between an anti-phosphonate antibody, an anti-phosphate antibody and two hydrolytic enzymes

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The substrate selectivities of an anti-phosphate and an anti-phosphonate kinetically homogeneous polyclonal catalytic antibody preparation and two hydrolytic enzymes were compared by using hapten-analogous and truncated carbonate and ester substrates each containing a 4-nitrophenolate leaving group. Syntheses of the truncated substrates devoid of recognition features in the non-leaving group parts of the substrates are reported. The relatively high kinetic selectivity of the more active anti-phosphonate antibody preparation is considered to depend on a relatively rigid catalytic site with substantial reaction centre specificity together with other important recognition interactions with the extended non-leaving group part of the substrate. In contrast, the less catalytically active, more flexible anti-phosphate antibody exhibits much lower kinetic selectivity for the substrate reaction centre comparable with that of the hydrolytic enzymes with activity much less dependent on recognition interactions with the non-leaving group part of the substrate. The ways in which haptenic flexibility and IgG architecture might contribute to the differential kinetic selectivities are indicated.

Key words: catalytic site rigidity, hydrolytic catalytic antibody, kinetic characterization, lock and key, substrate selectivity, truncated carbonate and carboxylic ester substrate synthesis.

INTRODUCTION

There is growing awareness of the importance of structural dynamics in catalysis by some enzymes, including the interconversion of protein conformers and the dynamic aspect of substrate recognition. Recent clearly demonstrated examples of these phenomena have involved stopped-flow kinetic studies on natural variants of the papain family of cysteine proteinases using substrate-derived disulphide reactivity probes and a specific thionoester substrate [1–4]. Discussion of the relative catalytic effectiveness of enzymes and catalytic antibodies (e.g. [5]) has included consideration of architectural differences between these two types of protein catalyst and their consequences for structural dynamics [6–11].

In the present paper, we compare the kinetic selectivities of two kinetically homogenous, enzyme-free, polyclonal catalytic antibody preparations. Each was generated by one of two closely related immunogens differing only in the flexibility of the atomic framework around the structural motifs of the hapten analogous to the reaction centres of the corresponding (cognate) substrates. As discussed in [12], the systematic study of the relationships between haptenic structure, antibody recognition and catalytic activity is facilitated by the use of polyclonal preparations. Of particular note is that: (i) generation of polyclonal catalytic antibodies samples the entirety of the immune response, (ii) polyclonal IgG preparations investigated to date in different laboratories have not deviated from single-phase (Michaelis–Menten) saturation kinetics, and (iii) polyclonal preparations, at least in our laboratories, are free from contamination by enzymes. The present study uses as its basis our recent demonstration [12] of the kinetic advantage of the antibody preparation produced by using the more rigid, phosphonate, immunogen (1, Figure 1) towards its cognate ester substrate (2, Figure 1) over that produced by using the less rigid, phosphate, immunogen (3, Figure 1) towards its cognate carbonate substrate (4, Figure 1). At pH 8.0, there is a 13-fold advantage in \( k_{\text{cat}} / k_{\text{non-cat}} \) and a 100-fold advantage in the proficiency constant, \( k_{\text{cat}} / k_{\text{non-cat}} \cdot K_m \). These kinetic data show that increasing the rigidity from that of the phosphate/carbonate system (3/4) to that of the phosphonate/ester system (1/2) results in substantial enhancement of the catalytic activity towards the substrate with a structure analogous to that of the relevant hapten. A haptenic structure that has fewer degrees of freedom around the reaction centre determinant would be expected to elicit antibodies with fewer relevant conformational

Abbreviations used: b.p., boiling point; m.p., melting point; 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.); PLE, pig liver esterase.

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variants associated with the reaction centre binding site. It seemed possible that the anti-phosphonate antibody might possess more 'lock and key' character in substrate binding than the anti-phosphate antibody and thus exhibit greater selectivity for a substrate with appropriate reaction centre structure. The present paper provides evidence for this hypothesis and reports also a greater dependence of catalytic activity on non-reaction centre recognition in the case of the anti-phosphonate antibody. The kinetic selectivity characteristics of the two types of catalytic antibody are compared also with those of two hydrolytic enzymes, serine proteinase \( \alpha \)-chymotrypsin (reviewed in [13]) and the active enzyme were 95–100\% in both cases.

**Enzymes**

The bovine pancreatic enzyme \( \alpha \)-chymotrypsin was obtained from Fluka, Gillingham, Dorset, U.K., as a 3\% crystalized product stated to be free from low \( M_r \) peptide fragments. Immediately before use, the solid enzyme (40 mg) was dissolved in 0.1 M KCl (2 ml containing 1 mM EDTA) and subjected to gel filtration on a Sephadex G-25 column (25 cm \( \times \) 2 cm) to remove residual low \( M_r \) material. Elution was with 0.1 M KCl solution containing 1 mM EDTA, and the concentration of enzyme protein was determined spectrophotometrically at 282 nm (\( \epsilon_{282} = 5.0 \times 10^{4} \text{ M}^{-1} \cdot \text{cm}^{-1} \) [19]).

The source of PLE (EC 3.1.1.1) was freeze-dried powder supplied by Fluka, and the purification was similar to that described for the rat kidney carboxylesterase [20] with DEAE-cellulose replaced by DEAE-Sephadex and the introduction of FPLC technology. The concentration of enzyme protein was determined spectrophotometrically at 280 nm with \( \epsilon_{280} = 2.32 \times 10^{4} \text{ M}^{-1} \cdot \text{cm}^{-1} \) calculated from the published value of \( A_{1%}^{10} = 13.8 \) and \( M_r = 1.68 \times 10^{5} \) [21].

Active-site titration of both enzymes was performed by using \( N \)-trans-cinnamoylimidazole as titrant [22]. Typical contents of active enzyme were 95–100\% in both cases.

**Immunogens and full-length cognate substrates**

The syntheses of these compounds have been reported previously: the phosphonate immunogen 1 and the carboxylic ester substrate 2 in [12], the phosphate immunogen 3 in [15], and the carbonate substrate 4 in [23].

**New syntheses: the truncated substrates**

Truncated carboxylic ester substrate 4-nitrophenyl propanoate 5 (Scheme 1a) and phosphate immunogen 2 (Scheme 1b) were produced as described in [15] and [12] respectively. The upper limits of the active-site contents of the PCA preparations were shown to be approx. 10\% of the total IgG content by using a combination of pre-steady-state and steady-state kinetics described previously [16,17] and reviewed in [18].

**Chemicals**

Unless otherwise stated, chemicals were from Sigma-Aldrich, Poole, Dorset, U.K.

**Antibodies**

The PCA (polyclonal catalytic antibody) preparation (PCA 271-22, isolated from the antiserum of sheep no. 271 in week 22 of the immunization programme) elicited using the 4-nitrophenyl phosphate immunogen 3 (Figure 1) and PCA 2649-16 (designated in an analogous way) elicited by using the 4-nitrophenyl phosphate immunogen 1 (Figure 1) were produced as described.
temperature (22°C). The mixture was washed with cold water (3 × 60 ml) and dried over anhydrous MgSO4, which was then removed by filtration. Evaporation of the solvent left the required product as a white solid. This was recrystallized twice from ethyl acetate/petroleum ether, b.p. (boiling point) 60–80°C (50:50). The white crystals were dried in vacuo and had a m.p. (melting point) of 58–62°C. TLC (ethyl acetate): Rf = 0.75. 1H-NMR (360 MHz, deuterated chloroform): δ 8.3 (2H, d, J 9.2 Hz, H2 of Abq), 7.3 (2H, d, J 9.2 Hz, H8 of Abq), 2.6 (2H, q, J 7.5 Hz, -COCH2), 1.3 (3H, t, J 7.5 Hz, -COCH2CH3). MS: m/z = 195 (M+).

Truncated carbonate substrate methyl 4-nitrophenyl carbonate 6 (Scheme 1b)

4-Nitrophenyl chloroformate (1 g, 5 mmol) was added over 20 min to a stirred mixture of anhydrous acetonitrile (10 ml), methanol (150 mg, 5 mmol, 201 µl) and triethylamine (502 mg, 5 mmol, 692 µl) at room temperature. The mixture was then left to stir for 1 h, during which time triethylamine hydrochloride precipitated. Ethyl acetate was added to the mixture which was then washed with cold water (3 × 15 ml). The organic layer was dried over anhydrous MgSO4, which was then removed by filtration. Evaporation of the filtrate left the required product as a white solid, which was recrystallized from ethyl acetate/petroleum ether, b.p. 60–80°C (7:3) and had a m.p. of 112–116°C. TLC (ethyl acetate/petroleum ether 1:5): Rf = 0.41. 1H-NMR (360 MHz, deuterated chloroform): δ 8.3 (2H, d, J 8.7 Hz, H2 of Abq), 7.4 (2H, d, J 8.7 Hz, H8 of Abq), 4.0 (3H, s, -OCOCH2CH3). MS: m/z = 196 (M-H+).

Kinetics

Kinetic studies of the hydrolysis of the 4-nitrophenyl carboxylic ester substrates 2 and 5 and of the 4-nitrophenyl carbonate substrates 4 and 6 catalysed by PCA 271-22, PCA 2649-16, α-chymotrypsin and PLE were performed in potassium phosphate buffer pH 8.0 (devoid of Cl−, see [12]) containing 10% (v/v) acetonitrile at 25°C and J 0.3. It is important to note that there is no evidence for inhibition by phosphate [12]. The values of IgG [catalytic activity] (in µM) for the various catalyses were as follows: for PCA 271-22 and PCA 2649-16, with 4, 1.2, with 2, 8.0, and with 5 and 6, 10.0; for α-chymotrypsin, 0.48 in all cases; for PLE, with 4, 0.048, and with the other three substrates, 0.48. The enzyme-catalysed reactions were studied also over pH ranges approx. 4–10 or 5–9 in acetate, phosphate and carbonate buffers under otherwise similar conditions. The reactions were initiated by the addition of various volumes of solutions of substrate in pure anhydrous acetonitrile to produce ranges of concentration in 1 ml reaction mixtures containing a total acetonitrile content of 10% (v/v). The release of 4-nitrophenolate from the substrate was monitored at 410 nm using a Cary 1 spectrophotometer over 2–5 min. Initial rates (v1) were calculated in M·s−1 from absorbance-time data by using the value of ε410 calculated from the relationship ε410 = 16963/(1 + [H+]2/Kcat) M−1·cm−1, where Kcat = 10−7.15 M. The rates of antibody-catalysed reactions were corrected by subtracting the rates of reaction determined in the presence of identical concentrations of IgG from NSS (normal sheep serum). Those of the enzyme-catalysed reactions were corrected by subtracting the rates of the aqueous hydrolysis. In all cases, the rates of aqueous hydrolysis were used to calculate the first-order rate constants to provide the value of kH2O (≈ kcat).

Parameter evaluation

The adherence of each set of the corrected initial rate, (v) 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 [24] and the linearity of ln [S]0/v against [S]0 plot [25]. Values of the parameters Vmax and Km 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 v2. Values of the catalytic rate constant (kcat) were calculated from kcat = 10Vmax/2[IgG] = 5Vmax/2[IgG] (10% of the IgG catalytic and two potential active centres per molecule) to provide lower limits for this parameter [16–18].

Preliminary characterization of the pH-dependence of kcat and kcat/Km was carried out by using the multitasking application program SKETCHER [26] written in ANSI C running under RISCOS on an Acorn Archimedes microcomputer [12].

RESULTS AND DISCUSSION

Design and synthesis of the truncated substrates 5 and 6

The objective of the present study was to investigate the substrate selectivities of the anti-phosphonate (PCA 2649-16) and anti-phosphate (PCA 271-22) catalytic antibody preparations and compare them with those of relevant enzyme preparations as discussed in the Introduction. One aspect of this involved the hypothesis that the anti-phosphonate antibody might exhibit greater reaction centre selectivity than either the anti-phosphate antibody or the enzymes. The other was concerned with the dependence of catalytic activity on non-reaction centre recognition. The latter was addressed by comparing the kinetic characteristics of the two types of antibody preparation, and the proteinase and esterase enzymes towards the “full-length” hapten-analogue carboxylic ester and carbonate substrates, 2 and 4 respectively, with those towards the truncated substrates 5 and 6. In 5 and 6, the potential recognition features in the non-leaving group parts of 2 and 4 (the phenyl ring and amide group) have been deleted. The 4-nitrophenyl group, which provides a chromogenic leaving group in all four substrates and a strongly antigenic group in the immunogens 1 and 3, is retained. The inclusion of identical leaving group determinants in the haptons and identical leaving groups in the corresponding substrates was essential to the investigation of the kinetic consequences of the variation in the flexibility of the atomic framework of the reaction centre and the analogous structural motif of the haptons [12]. Equally, the retention of the 4-nitrophenyl leaving group is essential to the investigations reported in the present paper. The synthesis of the truncated carboxylic ester 5 was achieved by condensation of propanoyl chloride with 4-nitrophenol and that of the truncated carbonate ester 6 by condensation of 4-nitrophenyl chloroformate with methanol [Scheme 1, (a) and (b) respectively].

Substrate selectivities: the data

The kinetic characteristics of the two hydrolytic antibody preparations and the two hydrolytic enzymes at pH 8.0 are collected in Table 1. In all cases, single-phase (Michaelis–Menten) saturation kinetics were observed, as demonstrated for the antibody preparations towards 2 and 4 in [12]. Substrates are designated cognate if the reaction centre is analogous to the corresponding haptenic determinant, i.e. carbonate for the anti-phosphonate antibody and carboxylic ester for the anti-phosphate antibody, and non-cognate if the opposite is true. As in [12], values of kcat for the antibody-catalysed reactions were calculated by assuming that 10% of the IgG is catalytic (see the Materials
Comparison of the kinetic characteristics of the examples of the two types of catalytic antibody preparation (anti-phosphonate and anti-phosphate) used (i.e. PCA 2649-16 and PCA 271-22 respectively), each with its own full-length cognate substrate (ester 2 and carbonate 4 respectively), demonstrated the kinetic advantage of the former, 13-fold in terms of $k_{cat}/k_{non-cat}$ and 100-fold in terms of $k_{cat}/K_M \cdot K_m$ [12]. These data suggest that, in the examples studied, decreasing the flexibility of the phosphate/carbonate system (3/4) to that of the phosphonate/ester system (1/2) results in substantial enhancement of the catalytic effectiveness of the catalytic antibody preparation. This conclusion accords with the concept that antibodies with fewer relevant conformational variants associated with reaction centre binding would be expected to result from the use of a haptenic structure with fewer degrees of freedom around the reaction centre determinant. Improvement in catalytic effectiveness would be expected to be the consequence of the production of at least one variant with better complementarity to the reaction transition state.

Presumably in the case of the antibody preparations used in the present work, one catalytically effective conformational variant predominates substantially in each preparation in view of the kinetic homogeneity observed for both preparations. The apparent generality of kinetic homogeneity in polyclonal preparations is discussed in [12].

The possible relationship between decreased conformational flexibility and catalytic effectiveness discussed above predicted greater substrate selectivity when the reaction centre is varied in the case of the less flexible anti-phosphonate antibody (PCA 2649-16). Support for this predication is provided by comparison of ratios of the relevant parameters collected in Table 1. Thus the anti-phosphonate antibody discriminates in favour of its own full-length cognate substrate (ester 2), relative to that (carbonate 4) of the other antibody, more effectively than the anti-phosphate antibody in terms of both $k_{cat}/k_{non-cat}$ [(1.2 × 107)/(900/250 = 3.6) = 43] and $k_{cat}/k_{non-cat} \cdot K_m$ [(3.1 × 102)/7.9 × 102 = 392]/(3.1 × 105/6.9 × 104 = 0.45) = (871) ratios.

The difference in the reaction centre selectivity between these examples of the two types of antibody is demonstrated also by the parameter ratios for the catalysis by each antibody with the same full-length substrate (ester 2 or carbonate 4). The relevant ratios show that when each antibody is presented with the cognate substrate for the anti-phosphonate antibody (ester 2), the anti-phosphonate antibody is more effective than the anti-phosphate antibody (by 48-fold in terms of $k_{cat}/k_{non-cat}$ and by 45-fold in terms of $k_{cat}/k_{non-cat} \cdot K_m$). In contrast, when each antibody is presented with the cognate substrate for the anti-phosphate antibody (carbonate 4), the anti-phosphate antibody is the more effective, but to a much smaller degree (by only 11-fold in terms of $k_{cat}/k_{non-cat}$ and by only 4-fold in terms of $k_{cat}/k_{non-cat} \cdot K_m$). Once again, this demonstrates greater substrate selectivity in the case of the less flexible, anti-phosphonate antibody (PCA 2649-16). This suggests that, whereas the relative rigidity of the catalytic site of the anti-phosphonate antibody is advantageous in binding an appropriate conformation of the cognate ester

### Table 1 Kinetic parameters for hydrolysis at 25 °C in phosphate buffer, pH 8.0, $I = 0.3$, containing 10% (v/v) acetonitrile catalysed by anti-phosphate PCA 271-22, anti-phosphonate PCA 2649-16, PLE and α-chymotrypsin (α-Ch)

<table>
<thead>
<tr>
<th>Catalyst</th>
<th>Substrate</th>
<th>Reaction centre: cognate (C) or non-cognate (N)</th>
<th>Full-length (FL) or truncated (T)</th>
<th>$K_M$ (µM)</th>
<th>$k_{cat}$ (-)</th>
<th>$k_{cat}/k_{non-cat}$</th>
<th>Proficiency constant $k_{cat}/K_M \cdot K_m$ (µM⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCA 271-22</td>
<td>Carbonate 4</td>
<td>FL</td>
<td>C</td>
<td>290 ± 44</td>
<td>1.8 × 10⁻¹ ± 0.2 × 10⁻¹</td>
<td>900</td>
<td>3.1 × 10⁶</td>
</tr>
<tr>
<td>PCA 271-22</td>
<td>Carbonate 6</td>
<td>T</td>
<td>C</td>
<td>104 ± 116</td>
<td>1.6 × 10⁻² ± 0.5 × 10⁻³</td>
<td>29</td>
<td>2.8 × 10⁵</td>
</tr>
<tr>
<td>PCA 271-22</td>
<td>Ester 2</td>
<td>FL</td>
<td>N</td>
<td>36 ± 3</td>
<td>7.8 × 10⁻³ ± 0.009 × 10⁻³</td>
<td>250</td>
<td>6.9 × 10⁶</td>
</tr>
<tr>
<td>PCA 271-22</td>
<td>Ester 5</td>
<td>T</td>
<td>N</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>PCA 2649-16</td>
<td>Ester 2</td>
<td>C</td>
<td>FL</td>
<td>39 ± 2</td>
<td>3.8 × 10⁻¹ ± 0.08 × 10⁻¹</td>
<td>1.2 × 10⁴</td>
<td>3.1 × 10⁴</td>
</tr>
<tr>
<td>PCA 2649-16</td>
<td>Ester 5</td>
<td>C</td>
<td>T</td>
<td>237 ± 65</td>
<td>9.0 × 10⁻² ± 2.0 × 10⁻³</td>
<td>160</td>
<td>6.5 × 10⁵</td>
</tr>
<tr>
<td>PCA 2649-16</td>
<td>Carbonate 4</td>
<td>N</td>
<td>FL</td>
<td>97 ± 65</td>
<td>1.5 × 10⁻² ± 0.4 × 10⁻²</td>
<td>77</td>
<td>7.9 × 10⁴</td>
</tr>
<tr>
<td>PCA 2649-16</td>
<td>Carbonate 6</td>
<td>N</td>
<td>T</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>PLE</td>
<td>Carbonate 4</td>
<td>FL</td>
<td>NA</td>
<td>7.1 ± 0.4</td>
<td>7.1 ± 0.1</td>
<td>3.5 × 10⁴</td>
<td>5.0 × 10⁴</td>
</tr>
<tr>
<td>PLE</td>
<td>Carbonate 6</td>
<td>T</td>
<td>NA</td>
<td>190 ± 39</td>
<td>3.3 ± 0.5</td>
<td>1.6 × 10⁵</td>
<td>8.4 × 10⁴</td>
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<tr>
<td>PLE</td>
<td>Ester 2</td>
<td>FL</td>
<td>NA</td>
<td>25 ± 2</td>
<td>3.8 ± 0.04</td>
<td>1.2 × 10⁵</td>
<td>4.7 × 10⁵</td>
</tr>
<tr>
<td>PLE</td>
<td>Ester 5</td>
<td>T</td>
<td>NA</td>
<td>72 ± 8</td>
<td>23 ± 2</td>
<td>4.0 × 10⁵</td>
<td>5.6 × 10⁵</td>
</tr>
<tr>
<td>α-Ch</td>
<td>Carbonate 4</td>
<td>NA</td>
<td>FL</td>
<td>10 ± 1</td>
<td>1.3 ± 0.04</td>
<td>6.5 × 10⁵</td>
<td>6.8 × 10⁵</td>
</tr>
<tr>
<td>α-Ch</td>
<td>Carbonate 6</td>
<td>NA</td>
<td>T</td>
<td>74 ± 13</td>
<td>0.03 ± 0.004</td>
<td>1.5 × 10⁵</td>
<td>2.1 × 10⁵</td>
</tr>
<tr>
<td>α-Ch</td>
<td>Ester 2</td>
<td>NA</td>
<td>FL</td>
<td>21 ± 0.02</td>
<td>0.03 ± 0.01</td>
<td>9.0 × 10⁵</td>
<td>4.2 × 10⁵</td>
</tr>
<tr>
<td>α-Ch</td>
<td>Ester 5</td>
<td>NA</td>
<td>T</td>
<td>33 ± 4</td>
<td>0.02 ± 0.001</td>
<td>3.8 × 10⁵</td>
<td>1.2 × 10⁵</td>
</tr>
</tbody>
</table>
substrate, an analogous conformation is achieved with difficulty for the carbonate substrate.

**Contributions of the non-reaction centre recognition features of the substrates to the catalytic effectiveness of the antibodies**

There is considerable evidence in enzyme catalysis that some binding interactions not only locate substrates in appropriate enzyme loci, but also transmit signals with a chemical consequence to catalytic sites (e.g. [28] and references therein). In the present work, we investigated the extent to which the potential non-reaction centre recognition features of the full-length substrates 2 and 4, with retention of the common 4-nitrophenolone leaving group, contribute to the catalytic effectiveness of both the anti-phosphonate and anti-phosphate antibodies. It is particularly noteworthy that the value of the proficiency constant for catalysis by the anti-phosphonate antibody of hydrolysis of its full-length cognate (ester) substrate 2 is 477-fold greater than that for its catalysis of the truncated ester substrate 5. In contrast, the analogous ratio for the anti-phosphate antibody and its full-length cognate (4) and truncated (6) carbonate substrates is only 11 (43 times less). Thus not only does the antibody with the more rigid catalytic site exhibit greater reaction centre selectivity with full-length substrates, but also it appears to make more effective kinetic use of binding the recognition features in the non-leaving group part of the substrate. It appears, therefore, that the more rigidly defined active centre region postulated for the anti-phosphonate antibody requires specific interactions involving much of the substrate structure to produce the enhanced catalytic activity relative to that of the anti-phosphate antibody. This conclusion is supported by the finding that the anti-phosphonate antibody did not provide observable catalysis of the hydrolysis of the truncated substrate with the non-cognate (carbonate) reaction centre 6 even when the [IgG] was increased to 10 μM.

**Comparison of the substrate-selectivity characteristics of PCA 271-22 and PCA 2649-16 with those of PLE and α-chymotrypsin**

To show how the substrate-selectivity characteristics of the two antibody preparations differed not only from each other, but also from those of two hydrolytic enzymes (an esterase and a proteinase), we evaluated the catalytic characteristics of PLE and α-chymotrypsin using the same full-length (2 and 4) and truncated (5 and 6) substrates designed for use with the antibodies. The data are collected in Table 1.

α-Chymotrypsin is known to exert specificity for substrates with a hydrophobic substituent in the side chain of the amino acid residue contributing the carbonyl group of the scissile bond (the P1-position) as an occupant for the S1-subsite. Substrates 2 and 4 were therefore predicted to serve as reasonably effective substrates for α-chymotrypsin. This is confirmed by the values of the proficiency constant (Table 1). The fact that preliminary data for the forms of the pH-dependence of \( k_{cat} \) and \( k_{cat}/K_m \) for the catalysed hydrolysis of both 2 and 4 are those for specific substrates (sigmodial with \( \mathrm{pK}_a \) approximation). 7 for \( k_{cat} \) and bell-shaped for \( k_{cat}/K_m \) with \( \mathrm{pK}_a \)s of approx. 7 and 8.5 [13]) suggests that the well-known mechanism of catalysis by α-chymotrypsin is applicable also to these substrates.

PLE catalyses the hydrolysis of low \( M_r \) aliphatic and aromatic esters with broad specificity [14], and the values of the proficiency constant (Table 1) confirm that 2 and 4 serve as effective substrates. The pH-dependent kinetic characteristics of PLE are not well established, but our preliminary kinetic data indicate that both \( k_{cat} \) and \( k_{cat}/K_m \) for both 2 and 4 are close to optimal at pH 8, the pH at which the present studies were performed.

It is particularly noteworthy that neither enzyme exhibits the high selectivity shown by the anti-phosphonate antibody (PCA 2649-16). This is the case both for one full-length substrate relative to the other and for a full-length substrate relative to the related truncated substrate. PLE exhibits little preference for either full-length substrate [(PLE and 2)/(PLE and 4): 3.6 for \( k_{cat}/K_m \) values] and the preference of α-chymotrypsin for the full-length carbonate [(α-chymotrypsin and 4)/(α-chymotrypsin and 2): 7 for \( k_{cat}/K_m \) values] is small relative to the preference shown by PCA 2649-16 for ester substrate 2 [(PCA 2649-16 and 2)/(PCA 2649-16 and 4): 156 for \( k_{cat}/K_m \) values and 392 for \( k_{cat}/K_m \) values]. The greater selectivity of α-chymotrypsin than that of PLE for the full-length carbonate substrate (as opposed to the full-length ester substrate) might reflect the better fit of the phenyl ring of the non-leaving group in or near the S1-subsite. This is supported by the larger effect of truncation in the case of α-chymotrypsin than in the case of PLE.

**Concluding comment**

The relatively high kinetic selectivity of the anti-phosphonate antibody preparation (PCA 2649-16) is suggested to depend on a relatively rigid catalytic site with substantial reaction centre specificity together with other important recognition interactions with the extended non-leaving group part of the substrate. In contrast, the less catalytically active anti-phosphate antibody (PCA 271-22) exhibits much lower kinetic selectivity for the substrate reaction centre, comparable with that of the hydrolytic enzymes, with activity much less dependent on recognition interactions with the non-leaving group part of the substrate. One way in which haptenic flexibility could affect catalysis, discussed in [29], is that a flexible hapten might elicit an antibody-binding pocket that binds substrates partly in ground-state conformations. These might not proceed readily to transition state and/or products due to conformational constraints. Flexible haptens might ‘collapse’ into low-energy structures that are recognized by the antibodies, but may not proceed along the entire reaction coordinate. Such considerations could have implications also for substrate selectivity. The ‘lock and key’ character deduced for substrate binding by PCA 2649-16, which is not usually associated with enzyme catalysis, presumably derives from the IgG architecture. In anti-4-nitrophenyl phosphate/phosphonate catalytic antibodies, active sites are located in β-sandwich pockets, whereas in many hydrolytic enzymes they are in extended clefts which would be expected to provide more opportunities for conformational mobility (e.g. [30]). The hypothesis suggested by the results of the present study needs to be investigated with other antibody–substrate combinations with a view to revealing any general concepts that may exist.

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