Polyclonal antibody-catalysed amide hydrolysis

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1. The activated amide (4-nitroanilide), *N*-(4-nitrophenyl) *N*'-butyl-1,4-phenylenediacetamide (III) was synthesized. 2. A polyclonal antibody preparation (PCA 270-29) was elicited in a multigeneration cross-bred sheep (no. 270) and isolated 29 weeks into the immunization schedule by procedures described previously for PCA 270-22 [Gallacher, Jackson, Searcey, Badman, Goel, Topham, Mellor & Brocklehurst (1991) *Biochem J.* 271, 871–881]. These involved the use of an amide conjugate bonded through the carboxy group of 4-nitrophenyl 4'-carboxymethylphenyl phosphate and an amino group of keyhole-limpet haemocyanin as the immunogen. 3. PCA 270-29 was shown to catalyse the hydrolysis of both the carbonate ester substrate 4-nitrophenyl 4'-(3-aza-2-oxoheptyl)phenyl carbonate (I) and the amide substrate (III). Both catalyses obeyed the Michaelis–Menten equation with the following values of the parameters at 25 °C: for the hydrolysis of (I) at pH 8.0, $K_m = 3.96 \pm 0.28 \mu\text{M}$ and $k_{cat} = 0.135 \pm 0.004 \text{ s}^{-1}$ ($k_{non-cat.} = 1.99 \times 10^{-4} \text{ s}^{-1}$); for the hydrolysis of (III) at pH 9.0, $K_m = 5.4 \pm 1.4 \mu\text{M}$ and $k_{cat} = (5.95 \pm 0.75) \times 10^{-5} \text{ s}^{-1}$ ($k_{non-cat.} = \text{approx. } 2 \times 10^{-7} \text{ s}^{-1}$). 4. The finding that PCA 270-29 is almost equally effective as a catalyst for the hydrolysis of the amide (III) as for that of the carbonate ester (I) when allowance is made for the different intrinsic reactivities of the two types of substrate is discussed. The catalytic characteristics of PCA 270-29, the first example of a polyclonal catalytic antibody preparation shown to catalyse the hydrolysis of an amide and the first example of an antibody preparation (monoclonal or polyclonal) with such catalytic character to be produced by use of a phosphate immunogen, are compared with those of the small number of other antibody-mediated hydrolyses of amides in the literature.

INTRODUCTION

Antibody-mediated catalysis was first demonstrated in 1986 when it was shown that monoclonal antibodies that bound charged tetrahedral phosphorus analogues of reaction transition states catalysed hydrolysis of corresponding ester [1] and carbonate [2] compounds. Before this, attempts to obtain polyclonal catalytic antibodies had been unsuccessful [3]. The number of reaction types for which monoclonal catalytic antibodies have been produced continues to increase and these developments have been reviewed [4].

We recently reported the production of polyclonal catalytic antibodies that catalyse the hydrolysis of the activated carbonate (I), with Michaelian kinetic characteristics [5,6]. They were raised against the phosphate immunogen (II), an analogue of the postulated transition state for the hydrolysis of the carbonate (I). This was the first demonstration of the production of polyclonal catalytic antibodies.

Considerable effort, in a number of laboratories, is currently being directed towards the development of antibodies that catalyse amide hydrolysis, and we here report that the polyclonal antibodies raised against the immunogen (II) also catalyse the hydrolysis of the activated amide (III). This remarkable result is compared with the relative lack of success of experiments reported in the literature in which attempts have been made to produce monoclonal catalytic antibodies capable of catalysing amide hydrolysis.

MATERIALS AND METHODS

Materials

Solvents (AR) were from BDH, Dagenham, Essex, U.K. All other chemicals were from Aldrich, Gillingham, Dorset, U.K.

Methods

Synthetic reactions were carried out at room temperature (approx. 22 °C), and after extractions of aqueous systems, organic solutions were dried with anhydrous Mg_2SO_4 and filtered. N.m.r. spectra (400 MHz) were provided by The University of London Intercollegiate Research Service at Queen Mary and Westfield College.

Synthesis of the carbonate substrate, 4-nitrophenyl 4'-(3-aza-2-oxoheptyl)phenyl carbonate (I). This was reported previously [6].

Synthesis of the amide substrate, *N*-(4-nitrophenyl) *N*'-butyl-1,4-phenylenediacetamide (III) (Scheme 1). Ethyl dimethylamino-propylcarbodi-imide (99 mg, 0.5 mmol) was added to a stirred solution of 1,4-phenylenediacetic acid (100 mg, 0.5 mmol) and

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Scheme 1. Synthetic route to the 4-nitroanilide (III)

1,4-Phenylenediacetic acid was transformed, via the activated ester (IV), into the mono-amide/mono-acid (V) which was then converted into the acid chloride (VI), and treated with 4-nitroaniline to give the 4-nitroanilide (III). See the text for reagents and conditions.

N-hydroxysuccinimide (59 mg, 0.5 mmol) in dimethylformamide (0.3 ml). The reaction mixture was stirred for 45 min, after which time was added butylamine (237 mg, 2.5 mmol). The resulting solution was stirred for 16 h, basified with aqueous NaHCO_3 (0.1 M, 5 ml), extracted with ethyl acetate and the extract discarded. The solution was then acidified with HCl (1 M, 1 ml) and extracted with ethyl acetate (5×5 ml). These extracts were combined, dried and concentrated *in vacuo*. Recrystallization from water gave pure 4-(3-aza-2-oxoheptyl)phenyl acetic acid (V) (65 mg, 52%) as white crystals (m.p. 167–168 °C). ^1H n.m.r. (80 MHz; $[\text{H}]$ chloroform) δ 7.20(4H, s, ArH), 3.53(2H, s, ArCH_2), 3.40(2H, s, ArCH_2), 3.06(2H, m, NHCH_2), 1.43(4H, m, CH_2CH_2), 0.90(3H, t, J 8 Hz, CH_3).

Thionyl chloride (61 μl , 8.8 mmol) was dissolved in anhydrous acetonitrile (3 ml) and 4-(3-aza-2-oxoheptyl)phenylacetic acid (200 mg, 8 mmol) was added. The mixture was heated under reflux for 1 h, cooled, and 4-nitroaniline (110 mg, 8 mmol) and pyridine (132 μl , 24 mmol) were added. The resulting mixture was heated under reflux for 1 h, cooled, and stored at -20 °C for 3 days during which time the crude product crystallized from the solution. Recrystallization from methanol/water (4:1, v/v) gave

the pure amide (III) (224 mg, 74%) as white crystals, (m.p. 188–189 °C). ^1H n.m.r. (400 MHz; $[\text{H}]$ dimethyl sulphoxide) δ 10.78(1H, bs, NHCH_2), 8.20(2H, d, J 10 Hz, *o*- HArNO_2), 7.84(2H, d, J 10 Hz, *m*- HArNO_2), 7.30(1H, s, HNAr), 7.22(2H, d, J 8 Hz, ArH), 7.20(2H, d, J 8 Hz, ArH), 3.70(4H, 2 $\times$ s, 2 $\times$ ArCH_2), 3.00(2H, m, NHCH_2), 1.30(4H, m, CH_2CH_2), 0.82(3H, t, J 8 Hz, CH_3).

Purification of IgG. IgG was isolated by salt fractionation and purified as described previously [6]. In one case IgG was obtained from a sheep (sheep 270) immunized with the phosphate immunogen (II). In another case IgG was obtained from a non-immunized sheep. The present polyclonal catalytic antibody (PCA) preparation raised against the phosphate immunogen (II) in sheep 270, 29 weeks into the immunization programme, is designated PCA 270–29 and the analogous IgG preparation reported in [6] is now designated PCA 270–22 to indicate that it was obtained 22 weeks into the immunization programme.

Kinetics. Initial rates (v) of hydrolysis of the amide (III), in the presence of 1 μM -IgG from either sheep 270 or a control (non-immunized) sheep, were determined for a range of amide substrate concentrations between 2 and 15 μM . Production of 4-nitroaniline was monitored as an increase in A_{410} (ϵ_{410} $8.8 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$) for each substrate concentration over a period of 6 h by using a Cary 1 spectrophotometer. The reactions were carried out in 50 mM-sodium borate buffer (pH 9.0, 4.5 ml

Fig. 1. Plots of typical data showing progress curves and the linear dependence of v on $[\text{IgG}]$ for hydrolytic reactions catalysed by PCA 270–29

For (a), ($\bullet$), $[\text{III}]_0 = 5 \mu\text{M}$; ($\blacktriangle$), $[\text{III}]_0 = 15 \mu\text{M}$; for (b), $[\text{I}]_0 = 15 \mu\text{M}$. For further details see the text.

Fig. 2. Demonstration of adherence to the Michaelis–Menten equation by, and determination of the characteristic parameters of, the hydrolysis at 25 °C of (a) and (b) the carbonate ester (I) at pH 8.0 and (c) and (d) the 4-nitroanilide (III) at pH 9.0

(a) and (c) The linearity of the plots of $[S]/v$ against $[S]$ demonstrates the adherence of the catalysed reaction to the Michaelis–Menten equation. This type of plot is particularly effective in demonstrating any non-linearity that may exist. The lack of convincing evidence for non-linearity in the plots suggests that whatever structural heterogeneity exists in the IgG molecules that possess catalytic properties, it does not provide for significant variation in catalytic characteristics. Mixtures of catalysts with different characteristics provide for downward concavity in this type of plot [14]. (b) and (d) Best-fit value of $V_{\max}$ and K_m were obtained by fitting the v versus $[S]$ data to the Michaelis–Menten equation by weighted non-linear regression analysis as described previously [6]. Constant relative error was assumed with weights inversely proportional to v^2 . The continuous lines correspond to $v = V_{\max} [S]/(K_m + [S])$ with for (b) $V_{\max} = (5.40 \pm 0.17) \times 10^{-8} \text{ M} \cdot \text{s}^{-1}$ and $K_m = 3.96 \pm 0.28 \mu\text{M}$ and for (d) $V_{\max} = (1.19 \pm 0.15) \times 10^{-10} \text{ M} \cdot \text{s}^{-1}$ and $K_m = 5.40 \pm 1.41 \mu\text{M}$.

containing 0.67% acetonitrile) at 25 °C. The hydrolysis of the carbonate ester (I) catalysed by PCA 270-29 was studied as described previously for the catalysis of PCA 270-22 [6] by monitoring the increase in A_{400} ($\epsilon_{400} = 1.65 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$). Data analysis was carried out as described previously [6].

RESULTS AND DISCUSSION

Synthesis of the amide substrate (III)

1,4-Phenylenediacetic acid was transformed into the activated amide (4-nitroanilide) (III) in two stages (Scheme 1). Reaction with 1 mole equivalent each of carbodi-imide and *N*-hydroxysuccinimide followed by reaction with excess butylamine gave the mono-amide/mono-acid (V) presumably via the activated ester (IV). Compound (V) was isolated in acceptable (52%) yield overall from phenylenediacetic acid. Attempts to obtain the 4-nitroanilide (III) via formation of activated esters were unsuccessful, possibly due to decreased nucleophilicity of the 4-nitroaniline nitrogen with respect to alkylamine nitrogen. The mono-amide/mono-acid (V) was therefore converted into the acid chloride (VI), which was not isolated but was treated with 4-nitroaniline to give the desired 4-nitroanilide (III) in good (74%) yield.

Comparison of the hydrolyses of the carbonate ester and amide substrates [(I) and (III)] catalysed by PCA 270-29

Typical sets of kinetic data for the hydrolysis of the carbonate

ester substrate (I) and of the amide substrate (III) catalysed by PCA 270-29 are shown in Figs. 1 and 2, which demonstrate linearity of progress curves and of the dependence of v on $[IgG]$, and the adherence to the Michaelis–Menten equation. The kinetic data for the catalysed hydrolysis of substrate (I) are corrected for background hydrolysis as described previously for the reaction catalysed by PCA 270-22 [6]. As was found for PCA 270-22, the reaction catalysed by PCA 270-29 obeys the Michaelis–Menten equation (see Fig. 2). As was discussed previously [6], the adherence to the Michaelis–Menten equation provides no evidence for major differences in the catalytic characteristics of the IgG population whatever structural heterogeneity may exist. The value of K_m ($3.96 \mu\text{M}$) for the hydrolysis of substrate (I) catalysed by PCA 270-29 obtained by weighted non-linear regression analysis is closely similar to that ($3.34 \mu\text{M}$) for the reaction catalysed by PCA 270-22. The apparent value of k_{cat} , however, is almost five times larger than that for the PCA 270-22-catalysed reaction, possibly reflecting a higher proportion of anti-haptenic IgG with catalytic properties in the serum collected in the later bleed. Kinetics of the hydrolysis of the amide substrate (III) catalysed by PCA 270-29 did not require correction. Hydrolysis of (III) was not observed over a 6 h time-scale in the presence of IgG from the control sheep under the conditions given in the Materials and methods section. In the presence of PCA 270-29, however, the catalysed hydrolysis was readily monitored spectrophotometrically over a 6 h time-scale (see Fig. 1). As was found for the catalysed

Table 1. Comparison of the kinetic parameters for the polyclonal antibody-catalysed hydrolysis of the carbonate substrate (I) and of the amide substrate (III) in 0.67% (v/v) acetonitrile at 25 °C

The results for K_m and k_{cat} are means $\pm$ S.E.

Catalytic antibody	Substrate	pH	K_m (μ M)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)	$k_{non-cat}^*$ (s^{-1})	Rate-enhancement factor $k_{cat}/k_{non-cat}$	Reference
PCA 270-22	Carbonate (I)	8.0	3.34 ± 0.24	0.029 ± 0.001 (if [CA] = $2 \times [IgG]$) 2.9 ± 0.1 (if [CA] = $0.02 \times [IgG]$)	8.8×10^3 8.8×10^5	1.99×10^{-4}	1.5×10^2 1.5×10^4	[6]
PCA 270-29	Carbonate (I)	8.0	3.96 ± 0.28	0.135 ± 0.004 (if [CA] = $2 \times [IgG]$) 13.5 ± 0.4 (if [CA] = $0.02 \times [IgG]$)	3.4×10^4 3.4×10^6	1.99×10^{-4}	6.8×10^2 6.8×10^4	Present work
PCA 270-29	Amide (III)	9.0	5.4 ± 1.4	$(5.95 \pm 0.75) \times 10^{-5}$ (if [CA] = $2 \times [IgG]$) $(5.95 \pm 0.75) \times 10^{-3}$ (if [CA] = $0.02 \times [IgG]$)	11 1.1×10^3	Approx. 2×10^{-7}	Approx. 3×10^2 Approx. 3×10^4	Present work

* $k_{non-cat}$ is the first-order rate constant for the reaction of the substrate with hydroxide ion under the conditions used for the determination of k_{cat} ; [CA], concentration of catalytically active IgG.

hydrolysis of (I), the hydrolysis of (III) catalysed by PCA 270-29 obeys the Michaelis–Menten equation (Fig. 2c and d), and weighted non-linear regression gave the following values for the parameters ($\pm$ S.E.): $K_m = 5.40 \pm 1.41 \mu$ M, and $V_{max} = (1.19 \pm 0.15) \times 10^{-10} M \cdot s^{-1}$.

If it is assumed that all the IgG is catalytically active antibody (CA) and therefore [CA] = 2[IgG], then $k_{cat} = (5.95 \pm 0.75) \times 10^{-5} s^{-1}$. A more reasonable estimate that only between 10 and 1% of the IgG is catalytically active results in proportionately larger values of k_{cat} (approx. 6×10^{-4} – $6 \times 10^{-3} s^{-1}$). This range of values is similar to that reported by Janda *et al.* [7] (8×10^{-4} – $1.3 \times 10^{-3} s^{-1}$) for the hydrolysis of the 4-nitroanilide substrate (VII) catalysed by a

monoclonal catalytic antibody (NPN43C9) under somewhat different conditions of organic co-solvent and temperature (37 °C as against 25 °C used in the present work). The value of K_m for the PCA 270-29-catalysed hydrolysis of the amide substrate (III) is similar to that for the catalysis of the hydrolysis of the carbonate substrate (I) by this IgG preparation. This value of K_m (5.5μ M) for PCA 270-29-catalysed hydrolysis of amide (III) is approx. 70 times smaller than that (370μ M) for monoclonal antibody NPN43C9-catalysed hydrolysis of the structurally similar amide (VII), but is approx. 140 times larger than that (0.0379μ M) for human autoantibody-catalysed hydrolysis of vasoactive intestinal peptide (see Table 2).

A lower value of k_{cat} for the catalysed hydrolysis of the amide substrate than for that of the carbonate substrate would be predicted from the very different reactivities of these compounds towards hydroxide ion. It proved possible to estimate the very low reactivity of the amide substrate (III) towards hydroxide ion (in the presence of IgG from the control sheep) by monitoring the reaction spectrophotometrically over a 48 h time-scale. This produced an upper limit for $k_{non-cat}$ for the hydrolysis of (III) of approx. $2 \times 10^{-7} s^{-1}$ at pH 9.0, which is 1000 times smaller than

the value for $k_{non-cat}$ for the hydrolysis of the carbonate ester (I) at pH 8.0 (Table 1). It was not possible to make a direct comparison of both non-catalysed and PCA 270-29-catalysed reactions of these two substrates at the same pH. At pH 8 the aqueous hydrolysis of (III) is immeasurably slow, even over a 48 h time-scale, and the PCA 270-29-catalysed hydrolysis is too slow for reliable kinetic analysis. In addition, the aqueous hydrolysis of (I) at pH 9 makes an unacceptably large contribution to the observed rate of hydrolysis in the presence of 0.2μ M-PCA 270-29. On the reasonable assumption that the aqueous hydrolysis of both (I) and (III) is due to reaction with OH^- , an upper limit for the rate constant for the non-catalysed hydrolysis of (III) ($k_{non-cat}$) at pH 8 may be estimated to be approx. $2 \times 10^{-8} s^{-1}$. Thus $k_{non-cat}$ (I)/ $k_{non-cat}$ (III) is at least 1×10^4 . If the assumption is made that the PCA 270-29-catalysed hydrolyses of both (I) and (III) involve reaction of OH^- (which is the case for hydrolytic reactions catalysed by some monoclonal catalytic antibodies [8]) the value of k_{cat} (I)/ k_{cat} (III) (at the same pH) may be estimated as 2×10^4 . It is interesting that the

Scheme 2. Postulated transition states for the PCA 270-29-catalysed hydrolysis of (a) the 4-nitrophenylcarbonate substrate (I) and (b) the 4-nitroanilide substrate (III)

(a) In the catalysed hydrolysis of (I) the rate-determining step is considered to involve nucleophilic attack of OH^- and stabilization of the developing negative charge on the carbonyl oxygen by electrostatic/hydrogen-bonding effects provided by the postulated (His)-ImH $^+$. (b) In the catalysed hydrolysis of (III) the rate-determining step is considered to involve elimination of the 4-nitroaniline product from the anionic tetrahedral intermediate assisted by general acid catalysis provided by the postulated (His)-ImH $^+$.

rate enhancement, ($k_{\text{cat.}}/k_{\text{non-cat.}}$), provided by the active components of PCA 270-29 in the hydrolysis of the amide (III) is not very much smaller (a factor of 3–4) than that provided by the antibody preparation in the hydrolysis of the carbonate ester (I) (see Table 1). Thus, despite the fact that the antibodies were elicited by an immunogen designed as an analogue of the transition state for carbonate hydrolysis, the same antibody preparation is about equally effective towards an analogous anilide substrate (when allowance is made for the different intrinsic reactivities of the two substrates). If, as seems possible, the catalytic site of PCA 270-29 contains a potential proton donor [such as (His)-Im⁺H], its location may permit stabilization of the predicted transition state for anilide hydrolysis [tetrahedral intermediate (THI) breakdown in Scheme 2*b*] as well as that for carbonate ester hydrolysis (THI formation in Scheme 2*a*).

Antibody-catalysed hydrolysis of amides: the current position

There are now more than 15 papers describing antibodies that catalyse hydrolysis of esters or carbonates [4]. These antibodies have not been shown to catalyse amide hydrolysis, and, where details have been reported, in almost all cases ester hydrolysis is catalysed but not the corresponding amide hydrolysis [9,10]. There are only three papers describing antibody catalysis of amide hydrolysis. The first involved a monoclonal antibody raised against a phosphonoamidate immunogen [7] and it was recently reported that this antibody catalyses the hydrolysis also of the corresponding ester [4]. The second involved a monoclonal antibody that simultaneously bound a tetrapeptide and a metal cofactor [11]. The third involved human autoantibody(ies), of uncertain origin, to vasoactive intestinal peptide [12]. The properties of these catalytic antibodies are summarized and compared with those of the present polyclonal catalytic antibody preparation in Table 2.

Concluding comments

We originally designed the phosphate immunogen (II) to produce antibodies that would catalyse hydrolysis of the corresponding carbonate (I). We investigated these polyclonal antibodies for amidase activity with the amide (III) despite the discouraging monoclonal antibody results discussed above. We reasoned firstly that it is known that transition-state analogue inhibitors of peptidase enzymes are not confined to phosphonamidates but encompass other phosphorous derivatives including phosphates [13], and secondly that the polyclonal antibody preparation will contain many clones of antibodies against the phosphate (II), some of which might exhibit amidase

activity. The polyclonal antibodies do catalyse hydrolysis of the amide (III) and their catalytic characteristics ($k_{\text{cat.}}$ and $k_{\text{cat.}}/K_m$) compare favourably with those of the monoclonal antibody raised against the phosphonamidate immunogen (Table 2).

In contrast with results from monoclonal antibody studies, we have obtained antibodies that catalyse amide hydrolysis via immunization with a phosphate immunogen. It may be that by raising polyclonal antibodies we more effectively survey the diversity of the immune response. In addition the demonstration that it is possible to design and synthesize immunogens that will induce the production of catalytic antisera capable of amide hydrolysis may encourage the developments of new therapeutic strategies.

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