# A general kinetic approach to investigation of active-site availability in macromolecular catalysts

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A potentially general kinetic method for the investigation of active-site availability in preparations of macromolecular catalysts was developed. Three kinetic models were considered: (a) the conventional two-step model of enzyme catalysis, where the preparation contains only active catalyst ($E_a$) and inert (i.e. non-binding, non-catalytic) material ($E_i$); (b) an extension of the conventional model (a) involving only $E_a$ and $E_i$, but with non-productive binding to $E_a$ (in addition to productive binding); (c) a model in which the preparation contains also binding but non-catalytic material ($E_b$), predicted to be present in polyclonal catalytic antibody preparations. The method involves comparing the parameters $V_{max}$ and $K_m$ obtained under catalytic conditions where substrate concentrations greatly exceed catalyst concentration with those ($k_{obs}^{lim}$, the limiting value of the first-order rate constant, $k_{obs}$, at saturating concentrations of catalyst; and $K_m^{app}$) for single-turnover kinetics, in which the reverse situation obtains. The active-site contents of systems that adhere to model (a) or extensions that also lack $E_b$, such as the non-productive binding model (b), may be calculated using $[E_a]_T = V_{max}/k_{obs}^{lim}$. This was validated by showing that, for $\alpha$-chymotrypsin, identical values of $[E_a]_T$ were obtained by the kinetic method using Suc-Ala-Ala-Pro-Phe-4-nitroanilide as substrate and the well-known 'all-or-none' spectroscopic assay using *N-trans*-cinnamoylimidazole as titrant. For systems that contain $E_b$, such as polyclonal catalytic antibody preparations, $V_{max}/k_{obs}^{lim}$ is more complex, but provides an upper limit to $[E_a]_T$. Use of the kinetic method to investigate PCA 271-22, a polyclonal catalytic antibody preparation obtained from the antiserum of sheep 271 in week 22 of the immunization protocol, established that $[E_a]_T$ is less than approx. 8 % of [IgG], and probably less than approx. 1 % of [IgG].

Key words: active-site titration, catalytic antibodies, enzymes.

## INTRODUCTION

Macromolecules that provide specific and efficient catalysis under mild conditions are typified by enzymes [1], but include also catalytic antibodies [2,3] and synthetic polymers [4,5] for which, in some cases, cavities of defined geometry containing binding and catalytic sites can be created [6,7]. The investigation of all of these types of catalyst promises advances in understanding molecular recognition [8] and the nature of transition states in both enzymic and non-enzymic reactions [9,10]. Such advances are anticipated to aid the design of tailor-made catalysts with valuable potential applications in biotechnology and medicine.

Kinetic characterization is an essential aspect of almost all experimental studies on catalyst preparations [11], and this requires knowledge of the molarity of functional sites ('operational molarity') in order to permit calculation of values of $k_{cat}$ and $k_{cat}/K_m$ from experimentally determined values of $V_{max}$ (and, for the latter, of $K_m$). In some cases site concentrations may be determined by making use of stoichiometric reaction of the intact site with a substrate or site-specific inhibitor, i.e. an 'all-or-none' assay, to produce a product that is readily observable by a physico-chemical technique [11,12]. These approaches require the existence of either particular intermediates or particular active-site residues that are susceptible to specific covalent modification. Methods involving measurement of loss of catalytic activity during titration with sub-stoichiometric amounts of inhibitor [12] require rapidly reacting site-specific inhibitors. This approach has been applied to monoclonal catalytic antibodies using a hapten as inhibitor [13], but cannot be used for polyclonal preparations because of the suspected presence of binding but non-catalytic IgG. The need to determine active-site concentrations generally in all types of enzyme and analogous catalysts and the growing interest in catalytic antibodies (both monoclonal and polyclonal) and imprinted polymers requires a method that may be widely applied. The obvious alternative to 'all-or-none' assays is a kinetic assay of catalysis, which should have general applicability. A simple conventional kinetic assay, however, is unsatisfactory, principally because it would require a sample of 100 % pure active enzyme or other catalyst as a primary standard, which is difficult if not impossible to obtain [14].

In the present paper a kinetic assay that has its origin in a symmetrical characteristic of the Michaelis–Menten/Briggs–Haldane equation in which concentrations of enzyme and substrate may be interchanged [15], and which does not require a primary standard, is examined mathematically and exper-

Abbreviations used: $E_a$, active catalyst; $E_i$, inert (non-binding, non-catalytic) material; $E_b$, binding but non-catalytic material; $[E]_T$, total concentration of protein in the catalyst preparation; $[S]_0$, initial substrate concentration; $k_{obs}$, first-order rate constant; $k_{obs}^{lim}$, limiting value of $k_{obs}$ at saturating concentrations of catalyst; $v_i$, initial rate; PCA 271-22, polyclonal antibody preparation obtained from the antiserum of sheep 271 in week 22 of the immunization protocol; NTCI, *N-trans*-cinnamoylimidazole; TFA, trifluoroacetic acid.

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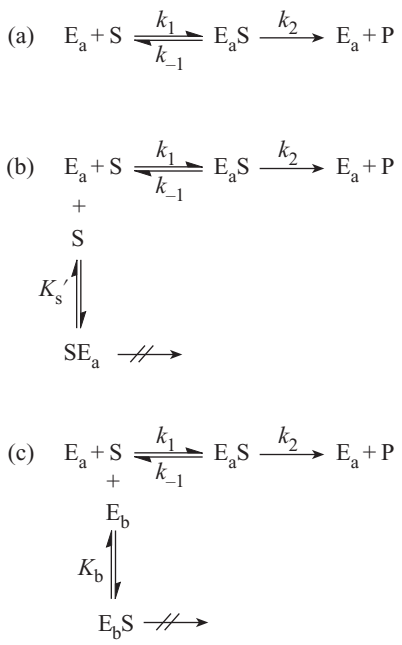


**Scheme 1 Kinetic models for catalysis**

$E_a$ is the active catalyst; $SE_a$ is a complex in which the substrate is bound non-productively to the active catalyst $E_a$ such that its conversion into product does not occur; $E_b$ is a component that binds substrate as $E_bS$ but does not catalyse its conversion into product; $K_b$ and $K_s'$ are the dissociation equilibrium constants of $E_bS$ and $SE_a$ respectively. (**a**) Simple model for enzyme catalysis written in terms of active enzyme, $E_a$, for which $V_{max}[E_a]_T = k_2$, where $[E_a]_T = [E_a] + [E_aS]$; when this model is written in terms of total protein [E] (i.e. $[E_a] + [E_i]$, where $E_i$ is an inert component), $V_{max}/[E]_T = k_{cat}$. (**b**) Extension to (**a**) that recognizes the existence of non-productive binding. (**c**) Model that acknowledges the existence of $E_b$ as well as $E_a$, and is appropriate for catalysis by polyclonal catalytic antibody preparations.

imentally. The technique involves a combination of steady-state kinetics, with excess of substrate over catalyst, to determine $V_{max}$ (and $K_m$) and single-turnover kinetics, with excess of catalyst over substrate, to determine the limiting value ($k_{obs}^{lim}$) of the observed first-order rate constant ($k_{obs}$), corresponding to saturation of substrate by catalyst (and $K_m^{app}$). We report the results of mathematical analysis of single-turnover kinetics using a technique that provides a basis for dealing with two elaborations of the simple enzyme catalysis model (Scheme 1a) that takes into account the possibility of non-productive binding [16–18] to the active catalytic species ($E_a$) (Scheme 1b) and the presence of a macromolecular species ($E_b$) that binds substrate but does not catalyse its conversion into products (Scheme 1c). The analysis reveals the basis of this simple, general approach to active-site determination even if non-productive binding exists, as well as the special circumstances that relate to its use with polyclonal catalytic antibody preparations.

The kinetic method using Scheme 1(a) (or Scheme 1b) was validated by showing that the value of the active-site concentration of $\alpha$-chymotrypsin determined by using Suc-Ala-Ala-Pro-Phe-4-nitroanilide as substrate was identical with that determined by the well-known 'all-or-none' titration of this enzyme using *N-trans*-cinnamoylimidazole (NTCI) [19]. In the presence of the binding but non-catalytic macromolecule, the result of the mathematical analysis is more complex, but still provides an upper limit of the active-site concentration. The kinetic method relating to Scheme 1(c), involving a catalyst preparation also containing a substrate-binding but non-catalytic component ($E_b$), was investigated experimentally by using a polyclonal antibody preparation PCA 271-22 obtained from the antiserum of sheep no. 271 in week 22 of the immunization protocol. This established the upper limit of active-site availability as approx. 8 % of the IgG concentration, and probably $\leqslant 1$ % of IgG concentration. The fact that the value of $K_m^{app}$ ($15.8 \pm 1.3$ $\mu$M) determined using values of IgG concentration corrected for the presence of non-binding IgG ($E_i$) was found to be greater than the value of $K_m$ ($4.36 \pm 0.04$ $\mu$M) demonstrates for the first time the presence of binding but non-catalytic antibody $E_b$ in a polyclonal preparation.

## EXPERIMENTAL

### Materials

Imject® BSA, keyhole limpet haemocyanin and ImmunoPure® rabbit anti-[sheep IgG (H + L)] were from Pierce and Warriner Ltd. (Chester, U.K.). Protein G–Sepharose (4 Fast Flow), Sephadex G-25 and PD10 columns were from Pharmacia Biotech (Uppsala, Sweden). Microtitre plates (flat bottomed; Immunoplate Maxisorp F96) were from Nunc (Rosekilde, Denmark). $\alpha$-Chymotrypsin was the bovine pancreas 3 × crystallized product stated to be free from low-$M_r$ peptide fragments from Fluka (Gillingham, Dorset, U.K.; no. 27267). Suc-Ala-Ala-Pro-Phe-4-nitroanilide, the $\alpha$-chymotrypsin substrate from Bachem, was used without further purification. Stock solutions were prepared in 100 % DMSO. The exact concentrations of the stock solutions were determined from infinity values of $A_{410}$ following complete enzyme-catalysed or alkaline hydrolysis resulting in the release of 4-nitroaniline ($\epsilon_{410}$ 8800 $M^{-1} \cdot cm^{-1}$). NTCI was purchased from Sigma Chemical Co. and was used without further purification. Solvents (analytical grade) and silica gel 60 for flash column chromatography (particle size 0.040–0.063 nm) were from Merck Ltd. (Lutterworth, Leicestershire, U.K.). All other chemicals were from Sigma–Aldrich Co.

### Synthesis of immunogen (1), hapten (2), carbonate substrate (3) and hapten analogue (4)

The synthesis of 4-nitrophenyl 4′-(carboxymethyl)phenyl hydrogen phosphate (**2**), preparation of the phosphate immunogen (**1**) and synthesis of the carbonate substrate 4-nitrophenyl 4′-(3-aza-2-oxoheptyl)phenyl carbonate (**3**) (see Figure 1) were carried out as described previously [20] .

The synthesis (Scheme 2) of the 4-nitrophenylphosphate hexylamine ligand (the hapten analogue; **4**), involving synthesis of *N*-t-butoxycarbonyl-6-amino-1-hexanol, was carried out as follows. To a solution of 6-amino-1-hexanol (2 g, 17 mmol) in 50 % (v/v) aqueous dioxane (50 ml) and triethylamine (4.14 ml, 25.55 mmol) was added 4.19 g (17 mmol) of 2-(t-butoxycarbonyloxyimino)-2′-phenylacetonitrile. The reaction mixture was stirred at room temperature; after 12 h, TLC monitoring (eluent dichloromethane/methanol, 5:1, v/v) indicated that formation of *N*-t-butoxycarbonyl-6-amino-1-hexanol was complete. The solvent was removed *in vacuo* to give a yellow oil which was purified by flash chromatography (eluent dichloromethane/methanol, 7:1, v/v) to give pure *N*-t-butoxycarbonyl-6-amino-1-hexanol as a white solid (2.37 g; 64 % yield; m.p. 34 °C). The compound had the following properties: [nujol mull (i.e. sample dispersed in liquid paraffin)] IR spectroscopy, $\nu$ ($cm^{-1}$) 3280, 1720, 1140; MS, $m/e$ 219 $(MH_2)^+$, 116; $^1$H-NMR ($C^2HCl_3$, 250 MHz), $\delta$ 1.4 (s, 9H), 1.53 (m, 8H), 3.1 (m, 2H), 3.47 (s, 1H), 3.6 (t, 2H), 4.6 (s, 1H).

4-Nitrophenylphosphodichloridate (0.8 g, 3.12 mmol) in tetrahydrofuran (12 ml) was stirred at −15 °C under a nitrogen atmosphere. *N*-t-Butoxycarbonyl-6-amino-1-hexanol (0.68 g,

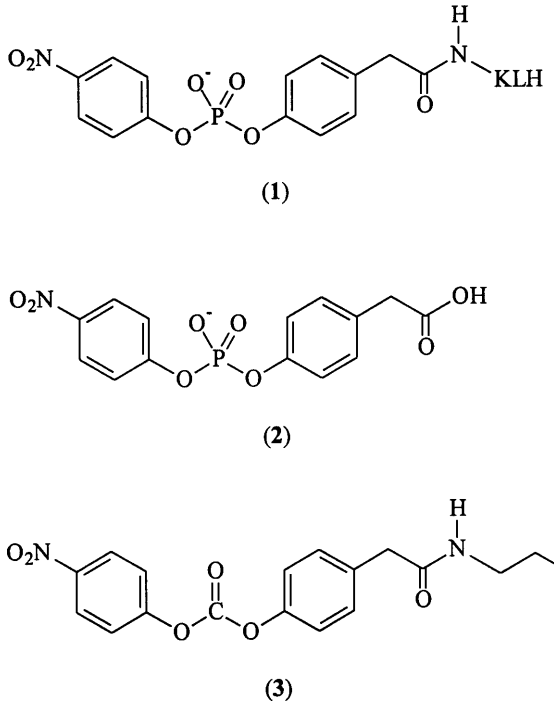
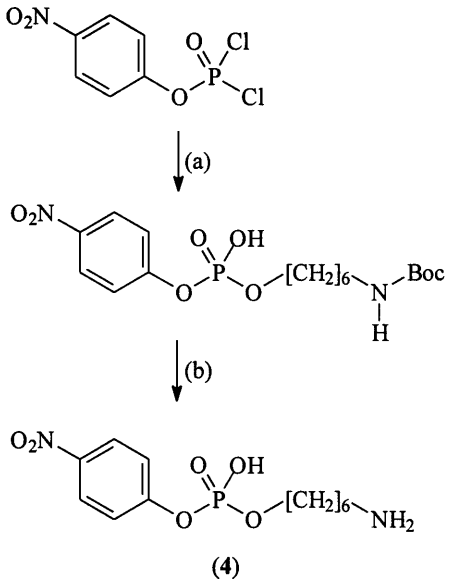



**Figure 1 Structures of phosphate immunogen (1), phosphate hapten (2) and carbonate substrate (3)**

13.2 mmol) and pyridine (256 ml, 3.12 mmol) in tetrahydrofuran (4 ml) were added dropwise over 90 min. The reaction mixture was then allowed to reach room temperature (approx. 22 °C) over 12 h. The tetrahydrofuran was removed *in vacuo* and the solution was then basified with $NaHCO_3$ to pH 9 and washed with diethyl ether ($2 \times 15$ ml). The aqueous layer was acidified with 2 M HCl to pH 1 and extracted with ethyl acetate ($3 \times 120$ ml). The combined organic extracts were dried over $MgSO_4$, filtered and the solvent removed *in vacuo* to give a yellow oil. Flash chromatography (eluent dichloromethane/methanol, 9:1, v/v) gave pure *N*-t-butoxycarbonyl-4-nitrophenylhexylamine hydrogen phosphate as a white solid (0.68 g; 52 % yield; m.p. 114 °C). The compound had the following properties: (nujol mull) IR spectroscopy, $\nu$ ($cm^{-1}$) 3400, 1720, 1510, 1190; MS, *m/e* 419, 319, 279; $^1$H-NMR ($C^2HCl_3$, 250 MHz), $\delta$ 1.4 (s, 9H), 1.5 (m, 8H), 2.9 (m, 2H), 4.0 (m, 2H), 5.1 (m, 1H), 7.4 (d, 2H, $J_{H-H}$ 8.4 Hz), 8.1 (d, 2H, $J_{H-H}$ 8.4 Hz).

*N*-t-Butoxycarbonyl-4-nitrophenylhexylamine hydrogen phosphate (0.45 g, 1.1 mmol) was dissolved in 50 % (v/v) trifluoroacetic acid (TFA) in dichloromethane (15 ml) and stirred at room temperature. After 30 min, TLC monitoring (eluent ethyl acetate/methanol, 1:1, v/v) indicated that formation of 4-nitrophenylhexylamine hydrogen phosphate TFA salt was complete. The solvent was removed *in vacuo*. Toluene (30 ml) was added to the mixture and then removed *in vacuo*. This procedure was repeated twice more. The resulting oil was triturated with diethyl ether ($3 \times 30$ ml) and the residual solvent was removed to give pure 4-nitrophenylhexylamine hydrogen phosphate TFA salt as a yellow solid (0.326 g; 93 % yield; m.p. 97 °C). The compound had the following properties: (nujol mull) IR spectroscopy, $\nu$ ($cm^{-1}$) 3450, 1730, 1500, 1200; MS, *m/e* 319 $[M]^+$; $^1$H-NMR ($^2H_6$-DMSO, 250 MHz), $\delta$ 1.45–1.86 (m, 8H), 3.54 (m, 2H), 4.2 (m, 2H), 7.55 (d, 2H, $J_{H-H}$ 8.2 Hz), 8.21 (d, 2H, $J_{H-H}$ 8.2 Hz).




**Scheme 2 Synthetic route to the hapten analogue (4)**

Step (a): (i) *N*-t-butoxycarbonyl-6-amino-1-hexanol, tetrahydrofuran/pyridine, −15 °C; (ii) water. Step (b): TFA/dichloromethane (1:1, v/v), room temperature.

### Preparation of affinity column for use in the determination of the content of non-antigen-binding antibodies

*N*-Hydroxysuccinimide-activated CH-Sepharose 4B from Pharmacia (8 g) was washed with 1 M HCl ($3 \times 50$ ml). The 4-nitrophenylphosphate hexylamine ligand (**4**) (12 mg) was dissolved in $NaHCO_3$ (5 ml; 0.1 M containing 0.1 M NaCl, pH 8.5) and added to the wet gel. The gel/ligand mixture was suspended in bicarbonate buffer (30 ml; 0.1 M; pH 8.5) and rotated at 4 °C for 24 h. It was then thoroughly washed with bicarbonate buffer ($10 \times 50$ ml) and the remaining reactive groups were blocked by reaction with ethanolamine (30 ml; 1 M in 0.1 M $NaHCO_3$, pH 8) with further rotation of the mixture for 2 h at room temperature. The resulting gel was then washed thoroughly with three cycles of alternating solutions [acetate buffer (0.1 M containing 0.5 M NaCl, pH 4), followed by Tris/HCl buffer (0.1 M containing 0.5 M NaCl, pH 8)], and finally with sodium phosphate buffer (0.05 M, pH 7.5), and then packed in a column (10 cm × 1 cm). After each use the column was stored at 4 °C in 20 % (v/v) ethanol in water. The number of available ligand sites was determined by suspending the gel (0.5 ml) in 3 M NaOH (0.5 ml) for 24 h, filtering the mixture and quantifying the amount of 4-nitrophenolate released by spectrophotometric measurement at 410 nm ($\epsilon_{410}$ 16963 $M^{-1} \cdot cm^{-1}$).

### Preparation of the BSA–(hapten 2) conjugate

Hapten (**2**) (5 mg) was dissolved in 50 % (v/v) aqueous dimethyl formamide (2 ml) and added to acetate buffer (3 ml; 0.1 M containing 10 mg of BSA, pH 5). 1-Ethyl-3-(3-dimethylaminopropyl)carbodi-imide hydrochloride (20 mg) was added and the mixture was left to react at room temperature for 16 h. The solution was then dialysed overnight against sodium phosphate buffer (0.05 M; containing 0.1 M NaCl, pH 7.2). Determination of the number of mol of hapten bonded per mol of BSA (hapten density) was performed as follows. An 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 (2000 ***g*** for 20 min) and the amount of 4-nitrophenolate released from the supernatant was determined spectrophotometrically at 410 nm ($\epsilon_{410}$ 16963 $M^{-1}\cdot cm^{-1}$). The hapten density of the conjugate was calculated as mol of 4-nitrophenolate released/mol of BSA.

### Production and purification of antibodies

The polyclonal antibody preparation PCA 271-22 was obtained from sheep 271 which had been immunized with the conjugate of keyhole limpet haemocyanin and immunogen (**1**). The IgG was isolated by salt fractionation using $Na_2SO_4$ and purified by chromatography on a Protein G–Sepharose 4 Fast Flow column, as described previously [20], with the following modification. To obtain antibody samples of sufficiently high concentration for the single-turnover kinetic experiments, the fractions (2.3 ml) collected from the Protein G–Sepharose 4 Fast Flow column in glycine buffer (0.1 M, pH 2.5) were adjusted to approximately neutral pH by addition of Tris/HCl buffer (200 $\mu$l; 1 M; pH 8.0). These fractions (each of 2.5 ml) were then passed through a PD10 column using sodium phosphate buffer (0.08 M; pH 9.0) as eluent, and three 1.2 ml fractions were collected. The protein concentration of each fraction was determined from the $A_{280}$ using a value of $\epsilon_{280} = 1.986\times10^5\ M^{-1}\cdot cm^{-1}$, determined as follows. The IgG of PCA 271-22 isolated as described above was dialysed for 3 days using deionized water ($3\times10$ litres) to exchange the buffer, and the salt-free IgG solution was freeze-dried. To 10 ml of 0.1 M Tris/HCl buffer (pH 8.0) was added the freeze-dried IgG (5 mg; 0.03 $\mu$mol), and the $A_{280}$ of the solution was determined as 0.662, which corresponds to $1.986\times10^5\ M^{-1}\cdot cm^{-1}$ as the value of $\epsilon_{280}$. This compares well with the commonly cited value ($2.0\times10^5\ M^{-1}\cdot cm^{-1}$) [21].

### Affinity chromatography

An affinity column (10 cm × 1 cm) containing ligand (**4**)–Sepharose was connected to a Pharmacia LKB pump, a dual-path UV2 monitor set at 280 nm and a Pharmacia chart recorder. The column was washed with sodium phosphate buffer (500 ml; 0.05 M containing 0.1 M NaCl, pH 7.2). Purified antibody samples of known concentration (1 ml) were loaded on to the column over 20 min, with the eluate monitored continuously at 280 nm. The fraction eluted by the sodium phosphate buffer was collected and the baseline allowed to return to zero. Glycine buffer (0.1 M; pH 2.7) was then applied to the column, with the pH of the eluate containing protein being readjusted to approximately neutral pH by addition of Tris/HCl buffer (1 M; pH 8). Both fractions were tested with the ELISA to determine the presence of antigen-specific antibodies.

### ELISA procedure

Microtitre plates were coated with the BSA–(hapten **2**) conjugate (hapten density 12 mol of hapten/mol of BSA; 1.5 $\mu$g of hapten/ml; 100 $\mu$l/well) in sodium phosphate buffer (50 mM containing 150 mM NaCl, pH 7.4) at 4 °C overnight, and the protocol described elsewhere [3] was followed.

### Enzyme preparation

$\alpha$-Chymotrypsin from bovine pancreas was a 3× crystallized product stated to be free from low-$M_r$ peptide fragments. Prior to experiments, the solid enzyme (40 mg) was dissolved in 0.1 M KCl (2 ml; containing 1 mM EDTA) and passed through a Sephadex G-25 column (25 cm × 2 cm) to remove residual low-$M_r$ material. Elution of the protein was performed using KCl (0.1 M; containing 1 mM EDTA) and the concentration of the purified enzyme solution was determined spectrophotometrically at 282 nm ($\epsilon_{282}$ $5\times10^4\ M^{-1}\cdot cm^{-1}$) [22].

### Spectroscopic titration of active sites

Stock solutions (3 mM) of NTCI were prepared by dissolving 8 mg in acetonitrile (10 ml). Addition of NTCI stock solution (10 $\mu$l) to acetate buffer (790 $\mu$l; *I* 0.1; pH 5.0) resulted in a slow decrease in $A_{335}$ due to the hydrolysis of NTCI. Further addition of enzyme solution (200 $\mu$l in 0.1 M KCl containing 1 mM EDTA) resulted in a rapid drop in absorbance due to the rapid acylation of $\alpha$-chymotrypsin. The value of $\Delta A_{335}$ obtained (typically in the region of 0.1–0.2) and a value for $\epsilon_{335}$ of 9370 $M^{-1}\cdot cm^{-1}$ [19] were used to determine the concentration of catalytically active enzyme.

### Kinetics of the reaction of α-chymotrypsin with Suc-Ala-Ala-Pro-Phe-4-nitroanilide

All reactions were carried out at 25 °C in sodium phosphate buffer [0.1 M; containing 2 % (v/v) DMSO, pH 7.0] using a Hi-Tech Scientific (Salisbury, U.K.) SF-61 stopped-flow spectrophotometer, kinetics workstation, data acquisition and analysis software. Temperature maintenance was attained by thermostatically cycling water using a Grant LTD6 water bath. Monochromator entrance and exit slit widths were set at 0.5 mm and fitted with a UG5 bandpass filter. The release of 4-nitroaniline was measured at 410 nm and quantified using $\epsilon_{410} = 8.8\times10^3\ M^{-1}\cdot cm^{-1}$ [23]. For reactions with $[S]_0 \gg [E]_T$ (where $[S]_0$ is initial substrate concentration and $[E]_T$ is total catalyst concentration), the concentrations in the mixing chamber were $[E]_T = 0.5\ \mu M$ and $[S]_0 = 4.8$–$48.0\ \mu M$ and initial rates were measured. For reactions with $[E]_T \gg [S]_0$ the analogous concentrations were $[S]_0 = 1.6\ \mu M$ and $[E]_T = 7.9$–$98.8\ \mu M$. First-order rate constants ($k_{obs}$) were obtained by fitting the (*A*)–*t* data collected by the microcomputer of the stopped-flow machine to an equation for a single-exponential process, i.e. $A = P_1e^{-P_2t} + P_3$, where $P_1 = A_0 - A_\infty$, $P_2 = k_{obs}$ and $P_3 = A_\infty$.

### Kinetics of the reaction of PCA 271-22 (E) with carbonate substrate (3)

The release of 4-nitrophenolate from carbonate substrate (**3**) was monitored in sodium phosphate buffer [0.1 M; containing 0.7 % (v/v) acetonitrile, pH 8.45] at 25 °C. Reactions with $[S]_0 \gg [E]_T$ were performed using the stopped-flow system, with initial rates of release of 4-nitrophenolate measured at 410 nm and quantified using $\epsilon_{max} = 16963\ M^{-1}\cdot cm^{-1}$. Stock solutions of the carbonate substrate at different concentrations were prepared in pure anhydrous acetonitrile. Aliquots of purified IgG (PCA 271-22) were added to sodium phosphate buffer (0.08 M, pH 9.0) to give a volume of 1 ml and a protein concentration of 2.4 $\mu$M, and loaded into one syringe of the stopped-flow sampling handling unit. The other syringe of the stopped-flow sampling handling unit contained substrate (ranging in concentration between 2.8 $\mu$M and 26 $\mu$M) in sodium phosphate buffer (2 mM, pH 5.6) containing 1.4 % (v/v) acetonitrile. Equal volumes of these solutions were allowed to mix in the reaction chamber, where the resulting mixture had a pH of 8.45. Initial rates were determined from the linear increase in $A_{410}$ and were calculated in units of $M\cdot s^{-1}$. The rates of the non-enzymic reaction under these experimental conditions were negligible (< 1 %) and thus did

not require a correction to be applied to the reaction catalysed by IgG. The exact initial concentration of the substrate in each kinetic run was determined from the infinity value of $A_{410}$ after complete IgG-catalysed hydrolysis of the substrate.

Reactions with $[E]_T > [S]_0$ were also performed using the stopped-flow system. Solutions of carbonate substrate (1.6 $\mu$M) in sodium phosphate buffer (2 mM, pH 5.6) containing 1.4% (v/v) acetonitrile were prepared and loaded in one syringe of the stopped-flow sampling handling unit. The other syringe of the stopped-flow sampling handling unit contained IgG solutions of different concentrations (10.6–58.8 $\mu$M total IgG) in sodium phosphate buffer (0.08 M, pH 9.0). Equal volumes of these solutions were allowed to mix in the reaction chamber, where the resulting mixture had a pH of 8.45.

First-order rate constants ($k_{obs}$) were obtained by fitting the ($A$)–$t$ data collected as described above for the $\alpha$-chymotrypsin reaction.

### Analysis of kinetic data

The adherence of the initial-rate ($v_i$) versus $[S]_0$ 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 an $[S]_0/v_i$ against $[S]_0$ plot [25,26]. Values of the parameters $V_{max}$ and $K_m$ were then determined by using the weighted non-linear regression program in SIGMAPLOT 2.01 (Jandel Scientific) running under Windows 95. An error structure of constant relative error was assumed and weighting factors were inversely proportional to $v_i^2$. The $k_{obs}$ versus $[E]_T$ or $[E']_T$ data from the single-turnover experiments were used to determine values of $k_{obs}^{lim}$ and $K_m^{app}$ using the non-linear regression program in SIGMAPLOT 2.01. An error structure of constant relative error was assumed and weighting factors were inversely proportional to $(k_{obs})^2$. Fits to the ($A$)–$t$ data collected by the microcomputer of the stopped-flow machine were obtained as described for the $\alpha$-chymotrypsin reactions.

## RESULTS AND DISCUSSION

### First-order kinetic behaviour of single turnover for the models of Scheme 1

The possibility of the kinetic study of enzyme reactions with enzyme in excess was mentioned in the 1940s [27,28] and shown to result in saturation of substrate by enzyme in the 1950s and 1960s [15,29,30]. Use was made of single-turnover kinetics to determine active-site concentrations in synthetic polymers [5,31], but both the simplest kinetic model (Scheme 1a) and first-order kinetic behaviour were assumed. In fact, first-order behaviour for Scheme 1(a) had effectively been predicted by treatment of the acylation phase of the three-step acyl-enzyme model reported by Kasserra and Laidler [32] (see also [33]).

In the present work it was necessary to demonstrate first-order behaviour in single turnover for the extended models also (Schemes 1b and 1c) and to reveal the significance of $k_{obs}^{lim}$, the limiting value of the first-order rate constant, $k_{obs}$, at high $[E]_T$, as well as of $K_m^{app}$, the parameter for single-turnover kinetics corresponding to $K_m$ for kinetics of catalysis. The significance in terms of kinetic constants of these parameters and of $V_{max}$ and $[E_a]_T$ is summarized in Table 1. These objectives were achieved by solution of the sets of differential equations for the three models by using the Laplace–Carson operator method [34,35]. The manual solution of a given set of linear differential equations involves finding the unknown function that satisfies the differential obtained by solving for each dependent variable separately. The process of eliminating one or more of the dependent variables can often be facilitated by using the Laplace–Carson operator method, wherein the essential objective is to produce an integral operator transform for each reaction species, the solution to which can be found in standard mathematical texts. Thus the method conveniently provides constants of integration, and hence reaction amplitudes, directly. Imposition of the condition $[E_a]_T \gg [S]_0$ enables $[E_a]$ to be treated as a constant approximated by $[E_a]_T$, and permits the reduction of the systems of non-linear differential equations describing the models in Schemes l(a) and 1(b) to a set of linear equations that can then be solved analytically. For the mechanism that acknowledges the existence of the binding, but catalytically inactive, component $E_b$ (Scheme 1c), it is necessary to make the further assumption $[E_b]_T \gg [S]_0$. In practice it is possible to include experimental data corresponding to values of $[E_a]_T$ or $[E_b]_T$ that are not $\gg [S]_0$, provided that $K_m^{app}$ is significantly greater than $[S]_0$. Adherence to first-order kinetics under these conditions follows from the modified form of the Michaelis–Menten equation discussed by Laidler [33].

Transforms of the differential equations are obtained by replacing the derivative with respect to time ($d/dt$) by the Laplace–Carson operator, $\Psi$. Because $\Psi$ is independent of the variable, $t$, it can be manipulated algebraically in the same way as a constant. It was necessary to investigate non-productive binding (Scheme 1b), because this is known to occur in some enzyme reactions, as well as the effect of the presence of a binding but non-catalytic component (Scheme 1c), because this is expected to exist in polyclonal catalytic antibody preparations. The reasons for the growing interest in the latter [3,20,36–42] include: the relative simplicity of producing them, their potential value in technological applications, the rapid and cost-effective application in evaluating a range of haptens for catalyst production, and their potential value in the development of novel therapies when produced by active immunization. Because polyclonal IgG preparations necessarily represent the entirety of the immune response, the relative immunogenic capabilities of a series of haptens may be assessed more effectively by using polyclonal catalytic antibody preparations than by studies on a small selection of isolated monoclonal catalytic antibodies. By use of the Laplace–Carson operator method it was demonstrated that, for all three models, product (P) formation in the observable transient phase is described by the first-order expression given in eqn. (1) below, for which $k_{obs}$ for Schemes 1(a)–1(c) is given by eqns. (2)–(4) respectively, where $[E_a]_T$ = total concentration of active catalyst (i.e. $[E_a]+[E_aS]$), $[E_b]_T$ = total concentration of the binding, non-catalytic component, $K_b$ and $K_s'$ are the dissociation constants of $E_bS$ and the non-productive complex, $SE_a$, respectively and $K_m$ is the steady-state assembly around $E_aS$, i.e. $(k_{-1}+k_2)/k_1$, which may approximate to the dissociation equilibrium constant $K_s = k_{-1}/k_1$.

$$[P] = [S]_0(1-e^{k_{obs}t}) \quad (1)$$

For Scheme 1(a):

$$k_{obs} = \frac{k_2[E_a]_T}{K_m+[E_a]_T} \quad (2)$$

For Scheme 1(b):

$$k_{obs} = \frac{k_2[E_a]_T/(1+K_m/K_s')}{K_m/(1+K_m/K_s')+[E_a]_T} \quad (3)$$

For Scheme 1(c):

$$k_{obs} = \frac{k_2[E_a]_T}{K_m(1+[E_b]_T/K_b)+[E_a]_T} \quad (4)$$

**Table 1 Significance in terms of kinetic constants of the parameters $V_{max}$, $k_{obs}^{lim}$ and $K_m^{app}$ and of $[E_a]_T$ for the models of Schemes 1(a)–1(c)**

The model of Scheme 1(a) is the conventional two-step model of enzyme catalysis, in which $[E]_T = [E_a] + [E_aS] + [E_i]$, where $E_a$ is active enzyme and $E_i$ is inactive, non-binding protein, and $[E_a]_T = [E_a] + [E_aS]$. The model of Scheme 1(b) is an extension of that in Scheme 1(a) in which substrate binds non-productively to $E_a$ to form $SE_a$ with dissociation constant $K_s'$, as well as productively to form $E_aS$ which leads to product formation. In the model of Scheme 1(c), the substrate binds to a binding non-catalytic component ($E_b$) as well as to the catalytically active component ($E_a$); $[E]_T = [E_a] + [E_aS] + [E_b] + [E_bS] + [E_i]$ and $[E']_T = [E]_T - [E_i]$; $K_b$ is the dissociation constant of $E_bS$. $k_{obs}^{lim}$ is the saturation value of the observed first-order rate constant for single-turnover reactions with excess of catalyst over substrate. $K_m^{app}$ is the value of $[E]_T$ (models a and b) or $[E']_T$ (model c), corresponding to $k_{obs}^{lim}/2$.

| Model | $V_{max}$ | $k_{obs}^{lim}$ | $[E_a]_T$ | $K_m^{app}$ |
|---|---|---|---|---|
| Scheme 1(a) | $k_{cat}[E]_T = k_2[E_a]_T$ | $k_2$ | $\frac{V_{max}}{k_{obs}^{lim}}$ | $K_m(1 + [E_i]/[E_a]_T)$ |
| Scheme 1(b) | $k_{cat}[E]_T = k_2[E_a]_T/(1 + K_m/K_s')$ | $k_2/(1 + K_m/K_s')$ | $\frac{V_{max}}{k_{obs}^{lim}}$ | $\frac{K_m(1 + [E_i]/[E_a]_T)}{(1 + K_m/K_s')}$ |
| Scheme 1(c) | $k_{cat}[E']_T = k_2[E_a]_T$ | $k_2 \Big/ \left(1 + \frac{[E_b]_T}{[E_a]_T}\right)$ | $\frac{V_{max}}{k_{obs}^{lim}} - \frac{K_m}{K_b}[E_b]_T$ | $\frac{K_m(1 + [E_b]_T/[E_a]_T)}{(1 + K_b[E_b]_T/K_m[E_a]_T)}$ |

## Equations for evaluation of experimental data in terms of Schemes 1(a) and 1(b)

We consider first the simplest model of catalysis (Scheme 1a). The single-turnover kinetic equation for this model, eqn. (2), is in terms of $[E_a]_T$. This is not of practical use because measurement of protein concentration provides only $[E]_T$, i.e. $[E_a]_T + [E_i]$, where $E_i$ is inert (i.e. a non-catalytic, non-binding protein). Nevertheless, the combination of single-turnover kinetics and the more commonly used kinetics of catalysis, where the concentration of substrate in excess over enzyme is varied, allows determination of $[E_a]_T$. Single-turnover experiments provide $k_{obs}$-$[E]_T$ data pairs which are processed to provide values of $k_{obs}^{lim}$ and $K_m^{app}$ by using eqn. (5), where $k_{obs}^{lim}$ is the limiting value of $k_{obs}$ when substrate is saturated by enzyme:

$$k_{obs} = \frac{k_{obs}^{lim}[E]_T}{K_m^{app} + [E]_T} \quad (5)$$

The significance of the parameters of eqn. (5) is revealed by using $[E]_T = [E_a]_T + [E_i]$, writing $[E_i]$ as $\alpha[E_a]_T$, replacing $[E_a]_T$ in eqn. (2) by $[E]_T/(1+\alpha)$ and rationalizing the resulting expression to provide eqn. (6):

$$k_{obs} = \frac{k_2[E]_T}{K_m(1 + [E_i]/[E_a]_T) + [E]_T} \quad (6)$$

Comparison of eqns. (5) and (6) establishes that, for the model of Scheme 1(a), $k_{obs}^{lim} = k_2$ and $K_m^{app} = K_m(1 + [E_i]/[E_a]_T)$. Thus when the enzyme preparation contains only active enzyme, $[E_i] = 0$ and $K_m^{app} = K_m$. If, however, $[E_i] \neq 0$, the value of $K_m^{app}$, determined from single-turnover kinetics, will be greater than the value of $K_m$ determined from kinetics of catalysis by the factor $(1+\alpha)$, i.e. $(1 + [E_i]/[E_a]_T)$. The single-turnover data are complemented by initial-rate data for catalysis, expressed in terms of $[E_a]_T$ by eqn. (7) and in terms of protein concentration $[E]_T$ by eqn. (8), where $k_2[E_a]_T = k_{cat}^{app}[E]_T = V_{max}$.

$$v_i = \frac{k_2[E_a]_T[S_0]}{K_m + [S]_0} \quad (7)$$

$$v_i = \frac{k_{cat}^{app}[E]_T[S]_0}{K_m + [S]_0} \quad (8)$$

Clearly, the value of $k_{cat}^{app}$, determined experimentally as $V_{max}/[E]_T$, will not be that of $k_2$ unless the enzyme preparation is fully active. For Scheme 1(a) the single-turnover data provide the value of $k_2$ as that of $k_{obs}^{lim}$, and the initial-rate data provide the value of $V_{max}$. Together, therefore, they provide the value of $[E_a]_T$ as $V_{max}/k_{obs}^{lim}$. The values of $\alpha$ and thus $[E_a]_T$ are provided also by using the relationship of the concentration of substrate needed for half-saturation of the enzyme (the steady-state parameter, $K_m$) and the concentration of total protein (active and inactive) needed for half-saturation of the substrate (the single-turnover parameter, $K_m^{app}$) i.e. $K_m^{app} = K_m(1+\alpha)$ (see eqn. 6), so that $\alpha = (K_m^{app} - K_m)/K_m$.

We next consider non-productive binding [16–18], in which substrate binds to $E_a$ not only as $E_aS$, which leads to product formation, but also as $SE_a$, which does not (Scheme 1b). This is an extension of the simple model (Scheme 1a). The kinetic equation for Scheme 1(b) under conditions of catalytic turnover is well known [17,18] and is shown as eqn. (9), where $k_2/(1 + K_m/K_s')[E_a]_T = V_{max}$, $K_m$ is the steady-state assembly around $E_aS$, which may approximate to the dissociation equilibrium constant of $E_aS$ (i.e. $K_s = k_{-1}/k_1$), and $K_s'$ is the dissociation equilibrium constant of the non-productive complex $SE_a$:

$$v_i = \frac{k_2/(1 + K_m/K_s')[E_a]_T[S]_0}{K_m/(1 + K_m/K_s') + [S]_0} \quad (9)$$

In the present work, $k_{obs}$ determined by single-turnover kinetics was shown to be given by eqn. (3). Re-writing eqn. (3) in terms of $[E]_T$ rather than $[E_a]_T$, by making use of $[E_a]_T = [E]_T/(1+\alpha)$, as was done to produce eqn. (6) from eqn. (2) for Scheme 1(a), provides eqn. (10):

$$k_{obs} = \frac{k_2/(1 + K_m/K_s')[E]_T}{\frac{K_m/(1 + [E]_i/[E_a]_T)}{(1 + K_m/K_s')} + [E]_T} \quad (10)$$

Thus $k_{obs}^{lim} = k_2/(1 + K_m/K_s')$, with $K_m^{app}$ modified by the factor $(1+\alpha)$. Because the expressions for both $V_{max}$ and $k_{obs}^{lim}$ contain the factor $(1 + K_m/K_s')$ in equivalent positions, $[E_a]_T$ may be calculated as $V_{max}/k_{obs}^{lim}$ for the model of Scheme 1(b), as in the case of the simple enzyme catalysis model (Scheme 1a).

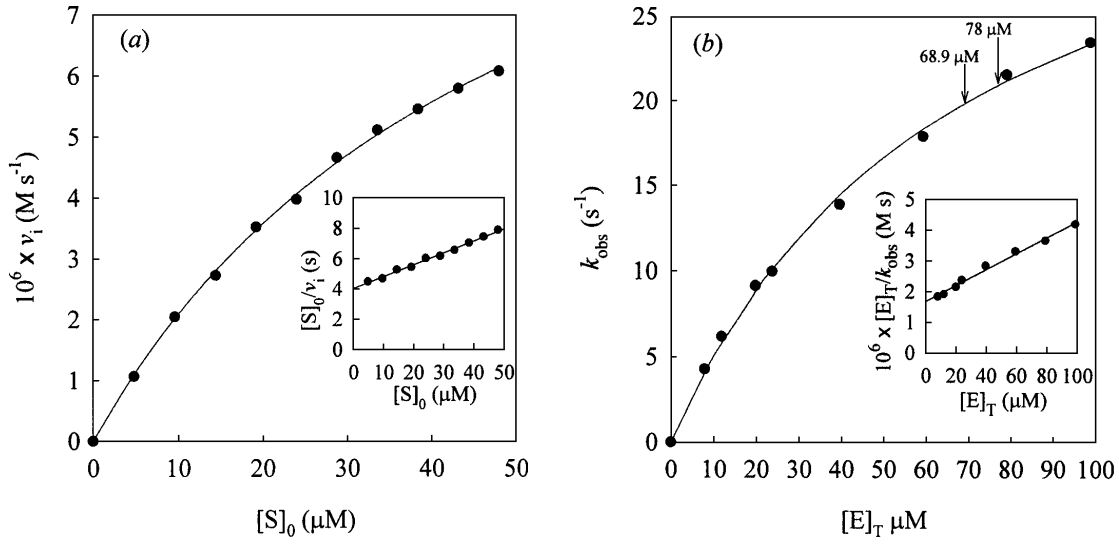



**Figure 2 Demonstration of adherence to hyperbolic kinetics involving saturation of (a) enzyme (0.5 μM protein) by substrate and (b) substrate (1.6 μM) by enzyme**

The characterizing parameters for the reaction of α-chymotrypsin within a commercial preparation with Suc-Ala-Ala-Pro-Phe-4-nitroanilide were determined at 25 °C in 0.1 M sodium phosphate buffer, pH 7.0, containing 2% (v/v) DMSO. The points were determined experimentally, and the best-fit values of $V_{max}$ and $K_m$ (**a**) and of $k_{obs}^{lim}$ and $K_m^{app}$ (**b**) were obtained by fitting the $v_i$ versus $[S]_0$ and $k_{obs}$ versus $[E]_T$ data to hyperbolic saturation curves by weighted non-linear regression, as described in the Experimental section. The curves correspond to (**a**) $v_i = V_{max}[S]_0/(K_m + [S]_0)$, with $V_{max} = 1.25 \times 10^{-5}$ M·s$^{-1}$ (S.E.M. $\pm 3.87 \times 10^{-7}$ M·s$^{-1}$) and $K_m = 49.8$ μM ($\pm 2.50$ μM); and (**b**) $k_{obs} = k_{obs}^{lim}[E]_T/(K_m^{app} + [E]_T)$, i.e. eqn. (5) of the text, with $k_{obs}^{lim} = 39.6$ s$^{-1}$ ($\pm 2.50$ s$^{-1}$) and $K_m^{app} = 69$ μM ($\pm 8$ μM). The arrows in (**b**) indicate the values of $K_m^{app}$ obtained by analysis of the data (68.9 μM) and calculated as $K_m(1 + [E_i]/[E_a]_T) = 49.8(1 + 0.36/0.64) = 78$ μM. The linearity of the plots in the insets emphasizes the adherence of the catalysis data in (**a**) to the Michaelis–Menten equation and of the single-turnover data in (**b**) to the analogous equation (eqn. 5). For the conventional model of enzyme catalysts (Scheme 1a), $k_{obs}^{lim} = k_2$ (cf. eqns. 2 and 5) and $[E_a]$ ($= V_{max}/k_2$) is calculated to be 0.32 μM, i.e. 64% of $[E]_T$, which is the value determined using the well-known 'all-or-none' spectroscopic assay with NTCI as titrant. The fact that $K_m^{app} > K_m$ is predicted for a less than fully active enzyme preparation (see the text).

### Determination of the active-site content of a commercial α-chymotrypsin preparation using Suc-Ala-Ala-Pro-Phe-4-nitroanilide as substrate, and validation of the kinetic method by using NTCI as an 'all-or-none' titrant

The hyperbolic dependences of the initial rate ($v_i$) on substrate concentration ($[S]_0$) at constant enzyme concentration (with $[S]_0 \gg [E]_T$) and of the experimentally determined first-order rate constant ($k_{obs}$) on total protein concentration ($[E]_T = [E_a]_T + [E_i]$) at constant substrate concentration (with $[E]_T \gg [S]_0$) are demonstrated by typical data sets in Figures 2(a) and 2(b) respectively. For the data in Figure 2(a), the protein concentration $[E]_T$ is 0.5 μM and $V_{max} = 1.25 \times 10^{-5}$ ($\pm 3.87 \times 10^{-7}$) M·s$^{-1}$. Figure 2(b) provides the value of $k_{obs}^{lim} = 39.58$ ($\pm 2.50$) s$^{-1}$. For the conventional model of enzyme catalysis (Scheme 1a), $k_{obs}^{lim} = k_2$ and the active-site concentration $[E_a]_T$ is calculated as $V_{max}/k_2 = 0.32$ μM. Thus $[E_a]_T/[E]_T \times 100 = 64\%$, which is identical with the value determined by the well-known 'all-or-none' assay using NTCI as titrant, as described in the Experimental section. The values $K_m = 49.8$ ($\pm 2.50$) μM (Figure 2a) and $K_m^{app} = 68.9$ ($\pm 8.1$) μM (Figure 2b) differ, as expected for a partially active enzyme preparation, as was discussed above. The value of $K_m^{app}$, calculated as $K_m(1 + [E_i]/[E_a]_T) = 49.8(1 + 0.36/0.64) = 78$ μM, is in reasonable accord with the value determined from the data in Figure 2(b). Calculation of $\alpha$ ($= [E_i]/[E_a]_T$) as $(K_m^{app} - K_m)/K_m = (68.9 - 49.8)/49.8 = 0.38$ corresponds to $[E_a]_T/[E]_T \times 100 = 62\%$, which is in good agreement with the value of 64% calculated using $V_{max}$ and $k_{obs}^{lim}$.

### Equation for the evaluation of experimental data in terms of Scheme 1(c)

Scheme 1(c) is the model appropriate to the investigation of polyclonal catalytic antibody preparations where substrate binds not only to the catalytically active component, $E_a$, but also to a binding non-catalytic component, $E_b$.

Just as evaluation of data using Schemes 1(a) and 1(b) needs eqns. (6) and (10), where $k_{obs}$ is a function of $[E]_T$ rather than $[E_a]_T$ [as it is in eqns. (2) and (3)], so data evaluation using Scheme 1(c) requires the parameters of eqn. (4) to be related to those of eqn. (11), in which $[E_a]_T$ is replaced by $[E']_T$ ($= [E_a]_T + [E_b]_T$).

$$k_{obs} = \frac{k_{obs}^{lim}[E']_T}{K_m^{app} + [E']_T} \quad (11)$$

The procedure is simplified by using $[E']_T$ (i.e. $[E_a]_T + [E_b]_T$) rather than $[E]_T$ (i.e. $[E_a]_T + [E_b]_T + [E_i]$), from which it may be obtained by subtracting $[E_i]$, which is readily determined for polyclonal catalytic antibody preparations by analytical affinity chromatography, as described below. By making use of $\beta = [E_b]_T/[E_a]_T$, which allows $[E_a]_T$ to be written as $[E']_T/(1 + \beta)$ and $[E_b]_T$ to be written as $\beta[E']_T/(1 + \beta)$, and of $\gamma = K_m/K_b$, eqn. (4) may be written as eqn. (12):

$$k_{obs} = \frac{[k_2/(1 + \beta\gamma)][E']_T}{K_m(1 + \beta)/(1 + \beta\gamma) + [E']_T} \quad (12)$$

Thus the parameters of eqn. (11) are given by eqns. (13) and (14):

$$k_{obs}^{lim} = \frac{k_2}{(1 + \beta\gamma)} \quad (13)$$

$$K_m^{app} = \frac{K_m(1 + \beta)}{(1 + \beta\gamma)} \quad (14)$$

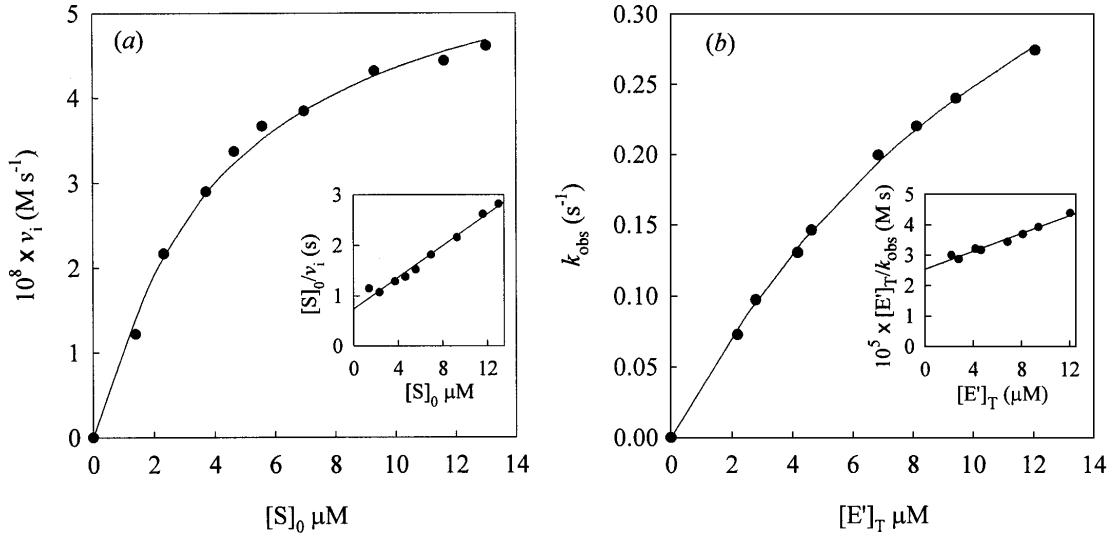



**Figure 3 Demonstration of adherence to hyperbolic kinetics involving saturation of (a) polyclonal catalytic antibody preparation PCA 271-22 (E) by substrate and (b) substrate (0.8 μM) by PCA 271-22**

The substrate used was 4-nitrophenyl 4′-(3-aza-2-oxoheptyl)phenyl carbonate (**3**) (see Figure 1). The reactions were carried out at 25 °C in 0.1 M sodium phosphate buffer, pH 8.45, containing 0.7% (v/v) acetonitrile. The characterizing parameters for the reaction under both sets of conditions and the upper limit of the concentration of catalytically active IgG ($E_a$) were determined. The points were determined experimentally and are plotted in (**b**) using the term $([E']_T = [E]_T - [E_i]) = [E_a] + [E_b] - [E_i]$, where $E_a$ is catalytic IgG, $E_b$ is binding but non-catalytic IgG and $E_i$ is non-binding IgG (determined by affinity chromatography and an ELISA procedure to represent 59% of $E_T$); in (**a**), $[E']_T = 0.5\ \mu$M. Best-fit values of $V_{max}$ and $K_m$ (**a**) and of $k_{obs}^{lim}$ and $K_m^{app}$ (**b**) were obtained by fitting the $v_i$ versus $[S]_0$ and $k_{obs}$ versus $[E']_T$ data to hyperbolic saturation curves by weighted non-linear regression, as described in the Experimental section. The curves correspond to (**a**) $v_i = V_{max}[S]_0/(K_m + [S]_0)$, with $V_{max} = 6.26 \times 10^{-8}$ M·s$^{-1}$ (S.E.M. $\pm 0.26 \times 10^{-8}$ M·s$^{-1}$) and $K_m = 4.36\ \mu$M ($\pm 0.04\ \mu$M); and (**b**) $k_{obs} = k_{obs}^{lim}[E']_T/(K_m^{app} + [E']_T)$, i.e. eqn. (11) of the text, with $k_{obs}^{lim} = 0.64$ s$^{-1}$ ($\pm 0.03$ s$^{-1}$) and $K_m^{app} = 15.8\ \mu$M ($\pm 1.3\ \mu$M). The linearity of the plots in the insets emphasizes the adherence of the catalysis data in (**a**) to the Michaelis–Menten equation and of the single-turnover data in (**b**) to the analogous equation (eqn. 11). The adherence of the data in (**a**) to the Michaelis–Menten equation 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 the $[S]_0/v_i$ versus $[S]_0$ plot (see [26]). For the kinetic model in Scheme 1(c) that acknowledges the existence of binding but non-catalytic IgG ($E_b$), $k_{obs}^{lim} = k_2/(1+\beta)$ and $K_m^{app} = K_m(1+\beta)/(1+\beta\gamma)$, where $\beta = [E_b]_T/[E_a]_T$, $\gamma = K_m/K_b$ and $K_b$ is the equilibrium dissociation constant of $E_bS$ (cf. eqns. 11 and 12). The upper limit of $[E_a]_T$ given by $V_{max}/k_{obs}^{lim}$ (eqn. 15) is approx. 8% of $[E]_T$; however, if, for example, $K_m/K_b = \sim 0.25$, then $[E_a] \leqslant 1\%$ of $[E]_T$. The fact that $K_m^{app} > K_m$ establishes the existence of $E_b$ in PCA 271-22.

Combination of $[E_a]_T = V_{max}/k_2$ with eqn. (13) provides eqn. (15):

$$[E_a]_T = \frac{V_{max}}{k_{obs}^{lim}} - \frac{K_m}{K_b}[E_b]_T \qquad (15)$$

It follows from eqn. (15) that, as expected, $k_{obs}^{lim} = k_2$ when $[E_b]_T$ is zero (i.e. $[E_a]_T = V_{max}/k_{obs}^{lim}$), and that the effect of the presence of $E_b$ is modulated by the relative values of $K_m$ and $K_b$. The additional condition when $[E_b]_T = 0$ is that the value of $V_{max}/k_{obs}^{lim}$ must equal that of $[E']_T$. Eqn. (14) reveals that if either $[E_b] = 0$ (i.e. $\beta = 0$) or $K_m = K_b$ (i.e. $\gamma = 1$), then $K_m^{app} = K_m$. If, however, $K_m^{app} \neq K_m$, eqn. (14) rearranged as eqn. (16) allows values of $[E_b]_T/[E_a]_T$ to be calculated for particular values of $K_m/K_b$:

$$\frac{[E_b]_T}{[E_a]_T} = \frac{(K_m^{app}/K_m) - 1}{1 - (K_m^{app}/K_m)(K_m/K_b)} \qquad (16)$$

Since the right-hand side of eqn. (16) must be positive, both $K_m$ and $K_m^{app}$ must be $< K_b$ if $K_m^{app} > K_m$.

## Investigation of the active-site content of polyclonal catalytic antibody preparation PCA 271-22

Polyclonal catalytic antibody preparations would be expected to contain catalytic IgG ($E_a$), binding but non-catalytic IgG ($E_b$) and IgG that neither binds substrate nor catalyses its conversion into product ($E_i$), some of which might bind the carrier protein (keyhole limpet haemocyanin). Thus they probably differ from the α-chymotrypsin preparation in containing three types of protein component rather than two. This hypothesis is based on the results of studies on monoclonal antibodies reported by different groups which show that, during the screening phase of antibody production, of the hundreds of hybridomas obtained from the fusion, only a small fraction produces hapten-binding antibodies and an even smaller fraction has catalytic activity. For example, Tawfik et al. [43] reported that 1590 hybridomas produced 970 clones that showed binding above background, but only nine clones had catalytic activity above background. Accordingly it was appropriate to investigate PCA 271-22 by making use of the kinetic model of Scheme 1(c).

To determine the $E_i$ content of the polyclonal preparation resulting from Protein G chromatography, the preparation was subjected to affinity chromatography using a Sepharose column bonded to the 4-nitrophenylphosphate hexylamine hapten analogue, ligand (**4**), synthesized as indicated in Scheme 2 and as described in the Experimental section. The clean separation of the $E_i$ component in the breakthrough fraction from IgG that binds substrate (i.e. a mixture of $E_a$ and $E_b$) in the fraction eluted by the low-pH buffer was demonstrated by the ELISA procedure using hapten (**2**) conjugated to BSA. Spectral analysis at 280 nm of the IgG applied to the column and of the breakthrough fraction established the $E_i$ content of PCA 271-22 to be 59% of the total IgG, which was used to calculate $[E']_T$ as $[E]_T - [E_i] = [E_a] + [E_b]$.

The hyperbolic dependences of the initial rate ($v_i$) on substrate concentration ($[S]_0$) at constant $[E']_T$ (i.e. $[E]_T - [E_i]$) and of the experimentally determined first-order rate constant ($k_{obs}$) on $[E']_T$ at constant $[S]_0$ are demonstrated by typical data in Figures 3(a)

and 3(b). The adherence of the data to the Michaelis–Menten equation (Figure 3a, where $[E']_T = 0.5\ \mu M$) demonstrates the kinetic homogeneity of the polyclonal preparation and provides values of $V_{max} = 6.26 \times 10^{-8}$ ($\pm 0.26 \times 10^{-8}$) $M \cdot s^{-1}$ and $K_m = 4.36$ ($\pm 0.04$) $\mu M$. The data in Figure 3(b) provide values of $k_{obs}^{lim} = 0.64$ ($\pm 0.03$) $s^{-1}$ and $K_m^{app} = 15.8$ ($\pm 1.3$) $\mu M$.

As discussed above, the fact that $K_m^{app} > K_m$ establishes the existence of $E_b$ in PCA 271-22. This conclusion is in accord with the fact that the upper limit of $[E_a]_T$ defined by eqn. (15) as $V_{max}/k_{obs}^{lim}$ is $< 0.1\ \mu M$, which is less than the value of $[E']_T$ ($= [E_a] + [E_b] = 0.5\ \mu M$), the value that it would be if $[E_b] = 0$. The upper limit of $[E_a]_T$ thus defined (20% of $[E']_T$ or 8% of $[E]_T$) may be revised downwards by consideration of probable values for $K_m/K_b$. There is no reason to suggest that the values of $K_m$ and $K_b$ might be very different. If, for example, this ratio is taken to be approx. 0.25, the value of $[E_a]_T$ calculated by using eqn. (16), $[E']_T = [E_a]_T + [E_b]_T$ and $[E_i]$ determined by affinity chromatography using the high-affinity immobilized hapten analogue must be $\leqslant 1\%$ of the concentration of total IgG, i.e. of $[E]_T$.

## Concluding comment

The result of the analysis of PCA 271-22 demonstrates the existence of $E_b$ and $E_i$, and is in accord with our suggestion [20,36] that the content of catalytically active antibody, $E_a$, within a polyclonal preparation might be rather low. The procedures described here now permit for the first time realistic assessment of the content of each of the components ($E_a$, $E_b$ and $E_i$) of a polyclonal catalytic antibody preparation, and consequently of the intrinsic catalytic ability of the $E_a$ component. The kinetic method may be widely applied to preparations of enzymes and enzyme-like catalysts and, like the well-known use of $k_{cat}/K_m$ for the characterization of kinetic specificity, is not affected by non-productive binding.

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