Mechanism of an antibody-catalysed allylic isomerization

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The catalytic antibody 4B2, which was generated against a substituted amidine 1, catalyses the allylic isomerization of β,γ-unsaturated ketones with an acceleration factor (k_{cat}/k_{uncat}) of 1.5×10^3. On the basis of the 'bait and switch' strategy, it was reasoned that the positively charged hapten could elicit, by charge complementarity, an acidic residue (Asp or Glu) in the antibody-binding site in the right position to catalyse this proton transfer reaction. The pH dependence curve of k_{cat}/K_m shows a bell-shaped feature with an optimum at approx. pH 4.5. By cloning and sequencing the light and heavy chains of the 4B2 antibody, we confirmed the presence of several Asp and Glu residues in the complementarity-determining region loops. The antibody catalyses the α-proton exchange on the same substrates, demonstrating the involvement of a dienol intermediate in the reaction mechanism. Kinetic studies with ^{2}H-NMR provide evidence that α-proton abstraction is stereospecific. Whether the process involves one or two acid/base residues in this simple proton transfer or whether it is a concerted mechanism is discussed.

Key words: abzymes, bait and switch strategy, catalysis, proton exchange.

INTRODUCTION

Allylic isomerization has a central role in biochemical pathways such as the biosynthesis of terpenoids and steroid hormones and in the biodegradation of fatty acids. This rearrangement occurs within a non-heteroatomic allylic system and involves a 1,3 sigmatropic hydrogen migration. From a mechanistic point of view, these reactions are simple because they are one-substrate reactions and do not require any cofactors. They are also of particular interest in studying the mechanism and efficiency of proton transfer catalysis in enzymes or enzyme mimics [1]. Moreover, the mechanism of some enzymic allylic rearrangements (at least those involving suprafacial rearrangement) seems to involve only one catalytic residue in the active site [2,3]. For these reasons, this reaction is a particularly interesting target for antibody catalysis because it might require only a precise positioning of a carboxy group within the antibody-combining site. Previous work on antibody catalysis has shown that such a residue can be induced in the antibody-combining site by a 'bait and switch' strategy' [4–6]. More recently, by means of reactive immunization, an antibody with steroid isomerase activity that uses an enamine mechanism has been obtained [7].

In the course of experiments to prepare glycosidase catalysts, we produced monoclonal antibodies against the amidinium hapten **1** (see Scheme 1), designed to mimic the half-chair conformation of the transient oxocarbocation and to induce a complementary charged group in the antibody pocket [8]. Although none of the monoclonal anti-**1** antibodies accelerated the rate of hydrolysis of (aryloxy)tetrahydropyrans significantly, some of them promoted efficient catalysis of the Kemp elimination reaction [9]. These results suggest that these antibodies have a functional group, presumably a carboxy group, that could act as a general base to catalyse proton transfer efficiently [10]. We reasoned that some of these antibodies might also catalyse other proton transfer reactions involving a general acid/base residue.

In the present study we show that one of these anti-**1** antibodies efficiently catalyses proton transfer in the allylic isomerization of β,γ-unsaturated ketones. Furthermore, we demonstrate that the mechanism involved in this rearrangement occurs through the stabilization of an enol intermediate associated with a stereospecific proton exchange with the solvent, as observed in most enzyme-catalysed natural reactions.

EXPERIMENTAL

General methods

Unless noted otherwise, materials were obtained from Sigma or Aldrich and were used without further purification. Analytical TLC was performed on precoated silica gel plates (Merck). Flash chromatography was performed with Kieselgel 60 (230–400 mesh) silica gel. NMR spectra were recorded on a Brüker DRX400 (400 MHz) or a DPX500 (500 MHz) spectrometer.

2-(4-Acetamidobenzylamino)-3,4,5,6-tetrahydropyridinium (1b)

The cyclic guanidinium salt (**1b**) and its protein conjugate (**1a**) (Scheme 1) were prepared from the commercial 2-piperidone in four steps as described previously [11].

α-Cyclopent-1-en-1-yl-*p*-acetamidoacetophenone (2a)

To a solution of methylenecyclopentane (500 mg, 6.1 mmol) and 4-acetamidobenzaldehyde (1.1 g, 6.7 mmol) in 20 ml of CH_2Cl_2 was added dropwise 9.2 ml of a 1 M dimethyl aluminium chloride in hexane. After 30 min a pale yellow precipitate had formed; the reaction was quenched by the addition of 20 ml of KH_2PO_4 (0.5 M). The resulting mixture was extracted with methanol, filtered on Celite and concentrated. The residue was purified by flash chromatography [3 cm (internal diameter) × 20 cm, ethyl acetate/light petroleum (boiling range 45–65 °C) (1:1, v/v)] to yield 340 mg (25 %) of 1-*p*-acetamidophenyl-2-cyclopent-1-en-1-ylethanol as a white solid.

^{1}H NMR (C^2HCl_3): δ 1.89 (m, 2H), 2.19 (s, 3H), 2.34 (m, 4H), 2.46 (dd, 1H), 2.55 (dd, 1H), 4.79 (m, 1H), 5.55 (s, 1H), 7.34 (d, 2H), 7.48 (d, 2H). A 200 mg sample of this allylic alcohol was

Abbreviations used: CDR, complementarity-determining region; OSI, oxosteroid isomerase.

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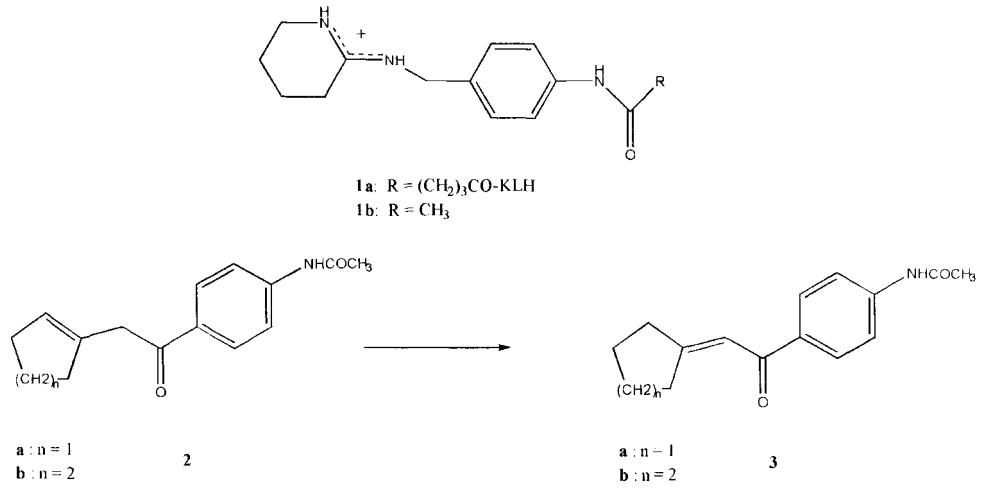
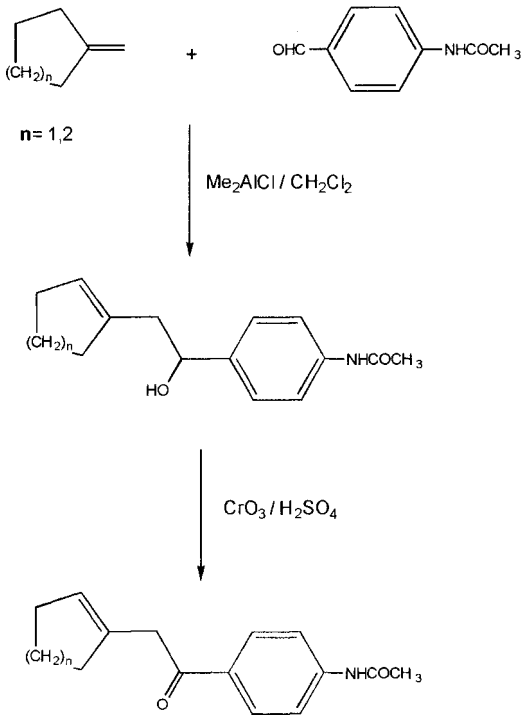


Scheme 1 Structure of the hapten and substrates



Scheme 2 Synthesis of substrates

dissolved in 10 ml of acetone and cooled to 0 °C. To this solution was added dropwise 400 μl of Jones reagent (5.34 g of CrO_3 in 20 ml of 4 M H_2SO_4) (Scheme 2). The resulting brown solution was stirred for a further 15 min and the excess oxidant was destroyed by the addition of 100 μl of methanol. Chromium salts were removed by filtration through Celite and the acetone solution was diluted with 30 ml of ethyl acetate, washed several times with brine, dried and evaporated. The residue was purified by flash chromatography [1 cm × 20 cm, ethyl acetate/light petroleum (boiling range 45–65 °C) (1:1, v/v)] to yield 80 mg (66 %) of **2a** as a white solid, m.p. 125 °C. ^{1}H NMR ($C^2H_3O^2H$): δ 1.90 (m, 2H), 2.17 (s, 3H), 2.34 (m, 4H), 3.77 (s, 2H), 5.53 (s, 1H), 7.70 (d, 2H), 7.98 (d, 2H). High resolution MS (fast atom bombardment): calcd m/z for $C_{15}H_{18}NO_2$ ($M-H^+$) 244.1337; found 244.1322.

α-Cyclohex-1-en-1-yl-*p*-acetamidoacetophenone (2b)

This compound was prepared as described above for **2a** by using methylenecyclohexane as starting material; m.p. 112 °C. ^{1}H NMR ($C^2H_3O^2H$): δ 1.65 (m, 2H), 1.73 (m, 2H), 2.07 (m, 2H), 2.11 (m, 2H), 2.24 (s, 3H), 3.68 (s, 2H), 5.66 (s, 1H), 7.77 (d, 2H), 8.06 (d, 2H). High resolution MS (fast atom bombardment): calcd m/z for $C_{16}H_{20}NO_2$ ($M-H^+$) 258.1493; found 258.1453.

α-Cyclopentylidien-*p*-acetamidoacetophenone (3a)

This product, resulting from the acid- or antibody-catalysed allylic isomerization of **2a**, was purified by HPLC (C_{18} reverse-phase) by using an elution solvent of 50 % CH_3OH in water. ^{1}H NMR (C^2HCl_3): δ 1.75 (m, 2H), 1.85 (m, 2H), 2.35 (s, 3H), 2.63 (t, 2H), 2.93 (t, 2H), 7.01 (s, 1H), 7.66 (d, 2H), 7.93 (d, 2H).

1-*p*-Acetamidophenyl-2-cyclopent-1-en-1-ylethanone-2,2-d_2 (8a) (see Figure 5)

To a stirred solution of **2a** (10 mg) dissolved in 1.5 ml of methanol-d_4 (Eurisotop, CEA France) was added 200 μl of potassium ter-butoxide (18 mM) in $C^2H_3O^2H$. The resulting mixture was stirred at room temperature for 30 min. The reaction was quenched with 100 μl of ^{2}HCl (38 mM) and the reaction mixture was concentrated, extracted with ethyl acetate and washed with brine. The organic solution was concentrated to dryness *in vacuo*. Complete deuterium exchange with the α-proton was confirmed by ^{1}H-NMR, with the disappearance of the peak at 3.77 p.p.m.

1-*p*-Acetamidophenyl-2-cyclohex-1-en-1-ylethanone-2,2-d_2 (8b)

The same procedure as that described for **8a** was used but the exchange was completed at room temperature within 8 h.

Antibody production and purification

Monoclonal antibodies were generated against hapten **1a** by using standard hybridoma technology [12]. Three Balb/c mice

were injected intraperitoneally with keyhole-limpet haemocyanin-1 (30 μg) emulsified in complete Freund's adjuvant. Boosters were given at intervals of 3 weeks. At 3 days after the final intravenous injection, the spleen cells of the mouse presenting the highest antiserum titre for BSA–1 conjugate were fused with SP_2O myeloma cells. Screening by ELISA for binding to BSA–1 identified 20 cell lines specific for the hapten, which were then cloned by limiting dilution. Five antibodies displaying the highest affinity for the hapten were expanded as ascitic fluids in mice.

Monoclonal antibodies were purified by precipitation with 50 %-satd $(NH_4)_2SO_4$ and Protein A affinity chromatography. For antibody 4B2, purification on Protein A was performed with a high-salt binding buffer [aqueous 1.5 M glycine/3 M NaCl (pH 8.9)]. Antibody was eluted from the column with 0.1 M citrate buffer, pH 3. The eluted antibody was dialysed exhaustively against PBS and stored at 4 °C after sterile filtration (0.2 μm pore size). The homogeneity of the purified antibodies was verified by gel electrophoresis.

The dissociation constants (K_d) of the antigen–antibody complexes were determined by competitive ELISA on plates coated with BSA–**1a** in accordance with published methods [13,14].

Determination of active antibody concentration

The concentration of active sites was determined by using the inhibition of the Kemp elimination by hapten **1b** [9]. Antibodies (0.45 mg/ml) were incubated in the presence of **1b** at various concentrations (0–10 μM). 5-Nitrobenzisoxazole (0.4 mM) was then added and the rate of ring opening was followed spectroscopically at 405 nm (iEMS; Labsystem) in 20 mM phosphate buffer/50 mM NaCl (pH 7.0). Active-site concentration was determined by extrapolating to 100 % inhibition. The active-site concentrations determined by titration with the hapten corresponded to twice the IgG concentration determined by A_{280} (ϵ 1.34 litre$\cdot g^{-1}\cdot cm^{-1}$).

Kinetic assays

Rates for the antibody-catalysed isomerization of **2a** to **3a** were measured with a Uvikon 860 spectrophotometer equipped with a constant-temperature cell held at 30 °C. Stock solutions of the substrate **2a** at various concentrations were prepared in methanol. Velocities were determined by measuring the initial increase in A_{313} ($\Delta\epsilon$ 8000 $M^{-1}\cdot cm^{-1}$) indicating the release of product **3a**. Reactions were initiated by the addition of a concentrated stock solution of antibody in PBS to an aqueous solution, so that the final solution (400 μl) contained 50 mM acetate buffer, pH 4.5, 7 % methanol, 10 μM antibody and 0–1.5 mM substrate. Ionic strength was maintained at 0.16 with NaCl. In all cases the reported rates were calculated by measuring the initial slopes (less than 10 % conversion, more than 30 points) and standard errors were estimated from the linear regression slopes. HPLC (Waters) kinetic measurements were performed on a C_{18} reverse-phase Hyperpep 5 μm column with an elution solvent of 50 % (v/v) methanol at a flow rate of 0.5 ml/min and detection with UV at 290, 305 and 313 nm. Retention times for the substrate **2a** and the product **3a** were 30 and 35 min respectively. Kinetic analyses of uncatalysed reactions were performed in the absence of antibodies under otherwise identical conditions.

Antibody modification

A solution of 1 mg/ml IgG 4B2 (1 ml) in 50 mM Mes/1 M glycine ethyl ester (pH 5.5)/100 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide was incubated in the presence or absence of 1 mM hapten **1b** for 15 h at 20 °C, then treated with 0.4 M sodium acetate (0.25 ml) at 20 °C for 3 h. The two samples were then dialysed extensively against PBS and concentrated on a Centrisart (Sartorius). The antibody concentration was determined by A_{280}; each sample was diluted to a standard concentration of 10 μM before the assay for catalysis.

Antibody cloning and sequencing

Total RNA was isolated from the hybridoma cell line (1.4×10^8 cells) producing monoclonal antibody (4B2) by using a Fast Track kit (Invitrogen). Total RNA was enriched for mRNA by affinity chromatography with oligo(dT)-cellulose. The oligonucleotide primers used for reverse-transcriptase-mediated PCR of the V regions of H-chain and L-chain cDNA [15] were obtained from Gibco BRL. Single-stranded cDNA was synthesized from 2 μg of the purified mRNA, 250 ng of oligo(dT), 0.5 mM dNTPs and 16 units of reverse transcriptase in a total volume of 24 μl. Reverse-transcriptase-mediated PCR products were subjected to electrophoresis in a 2 % (w/v) agarose gel and then stained with ethidium bromide. PCR amplification yielded sufficient DNA for cloning and sequencing. In brief, 2 μl of synthetic cDNA mixture, 0.4 μM PCR primers and 5 units of *Taq* polymerase (Gibco BRL) were incubated in a final volume of 100 μl; 30 cycles of 94 °C (40 s), 60 °C (30 s), 72 °C (2 min) were performed. PCR products were subjected to electrophoresis in a 2 % (w/v) agarose gel and then stained with ethidium bromide. The V_L and V_H genes were cloned into plasmid Bluescript SK^+ (Stratagene). Sequencing of the cDNA was performed by Eurogentec (Herstal, Belgium). The N-terminal amino acid sequences of the V regions of the H-chain and the L-chain were determined by the cycle sequencing method of Edman on a 470A automatic sequencer (Applied Biosystem) and proved to match the corresponding cDNA sequences cloned in the vector. The numbering of residues follows the convention of Kabat et al. [16].

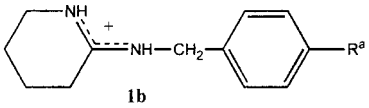
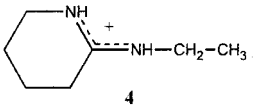
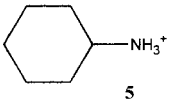
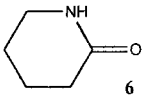
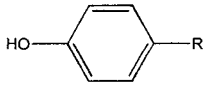
RESULTS

Monoclonal antibody characterization

To characterize in detail the monoclonal antibodies that were produced against hapten **1**, their binding properties were studied by competitive immunoassay. Table 1 reports the dissociation constants determined for five of the best binders to the hapten **1b**. These results show that the binding site of the antibodies covers the entire surface of the hapten because all parts of the molecule contributed to the affinity. The presence of a positive charge is an essential factor for recognition because the neutral compounds **6** and **7** exhibited a much lower affinity than **1b** and **4**. This was expected for an antibody-binding site containing a negatively charged residue. The contribution of the acetamidophenyl group to the binding is less important than the cyclic amidinium moiety because the decrease in affinity was greater for **7** than for **4**.

Those results prompted us to design new substrates mimicking the hapten structure for proton transfer reactions. In an attempt to test the antibody catalysis of allylic isomerization, the potential substrates **2a** and **2b** were synthesized as shown in Scheme 2 [17]. These substrates contain some of the essential structural features of the hapten, i.e. a partly constrained aliphatic cycle, an acetamidophenyl group and a similar steric hindrance. It was expected that these substrates would bind to the antibodies although they lacked a positive charge and that the decreased affinity in comparison with that for the cationic hapten would prevent an eventual inhibition of catalysis by the product of the reaction.

Table 1 Binding characteristics (K_d) of monoclonal antibodies elicited against 1a at pH 7.4

Ligand	K_d (M)				
	10G7	8D4	6C2	5D6	4B2
 **1b**	2×10^{-8}	2×10^{-8}	9×10^{-9}	6×10^{-9}	4×10^{-10}
 **4**	5×10^{-5}	10^{-5}	2×10^{-5}	5×10^{-6}	1×10^{-6}
 **5**	2×10^{-2}	10^{-2}	6×10^{-5}	4×10^{-4}	3×10^{-4}
 **6**	9×10^{-3}	3×10^{-3}	10^{-2}	7×10^{-3}	3×10^{-3}
 **7**	–[b]	–	3×10^{-3}	–	10^{-2}

[a]R = $NHCOCH_3$; [b]$K_d > 10^{-2}$ M

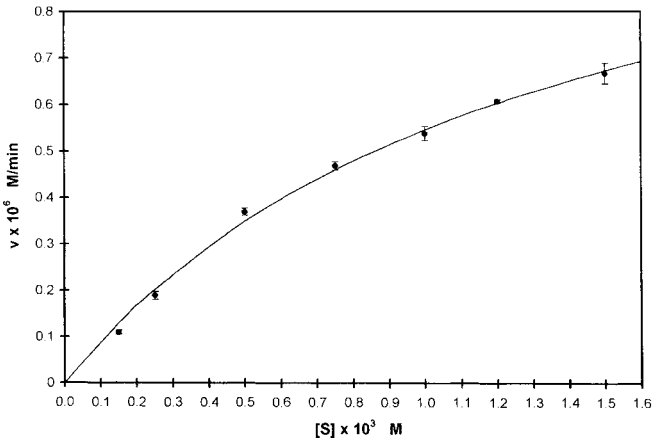


Figure 1 Activity of 4B2

Michaelis–Menten plot of the initial rates for the catalysis of the allylic isomerization of substrate **2a** by antibody 4B2 (10 μM) in 50 mM acetate buffer, pH = 4.5. The kinetic constants determined by non-linear regression fit are: $V_{max} = 1.2 \pm 0.09$ μM/min and $K_m = 1.19 \pm 0.15$ mM. Results are means ± S.D.

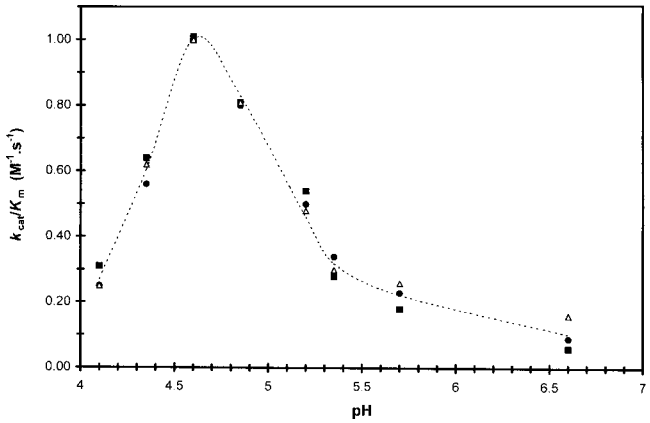


Figure 2 Plot of k_{cat}/K_m against pH for the conversion of 2a into 3a catalysed by 4B2

Kinetics were performed under pseudo-first-order conditions ($[S]_0 < 0.20$ mM) at 30 °C in the following buffers, each at 50 mM and containing 150 mM NaCl and 10% (v/v) ethanol: sodium acetate (pH < 6) and sodium phosphate (6 < pH < 7.5). Each point corresponds to an independent measurement.

Kinetics of the antibody-catalysed allylic isomerization

From the panel of five monoclonal antibodies, selected for their high affinity for **1b**, two (6C2 and 4B2) were found to catalyse the allylic isomerization of **2a** to **3a** (Scheme 1). 4B2 was the most efficient catalyst and was therefore selected for further characterization. However, neither the uncatalysed nor the catalysed conversion of **2b** into **3b** was observed.

The catalysed allylic isomerization of **2a** by 4B2 followed Michaelis–Menten kinetics (Figure 1) with a k_{cat} of 1.6×10^{-3} min^{-1} and a K_m of 1.3 mM at pH 4.5, corresponding to a rate enhancement (k_{cat}/k_{uncat}) of 1500 over the uncatalysed reaction ($k_{uncat} = 1.06 \times 10^{-6}$ min^{-1}). The rate constant for the acetic acid-catalysed conversion of **2a** into **3a** was 2.8×10^{-4} $M^{-1} \cdot min^{-1}$ at 30 °C. Thus the specificity constant k_{cat}/K_m was 4.4×10^3-fold that of acetic acid in solution.

The pH dependence of the antibody-catalysed reaction with **2a** was examined between pH 4.0 and 7.0 (Figure 2). The curve shows that 4B2 exhibits its maximal efficiency (k_{cat}/K_m) at approx.

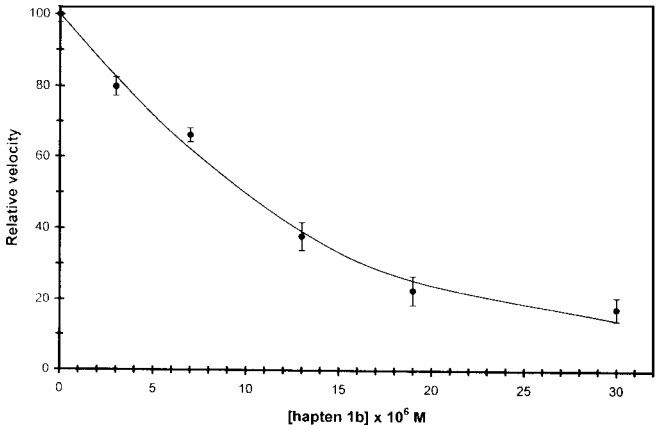


Figure 3 Active-site titration

Initial velocity of catalysis by 4B2 (10 μM) with substrate **2a** (1 mM) in the presence of various concentrations of hapten **1b** at pH 4.7. Initial velocities are presented as relative activities. Kinetic data were fitted to the equation for tight binding inhibition:

$v/v_0 = \{n - l - K_i + [(K_i + n - l)^2 + 4K_i l]^{\frac{1}{2}}\}/2n$

where n is the concentration of active sites. The line corresponds to the best fit of the data obtained with $n = 13.7$ μM and $K_i = 3$ μM. Results are means $\pm$ S.D.

pH 4.5. We checked the antibody stability at these pH values so as to eliminate a possible irreversible denaturation effect. Initial velocities were constant during the overall period of incubation (0.5–2 h) at all pH values tested. Furthermore, measurements of the residual activity of 4B2 on the Kemp elimination reaction at the optimal pH of 7.5, after preincubation (30, 45 and 120 min at 30 °C) at pH values ranging from 3.5 to 7, showed that full activity was recovered in all cases (results not shown). These results demonstrate that the decline in k_{cat}/K_m below and above pH 4.5 is not due to denaturation of the antibody but is instead consistent with an activity of 4B2 on allylic isomerization depending on the protonation and deprotonation state of at least one acid/base residue. A striking feature of this pH-dependent 'bell-shaped' curve is its narrowness at half-height, close to the theoretical limit of 1.14 [18]. According to these authors, the mid-height width of such curves becomes insensitive to changes in pK_a differences as it approaches this value. This precludes the accurate determination of the pK_a value(s) of the ionizing groups on which the catalytic activity of the antibody depends. Nevertheless, a pK_a value of between 4 and 5 is a reasonable estimate and is consistent with the involvement of at least one acid/base group in the antibody activity. To verify whether aspartic or glutamic residues have a role in catalysis, chemical modification experiments were performed with the carboxy-specific reagent 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride/1 M glycine ethyl ester. Modification in the absence of hapten **1b** resulted in the complete inactivation of the antibody, whereas partial protection (50 %) of the activity was observed in the presence of **1b**.

The antibody-catalysed isomerization of **2a** proceeded with multiple turnovers (where one antibody molecule accomplishes several catalytic cycles, $> 10^2$, without inactivation) and was progressively inhibited on the addition of hapten **1b** (Figure 3). This ensured that catalysis occurred within the binding site. The calculated inhibition constant ($K_i = 3$ μM) of the hapten **1b** at pH 4.7 was 10^4-fold its dissociation constant ($K_d = 400$ pM) measured at pH 7.4 by competitive ELISA (Table 1). This lower affinity for the positively charged hapten at low pH also suggests that the antibody-binding site contains a side chain that should be neutral in its protonated form and negatively charged as a free base.

Cloning and sequencing of 4B2 Fab

To verify the presence of one or several carboxy groups in the antibody-binding site, we cloned the Fab region of 4B2 antibody. The deduced amino acid sequences of the 4B2 V_L and V_H regions are shown in Figure 4. V_L possesses the highest similarity to the $V_\kappa 1$ family group [19] and is joined in frame to $J_\kappa 2$. The V_H

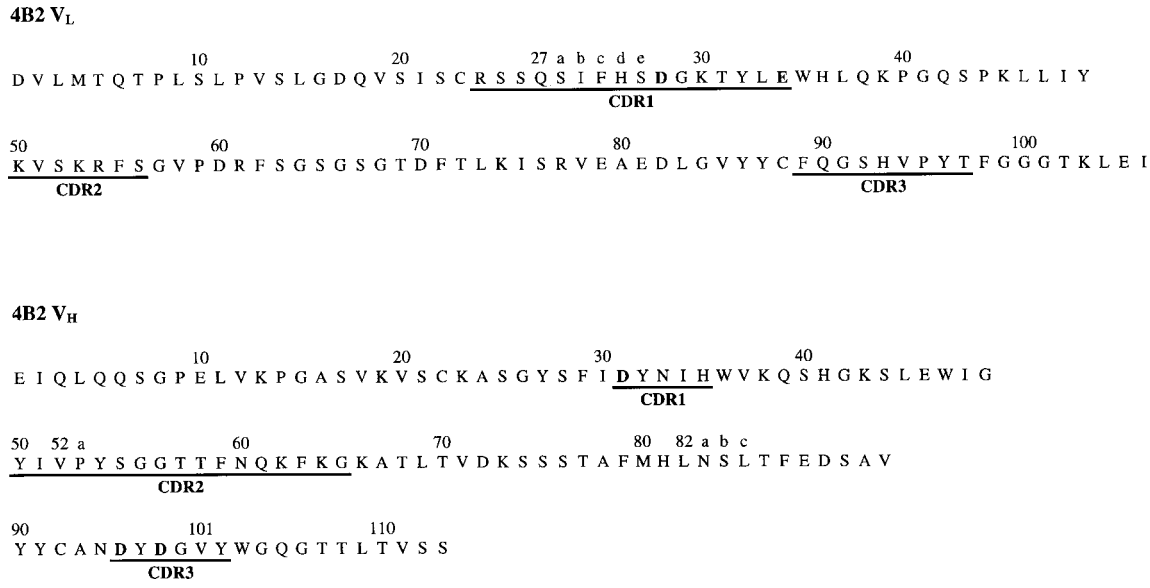


Figure 4 Amino acid sequences (single-letter codes) of 4B2 VL and VH regions

The CDRs are underlined and the five dicarboxylic amino acid residues in the CDRs are shown in bold.

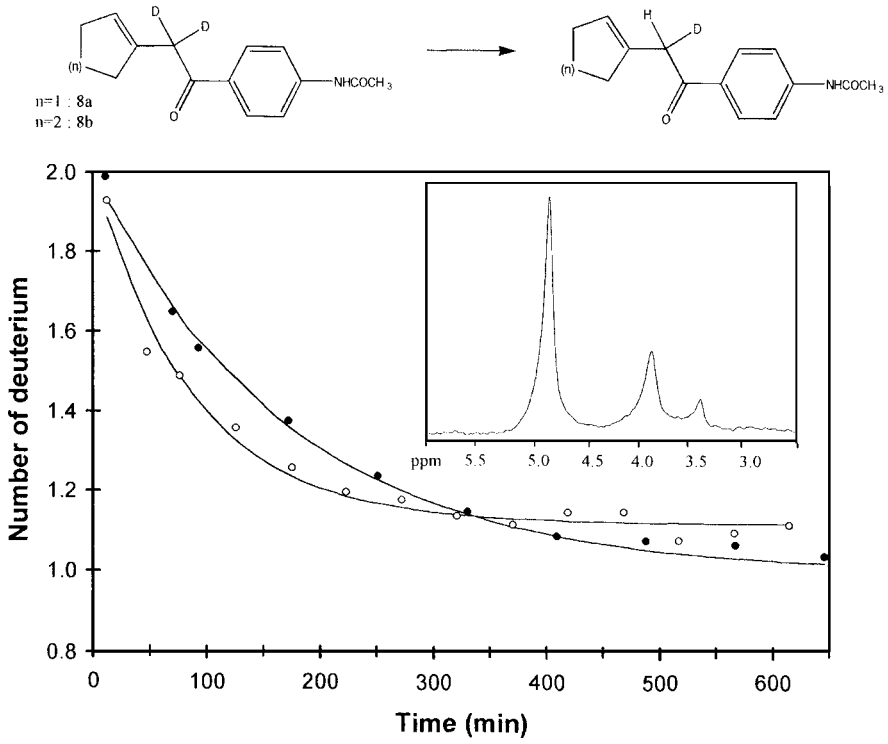


Figure 5 Kinetics of the substrate deuterium exchange

Inset: A 61.42 MHz deuterium spectrum of substrate **8a** (0.5 mM) in 50 mM acetate buffer (pH 4.5)/10% (v/v) methanol before the addition of antibody. The α-deuterium resonance is at 3.7 p.p.m.; the resonances at 3.2 and 4.7 p.p.m. arise from methanol and HO^2H respectively. Main panel: rate of 4B2-catalysed α-deuterium exchange of substrate **8a** (●) and **8b** (○) with solvent proton. Each time point represents the fraction of α-deuterium remaining, calculated by setting the initial intensity of the signal at 3.7 p.p.m. equal to two deuterium atoms in both experiments. The subsequent measurements were divided by this initial intensity to give the fraction of deuterium remaining. The spectra were acquired in 16 min on a sample containing 50 mM acetate buffer, pH 4.5, 200 μl of methanol, 0.5 mM deuterated substrates and 10 μM antibody 4B2 in a final volume of 2 ml.

sequence is closest to the V_H1 (J558) family. It is interesting to see that five acidic residues (Glu and Asp) are present in the hypervariable loops. Three of them (Asp-28^L, Asp-31^H and Asp-95^H) are not present in most of the germline genes and therefore probably result from somatic mutations. It is therefore likely that some of these residues are involved in the catalytic activity of the antibody.

α-Proton exchange and isotope effect

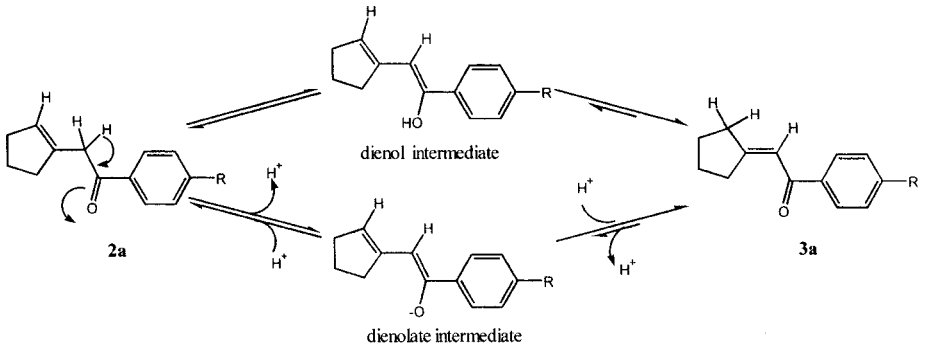
Most of the enzymes that catalyse the allylic isomerization of β,γ-unsaturated ketones involve a dienol or dienolate intermediate that results from the abstraction of a proton α to the carbonyl carbon. In an attempt to understand the mechanism of the allylic isomerization by 4B2, the kinetics of the exchange of the α-proton were studied with deuterated substrates **8** (Figure 5) and ^{2}H-NMR.

Monitoring of the decrease in deuterium signal intensity at 3.7 p.p.m. as a function of time allowed a first-order rate constant for uncatalysed and catalysed reactions to be obtained. In the absence of enzyme, the rate was 2×10^{-4} min^{-1} in acetate buffer at pH 4.5 for both **8a** and **8b**. For the antibody-catalysed process the rates were 5×10^{-3} and 1.1×10^{-2} min^{-1} with substrates **8a** and **8b** respectively (with the use of 10 μM antibody). Hence the antibody 4B2 accelerates the exchange of the α-proton significantly. Interestingly, as shown in Figure 5, the antibody-catalysed exchange levelled off after the exchange of only one proton with both substrates, suggesting a stereospecific control of the reaction. Complete disappearance of the signal was observed in a control experiment using catalysis with acetic acid. In addition, no product with deuterium at the γ-position was obtained in the presence of 4B2 even after 30% conversion (monitored by HPLC) of substrate **8a** into product **9a**.

A substrate kinetic effect of deuterium of 2.2 for allylic isomerization was measured by a comparison of the k_{cat}/K_m determined with protonated substrate **2a** and deuterated substrate **8a**.

DISCUSSION

In the present study we have characterized five monoclonal antibodies that were generated against the cationic amidinium hapten **1a** and we have selected two antibodies for their ability to catalyse the allylic isomerization of the β,γ-unsaturated ketone **2a**. Antibody 4B2 was the best catalyst, exhibiting a significant rate enhancement ($k_{cat}/k_{uncat} = 1.5 \times 10^3$) and multiple turnovers. However, antibody 4B2 was unable to catalyse the allylic isomerization of **2b**. Attempts to isomerize **2b** to **3b** in acidic or basic media without antibodies were also unsuccessful. It seems that this substrate was unreactive because of the thermodynamic equilibrium of the isomerization reaction, which favours **2b**. The six-membered ring seems to stabilize the endocyclic rather than the exocyclic double bond. With the five-membered ring, as in substrate **2a**, the thermodynamic equilibrium of the rearrangement is largely in favour of **3a** (more than 90%) as measured after a long reaction time.



Scheme 3 Mechanism of the allylic isomerization of 2a

In enzyme-catalysed allylic isomerization, active-site residues such as aspartate or glutamate can act either as general bases to abstract the α-proton of a β,γ-unsaturated ketone [3,20] or as acid catalysts to protonate the oxo oxygen of the substrate. In Δ^5-3-oxosteroid isomerase (OSI) from *Pseudomonas testosteroni*, which can be considered as a catalytically 'perfect enzyme', two carboxylic acids, Asp-38 and Asp-99, and an additional acid group, Tyr-14, act in a concerted manner to isomerize the β,γ-unsaturated ketone to enone by stabilizing the dienolate intermediate [21]. In our 'bait and switch' approach to induce a desired catalytic residue, presumably a carboxylic acid, inside the binding site of an antibody, we could expect this group in the right position to act either as a general base or a general acid catalyst.

The mechanism for allylic isomerization requires that a substrate proton be lost from the α position and a solvent proton be incorporated at the γ position. Greater insight into the mechanism of the antibody-catalysed reaction was gained by showing that 4B2 also accelerates the α-proton exchange, even on the substrate **2b**, which does not evolve to the conjugated product **3b**. An interesting feature of this proton exchange was illustrated by following deuterium exchange quantitatively by NMR. Although both α-protons of **8a** and **8b** were exchanged in the time course of the reaction without 4B2, only one of them was exchanged within the antibody-binding site, suggesting that the deuterium abstraction and the subsequent re-protonation occur on the same face of the dienol intermediate of both substrates. This demonstrates that the antibody ensures a stereoselective exchange, mimicking that observed with most known enzymes catalysing enol formation [2,22]. Whether the stereoselectivity of the α-proton exchange is induced by a preferential channelling of the proton in the binding site or by a residue acting as a base remains unclear. The second mechanism, in which the α-proton abstraction relies on the presence of a deprotonated residue in the binding site, could account for the decrease in the k_{cat}/K_m below pH 4.5.

Kinetic studies by NMR of the antibody-catalysed allylic isomerization of the deuterated substrate **8a** revealed that the α-proton exchange is faster than the formation of the conjugated product. In addition, the proton transfer from the α to the γ position is not direct but instead involves a proton exchange with the solvent because no product with deuterium at the γ position was obtained from substrate **8a**. Thus, in the catalysed reaction, the antibody efficiently accelerates the rate of formation of the dienol or dienolate intermediate (Scheme 3).

This assumption is also supported by the substrate primary isotope effect (2.2) on the kinetic constant k_{cat}/K_m, which is far from the maximum possible value (8–10 for a symmetrical transition state) [23]. This finding is not surprising considering the energy barrier associated with the abstraction of a proton adjacent to a ketone, for which the pK_a is expected to be approx. 12 [24]. As a result, the transition state for the transfer of the α-proton is asymmetrical and late on the reaction co-ordinate for the formation of the dienol/dienolate intermediate [25]. Considering the two possible forms for the intermediate, dienol or dienolate, we can reasonably assume that the dienolate would be highly destabilized in a binding site that was selected to bind a positive hapten. The involvement of the more favoured neutral dienol implies that a concomitant protonation at the carbonyl oxygen involving either a solvent hydronium ion or a protonated antibody side chain should occur. Both protonation mechanisms yielding the neutral dienol intermediate could account for the pH dependence of k_{cat}/K_m above pH 4.5.

No definite conclusion can be drawn from the kinetic results on the role, number and nature of the antibody residues involved in the reaction mechanism. Nevertheless, the results obtained strongly suggest that at least one carboxylic acid, which may be responsible for the antibody activity, has been induced by charge complementarity in the antibody-binding site. First, the antibody is inactivated by selective chemical modification of carboxy groups. Secondly, a positive charge in the hapten is essential for strong antibody binding (Table 1), suggesting the presence of a negatively charged residue in the binding pocket. Thirdly, the catalytic activity depends on at least one ionizable protein side chain with a pK_a of approx. 4.5. Finally, the amino acid sequences of the 4B2 V_L and V_H chains reveal the presence of several Asp and Glu residues in the complementarity-determining region (CDR) loops. To assess the eventual presence of these residues within the binding site, a computational model of the Fab was generated by homology modelling with antibody framework regions and a crystallographic database of antibodies sharing canonical loop structures with Fab 4B2 [26]. The model (results not shown) suggests that CDR loops form a deep binding pocket containing two carboxylic acid residues, Glu-34^L and Asp-95^H.

Several mechanisms in which a carboxylate could have a role can be envisaged: (1) direct or indirect proton transfer from the protonated form to the carbonyl oxygen to favour the formation of the dienol intermediate, (2) direct or indirect proton abstraction at the α-carbon by the deprotonated form, and (3) proton transfer at the γ position. The pH-dependent kinetic results are ambiguous and the bell-shaped feature of the pH dependence of k_{cat}/K_m can be explained either by the involvement of a second group with a similar pK_a in the active site acting in a concerted way similar to the mechanism depicted for OSI-catalysed allylic rearrangement, or by the same group's acting as a general base at one point in the mechanism and as a general

acid in another. Nevertheless, it is unlikely that a concerted mechanism is involved in the antibody-catalysed allylic isomerization of **2a**. Site-directed mutagenesis studies on OSI have shown that each active-site residue contributes 10^4–10^5-fold to catalysis on an individual basis [27]. For example, mutation of the acid group Asp-99 resulted in a decrease in k_{cat} to $1/10^{3.7}$ [28]. The acceleration factor observed in 4B2 ($10^{3.2}$) is of the same order of magnitude as that observed for the contribution of an individual residue in the mutated OSI. We would therefore expect a greater acceleration factor for a concerted mechanism involving two acid–base residues. Furthermore, this concerted mechanism, depending on the presence in the right position in the binding site of two acid–base residues, is hardly compatible with the structural information available in the hapten used to generate the antibody 4B2.

To allow direct comparison with other reported catalytic antibodies that catalyse similar reactions with different substrates, we can calculate the catalytic proficiency, $k_{cat}/(K_m k_{uncat})$, as defined by Wolfenden [29]. Antibody 4B2 has a proficiency of 1.2×10^6 M^{-1}, which is higher than that reported for an OSI-like catalytic antibody with a similar mechanism [30] and is of the same order of magnitude as that reported recently for an antibody with a completely different mechanism [7].

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