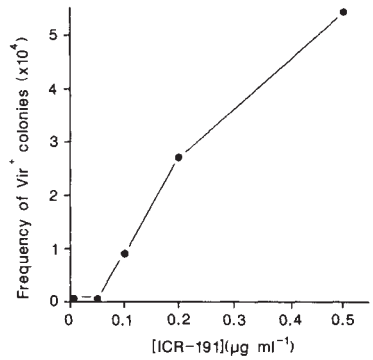

FIG. 4 Reversion of the avirulent phase phenotype of BP326 by treatment with the frameshift mutagen ICR-191. The procedure was essentially as previously described[24]. A culture of BP326 grown in Stainer–Scholte broth was diluted by 10^{-4}. Aliquots (0.1 ml) were added to 3 ml of the same medium containing different concentrations of ICR-191 and incubated in the dark at 35 °C, with shaking, for 5–7 days. Cultures showing visible turbidity were diluted and plated on Bordet–Gengou agar to obtain 10^3–10^4 colonies per plate. After growth for 5 days, plates were examined for haemolytic colonies.

Similar results were obtained for BP369-3 and BP371-1. These data support the hypothesis that phase variation in this series of strains occurs via a frameshift mutation in a *vir* gene.

An important question arising from our results is whether this frameshift mutation represents a true 'programmed' genetic event reflecting an evolved site-specific mechanism. The exact nucleotide sequence requirements at the site of the mutation are not yet known. It is worth noting that in *B. pertussis*, an organism with a relatively high (G+C) content, a run of six G or six C residues is, as expected, not uncommon. The sequence around the site of the frameshift mutation is striking however, in its abundance of GC pairs and in its near symmetry. It will be interesting to test whether the groups of C residues outside the core six C residues affect the frequency of phase variation. This frequency varies with the strain used and is relatively high in the strains which have descended from Tohama III (about 10^{-3} for the vir^+ to vir^- transition[4]). In contrast, the frequency at which BP338 undergoes this transition is about 10^{-6}. In an attempt to determine the basis for this difference, we are examining the reversion of other frameshift mutations in these two backgrounds to determine whether this is a general effect on frameshift mutation/reversion. The relevance of our results, derived from work on laboratory strains, to bacteria in their natural environment is an important issue. It is unclear what the role of phase variation is in the natural biology of this highly adapted pathogen. This phenomenon has been observed after passage of *B. pertussis* isolates *in vitro* in most cases. The isolation of Vir^-, or phase III, organisms from patients late in infection has been reported however[6]. We are currently examining vir^+/vir^- pairs derived from other isolates to determine if the frameshifting we have described represents a common mechanism for phase variation in *Bordetella* species. □

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A new strategy for the generation of catalytic antibodies

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THE high binding affinity and specificity of antibodies for a wide range of ligands has recently been exploited in the generation of catalysts for acyl-transfer reactions[1–7], carbon–carbon bond forming[8,9] and carbon–carbon bond cleaving reactions[10]. In addition, a number of strategies are emerging for the generation of catalytic antibodies including transition state stabilization[1–6], catalysis by approximation[7,8], and the introduction of catalytic groups or cofactors into antibody combining sites[10–13]. An important goal in the design of catalytic antibodies is the development of general rules relating hapten structure to the corresponding catalytic groups in the antibody combining site. We report here that electrostatic interactions between a hapten and the complementary antibody[14,15] can be exploited to generate catalytic amino-acid side chains in an antibody-combining site. The antibody-catalysed reaction, a β-elimination reaction, exhibits saturation kinetics, substrate specificity, competitive inhibition by hapten, and specific inactivation by a reagent that modifies carboxylate residues.

To expand the scope of antibody catalysis we targeted reactions involving proton abstraction from a carbon centre. This class of reactions includes elimination and isomerization reactions and aldol and Claisen condensations[16–18]. Proton abstraction in enzymes is often performed by carboxylate side chains of glutamate or aspartate amino acids. These residues typically display higher than normal pKa values (6.5–8.2) in the hydrophobic active sites of enzymes[19]. Aspartate and glutamate residues have been generated in the combining sites of antibodies by taking advantage of charge complementarity between haptens and the corresponding antibodies[14,15]. Consequently, we reasoned that an antibody-combining site could be generated that contains a carboxylate group within bonding distance of an abstractable substrate proton by using the appropriate positively charged hapten. Importantly, the position of a charged group such as alkyl ammonium ion in the hapten should reflect that of the target C–H group in the substrate.

To test these notions, monoclonal antibodies generated to hapten H1 (Fig. 1) were assayed for their ability to catalyse H–F elimination from the fluorinated substrate S2 (Fig. 1). The hapten contains a positively charged ammonium ion replacing the α-CH_2 group of the substrate (the tertiary nitrogen obviates competing lactam formation during the coupling of H1 to pro-

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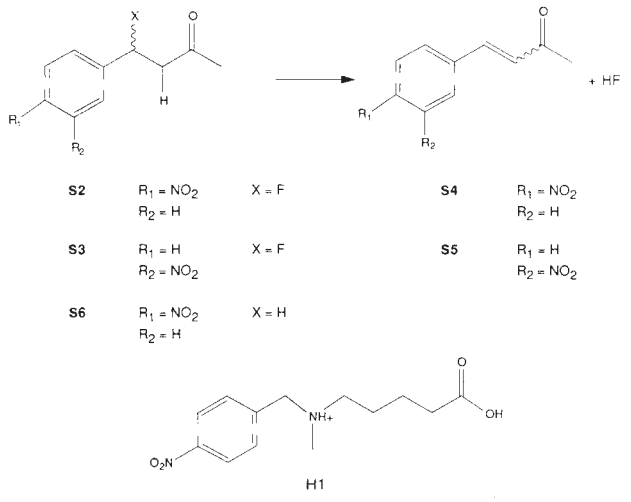

FIG. 1 Monoclonal antibodies elicited against the (KLH) conjugate of H1 catalyse the conversion of S2 or S4. Synthesis of H1 was accomplished by treatment of *p*-nitrobenzaldehyde and ∂-aminovaleric acid with 0.7 equivalents (eq.) of sodium cyanoborohydride ($NaCNBH_3$) in methanol at 25 °C to afford N(*p*-nitrobenzyl)-∂-aminovaleric acid which was purified by recrystallization from H_2O/acetone. This compound was then treated with 37% aqueous formaldehyde and 2 eq. $NaCNBH_3$ in H_2O at 25 °C to afford H1 which was purified by silica-gel chromatography. Compound H1 was coupled through amide linkages to KLH by treatment with the *N*-hydroxysuccinimide ester of H1 to afford the KLH conjugate with an epitope density of 18 per carrier monomer as determined by the method of Habeeb[24]; protein concentration was determined by the method of Lowry[25]. Synthesis of S2 was carried out by treatment of *p*-nitrobenzaldehyde with a catalytic amount of 1% aqueous NaOH in acetone at 0 °C to give the intermediate (*R, S*) 4-hydroxy-4-*p*-nitrophenylbutan-2-one which after purification by silica-gel chromatography, treatment with diethylaminosulphur trifluoride in CH_2Cl_2 at −78 °C and aqueous extraction afforded racemic S2. Treatment of the intermediate alcohol with 1.1 eq of H_2SO_4 (1N) in acetone at 25 °C and purification by silica gel chromatography afforded the *trans* isomer of S4. Compounds S3 and S5 were synthesized analogously to S2 and S4 starting with 3-nitrobenzaldehyde. Substrate analogue S6 was obtained by nitration of benzylacetone with $Cu(NO_3)_2 \cdot 3H_2O$ in neat trifluoroacetic anhydride at 39 °C and purified by silica gel chromatography. All compounds were characterized by NMR, mass spectrometry, infrared and ultraviolet spectroscopy and elemental analysis.

tein carriers for immunization). The fact that the hapten and substrate share a common recognition element, the *p*-nitrophenyl group, ensured that the antibody-binding affinity for the substrate would be reasonable. Moreover, replacement of hapten by substrate in the antibody-combining site should lead to an increase in the basicity of the catalytic carboxylate since a stabilizing salt bridge interaction is lost.

Antibodies were generated against hapten H1, which at physiological pH exists as a positively charged alkyl ammonium ion. The hapten was conjugated to the carrier proteins bovine serum albumin (BSA) and keyhole limpet haemocyanin (KLH) using the activated *N*-hydroxysuccinimide ester of H1 (ref. 20). Six monoclonal antibodies specific for H1 were produced *in vivo* and isolated by affinity chromatography on protein A-coupled Sepharose 4B as decribed previously[3]. Antibody purity was determined by 10–15% SDS polyacrylamide gel electrophoresis with Coomasie blue staining[21]. Of these six IgGs isolated, four accelerated the β-elimination reaction of S2 to S4 (Fig. 1) and were completely inhibitable by the addition of free

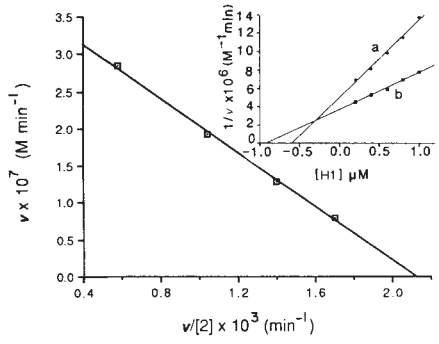

FIG. 2 Eadie–Hofstee plot for the β-elimination conversion of S2 to S4, catalysed by 43D4-3D3. Velocities were determined spectrophotometrically by measuring the initial absorbance increase at 330 nm: S2, λ_{max} = 282 nm (3.99); S4 λ_{max} = 312 nm (4.43); ΔE(S4–S2) (330 nm) = 16,820 cm^{-1} M^{-1}. The concentration of 43D4-3D3 was 2.00 μM as determined by absorbance at 280 nm using E(1 cm, 0.1%) = 1.37 and M_r = 150,000 for IgG. 43D4-3D3 was preincubated at 37 °C in 10 mM bis [2-hydroxyethyl]imino-tris-[hydroxymethyl]-methane (bis-Tris), 100 mM NaCl, pH 6.0. The reaction was initiated by adding 10 μl of a stock solution of S2 in CH_3CN to give a final CH_3CN concentration of 2%. The uncatalysed rate was measured under the same conditions, affording a $k_{uncat} = 1.30 \times 10^{-4}$ min^{-1}. Inset: Dixon plot[26] of inhibition of 43D4-3D3 by H1. Antibody concentration, 2.01 μM. Data was obtained at two concentrations of S2: *a*, 208 μM; *b*, 514 μM. Buffer conditions as above.

hapten H1. Such hapten-inhibitable rate acceleration suggests that the catalysis is due to binding of the substrate in the antibody-combining site (non-specific antibodies did not catalyse the elimination reaction). The fact that 66% of these antibodies were catalytic demonstrates the generality of this strategy for producing catalytic antibodies. One of the four antibodies (43D4-3D3) was characterized further.

The reaction catalysed by 43D4-3D3 obeys classical Michaelis–Menten kinetics: a k_{cat} of 0.193 min^{-1} and a K_M of 182 μM for substrate S2 were measured at pH 6.0 (Fig. 2). Both the *cis* and *trans* isomers of enone S4 are formed in the antibody-catalysed conversion of racemic S2, as determined by high pressure liquid chromatography (HPLC) analysis. The catalysed reaction is also competitively inhibited by hapten H1 (K_i = 290 nM) (Fig. 2 inset) demonstrating that the catalytic activity is associated with binding in the antibody-combining site. An unreactive substrate analogue *p*-nitrobenzylacetone (S6) is also a competitive inhibitor of the catalysed reaction (K_i = 280 μM). As expected, the antibody-catalysed reaction is substrate-specific, in accordance with the characteristic specificity of antibodies for their ligands[15]. The K_M and k_{cat} values for 4-fluoro-4-*m*-nitrophenylbutan-2-one (S3) are 571 μM and 0.079 min^{-1}, respectively ($k_{cat}/K_M = 1.38 \times 10^2$ M^{-1} min^{-1}) at pH 6.50, compared with a k_{cat} of 0.304 min^{-1} and K_M of 214 μM ($k_{cat}/K_M = 1.42 \times 10^3$ M^{-1} min^{-1}) for S2 at this pH.

The identity of the catalytic amino-acid side chain in the antibody combining site was probed by two methods. The pH dependence of k_{cat} shows the classical profile for catalysis attributable to a single titratable group (Fig. 3). The pK_a for the active site residue was determined to be 6.2. This value is very close to the pK_a for the active-site Glu 135 in carboxypeptidase A (pK_a = 6.5), which is known to be responsible for the catalysis of a similar β-elimination reaction (not the physiologically relevant reaction for this enzyme)[22]. The maximum k_{cat} value obtainable by the antibody results when the residue responsible

TABLE 1 Effect of DAA treatment of 43D4-3D3 on catalysis of S2 to S4

Sample	v (M^{-1} min^{-1})*	% activity remaining
Untreated	3.41×10^{-7}	100
DAA treated†	7.97×10^{-8}	23.4
DAA treated† + 2 mM S6	2.79×10^{-7}	82.0

* 1.5 μM 43D4-3D3, 540 μM S2, pH 6.0, 10 mM Bis-Tris, 100 mM NaCl.
† Following the procedure described in ref. 14.

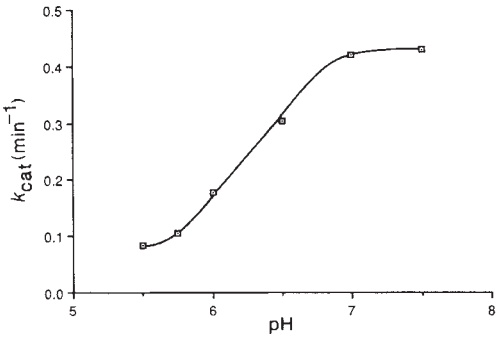

FIG. 3 The k_{cat} versus pH profile for conversion of S2 to S4 catalysed by 43D3-3D3. At each pH the k_{cat} value was obtained from a Lineweaver–Burke plot. The buffer used was 10 mM bis-Tris, in the presence of 100 mM NaCl.

for base catalysis is fully deprotonated. The value of k_{cat}(max), determined from the pH profile by plotting k_{cat} versus $k_{cat}[H^+]$, is 0.458 min^{-1} (ref. 23).

Chemical modification confirmed that an aspartate or glutamate residue is involved in the elimination reaction. Specific chemical modification of carboxylate residues was effected by treatment of 43D4-3D3 with diazoacetamide (DAA). DAA has been shown to react almost exclusively with carboxylate groups to form glycolamide ester linkages[14]. The antibody 43D4-3D3 retains only 23% of its catalytic activity after treatment with DAA. However, in the presence of competitive inhibitor S6 (2.0 mM), under otherwise identical conditions, 82% of its catalytic activity is retained. These results indicate that a carboxylate group could be located in or very near the binding site of 43D4-3D3.

The ability to generate antibody binding sites with specific catalytic groups or microenvironments provides a method for dissecting the contribution of various factors involved in enzymatic catalysis, such as transition-state stabilization, proximity effects, general acid-base, nucleophilic catalysis and ground state strain. Hapten H1 bears very little resemblance to the transition state for the conversion of S2 to S4; in fact it has the opposite charge of an E2 elimination transition state. This observation, together with the pH profile (Fig. 3), suggests that the observed catalysis is largely due to the presence of a catalytic base. The rate constant for acetate-catalysed conversion of S2 to S4, k_{OAc^-}, is 1.13×10^{-2} M^{-1} min^{-1} at 37 °C in 100 mM NaCl. The k_{cat}/K_M value of the antibody-catalysed reaction at pH 6 is 9.95×10^{2} M^{-1} min^{-1}. The rate enhancement obtained by introduction of a carboxylate residue in a hydrophobic binding site compared with acetate free in solution is therefore 8.80×10^{4} at the pH. This value is similar to that of 6×10^{4} reported for an esterolytic antibody that ccontains an active site nucleophilic thiol[12]. The large rate enhancement due solely to base catalysis by a carboxyliate residue suggests that an antibody which combines transition state stabilization and base catalysis might attain rate accelerations similar to those of enzymes. Nuclear magnetic resonance experiments and resolution of the *R*, *S* stereoisomers of S2 are currently in progress to determine the stereochemistry of proton abstraction (pro*R*, pro*S*) and elimination (*syn*-coplanar, *anti*-coplanar).

This work represents the extension of antibody catalysis to a new class of reactions. Moreover, it should be possible to exploit hapten complementarity to generate antibodies which contain other catalytic groups and catalyse a variety of reactions of interest, including aldol and Claisen condensations, as well as amide and sugar hydrolyses.

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Three amino acids of the oestrogen receptor are essential to its ability to distinguish an oestrogen from a glucocorticoid-responsive element

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STEROID hormone receptors activate specific gene transcription by binding as hormone-receptor complexes to DNA enhancer elements termed hormone responsive elements (refs 1, 2 and references therein). A highly conserved 66-amino-acid region of the oestrogen and glucocorticoid receptors which corresponds to part of the receptor DNA-binding domain (region C) determines the specificity of target gene recognition[3–5]. This region contains two subregions (CI and CII), encoded in two separate exons (refs 1, 7 and references therein), that are analogous to the 'zinc fingers' of the transcription factor TFIIIA (reviewed in ref. 6). The N-terminal CI finger determines the recognition specificity of the hormone responsive element[8]. A chimaeric oestrogen receptor, in which the CI finger is replaced with the corresponding glucocorticoid receptor CI finger region, activates transcription from a reporter gene containing a glucocorticoid-responsive element, but not from a reporter gene containing an oestrogen-responsive element[8]. We report here that three amino acids located at the C-terminal side of the oestrogen receptor CI finger play a key part in this specificity.

In the reporter gene *vit-tk-CAT* (ref. 9), the 5′-flanking region of the *Xenopus* vitellogenin A2 gene (*vit*), which contains a consensus palindromic oestrogen-responsive element (ERE), is inserted upstream of the herpes simplex virus promoter for thymidine kinase (*tk*). In the mouse mammary tumour virus (MMTV) reporter gene MMTV-*CAT*, the glucocorticoid-

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