Antibody catalyzed modification of amino acids. Efficient hydrolysis of tyrosine benzoate†

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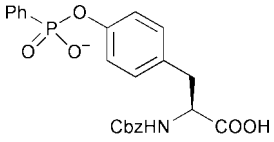
Esterase antibody 522c2, the first example of a catalytic antibody specifically programmed to control the reactivity of functional groups on the side chain of tyrosine, accelerates the hydrolysis of benzoate esters of L-tyrosine and tyrosine-containing dipeptides by a factor of 10^4 and is moderately active against other benzoate esters.

The control of reactivity of functional groups on the side chains of amino acids and peptides is a potentially attractive field for applications of catalytic antibodies.[1] The selective modification of amino acid side chains in peptides and proteins is important for many areas of chemistry, from organic synthesis to the irreversible inhibition of enzymes and the improvement of pharmacokinetic properties of peptide drugs.[2] However, the high level of control over chemo- and regioselectivity which is required for these transformations is not easily obtained by chemical or enzymatic approaches.[3]

While a number of antibody catalyzed reactions taking place on amino acid substrates have been described, there are no examples to date dealing specifically with the reactivity of amino acid side-chains. In this communication we describe the activity of esterase antibody 522C2, the first catalytic antibody specifically designed to hydrolyze an ester group on the tyrosine phenolic side chain. As benzoate is a common modifier and protecting group in amino acid and peptide chemistry, we chose the hydrolysis of tyrosine benzoate **1** as the target reaction (Scheme 1).

Hapten **2**, a classical phosphonate transition state analog, was synthesized by a conventional approach,[4] *via* the corresponding monoester monochloride.† The carboxylate group of tyrosine



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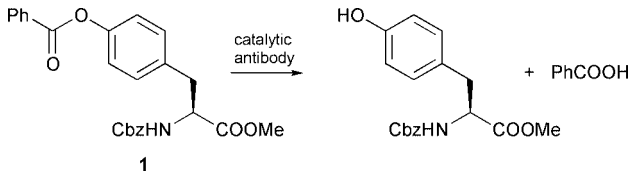
was used to conjugate directly the phosphonate to cationised bovine serum albumin (cBSA)†. We reasoned that holding the hapten in close contact with the carrier protein surface would result in a relatively 'open' antigen binding site, able to recognize and hydrolize the substrate also when tyrosine is part of a peptide chain. For this reason the use of a linker between the hapten and the carrier was avoided.

The cBSA conjugate was used to immunize three Balb/c mice following a standard protocol.[5] Hybridomas obtained from the



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Scheme 1

fusion of spleen cells were selected on the basis of the affinity of the antibodies for an ovalbumin (OVA) conjugate of **2** and cloned. Only three good binders for the hapten **2** were obtained, but two of the selected antibodies (517A41 and 522C2) exhibited catalytic activity for the hydrolysis of benzoate **1** after purification by protein G and ion exchange chromatography.† Antibody 517A41 was only a weak catalyst (k_{cat}/k_0 = 148), while 522C2 accelerated the hydrolysis of **1** by a factor of over 10^4 and was selected for further studies (Table 1). After the preliminary kinetic assay, carried out on the antibody isolated from hybridoma supernatants, 522C2 was subcloned and larger quantities of the antibody were obtained from ascitic fluids. A fully functional Fab fragment was also obtained by papain digestion and purification by gel exclusion and ion exchange chromatography. The observed catalytic activity was always reproducibile and did not depend on the antibody's preparation.

The kinetic parameters for the catalyzed hydrolysis of ester **1** were calculated at pH 8 in TRIS buffer at 25 °C (k_{cat} = 0.063), and at pH 7.5 in PBS at 30 °C (k_{cat} = 0.017), showing in both cases a rate enhancement factor in excess of 10^4 (Table 1).† This allows the observation of a net enhancement of more than 150-fold in the initial rates of hydrolysis under typical experimental conditions (5 μM antibody, 50 μM substrate **1**), in spite of the rather high value of K_M (370–500 μM), which does not allow the maximum rate to be reached within the solubility limit of this substrate.

The antibody is inhibited by an equimolar amount of the phosphonate **2**. The apparent dissociation constant of the antibody–hapten complex obtained by a competitive ELISA assay[6] ($K_{d,app}$ = 75 nM) is in reasonable agreement with the upper limit for the inhibition constant ($K_i \leq$ 100 nM) estimated from kinetic measurements. Inhibition by the hapten demonstrates that the catalyzed process takes place in the antibody combining site and is not due to interactions between the substrate and the protein surface. Nonspecific interactions of this type can indeed be observed when the hydrolysis of **1** is carried out in the presence of BSA or unrelated mouse IgGs, but result in a modest acceleration by a factor of less than 1.5 in the initial rates (at 5 μM protein and 50 μM substrate **1**).

For an antibody accelerating a reaction by simple transition state complementarity, it has been derived that $K_M/K_i = k_{cat}/k_0$.[7] In the case of 522C2, the values of k_{cat}/k_0 (11 000) and K_M/

† Electronic supplementary information (ESI) available: the synthesis and characterization of compound **2**, preparation of the immunogenic conjugate, preparation and purification of monoclonal antibodies, experimental details for kinetic studies and Lineweaver-Burk plots. See http://www.rsc.org/suppdata/cc/b1/b100450f/

Table 1 Kinetic parameters for the 522C2 catalyzed hydrolysis of tyrosine benzoate and other esters

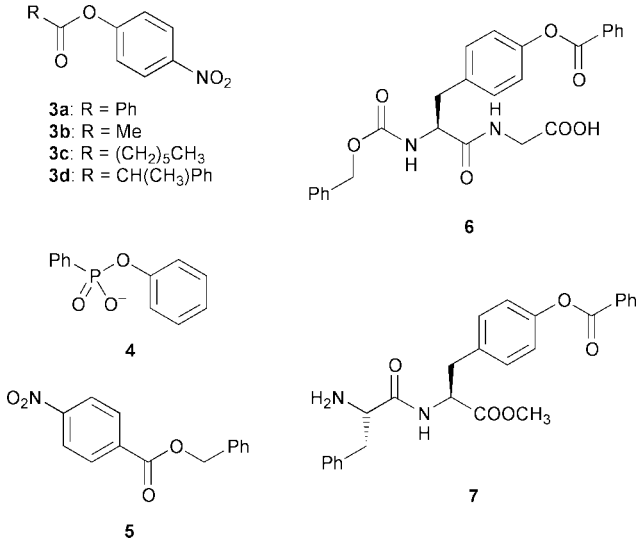
Substrate	k_0/s^{-1}	k_{cat}/s^{-1}	K_M (μmol dm^{-3})	k_{cat}/k_0	k_{cat}/K_M (dm^3 s^{-1} mol^{-1})	$k_{cat}/K_M k_0$ (dm^3 mol^{-1})
(*S*)-**1**[a]	5.06×10^{-6}	0.063	500	12 400	126	2.5×10^7
(*S*)-**1**[b]	1.53×10^{-6}	0.017	370	11 100	45.9	3.0×10^7
(*S*)-**1**[b,c]	1.53×10^{-6}	0.020	410	13 100	48.8	3.2×10^7
3a[b]	8.16×10^{-6}				44.9	5.5×10^6
3b[b]	1.04×10^{-4}				35.4	3.4×10^5
3c[b]	4.49×10^{-5}				8.5	1.9×10^5
3d[b]	1.94×10^{-4}				—	—
5[b]	2.90×10^{-6}				0.39	1.4×10^6
6[d]	5.70×10^{-8}	5.5×10^{-4}	460	9 700	1.2	2.1×10^7
7[d]	8.21×10^{-8}	7.5×10^{-4}	650	9 100	1.2	1.4×10^7

[a] TRIS 10 mM, pH 8, dioxane 10%, 25 °C. [b] PBS, pH 7.5, 10% dioxane, 30 °C. [c] Fab fragment. [d] PBS, pH 7.5, 30% DMSO, 30 °C.

$K_{d,app}$ (4900) indicate that selective recognition of the transition state significantly contributes to the catalytic activity. Thus 522C2 behaves similarly to other ester hydrolyzing antibodies for which oxyanion stabilization has been recognized as the main source of catalytic activity.[8]

Antibody 522C2 is highly specific for the *S*-enantiomer of tyrosine benzoate, mirroring the configuration of the hapten, and no acceleration over background is observed in the hydrolysis of the corresponding *R*-ester. While displaying such a high enantiospecificity, antibody 522C2 is able to hydrolyze a number of simplified esters **3** in which tyrosine is replaced by *p*-nitrophenol. Solubility, and the high values of K_M for these substrates, restrict the accessible substrate concentrations to a range in which [*S*] << K_M. Therefore, second order rate constants, corresponding to k_{cat}/K_M ratios assuming Michaelis–Menten behaviour, were obtained for this set of esters and the ratio $k_{cat}/K_M \cdot k_0$ (catalytic proficiency) was chosen to compare the efficiency of the antibody on these substrates (Table 1).[9]

Data in Table 1 show that one order of magnitude in the catalytic proficiency is lost on going from *N*-Cbz-tyrosine benzoate **1** to *p*-nitrophenyl benzoate **3a**. This decrease in the catalytic activity of the antibody is paralleled by its affinity for the corresponding phosphonates. In fact, while the tyrosine phosphonate **2**, as we have seen, is a strong binder, the simple phenyl phenylphosphonate **4** does not inhibit 522C2. This



indicates that in the substrate **1** and hapten **2** the region of the molecule corresponding to the protected α-amino acid group plays a significant role in recognition by the antibody.

Replacing the benzoyl group by aliphatic residues, as in the hydrolysis of esters **3b** and **3c**, results in a further decrease of catalytic proficiency by one order of magnitude (Table 1). The catalytic activity is completely lost when branching is introduced in the ester **3d**. Surprisingly however, antibody 522C2 shows good efficiency in the hydrolysis of benzyl *p*-nitrobenzoate **5**.

It is noteworthy that the substrate selectivity exhibited by 522C2 is reversed with respect to that displayed by a hydrolytic enzyme such as α-chymotrypsin. This protease is known to hydrolyze esters with a broad specificity. However, under similar conditions, the acceleration in the hydrolysis of *p*-nitrophenyl ester **3a** is six times higher than the acceleration in the hydrolysis of tyrosine ester **1**. Another major difference between α-chymotrypsin and 522C2 is represented by the pre-steady state burst which is observed with the enzyme but not with 522C2, in agreement with the mechanism proposed for the antibody.

Finally, the activity of 522C2 was also tested on the dipeptides **6** and **7** (Table 1). 522C2 retains most of its catalytic activity on both the dipeptide benzoates even in the presence of 30% DMSO, which is necessary for solubility. Replacement in **7** of the *N*-Cbz protecting group with the somewhat isosteric residue of phenylalanine does not lead to a loss of activity.

In conclusion, 522C2 is an efficient and selective catalyst for the hydrolysis of (*S*)-tyrosine benzoate and retains its activity when the substrate is part of a simple dipeptide. This preliminary result is promising in view of a possible extension of this approach to the selective deprotection of tyrosine benzoate in more complex peptide structures, which would be a valuable application and is currently being investigated.

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Notes and references

1 D. Hilvert, *Annu. Rev. Biochem.*, 2000, **69**, 751; G. M. Blackburn and A. Garçon, in *Biotechnology*, ed. D. R. Kelly, Wiley-VCH, Weinheim, 2nd edn., vol. 8b, 2000, p. 403; J. D. Stevenson and N. R. Thomas, *Nat. Prod. Rep.*, 2000, **17**, 535; J. L. Reymond, *Top. Curr. Chem.*, 1999, **200**, 59; G. M. Blackburn, A. Datta, H. Denham and P. Wentworth Jr., *Adv. Phys. Org. Chem.*, 1998, **31**, 249.

2 S. P. Vyas, P. Venugopalan, A. Sood and N. Mysore, *Pharmazie*, 1997, **52**, 339; D. K. Clodfelter, A. H. Pekar, D. M. Rebhun, K. A. Destrampe, H. A. Havel, S. R. Myers and M. L. Brader, *Pharmacol. Res.*, 1998, **15**, 254; M. Baudys, D. Letourneur, F. Liu, D. Mix, J. Jozefonvicz and S. W. Kim, *Bioconjugate Chem.*, 1998, **9**, 176.

3 B. T. Miller, *Biochem. Biophys. Res. Commun.*, 1996, **218**, 377; R. D. Lundbald, *Techniques in Protein Modification*, CRC Press, Boca Raton, 1995; A. W. Yem, H. A. Zurcher-Neely, K. A. Richard, N. D. Staite, R. L. Heinrikson and M. R. Deibel, *J. Biol. Chem.*, 1989, **264**, 17691; L. Gardossi, D. Bianchi and A. Klibanov, *J. Am. Chem. Soc.*, 1991, **113**, 6328.

4 M. F. Hersman and L. F. Audrieth, *J. Org. Chem.*, 1958, **23**, 1889.

5 E. Harlow and D. Lane, *Antibodies, a Laboratory Manual*, Cold Spring Harbor Laboratory, 1988, p. 139.

6 B. Friguet, A. F. Chaffotte, L. Djavadi-Ohaniance and M. E. Goldberg, *J. Immunol. Methods*, 1985, **77**, 305.

7 J. D. Stewart and S. J. Benkovic, *Nature*, 1995, **375**, 388.

8 J. L. Buchbinder, R. C. Stephenson, T. S. Scanlan and R. J. Fletterick, *J. Mol. Biol.*, 1998, **282**, 1033; J. B. Charbonnier, B. Gigant, B. Golinelli-Pimpaneau and M. Knossow, *Biochimie*, 1997, **79**, 653; G. MacBeath and D. Hilvert, *Chemistry & Biology*, 1996, **3**, 433.

9 A. R. Radzicka and R. A. Wolfenden, *Science*, 1995, **267**, 90; C. L. Lo, P. Wentworth, K. W. Jung, J. Yoon, J. A. Ashley and K. D. Janda, *J. Am. Chem. Soc.*, 1997, **119**, 10 251.