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# Catalytic and binding poly-reactivities shared by two unrelated proteins: The potential role of promiscuity in enzyme evolution

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## Abstract

It is generally accepted that enzymes evolved via gene duplication of existing proteins. But duplicated genes can serve as a starting point for the evolution of a new function only if the protein they encode happens to exhibit some activity towards this new function. Although the importance of such catalytic promiscuity in enzyme evolution has been proposed, little is actually known regarding how common promiscuous catalytic activities are in proteins or their origins, magnitudes, and potential contribution to the survival of an organism. Here we describe a pattern of promiscuous activities in two completely unrelated proteins—serum albumins and a catalytic antibody (aldolase antibody 38C2). Despite considerable structural dissimilarities—in the shape of the cavities and the position of catalytic lysine residues—both active sites are able to catalyze the Kemp elimination, a model reaction for proton transfer from carbon. We also show that these different active sites can bind promiscuously an array of hydrophobic negatively charged ligands. We suggest that the basic active-site features of an apolar pocket and a lysine residue can act as a primitive active site allowing these promiscuous activities to take place. We also describe, by modelling product formation at different substrate concentrations, how promiscuous activities of this kind—inefficient and rudimentary as they are—can provide a considerable selective advantage and a starting point for the evolution of new functions.

**Keywords:** Promiscuous; promiscuity; substrate ambiguity; cross-reactivity; catalytic antibody; serum albumin; directed evolution; enzyme evolution; medium effects

Specificity is considered a hallmark of biological activity. But there exists evidence (most of which is sporadic in nature) suggesting that proteins can react with substrates for which they have been neither evolved nor designed. Although such promiscatalytic activities (be they binding or catalysis) are often discarded as undesirable side effects or experimental artifacts, they may in fact be of fundamental importance. In the course of evolution, promiscuity may provide a vital springboard from which new catalytic ac-

tivities can emerge out of existing folds and active sites. Indeed, as indicated by the relatively small number of enzyme superfamilies, evolution seems to have solved a diverse array of chemical problems with relatively few solutions (Babbitt and Gerlt 2000). However, the concept of promiscuity as a general, inherent property of protein active sites is at odds with the exquisite specificity that is at the heart of biological activity. Consequently, whereas the specific activity or even cross-reactivity of many thousands of proteins has been analyzed in much detail, promiscuous activities or poly-reactivities have been studied with only few proteins, and almost never in a systematic manner. There are even fewer cases where close structural resemblance has led to the identification of promiscuous activities

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(Nagahara et al. 1995; Palmer et al 1999), or where such activities were the result of directed evolution (Jurgens et al. 2000; Matsumura and Ellington 2001). Thus, to ascertain a conclusive role for promiscuity in enzyme evolution (Jensen 1976; O'Brien and Herschlag 1999), we would need to systematically determine how common promiscuous activities are and correlate their type, mechanistic origins, and magnitude with prototypic active-site features.

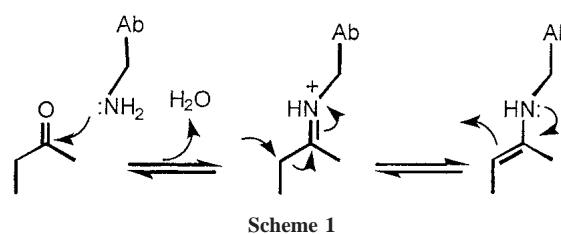
We distinguish here between promiscuity (or poly-reactivity) versus cross-reactivity in the following manner. Cross-reactivity is related to the original activity (i.e., the activity for which the active site evolved or was designed). Thus, a cross-reactivity would typically overlap to a significant extent with the original activity—for example, the substrate or ligand would be a derivative of the original one. In the case of binding, cross-reactivity relies on the same central binding site interactions to bind analogous ligands (Kramer et al. 1997). In contrast, poly-reactivity (or promiscuity) applies when the original and the promiscuous activities occur with dissimilar substrates or ligands and proceed via completely different mechanisms. Whereas cross-reactivities are typically identified by a rationale choice of an analog of the original substrate or ligand, promiscuous activities are typically found by a search of a random library of ligands (Varga et al. 1991; Kramer et al. 1997) or coincidence (Hollfelder et al. 1996).

In the case of binding, theoretical models that predict the probability and magnitude of promiscuity have been proposed and largely verified experimentally (Varga et al. 1991; Griffiths and Tawfik 2000). These suggest that any site, if screened against a large, random diversity of ligands will bind a certain fraction of these ligands. In general, there will be few ligands binding with high affinity and more ligands with lower affinity; and, the larger the number of screened ligands, the higher the frequency of binders for any given affinity threshold. Importantly, the frequency and affinity of promiscuous ligands for a given site is generally independent of the affinity this site exhibits towards its original ligand. It is tempting to assume that a similar pattern would be applicable to catalysis, but catalytic promiscuity has yet to be systematically addressed—theoretically and experimentally. Most importantly, for both binding and catalysis, little is known regarding the molecular mechanisms that give rise to both promiscuity and fine specificity within the very same active site.

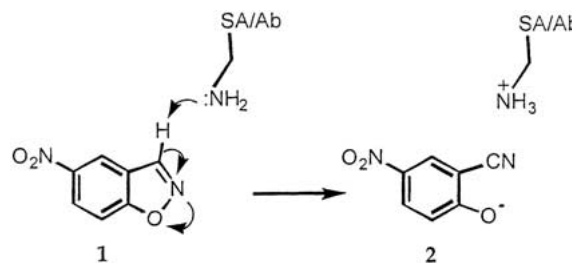
Here we describe the characteristics of what we believe to be one possible group of promiscuous activities for which the only requirement seems to be a site on the surface of a protein comprising an apolar, hydrophobic pocket and a lysine side chain. We do so by identifying functional similarities and analogies between the active site of an antibody (aldolase antibody 38C2; Barbas et al. 1997) and site IIA of human serum albumin (HSA) that satisfy these requirements.

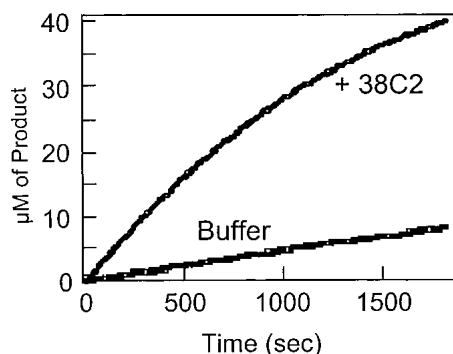
## Results

Antibody 38C2 belongs to a family of highly homologous antibodies raised against a reactive diketone hapten (Wagner et al. 1995) and possessing an active site consisting of a deep hydrophobic pocket with a conserved lysine residue (Barbas et al. 1997; Karlstrom et al. 2000). It has been established that the lysine's amine attack on the carbonyl group of various substrates (including the immunizing hapten) leads to the formation of a covalent enamine intermediate (Scheme 1). Consequently, a variety of reactions that proceed via an enamine intermediate are catalyzed by these antibodies: aldol and retro-aldols (Wagner et al 1995; Barbas et al. 1997), decarboxylation of  $\beta$ -keto acids (Bjornstedt et al. 1996), and an allylic rearrangement (Lin et al. 1997).



The active site of the aldolase antibodies is broadly reminiscent of site IIA of HSA, which also consists of a hydrophobic pocket surrounding a reactive lysine (Carter and Ho 1994; Curry et al. 1998). The IIA site has been shown to catalyze several reactions (Hollfelder et al. 1996, 2000, and references therein). These include the base-catalyzed elimination of 5-nitrobenzoxazole (the Kemp elimination; Kemp et al. 1975; Scheme 2) where a lysine side chain is thought to act as the catalytic base (Lys 222 in bovine serum albumin [BSA]) (Kikuchi et al. 1996) or the homologous Lys 199 in HSA (Hollfelder et al. 2000). We examined whether the active site of 38C2, although selected specifically for the formation of an enamine intermediate with carbonyl substrates, can also catalyze other reactions via a different mechanism—for example, general-base (Scheme 2), rather than nucleophilic catalysis (Scheme 1)—and bind ligands similar to the ones known to promiscuously react with HSA.





**Fig. 1.** Catalysis of the elimination benzisoxazole **1** by antibody 38C2. Substrate (**1**, 40  $\mu\text{M}$ ) was added to 50 mM Tris buffer (pH 8.9) or to the same buffer containing antibody 38C2 (4.4  $\mu\text{M}$ ). The rate of elimination was followed by monitoring the appearance of the phenol product **2** at 405 nm.

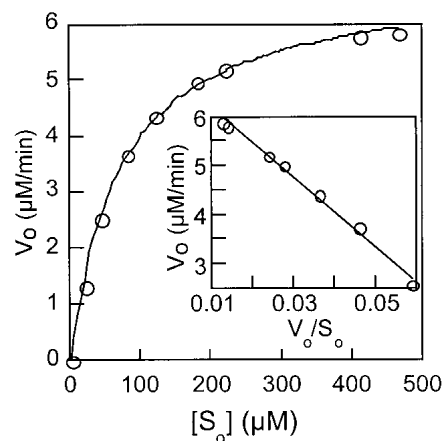
Antibody 38C2 was found to accelerate the rate of elimination of benzisoxazole **1** (Fig. 1). The kinetic parameters for the antibody-catalyzed reaction were determined using the Michaelis-Menten model (Fig. 2; Table 1). Although  $k_{\text{cat}}$  for 38C2 is around 20-fold lower than that of HSA (and so is the rate acceleration,  $k_{\text{cat}}/k_{\text{uncat}}$ ),  $K_{\text{M}}$  is also lower and by the same degree, thus giving rise to a very similar  $k_{\text{cat}}/K_{\text{M}}$ .

Catalysis by 38C2 was measured over a range of pHs between 7.4 and 10.4 (Fig. 3) and the kinetic  $\text{pK}_{\text{a}}$  (8.9) found to be very similar to that of HSA (9.2). These (kinetic)  $\text{pK}_{\text{a}}$ s are consistent with the catalytic base being an  $\epsilon$ -amino group of a lysine side chain, the  $\text{pK}_{\text{a}}$  of which is normally around 10.5, reduced because of an apolar micro-environment (Hollfelder et al. 2000). There exists however, a difference of 3 units from the kinetic  $\text{pK}_{\text{a}}$  of 6.0 observed with antibody 38C2 for the formation of an enamine with 3-methyl-2,4-pentanedione (Barbas et al. 1997). A kinetic  $\text{pK}_{\text{a}}$  reflects the effect of pH on the sum of all of the rate constants affecting the reaction (Fersht 1985). Different substrates will show different  $\text{pK}_{\text{a}}$ s depending on the rate constants leading to the formation and breakdown of their complexes, although the actual ionization state of the catalytic residue in the free (uncomplexed) active site is obviously the same. The difference of 3 logarithmic units between the  $\text{pK}_{\text{a}}$ s of the two 38C2 catalyzed reactions appears to be consistent with the different rate enhancements: the  $k_{\text{cat}}/k_{\text{uncat}}$  for the aldol reactions of antibodies of the 38C2 family range between  $10^4$  and  $10^7$  (Barbas et al. 1997; Karlstrom et al. 2000), whereas for the Kemp elimination the  $k_{\text{cat}}/k_{\text{uncat}}$  is  $0.27 \times 10^3$  (Table 2). Thus, the difference in reactivities of the catalytic lysine side chain, between the aldolase reaction for which the antibody has been selected and the promiscuous Kemp elimination, is reflected not only in the rate but also in the  $\text{pK}_{\text{a}}$  of these reactions. This may be due to differences in the positioning of the reactive lysine between the different substrates and the equilibrium shift

that follows the covalent modification of the reactive lysine in the reaction of 38C2 with 3-methyl-2,4-pentanedione.

The observed kinetic  $\text{pK}_{\text{a}}$  (Fig. 3) could also be the result of a residue with a  $\text{pK}_{\text{a}}$  of 8.9, which is essential for the integrity of the active site or binding of the benzisoxazole substrate. The catalytic base could in this case be hydroxide coming from solution. However, no further increase in rate of product release is observed when exogenous bases (e.g., formate or ammonia, which is also of similar size to hydroxide, at 25–50  $\mu\text{M}$  concentration) are added to either antibody 38C2 or HSA (data not shown).

To verify that the Kemp elimination and aldol reactions both take place in the same site and are catalyzed by the same lysine residue of antibody 38C2, we have tested the blocking effect of three different ketones on the eliminative activity of 38C2. These ketones were shown to form stable enamines with the catalytic lysine in the aldol reaction (Barbas et al. 1997). We found that these ketones do inhibit the catalysis of the Kemp elimination. At a concentration of 50  $\mu\text{M}$ , the eliminative activity of 38C2 was partially inhibited by pyridoxal and 3-methyl-2,4-pentanedione (40% and 53%, respectively) and completely inhibited by pentadione (>98%). Serum albumins have also been shown to react with pyridoxal phosphate via an enamine mechanism, and the formation of this Schiff-base inhibits their catalysis of the Kemp elimination (80% for BSA and 40% to HSA). With BSA, the same lysine residue seems to be involved in Schiff-base formation and in catalysis of the Kemp elimination. However, the precise interpretation of these results (and in particular with regard to HSA) is complicated by the fact that there appears to be more than one site for pyridoxal phosphate binding (Hollfelder et al. 2000).



**Fig. 2.** The kinetic parameters for the 38C2-catalyzed elimination of benzisoxazole **1**. Initial rates of product release ( $v_o$ ) were determined at a range of substrate concentrations ( $[\text{S}_o] = 20\text{--}500 \mu\text{M}$ ;  $[\text{Ab}_c] = 4.4 \mu\text{M}$ ; in 50 mM Tris at pH 8.9;  $T = 25^\circ\text{C}$ ). Data were fitted directly to the Michaelis-Menten model ( $v_o = [\text{Ab}_c] \times k_{\text{cat}} \times [\text{S}_o]/([\text{S}_o] + K_{\text{M}})$ ) and also to the Eadie-Hofstee fit (inset) to give  $k_{\text{cat}}$  of  $1.56 \pm 0.02/\text{min}$  and  $K_{\text{M}}$  of  $70.9 \pm 3.3 \mu\text{M}$ .

**Table 1.** Kinetic parameters for antibody 38C2 and HSA

Protein <sup>a</sup>	pK <sub>a</sub>	K <sub>M</sub> (μM)	k <sub>cat</sub> (min <sup>-1</sup> )	k <sub>cat</sub> /K <sub>M</sub> (M <sup>-1</sup> min <sup>-1</sup> )	k <sub>cat</sub> /k <sub>uncat</sub>	°Turnover
38C2	8.9	70.9 ± 3.3	1.56 ± 0.024	2.23 × 10 <sup>4</sup>	0.27 × 10 <sup>3</sup>	≥10
HSA <sup>b</sup>	9.2	3060 ± 1200	28.8 ± 9.7	2.37 × 10 <sup>4</sup>	5.0 × 10 <sup>3</sup>	~30

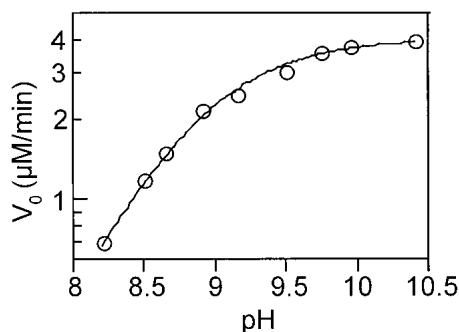
<sup>a</sup> Reactions for 38C2 and HSA were carried out in 50mM Tris at pH 8.9 and 9 respectively.

<sup>b</sup> The determination of HSA's kinetic parameters is described in Hollfelder et al. (1996, 2000).

<sup>c</sup> Minimum number of product molecules produced per enzyme molecule (see Materials and Methods).

To assess the degree of overlap between HSA and antibody 38C2 in their promiscuous binding activities, we screened a series of compounds for inhibition of catalysis by both proteins. Many of these had been shown to bind HSA (Carter and Ho 1994) and inhibit its catalysis of the Kemp elimination (Hollfelder et al. 2000); others were chosen because they possess a negative charge and a hydrophobic moiety (Table 2). We identified a large number of compounds that significantly inhibit catalysis in both 38C2 and HSA. Although most of the HSA inhibitors have only a modest effect on 38C2 those that show greater inhibition share the same general chemical features—hydrophobic moiety and a negative charge. When the negative group is blocked, inhibition both of HSA and 38C2 is generally reduced (Table 2, cf. 4-Iodophenol and 4-Iodoanisole or phenyl acetic acid and its methyl ester).

The pattern of inhibition suggests a considerable overlap in the ligand binding of antibody 38C2 and HSA, with both electrostatic interactions (presumably with the lysine amino side chains) and hydrophobicity playing major roles. Indeed, hydrophobicity has been shown to be a major driving force for ligand binding to antibody 38C2 (Barbas et al. 1997). The exception to this is Fmoc-glycine, the methyl ester of which binds more tightly in both HSA and 38C2.



**Fig. 3.** The kinetic pK<sub>a</sub> for the 38C2-catalyzed elimination of benzisoxazole **1**. Initial rates of product release ( $v_o$ ) were determined at pH 8.2–10.4 under pseudo-first-order conditions ( $[S_o] = 25 \mu\text{M}$ ,  $[\text{Ab}_o] = 4.4 \mu\text{M}$ , Buffer: 50 mM Tris at pH 7.4–8.9, 50 mM AMP at pH 9.15–10.4,  $T = 25^\circ\text{C}$ ). Data were fit to the equation:  $v_o = v_o^H / (10^{(\text{pK}_a - \text{pH})} + 1)$ , where  $1/(10^{(\text{pK}_a - \text{pH})} + 1)$  is the active fraction of antibody at a given pH,  $v_o^H$  is the rate of the fully active (deprotonated) form of 38C2 ( $4.17 \pm 0.1 \mu\text{M}/\text{min}$ ) and pK<sub>a</sub> is  $8.9 \pm 0.04$ .

This reflects that, regardless of the driving force, the shape of the ligand affects affinity and binding is specific. A non-specific mode of binding (e.g., hydrophobic stickiness; Padlan 1994) is also contradicted by the relative inhibition efficiency of different Fmoc amino acids. Fmoc-L-alanine and Fmoc-L-phenylalanine exhibit similar inhibition despite their very different hydrophobicities; and Fmoc-tryptophan, which is even more hydrophobic than the preceding two, shows the lowest inhibitory effect.

## Discussion

The binding and catalytic activities described above for both antibody 38C2 and HSA genuinely can be defined as promiscuous (see definition in introduction). In the case of HSA, although it is unclear what the physiological role and hence the driving force for the evolution of site IIA is (Carter and Ho 1994), it clearly could not have been the elimination of benzisoxazoles—a physiologically meaningless reaction. Antibody 38C2 was selected *in vivo*, by the immune system, to bind and react covalently with a  $\beta$ -diketone hapten via an enamine mechanism (Wagner et al. 1995). Consequently, it exhibits efficient catalysis of several reactions proceeding via an enamine intermediate (Scheme 1) (Wagner et al. 1995; Bjornestedt et al. 1996; Barbas et al. 1997; Lin et al. 1997; Karlstrom et al. 2000). In a sense, these reactions can be defined as cross-reactivities as they all follow the antibody's ability to form an enamine intermediate—an activity for which it was selected. In the promiscuous catalysis of the Kemp elimination, however, the same lysine side chain acts in a different context and mechanism, namely as a general base rather than a nucleophile, and in a concerted, proton transfer reaction rather than via a covalent antibody-substrate intermediate (compare Schemes 1 and 2).

Serum albumins are highly conserved—from sea lamprey to humans (Gray and Doolittle 1992; Carter and Ho 1994). Indeed, every serum albumin tested thus far can catalyze the Kemp elimination (Hollfelder et al. 2000), including bovine serum albumin where site IIA, and Lys 222 within it, could be directly implicated in catalysis (Kikuchi et al. 1996). Similarly, antibody 38C2 is part of a family of highly homologous antibodies sharing the conserved reactive lysine

**Table 2.** Inhibition of catalysis by 38C2 and HSA

Inhibitor	% Inhibition <sup>a</sup>	
	HSA	38C2
Fmoc-glycine <sup>b</sup>	67	69
Fmoc-glycine methyl ester	70	85
Fmoc-L-alanine	63	23
Fmoc-L-phenylalanine	59	25
Fmoc-L-tryptophan	26	0
4-iodophenol	61	54
4-iodoanisole	45	17
Caprylic acid	11	42
3-nitrobenzoic acid	4	48
Phenylacetic acid	65	64
Methyl phenyl acetate	35	28
4-toluene sulphonic acid	22	50
Ibuprofen	33	48
4-iodophenoxyacetic acid	–	35
Warfarin	44	42
2,3,5-tri-iodobenzoic acid	19	53
3-nitrobenzeneboronic acid	15	51

<sup>a</sup> Inhibitions were determined at 500  $\mu$ M ligand (see Materials and Methods).

<sup>b</sup> FMOC = N-(9-fluorenylmethoxycarbonyl).

and other active-site residues (Karlstrom et al. 2000); we have tested only the commercially available 38C2 but other members of the family are likely to exhibit activity. It therefore seems that two unrelated protein families—each evolved under a completely different selection pressure—can maintain a very similar prototypic pattern of promiscuous activities. To assess the general abundance of this (and other) active-site prototypes, systematic screens will have to be performed with large numbers of proteins. Such screens for very weak activities are, however, technically quite demanding (Tawfik et al. 1992; O'Brien and Herschlag 1999).

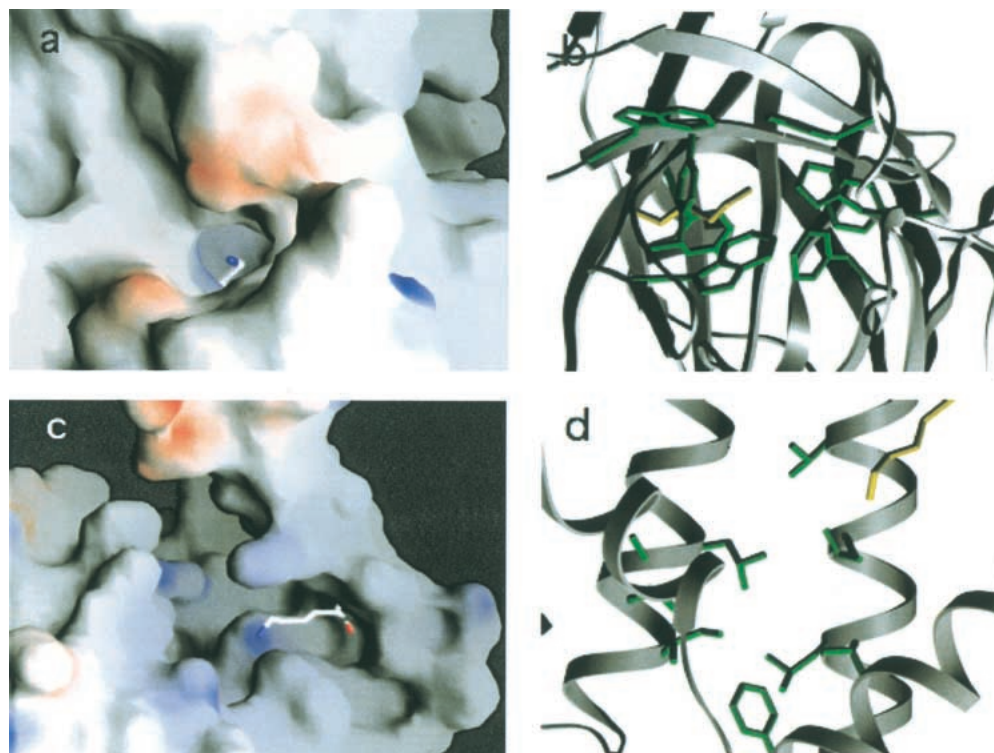
#### *Serum albumins and aldolase antibody 38C2 share prototypic active-site features*

The common pattern of activities in serum albumins and antibody 38C2 might imply a strong degree of structural similarity. However, these proteins differ dramatically not only in their overall structure but also in the fine architecture of their active sites. The structure of 38C2 is not known but the Fab structure of another aldolase antibody, 33F12, which is highly homologous to 38C2 in sequence and activity (Karlstrom et al. 2000), has been solved (Barbas et al. 1997). The binding site of 33F12 consists of a narrow cleft formed at the interface between the heavy and light chains and enclosed by the CDR loops (Fig. 4a,b). The base of the cleft is highly hydrophobic, and the catalytic Lys 93H is surrounded by a number of aromatic residues. In contrast to the all- $\beta$ -sheet antibodies, HSA is entirely  $\alpha$ -helical. The IIA site consists of an extended hydrophobic

channel bordered by a number of aromatic residues, with the catalytic lysine at one end (Lys 199; homologous to Lys 222 in BSA; Fig. 4c,d) (Curry et al. 1998). Despite these dissimilarities—in orientation and composition of the side chains constituting the walls of the two active sites, in the general shape of the cavities, and even in the position of the lysines relative to the cavities—the two active sites share an essential feature: a quite simple arrangement of hydrophobic residues surrounding a catalytic lysine amino side chain (Figure 4a,c).

The origins of serum albumin catalysis of the Kemp elimination previously has been ascribed to specific medium effects (Hollfelder et al. 1996). This stems from the fact that the amine-catalyzed Kemp elimination (unlike the carboxyl-catalyzed reaction) is not affected by nonspecific medium effects of type exerted by organic solvents (e.g., by desolvation and activation of the base catalyst) (Kemp et al. 1975). However, this reaction is affected by specific, localized medium effects exerted by the active-site microenvironment that stabilize the transition state and thereby accelerate its rate (Hollfelder et al. 1996, 2001). The same mechanism probably prevails not only in HSA but also in antibody 38C2.

Beyond the particular similarities between 38C2 and HSA, active sites, regardless of how they evolved and for what, possess common characteristics. The heterogeneous microenvironment of the active site, an organized matrix of apolar, hydrophobic residues next to polar or charged ones, induces specific medium effects that contribute to the catalysis of the Kemp elimination as in essentially any other reaction (Hotta et al. 2000 and references therein). Such microenvironments seem to be common at the interfaces of proteins' surfaces and their hydrophobic core (Perutz 1967). These microenvironments can, for example, desolvate a substrate and thereby activate it, render a catalytic side chain more basic, acidic, or nucleophilic (and thus affect its  $pK_a$ ), stabilize charged transition states via dispersion and stacking interactions, or immobilize water molecules to serve as catalysts or charge stabilizers (Jencks 1969; Fersht 1985; Cannon and Benkovic 1998). In other words, active sites possess features that are inherently catalytic and this explains why promiscuity is both possible and probable. Because these features are of course under selection in every biological active site to provide optimal catalysis of a specific reaction, they also provide the necessary forces to potentially catalyze reactions on any substrate that can be bound. A clear demonstration of this point is the fact that the two proteins described here have evolved under completely different selection pressures but have still ended up with an active site capable of promiscuously catalyzing the same (physiologically irrelevant) reaction. The generally catalytic nature of active sites, in fact, explains why, for example, active-site residues are so often labeled by generic amino acid modifiers, whereas other residues on



**Fig. 4.** Secondary structures and surface representations of the active sites of aldolase antibody 33F12 (a homolog of 33C2) (Barbas et al. 1997) and HSA (Curry et al. 1998). (a) The active-site surface of 33F12 takes the form of a cleft with the catalytic lysine residue (H93) at the bottom. (b) The main chain and residues surrounding Lys 93 (in yellow) and forming the cavity are depicted (in green) (H33W, L96Y, L27dH, L98F, L32F, H103W, H98Y, H93K, H95Y); these come from the  $\beta$ -strands of both the heavy (H) and the light chain (L). (c) The IIA site of HSA consists of a deep channel into which the  $\epsilon$ -amino group of Lys 199 projects. (d) The main chain of the  $\alpha$ -helices and the side chains forming the walls of the IIA site are depicted (in green and lysine in yellow) (V241, L238, L219, F223, L234, I264, I290, A261, L260, K199). PDB files 1AXT (Barbas et al. 1997) and 1BKE (Curry et al. 1998) were used for depicting the sites of aldolase antibody 33F12 and HSA, respectively. Surfaces (A and C) were calculated using a 1.4 Å probe in GRASP (Nicholl and Honig 1991). Electrostatic potentials are marked, with red corresponding to an overall negative charge and blue for positive. Ribbon representations were created using SETOR (Evans 1993).

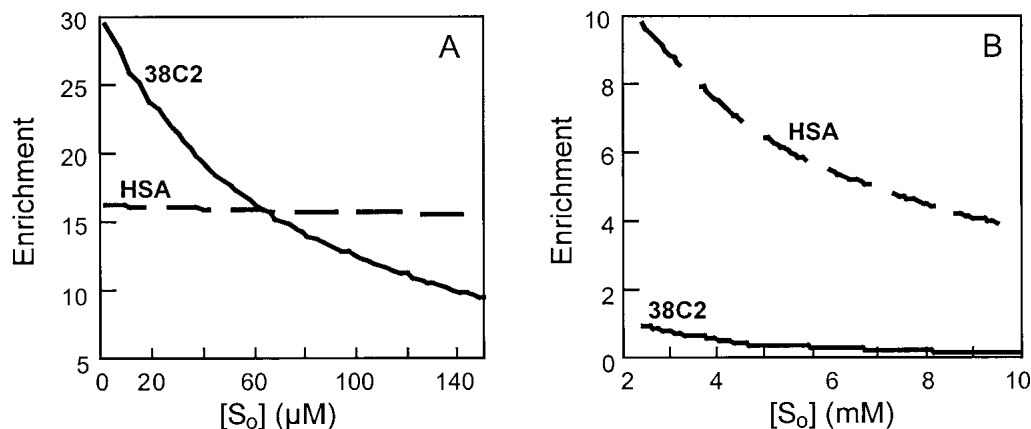
the surface of the protein are not (O'Brien and Herschlag 1999); or, why certain regions of proteins comprise hot spots for binding of natural and in vitro-selected ligands whereas the rest of the protein surface seems inert (DeLano et al. 2000).

#### *The potential selective advantage of catalytic promiscuity*

In a wider context, one should be asking how relevant are promiscuous activities of the type and magnitude described above as a basis for enzyme evolution? The reaction described in this work, the Kemp elimination, admittedly bears no physiological relevance, although it does model proton transfer from carbon, a critical step in numerous bioreactions. And, the catalytic activity of HSA, and in particular of antibody 38C2 is rather low. The rate acceleration exhibited by the latter—270-fold—is orders of magnitude lower than the weakest of natural enzymes (Griffiths

and Tawfik 2000). To assess the hypothetical selective advantage of a protein exhibiting the level of promiscuous activity of HSA or antibody 38C2, we have calculated the ratio of product produced in the presence versus the absence of these promiscuous proteins (Fig. 5). We assume, for the sake of this demonstration, that the product is contributing to the survival or growth of the organism and that the above ratio therefore reflects the advantage provided to an organism carrying these promiscuously active proteins over other organisms that do not. Our analysis is based on a protein concentration of 10  $\mu$ M. This may seem unusually high as in vitro assays are typically performed at nM or even pM enzyme concentrations. In the cell, however, numerous proteins, in particular house-keeping enzymes are present at very high concentrations, sometimes exceeding 1 mM (Price and Stevens 1999).

There are many factors on which the evolution of a new protein may depend. The results of our hypothetical analysis obviously relate to a simplified case where the promiscuous



**Fig. 5.** The putative enrichment for product in the presence of promiscuous catalysts HSA and antibody 38C2. The enrichment is the ratio of product obtained in the presence versus the absence of a promiscuous catalyst, at different substrate concentrations. Assuming initial velocity conditions ( $[\text{Product}] \leq 0.1[\text{S}_o]$ ), the enrichment is given by:  $[\text{E}_o](k_{\text{cat}}/k_{\text{buffer}})/([\text{S}_o] + K_M)$ ; where  $[\text{E}_o]$  is the concentration of the promiscuous catalyst (10  $\mu\text{M}$ ; see text);  $k_{\text{cat}}$  and  $K_M$  are the kinetic parameters for HSA and 38C2 (Table 1); and,  $k_{\text{buffer}}$  is the first-order rate constant for the background, buffer-catalyzed reaction under the same conditions. Results are presented for two ranges of substrate concentrations:  $[\text{S}_o] = 1\text{--}150 \mu\text{M}$  (A) and  $[\text{S}_o] = 2\text{--}10 \text{mM}$  (B).

starting point exhibits substrate binding and catalysis. These indicate that at low substrate concentrations ( $<0.2 \text{mM}$ ), both proteins, including 38C2 with a rate acceleration of 270, can potentially provide a substantial selective advantage (10- to 30-fold). At high substrate concentrations ( $>0.25 \text{mM}$ ), only HSA with a rate acceleration of 5000 is providing a conspicuous selective advantage. But as the concentration of the promiscuous protein has a direct (linear) effect on its selective advantage, antibody 38C2 could, in principle, also become selectable at concentrations higher than 10  $\mu\text{M}$ . Interestingly, 38C2 and HSA have almost identical catalytic efficiencies ( $k_{\text{cat}}/K_M$ ; Table 1) although the contribution from either  $k_{\text{cat}}$  or  $K_M$  significantly differs. Antibody 38C2 exhibits high affinity (low  $K_M$ ) for its promiscuous substrate but poor rate acceleration ( $k_{\text{cat}}$ ). Our modelling suggests that this type of promiscuity can provide a selective advantage but primarily at low substrate concentrations (Fig 5). On the other hand, HSA exhibits lower substrate affinity (high  $K_M$ ) but higher  $k_{\text{cat}}$ . The latter seems to become particularly essential at high substrate concentrations (Fig 5).

#### Implications for *in vitro* evolution

It has been shown recently, that during the *in vitro* evolution of an enzyme with a new specificity, intermediates are generated with promiscuous characteristics (Matsumura and Ellington 2001). Such studies may provide insights regarding the role of promiscuity in enzyme evolution in nature and also suggests that in the future promiscuous precursors may play a key role in the directed evolution (Merz et al. 2000; Arnold et al. 2001) of new enzymes in the laboratory. Thus, perhaps in the future, *in vitro* selection strategies may make

use of libraries of existing proteins with a range of promiscuous activities. To take advantage of such starting points, marginal activities will have to be detected, thus making the threshold for selection of an active protein mutant as low as possible (Griffiths and Tawfik 2000). But the availability of promiscuous starting points should allow the evolutionary process to proceed with a rather limited diversity thus circumventing the need for very large libraries.

## Materials and methods

### Materials

Antibody 38C2 and HSA were purchased from Sigma-Aldrich Ltd. (A-47,995-0 and S-47,995-0, respectively). The antibody samples were reconstituted in deionized water, aliquoted, and stored at  $-20^\circ\text{C}$ . Before each assay, the sample was centrifuged to remove precipitated material and the antibody concentration in the supernatant determined by measuring the optical density at 280 nm ( $\epsilon^{280\text{nm}} = 1.4 \text{mg/mL per cm}$ ). Benzisoxazole substrate **1** and phenol product **2** were prepared as described (Kemp et al. 1975; Hoffelder et al. 2000).

### Kinetic assays

Initial velocities were determined by monitoring the release of product **2** at 405 nm in a microtitre plate reader (Molecular Devices) and rates were calculated using  $\epsilon^{405\text{nm}} = 4300 \text{M}^{-1}\text{cm}^{-1}$  at 0.2-mL reaction volumes per well. The 5-nitrobenzisoxazole substrate **1** was dissolved in methanol, freshly diluted (1:10) into water, and added to the protein in buffer. The percentage of methanol in the final reaction mixture was 2%. Rate of the background, buffer-catalyzed elimination of **1** ( $k_{\text{buffer}}$ ) was determined to be  $7.3 \times 10^{-3}/\text{min}$ , by measuring initial velocities at substrate concentrations ranging from 20  $\mu\text{M}$  to 0.5 mM in 50 mM Tris buffer (pH 8.9). For obtaining the value of  $k_{\text{uncat}}$ , rates at different buffer

concentrations were extrapolated to zero buffer concentration yielding a value of  $5.75 \times 10^{-3}$ /min at pH 9.0 (Hollfelder et al. 1996). To test inhibition by various ligands, antibody 38C2 (4.4  $\mu$ M) or HSA (7.5  $\mu$ M) were incubated with the various ligands (typically at 500  $\mu$ M) for 1 h in 50 mM Tris (pH 8.9). The substrate (**1**) was added to a final concentration of 25  $\mu$ M and the rate of reaction monitored at 405 nm and  $T = 25^\circ\text{C}$ . The rate of the buffer-catalyzed reaction was deducted from all rates and percentage of inhibition calculated from the ratio of rates observed in the presence and in the absence of the protein.

### Determination of turnover

The appearance of reaction product **2** was followed at  $[\text{Ab}_0] = 4.4 \mu\text{M}$ ,  $[\text{1}] = 40 \mu\text{M}$  in 50 mM Tris (pH 8.9). The turnover limit is determined at the point where the rate of the antibody-catalyzed reaction matches the rate of the background reaction (followed in parallel in the same buffer without the antibody). The concentration of product released up to that limit was divided by the concentration of antibody active sites to yield the number of turnovers.

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### References

- Arnold, F.H., Wintrode, P.L., Miyazaki, K., and Gershenson, A. 2001. How enzymes adapt: Lessons from directed evolution. *Trends Biochem Sci.* 26: 100–106.
- Babbitt, P.C. and Gerlt, J.A. 2000. New functions from old scaffolds: How nature reengineers enzymes for new functions. *Adv. Protein Chem.* 55: 1–28.
- Barbas, C.F., 3rd, Heine, A., Zhong, G., Hoffmann, T., Gramatikova, S., Bjornestedt, R., List, B., Anderson, J., Stura, E.A., Wilson, I.A., and Lerner, R.A. 1997. Immune versus natural selection: antibody aldolases with enzymic rates but broader scope. *Science.* 278: 2085–2092.
- Bjornestedt, R., Zhong, G.F., Lerner, R.A. and Barbas, C.F. 1996. Copying nature's mechanism for the decarboxylation of beta-keto acids into catalytic antibodies by reactive immunization. *J. Amer. Chem. Soc.* 118: 11720–11724.
- Cannon, W.R. and Benkovic, S.J. 1998. Solvation, reorganization energy, and biological catalysis. *J. Biol. Chem.* 273: 26257–26260.
- Carter, D.C. and Ho, J.X. 1994. Structure of serum albumin. *Adv. Protein. Chem.* 45: 153–203.
- Curry, S., Mandelkow, H., Brick, P., and Franks, N. 1998. Crystal structure of human serum albumin complexed with fatty acid reveals an asymmetric distribution of binding sites. *Nat. Struct. Biol.* 5: 827–35.
- DeLano, W.L., Ultsch, M.H., de Vos, A.M. and Wells, J.A. 2000. Convergent solutions to binding at a protein–protein interface. *Science.* 287: 1279–1283.
- Evans, S.V. 1993. SETOR: Hardware lighted three-dimensional solid model representations of macromolecules. *J. Mol. Graphics.* 11: 134–138.
- Fersht, A.R. 1985. *Enzyme structure and mechanism.* W.H. Freeman & Co., New York.
- Gray, J.E. and Doolittle, R.F. 1992. Characterization, primary structure, and evolution of lamprey plasma albumin. *Protein Sci.* 1: 289–302.
- Griffiths, A.D. and Tawfik, D.S. 2000. Man-made enzymes—from design to in vitro compartmentalisation. *Curr. Opin. Biotechnol.* 11: 338–353.
- Hollfelder, F., Kirby, A.J., and Tawfik, D.S. 1996. Off-the-shelf proteins that rival tailor-made antibodies as catalysts. *Nature.* 383: 60–2.
- Hollfelder, F., Kirby, A. J. and Tawfik, D. S. 2001. On the Magnitude and Specificity of Medium Effects in Enzyme-like Catalysts for Proton Transfer. *J. Org. Chem.* 66: 5866–5874.
- Hollfelder, F., Kirby, A. J., Tawfik, D. S., Kikuchi, K. and Hilvert, D. 2000. Characterization of proton-transfer catalysis by serum albumins. *J. Am. Chem. Soc.* 122: 1022–1029.
- Hotta, K., Lange, H., Tantillo, D. J., Houk, K. N., Hilvert, D. and Wilson, L. A. 2000. Catalysis of decarboxylation by a preorganized heterogeneous micro-environment: Crystal structures of abzyme 21D8. *J. Mol. Biol.* 302: 1213–1225.
- Jencks, W.P. 1969. *Catalysis in chemistry and enzymology.* McGraw Hill, New York.
- Jensen, R.A. 1976. Enzyme recruitment in evolution of new function. *Annu. Rev. Microbiol.* 30: 409–25.
- Jurgens, C., Strom, A., Wegener, D., Hettwer, S., Wilmanns, M., and Sterner, R. 2000. Directed evolution of a (beta alpha)8-barrel enzyme to catalyze related reactions in two different metabolic pathways. *Proc. Natl. Acad. Sci.* 97: 9925–9930.
- Karlstrom, A., Zhong, G., Rader, C., Larsen, N.A., Heine, A., Fuller, R., List, B., Tanaka, F., Wilson, I.A., Barbas, C.F., 3rd, and Lerner, R.A. 2000. Using antibody catalysis to study the outcome of multiple evolutionary trials of a chemical task. *Proc. Natl. Acad. Sci.* 97: 3878–3883.
- Kemp, D.S., Cox, D.D., and Paul, K.G. 1975. The physical organic chemistry of benzisoxazoles. IV. The origins and catalytic nature of the solvent rate accelerations for the decarboxylation of 3-carboxybenzisoxazoles. *J. Amer. Chem. Soc.* 97: 7312–7318.
- Kikuchi, K., Thorn, S., and Hilvert, D. 1996. Albumin-catalyzed proton transfer. *J. Am. Chem. Soc.* 118: 8184–8185.
- Kramer, A., Keitel, T., Winkler, K., Stocklein, W., Hohne, W., and Schneider-Mergener, J. 1997. Molecular basis for the binding promiscuity of an anti-p24 (HIV-1) monoclonal antibody. *Cell.* 91: 799–809.
- Lin, C.H., Hoffman, T.Z., Wirsching, P., Barbas, C.F., Janda, K.D., and Lerner, R.A. 1997. On roads not taken in the evolution of protein catalysts: Antibody steroid isomerases that use an enamine mechanism. *Proc. Natl. Acad. Sci.* 94: 11773–11776.
- Matsumura, I. and Ellington, A.D. 2001. In vitro evolution of beta-glucuronidase into a beta-galactosidase proceeds through non-specific intermediates. *J. Mol. Biol.* 305: 331–339.
- Merz, A., Yee, M.C., Szadkowski, H., Pappenberger, G., Cramer, A., Stemmer, W.P., Yanofsky, C., and Kirschner, K. 2000. Improving the catalytic activity of a thermophilic enzyme at low temperatures. *Biochemistry.* 39: 880–889.
- Nagahara, N., Okazaki, T., and Nishino, T. 1995. Cytosolic mercaptopyruvate sulfurtransferase is evolutionarily related to mitochondrial rhodanase. Striking similarity in active site amino acid sequence and the increase in the mercaptopyruvate sulfurtransferase activity of rhodanase by site-directed mutagenesis. *J. Biol. Chem.* 270: 16230–16235.
- Nicholl, A. and Honig, B. 1991. GRASP: A program for the graphical representation and analysis of surface properties. *J. Comp. Chem.* 12: 435–445.
- O'Brien, P.J. and Herschlag, D. 1999. Catalytic promiscuity and the evolution of new enzymatic activities. *Chem. Biol.* 6: R91–R105.
- Padlan, E.A. 1994. Anatomy of the antibody molecule. *Mol. Immunol.* 31: 169–217.
- Palmer, D.R., Garrett, J.B., Sharma, V., Meganathan, R., Babbitt, P.C., and Gerlt, J.A. 1999. Unexpected divergence of enzyme function and sequence: "N-acetylamino acid racemase" is o-succinylbenzoate synthase. *Biochemistry.* 38: 4252–4258.
- Perutz, M. 1967. Concluding remarks. *Proc. Roy. Soc. B.* 167: 349.
- Price, N.C. and Stevens, L. 1999. *Fundamentals of enzymology.* pp. 355–366. Oxford University Press, Oxford, UK.
- Tawfik, D.S., Green, B.S. and Eshhar, Z. 1992. Detection of catalytic monoclonal antibodies. *Anal Biochem.* 202: 35–39.
- Varga, J.M., Kalchschmid, G., Klein, G.F., and Fritsch, P. 1991. Mechanism of allergic cross-reactions—I. Multispecific binding of ligands to a mouse monoclonal anti-DNP IgE antibody. *Mol. Immunol.* 28: 641–654.
- Wagner, J., Lerner, R.A., and Barbas, C.F. 1995. Efficient aldolase catalytic antibodies that use the enamine mechanism of natural enzymes. *Science.* 270: 1797–1800.