Highly Efficient Antibody-Catalyzed Deuteration of Carbonyl Compounds

Avidor Shulman,[a] Danielle Sitry,[a] Hagit Shulman,[a] and Ehud Keinan*[a, b, c]

Abstract: Antibody 38C2 efficiently catalyzes deuterium-exchange reactions at the α position of a variety of ketones and aldehydes, including substrates that have a variety of sensitive functional groups. In addition to the regio- and chemoselectivity of these reactions, the catalytic rates (k_{cat}) and rate-enhancement values (k_{cat}/k_{un}) are among the highest values ever observed with catalytic antibodies. Comparison of the substrate range of the catalytic antibody with highly evolved aldolase enzymes, such as rabbit-muscle aldolase, highlights the much broader practical scope of the antibody, which accepts a wide range of substrates. The hydrogen-exchange reaction was used for calibration and mapping of the antibody active site. Isotope-exchange experiments with cycloheptanone reveal that the formation of the Schiff base species (as concluded from the $^{16}O/^{18}O$ exchange rate at the carbonyl oxygen) is much faster than the formation of the enamine intermediate (as concluded from the H/D exchange rate), and both steps are faster than the antibody-catalyzed aldol addition reaction.

Keywords: aldol reaction · catalytic antibodies · deuterium · isotopic labeling · reaction mechanisms

Introduction

Deuterium- and tritium-labeled organic compounds have become increasingly important for the role they play in structure determination, in mechanistic studies, in the elucidation of biosynthetic pathways and in biochemical studies. One of the most commonly used deuteration/tritiation methods is the acid- or base-catalyzed exchange of protons α to a carbonyl function by using D_2O or tritiated water. Unfortunately, the fairly strenuous aqueous conditions required to complete this exchange reaction are incompatible with substrates that contain acid/base-sensitive functional groups.[1, 2] These difficulties have inspired much activity and innovation at the level of process engineering.[3] Deuteration of aldehydes is a particularly difficult task because most aldehydes are incompatible with both the basic and acidic conditions required for the exchange reaction.[4] The use of amine catalysts that form intermediate enamines could represent a solution to this problem. This approach, however, is applicable only in rare cases in which the enamine can be formed in water.[5]

Biocatalysis could provide an attractive strategy for achieving these goals. Biocatalysts have already proven to be useful tools in synthetic organic chemistry, mainly due to their high levels of catalytic efficiency under mild reaction conditions.[6] Catalytic antibodies, in particular, have been shown to catalyze a remarkable range of chemical reactions with impressive rates.[7] Of special interest are the aldolase antibodies, such as 38C2 and 33F12, which have been obtained by reactive immunization.[8] These antibodies were elicited against a 1,3-diketone hapten to evoke covalent bonding between the hapten and an active-site lysine residue. One of the most remarkable features of these antibodies is their ability to accept a broad range of substrates.[1e, 9] It has been shown that this feature reflects termination of the immunization process before the antibody shape complementarity has been fully optimized by somatic mutations.[10]

We reasoned that aldolase antibodies could provide an opportunity to solve the above-mentioned problems, since the deuterium-exchange reaction could be carried out under neutral conditions. Here we report that antibody 38C2 efficiently catalyzes the exchange reaction of α-protons in a variety of ketones and aldehydes. The observed catalytic rates (k_{cat}) and rate-enhancement values (k_{cat}/k_{un}) are among the highest values ever observed with catalytic antibodies. Furthermore, we show that, unlike natural aldolase enzymes, this catalytic antibody is much more able to accept a wide range of

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  0947-6539/02/0801-0229 $ 17.50+.50/0

substrates, including a variety of functionalized aldehydes or ketones. This study also highlights mechanistic details of the catalytic machinery of antibody 38C2. For example, it shows that the deuteration reaction proceeds by the Schiff base mechanism, that the formation of the Schiff base is rapid in comparison with the tautomerization to the enamine intermediate, and that both steps are faster than the antibody-catalyzed aldol addition reaction.

Results and Discussion

Antibody-catalyzed deuterium-exchange reactions were studied with a broad range of ketones and aldehydes (Tables 1–4, below). All reactions were carried out in D_2O under neutral conditions and in the presence of catalytic amounts of the commercially available antibody 38C2.[11] The exchange reaction was followed either by mass spectrometry (GCMS) or by ^1H NMR. The high sensitivity of the GCMS method with respect to substrate and product concentrations allowed for the determination of the kinetic parameters of these reactions (k_{cat} and K_M) by using Lineweaver–Burk analysis (Figure 1).[12] In all cases that were studied by GCMS, the

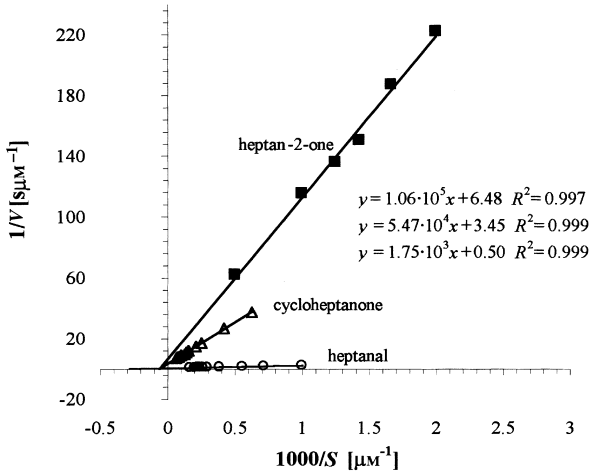


Figure 1. Lineweaver–Burk plots of the 38C2-catalyzed deuterium exchange reaction with three representative substrates: heptan-2-one (Table 1, entry 6), cycloheptanone (Table 2, entry 4), and heptanal (Table 3, entry 3).

deuterium exchange reaction followed Michaelis–Menten kinetics and was effectively inhibited by acetylacetone, which is a mechanism-based inhibitor of antibody 38C2.[8] All NMR measurements were carried out with a high substrate concentration (100 mM) in order to approach substrate saturation and thereby determine v_{max} and k_{cat}. These experiments also provided useful regioselectivity information with nonsymmetrical ketones (vide infra), and chemoselectivity data with polyfunctional carbonyl compounds. The NMR approach, however, is limited in the case of substrates with large K_M values (e.g. Table 1, entries 3, 4). In such cases the apparent v_{max} measured by NMR is actually smaller than the more accurate v_{max} measured by MS. For example, in cases where K_M is nearly 100 mM, v_{max} (as measured by MS) was found to be twice the value of the apparent reaction velocity measured by NMR, in accordance with the Michaelis–Menten kinetic theory. For the substrates with lower K_M values (e.g. Table 1, entries 5, 10, 12), an excellent agreement was obtained between the NMR and MS kinetic data.

It is difficult to assess the actual rate enhancement of the deuteration reaction because of the divergence in the reported uncatalyzed background rates.[13] We found it necessary to run the uncatalyzed reaction of many substrates for periods of time up to five months in order to detect measurable quantities of products. Thus, even taking the most generous estimate for the uncatalyzed rates, we found that, in some cases, the rate enhancement (k_{cat}/k_{un}) of the deuteration reaction exceeded 10^8-fold.

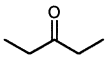
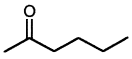
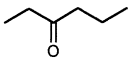
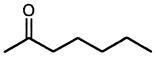
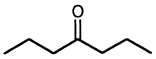
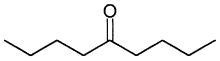
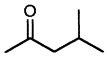
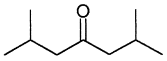
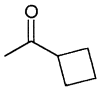
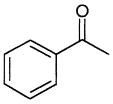
Beyond the practical applicability of the isotopic labeling of organic compounds, the kinetic parameters for the substrates point to important features of the antibody active site and suggest mechanistic details of the exchange reaction. The remarkably broad scope of carbonyl substrates accepted by antibody 38C2 ranges from acyclic and cyclic ketones (Tables 1 and 2, below) to aldehydes (Table 3, below) and polyfunctional ketones (Table 4, below).

Acyclic ketones: Table 1 exhibits a variety of symmetrical and nonsymmetrical ketones that undergo the 38C2-catalyzed deuterium-exchange reaction. The regioselectivity of exchange at the α versus α' positions of nonsymmetrical ketones is of particular interest because it may provide useful information about the exchange mechanism. For example, with linear ketones such as butanone (entry 2), the regioselectivity is known to depend on the nature of the catalyst used. Under strong acid catalysis, exchange of the methylene protons is generally preferred over that of the methyl protons ($k_{CH_2}/k_{CH_3} = 2.5$). A similar trend was reported for neutral conditions ($k_{CH_2}/k_{CH_3} = 1.6$).[14] Conversely, the opposite regioselectivity has been observed under strong base catalysis ($k_{CH_2}/k_{CH_3} = 0.7$).[14] Other ratios within this range ($k_{CH_2}/k_{CH_3} = 1.0–2.0$) have been reported for a variety of weak acid and base catalysts, such as acetate buffer solutions.[5a, 15] We found that the antibody-catalyzed exchange with butanone at neutral pH is significantly more regioselective ($k_{CH_2}/k_{CH_3} = 3.0$) than the highest value reported for acid catalysis. With larger ketone substrates, in which the two alkyl groups are considerably different from one another such as hexan-2-one (entry 4), the discrimination in favor of the methylene is even higher ($k_{CH_2}/k_{CH_3} = 5.8$, Figure 2). These observations show that although the reaction is carried out under neutral aqueous conditions, its mechanism is reminiscent of a typical acid-catalyzed exchange reaction. This is consistent with a protonated Schiff base intermediate that is formed from the carbonyl moiety and the lysine residue in the antibody active site (**I** in Scheme 1). We have already reported that the 38C2-catalyzed aldol reaction has characteristics that resemble those of an acidic mechanism.[10]

That the observed regioselectivity under antibody catalysis is higher than that expected for simple acid catalysis could reflect a nonsymmetrical binding mode of the substrate with subsequent formation of a nonsymmetrical Schiff base intermediate, **I**. This covalent interaction could define two

  0947-6539/02/0801-0230 $ 17.50+.50/0 *Chem. Eur. J.* **2002**, *8*, No. 1

Table 1. Kinetic data for the antibody 38C2-catalyzed deuteration of acyclic ketones.

	Substrate	k_{cat} (apparent) (NMR)[a] [min^{-1}]	k_{cat} (MS)[a] [min^{-1}]	K_M [mM]	k_{cat}/k_{un}[b]
1		2.6			2.6×10^7
2		2.36 (CH_2)			2.4×10^7
		0.8 (CH_3)			
3		4.8	10.7	79	1.1×10^8
4		3.5 (CH_2)	9.7	105	9.7×10^7
		0.6 (CH_3)			
5		7.4	8.8	47	8.8×10^7
6			3.5	16	6.9×10^7
7			4.4	13	4.4×10^7
8			nd		
9		nd			
10		0.4 (CH_2)	1.4	45	1.4×10^7
		0.9 (CH_3)			
11			nd		
12		0.1	0.1	31	1.0×10^6
13		nd	< 0.1		
14		nd	nd		
15			1[c]	10[c]	1.0×10^7

[a] All kinetic parameters refer to the exchange of one hydrogen atom. [b] The consistent k_{un} value (10^{-7} min^{-1}), which was measured for heptan-2-one (entry 4), cyclohexanone and cycloheptanone (Table 2, entries 3, 4), was applied for all substrates. [c] Estimated value on the basis of relative rates. nd = not detected.

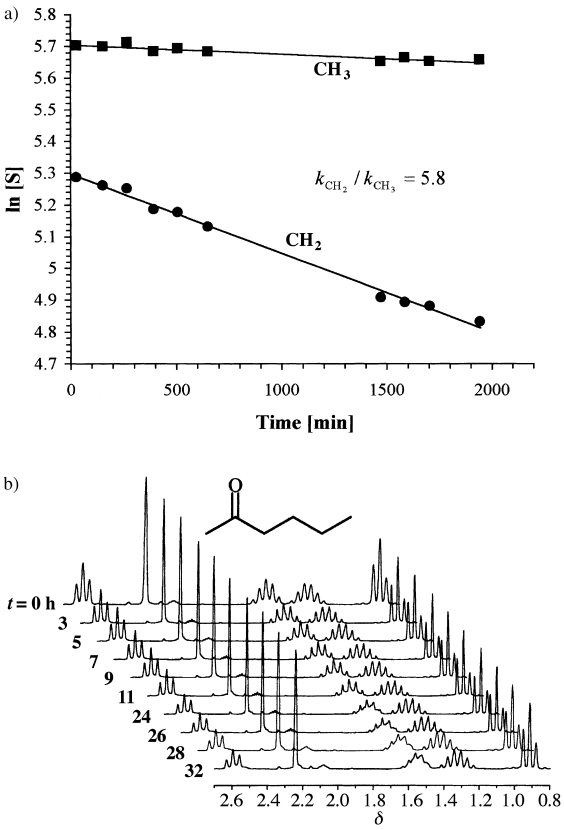
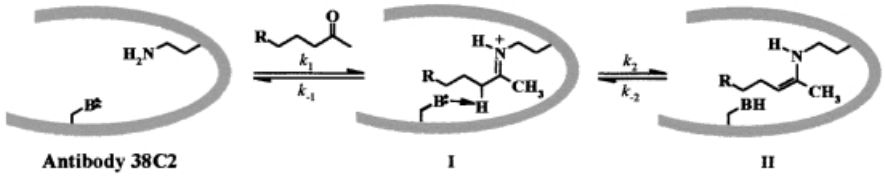


Figure 2. a) Regioselective 38C2-catalyzed deuteration of hexan-2-one (Table 1, entry 4) as monitored by ^{1}H NMR. b) Time-resolved ^{1}H NMR monitoring of the regioselective 38C2-catalyzed deuteration of hexan-2-one. The methylene signal ($\delta = 2.6$) decreased with time while that of the methyl group ($\delta = 2.22$) remained unchanged.

binding regions in the protein that accommodate the large and small alkyl groups of the ketone substrate.[16] Considering the known structure of the hapten used to raise antibody 38C2, it seems likely that the large binding region is positioned away from the lysine group towards the solvent. Our results suggest that a general base residue located between the lysine group and the solvent is responsible for the regioselective deprotonation (Scheme 1). This general base may preferentially deprotonate the α' position directly (as shown in the Scheme) or the α position by a relay of water molecules. This assumption is supported by the consistent exchange rates at the methyl group of several methyl ketones (approximately 0.8 min^{-1}, Table 1 entries 2, 4, 10, 15) in contrast to the higher rate with acetone (2.6 min^{-1}), in which one methyl group is positioned in close proximity to the general base.

Therefore, it is interesting to compare the kinetic parameters of different symmetrical ketones, R_2CO (Table 1, entries 1, 3, 7, 8, 11), which can have only one binding mode. This comparison provides a rough estimate of the size of the smaller binding region. Thus, while the reactivity of the smaller substrates, with R being a methyl, ethyl, or *n*-propyl group is quite similar, symmetrical ketones with larger alkyl



Scheme 1.

groups (*n*-butyl or *i*-butyl) are nonsubstrates. Therefore we conclude that the smaller binding region in the antibody can accommodate fragments no larger than an *n*-propyl group. This is further supported by the finding that di-*n*-propyl ketone has a smaller K_M value than diethyl ketone.

Our structural hypothesis was reinforced by computational modeling (Figure 3) with the crystal structure of another aldolase antibody, 33F12,[8b] which is a very close homologue

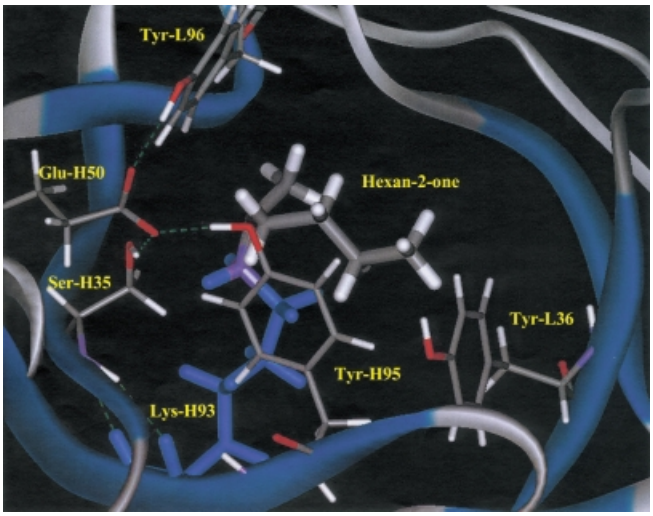


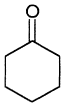
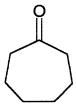
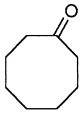
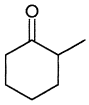
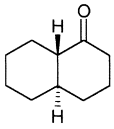
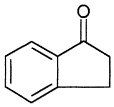
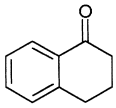
Figure 3. Hexan-2-one bound in the active site of antibody 33F12, after energy minimization with the Discover module of Insight II.

of antibody 38C2. That structure reveals several active-site residues that could function as a general base: a carboxylate (Glu H50), an alcohol (Ser H35), and three phenols (Tyr H95, Tyr L96, Tyr L36) all positioned within 7 Å of the active-site lysine (Lys H93). To gain more information about this catalytic machinery we used the Insight II software package to carry out energy minimization of the lysine-bound hexan-2-one within the active site of antibody 33F12. We chose to model the reduced Schiff base intermediate so as to allow maximum conformational flexibility during the calculation. This model suggests that Tyr H95, which is positioned in close proximity to the substrate α-protons, is the catalytic base. All the other basic residues are positioned further away from the substrate. Moreover, this calculated model suggests that the general basicity of Tyr H95 is enhanced by hydrogen bonding to Glu H50. The observed regioselectivity with this substrate could be explained by the close proximity (3.3 Å) between the C3 hydrogens and Tyr H95 (Figure 3). By contrast, the approach of this base to the C-1 hydrogens (which are positioned 6–7 Å away from Tyr H95) is obstructed by the substrate itself.

An interesting experimental observation that further supports our hypothesis about the location and function of the general base is the low reactivity of branched substrates (entries 9–14). This trend probably reflects a sterically hindered approach of the general base towards the acidic proton, for example, the C-3 protons in 4-methylpentan-2-one (entry 10). In this case the steric hindrance seems to be sufficiently large to cause reversal of regioselectivity ($k_{CH_2}/k_{CH_3} = 0.44$).

Cyclic ketones: As can be seen in Table 2, the unsubstituted monocyclic substrates (entries 2–5) are generally more reactive than the acyclic ketones. The lack of reactivity of cyclobutanone (entry 1) can be attributed to the relatively high energy required to form its enamine derivative. The highest reactivity was observed by NMR (Figure 4) with

Table 2. Kinetic data for the antibody-38C2-catalyzed deuteration of cyclic ketones.

	Substrate	k_{cat} (MS)[a] [min^{-1}]	K_M [mM]	k_{cat}/k_{un}[b]
1		nd		
2		29.9	25	3.0×10^8
3		84.9 (194)[d]	5	5.9×10^8
4		13.1	16	1.0×10^8
5		47.4	23	4.7×10^8
6		8.8	14	8.8×10^7
7		0.07	20	7.0×10^5
8		0.95	13.5	9.5×10^6
9		1[c]		

[a–c] See notes to Table 1. [d] The number in parentheses, which refers to saturation conditions in NMR experiment, is more accurate than the value obtained by MS. The error limit is approximately 15%.

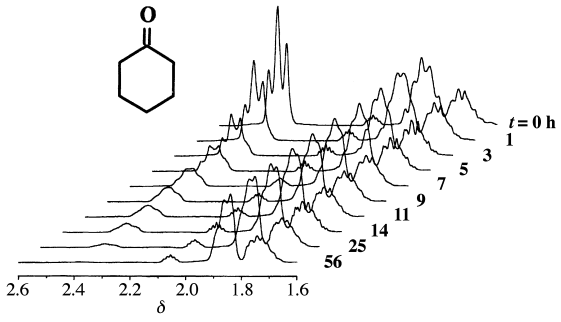


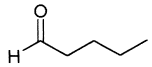
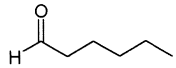
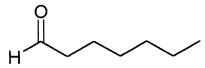
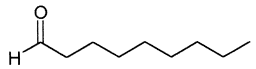
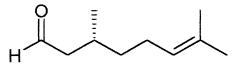
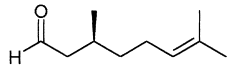
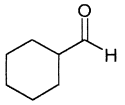
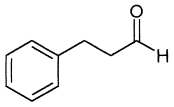
Figure 4. ^{1}H NMR trace of the antibody-38C2-catalyzed deuteration of cyclohexanone (Table 2, entry 3).

0947-6539/02/0801-0232 $ 17.50+.50/0 *Chem. Eur. J.* **2002**, *8*, No. 1

cyclohexanone, which exhibits a record value of k_{cat} in the order of 200 min^{-1} ($k_{cat}/k_{un} \approx 6 \times 10^8$). As was the case with the branched acyclic ketones (vide supra), substituted cyclic ketones (entry 6) and bicyclic substrates (entries 7–9) are much less reactive than the simple monocyclic ketones.

Aldehydes: The efficient antibody-catalyzed deuteration of aldehydes (Table 3) is of particular synthetic significance because aldehydes are less chemically stable than ketones. The aldehyde substrates we used are incompatible with even mild reaction conditions (room temperature, neutral aqueous medium) over the prolonged periods of time required for the formation of any detectable amounts of deuterated products in the uncatalyzed reaction. The data in Table 3 indicate that aldehydes are generally much better substrates than ketones, as reflected by their relatively higher k_{cat} and lower K_M values. Although aldehydes are known to undergo rapid self-aldol condensations, neither uncatalyzed nor 38C2-catalyzed self condensations were observed with valeraldehyde and higher homologues under the reaction conditions. Thus, the only detectable reaction with such aldehydes was the deuterium exchange.

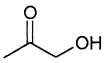
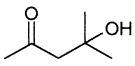
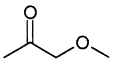
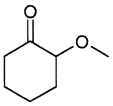
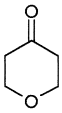
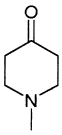
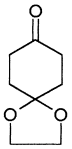
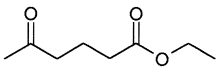
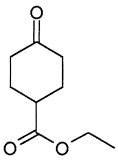
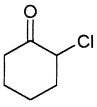
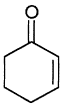
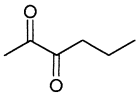
Table 3. Kinetic data for the antibody-38C2-catalyzed deuteration of aldehydes.

	Substrate	k_{cat} (MS)[a] [min^{-1}]	K_M [mM]	k_{cat}/k_{un}[b]
1		19.0	0.7	[d]
2		27.6	0.6	[d]
3		45.2	4	[d]
4		127	1	[d]
5		50[c]	1[c]	[d]
6		50[c]	1[c]	[d]
7		5.8	0.5	[d]
8		32.5	3	[d]

[a–c] See notes to Table 1. [d] d) No background rate could be determined due to the complete loss of starting material before any exchange product could be detected. The error limit is approximately 15%.

Bifunctional ketones: Chemoselective deuteration of bifunctional carbonyl compounds (Table 4) not only affords synthetically useful intermediates but also provides insight into the catalytic machinery of the antibody. Under neutral conditions, antibody 38C2 catalyzes the deuteration of acid-/base-sensitive substrates, such as ketoketals (entry 8), keto-

Table 4. Kinetic data for the antibody 38C2-catalyzed deuteration of bifunctional ketones.

	Substrate	k_{cat} (*apparent*) (NMR)[a] [min^{-1}]	k_{cat} (MS)[a] [min^{-1}]	K_M [mM]
1		0.4 (CH_2) 0 (CH_3)	0.08	3
2		~0.4 (CH)[c] 0 (CH_3)		
3			nd	
4		< 0.1	< 0.1	
5			1–5[c]	
6			1–10[c]	
7			246	340
8			1–10[c]	
9		0.66 (CH_2) 0.14 (CH_3)	0.4	10
10			1–10[c]	
11		0.2 (CH_2) 0.2 (CH_3)		
12		nd		
13			1–5[c]	
14			1[c]	
15			10[c]	

[a and c] See notes to Table 1. The error limit is approximately 15%.

esters (entries 9, 10), and α-haloketones (entries 11, 13). No background reaction could be detected with these substrates. For example, with ethyl 5-oxohexanoate (entry 9) we failed to detect any product of the uncatalyzed deuteration reaction due to the faster consumption of the substrate in various side reactions even in a neutral aqueous medium. An attempt to catalyze this reaction with *n*-butylamine yielded the corresponding butylamide as the only detectable product. With 38C2, however, highly chemoselective deuterium exchange occurred at the position α to the carbonyl but not α to the ester group (Figure 5). Furthermore, as in the case of the

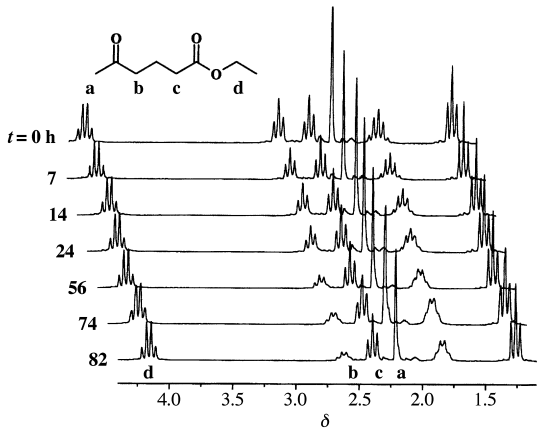


Figure 5. ^{1}H NMR trace of the regioselective 38C2-catalyzed deuteration of ethyl 5-oxohexanoate (Table 4, entry 9). The C4 methylene signal ($\delta =$ 2.7) decreased with time, while that of the methyl group ($\delta = 2.24$) decreased very slowly. All other signals were totally preserved during this reaction.

nonsymmetrical acyclic ketones, deuteration of the methylene hydrogens was highly favored ($k_{CH_2}/k_{CH_3} = 4.7$). Interestingly, although fluoroacetone (entry 11) is a moderate substrate, trifluoroacetone (entry 12) is a nonsubstrate in this reaction. Other functional groups that were found to be compatible with the antibody-catalyzed reaction conditions are ethers (entries 4–6), unsaturated ketones (entry 14) and 1,2-diketones (entry 16).

Of particular interest is the behavior of the hydroxyketones (entries 1–3), in which the presence of a hydroxy substituent in the molecule significantly impairs the deuterium exchange reaction. This observation is intriguing, particularly when considering the high reactivity of hydroxyacetone and hydroxybutanone in the 38C2-catalyzed aldol addition.[8] To gain more information about the interaction of hydroxyketones with 38C2 we carried out inhibition experiments in which the deuteration of cycloheptanone took place in the presence of 25 mol% (with respect to the substrate) of acetylacetone, 3-hydroxybutanone, cyclohex-2-en-1-one, and fluoroacetone (Figure 6). As expected, acetylacetone caused almost complete inhibition of the deuteration reaction. The low levels of inhibition by cyclohex-2-en-1-one and fluoroacetone indicate that these two ketones are competitive inhibitors, whose binding affinities are similar to that of cycloheptanone. Yet, the much stronger inhibition with 3-hydroxybutanone suggests that the active site binds this compound more efficiently than a simple ketone. We suggest that the hydroxyl group, unlike simple ketones, may interact with an active

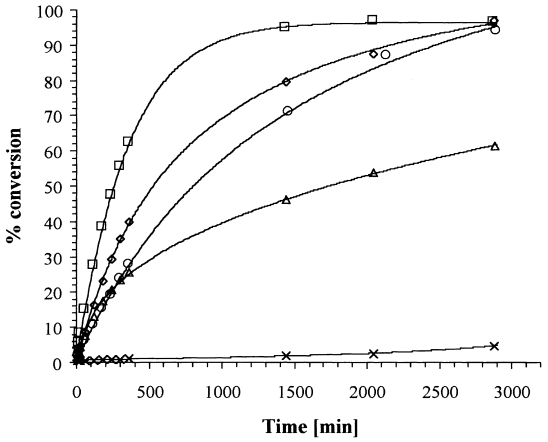


Figure 6. Inhibition of the deuteration of cycloheptanone (Table 2, entry 4) by various ketones. □ no inhibitor; ◇ cyclohex-2-en-1-one; ○ fluoroacetone; △ 3-hydroxybutanone; × acetylacetone.

site residue by additional hydrogen bonding. In hydroxyketone substrates, this additional bonding could stabilize the Schiff base intermediate and inhibit its tautomerization to the enamine. Yet hydroxyketones may still be active in the aldol addition reaction because their binding mode and propensity to form an enamine intermediate may change upon addition of an aldol acceptor molecule.

An interesting case of high regioselectivity was exhibited by 2-methoxycyclohexanone (Table 4, entry 5). We had initially observed in preliminary mass spectrometry experiments that the exchange reaction does not progress beyond 35% conversion. This observation could reflect exchange of just one out of the three acidic protons in the molecule. Indeed, further ^{1}H NMR experiments revealed that, even after long reaction times, only one proton (at the C-6 methylene) was exchanged for deuterium. Closer inspection of the ^{1}H NMR spectra revealed that only the axial proton at C-6 (td, $J = 13.2$, 5.9 Hz) was exchanged by deuterium. Similar regioselectivity was observed with 2-methylcyclohexanone (Table 2, entry 6). Further studies on the use of antibody 38C2 for the kinetic resolution of these substrates and other substituted cyclic ketones are currently underway.

Mechanistic studies:

The 38C2-catalyzed deuteration reaction: It is interesting to compare the mechanistic details of the antibody-catalyzed exchange reaction with similar reactions catalyzed by natural enzymes. Prior observations with antibody-38C2-catalyzed aldol, retroaldol, and decarboxylation reactions[17] suggest that all of these reactions involve the formation of a Schiff base intermediate with an active-site lysine residue. Therefore it is likely that the deuterium exchange reaction involves a multistep process in which every proton is exchanged individually in a series of equilibria, as exemplified by Scheme 1. An important question relates to the relative rates of the two main steps, that is, formation of the protonated Schiff base, **I**, and tautomerization of the latter to enamine **II**. If the first step is faster than the second, the expected kinetic behavior of the entire process is that of consecutive pseudo-first-order reactions with the formation and disappearance of

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partially deuterated products, $[D_1]$, $[D_2]$, $[D_3]$ etc. Alternatively, if the second step is faster than the first one, it is to be expected that no partially deuterated intermediates would be observed during the overall reaction.

To check this issue, we followed the progress of the exchange reaction of cycloheptanone, **1**, by GCMS using a low concentration of catalyst to allow detailed monitoring of this multistep reaction (Figure 7a). It is noteworthy that the antibody-catalyzed deuteration reaction was complete before any background reaction could be detected. The data in Figure 7a fit well with a kinetic model of four consecutive first order reactions where each step is independent of the others.[18] A single rate constant with statistical corrections describes the seemingly complicated array of data. Thus, the first-order rate constant (k) related to the first step (monodeuteration of **1** to produce $[D_1]$**1**) was easily obtained from the exponential curve, which describes the disappearance of $[D_0]$**1**. The rate constant for the next step (formation of $[D_2]$**1**) was determined as $\frac{3}{4}k$. The rate constant for the third step (formation of $[D_3]$**1**) was $\frac{1}{2}k$. Accordingly, the rate constant for the fourth step (formation of $[D_4]$**1**) was $\frac{1}{4}k$. The excellent agreement between the experimental data and the calculated curves in Figure 7a confirmed that the exchange reaction occurs in a stepwise manner. This agreement justifies the neglect of a second-order isotope effect. Thus, referring to Scheme 1, k_{-1} is large compared with k_2, and the process that corresponds to k_1/k_{-1} can be treated as a rapid pre-equilibrium. Similar behavior has been observed by Pratt in the RMA-catalyzed (RMA = rabbit-muscle aldolase) deuteration of hydroxyacetone phosphate[19] and by Westheimer in the acetoacetate decarboxylase-catalyzed de-deuteration reaction with $[D_6]$acetone.[20] This model of a rapid pre-equilibrium fits the kinetics of most of the ketone and aldehyde substrates shown in Tables 1–4.

To further verify the assumption that the Schiff base intermediate is formed in a rapid pre-equilibrium, we examined the antibody-catalyzed $^{16}O/^{18}O$ exchange of the carbonyl oxygen in cycloheptanone. The reaction, carried out with cycloheptanone in $H_2{}^{18}O$, was found to be very fast and to require a low antibody concentration (0.1 mg mL^{-1}) and a short reaction time (2 min). An excellent fit with Michaelis–Menten kinetics was observed ($k_{cat} = 418$ min^{-1}, $K_M = 21$ mM). Comparing these results with the kinetic parameters for the 38C2-catalyzed H/D exchange with the same substrate ($k_{cat} = 13$ min^{-1}, $K_M = 16$ mM), one may conclude that the formation of intermediate **I** is indeed faster (by a factor of at least 30) than the formation of intermediate **II**. Thus, we can conclude that the rate-determining step in the deuterium exchange reaction is the formation of the enamine intermediate, **II**.

The 38C2-catalyzed aldol reaction: This reaction is a multistep cascade of events in which the formation of the Schiff base (**I**) and enamine (**II**) intermediates represent the first two steps. The slowest step in the enzyme-catalyzed aldol addition is the reaction of **II** with the carbonyl acceptor to form a new C–C bond. Accordingly, the antibody-catalyzed aldol addition is expected to be a slower process in comparison with the deuterium-exchange reaction. Indeed, comparison of the reported 38C2-catalyzed aldol reaction rates with our exchange reaction rates for the same ketone substrates reveals that the aldol reactions are generally 100–10000-fold slower.[1e, 9] The aldol reaction has a much more sterically demanding transition state than the exchange reaction. This

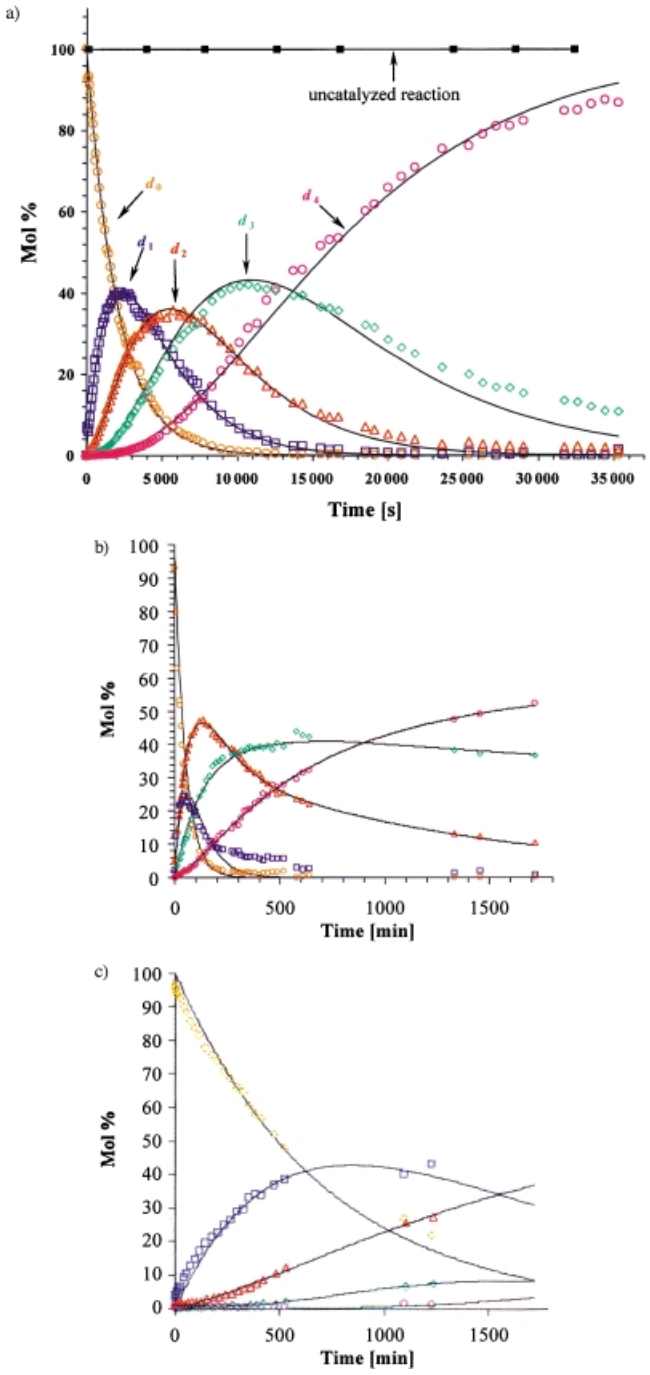
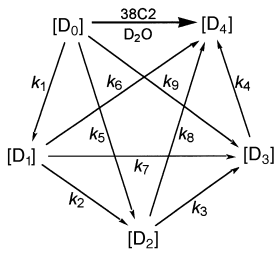


Figure 7. a) Antibody-38C2-catalyzed deuteration of cycloheptanone (Table 2, entry 4). All data points were obtained from time-resolved GCMS measurements. The calculated curves are based on a four-step series of consecutive first-order reactions by using a single rate constant ($k_{obs} = 0.00045$ s^{-1}) multiplied by the appropriate statistical factor. The background rate is undetectable under the reaction conditions. b) Antibody-38C2-catalyzed deuteration of 1-methylpiperid-4-one (Table 4, entry 7). All data points were obtained from time-resolved GCMS measurements. The calculated curves are based on the kinetic model shown in Scheme 2. The kinetic behavior of the background (uncatalyzed) reaction (c) agrees with the simple stepwise model (calculated lines).

explains why the difference between the aldol and exchange reaction rates for a given substrate depends strongly on the substrate size. For example, the deuterium-exchange reaction with acetone is 500-fold faster than the aldol reaction between acetone and 4-acetamidodihydrocinnamaldehyde. Yet, with more sterically demanding donors, the difference between the corresponding reaction rates increases. For example, the exchange reaction with cyclohexanone is 30000 fold faster than the aldol reaction between cyclohexanone and the above-mentioned aldehyde acceptor. Cyclooctanone, which is an even more sterically demanding ketone, is an active substrate for the exchange reaction (Table 2, entry 5) but is a nonsubstrate for the aldol reaction.[9]

The 38C3-catalyzed deuteration of bifunctional compounds: While the above-described rapid pre-equilibrium results in stepwise deuteration of simple ketones, the situation could change with bifunctional substrates. For example, the kinetic behavior exhibited by 1-methylpiperid-4-one (Figure 7b) represents a different mechanistic model in which a rapid pre-equilibrium no longer exists. While the background deuteration reaction of 1-methylpiperid-4-one follows the stepwise kinetics described above (see Figure 7c), the kinetic behavior of the antibody-catalyzed reaction with this substrate reflects a more complex sequence of events.

In principle, all substrates may undergo more than a single proton exchange in every encounter with the antibody active site. While in the case of cycloheptanone (Figure 7a) such double and triple deuteration steps seem negligible, multiple deuteration reactions become significant in the case of 1-methylpiperid-4-one. As can be seen from Figure 7b, the kinetic behavior of this substrate, whose catalytic conversion is much more efficient than that of cycloheptanone, does not fit the simple stepwise kinetic model. In order to estimate the rate constants in this case, we developed a set of integrated rate equations on the basis of the kinetic model shown in Scheme 2. To simplify this rather complex kinetic model, we did not take the background reaction into account. Additionally, all the reverse reactions and the triple deuteration reactions (represented in Scheme 2 by k_6 and k_9) were neglected. Although this treatment should be considered as a qualitative analysis, it yielded an interesting conclusion that the values of the rate constants of the single and double deuteration steps are approximately of the same magnitude. This finding suggests that k_{-1} (Scheme 1) is no longer much larger than k_2. This, in turn, results in an increased residence time of this substrate in the active site of 38C2. The increased reactivity of this substrate could also result from the close proximity between the tertiary amine, which is probably protonated under neutral pH, and the Schiff base. This proximity could facilitate the conversion of the protonated Schiff base intermediate (**I** in Scheme 1) to the enamine intermediate **II**.



Scheme 2.

The H/D kinetic isotope effect in the exchange reaction (Figure 8) was studied by comparing the rate of the transformation of **1** into [D_4]**1** (in D_2O) with the rate of the reverse transformation ([D_4]**1** into **1**, in H_2O). No significant primary

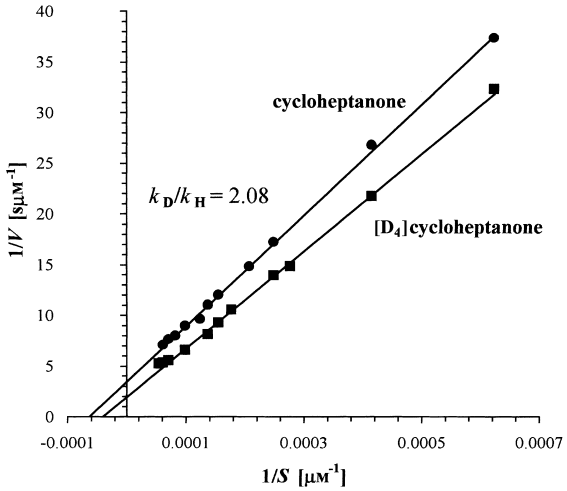


Figure 8. Lineweaver–Burk plots of the 38C2-catalyzed deuteration reaction of cycloheptanone in D_2O ($k_{cat} = 13\,min^{-1}$, $K_M = 16$ mM), and deuteration of [D_4]cycloheptanone in H_2O ($k_{cat} = 27\,min^{-1}$, $K_M = 29$ mM).

kinetic hydrogen isotope effect was found to be associated with the exchange reaction. The small isotope effect ($k_D/k_H = 2$, $k_{cat}/K_M(D)/k_{cat}/K_M(H) = 1.1$) can be attributed to solvent and to equilibrium isotope effects. The lack of a large isotope effect, which would be expected if the formation of the enamine occurred in the rate determining step, probably reflects a nonsymmetrical transition state. Similar observations and interpretations have already been reported for RMA.[19] It is remarkable that a catalytic antibody and highly evolved enzymes share the fine details of their catalytic mechanisms.

Comparison with enzymes: Although there are a few reported cases of an enzyme-catalyzed exchange of protons α to carbonyl groups under mild conditions, practically all of them were limited to very few substrates. For example, pyruvic acid was deuterated by using oxaloacetate decarboxylase,[21] acetone and butanone were deuterated by using acetoacetate decarboxylase under slightly acidic conditions (pH 5.9),[22] dihydroxyacetone phosphate was tritiated by using RMA,[23] and enantioselective deuteration of 2-carboxycyclohexanone was achieved with acetoacetate decarboxylase.[24] Few other examples of substrate-specific deuterium exchange reactions have been reported.[25]

The results summarized in Tables 1–4 highlight the remarkably broad range of substrates that are accepted by antibody 38C2. To compare the substrate range of 38C2 with that of the widely studied RMA, we studied the biocatalyzed deuterium-exchange reaction with a representative selection of substrates: heptan-2-one, heptan-4-one, cycloheptanone, cyclooctanone, octanal, hydroxyacetone, 3-hydroxybutanone, and ethyl-5-oxohexanoate. No RMA-catalyzed reaction was observed with any of these substrates. Very modest catalytic

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activity (significantly lower than that of 38C2) was observed with heptanal and cyclohexane carboxaldehyde. RMA exhibited high reactivity only with its specific substrate, dihydroxyacetone phosphate.

Conclusion

We have shown here that antibody 38C2 catalyzes deuterium exchange reactions with a broad variety of ketones and aldehydes. The catalytic rates and rate-enhancement values are among the highest values ever observed with catalytic antibodies. Since all reactions were carried out under neutral conditions the method was compatible with aldehydes and other sensitive functional groups. The usefulness of this catalytic reaction is evident from the ability to carry out the reaction with a broad scope of substrates, from the regioselectivity with nonsymmetrical ketones, and from the chemoselectivity observed with polyfunctional substrates. A relative reactivity study with many substrates represents a useful method of mapping and calibrating the active site. The observed regioselectivity suggests the existence of two binding regions in the antibody that accommodate the small and large alkyl groups of unsymmetrical ketones. Furthermore, qualitative computational modeling suggests that either Tyr H95 or Glu H50 or a combination of both residues could function as the general base in the catalytic machinery.

Isotope-exchange experiments with cycloheptanone reveal that the formation of the Schiff base species (as concluded from the $^{16}O/^{18}O$ exchange rate at the carbonyl oxygen) is much faster than the formation of the enamine intermediate (as concluded from the H/D exchange rate), and both steps are faster than the antibody-catalyzed aldol addition. These findings reinforce the hypothesis that the slowest step in the catalytic aldol addition reaction is the formation of a new C–C bond. Time-resolved studies show that polydeuteration of simple ketones and aldehydes occurs by a stepwise mechanism in which each deuteration step is independent. By contrast, with bifunctional substrates, such as 1-methylpiperid-4-one, multiple exchanges may occur before substrate dissociation. Comparison of the substrate range of the catalytic antibody with highly evolved aldolase enzymes, such as rabbit-muscle aldolase, highlights the much broader practical scope of the antibody. Further applications of these results are currently being developed, particularly with respect to enantioselectivity and preparative-scale reactions.

Experimental Section

General methods: All kinetic studies that were based on mass spectrometry analysis were carried out with a Finnigan TSQ-70 GCMS-CI linked to a Varian GC equipped with a DB-5ms capillary column. ^1H NMR kinetic measurements were recorded on a Bruker AM200 operating at 200 MHz or a Bruker AM400 operating at 400 MHz. UV/Vis measurements were recorded on a Shimadzu UV-1601 spectrophotometer with a UV/Vis microcuvette (100 μL). Long-period reactions were maintained at 25 °C by using a Friocell incubator. All substrates (analytical grade) and deuterated solvents were purchased from Aldrich and were used without further purification. All solvents (HPLC grade) were purchased from either Aldrich or Merck.

Catalysts: Catalytic antibody 38C2 was purchased from Aldrich in the form of a lyophilized powder. For all deuteration experiments, the antibody was dissolved in a solution of D_2O containing 0.1M NaCl. The antibody final concentration was established by its absorbance at 280 nm. The same results were obtained with an antibody solution that was obtained from dialysis of a concentrated antibody sample in phosphate-buffered saline (PBS – H_2O, pH 7.4) against a solution of 0.1M NaCl in D_2O. In the reactions in which a deuterium atom was exchanged for hydrogen, the antibody was dissolved in H_2O (HPLC grade) containing 0.1M NaCl. For the oxygen exchange experiments, the antibody (1 mg) was dissolved in 1 mL of $H_2{}^{18}O$ (97%, purchased from Rotem Industries Ltd., Israel) containing 0.1M NaCl.

Determination of the background reactions: The background deuteration reaction was monitored along with the catalytic reaction. In most cases, during the time period suitable for obtaining the kinetic parameters of the catalyzed reaction, no background products could be detected. For entries 6 in Table 1; 3, 4 in Table 2; 1, 3, 4 in Table 3; and 9 in Table 4, the background reaction was measured over a period of 5 months. Five different samples were prepared for each substrate (0.5mM in D_2O containing 10% acetonitrile and 0.1M NaCl). These samples were maintained at room temperature for a period of 5 months and then analyzed by GCMS-CI. The average rate of these measurements was calculated as the uncatalyzed rate of the deuteration reaction.

NMR kinetics: All deuteration reactions were conducted by using 0.1M of substrate and 1 mg mL^{-1} of antibody in D_2O containing 0.1M NaCl. The control samples were measured in D_2O containing 0.1M NaCl without antibody. The reaction mixtures contained 10% *v*/*v* of deuterated acetonitrile, except in the case of acetone, butanone, acetol, fluoroacetone, and 1,1,1, trifluoroacetone.

With most substrates analyzed by NMR, a peak resulting from nonexchangeable protons was used as an internal standard to calibrate the integration. This allowed monitoring of the decrease in peak area of the α-hydrogens. With substrates containing only exchangeable protons, the reaction mixture contained 0.1M of acetonitrile, and the acetonitrile signal was used as an internal standard.

The initial rate of hydrogen exchange was monitored during the first 24 h at time intervals of 2–3 h. Each sample was analyzed by 24 scans by using a relaxation delay of 10 s and file size of 16 K. In the case of cyclohexanone, due to the extremely high exchange rate, the reaction was monitored only during the first hour at time intervals of 2 min by using 16 scans with a relaxation delay of 5 s.

MS-CI kinetics: In all cases, a preliminary investigation of the reaction rate was conducted by monitoring the deuteration reaction with 2mM substrate and 0.2 mg mL^{-1} antibody. This allowed for the determination of the time needed to measure the initial rates (approximately 5% conversion) with a good signal to noise ratio. Samples of the catalyzed and uncatalyzed reactions (70 μL each) were withdrawn at different time intervals during a two day period. These samples were quenched with dichloromethane (1 mL) and shaken vigorously for 1 min to allow efficient extraction of the substrate and products to the organic solvent and to destroy all antibody activity. The organic phase was separated and analyzed by GCMS-CI.

The kinetic measurements for each substrate were carried out by monitoring a set of 15 reaction mixtures with a constant antibody concentration (0.1 to 1 mg mL^{-1} for different substrates) and various substrate concentrations (0.1mM to 10mM). All kinetic measurements were conducted in D_2O containing 0.1M NaCl and 10% acetonitrile. The reactions were allowed to reach 5% conversion, and then quenched with dichloromethane as described for the preliminary experiment. Analysis of the product distribution was carried out by MS. The rate of the uncatalyzed reaction was subtracted when necessary. The kinetic parameters k_{cat} and K_M were elucidated from Lineweaver–Burk plots.

Time resolved MS experiments: The exchange reaction was followed as a function of time. Each reaction was carried out as described above for the preliminary kinetic experiments, by using 0.1 mg mL^{-1} of antibody and a substrate concentration of 0.2mM. Samples were withdrawn at various time intervals over a period of 24 h and analyzed by MS.

Kinetic models for the polydeuteration reactions: The time-resolved MS technique allowed for the determination of each of the partially deuterated species in terms of molar ratio. This analysis reflected the product distribution as a function of time. The following integrated rate equations

were developed[18] in accordance with the consecutive pseudo first order reactions model ($[D_0] \rightarrow [D_1] \rightarrow [D_2] \rightarrow [D_3] \rightarrow [D_4]$) in which d_0^0 is the initial concentration of the molecule with no deuterium atoms and d_n refers to the concentration of molecules with n deuterium atoms:

$$d_0 = d_0^0 e^{-k_0 t}$$
$$d_1 = 4\, d_0^0 (e^{-k_1 t} - e^{-k_0 t})$$
$$d_2 = 6\, d_0^0 (e^{-k_0 t} - 2\, e^{-k_1 t} + e^{-k_2 t})$$
$$d_3 = 4\, d_0^0 (-e^{-k_0 t} + 3\, e^{-k_1 t} - 3\, e^{-k_2 t} + e^{-k_3 t})$$
$$d_4 = d_0^0 (1 + e^{-k_0 t} - 4\, e^{-k_1 t} + 6\, e^{-k_2 t} - 4\, e^{-k_3 t})$$

These equations were used to produce the lines shown in Figure 7a, representing the best fit between the calculated model and the experimental results. For the case of 1-methylpiperid-4-one (Figure 7b), another set of integrated rate equations, developed on the basis of the kinetic model shown in Scheme 2, was used.

Preparation of [D_4]cycloheptanone: Cycloheptanone (50 mmol, 5.9 mL) was dissolved in anhydrous ether (30 mL) and D_2O (20 mL) under argon. Aqueous NaOD (8 g, 40% in D_2O, 80 mmol) was added, and the mixture was stirred vigorously for 5 days. D_2O and NaCl were added, and the mixture was extracted twice with diethyl ether. The combined organic phase was dried over magnesium sulfate and filtered, and the solvent was removed under reduced pressure to yield 5 g of partially deuterated cycloheptanone (by ^{1}H NMR analysis). This procedure was repeated two more times to yield 3.9 g [D_4]cycloheptanone (isotopic purity above 98%). ^{1}H NMR ($CDCl_3$): $\delta = 1.62$ (brs).

Kinetic H/D isotope effect: Two kinetic experiments were carried out according to the general procedure described above by using GCMS. In the first experiment, nondeuterated cycloheptanone was treated with antibody 38C2 (0.1 mg mL^{-1}) in saline solution (D_2O containing 0.1M NaCl). The second experiment was carried out in the same way with [D_4]cycloheptanone in H_2O containing 0.1M NaCl. Both experiments were carried out with substrate concentrations between 0.4 and 4.5 mM. The reaction mixtures were quenched after 40 min at room temperature.

Oxygen-exchange experiments: The rate of Schiff base formation was measured by following the $^{16}O/^{18}O$ exchange rate of the carbonyl oxygen of cycloheptanone. A set of nine reaction mixtures, all containing antibody 38C2 (0.1 mg mL^{-1}) and cycloheptanone (0.4–4.0 mM) in $H_2{}^{18}O$ (containing 0.1M NaCl), was kept at room temperature. All reactions were quenched after 2 min as described above and analyzed by MS. The background reaction in the absence of 38C2 was allowed to stand for 40 minutes at room temperature before being quenched. A Lineweaver–Burk plot of the results (9 points) afforded a good linear fit with $k_{cat} = 418\,min^{-1}$ and $K_M = 21$ mM ($R^2 = 0.997$).

Enzymatic reactions: Rabbit-muscle aldolase (EC 4.1.2.13), which was purchased from Sigma in the form of a lyophilized powder, was dissolved in D_2O containing 0.1M NaCl. The kinetic measurements with this enzyme were based on ^{1}H NMR and GCMS analyses as described above for antibody 38C2.

Inhibition experiments: Preliminary inhibition experiments were conducted as described above for the preliminary kinetic experiments with the following substrates: heptan-2-one, heptanal, and cycloheptanone. The inhibitors used were: acetylacetone, cyclohex-2-en-1-one, 3-hydroxybutanone and fluoroacetone. In all cases a substrate concentration of 2 mM and an inhibitor concentration of 0.5 mM were used. The reactions were monitored over a period of 48 h.

Stereoselective deuteration of 2-methoxycyclohexanone: The deuterium exchange reaction was monitored by ^{1}H NMR (400 MHz) according to the general procedure described above. Both the antibody-catalyzed reaction and the corresponding uncatalyzed reaction were carried out for 42 days at room temperature. ^{1}H NMR (D_2O): $\delta = 4.15$ (dd, $J = 12.0$, 6.2 Hz, 1H; H_{2ax}), 3.44 (s, 1H; H_7), 2.54 (td, $J = 13.2$, 5.9 Hz, 1H; H_{6ax}), 2.44 (m, 2H; H_{6eq}, H_{3eq}), 2.11 (dm, 1H; H_{5eq}), 1.93 (dm, 1H; H_{4eq}), 1.78 (qt, $J = 13.0$, 3.59 Hz, 1H; H_{4ax}), 1.62, (qt, $J = 13.0$, 4.0 Hz, 1H; H_{5ax}), 1.55 (qd, $J = 12.4$, 4.0 Hz, 1H; H_{3ax}).

Molecular mechanics calculations: The molecular mechanics studies were accomplished by using the Discover module with CVFF force field within the Insight-II molecular simulation package. The calculations were carried out on a Silicon Graphics Indigo work station. The initial coordinates for antibody 33F12 were acquired from the Brookhaven Protein Data Bank (entry 1axt). All water molecules and all residues except those amino acids that construct the Fv region of the protein were deleted from the model. The geometry of the Fv region (Light 1–107, Heavy 5–110) was optimized with no constraints. A three-carbon unit simulating acetone was then bound to the ε-nitrogen of lysine H-93 by using the Builder module. The resultant amine was protonated to simulate the positive charge of the protonated Schiff base intermediate in the antibody-catalyzed reaction. The geometry of the structure was optimized until the root-mean-square (rms) energy gradient was less than 0.01 Kcal mol^{-1}. The larger substrate molecules were built stepwise onto the resultant scaffold. The geometry of the structures was repeatedly optimized after each modification. The dielectric constant was set to 1 throughout the calculations. Glutamate H50 was deprotonated by setting the pH to 7.0, which was the pH of all antibody-catalyzed reactions. In all calculations with the modified protein, the bound substrate as well as all six CDR regions of the Fv (H31–37, H50–65, H90–102; L-24–36, L-50–56, L-89–98) were not constrained.

Acknowledgement

We thank Dr. Noam Adir for assistance with the computational methods. We are grateful to Jacob Katzir and Alex Etinger of the Israel Center for Mass spectrometry, Technion-Israel Institute of Technology for their dedicated assistance with MS measurements. We thank the Israel Science Foundation and the Skaggs Institute for Chemical Biology for financial support. A.S. thanks the Clore Foundation for a graduate fellowship.

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Received: May 25, 2001 [F3282]