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The effect of the hydrophobic environment on the retro-aldol reaction: Comparison to a computationally-designed enzyme

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Abstract

Recent work on a computationally-designed retroaldolase RA-61 suggested that most of the rate-acceleration brought about by this enzyme was due to non-specific interactions with the aromatic substrate. To provide a benchmark for the role of non-specific interactions in this system, we measured the second-order rate constant for the amine-catalysed retro-aldol reaction of methodol in the presence of non-specific hydrophobic pockets such as micelles. We found that a simple micellar system, that consists of a positively-charged surfactant and a long-chain amine, can accelerate the retro-aldol reaction of methodol by 9,500-fold. This effect rivals the 10⁵-fold rate acceleration of RA-61. Similar results were obtained with BSA used as the catalyst, implying that the retro-aldol reaction of methodol can be greatly accelerated by non-specific hydrophobic pockets that contain an amino group.

Introduction

Computation is emerging as a powerful tool to complement experimental research in many fields of chemistry and biology, including enzymology.^{1, 2} Recent progress in the field has allowed the de-novo design of a handful of enzymes, with rate accelerations ranging from two to five orders of magnitude relative to the uncatalyzed reactions.^{3–7} Although these figures are still considerably smaller than the ones associated with rate accelerations achieved by natural enzymes, they represent significant improvements relative to the uncatalyzed reactions.

In case of the computationally-designed retroaldolase (RA), a key catalytic element in the design was an active site lysine, which provided the nucleophilic group needed for the formation of the iminium electron sink intermediate that facilitates the retro-aldol cleavage, as observed in type I aldolases.³ Functional and structural work validated the importance of this residue in catalysis.^{8, 9}

Additionally, the computational design engineered a hydrophobic pocket to lower the catalytic lysine pK_a, a positioned water molecule to facilitate proton transfer, and binding

interactions with the substrate. However, subsequent functional work on a particular retroaldolase variant (RA-61) showed that removal of residues involved in positioning of the water molecule did not affect reactivity, and that alteration of the lysine pK_a contributes only 10-fold to the rate acceleration.⁸ Thus, much of the catalytic power of RA-16 seems to derive from binding interactions with the substrate, probably in a non-specific fashion.⁸ However, it is still unclear to what extent a very simple system, with no special design, can accelerate the same reaction.

To provide a benchmark for the role of non-specific interactions in the reaction catalysed by RA-61 (shown in Figure 1), we decided to study the amine-catalysed retro-aldol reaction of 4-hydroxy-4-(6-methoxy-2-naphthyl)-2-butanone (also called methodol) in the presence of micelles. Micelles are aggregates of molecules in a colloidal solution, and often possess a hydrophobic cavity that can bind hydrophobic compounds through non-specific, entropy-driven forces (commonly referred to as 'hydrophobic interactions'). Thus, micelles can be thought as very simple hydrophobic patches in water, which resemble the hydrophobic core of enzymes stripped of any functional group. Indeed, it is known that the rates of many chemical reactions involving aromatic substrates can be accelerated by micelles,^{11, 12} including the aldol and retro-aldol reactions.^{13–16} However, no specific figures for rate accelerations were given in these reports.

In this paper, we determined the magnitude of the rate acceleration of the retro-aldol reaction of methodol that can be achieved by such simple micellar systems, where no specific interactions are made with the substrate. We also measured the rate of this reaction in the presence of the protein BSA, which is known to accelerate several chemical reactions in a non-specific manner.

Results and Discussion

To study the retro-aldol cleavage of methodol, shown in Figure 1, we took advantage of the well-known fluorescent properties of the reaction product, 6-methoxy-2-naphthylaldehyde.¹⁷ Appearance of this compound can be conveniently monitored by the quantitation of the emission at 452 nm, allowing straightforward determination of its concentration over time, as done for the reaction of RA-61 and related enzymes.^{3, 8}

We first screened different detergents to establish their effects on the amine-catalysed retro-aldol reaction of the aromatic substrate. In particular, we used detergents with different ionic characteristics, as shown in Figure 2: hexadecyltrimethylammonium chloride (CTAC, a cationic detergent), sodium dodecyl sulfate (SDS, anionic), sulfobetaine-12 (zwitterionic), Brij-35 (neutral, linear) and Tween-20 (neutral, branched). All reactions were conducted at pH 7.5 to allow direct comparison with reactions of RA-61,⁸ and the detergents were present at concentrations close to their critical micelle concentration (c.m.c). We decided to work at concentrations of detergents equal or slightly higher than their c.m.c. values because it is known that rates of micelle-accelerated reactions can drop after such value.^{12, 18} Further, we used butylamine (BuAm) as the catalytic amine because its pK_a value (10.6)¹⁹ is similar to that of lysine in solution. Whereas the presence of 5% DMSO (needed to solubilize the substrate) and of 50 mM sodium phosphate can alter the c.m.c values reported in Table 1, we did see micelle formation in the experimental conditions used, as judged by surface tension measurements (data not shown).

The results reported in Table 1 show that the cationic detergent CTAC significantly accelerates the retro-aldol reaction of methodol by about 30-fold relative to the same reaction with no detergent present, while other detergents were less effective, at least at the concentrations used in our assays. Controls showed that at concentrations lower than the

c.m.c. or in the absence of stirring CTAC and SDS did not accelerate the reaction (data not shown), suggesting the reaction takes place in or around the micelles.

We did not specifically investigate the reason for the different rate acceleration provided by different detergents, but it is known that reactions that involve neutral or negatively-charged substrates are better accelerated by positively charged micelles, and that the opposite happens in case of positively-charged substrates. This is likely due to the electrostatic attraction (or repulsion) between the micelle and the charged reactive species.^{20–23} This interpretation is consistent with the results shown in the last column of Table 1, which indicate that the rate normalized per average micelle concentration is maximal in CTAC, intermediate in the neutral detergents, and minimal in SDS and sulfobetaine, which contain a negatively-charged head (Figure 2). This result is likely due to the positive interaction between CTAC and the neutral, electron-rich amine.

Collectively, the results reported in Table 1 show that at least one detergent provides significant rate acceleration. Thus, we decided to further characterize the reaction in the presence of CTAC by determining its second-order rate constant. In doing so, we considered only the concentration of amine in solution, not its effective concentration in the micellar phase. Because we worked at amine concentrations higher than the detergent concentration, our results likely represent a lower estimate of the real second-order rate constant for the reaction in micelles. Notice that due to the aggregation number of 170 for CTAC, the average micelle concentration in solution is only $\sim 6 \mu\text{M}$.

To determine the second-order rate constant (k_2) for the retro-aldol reaction of methodol, we first varied the amine concentration, keeping the CTAC and methodol concentrations fixed at 1.0 mM and 300 μM , respectively; then, we varied the methodol concentration keeping the amine fixed at 2.0 mM. As shown in Table 2, the calculated second-order for the reaction catalysed by BuAm in the presence of CTAC is $2.5 \times 10^{-4} \text{ M}^{-1}\text{s}^{-1}$, which represents a ~ 120 -fold rate-acceleration relative to the same reaction without detergent. In comparison, RA-61 catalyses the reaction with a second-order rate constant of $0.49 \text{ M}^{-1}\text{s}^{-1}$ in the same conditions,⁸ which corresponds to $\sim 230,000$ -fold rate acceleration (Table 2). Clearly, the simple micellar system is less efficient than RA-61 in promoting the retro-aldol reaction of methodol. Nevertheless, given the simplicity of this system with no designed elements, this rate acceleration represents a significant improvement relative to the reaction in the absence of detergents.

To test whether the rate acceleration observed in solutions containing CTAC was due to hydrophobic interactions with the substrate, we measured the reactivity of 4-hydroxy-4-methyl-2-pentanone (HMP, Figure 3), an alternative substrate that lacks the highly hydrophobic naphthyl group of methodol. The same experiment was done with RA-61 to account for the role of binding interactions.¹⁷ If hydrophobic interactions were responsible for the rate acceleration of methodol, we expected significantly reduced rate acceleration in case of HMP. Because HMP is not fluorogenic, we measured its reactivity by NMR.

HMP reacts with butylamine in the presence of CTAC with a second-order rate constant of $7.0 \times 10^{-7} \text{ M}^{-1}\text{s}^{-1}$. This value corresponds to less than a 2-fold rate acceleration (the second-order rate constant for reaction of HMP in the absence of CTAC was estimated to be $5.3 \times 10^{-7} \text{ M}^{-1}\text{s}^{-1}$ from ref. 8), suggesting that, as expected, hydrophobic interactions with the substrate are important for the rate-acceleration.

As mentioned above, the rate acceleration by BuAm may represent a lower estimate of the catalytic potential of the micellar system, because not all of the amine is associated with micelles. To determine whether increasing the concentration of micellar aggregates that

contain amino groups accelerates the reaction, we measured the rate acceleration in systems containing amines with longer alkyl chains, such as octylamine (OcAm) and dodecylamine (DoAm). These amines are more hydrophobic than butylamine, but have the same pK_a in solution.²⁴ Because of their low solubilities in water, we could not directly measure the contributions of these amines to the second-order rate constant of the retro-aldol reaction in the absence of micelles. However, because their pK_a values are identical to that of BuAm, we do not expect these contributions to be different from that of BuAm.

In the presence of micelles, changes in the rate constant with these amines may arise from a different partitioning between the aqueous and the micellar system, or alternatively, from the ability of longer amines to more effectively position the amino group within the hydrophobic core of the micelle. In addition, long-chain amines pK_a 's might be shifted towards lower values in presence of CTAC, as the amine can form co-micelles with the detergent,²⁵ and changes in rate may also reflect these pK_a changes. Importantly, we measured reactivity in the presence of amines at concentrations below their c.m.c. values.²⁶

When we measured the second-order rate constant for the retro-aldol reaction of methodol using these amines, we found a significant increase as the length of the alkyl chain increases (Table 2). In particular, the rate enhancements relative to reactions with no micelles present were 710- and 9,500-fold when OcAm and dodecylamine DoAm, respectively, were used as catalysts.

To separate the partitioning effect from the change in pK_a , we first determined whether the presence of CTAC affects the pK_a value for the reactive amine species. Previous results have shown that the bulk amine pK_a is only slightly altered in CTAC, at least at the low concentrations of OcAm and DoAm used herein, while the pK_a of these amines associated with CTAC micelles might be significantly altered, even to values lower than 7.²⁵

When we measured the pH dependence for the rate constant of the BuAm-catalysed retro-aldol reaction in the presence of CTAC, we found that the pK_a of the amine is not significantly altered relative to the value in solution (Figure 5, squares): the value for the kinetic pK_a is > 9.5 , a value similar to the solution pK_a of 10.6. In contrast, the pK_a of DoAm is lowered by 2.4 units, from 10.6 to 8.2 (Figure 5, circles). In case of DoAm the values at high pH (filled circles) were excluded from the fit, because these points measured the amine-independent, hydroxide-catalysed cleavage of methodol.

In reactions with no detergent present, a shift of 2.4 units in pK_a enhances the second-order rate constant for cleavage of methodol by ~6-fold (this value was calculated using the plot shown in Figure 4B of reference 8), while in our case DoAm reacts ~80-fold faster than BuAm at pH 7.5 (Table 2). However, the results regarding the effect of changing the pK_a of the catalytic amine were obtained in the absence of detergents, and we wanted to more rigorously establish whether a similar shift in pK_a values would produce the same rate acceleration in the system containing CTAC.

To determine the effect of alteration of the pK_a of the catalytic amine in our system, we measured the reactivity of methodol in the presence of CTAC and ethylamine (EtAm, $pK_a = 10.6$), 2-fluoroethylamine (FEtAm, $pK_a = 9.0$),²⁷ or 2,2-difluoroethylamine (FFEtAm, $pK_a = 7.3$).²⁷ These amines are relatively small and thus are not likely to form co-micelles with CTAC; thus, their pK_a values are not expected to be significantly affected in the presence of CTAC. Further, they are predicted to have very similar octanol/water partition coefficients (P), suggesting that any alteration of pK_a due to interaction with the micelle should be very similar across the three amines (calculated values of log P are -0.20, -0.28, and -0.13 for EtAm, FEtAm, and FFEtAm, respectively).²⁸

As shown in Table 3, changing the amine pK_a from 10.6 to 7.3 produces less than a 3-fold change in the second-order rate constant of the reaction of methodol, which is somehow smaller than the 10-fold previously measured in aqueous solution.⁸ This could be due to an electronic requirement in the micellar reaction different than that in the absence of the detergent, but we cannot rule out that the small difference in partitioning coefficients leads to an offset that results in the reduction of the overall effect of the pK_a shift. However, the good correlation between the number of carbon atoms present in the amine and the observed second-order rate constant for the reaction of methodol in the presence of CTAC (Figure 6) suggests that most of the rate acceleration observed in the presence of CTAC is due to localization of the amine within the micelle, and that the shift in pK_a has little effect on catalysis. Using the relationship $\Delta\Delta G = -2.303 \times RT \times \text{slope}$, we can estimate a contribution of 1.6 kJ/mol, or 0.39 kcal/mol, per CH_2 unit to the rate acceleration. This result strikingly resembles the value of 0.44 kcal/mol measured in the nucleophilic displacement of long-chain *p*-nitrophenyl esters in the presence of CTAB micelles.²⁹

To provide an alternative estimation of the effect of non-specific interactions in the retro-aldol reaction of methodol, we determined to what extent bovine serum albumin (BSA), a carrier protein that possesses a lysine buried within a hydrophobic core, can accelerate this reaction. The pK_a of the buried lysine in BSA has been estimated to be 9.3,³⁰ and it is known that BSA and its homologue human serum albumin (HSA) can accelerate chemical reactions that involve hydrophobic substrates, including hydrolysis of *p*-nitrophenyl esters,^{30, 31} Kemp eliminations,^{32–35} and aldol reaction of 6-methoxy-2-naphthaldehyde and acetone.³⁶ In the latter case, the rate acceleration was about 10^5 -fold.³⁶ Because the aldol and retro-aldol reaction must proceed through the same transition state, as any forward and reverse reactions,^{37, 38} we anticipated that BSA would accelerate the retro-aldol reaction shown in Figure 1. However, while the aldol reaction of 6-methoxy-2-naphthaldehyde was followed at 37 °C in the presence of 10% acetone,³⁶ our reactions were monitored at 22 °C in the absence of acetone. Thus, the rate acceleration provided by BSA under our conditions had to be determined.

When we carried out the reaction in the presence of BSA obtained from two different commercial sources, we found a significant rate acceleration of about 8,500-fold (Table 2), comparable to the rate acceleration brought about by the simple micellar system with CTAC and dodecylamine. This reaction was abolished when acetylated BSA was used (data not shown). These results show that BSA, or a contaminant present in the BSA preparations, is capable of accelerating the retro-aldol reaction of methodol. Because two different preparations of non-acetylated BSA gave the same result and the mentioned report describing an accelerated aldol reaction in the presence of BSA,³⁶ we favour the interpretation that this effect can be attributed to the protein and not a contaminant.

Computational design has recently established a series of different retroaldolases,³⁹ one of which has been evolved through error-prone PCR and gene shuffling to produce an enzyme capable of about 8 orders of magnitude rate-acceleration.⁴⁰ Some of the structural changes that arose from such an evolution process resulted in the formation of an extended hydrophobic pocket and in the introduction of a second lysine in the active site. These results are consistent with our findings concerning the simpler micellar system, where the use of the more hydrophobic DoAm significantly increases reactivity. Because DoAm can form co-micelles with CTAC, a larger concentration of amine in solution likely increases the number of amino groups in the micelle, thereby increasing the number of amino groups capable to interact with the substrate, which ultimately results in an overall enhancement of reactivity.

Retro-aldol reaction of methodol or related compounds can be significantly accelerated by computationally-designed and evolved retroaldolases,^{3, 39, 40} catalytic antibodies,¹⁷ small peptides,⁴¹ foldamers,⁴² cyclodextrins,⁴³ and, as shown herein, micelles and BSA. Collectively, these systems possess a hydrophobic binding pocket and an amino group localized within this pocket. This rather simple design principle suggests that it is relatively easy to find a system that could be further optimized, for example by random mutagenesis,⁴⁰ to achieve enzyme-like rate accelerations for the retro-aldol reaction of methodol.

Conclusions

Our results have shown that the retro-aldol reaction of methodol can be accelerated ~10,000-fold using a simple micellar system in the presence of a long-chain amine. Similar rate acceleration can be achieved by a non-specific proteinaceous binding pocket that contains a lysine residue, such as that one of BSA. In conjunction with previous functional studies, our results support the prior conclusions that most of the rate acceleration brought about by the computationally-designed retroaldolase RA-61 can be ascribed to non-specific rather than specific interactions, which represent one of the hallmarks of enzyme catalysis.

Experimental

General

Diisopropylamine was purchased from Acros and dried and distilled over calcium hydride. All other chemicals were used as received. Brij-35, 4-hydroxy-4-methyl-2-pentanone, and 6-methoxy-2-naphthaldehyde were purchased from Alfa Aesar. Butylamine and n-butyllithium were purchased from Acros. Sodium dodecyl sulphate (SDS), sulfobetaine-12, and Tween-20 were purchased from Amresco. Hexadecyltrimethylammonium chloride (CTAC), dodecylamine, and octylamine were purchased from TCI America. Bovine serum albumin (BSA) was from NEB and Amresco (fraction V); acetylated BSA was from Promega.

Fluorescence measurements were taken using a LS-50 fluorometer from Perkin-Elmer. Appearance of 6-methoxy-2-naphthaldehyde was monitored with excitation at 330 nm and emission at 452 nm. For each detergent condition, product concentration was calibrated by using a standard curve of 6-methoxy-2-naphthaldehyde fluorescence.

NMR spectroscopy

Reactions of 4-hydroxy-4-methyl-2-pentanone were monitored by proton ¹H NMR at 25 °C on a Bruker Avance III 600 MHz NMR spectrometer equipped with a 5 mm cryogenically-cooled QCI-inverse probe on a 900 MHz system with PRESAT water suppression, with 10% D₂O added for signal lock. Solvent suppression was achieved using the excitation sculpting scheme.⁴⁴ Spectra were acquired employing 3 s recycle delays with a total of 32 transients in addition to 4 dummy scans. The acetone peak was integrated using Topspin 3.1 (Bruker-BioSpin, Billerica, MA) and compared to the internal standard given by CTAC signal resonating at 3.195 ppm (1.0 mM amine) or the internal reference at 0 ppm (2.0 and 4.0 mM amine).

Synthesis of methodol

Methodol was prepared according to a literature procedure,¹⁷ slightly modified in the final work-up. All reactions were carried out under argon. Tetrahydrofuran (THF) was dried over lithium aluminium hydride. To a solution of diisopropylamine (1.66 mL, 11.8 mmol) in dry THF (20 mL) at 0 °C was added n-butyllithium (5.15 mL, 2.29 M in hexanes) to obtain lithium diisopropylamide (LDA). After 20 min the solution of LDA was cooled to -78 °C and a solution of freshly distilled acetone (0.818 mL, 11.3 mmol) was added dropwise. After

30 min a solution of 6-methoxy-2-naphthaldehyde (1.00 g, 5.37 mmol) in dry THF (10 mL) was added dropwise via cannula. After 15 min a saturated ammonium chloride solution (2 mL) was added dropwise and the mixture was warmed to room temperature. The mixture was diluted with diethyl ether (150 mL) and the organic layer was washed with water (50 mL), dried over MgSO₄, filtered, concentrated and purified via flash chromatography (SiO₂, gradient 35% ethyl acetate/hexane to 55% ethyl acetate/hexane) to give 0.976 g (3.99 mmol, 74% yield) of methodol as a colourless solid. NMR spectra of product was identical to that reported in the literature for methodol.

General kinetic methods

All reactions were run in a closed vessel at 22 °C, 50 mM buffer (sodium phosphate, pH 7.5, unless otherwise stated) and in the presence of 5% DMSO. Reactions were stirred at 600 rpm. Detergent concentrations are given in Table 1. In reactions with amines, the amine concentrations were varied from 0.20 mM to 4.0 mM (butylamine), 0.20 mM to 2.0 mM (octylamine) and 50 µM to 200 µM (dodecylamine). Substrate concentrations were varied from 30 to 350 µM. The typical reaction volume was 5.0 mL, and a 1-mL aliquot was withdrawn at different times to quantitate the product concentration. Product concentration was determined by fluorescence or by NMR, as described above. BSA-catalysed reactions were run under the same conditions, except that BSA was 4 µM, methodol was varied from 0 to 400 µM, and no amine was present.

To determine the amines pK_a in the presence of CTAC micelles, data from the pH-rate profile were fit to equation (1), that derives from a reaction scheme that involves a single deprotonation event of the catalytic amine and a contribution from the hydroxide-catalysed reaction:

$$k_{obs} = \frac{k_{max}}{1 + 10^{(pK_a - pH)}} + A \frac{1}{10^{(-14 + pH)}} \quad (1)$$

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Retro-aldol cleavage of methodol.

Figure 2.
Detergents screened in this work.

Figure 3.
Methodol and a non-aromatic analogue, HMP.

Figure 4.
Different effect of CTAC in the BuAm-catalysed reactions of methodol and HMP.

Figure 5. pH-rate profiles for retro-aldol reactions of methodol with 50 μM DoAm (circles) or 2.0 mM BuAm (squares) and 200 μM methodol. Fitting to equation 1 gives pK_a values of (8.2 ± 0.2) and >9.5 for DoAm and BuAm, respectively. The upward curvature above pH 9 arises from hydroxide contribution.

Figure 6. Correlation between the number of carbon atoms in the amine catalyst and the logarithm of the second-order rate constant of methodol in the presence of CTAC at 22 °C. Fitting to a linear relationship gives a slope of 0.286.

Table 1

Rate of appearance of 6-methoxy-2-naphthaldehyde in the presence of different detergents, 2 mM butylamine, and 250 μ M methodol at pH 7.5.

Detergent	Rate (μ M/s)	Relative rate	Detergent concentration (mM)	c.m.c. (mM)*	Aggregation number*	Average micelle concentration (M)	Rate/[M] _{av} ($s^{-1} \times 10^{-6}$)
-(no detergent)	3.0×10^{-6}	(1)	-	-	-	-	-
Brij-35	1.5×10^{-5}	5.0	0.10	0.10	30	3.3×10^{-6}	4.5
Sulfobetaine-12	2.6×10^{-5}	8.7	4.0	4.0	55	7.3×10^{-5}	0.36
Tween-20	2.1×10^{-5}	3.7	0.10	0.060	20	5.0×10^{-6}	4.2
SDS	3.4×10^{-5}	11	9.0	7.0	62	1.5×10^{-4}	0.23
CTAC [#]	9.8×10^{-5}	33	1.0	1.0	170	5.9×10^{-6}	17

* c.m.c. value and aggregation numbers are taken from http://www.sigmaaldrich.com/img/assets/15402/Detergent_Selection_Table.pdf, except for the aggregation number of Tween-20 which is from ref. 10.

[#] Values refer to those of CTAB, which contains a bromide counterion (rather than chloride). This substitution is not expected to significantly alter the parameters.

Table 2

Second-order rate constants for the retro-aldol reaction of methodol at pH 7.5 using different catalysts.

Catalyst	k_2 (M ⁻¹ s ⁻¹)	Relative k_2
BuAm	2.1×10^{-6}	(1)
CTAC/BuAm	2.5×10^{-4}	120
CTAC/OcAm	1.5×10^{-3}	710
CTAC/DoAm	2.0×10^{-2}	9,500
RA-61	0.49*	230,000
BSA	1.8×10^{-2}	8,500

* value from ref. 8.

Table 3

Second-order rate constants for the retro-aldol reaction of methodol in CTAC in the presence of different ethylamines at pH 7.5.

Amine	p <i>K</i> _a	<i>k</i> ₂ (M ⁻¹ s ⁻¹)	Relative <i>k</i> ₂
Ethylamine	10.6	1.8 × 10 ⁻⁵	(1)
2-Fluoroethylamine	9.0	3.4 × 10 ⁻⁵	1.9
2,2-Difluoroethylamine	7.3	4.3 × 10 ⁻⁵	2.4