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SUBSTRATE-SELECTIVE MECHANISMS IN BIOCATALYSIS DEMONSTRATED WITH A VERSATILE AND EFFICIENT ALDOLASE ANTIBODY

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Abstract: A structure–activity relationship study with a series of aldol substrates shows that the mechanism of the antibody 38C2-catalyzed retrograde aldol reaction depends on the nature of the substrate With electron-deficient substrates an early deprotonation precedes the C–C bond cleavage while with electron-rich substrates the catalytic mechanism involves an initial C–C bond cleavage leading to a positively charged intermediate.
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The importance of the aldol condensation reaction stems not only from the key role it plays in biosynthesis but also from the fact that this reaction represents one of the main carbon–carbon bond forming methodologies in organic chemistry.[1] The aldol condensation is a non-trivial multistep reaction that is catalyzed by either bases or acids as well as by metals. Mechanistic studies of biocatalyzed aldol reactions are useful because they can teach us how to design efficient synthetic aldol catalysts. Studies of substituent effects could be particularly beneficial for this purpose, as they provide important information about charge development in the transition state.[2] However, since the natural aldolase enzymes are highly substrate specific, the use of a broad range of substrates to obtain linear free-energy relationships is difficult or impossible.

Fortunately, this problem has been recently removed with the report on the antibody aldolases, such as 38C2 and 33F12.[3] These antibodies, which were obtained via the reactive immunization technique, exhibit high efficiency and enantioselectivity over a broad range of ketone and aldehyde substrates.[4] Antibody 38C2 catalyzes the aldol as well as the retrograde aldol reactions under neutral pH via protonated imine and enamine intermediates (**I** and **II** in Scheme 1). This mechanism, initiated by a reaction of the substrate with a low pKa lysine residue, is also characteristic of the Type-I aldolase enzymes.

The three major events in the 38C2-catalyzed aldol fragmentation are formation of a Schiff base intermediate, deprotonation of the alcohol group by a general base and cleavage of the C–C bond. We have already shown that the reversible formation of Schiff base intermediates with this antibody may be considered as a rapid pre-equilibrium.[5] Therefore, since protonation and deprotonation of heteroatoms are known to be fast steps, the rate determining step in the retrograde aldol reaction sequence is the cleavage of the carbon–carbon bond (**I** to **II**, Scheme 1).[6] Thus, one of the key issues concerns the timing of the proton abstraction by a general base. The relative timing of these two events may define two mechanistic pathways (Scheme 2): (a) initial C–C

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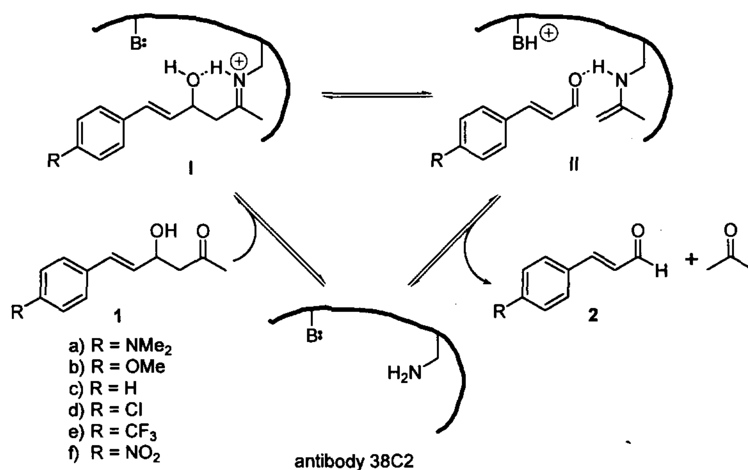
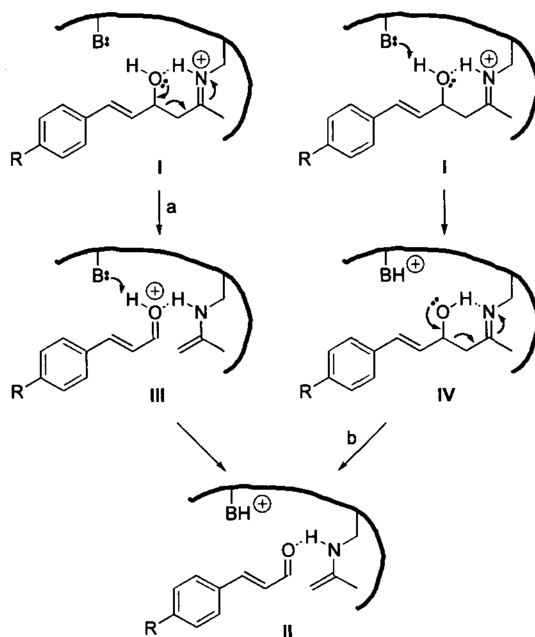
bond cleavage, leading from **I** to intermediate **III**, followed by deprotonation, and (b) deprotonation of **I** leading to intermediate **IV**, followed by C–C bond cleavage. If pathway (a) dominates then accumulation of positive charge in the transition state is expected. By contrast, if the reaction follows pathway (b) this transformation will involve a negatively charged intermediate, **IV**.



Scheme 1.



Scheme 2.

Here we use a structure–activity relationship study with a series of aldol substrates **1a–f** to show that the mechanism of the antibody 38C2-catalyzed retrograde aldol reaction depends on the nature of the substrate. With electron-rich substrates the catalytic mechanism involves a positively charged transition state while with electron-deficient substrates an early deprotonation precedes the C–C bond cleavage.

Substrates **1a–f** were prepared by an aldol condensation between the appropriate cinnamaldehydes and the lithium enolate of acetone.[7] The antibody 38C2-catalyzed retrograde aldol reactions were carried out in phosphate buffered saline, pH 7.4, 26 °C, and monitored by HPLC, exhibiting Michaelis Menten saturation kinetics.[8] The kinetic parameters (k_{cat} and K_M , Table 1) were extracted from Lineweaver–Burk analysis of the kinetic data. Figure 1 shows the correlation between $\log k_{cat}$ and the Hammett sigma substituent constant.[9]

Table 1. Kinetic parameters of the antibody catalyzed retrograde aldol reactions with substrates **1a–f**. All reactions were carried out in 50 mM PBS, pH 7.4. Sigma values were taken from ref 9.

Substituent	k_{cat} (1/min)	K_m (μM)	Sigma
NMe_2	2.52	1248	–0.63
OMe	0.4	522	–0.28
H	0.087	415	0
Cl	0.169	324	0.24
CF3	0.136	239	0.53
NO_2	0.078	140	0.81

As can be seen from Table 1 and Figure 1, the correlation of $\log k_{cat}$ and sigma in the antibody catalyzed reaction is not linear. In general, as we proceed from electron donating towards electron withdrawing substituents, reaction rates decrease. With electron-rich systems the reaction rate is sensitive to the nature of the substituent ($\rho = -2.3$). However, with electron-defficient systems reaction rates are essentially independent of the substituent ($\rho \approx 0$). The break in the Hammett line points at a change in mechanism when going from one group of substituents to the other.[10]

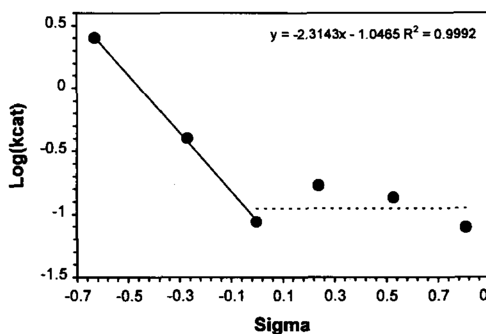


Figure 1

The negative Hammett correlation coefficient exhibited by the electron-donating substrates, **1a–c**, points at a positively charged transition state. Such a transition state is expected for the transformation of **I** into **III**, supporting the intermediacy of **III** for these substrates. A similar Hammett correlation coefficient ($\rho = -2.18$) characterizes the nucleophilic substitution of benzyl chlorides in aqueous ethanol to produce

benzyl alcohols.[11] These reactions also involve the development of a partial positive charge in the transition state. In contrast, with electron deficient substrates, **1d–f**, k_{cat} values are essentially independent of the substituent R. This observation stands in agreement with mechanistic pathway (b) (Scheme 2) where changes in the charge density in the transition state are not affected by the aromatic group for lack of conjugation. Under such circumstances ρ is expected to be essentially zero. The reason for the change in mechanism may stem from the fact that the positively charged transition state leading to intermediate **III** is no longer stabilized by the electron-withdrawing substituents on these substrates.

The above described interpretation of the experimental results for substrates **1a–c** is supported by literature precedents on the acid-catalyzed retrograde aldol reaction. For example, the fragmentation rate of 4-phenyl-4-hydroxy-3-methylbutan-2-one to benzaldehyde and butanone was qualitatively reported to be highly sensitive to the *p*-substituent on the aromatic ring.[12] This high sensitivity was explained by formation of a protonated aldehyde and an enol as the initial products. Conversely, the base-catalyzed retrograde aldol reaction is expected to be essentially independent of the nature of the substituent because changes in charge density (neutralization of the alkoxide anion) occur on an atom that is not conjugated with the substituted aromatic ring. Indeed, we measured the base-catalyzed rates of the retrograde aldol reaction with all substrates **1a–f** and found no significant differences.[13]

Interestingly, the Michaelis constant K_M is linearly correlated with the susbsituent parameter, sigma (Figure 2). The negative slope ($\rho = -0.6$) of this correlation indicates that substrates with electron withdrawing substituents on the aromatic ring are more tightly bound to the antibody active site. We therefore conclude that the interactions between the substrate's aromatic portion and the antibody contribute significantly to the binding phenomenon. It is likely that these interactions are mainly π-stacking and hydrophobic interactions with electron rich amino acid side chains, such as the aromatic portion of tyrosine and tryptophane.

In fact, the recently reported crystal structure of a closely related aldolase antibody, 33F12, and the aminoacid sequences of both 38C2 and 33F12 have confirmed that the binding pockets of these antibodies are indeed very rich in aromatic residues, and in tyrosines and tryptophanes in particular.[14] Moreover, considering the fact that the active site does not have positively charged residues other than the lysine group, the hypothesis of π-stacking and hydrophobic interactions rather than elctrostatic interaction between the protein and the substrate is further reinfoced.

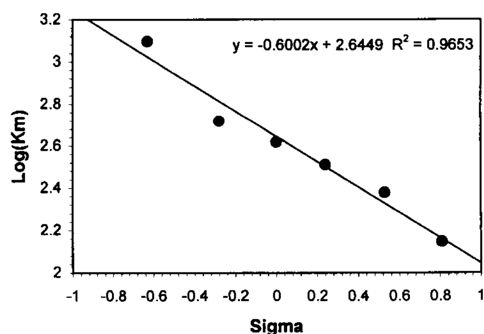


Figure 2

Positive charge stabilization by electron rich aromatic residues has been widely observed in antibodies and enzymes.[15] A recent example of this phenomenon is the active site of a polyene cyclase catalytic antibody which was designed to reduce the positive charges that develop along the reaction coordinate.[16]

In summary, we have used the Hammett correlation with a series of aldol substrates in order to learn about the mechanisms of the antibody 38C2-catalyzed retrograde aldol reaction. We found that *the mechanism depends on the nature of the substrate*. With electron-rich substrates the catalytic mechanism involves an initial C-C bond cleavage leading to a positively charged intermediate. Conversely, with electron-deficient substrates an early deprotonation precedes the C-C bond cleavage. Activation of a ketone in the form of a protonated Schiff base is analogous to its activation by direct protonation of the carbonyl oxygen. Thus, the catalytic apparatus of antibody 38C2 comprises both "acid" and base catalytic tools operating in concert. We have learned from this study that the catalytic retrograde aldol event is triggered by the "acidic" tool with one group of substrates and by the basic device with the other.

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Incumbent of the Benno Gitter & Ilana Ben-Ami chair of Biotechnology, Technion.

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8. All antibody-catalyzed reactions were performed in phosphate buffered saline (50 mM phosphate, 100 mM, NaCl pH 7.4) containing 10% acetonitrile. The progress of the reactions was monitored by HPLC and initial rates were calculated by regressional analysis. Antibody concentrations were between 4–8 uM active sites. Initial rates were measured within the first 1 h of the reaction. The uncatalyzed reactions were found to be too slow to be determined under these reaction times and conditions.

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