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Carbon−Carbon Bonds by Hydrolytic Enzymes

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Enzymes are efficient catalysts in synthetic chemistry, and their catalytic activity with unnatural substrates in organic reaction media is an area attracting much attention.[1] Protein engineering has traditionally been used to change properties of enzymes such as thermostability, chemostability, and substrate selectivity. In this communication, we show both by quantum chemical calculations and by chemical experiments that it is possible to introduce new reaction specificity in an enzyme by introducing changes among the catalytically active amino acids. We have used the well-studied protein scaffold offered by the serine hydrolase *Candida antarctica* lipase B (CALB, EC 3.1.1.3) to achieve catalytic activity for aldol reactions.

Carbon−carbon bond-forming reactions are fundamental in organic synthesis. A number of aldol reactions occur in the living cell, but no aldolase is known to follow the reaction mechanism we propose here. The aldolases in the central carbohydrate metabolism use either a Zn^{2+} or a lysine in the formation of an enolate. The enzymes are generally highly specific for the donor substrate, that is, the nucleophilic enolate, but relatively flexible with respect to the electrophile. In recent years, catalytic antibodies with an amino functionality mimicking aldolases have been developed. These have demonstrated the possibility of introducing new functions in existing protein scaffolds, and they also have the ability to accept a wider range of substrates.[2]

CALB belongs to the folding family of α/β hydrolases. This fold harbors a number of different enzyme activities such as carboxylic acid esterases, thioesterases, peptidases, dehalogenases, epoxide hydrolases, halo peroxidases, and enzymes cleaving C−C bonds.[3] A common feature of all of these reaction mechanisms involves the activation of a carbonyl function through an oxyanion hole. Another common feature is a nucleophilic elbow with a Ser, Cys, or Asp as the nucleophile. The serine hydrolases are hydrolytic enzymes which can hydrolyze both ester and peptide bonds. Their stability in organic solvents is well documented.[1] Three amino acid residues, the so-called catalytic triad Asp-His-Ser, play a key role in the catalytic process. The basic nitrogen of the Asp-His pair facilitates proton transfer during the catalytic process, while the serine assisted by the Asp-His pair functions as a nucleophile. It can be expected that the proton transfer and the charge stabilization capacity through hydrogen bonding in the oxyanion hole could be utilized for catalysis of reactions that are unnatural to the native enzyme, if the nucleophilic functionality is removed. We have investigated the ability of an enzyme where the serine is mutated for a nonpolar residue, such as alanine, to catalyze aldol additions of aldehydes and ketones.

A tentative reaction mechanism of an aldol addition involving charge stabilization by the oxyanion hole was first studied in detail by means of quantum chemical calculations in a minimal model system, as shown in Figure 1. In a previously published study, a similar model system was used to examine ester hydrolysis in the natural enzyme.[4] That study demonstrated the relevance of the minimal model system and that it could predict the effects of point mutations.

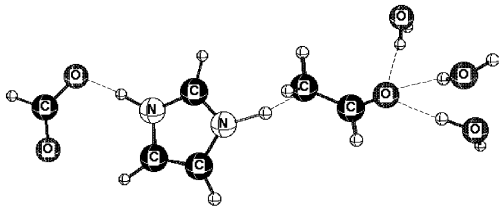

Figure 1. Model system used in the quantum chemical studies of the aldol additions, represented by the optimized structure of the first transition state (2TS). The aspartate, histidine, and oxyanion hole are represented by formate ion, imidazole, and three water molecules, respectively.

The catalytic pathways of the aldol addition of acetaldehyde and acetone were analyzed by geometry optimization of stationary points (minima and transition states) using the B3LYP method and a specially augmented 6-31G basis set. The positions of the water molecules relative to the imidazole were estimated by a comparison with representative structures from a MD simulation on the Ser105Ala mutant with an enolate intermediate bound to the active site. Limited constraints were used in the geometry optimizations to preserve this positioning. Single point calculations were performed at the B3LYP/6-31+G* and MP2/6-31+G* levels to obtain accurate energies. The Gaussian 98 software[5] was used for all quantum chemical computations.

To test the tentative reaction mechanism, experiments were run with wild-type and mutant lipases. Consequently, Ser105 in CALB was targeted by site-directed mutagenesis to create enzyme variants lacking the nucleophilic feature of the active site. Wild-type lipase and the mutants Ser105Ala and Ser105Gly were expressed and produced in recombinant yeast *Pichia pastoris*.[6] The produced enzymes were purified by hydrophobic interaction chromatography and then immobilized on the polypropylene carrier EP100.[7] The reactions were run at room temperature with 0.3−0.5 M aldehyde (propanal or hexanal) in cyclohexane with decane as the internal standard. Samples were taken for GC and GC-MS analyses using chiral capillary columns (Chrompack Chirasil-dex, 25 m × 0.32 mm i.d., d_f 0.25 μm and J&W CyclosilB, 30 m × 0.32 mm i.d., d_f 0.25 μm, respectively). The results for hexanal are shown in Figure 2.

The quantum chemical calculations support a mechanism where the enolate intermediate is stabilized in the oxyanion hole, according to the two-step mechanism presented in Scheme 1. In the first step, the Asp-His dyad and the oxyanion hole stabilize the formation of an enolate ion. This ion is subsequently attacked by a second substrate molecule. In this second step, a carbon−carbon bond is formed, and the proton is transferred to the oxygen in a concerted

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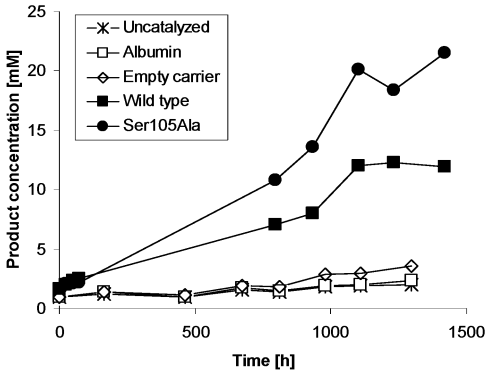

Figure 2. Progress curve of aldol addition product from hexanal catalyzed by CALB wild type and one serine mutant.

Scheme 1. Two-Step Mechanism of the Aldol Addition Reaction of Acetaldehyde (R = H), or Acetone (R = CH_3), in the Lipase Mutant Suggested by the Calculations

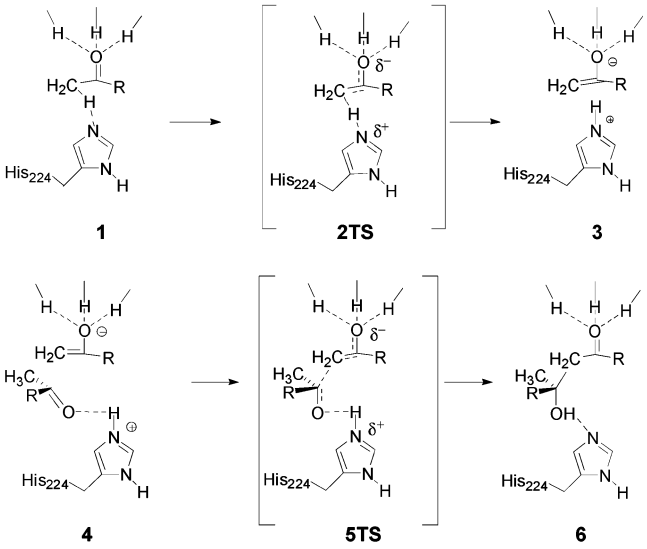

Table 1. Relative Energies (kcal/mol) of the Stationary Points in the Aldol Addition[a]

acetaldehyde	B3LYP/ 6-31+G*	MP2/ 6-31+G*	acetone	B3LYP/ 6-31+G*	MP2/ 6-31+G*
1	0.0	0.0	**1**	0.0	0.0
2TS	11.2	14.5	**2TS**	14.5	17.4
3	7.1	9.8	**3**	11.5	13.7
4	6.8	3.2	**4**	12.3	7.9
5TS	15.1	13.2	**5TS**	17.9	13.0
6	−3.7	−13.7	**6**	−0.2	−13.0

[a] The energies of **4**, **5TS**, and **6** have been corrected for the desolvation of the second substrate. Solvation energies in acetone were calculated at the PCM-B3LYP/6-31+G* level.

process. The energies of stationary points relative to the near attack complex **1** are presented in Table 1. The MP2/6-31+G* calculations predict the initial proton transfer to be rate-determining, whereas the B3LYP/6-31+G* values suggest that the addition step determines the rate. However, both levels of theory predict the overall activation energy to be close to 15 kcal/mol. This indicates that the enzyme has the potential to catalyze the aldol addition reaction with reaction rates close to those of natural enzymes.

The experiments clearly showed an increased reaction rate when the aldol reaction was catalyzed by the mutant enzymes as compared to the wild-type lipase. From the data in Figure 2, specific activities were calculated. The mutants Ser105Ala and Ser105Gly (data not shown) exhibited similar specific activities (0.0013 μmol min^{-1} mg^{-1}), 4 times higher than that of wild-type lipase and 300 times higher than those of albumin and carrier (without protein), as calculated after subtractions of the uncatalyzed reaction rate (Figure 2). Similar results were obtained using propanal (data not shown).

To further establish that the reaction takes place in the enzyme's active site, control reactions were run with covalently inhibited wild-type CALB using the inhibitor methyl *p*-nitrophenyl *n*-hexylphosphonate.[7] The inhibited enzyme did not show any lipase activity, and the specific activity for the aldol reaction was as that of albumin. Furthermore, altered diastereoselectivity from that of the uncatalyzed reaction (2:1 in favor of the first-eluting diastereomer) was observed by mutated lipase (1:3.2 in favor of the other diastereomer). These results clearly show that the active site is responsible for the aldol addition reaction.

In conclusion, we have shown the possibility to engineer the hydrolytic reaction specificity of CALB for catalysis of aldol reactions. The quantum chemical calculations predicting the possibility of aldol reactions could be experimentally confirmed. The reaction rate was on the same order as that of catalytic antibodies.[2b] Assuming that all protein is active, the Ser105Ala mutant has a specific rate of 65 day^{-1}. The increased rate expressed by the mutant enzymes as compared to that of wild type may be due to the lack of the hemiacetal formed by serine attacking the substrate in the wild type. The reactions may be possible to carry out both in organic as well as in aqueous solvents, and we expect that the new catalytic activity, harbored in the stable protein scaffold of the lipase, will allow aldol additions of substrates, which cannot be reached by traditional aldolases.

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References

(1) (a) Rogers, R. S. *Chem. Eng. News* **1999**, *19 July*, 87−91. (b) Patel, R. N., Ed. *Stereoselective Biocatalysis*; Marcel Dekker: New York, 2000. (c) Faber, K. *Biotransformations in Organic Chemistry*; Springer: Berlin-Heidelberg, 2000. (d) Bornscheuer, U. T.; Kazlauskas, R. J. *Hydrolases in Organic Synthesis, Regio- and Stereoselective Biotransformations*; Wiley-VCH: Weinheim, 1999.

(2) (a) Wentworth, P.; Janda, K. D. *Curr. Opin. Chem. Biol*. **1998**, *2*, 138. (b) Hoffmann, T.; Zhong, G.; List, B.; Shabat, D.; Anderson, J.; Gramatikova, S.; Lerner, R. A.; Barbas, C. F., III. *J. Am. Chem. Soc.* **1998**, *120*, 2768−2779.

(3) (a) Ollis, D. L.; Cheah, E.; Cygler, M.; Dijkstra, B.; Frolow, F.; Franken, S. M.; Harel, M.; Remington, S. J.; Silman, I.; Schrag, J.; Sussman, J. L.; Verschueren, K. H. G.; Goldman, A. *Protein Eng.* **1992**, *5*, 197−211. (b) Holmquist, M. *Curr. Protein Pept. Sci*. **2000**, *1*, 209−235.

(4) Hu, C.-H.; Brinck, T.; Hult, K. *Int. J. Quantum Chem*. **1998**, *69*, 89−103.

(5) Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Zakrzewski, V. G.; Montgomery, J. A., Jr.; Stratmann, R. E.; Burant, J. C.; Dapprich, S.; Millam, J. M.; Daniels, A. D.; Kudin, K. N.; Strain, M. C.; Farkas, O.; Tomasi, J.; Barone, V.; Cossi, M.; Cammi, R.; Mennucci, B.; Pomelli, C.; Adamo, C.; Clifford, S.; Ochterski, J.; Petersson, G. A.; Ayala, P. Y.; Cui, Q.; Morokuma, K.; Malick, D. K.; Rabuck, A. D.; Raghavachari, K.; Foresman, J. B.; Cioslowski, J.; Ortiz, J. V.; Stefanov, B. B.; Liu, G.; Liashenko, A.; Piskorz, P.; Komaromi, I.; Gomperts, R.; Martin, R. L.; Fox, D. J.; Keith, T.; Al-Laham, M. A.; Peng, C. Y.; Nanayakkara, A.; Gonzalez, C.; Challacombe, M.; Gill, P. M. W.; Johnson, B. G.; Chen, W.; Wong, M. W.; Andres, J. L.; Head-Gordon, M.; Replogle, E. S.; Pople, J. A. *Gaussian 98*, revision A.7.; Gaussian, Inc.: Pittsburgh, PA, 1998.

(6) Rotticci-Mulder, J.; Gustavsson, M.; Holmquist, M.; Hult, K.; Martinelle, M. *Protein Expression Purif.* **2001**, *21*, 386−392.

(7) Rotticci, D.; Norin, T.; Hult K.; Martinelle, M. *Biochim. Biophys. Acta* **2000**, *1483*, 132−140.

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