Electronic Supplementary Material (ESI) for Chemical Communications

Rational engineering of CALB for selective monoacylation of diols

Supporting information

Molecular modelling: All modelling were performed using YASARA[1] software and AMBER99 force field. A simulation cell of 10 Å and periodic wall was used. Molecular dynamic were run with the temperature control set at rescale velocities and the temperature at 298 K. The chrystal structure of *Candida antarctica* lipase B, 1tca, was retrieved from the RCSB Protein Data Bank. Hydrogens to the structure, followed by minemizations a series of iterative steps according to a previously defined protocol.[2] The tetrahedral intermediate was built onto the serine 105 residue in the active site. Four water molecules were removed to make room for the tetrahedral intermediate in the active site. Energy minimization followed by 1 ps dynamic and another round of energy minimization were performed with only the tetrahedral intermediate free and the rest of the enzyme fixed. Energy minimization followed by 1 ps molecular dynamic and another round of energy minimization were performed with only the tetrahedral intermediate free and the rest of the enzyme fixed. Thereafter, energy minimization followed by 10 ps molecular dynamic and another round of energy minimization were performed on the whole enzyme. The final structures were used for visual interpretation of the transition states of the modelled reactions.

Wild-type CALB: Immobilized wild-type CALB was obtained from in-house productions from previously described experiments.[3] Enzyme concentrations were determined by active site titration according to a previously defined protocol using the inhibitor methyl 4-methylumbelliferyl hexylphosphonate, fluorescent upon inhibition.[3] The level of inhibited enzyme was adjusted after determining remaining enzyme activity, measured after inhibition. A reaction solution was prepared containing 1-butanol (100 mM), decane (20 mM) and vinyl butyrate (1 M) dissolved in MTBE. Reaction solution (4 mL) was added to inhibited wild-type (19 mg) and T40A (20 mg) CALB for determination of the degree of inhibition. 5 Aliquots were taken from each reaction at reaction times between 50 and 350 seconds. Analyses were performed on GC. The reaction was repeated with wild-type CALB which had not been subjected to inhibition.

Preparation of CALB A282L mutant and immobilization: Freeze dried A282L mutated CALB was obtained from in-house productions from earlier experiments.[4] Approximately 2 mg of the freeze dried A282L CALB was dissolved in 10 ml 100 mM $K_xH_yPO_4$, *p*H 7.0. The enzyme was immobilized on 1 g of Accurel beads according to the general protocol described under A. Materials and Methods, 1. Enzyme Immobilisation, with the following exceptions: The immobilisation solution was left in end over end in room temperature for 24 hours. After immobilisation the beads were washed with NH4Ac and dried under vacuum overnight. Thereafter, the enzyme beads were subjected to an additional wash in 50 ml 10 mM MOPS, *p*H 7.5, for 24 hours in end over end in room temperature and dried under vacuum overnight. The beads were dried under vacuum overnight and stored over lithium chloride. The amount of enzyme loaded on the beads was analyzed by active site titration, as described above for wild-type CALB. An enzyme load of 0.17% (w/w) was confirmed.

Preparation of CALB A281V and A281E mutant and immobilization: The A281V and A281E mutated CALB variants were prepared by overlapping extension PCR using pET22b+ vector obtained from earlier studies.[5] In the first step forward and reverse PCR reactions were done for the A281V and A281E mutated CALB. For the forward reaction, reverse Not primer was used together with 5' CGGCTGCGCTCCTGGCTCCTGTAGCTG 3' for the A281V variant and 5' CGGCTGCGCTCCTGGCTCCTGAGGCTG 3' for the A281E variant. For the reverse reaction, forward NocI primer was used together with 5' CTGCAGCTACAGGAGCCAGGAGCGCAG 3' for the A281V variant and 5' CAGCCTCAGGAGCCAGGAGCGCAGCCG 3' for the A281E variant. Second PCR reactions were carried out for the A281V and A281E mutated CALB. 1 µl from the forward and 1 µl from the reverse reaction were used together with forward NcoI and reverse Not primers. The PCR temperature program was identical for both steps. 98 °C for 30s, 30 cycles with 10 s at 98 °C, 15 s at 65 °C and 20 s at 72 °C and 5 min 72 °C. Forward NcoI and reverse Not primers were from Thermo Scientific. The PCR products and pET22b+ were digested using FastDigest restriction enzymes NotI and NcoI, from Fermentas. The PCR products and linearised pET22b+ were purified on a 1% agarose gel and extracted using the QIAquick Gel Extraction Kit from QIAGEN. The PCR products were ligated back into the linearised pET22b+ using T4 DNA ligase from Fermentas. Incubations were done in room temperature for 1 hour and 20 min. Electro competent Rosetta strain *Escherichia coli* cells were transformed with the ligation solution. 1 µl of the solution containing A281V CALB and 0.5 µl of the solution containing A281E CALB were added to the cells. The mutation was confirmed by sequencing using T7 and T7-term primers. Expression and purification of the protein was done according to a predefined protocol for periplasmic expression of CALB using *Escherichia coli* strain Rosetta.[5] The purified enzymes were subjected to buffer exchange using PD-10 columns from GE healthcare. The buffer was changed into 10 mM MOPS, *p*H7.2, which was used as immobilisation buffer. For the A281V and A281E mutated CALB, 1.5 g and 2 g Accurel enzyme carrier beads were used, respectively. Prior to the immobilisation, he beads were washed for 2 hours in 95% ethanol and then washed in fresh ethanol. The ethanol was washed off with 3 x 10 ml immobilisation buffer, 10 mM MOPS, *p*H 7.2. The immobilisation was done in 10 ml 10 mM MOPS, pH 7.2, left in end over end in room temperature overnight. Thereafter, the beads were dried under vacuum overnight and stored over saturated LiCl. The amount of

enzyme loaded on the beads was analyzed by active site titration, as described above for wild-type CALB. Enzyme loads of 0.0084% and 0.0054% (w/w) were confirmed for the immobilisation of Ala281Glu and Ala281Val, respectively. The low amount of immobilized enzyme required a series of measurements with various amounts of enzyme for reliable values.

Determination of enzyme selectivities and specificities: Reactions were run with two different straight chain primary diols: 1,2-ethanediol, ≥99.5% from MERCK and 1,4-butanediol, ≥99% from Aldrich. Two different acyl donors were used: vinyl acetate, ≥99% from Fluka and vinyl butyrate, >99% from Fluka. Decane, ≥99% from Fluka, was used as internal standard. MTBE, 99.5 % from Lab-Scan was used as solvent. Each diol was dissolved to a concentration of 100 mM together with 1 M of the acyl donor and 20mM decane in MTBE. Reactions were started by adding 3 ml reaction solution to a specific amount of enzyme carrier beads. Different amounts of enzyme carrier beads were used for the different reactions. For reactions with 1,2-ethanediol and vinyl acetate, 19.7 mg wild type, 22.5 mg A282L, 50.5 mg A281V, and 45.5 A281E CALB were used. For reactions with 1,2-ethanediol and vinyl butyrate, 19.3 mg wild type, 19.7 mg A282L, 40.5 mg A281V, and 40.6 A281E CALB were used. For reactions with 1,4-butanediol and vinyl acetate, 17.6 mg wild type, 22.5 mg A282L, 69 mg A281V, and 54 mg A281E CALB were used. For reactions with 1,4-butanediol and vinyl butyrate, 20.3 mg wild type, 25.4 mg A282L, 52.2 mg A281V, and 53 mg A281E CALB were used. The enzymes were evenly distributed in the reaction vials by magnetic stirrers. The vials were kept in a 29° C water bath during the reactions for temperature control. Aliquots of 10 µl were taken, filtered through wool in a Pasteur pipette and diluted with 90 µl MTBE prior to analysis. The decane used as internal standard was present in the reaction solution throughout both reaction and analysis. 20 mM decane was used as internal standard in the reaction mixtures together with 100 mM diol. Aliquots were taken during the reaction by taking 10 µl of reaction sample. The samples were filtered though wool together with 90 µl MTBE, thereby any remaining enzyme was removed and the reaction samples were diluted by a factor of 10 prior to analysis. The reactions catalyzed by the A281V-mutant were rerun with higher amounts of enzyme carrier beads to test selectivity determinations. For the reactions with 1,2-ethanediol and vinyl acetate, 121 mg beads with A281V CALB were used. For the reactions with 1,2-ethanediol and vinyl butyrate, 123 mg beads with A281V CALB were used. For the reactions with 1,4-butanediol and vinyl butyrate, 128 mg beads with A281V CALB were used.

Analysis: The reactions for selectivity and activity determinations and measurement of remaining activity after inhibition stydies were analyzed on GC, Hewlett Packard 5890 series II. Two different 25 m x 0,32 mm WCOT fused silica columns were used, depending on the different substrates and products that were analyzed. A polar GC-column with CP Chirasil-Dex CB coating was used for the reactions with 1,2-ethanediol. A non-polar GC-column with CP-SIL 5CB coating was used for the reactions with 1,4-butanediol. The inhibition studies for the active-site titration were analyzed by fluorescence using a Perkin Elmer LS 50 B fluorimeter.

References

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