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RESEARCH ARTICLE

From Synthetic Chemistry and Stereoselective Biotransformations to Enzyme Biochemistry – The Bioorganic Chemistry Group at the Budapest University of Technology and Economics

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Abstract

The activity of Bioorganic Chemistry Group (BCG) within Department of Organic Chemistry and Technology at Budapest University of Technology and Economics is related to various areas of synthetic chemistry, biotechnology and enzymology. This review gives an overview on the research activity of the group covering development of synthetic organic chemistry methods; stereoselective biotransformations with lipases, ammonia-lyases and further biocatalysts in batch and continuous-flow reactions; novel enzyme immobilization methods; and enzyme structural and mechanistic studies by experimental and computational techniques.

Keywords

synthetic organic chemistry, stereoselective biotransformation, continuous-flow reaction, lipase, ammonia-lyase, enzyme immobilization, enzyme structure, enzyme mechanism, QM/MM calculation

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1 Introduction

1.1 Scientific background of the Bioorganic Chemistry Research Group

The activity of Bioorganic Chemistry Group (BCG) of Department of Organic Chemistry and Technology at Budapest University of Technology and Economics is related to various areas of synthetic chemistry, selective biocatalysis [1] and enzymology with major emphasis on development of novel tools for stereoselective synthesis [2].

One of the main challenges facing organic chemistry is the rational synthesis of an ever growing number of complex, optically active natural products and their analogues [3]. According to the regulation of FDA production of chiral drugs, agrochemicals, fine chemicals has been allowed in enantiomerically pure form, because it often happens that only one of the two enantiomers shows the required therapeutical effect [4].

Synthetic application of novel biocatalytic methods is a continuously growing area of chemistry, microbiology and genetic engineering, due to the fact that biocatalysts are selective, easy-to-handle and environmentally friendly [1,5,6] Biotransformations – catalyzed by biocatalysts – are already being used industrially to manufacture a wide range of products, including drugs, agricultural chemicals, organics, fine chemicals and plastics [7,8]. Pharmaceutical companies as well as chemical and food industries using biocatalysts are growing and developing due to the increasing demand for new, efficient and low cost processes with low environmental and energy burden.

Because the immobilized biocatalysts are recyclable, storable and easy to handle, immobilization is an important trend in biotechnology [9-14]. Selectivity, specificity, catalytic activity and enzyme stability are key factors affecting the efficiency of biocatalysts [1-8]. Immobilization can often enhance these key properties and also enabling recovery and reuse [9-14]. In connection to continuous-flow processes, enzyme immobilization is of primary importance because immobilized enzymes may show enhanced stability, activity and selectivity as compared to their native form. Although it

is well known that the state of the enzyme − e.g. the mode of immobilization − may significantly influence the properties of the biocatalysts [9-14] there are only a few immobilized forms of enzymes commercially available.

Although a large number of biocatalytic reactions has been studied – particularly in academia – the operational parameters of many biocatalysts described to date have been rather suboptimal. Therefore, there is still an urgent need to promote the viability of biocatalytic processes, thus creating the background for their faster transfer to industrial scale. Even today, the chemical industry is working mostly on a fixed, batch based single step infrastructure. As a result, scale up is time consuming resulting in deviation from environmental and economic efficiency. Although continuous processes could provide solutions to this problem, the main advantages of the flow-through approach [15-20] – such as facile automation, reproducibility, and safety – have not been fully exploited so far. Some of the chemical industry companies, however, started to implement measures to increase effectiveness through the advantages of continuous-flow processes [21].

There is an extremely rapid development in understanding enzyme catalyzed processes at molecular level. This enormous expansion is due to the recent developments in molecular genetics (PCR, sequencing, novel expression systems and genome projects), structural biology (protein crystallography, liquid-phase NMR protein structure determinations) and bioinformatics (protein modeling, ligand-docking and quantum chemical calculations within the active sites). The better understanding of the nature of biocatalysis can have a positive impact on the synthetic methods, or even human applications of proteins and thus can broaden the application of the biotransformation at novel areas [22-24].

1.2 Facilities and project history of the Bioorganic Chemistry Research Group

The Bioorganic Chemistry Research Group was established in 2005 at the Department of Organic Chemistry and Technology, Budapest University of Technology and Economics by Prof. László Poppe, Dr. József Nagy and Dr. Gábor Hornyánszky. In 2012, Zoltán Boros joined as assistant lecturer to our group. Furthermore, 8-10 PhD students and 30-40 undergraduate students contribute to our research results in each year.

At BME about 220 m^2 well-equipped laboratory space is available for bioorganic chemistry research (with equipment for synthetic, biotransformation and basic protein works; 2 HPLC's, 3 GC's, 3 UV-VIS, FTIR, gel-electrophoresis, bench-top centrifuges, pumps and continuous-flow reactors). In addition, high performance PC's and molecular modeling program packages are available for protein modelling and QM/MM investigations. Thus, BCG has the proper infrastructure to synthesize various substrates / substrate analogues / inhibitors for different enzymes, to perform enzyme immobilization, to carry out biotransformations even in high pressure continuous-flow reactors, to achieve various analytical tasks (enzyme activity, enantiomeric purity) or to investigate enzymes by computational methods.

The FLOWREAC project "*Development of environmental friendly technologies by applying and designing high capacity lab flow reactors*"(NKFP 07 A2 FLOWREAC, National Office for Research and Technology, Hungary, 2008-2010; with ThalesNano Inc, Hungary) allowed BCG to utilize the continuous-flow technology and to gain expertise in enzyme-immobilization.

The OTKA NN project "*Immobilized wild type and mutant ammonia lyases and amino mutases for production of β- and β-phenylalanine analogs*" (OTKA NN-103242, Hungarian Scientific Research Fund, 2012-2015) in co-operation with Dr. Csaba Paizs (Cluj-Napoca, Romania, project PN-II-ID-PCE-2011-3-0799) aims processes for novel enantiopure unnatural β- and β-amino acids with the aid of native and immobilized phenylalanine ammonia-lyases and/or with phenylalanine 2,3-aminomutases (i.e. with the so called MIO-enzymes [25-27]).

The participation of Prof. L. Poppe in of EU COST (Actions "*Cascade Chemoenzymatic Processes - New Synergies Between Chemistry and Biochemistry*" CM 0701, 2010-2012, as MC member of Hungary; and "*Systems Biocatalysis*" CM 1303, 2013-2017, as MC member of Hungary and leader of Working Group 5) has also contributed to the extension of knowledge of BCG in biocatalysis.

The present activites of BCG are supported also by KMR projects "*Development of novel types of security systems for origin and uniqueness check against forgery based on complex coding procedures*" (KMR_12-1-2012-0051, Hungarian Research and Technology Innovation Fund, 2012-2015; industrial partner: Pharmapress Inc, Hungary) and "*Novel support family and automated method development system to develop rapid affinity purification technology of recombinant proteins*" (KMR_12-1-2012-0140, Hungarian Research and Technology Innovation Fund, 2013-2015; industrial partner: Fermentia Ltd, Hungary).

Further aid to our activity is the 3DSTRUCT project "*3D Structural investigation of medical/biotechnological target proteins and inhibitor candidates: crystallization and crystallography*" (National Office for Research and Technology, Hungary; consortium leader: Prof. B. G. Vértessy, with participation of Prof. L. Poppe) which established protein crystallization and protein X-ray structure determination facilities at BME.

Last but not least the novel supercomputing facility at BME which is based on the project (TÁMOP-4.2.2/B-10/1-2010-0009; 2011–2013, National Office for Research and Technology, Hungary) helps to perform the planned sophisticated biocomputing tasks on a high performance computing environment.

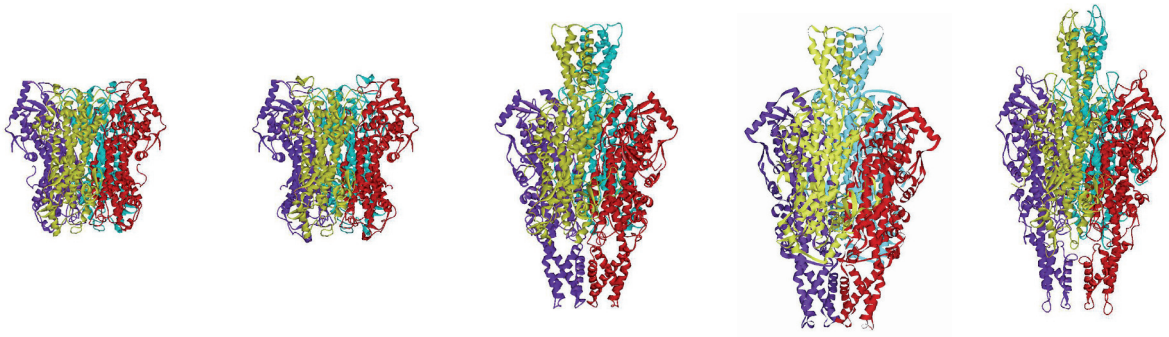


Fig. 1 Tetrameric structures of several MIO-containing enzymes from the protein databank [(A) *Pseudomonas putida* HAL; (B) *Anabaena variabilis* PAL; (C) *Rhodosporidium toruloides* PAL; (D) *Taxus canadensis* PAM and (E) *Petroselinum crispum* PAL]

2 From synthetic methods to enzyme mechanisms – Bioorganic Chemistry research at BME

The activity of Bioorganic Chemistry Group (BCG) covers a wide range of topics from development of novel synthetic methods, mostly using biocatalysis [1] for stereoselective synthesis [28] *via* novel enzyme immobilization methods and stereoselective hydrolase-catalyzed processes in continuous-flow mode [29] to sophisticated QM/MM calculations for exploring enzyme mechanisms [30]. The following parts of this review summarize the various activities of BCG from 2008 until recent days.

The most studied enzymes by BCG are lipases and the so-called MIO enzymes. Lipases (EC 3.1.1.3) are essential in the digestion, transport and processing of lipids (e.g. triglycerides, fats, oils) in most, if not all, living organisms. Lipases are versatile biocatalysts which can provide regio- and enantioselectivity in a wide range of reactions [7,31]. Consequently, they are one of the most extensively utilized biocatalysts in organic synthesis [32,33].

Among the so-called MIO-enzymes containing 3,5-dihydro-5-methylidene-4*H*-imidazol-4-one (MIO) as prosthetic group [25,26] (Fig. 1), phenylalanine and tyrosine ammonia-lyases (PAL: EC 4.3.1.24 and EC 4.3.1.25, and TAL: EC 4.3.1.24) can be utilized for synthesis of enantiopure L-β-amino acids [27]. Phenylalanine and tyrosine 2,3-aminomutase (PAM: EC 5.4.3.11 and TAM: EC 5.4.3.6) can extend the possibilities towards enantiopure β-amino acids.

2.1 Novel synthetic methods

Researchers of BCG were involved in development of a wide range of novel synthetic methods using purely chemical methods or even integrated chemoenzymatic processes (Fig. 2).

A recent observation revealed the potential of an ionic liquid (Fig. 2A) as an organocatalyst [34]. It was found, that carbene concentration in 1-ethyl-3-methylimidazolium acetate ionic liquid is sufficiently high to act as a catalyst in benzoin condensation, hydroacylation and also in oxidation of an alcohol by using CO_2 and air.

A substrate analogue, α,β-methylene-dUDP (Fig. 2B), was synthesized to characterize its interaction with dUTPase [35]. Crystal structure studies with *E. coli* dUTPase:α,β-methylene-dUDP and *E. coli* dUTPase:dUDP:Mn complexes revealed that methylene substitution at the α–β bridging position within the phosphate chain of dUDP profoundly perturbs ligand accommodation into the dUTPase active site.

As a part of the synthetic efforts towards efficient and stereoselective β-lactam-syntheses, a stable hydrate of a β-lactamcarbaldehyde (Fig. 2C) was prepared by hydrolysis of the corresponding imine [36]. The structural features of a stable aldehyde hydrate without a proximal electron withdrawing group were characterized by 2D NMR spectroscopy, thermogravimetry and chemical reactions.

Integrated enzymatic production of specifically structured lipid and phytosterol ester compositions was developed using an enzymatic one-pot process in organic-solvent-free medium starting from a mixture of phytosterol, caprylic acid and sunflower oil by the aid of (Fig. 2D) [37]. Two air-dried solid state fermentation (SSF) lipase preparations of *Aspergillus oryzae* NRRL 6270 and *Aspergillus sojae* NRRL 6271 were efficient as biocatalysts in this process.

To prepare racemic amine substrates **1a-i** for kinetic resolutions, novel one-step reductive amination of ketones were developed by transfer hydrogenations in batch and continuous-flow mode (Fig. 2E) [38]. The low-cost Zn dust method proved to be effective on ketones with carbonyl groups at the benzylic side-chain position of aromatic systems, whereas 10% Pd/C was an efficient catalyst even in a continuous-flow reactor for the reductive aminations of carbonyl groups non-conjugated with any π-system.

2.2 Stereoselective biotransformations and novel enzyme immobilization methods

One of BCG's major interest is to develop novel forms of immobilized enzymes and to perform kinetic resolutions or enantiotope selective biotransformations with them, preferably in continuous-flow mode (Fig. 3). The FLOWREAC project

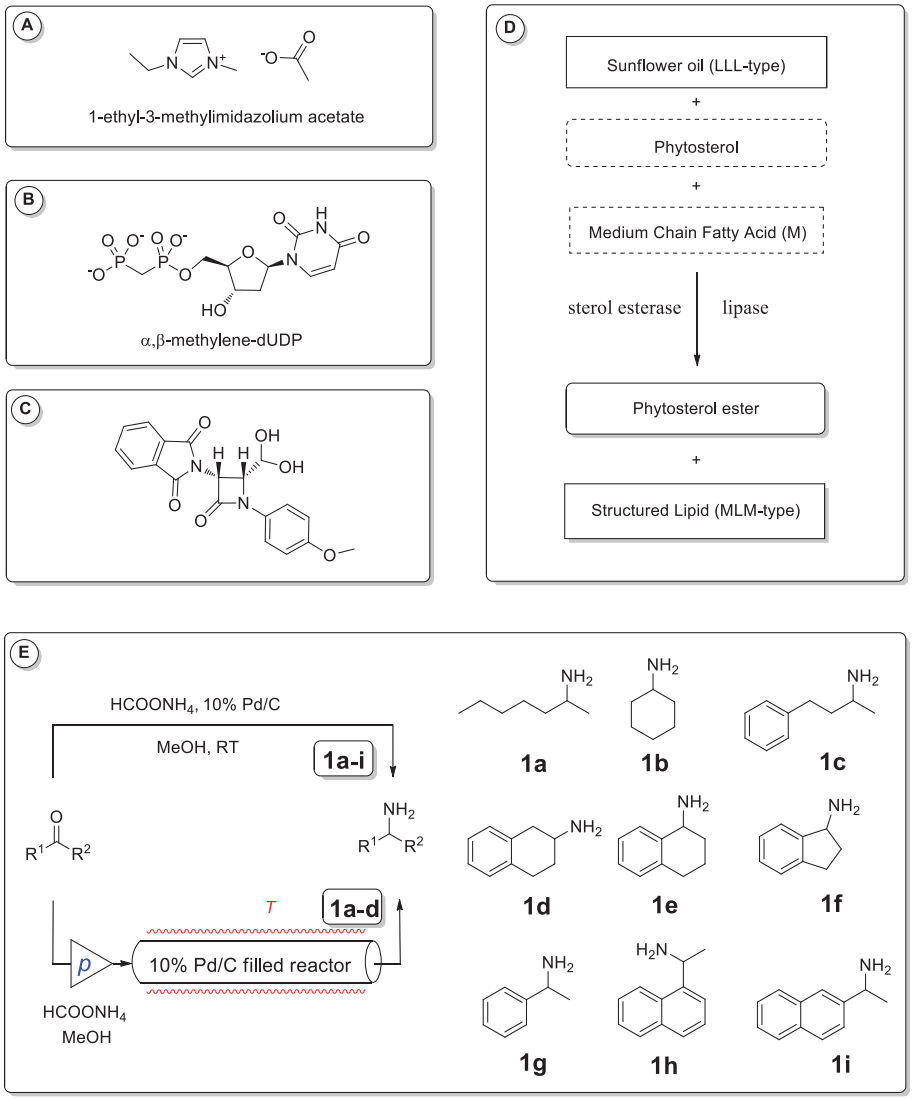
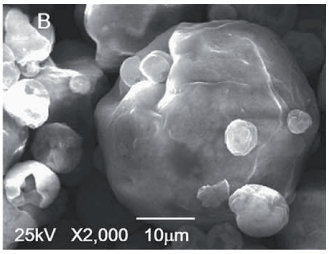
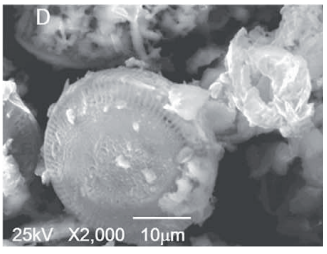


Fig. 2 Novel synthetic processes and compounds

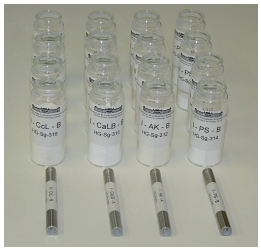
A) free lipase AK (SEM)



B) sol-gel immobilized lipase AK (SEM)



C) CatCart columns and sol-gel immobilized lipases



D) multicolumn reactor

Fig. 3 From free enzymes to continuous-flow bioreactor. (A) lipase AK (SEM); (B) sol-gel lipase AK (SEM); (C) CatCart columns filled with sol-gel lipases AK and PS, CcL, CaLB; (D) a continuous-flow multicolumn reactor

allowed us to utilize the continuous-flow technology of ThalesNano and gain expertise in enzyme-immobilization (Fig. 3B and 3C) and in development of continuous-flow biotransformations (Fig. 3C and 3D).

2.2.1 Novel enzyme immobilization methods

Immobilization of enzymes (Fig. 4) may significantly influence the beneficial properties of the biocatalysts [9-14]. All methods have favorable and disadvantageous properties and the balance between these may vary depending on the substrate and reaction conditions. Our recent investigations with the commercial and own made immobilized biocatalysts revealed that due to the large variations in substrates and conditions there is no "ideal" immobilization of a functional enzymes.

Enzyme immobilization on a solid support is one of the methods to use enzymes in industry because it confers easy

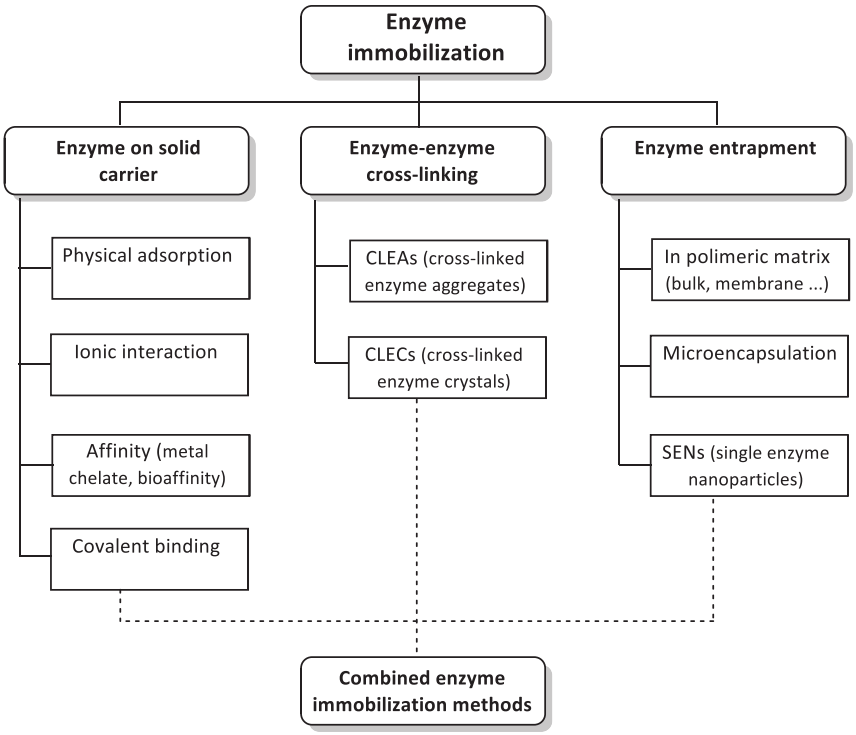


Fig. 4 Methods of enzyme immobilization

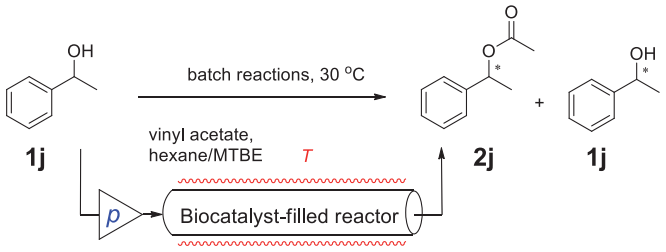


Fig. 5 Kinetic resolution of 1-phenylethanol as test reaction for the immobilized lipases

product separation and enhanced thermal and pH stability [9-14]. Ideal properties for immobilization support include chemical and mechanical stability, hydrophilicity, inertness toward enzymes, ease of derivatization, biocompatibility, resistance to microbial attack, and a large surface area. Among the many organic and inorganic supports our group focused mostly on mesoporous silica materials (MPs) for use in enzyme immobilization [39-41]. MPs have large surface areas with relatively large-pore diameters (5–100 nm) that match the sizes of the enzymes. The surfaces of MPs can also be functionalized, thus changing their properties [39-41].

Lipase adsorption on solid support is the most convenient, because the procedure is very simple and no modification of enzymes is required [42]. In addition, the surface functionalized adsorbents may be used in downstream processes even for protein separations [39]. To characterize lipase immobilization by adsorption on surface modified MPs [39-41], the kinetic resolution of 1-phenylethanol **1j** was selected as test reaction (Fig. 5).

A series of novel surface modified silica gels were systematically studied as carriers of lipases A and B from *Candida antarctica* (*Ca*L A and *Ca*L B) [39], and lipases from *Rhizomucor miehei* (*Rm*L) and *Thermomyces lanuginosus* (*Tl*L) [40] by using hydrophobic adsorption methodology. Many of the mechanically stable novel hydrophobic silica gels were found as efficient lipase carriers resulting in new biocatalysts with different degrees of activity and enantioselectivity. Selective adsorption preferring *Ca*L B from the mixture of the two lipases onto the novel supports showed that proper hydrophobic adsorbents can offer us a simple, efficient and low-cost technology to separate the desired lipases from their mixture or even from a crude extract. The usefulness of the methodology was demonstrated by production of a selective lipase from *Pseudozyma aphidis* (*Pa*L) as immobilized biocatalyst directly from the fermentation supernatant in a single step procedure [39]. The fact that different grafting methods led to the most efficient supports for *Ca*L A, *Ca*L B, *Rm*L and *Tl*L indicated that the selection of optimal support for the immobilization of a particular lipase cannot be predicted [39,40]. The well performing octyl- and phenyl-grafted silicagel supported *Ca*L B biocatalysts were also tested in continuous-flow reactors at various temperatures [41].

Entrapment is a useful technique of *enzyme immobilization*. The *sol-gel encapsulation of lipases* proved to be a particularly easy and effective way to enhance the mechanical and catalytic properties of biocatalysts. The sol-gel encapsulated enzymes usually retain their selectivity whereas their heat stability or specific activity may be significantly improved. BCG researchers investigated the lipase immobilization by entrapment in sol-gel

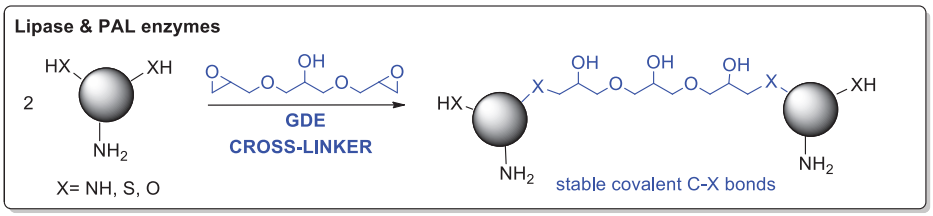


Fig. 6 Glycerol diglycidyl ether (GDE) as a convenient cross-linker for preparation of CLEAs

matrices to study the effect of enzymes, supports, enzyme/support ratios, silane precursor composition and bioimprinting effect of additives [43-47]. In addition to test the resulting sol-gel lipases in kinetic resolution of 1-phenylethanol **1j** using batch mode [43-47], the reactions were studied in continuous-flow systems as well [41].

The sol–gel immobilization of Celite-supported lipase from *Pseudomonas fluorencens* (Lipase AK) was systematically studied using *ternary silane precursor systems* consisting of alkyltriethoxysilane (alkyl-TEOS), phenyltriethoxysilane (PhTEOS) and tetraethoxysilane (TEOS) [45]. The best overall results in kinetic resolution of 1-phenylethanol **1j** were achieved with the medium-chain octylTEOS and perfluorooctylTEOS-containing ternary systems.

The *bioimprinting effect in sol-gel immobilization* of lipases was also studied to develop efficient novel immobilized biocatalysts with significantly improved properties [46]. Bioimprinting was studied with substrate mimicking surfactants already found in the active site of experimental lipase structures, such as polyethylene glycol or lauric acid. Four lipases (Lipase AK, Lipase PS, *Ca*L B and *Cr*L) were immobilized by a sol-gel process with nine bioimprinting candidates. In all but one case the most significant activity enhancement was found with the imprinting molecules already found as substrate mimics in X-ray structures of various lipases.

Disubstituted dialkoxysilane precursors such as dimethyldiethoxysilane (DMDEOS), methyl(phenyl)diethoxysilane (MPDEOS) and diphenyldiethoxysilane (DPDEOS) were used as precursor components in binary and ternary sol-gel systems for immobilization of lipase from *Pseudomonas fluorescens* (Lipase AK) [47]. Tests with kinetic resolutions of 1-phenylethanol **1j**, 1-phenylpropan-2-ol **1k** and 4-phenylbutan-2-ol **1l** indicated the catalytic properties and the operational stability of the DMDEOS-containing preparations as the best.

A comparative study of BGC researchers was performed on *hydrophobic adsorption and covalent immobilization of Candida antarctica lipase B* on grafted silica gel supports [41]. This study indicated that mesoporous silica gels grafted with various functions were ideal supports for both adsorptive and covalent binding for *Ca*L B. Adsorption of *Ca*L B on phenyl-functionalized silica gels improved in particular its specific activity. In addition, adsorption on silica gels modified by mixtures of phenyl- and aminoalkyl silanes increased the productivity of *Ca*L B further. Furthermore, *Ca*L B adsorbed onto a phenyl/aminoalkyl-modified surface and subsequently treated with glutaraldehyde (GA) as cross-linking agent provided a biocatalyst of enhanced durability. Adsorbed and cross-linked *Ca*L B was resistant to detergent washing that would otherwise physically deactivate adsorbed *Ca*L B preparations.

The effect of temperature on enantiomeric ratio (E) and specific reaction rate (r_{flow}) in the continuous-flow mode kinetic resolution of 1-phenylethanamine **1g**, 4-phenylbutan-2-amine **1c** and 1,2,3,4-tetrahydro-1-naphthalenamine **1e** by variously immobilized *Ca*L B biocatalysts was studied in the 0–70 ◦C range [48]. Alteration of E in the kinetic resolutions of three differently flexible amines **1c,g,e** as a function of temperature was rationalized by the various flexibility of the lipase in its

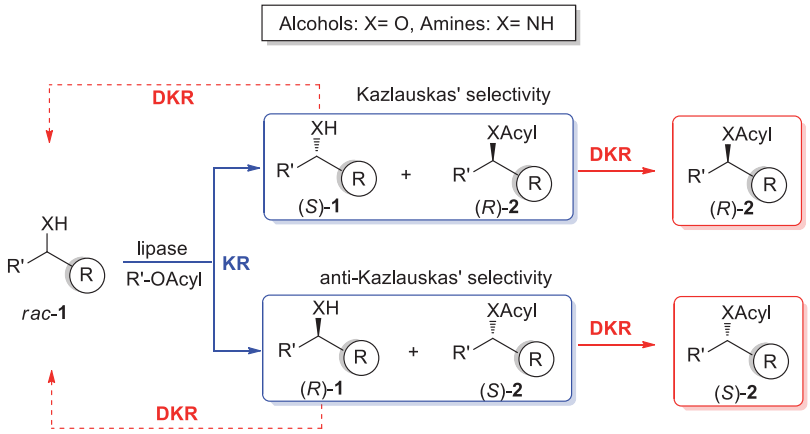


Fig. 7 Kinetic resolution (KR) and dynamic kinetic resolution (DKR) possibilities for alcohols and amines

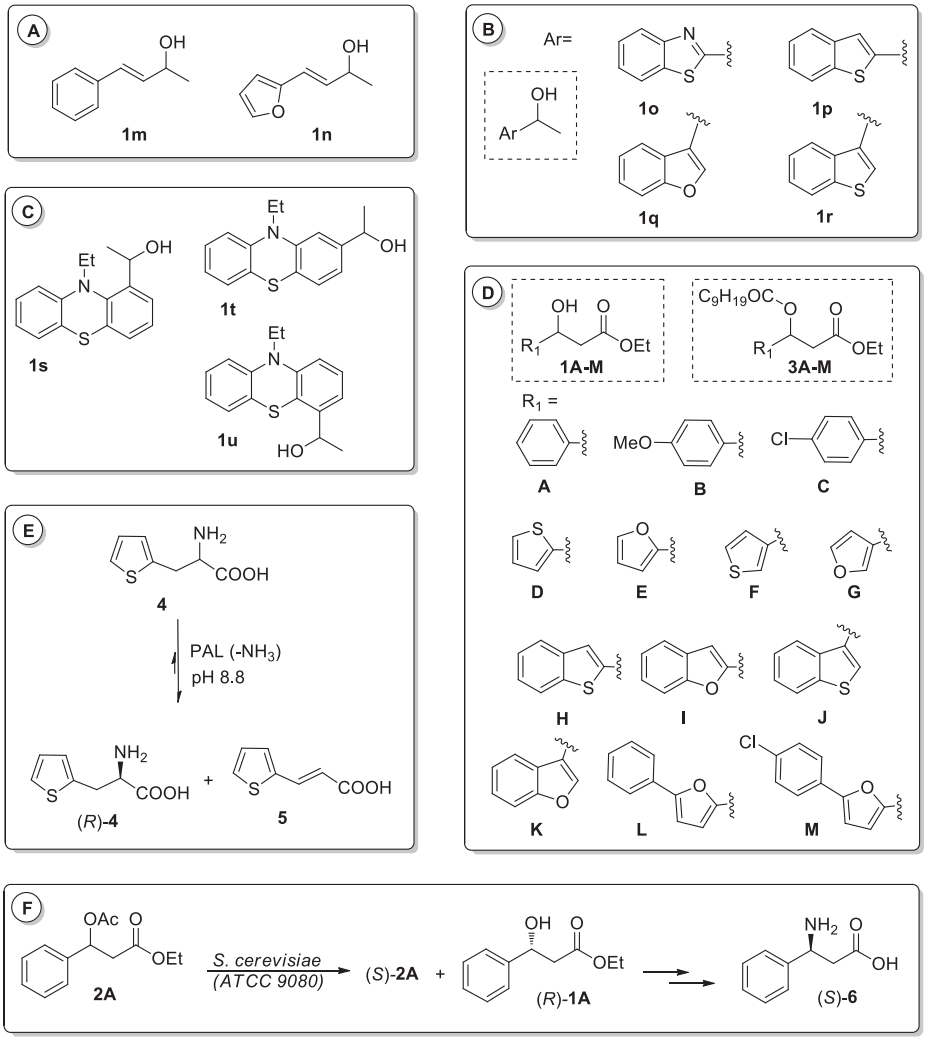


Fig. 8 Enzyme-catalyzed kinetic resolutions

different forms. Our results indicated that the *optimal method of immobilization depended both on the nature of the substrate and the reaction conditions*.

BCG demonstrated for the first time that the inexpensive *glycerol diglycidyl ether (GDE)* could be used conveniently as a bis-epoxide type cross-linking agent *in preparation of cross-linked enzyme aggregates (CLEAs)* from two different enzyme classes (Fig. 6) [49].

GDE cross-linked CLEAs of lipases from *Pseudomonas fluorescens* (Lipase AK), *Burkholderia cepacia* (Lipase PS) and lipase B from *Candida antarctica* (CaL B) and further of phenylalanine ammonia-lyase from *Petroselinum crispum* (*Pc*PAL) showed improved properties as compared to their glutaraldehyde (GA) cross-linked counterparts.

2.2.2 Kinetic resolutions

Chiral intermediates and fine chemicals are in high demand from the pharmaceutical and agrochemical industries for the preparation of bulk drug substances and agricultural products [3-8]. A number of multi-ton industrial processes use enzymatic kinetic resolution, often with lipases that tolerate different substrates. This initiated the activity of BCG to develop novel kinetic resolution (KR) and dynamic kinetic resolution (DKR) processes (Fig. 7 and Fig. 8).

Because efficient screening methods has increasing significance in rapid evaluation of novel biocatalysts BCG developed a *multi-substrate kinetic resolution screening method* for lipase biocatalysts based on enantioselective GC method [50].

KR of the alcohols (**1m** and **1n**) by vinyl acetate has been investigated with several crude enzyme preparations from solid state fermentation (SSF) of various fungi and with commercially available lipases (Fig. 8A) [51]. The majority of lipases catalyzed the formation of (*R*)-acetates [(*R*)-**2m,n**] according to the Kazlauskas' rule [52]. However, *several SSF preparations proved to act as selective anti-Kazlauskas catalysts*.

Another study aimed the *lipase-catalyzed KRs of racemic 1-heteroarylethanols* (Fig. 8B) [53]. Acylation of 1-(benzothiazol-2-yl)ethanol **1o**, 1-(benzo[b]thiophen-2-yl) ethanol **1p**, 1-(benzo[b]furan-3-yl)ethanol **1q** and 1-(benzo[b] thiophen-3-yl)ethanol **1r** was catalyzed with high enantiomer selectivity by a selection of commercial and in-house produced lipases. Alcoholysis of the corresponding racemic acetates **1o-r** catalyzed by *Ca*L B was also investigated.

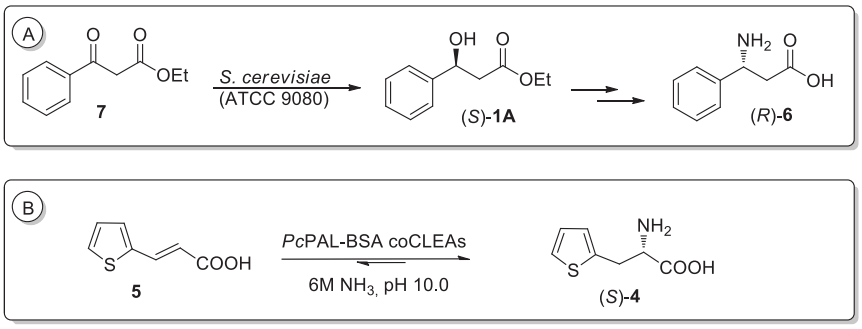


Fig. 9 Enantiotope selective biotransformations

Lipase-catalyzed KRs of racemic 1-(10-ethyl-10*H*-phenothiazin-1, 2, and 4-yl)ethanols **1s–u** (Fig. 8C) and their acetates **2s–u** were also studied [54]. By the aid of selective methanolysis of the esters **2s–u** with *Ca*L B and acylation of the racemic alcohols **1s–u** with *Ca*L A and *Ca*L B both enantiomers of the alcohols **1s–u** and their acetates **2s–u** were prepared.

The lipase-catalyzed reactions were successfully applied for KRs of a series of aromatic β-hydroxy esters **1A-M** in organic media (Fig. 8D) [55]. Decanoic acid and its esters were successfully used as acyl donors for selective *O*-acylation. The regio- and enantioselective enzymatic hydrolysis of the decanoate moiety of diesters **3A-M** was also investigated. A novel procedure was developed for the efficient and highly stereoselective synthesis of both enantiomers of the target compounds **1A-M** and **3A-M**.

The novel GDE-based *Pc*PAL-bovine serum albumin co-CLEAs (from phenylalanine ammonia-lyase of parsley origin) proved to be suitable a biocatalysts in KR of racemic 2-amino-3-(thiophen-2-yl)propanoic acid **4** (Fig. 8E), leading after digestion of the (*S*)-enantiomer of **4** to the mixture of (*R*)-**4** and the unsaturated compound **5** [49].

Besides native or immobilized forms of isolated enzymes, KRs can be performed by whole cell systems. Thus, KR of racemic ethyl 2-acetoxy-3-phenyl-propionate **3A** was performed with whole cells of yeast *Saccharomyces cerevisiae* (ATCC 9080) yielding after hydrolysis ethyl (*R*)-3-hydroxy-3-phenylpropionate (*R*)-**1A** and unreacted (*S*)-**3A** (Fig. 8F) [56]. The product (*R*)-**1A** was converted by a straightforward series of reactions to 3-amino-3-phenyl-propionic acid (*S*)-**6**.

2.2.3 Enantiotope selective biotransformations

Addition onto enantiotopic faces of achiral substrates is a straightforward way to obtain a single enantiomer of the product (Fig. 9).

The asymmetric reduction of ethyl 3-phenyl-3-oxopropionate **7** with the yeast *Saccharomyces cerevisiae* (ATCC 9080) belongs to this type of biotransformation (Fig. 9A) [56]. The produced ethyl (*S*)-3-hydroxy-3-phenylpropionate (*S*)-**1A** was converted to (*R*)-**6** by a similar series of reactions which were applied for the synthesis of (*S*)-**6** [49].

GDE-based PAL-bovine serum albumin co-CLEAs were active biocatalysts in stereoselective ammonia addition in 6M ammonia onto (*E*)-3-(thiophen-2-yl)acrylic acid **5** leading to the formation of enantiopure (*S*)-2-amino-3-(thiophen-2-yl) propanoic acid (*S*)-**4** (Fig. 9B) [49].

2.2.4 Stereoselective biotransformations in continuous-flow reactors

Enzymes – and thus hydrolases – can realize all kinds of selectivities (chemo-, regio-, diastereomer and diastereotope selectivity, enantiomer and enantiotope selectivity) [1,2]. Stereoselective biotransformations – such as asymmetric biotransformation, kinetic resolution and dynamic kinetic resolution – developed by BCG enabling the continuous-mode hydrolase-mediated production of compounds in high enantiomeric purity are discussed in this chapter.

Asymmetric acetylations of prochiral diol **8** with isopropenyl acetate in continuous-flow mode were investigated with sol-gel entrapped form of Lipase PS in small stainless steel packed-bed reactor (Fig. 10A) [57]. The effect of the temperature (0-60 °C) and flow rate (0.1-0.2 ml min^{-1}) on the conversion and enantiomer excess of the chiral monoester (*R*)-**9** was investigated. The best selectivity (91% ee) was achieved at 40 °C at a flow rate of 0.2 ml min^{-1}.

Continuous-flow KRs of alcohols were performed in stainless steel packed-bed reactors filled with different lipase preparations at analytical and at preparative scale (Fig. 10B) [41,46,47,58,59].

The effect of temperature (0–60 °C) and pressure (1–120 bar) on the continuous-flow acetylation of racemic **1k** was investigated in a *Ca*L B-filled reactor [58]. Whereas pressure had no significant effect on *r* and *E*, a monotonous increase of specific reaction rate (*r*) was observed within this temperature range. Most surprisingly, the enantiomer selectivity exhibited a maximum (*E* ~25, at 20 °C) and a minimum (*E* ~7, at 50 °C).

The continuous-flow and batch mode (shake flask) KRs of racemic alcohols **1j**, **1k** and **1O**, proceeded with similar enantiomer selectivities (*E*) but *productivities (specific reaction rate: r) were always higher in the continuous-flow reactions* (Fig. 10B) [58]. Similar results were observed in continuous-flow KRs of racemic **1j**, **1N** and **1P-T** with bioimprinted sol-gel lipases [46], of racemic **1j** and **1k** with

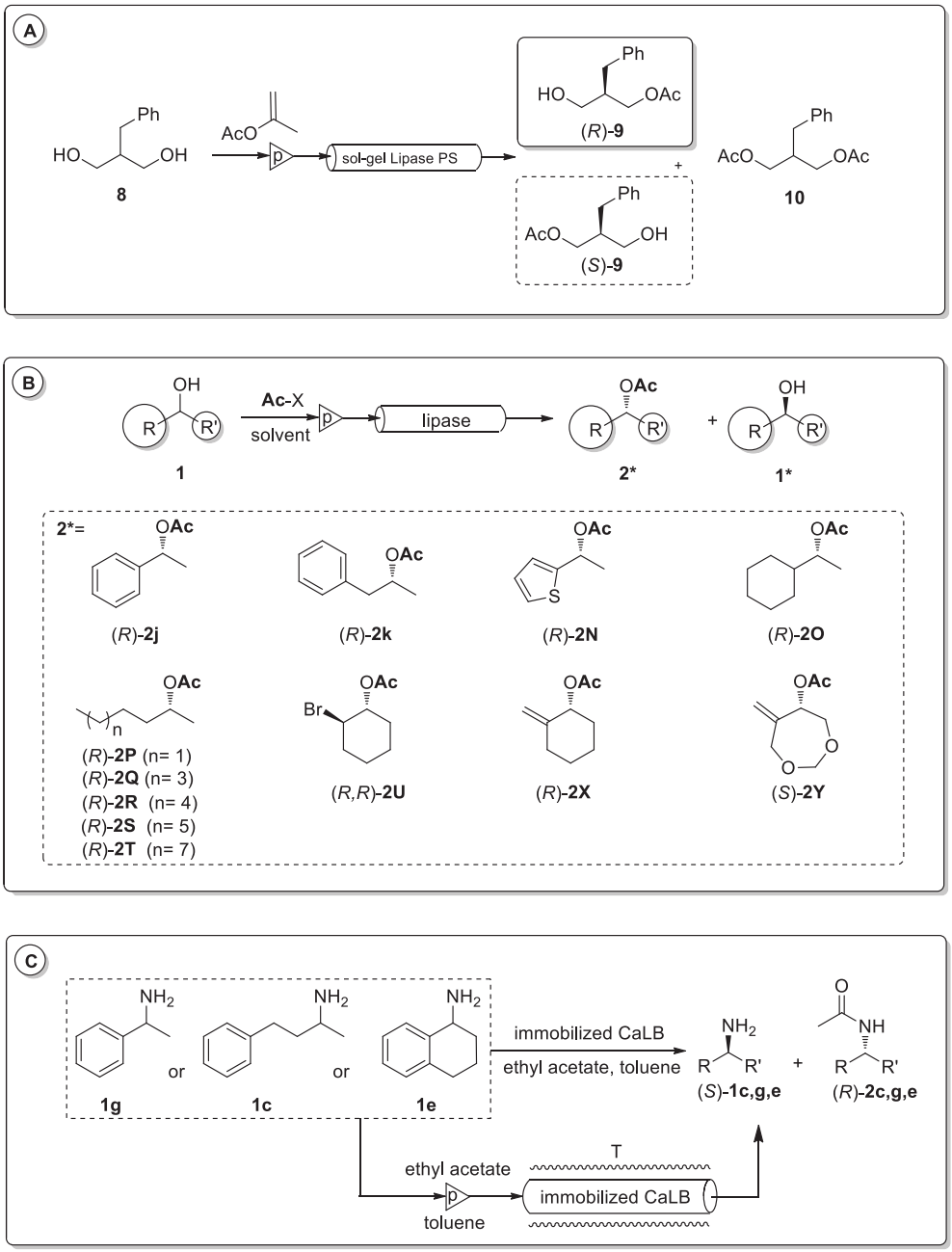


Fig. 10 Enzyme-catalyzed stereoselective reactions in continuous-flow mode

sol-gel entrapped Lipase AK [47], as well as of cyclic racemic secondary alcohols **1U**, **1X** and **1Y** with immobilized *Ca*L B and Lipase AK [59] (Fig. 10B).

The effect of temperature on enantiomeric ratio (E) and specific reaction rate (r_{flow}) in the continuous-flow KRs racemic amines **1c,e,g** by variously immobilized *Ca*L B biocatalysts was also studied in the 0–70 °C range [48,60]. In the continuous-flow KRs with differently immobilized *Ca*L B biocatalysts, the temperature effect depended significantly both on the nature of the substrate and on the mode of immobilization. Alteration of E in the KRs of three differently flexible amines **1c,e,g** as a function of temperature was rationalized by the various flexibility of the lipase in its different forms. Similar temperature dependence of the biocatalytic properties of *Ca*L B as a function of the mode of immobilization was found in continuous-flow KRs of the racemic alcohol **1j** [41].

The basic principles for a continuous-flow DKR process has been developed [61]. The continuous-flow DKRs of the racemic amine **1g** and racemic alcohol **1j** were performed using an alternating cascade of enzyme-filled resolution units and catalyst-filled racemization units arranged in two 10 column block thermostats at different temperatures (Fig. 3D).

2.3 Computer modeling for studies on enzyme structure and mechanism

As already stated structural biology (e.g. protein crystallography) can help the better understanding of the nature of biocatalysis and thus contribute to development of biotransformations at novel areas [22-24]. However, the

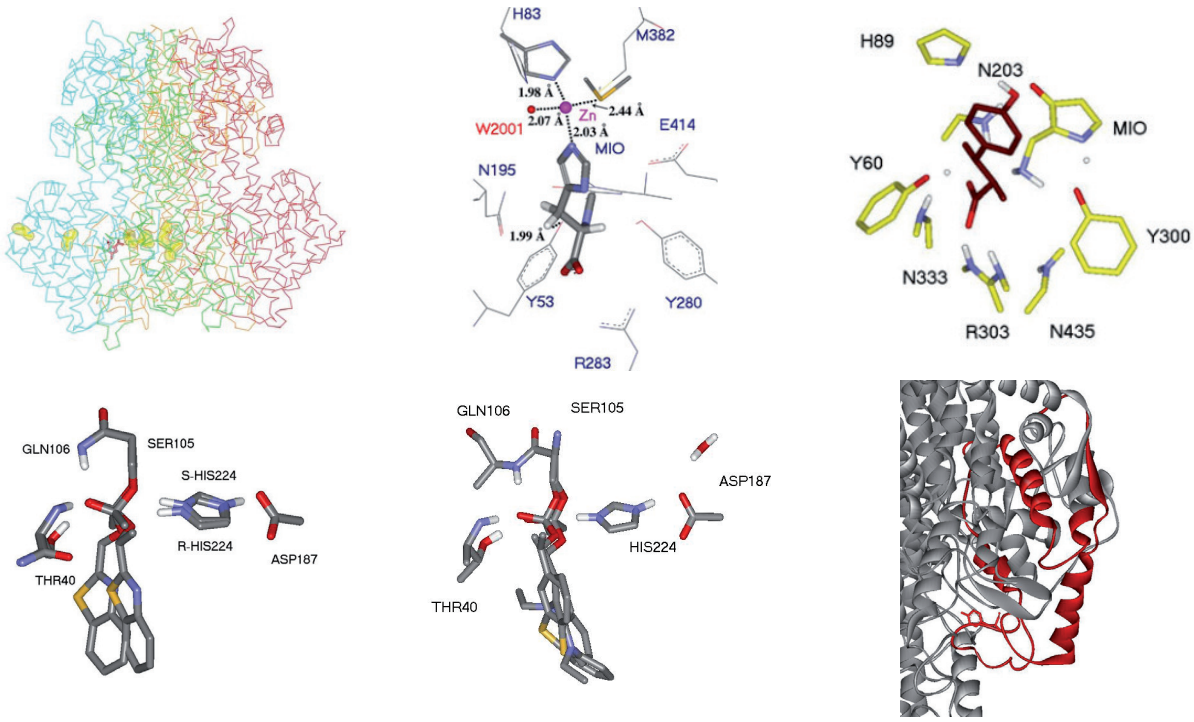


Fig. 11 Theoretical calculations on enzyme structures and on enzyme-catalyzed stereoselective reactions

X-ray structures with inhibitors may not fully reflect the fine details of structural changes during the enzymic reactions. For example, the product (*E*)-coumarate was present as inhibitor in two opposite zigzag arrangements in tyrosine ammonia-lyase (TAL) [62] allowing two different mechanisms. Similarly, a combined X-ray and QM/MM study within Lipase PS revealed that calculations on the covalently bound intermediates of the reaction can better explain the enantiomer selectivity of the lipase than the X-ray structures with co-crystallized (*S*)- or (*R*)-phosphonate inhibitors mimicking the ester forms of the acetylated products [63]. That is why BCG used theoretical calculation methods as potential tools in enzyme structural and mechanistic studies as well (Fig. 11).

2.3.1 Computer modeling to rationalize the stereoselectivity of lipase-catalyzed acylations

QM/MM calculations on the enantiomeric preference of *Ca*L B in the acylation of racemic heteroarylethanols **1o–r** allowed to determine the absolute configuration of the forming esters **2o–r** as (*R*) [53]. In this study, four possible tetrahedral intermediate (THI) states for acetylations of each alcohol **1o–r** were compared (Fig. 11A depicts the two lower energy THI states for acylation of **1o** within *Ca*L B determined by QM/MM [hf(3-21+g**)/uff] calculations).

The absolute configuration of enantiopure 1-(10-ethyl-10*H*-phenothiazin-1-yl)ethyl acetate **2s** was assigned similarly as (*R*) by using QM/MM(hf/3–21g:uff) calculations within the *Ca*L B (1LBT crystal structure) enzymic environment (Fig. 11B) [54].

2.3.2 Homology modeling of MIO-enzymes for structural studies

Among the so-called MIO-enzymes [25,26] (Fig. 1), phenylalanine ammonia-lyases (PALs) catalyzes the non-oxidative conversion of L-phenylalanine into (*E*)-cinnamate. Besides recombinant production [64] and experimental biochemical studies on PAL [25,26], BCG researchers performed theoretical calculations such as homology modeling, docking, molecular dynamics and QM/MM calculation on the MIO-enzymes.

Partial homology modeling of parsley PAL (*Pc*PAL) based on the X-ray structure (PDB code: 1W27) allowed to construct the catalytically active conformation of the essential Tyr-containing loop (Tyr-in) (Fig. 11C) having inactive conformation (Tyr-out) in the experimental structure (Fig. 1F). Molecular dynamics studies on *Pc*PAL's partial homology model and on a full model of a PAL from *Photorhabdus luminescens* PAL (*Pl*PAL) allowed to create hypothesis on the Tyr-loop modulating role of the *C*-terminal multihelix region in eukaryotic PALs [65].

The sequence of a PAL of the thermophilic bacterium *Rubrobacter xylanophilus* (*Rx*PAL) was identified by screening the genomes of bacteria for members of the PAL family and its gene was cloned and overexpressed in *E. coli* [66]. The extremely high pH optimum of *Rx*PAL could be rationalized by a homology model indicating possible disulfide bridges (Fig. 11D), extensive salt-bridge formation and an excess of negative electrostatic potential on the surface [66].

2.3.3 QM/MM studies on the mechanism of the reaction of ammonia-lyases

Histidine, phenylalanine and tyrosine ammonia-lyases (HAL, PAL and TAL) all catalyze ammonia elimination with the aid of a post-translationally formed electrophilic prosthetic group (MIO) [25-27]. For ammonia-lyases two significantly different mechanisms were suggested [25-27] implying either an *N*-MIO or a Friedel-Crafts (FC) type intermediate.

A common feature of both mechanisms is the formation of a covalent intermediate which allowed systematic confomational analysis of the ligand within the rigid active site. Comparative QM/MM calculations on the possible covalent intermediates of the TAL reaction demonstrated that the *N*-MIO intermediate has ~140 kcal mol^{-1} lower energy than the best FC state [30,67]. These results were confirmed by ligand docking and systematic conformational analysis in loop modified *Pc*PAL structure [68].

Partial homology modeling of the critical Tyr-loop of histidine ammonia-lyase (HAL) based on a more compact structure of *Anabaena variabilis* PAL (PDB code: 3CZO) allowed to investigate the Zn-containing active site of HAL by computational investigation [69,70]. Our calculations indicated the pathway *via* the *N*-MIO intermediate as the most plausible for the HAL reaction as well. Moreover, density functional theory (DFT) calculations on a small model of the *N*-MIO intermediate (Fig. 11E) elucidated the properties of Zn-complex playing a role in the reactivity and substrate specificity of HAL.

A detailed QM/MM study on the mechanism of the TAL reaction revealed also the *N*-MIO intermediate as the most plausible for the TAL reaction [30]. This study explored for the first time the role of Tyr300 in a tandem nucleophilic and electrophilic enhancement by a proton transfer. The second transition state (TS2) in the calculate pathway (Fig. 11E) showed a concerted C-N bond breaking of the *N*-MIO intermediate and deprotonation of the *pro-S* β position by Tyr60. This study elucidated the role of enzymic bases (Tyr60 and Tyr300) and other catalytically relevant residues (Asn203, Arg303, and Asn333, Asn435), which are fully conserved in the amino acid sequences and in 3D structures of all known MIO-containing ammonia lyases and 2,3-aminomutases.

3 Conclusions and outlook

The synergy and integration between various research interests of BCG involving novel stereoselective synthetic methods by chemoenzymatic techniques, intensification of biotransformations by novel immobilization methods of various enzymes and by continuous-flow reactions together with enzyme structural and mechanistic studies by experimental and computational techniques can result in novel, effective and ecofriendly synthetic methods. Thus, the complex research efforts of BCG can have positive impact on the development modern, integrated synthetic methods, or even on human applications of the biocatalysts.

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