

Project **BIOCAT**
**Modular biocatalyst platform for chiral synthesis of chemical compounds
by structure-based directed evolution**

Academy of Finland, project no. 117874

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Site of research: University of Oulu

Project webpage: <http://www.oulu.fi/bioprocess/biocatalysis.pdf>

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Technical staff: Ville Ratas (Biochemistry)

Other collaboration partners: Prof. J. Pursiainen (Dept. Chemistry, University of Oulu), Prof. K. Takkinen (Dept. Physiology, University of Oulu and VTT Biotechnology)

Project abstract:

BIOCAT is a new approach for the creation of tailor-made engineered biocatalysts to be used for the efficient, environmentally sound, biotransformation of chemical building blocks into highly pure enantiomers. It is based on the scaffold of the monomeric form of triosephosphate isomerase (TIM, see Inset 1).

The interdisciplinary consortium consisting of six research groups from three faculties of the University of Oulu, was established during an earlier Academy project where a new approach was developed to create a platform of non-natural enzyme catalysts. This concept was developed using as an example the well-known TIM-barrel framework of TIM which was successfully engineered to accept a wide range of chemical ligands. Now, the extended consortium aims to develop a series of

efficient biocatalysts for the cofactor-free synthesis of chirally pure chemical compounds. Therefore emerging techniques such as structure-based modeling and directed evolution of the biocatalyst in connection to chemical synthesis of a library of new compounds are jointly applied.

INSET I: Why TIM? TIM catalyses an important chiral conversion. A-TIM is a monomeric form of TIM being an ideal and very suitable starting protein for a biocatalytic platform:

- Small size (very suitable for NMR)
- Easily crystallized
- Soluble highly expressed in *E. coli*
- Stable molecule, which can tolerate many mutations at its “business” end
- The current set of X-ray structures suggest that it is a “flexible” molecule, and therefore an ideal starting point for directed evolution experiments
- Monomeric protein
- No cofactors needed
- In the closed conformation the binding site is an extended groove.
- Its wild type precursor is the extensively studied TIM. Knowledge of TIM properties will be continuously implemented in the protein engineering efforts.
- The TIM-barrel group represents the most common enzyme fold in the Protein Data Bank (PDB) database of known protein structures of structurally homologous enzymes. These enzyme families catalyze very different reactions but the active site is always located at topologically the same place.

BIOCAT clearly addresses to strengthen basic research in protein engineering and chemistry with a clearly applied relevance, namely strengthening scientific expertise and further developing research environments in biocatalysis, as a key area supporting sustainable production and products. Despite a solid past in industrial enzymes and chemistry, these areas have been separated in Finland. The project consortium creates a multidisciplinary team intertwining chemistry, biochemistry, molecular biology and bioprocessing. By the use of enzyme catalysts, working in aqueous solutions at intermediate temperatures and pressure, BIOCAT is directly targeting to new eco-efficient production concepts and chemical products. The project is well incorporated into national and international networks which are used to further strengthen the area and to disseminate research results. The project is closely connected to the interest in Finland for building up competence in industrial biotechnology and green chemistry.

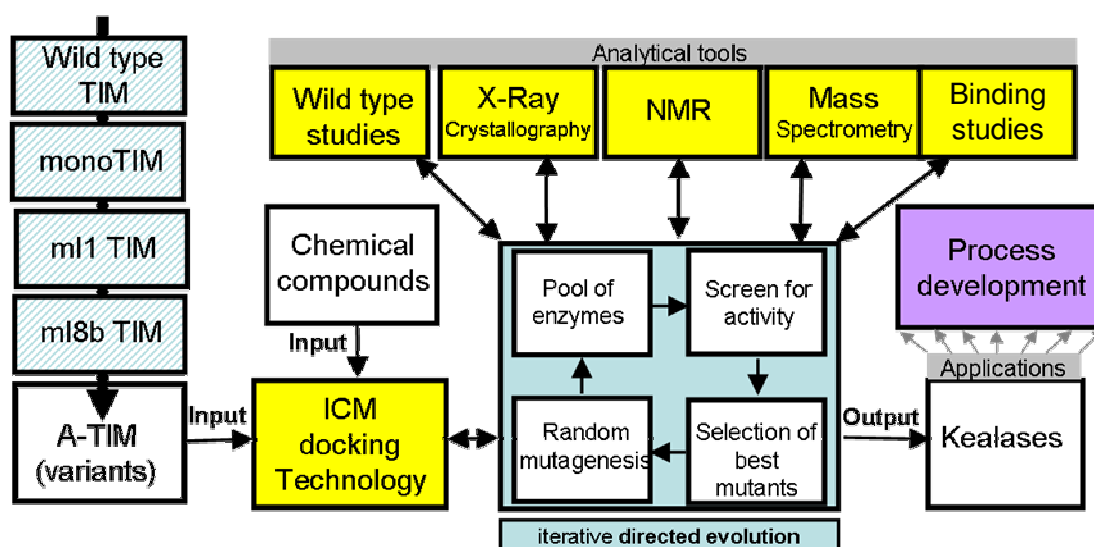


Figure 1. In- and output of BIOCAT. Analytical tools are shown in yellow, alterations in protein molecule are shown in blue (Striped is referring to directed mutagenesis and the history of A-TIM variants).

Results of research and future goals

BIOCAT will continue to focus on new binders and ligands, while understanding the underlying mechanisms, in so called proof of principle studies. In parallel BIOCAT will transform monomeric TIM variant from binding molecules to active enzymes, based on its original isomerisation reaction, using directed evolution approaches.

Results obtained:

- 16 randomized libraries of TIM variants have been created and are currently screened
- A biological selection system for finding improved TIM variants has been established and a first selected mutant is currently characterized
- Structures are available of complexes of A-TIM with the wild type TIM suicide inhibitor and the wild type TIM transition state analogue (Alahuhta et al, PEDS, in press)
- Structures are available of complexes of A-TIM with molecules completely different from the wild type TIM, like citrate(Alahuhta et al., PEDS in press) and maleate
- Structures are available of complexes of A-TIM with analogues of wild type TIM substrates and transition state analogues. Each of these analogues has been synthesized by the Lajunen group, and is characterized by having an additional tail, pointing into the new binding groove between loop-7 and loop-8, which is unique to A-TIM.
- 7 additional acid compounds have been synthesized. Altogether about 40 earlier unknown, self-synthesized compounds have been delivered to the other groups of the consortium for screening purposes
- 25 of the synthesized potential ligands have been screened by NMR
- 16 molecules have been confirmed to bind to A-TIM by NMR and MS
- Backbone NMR assignment of ATIM as an important basis [for further detailed binding studies](#) was finalised (BMRB entry 15588)
- Initial NMR measurements complemented by MS suggest that the starting ATIM variant can convert alpha hydroxyl ketones to alpha hydroxy aldehydes.

Publications:

1. Alahuhta M., Casteleijn M.G., Neubauer P., Wierenga R.K. 2008. The A178L mutation in the C-terminal hinge of the flexible loop-6 of dimeric and monomeric TIM favours the closed active site geometry. *Acta Crystallogr D Biol Crystallogr*. Accepted for publication.
2. Alahuhta M., Salin M., Casteleijn M.G., Kemmer C., El-Sayed I., Augustyns K., Neubauer P., Wierenga R.K. 2008. Structure-based protein engineering efforts with an inactive monomeric TIM variant: the importance of a single point mutation for generating a competent active site. PEDS. Accepted for publication.
3. Casteleijn M.G., Alahuhta M., Groebel K., El-Sayed I., Augustyns K., Lambeir A.M., Neubauer P., Wierenga R. 2006. The functional role of the conserved active site proline of triosephosphate isomerase. *Biochemistry*, 45 (51), 15483 – 15494
4. Vaismaa, M. J. P., Yliniemelä, S. M., Lajunen, M. K. 2007. An improved and green preparation of 3-(alkylthio)propionic acids. *Z. Naturforsch*, 62b: 1317 - 1323
5. Vaismaa, M. J. P., Lajunen, M. K. Microwave-Enhanced Preparation of Hydroxymethyl Ketones. Manuscript in preparation.
6. Vaismaa, M., Lajunen, M. K. A fast and facile synthesis of biologically active α -hydroxyketones. Manuscript in preparation.

Diploma and PhD theses:

1. Krause, M. 2007. Directed evolution of Triosephosphate Isomerase towards new Substrate Activity. Masters thesis. University of Göttingen, Germany. Available upon request

Posters:

1. Salin M, Alahuhta M, Casteleijn M, Vaismaa M, Lajunen M, Mattila S, Alho N, Neubauer P & Wierenga R (2007) Crystallographic docking studies with A-TIM: towards better binders and new enzyme activity. Presented in EMBO course on June 2007, and in BCO Discovery of the year happening.
2. M.G. Casteleijn, M. Alahuhta, M. Vaismaa, S. Mattila, M. Lajunen, J. Pursiainen, R.K. Wierenga, P. Neubauer. 2007. Creating new enzymes - From Triosephosphate Isomerase to Kealases. Poster at the European Congress of Biotechnology ECB13, Barcelona, September 16-19, 2007, J. Biotechnol. 131 (2), S114.
3. M. Ylianttila, M. Salin, M.G. Casteleijn, M. Krause, S. Mattila, M. Lajunen, R.K. Wierenga, P. Neubauer. 2007. Protein engineering of non-natural enzymes. Poster at the European Congress of Biotechnology ECB13, Barcelona, September 16-19, 2007, J. Biotechnol. 131 (2), S114.
4. Neubauer, P. 2007. High-throughput process development for recombinant proteins - possibilities, tools, and challenges. Invited oral presentation at the European Congress of Biotechnology ECB13, Barcelona, September 16-19, 2007.
5. M. Ylianttila, M.G. Casteleijn, M. Krause, T. Karppinen, S. Mattila, R.K. Wierenga, P. Neubauer. 2007. Protein engineering of non-natural enzymes. Poster at the Europacat, Turku, August 2007.
6. M.G. Casteleijn, M. Alahuhta, M. Vaismaa, R. Juvani, S. Mattila, M. Lajunen, J. Pursiainen, R.K. Wierenga, P. Neubauer. 2007. Creating new enzymes - From Triosephosphate Isomerase to Kealases. Poster at the Europacat, Turku, August 2007.
7. M.G. Casteleijn, M. Alahuhta, K. Groebel, K. Augustijns, A. Lambeir, P. Neubauer, R. Wierenga. 2007. The functional role of the conserved active site proline of triosephosphate isomerase. Poster at the Europacat, Turku, August 2007.
8. M. Salin, M. Alahuhta, M.G. Casteleijn, M. Vaismaa, M. Lajunen, S. Mattila, P. Neubauer and R. Wierenga. 2007. A-TIM: towards better binders and new enzyme activity. Poster at the Europacat, Turku, August 2007.
9. Silja Pelttari, Marco G. Casteleijn, Peter Neubauer, Sampo Mattila. Studies of kinetic properties of Uridine Phosphorylase. Poster at The XXIX Finnish NMR Symposium, Rymättylä (Finland), 13th -15th June 2007.
10. Marco G. Casteleijn, Silja Pelttari, Lilja Kusamo, Sampo Mattila, Peter Neubauer. Isolation and over expression of Uridine Phosphorylase from *Aeropyrum pernix* K1. Poster at OSSIBE4, Oulu (Finland), 11th – 15th June 2007.
11. Neubauer P. 2007. Towards high-throughput process development for recombinant proteins - possibilities, tools, and challenges. Invited speaker at the 160th SGM meeting in Manchester, March 26-29, 2007.

Cooperation network

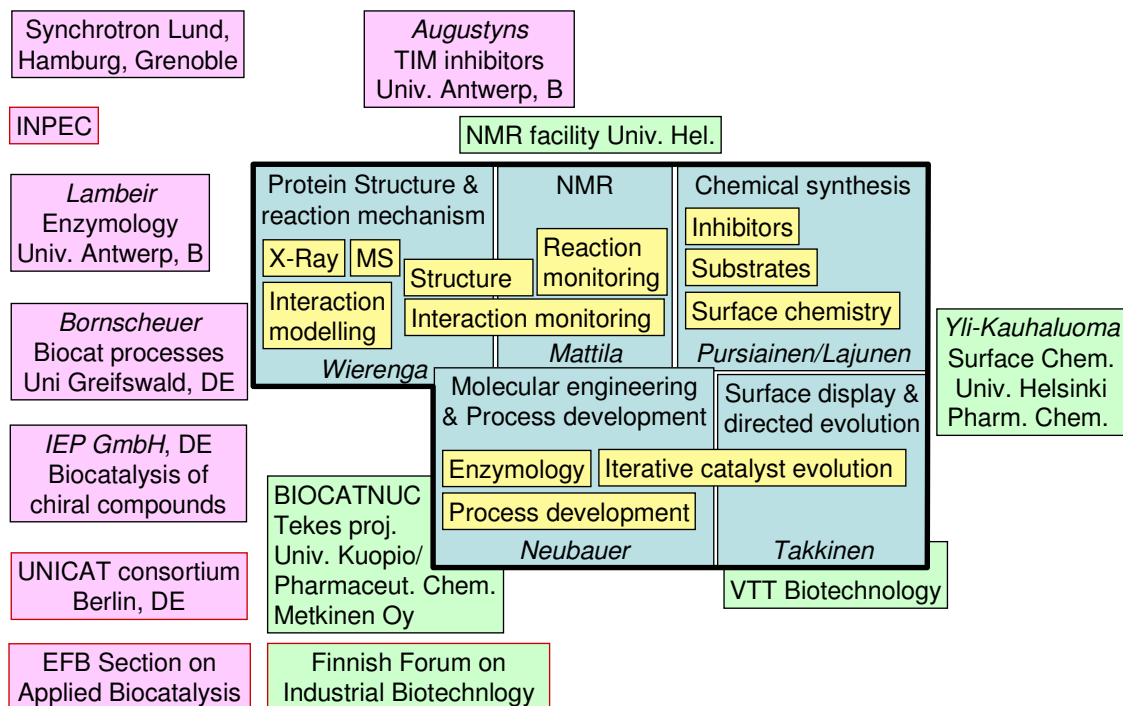


Figure 2: Cooperation framework of BIOCAT. Blue: Research consortium with the key methods applied; Green: National cooperation; Red: International cooperation; Striped: Organizations where consortium members are involved being used for dissemination of results.

Impact of the research

Organisation of courses in connection to the project

- 4th Oulu Summer School in Bioprocess Engineering (OSSIBE4) **Protein production - what can go wrong and how can you improve it fast?** 11.-15.6.2007, Oulu, Finland (Organiser Prof. P. Neubauer and BPEL)

Significance

- New engineered biocatalyst platform for synthesis of chemical building blocks.
- New non-standard competence in the iterative use of mutagenesis and screening /directed evolution of catalytically active molecules (standard technology is the search for binders only)
- New competence in use of the ICM modeling tool applicable for *in silico* modeling of possible enzyme binders (substrate, inhibitors), with wide application range including search for new drug compounds.
- Competence in high throughput optimization of biomolecules (which will be applied additionally in the new core-technology BPEL (Oulu) where a new robot system will be added for the HTS-Bio project).
- Competence of BPEL with directed evolution of biomolecules – being core technology also for creation of new binders, a key technology in our work with recombinant proteins and bioanalytics.
- Chemistry groups in Oulu will gain new competence in surface coupling of molecules and interdisciplinary knowledge on biomolecular opportunities.

Applicability of the research results

- Based on the new created enzymes, new industrial biocatalysts will be developed. A first application is the synthesis of modified sugars in cooperation with scientists of Univ. Kuopio and Metkinen Oy.
- Creation of reactive α -hydroxyaldehydes directly opens the question for a follow-up enzymatic reaction, such as carbon-bond formation performed by aldolases. Following this route of coupled biosyntheses is scientifically a highly interesting new field, where this consortium will take further lead.
- Competence obtained in frame of this project is directly applicable for generation of other biocatalysts.
- Competence obtained in surface coupling of chemical molecules and of generation of affine binding molecules will be highly relevant for bioanalytic developments at BPEL.
- New approaches will be tested in connection to NMR for the general screening strategies for new biocatalysts without establishing specific assays.
- Newly created kealases and competence obtained by the PhD students within the project may be directed directly to commercialization