SPRINGER
DESKTOP EDITIONS IN CHEMISTRY

T. Scheper (Ed.)

New Enzymes for Organic Synthesis

Screening, Supply and Engineering

Springer

T. Scheper (Ed.)

New Enzymes for Organic Synthesis

Screening, Supply and Engineering

Springer

Prof. Dr. Thomas Scheper
Universität Hannover
Institut für Technische Chemie
Callinstraße 3
D-30167 Hannover
Germany

Description of the Series

The Springer Desktop Editions on Chemistry is a Paperback series that offers selected thematic volumes from Springer chemistry series to graduate students and individual scientists in industry and academia at very affordable prices. Each volume presents an area of high current interest to a broad non-specialist audience, starting at the graduate student level.

Formerly published as hardcover edition in the review series
Advances in Biochemical Engineering/Biotechnolgy (Vol. 58) ISBN 3-540-61689-6

Cataloging-in-Publication Data applied for

ISBN 3-540-65549-2
Springer-Verlag Berlin Heidelberg New York

Cover: design & production, Heidelberg
Typesetting: Macmillan India Ltd. Bangalore-25
SPIN: 10711899 02/3020 -5 4 3 2 1 0 - Printed on acid-free paper

Springer Desktop Editions in Chemistry

L. Brandsma, S. F. Vasilevsky, H. D. Verkruijsse
Application of Transition Metal Catalysts in Organic Synthesis
ISBN 3-540-65550-6

H. Driguez, J. Thiem (Eds.)
Glycoscience, Synthesis of Oligosaccharides and Glycoconjugates
ISBN 3-540-65557-3

H. Driguez, J. Thiem (Eds.)
Glycoscience, Synthesis of Substrate Analogs and Mimetics
ISBN 3-540-65546-8

H. A. O. Hill, P. J. Sadler, A. J. Thomson (Eds.)
Metal Sites in Proteins and Models, Phosphatases, Lewis Acids and Vanadium
ISBN 3-540-65552-2

H. A. O. Hill, P. J. Sadler, A. J. Thomson (Eds.)
Metal Sites in Proteins and Models, Iron Centres
ISBN 3-540-65553-0

H. A. O. Hill, P. J. Sadler, A. J. Thomson (Eds.)
Metal Sites in Proteins and Models, Redox Centres
ISBN 3-540-65556-5

A. Manz, H. Becker (Eds.)
Microsystem Technology in Chemistry and Life Sciences
ISBN 3-540-65555-7

P. Metz (Ed.)
Stereoselective Heterocyclic Synthesis
ISBN 3-540-65554-9

H. Pasch, B. Trathnigg
HPLC of Polymers
ISBN 3-540-65551-4

T. Scheper (Ed.)
New Enzymes for Organic Synthesis, Screening, Supply and Engineering
ISBN 3-540-65549-2

Springer
Berlin
Heidelberg
New York
Barcelona
Hong Kong
London
Milan
Paris
Singapore
Tokyo

Professor M.-R. Kula

Preface

New enzymes for organic synthesis continue to represent a challenge both for those who prepare them as well as for those who finally apply them. The improved accessibility of enzymes as industrial catalysts has led to a large number of practical processes. The times are long past when it was believed that, at best, hydrolases would be suitable for industrial operations. Today, there are examples of practical applications for almost all enzyme classes.

Professor Dr. Maria-Regina Kula (Düsseldorf and Jülich, Germany) is a Pioneer in this field and it is therefore quite logical that this volume should appear on the occasion of her sixtieth birthday.

Professor Kula spent six month on sabbatical leave at the California Institute of Technology with Professor Frances H. Arnold, who has contributed a paper on the topic of "Optimizing Industrial Enzymes by Directed Evolution". Professor Arnold shows how enzymes usable in practice, for which in nature under "natural conditions" there has been no evolutionary stress, can result from directed evolution.

Profesor Kula has also been scientifically associated for many years with Professor Sakayu Shimizu, Department of Agricultural Chemistry, Kyoto University, Japan. Together with him and his predecessor, Professor Hideaki Yamada, she has organized the German-Japanese workshops on enzyme technology for many years. Professor Shimizu writes about "Screening of Novel Microbial Enzymes for the Production of Biologically and Chemically Useful Compounds". Many practical processes have originated from the Department of Agricultural Chemistry at Kyoto University and the technology transfer of this institute in particular is exemplary.

The other four chapters of this volume are contributed by heads of department from the Institute of Enzyme Technology at Heinrich Heine University, Düsseldorf, of which Prof. Kula is the director. First of all, Privatdozent Dr. Werner Hummel concerns himself with "New Alcohol Dehydrogenases for the Synthesis of Chiral Compounds". Up to just a few years ago, it was believed that due to the cofactor regeneration problem oxidoreductases could not be applied in practice. In his contribution, Dr. Hummel shows that an interesting pathway to chiral alcohols has been opened up by the provision of suffi-

cient quantities of alcohol dehydrogenases. These enzymes are used together with formate dehydrogenase and formate as a hydrogen source. Credit for the fact that formate dehydrogenase can today be termed an industrial biocatalyst is undoubtedly due to Professor Kula.

In the next paper, Privatdozent Dr. Lothar Elling is concerned with "Glycobiotechnology: Enzymes for the Synthesis of Nucleotide Sugars". Dr. Elling shows it is also true of glycobiotechnology that this field can only develop if corresponding enzymes are made available in sufficient quantities. Enzyme-catalysed oligosaccharide synthesis will undoubtedly receive great impetus from the inexpensive preparation of activated sugars.

Dr. Martina Pohl has entitled her contribution "Protein Design on Pyruvate Decarboxylase (PDC) by Site-Directed Mutagenesis". New enzymes cannot only be obtained by directed evolution but also by site-directed mutagenesis so that very interesting complementary approaches emerge here. Particularly in the past view years, success has been achieved in selectively modifying the substrate spectrum and reaction conditions for enzymes used in practical operations.

Finally, in a concluding paper Dr. Jörg Thömmes considers enzyme recovery in "Fluidized Bed Adsorption as a Primary Recovery Step in Protein Purification". As important as it is to track down new enzymes and selectively modify them, it remains equally important to actually make them available in the flask on the bench in adequate quantities at low cost with sufficient purity. Recovery is of central significance in this respect. Fluidized bed adsorption combines the process steps of cell separation, concentration and primary cleaning in recovery work. The procedure can also be excellently transferred from the laboratory to the pilot scale.

Professor Kula has performed really outstanding work in all these fields. Many of her collegues may be unaware that the subject of her doctoral dissertation concerned inorganic chemistry. She first came into intensive contact with enzymes during her postdoc time in the School of Medicine at John Hopkins University, USA. She later became head of a department and finally scientific director (1975-1979) at the Gesellschaft für Biotechnologische Forschung mbH (GBF), in Braunschweig, Germany.

To date she is, at least in Germany, the only woman ever to have been in charge of a national laboratory.

Since 1986 she has been engaged as a professor at the University of Düsseldorf and at the Research Centre Jülich. She has received numerous honours (including Order of Merit of the Federal Republic of Germany in 1979, the Technology Transfer Prize of the German Research Minister in 1983, and the Enzyme Engineering Award, USA, in 1995).

As a long-standing cooperation partner of Professor Kula, I feel the need to express my thanks as a kind of representative of the scientific community on the occasion of her sixtieth birthday for her numerous, fascinating scientific contributions and her exemplary willingness to cooperate.

Biotechnolgy is the integrated application of different scientific disciplines. Professor Kula has always exemplified this together with her staff. On the basis of long-term scientific work, real practical innovations have emerged due to her own qualities and her receptiveness for other scientific fields. Only a few poeple who have cooperated with her as long as I have will be aware of how much she has always enjoyed her work and the humour which accompanies her successes and setbacks.

We wish her continuing enthusiasm for her work and for us all more "new enzymes for organic synthesis".

Jülich, January 1997 Prof. Dr. C. Wandrey

Contents

Optimizing Industrial Enzymes by Directed Evolution

Frances H. Arnold and Jeffrey C. Moore
Division of Chemistry and Chemical Engineering 210-41,
California Institute of Technology, Pasadena, CA 91125, USA

Dedicated to Professor Dr. Maria-Regina Kula on the occcasion of her 60th birthday

Enzymes can be tailored for optimal performance in industrial applications by directing their evolution in vitro. This approach is particularly attractive for engineering industrial enzymes. We have created an efficient para-nitrobenzyl esterase over six generations of random point mutagenesis and recombination coupled with screening for improved variants. The best clones identified after four generations of sequential random mutagenesis and two generations of random recombination display more than 150 times the *p*-nitrobenzyl esterase activity of wild type towards loracarbef-*p*-nitrobenzyl ester in 15% dimethylformamide. Although the contributions of individual effective amino acid substitutions to enhanced activity are small (<2-fold increases), the accumulation of multiple mutations by directed evolution allows significant improvement of the biocatalyst for reactions on substrates and under conditions not already optimized in nature. The positions of the effective amino acid substitutions have been identified in a pNB esterase structural model. None appear to interact directly with the antibiotic substrate, further underscoring the difficulty of predicting their effects in a 'rational' design effort.

Advances in Biochemical Engineering/
Biotechnology, Vol. 58
Managing Editor: Th. Scheper

1 Introduction

Enzyme processes are making significant inroads in the production of foods, chemicals, and pharmaceuticals, thanks in large part to the sustained efforts of researchers like Professor Kula and her colleagues, who have identified enzymes to catalyze key transformations and developed viable processes to accompany them. Although the opportunities for using enzymes are numerous, identification of appropriate enzyme catalysts remains one of the key limiting steps.

Chemical engineers who design industrial processes using enzymes are constantly stymied by the fact that these catalysts have evolved over billions of years to perform very specific biological functions and to do so within the context of a living organism. Some of the features required for function in a complex chemical network are undesirable when the catalyst is lifted out of context (e.g. product inhibition). Conversely, many of the properties we wish an enzyme would have clash with the needs of the organism, or at least were never required: high stability, the ability to function in nonnatural environments and catalyze nonnatural reactions.

Despite intense research, there are enormous gaps in our understanding of the relationships between amino acid sequence, structure and function. As a result, the rational design of new proteins by the classical reductionist approach can be a frustrating, and often fruitless, exercise. Clues as to how to engineer better enzymes, however, come from studying how nature has done it. A study of protein evolution shows that enzymes are highly adaptable molecules, at least over evolutionary time scales. Many enzymes catalyzing very different reactions have come about by divergent evolution from a common ancestral protein of the same general structure, acquiring diverse capabilities by processes of random mutation, recombination, and natural selection. We also know that enzymes of a given function (for example, all catalyzing a particular step in a metabolic pathway) can exhibit widely different properties (stability, solubility, tolerance to pH, etc.), depending on where they are found.

The explosion of tools that has come out of molecular biology during the last 20 years has made it possible for us to evolve enzymes for features never required in nature. We can speed up the rate and channel the direction of evolution by controlling mutagenesis and the accompanying 'selection' pressures. By uncoupling the enzymes from the constraints of function within a living system, we can explore a variety of futures that include novel environments or even entirely new functions. Directed evolution is also a very practical approach to tailoring enzymes for a wide range of applications. Our recent review [1] describes just some of the protein properties that have been successfully altered by evolutionary approaches: substrate specificity, catalytic activity, activity in the presence of organic solvents, expression level and stability.

The methods are especially suitable for optimizing industrial enzymes. For example, we have recently directed the evolution of an enzyme to catalyze a nonnatural reaction, *p*-nitrobenzyl (pNB) ester hydrolysis in the presence of

polar organic solvents [2]. Scientists at a major pharmaceutical company, Eli Lilly, devoted significant effort to finding an enzyme that would selectively remove the pNB protecting group commonly used during large-scale synthesis of cephalosporin-type antibiotics [3, 4]. An enzyme with some pNB esterase activity was identified in *B. subtilis*, but its low activity, especially in the organic solvents required to solubilize these materials, made it a poor competitor to the existing zinc catalyst. We successfully evolved this enzyme to exhibit much higher activity towards the loracarbef pNB ester (LCN-pNB), both in aqueous buffer and in buffer-dimethylformamide solutions.

2 A Working Strategy for Directed Enzyme Evolution

The number of possible enzymes one can make is so vast than an exploration of their functions must be carefully guided in order to avoid becoming hopelessly lost. It is therefore important to develop a practical working strategy that will guide evolution in the laboratory. In nature, unfavorable mutations are winnowed out at the same time as beneficial mutations are amplified, by linking the organism's growth rate and reproductive success to the performance of its components. In this process of *selection*, those organisms which grow faster quickly dominate, allowing an efficient search of very large populations (10^6 and more for bacteria). Unfortunately, many of the features that are of interest for applications cannot be linked to the survival or growth of the host organism – the prerequisite to a selection. Therefore, mutant enzyme libraries must be screened. This unfortunate reality, a direct result of the first law of random mutagenesis ("You get what you screen for"), effectively limits the search for improvements to mutant libraries containing perhaps 10^4–10^6 variants [5].

The number of different sequences one can create by making mutations in an enzyme grows exponentially with the number of mutations. While there are only 5700 possible single mutants of a 300 amino acid enzyme, there are more than 30 billion different sequences that differ from the original enzyme at only three positions. While a rapid screen might be able to cover a large fraction of all single mutants, and even some significant fraction of all double mutants, screening would be unable to give more than a very sparse sampling of the enzymes with multiple amino acid substitutions. Beneficial mutations are generally rare; their frequency will depend on the extent to which the particular feature of interest has already been optimized. Because mutations are more likely to be harmful than beneficial, the probability that an enzyme will be improved with respect to its parent sequence decreases rapidly with increasing mutation rate. As a result, the search for effective mutations should be limited to proteins with sequences (and therefore properties) very similar to their parents. We try to tune the rate of mutation to produce enzyme libraries with primarily single (amino acid) substitutions.

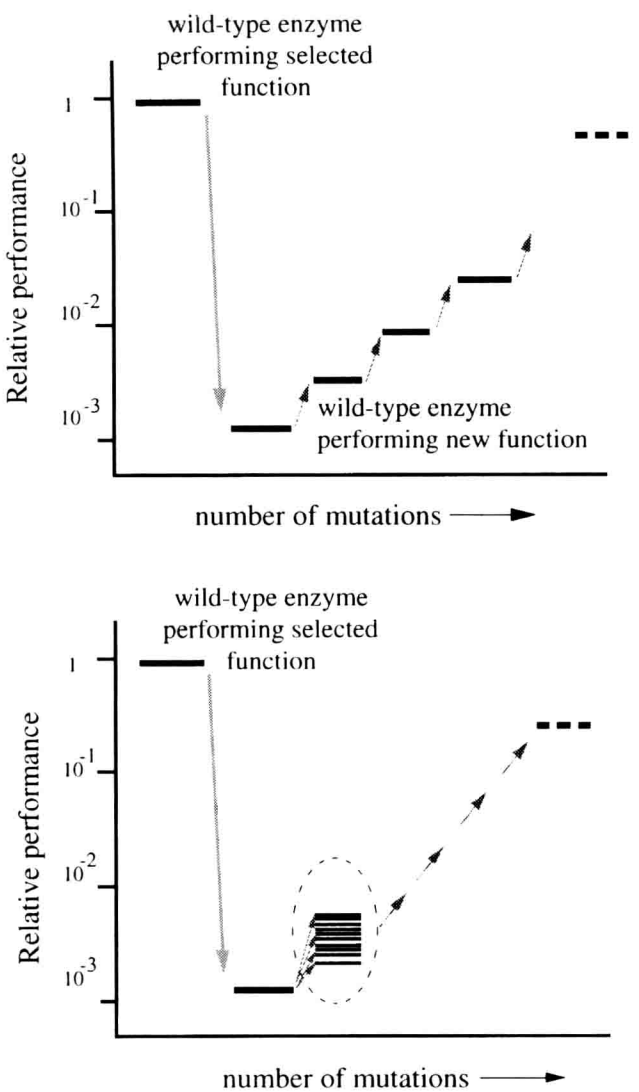

Fig. 1. A working strategy for directed enzyme evolution. The screening method should ensure that small enhancements brought about mainly by single mutations can be measured. The evolution of a new, useful enzyme requires an effective strategy for accumulating many such small improvements. Beneficial mutations can be accumulated in **a** sequential generations of random mutagenesis and screening or **b** by (random) recombination

Based on these arguments, we developed the simple directed evolution strategy illustrated in Fig. 1 [2, 6]. In comparison to the enzyme performing a function for which it is selected in nature, the new job is performed poorly indeed. Beneficial mutations will be identified during screening the products of

gene libraries containing relatively few (single or perhaps double) amino acid substitutions. Although these progeny will generally resemble their parents, the accumulation of mutations creates descendents quite different from their ancestor. Therefore, the generation of new, useful enzymes also relies on having an effective strategy for accumulating many such small improvements. One such strategy, illustrated in Fig. 1a, involves carrying out sequential generations of random mutagenesis to create a mutant library, coupled with screening of the resulting enzyme variants. In each generation, a single variant is chosen to parent the next generation, and sequential cycles allow the evolution of the desired features. Alternatively, effective mutations identified during one or more generations can be recombined, for example using the recently developed 'DNA shuffling' method described by Stemmer [7, 8] (Fig. 1b). Both approaches to accumulating beneficial mutations have proven effective for the evolution of the pNB esterase.

3 Screening for Improved Enzymes

The first requirement for successful laboratory evolution is the development of a sensitive screen to identify improved enzymes. The screen should ensure that the expected small enhancements brought about mainly by single amino acid substitutions can be measured. While a carefully-designed selection may be useful for making a first pass (i.e. to remove a large background of inactive clones [9]), most industrial enzyme systems will require screening to identify useful mutations. The resulting evolved enzymes will not be useful unless they exhibit a combination of features: high expression levels, stability and enantioselectivity, high activity on nonnatural substrates and/or the ability to carry out the reaction at a particular temperature, pH, substrate concentration, in organic solvents, etc. These features are unlikely to be reflected in an in vivo selection. Furthermore, if the screen does not reflect all desired criteria, one runs the risk of evolving one property at the expense of those not required to pass the screen.

If two properties are closely linked to one another, however, the screen may need only reflect one. Calculated risks of this type are almost always necessary in designing a screen suitable for dealing with large numbers of variants. The *p*-nitrobenzyl ester hydrolysis reaction, for example, is assayed by HPLC, which is unsuitable for rapid screening. We therefore devised a screening assay based on the loracarbef *p*-nitrophenyl ester, which provides a colorimetric signal upon hydrolysis [2]. The screening reactions could then be carried out in a microtiter plate, using a plate reader to analyze the enzyme kinetics of all wells simultaneously. To validate the screening method, we compared the activities of a set of mutants towards the *p*-nitrobenzyl and *p*-nitrophenyl substrates, as shown in Fig. 2. If the screening reaction perfectly mimicked the desired reaction, all the

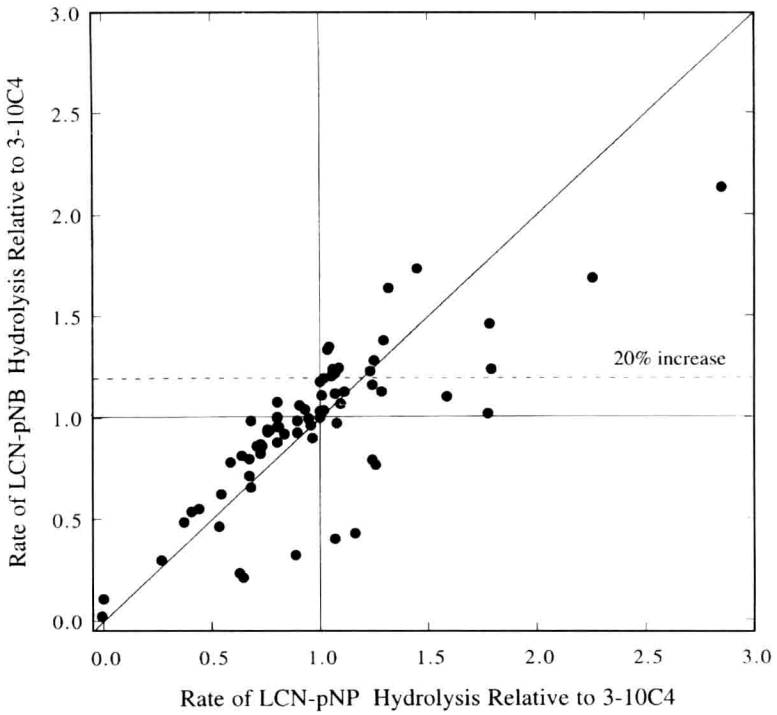

Fig. 2. Comparison of activities on target (*p*-nitrobenzyl) and screening (*p*-nitrophenyl) substrate of selected active pNB esterases isolated after fourth generation of random mutagenesis and screening, relative to parent enzyme from the 3rd generation. The five most active variants were pooled for random recombination

points would lie on the 45° line. Although there will be some false positives and false negatives using this screening reaction, the rapid screen provides sufficient information to make a rough cut of positive clones. Thus we need only test a small number of clones by HPLC to verify improved pNB esterase activity. Screening reactions were carried out at room temperature, in 15–20% DMF, with relatively high substrate concentrations (0.8 mM) to reflect the conditions under which the enzyme would be used industrially.

4 Evolution of pNB Esterase: Random Mutagenesis

Using the rapid colorimetric assay to screen about a thousand colonies per generation, we completed four generations of random mutagenesis (by error-prone PCR) and screening the *E. coli* colonies expressing the resulting gene libraries. The detailed methods and results have been described [2]; results are

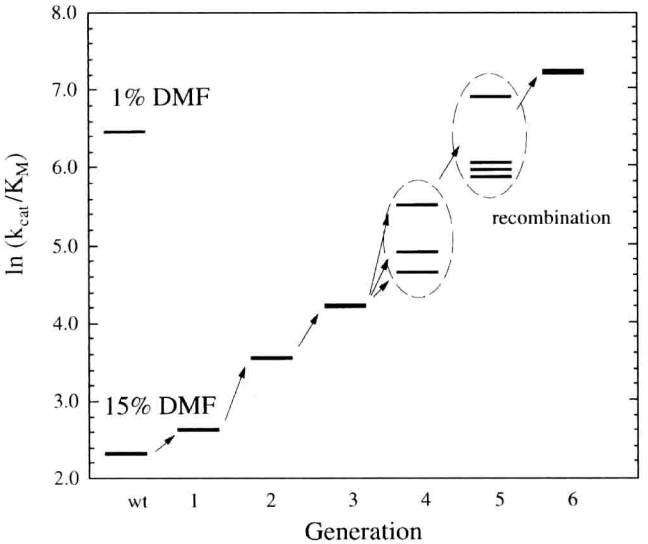

Fig. 3. Evolutionary progression of catalytic efficiency of pNB esterase (towards LCN-pNB) in 15% DMF through four generations of random mutagenesis, followed by two rounds of recombination by 'DNA shuffling' of circled populations.

summarized in Fig. 3. After four generations, the enzyme's catalytic efficiency in 15% DMF had improved 24-fold. Although not reflected in Fig. 3, expression level had also increased 2-fold.

The evolved enzymes were further characterized for their ability to carry out the desired pNB ester hydrolysis. Figure 4 shows the specific reaction rates for enzymes from the first four generations in 1% and 15% DMF. Each successive generation catalyst is more effective than its parent, and the best, pNB esterase 4-54B9, is 15 times more productive than wild type in 1% DMF. In 15% DMF, this enzyme makes product at 4 times the rate of the wild type enzyme in 1% organic solvent. The impact of this improvement is not only the increased productivity of the evolved enzyme, but also in the 4-fold increase in solubility of the substrate in 15% DMF. The increased solubility reduces the size of the reactor and the downstream processes required to produce and purify a given amount of product. The 2-fold increase in enzyme expression level further reduces process costs.

5 Evolution of pNB Esterase: In Vitro Recombination

In the fourth generation, we screened a larger number of clones ($\sim$7500) and picked a subset of those to analyze further, in order to validate the screening