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64

CENTRO DE REUNIONES INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Novel Biocatalysts

Organized by

S. J. Benkovic and A. Ballesteros

F. X. Avilés
A Ballesteros
S. J. Benkovic
S. A. Benner
T. C. Bruice
P. S. J. Cheetham
W. F. DeGrado
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D. Herschlag
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A. M. Klibanov
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Instituto Juan March (Madrid)

INDEX

PAGE

INTRODUCTION: Stephen J. Benkovic and Antonio Ballesteros. 9

Session I) ENZYMES
Chair: Alexander M. Klibanov............................ 13

Steven A. Benner: Evolution of enzymes................ 15

Peter S.J. Cheetham: Screening for novel biocatalysts.. 16

Short talk:
Yasuhisa Asano: Molecular aspects of newly discovered D-stereospecific peptide hydrolases, D-aminopeptidase and alkaline D-peptidase........... 18

Chair: Steven A. Benner................................. 19

Richard Wolfenden: Enzymes as time machines........... 21

Alexander M. Klibanov: Enzyme catalysis in nonaqueous media: control of enzymatic stereoselectivity by the solvent.. 23

Short talk:
Karl Hult: Molecular modelling as a tool in the understanding of lipase enantioselectivity.......... 24

Session II) ENGINEERED ENZYMES
Chair: Antonio Ballesteros.............................. 27

Antonio Ballesteros/Francisco J. Plou: Improving hydrolases by chemical modification.................... 29

Robin E. Offord: Semisynthetic proteins................ 31

Instituto Juan March (Madrid)

PAGE

Short talks:
Bernardo Herradón: Finding novel catalytic activities of enzymes: acylase as a catalyst for the selective synthesis of esters and amides.. 33

Angel Guerrero: Lipase-catalyzed enantioselective synthesis of methyl (R) and (S)-2-tetradecyloxiranecarboxylate through sequencial kinetic resolution................................ 34

Chair: Donald Hilvert................................ 35

Richard N. Perham: Engineering the coenzyme specificity of 2-oxo acid dehydrogenase multienzyme complexes: the role of protein domains................ 37

James A. Wells: Engineering enzymes and abzymes by rational and non-rational means........................ 39

Chair: Richard N. Perham................................ 41

Francesc Xavier Avilés: Engineering of protein inhibitors of metallocarboxypeptidases................ 43

William F. DeGrado: Analysis and design of ion channel proteins.. 44

Short talk:
Enrique Pérez-Payá: Design of functionalized protein-like structures using synthetic conformationally-defined libraries................ 45

Chair: Richard Wolfenden................................ 47

Sydney M. Hecht: Analogues of dihydrofolate reductase and firefly luciferase containing synthetic amino acids at defined positions............................ 49

Instituto Juan March (Madrid)

PAGE

Klaus Mosbach: Molecular and Bio-Imprinting - new techniques allowing formation of artificial "binders" and biocatalysts or of enzymes with altered substrate specificity.. 51

Short talk:
Jean-Baptiste Charbonnier: Structural convergence seen in the active sites of a family of catalytic antibodies.. 54

Session III) OTHER CATALYSTS
Chair: Javier de Mendoza................................ 55

A.J. Kirby: Efficiency in biocatalysis: lessons from model systems.. 57

Thomas C. Bruice: Ground state conformations and entropic and enthalpic factors in the efficiency of intramolecular and enzymatic reactions................. 59

Chair: Klaus Mosbach................................ 61

Jean-Marie Lehn: Supramolecular reactivity and catalysis.. 63

Javier de Mendoza: Design of artificial catalysts from supramolecular chemistry principles................ 64

Short talk:
Andrew E. Nixon: Fusion of protein modules in the generation of active enzymes........................ 65

Instituto Juan March (Madrid)

PAGE

Session IV) RIBOZYMES AND ABZYMES
Chair: A.J. Kirby 67

Daniel Herschlag: Biological catalysis: lessons from comparison of RNA and protein enzymes 69

Olke C. Uhlenbeck: Metal ions, phosphorothioates, and the hammerhead ribozyme 71

Stephen J. Benkovic: Enzymes and abzymes 72

Chair: Stephen J. Benkovic 75

Donald Hilvert: Evolution of enzymes and antibodies 77

Posters 79

Ana Arraztio: Construction of hybrid GlmS'S between *E. coli* and *Thermus thermophilus:* a tool to engineer proteins 81

Amir Avdagić: Long-distance control in stereoselective reduction of 3-[3-(4'-Bromo [1,1'-biphenyl]-4-yl)-3-keto-1-phenylpropyl]-4-hydroxy-2H-1-benzopyran-2-one; relative configuration of prevailing diastereomer and absolute configuration of its enantiomers 82

F. Javier Cañada/Alfonso Fernández-Mayoralas: Regio-selectivity of the transgalactosylation catalyzed by β-glycosidases 84

Zbigniew Chalecki: Baeyer-Villiger oxidation using *Candida antarctica* lipase 85

Jean-Baptiste Charbonnier: New formulae for folding catalysts make them multi-purpose enzymes 86

María Magdalena Cid: Approach to the synthesis of a hapten for a [1,7]-sigmatropic hydrogen shift 87

Instituto Juan March (Madrid)

PAGE

Oguz Ersoy: Antibody catalyzed cleavage of an amide bond using an external nucleophilic co-factor.......... 88

Mirjana Gelo-Pujic: Enzymatic reactions under extreme conditions.................................... 89

Béatrice Golinelli-Pimpaneau: Crystal structures of catalytic antibodies................................. 91

Angel Guerrero: Enzymatic synthesis of potential antipsychotic agents................................. 92

Xiao-Chuan Liu: A tailor-made catalyst for the diels-alder reaction.. 93

Vincent Monchois: Use of oligonucleotidic probes to isolate dextransucrase encoding genes from *Leuconostoc mesenteroides* NRRL B-1299.................. 94

Diana Pokorny: Studies on carbon-carbon bond hydrolyzing enzymes.................................. 95

Somsak Sarangbin: Studies of the citric acid production by 2-deoxyglucose-resistant mutant strain of *Aspergillus niger* using cellulose hydrolysate substrate.. 96

LIST OF INVITED SPEAKERS.............................. 99

LIST OF PARTICIPANTS.................................... 105

Instituto Juan March (Madrid)

INTRODUCTION

Stephen J. Benkovic and Antonio Ballesteros

Instituto Juan March (Madrid)

The finding of new catalysts and of molecules that rival natural enzymes in their substrate specificity and catalytic turnover is of a major importance in the production of compounds for use in pharmaceuticals, in the development of novel biosensors, and in the actual *in vivo* use of such molecules to treat human disease. Besides these practical considerations, the insights furnished by such efforts into the functioning and organization of catalytic biomolecules in cellular development and reproduction contribute to answering key contemporary questions in biology.

The present approaches to the creation of novel catalysts fall into two general categories: the *de novo* design and synthesis of catalysts from organic molecules such as macrocycles, polymers, cyclodextrins and peptides; and the modification of existing catalysts such as enzymes or ribozymes by genetic or chemical methods. Abzymes can be considered a hybrid of the two that uses design and synthesis to create a transition state analogue which in turn is used to tap the immunological response for putative proteinaceous catalysts. Advances in these areas include the construction of ribozymes capable of cleaving a viral DNA target; the induction of catalytic antibodies that promote stereospecific ester and peptide synthesis or disfavored chemical reactions; the formulation of enzymes to function in organic solvents; and the modification of enzymic activity to broaden substrate specificity to mention several. These advances have been associated with equally valuable "spin-off" technology: for example the cloning and expression of antibody fragments in bacteria, improvements in solid state peptide synthesis, and computer modeling of structures.

These and other recent advances set the stage for examining key issues such as: minimal catalyst size, the importance of long range molecular forces for substrate specificity and optimal turnover, the development of computer methods with predictive capabilities, the definition of novel medical targets within the scope of available catalysts and the applications of such catalysts to organic synthesis.

The present Workshop provided an opportunity for interdisciplinary researchers to discuss many of these issues:

- Sessions on enzymes provided an opportunity for examining the catalytic efficiency of enzymes relative to the spontaneous reaction for a given process. The range of catalytic efficiencies of enzymes has proven to be enormous, from a factor of 10^6 to one as high as 10^{24}. Interestingly, the enzyme catalyzed rates for all of these processes fall in the same time domain as would be required by the network of reactions in a living organism. How these marvelous machines evolved from ancestral families appears to be within our grasp, since powerful algorithms allow one to reconstruct the primordial genes and through modern recombinant techniques to express these proteins and to study their catalytic function. There are still a large number of organisms that have not been examined for their ability to catalyze specific reactions, but with the growing genomic databases the range of reactions catalyzed by enzymes will certainly expand. Consequently, powerful screening techniques need to be developed for not only exploring the nature of enzymes in various organisms but also the properties of recombinant enzymes that are members of large combinatorial libraries. Moreover, enzymic catalysis is no longer restricted to aqueous media, there is considerable evidence that enzymes function in nonaqueous media where their catalytic as well as stereospecificity can be manipulated.

Instituto Juan March (Madrid)

- The engineering of enzymes either by recombinant methods or chemical means holds considerable promise for extending the stereospecificity and regioselectivity of many enzymes. Recognizing that enzymes can often be divided up into discreet domains, the possibility of swapping these domains to create novel catalysts with changed cofactor specificity or reaction characteristics seems within reach. Many of these changes will be non-rational and cannot be predicted from existing structural databases. They will require phage display or other forms of combinatorial libraries. The resulting materials may be composites between naturally occurring amino acids and other organic reagents that can be introduced either by synthetic or recombinant methods. In fact, at one extreme molecular imprints can be created from polymeric materials that allow the formation of artificial binders and biocatalysts with altered substrate specificity.

- A fundamental tenet of enzymic catalysis is the principle of transition state stabilization that underlies many of the explanations for an enzyme's superior catalytic efficiency. Reexamination of this issue by both model systems as well as computational methods suggest that some of these beliefs may not be that well founded. For example, computational methods suggest that the enthalpic rather than the entropic term might be of paramount significance in the formation of the enzyme's substrate complex and its increased reactivity. In fact the ground state, enzyme substrate complex may be in a more reactive conformation than originally thought providing an impetus for the examination of such species by a variety of spectroscopic methods. The complexity of enzymic catalysis has always been subjected to dissection by a variety of model studies and those involving supermolecular complexes which follow enzyme-like kinetics in processing their substrates, may provide valuable insights as to the importance of rigidity vs. dynamics in the catalytic process.

- Ribozymes and abzymes have now appeared on the scene as another form of biological catalyst, the former naturally occurring and the latter manmade. A key question here is to whether these catalysts can be crafted to improved catalytic efficiencies, or perhaps more importantly what they tell us about enzyme function. Ribozymes and abzymes hold great promise as *in vivo* therapeutic agents.

Because catalysis cuts across so many disciplines, this meeting was particularly important because it brought together a number of practitioners that view this subject from different aspects. Some were grounded in the practicality of finding enzymes or catalysts for specific application, others were more interested in the fundamentals of how enzymes function, how they were designed, how they evolved, and still others were making a strong effort to build catalysts through synthetic or combinatorial methodologies. All agreed that the experience of finding a common language for communication as well as the opportunity to discuss issues in depth made this meeting most valuable and memorable.

Stephen J. Benkovic
Antonio Ballesteros

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SESSION I
ENZYMES

Chair: Alexander M. Klibanov

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EVOLUTION OF ENZYMES

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With the completion of the human genome project, together with allied genome projects from other organisms, chemistry as a science will move into its "post-genomic" phase. Organic chemistry has always been driven by the discovery of new natural products, elucidation of their structures, and exploration of their behaviors. Viewed from a chemical perspective, genome projects simply provide an enormous new collection of natural product structures to study. These display every behavior of interest to chemists: conformation, supramolecular organization, combinatorial assembly, photochemistry, and catalysis are just a few. The challenge of post-genomic chemistry is to infer information about conformation, reactivity, and physiological role of biological macromolecules from this massive amount of sequence data.

Evolutionary analyses are the keys to solving this challenge in a post-genomic world. Biological catalysts from contemporary organisms are the products of several billion years of biological evolution. Much of this evolutionary history can be reconstructed from the sequences of the descendent proteins, and ancient sequences from extinct organisms can then be prepared in the laboratory and studied. This permits us to follow in detail the origin and development of new physical and catalytic behavior in biomolecules. This facilitates "evolution-based" manipulation of protein structures. Further, it leads to the discovery of new mechanisms by which biological systems create new chemical function via natural "combinatorial chemistry.".

This talk will discuss three examples where an evolutionary analysis has been used to solve problems in the chemistry and physiology of biological catalysis: ribonuclease, alcohol dehydrogenase, and ribonucleotide reductase. This evolutionary analysis draws on the following tools:

(a) Methods for management of genome-sized database, developed in these laboratories in collaboration with Prof. Gaston Gonnet.

(b) Techniques for predicting the conformation of proteins starting from a set of aligned homologous protein sequences, techniques tested most recently in the "Critical Assessment of Structure Prediction" (CASP) project consummated in Asilomar in December, 1996.

(c) Paleontological information that, when coupled to an understanding of molecular evolution, allows one to assign physiological function to open reading frames in a genomic database.

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SCREENING FOR NOVEL BIOCATALYSTS
BY
Dr Peter S J Cheetham

The need to discover novel biocatalysts with new and improved activities provides interesting challenges for the design of effective screening methods with a high chance of success, together with a good likelihood of discovering exciting new biocatalysts of great scientific and commercial value. These opportunities arise because the enzyme or microorganism used to make a new or improved bioproduct is usually the lynchpin of the process, without which the product could not be made. This is despite the biocatalyst being only a relatively small element of the total cost of most of the processes that have achieved commercial success. Also new products demand new catalysts, and our current range of readily available and cost effective biocatalysts is still quite limited both in number and the range of reactions that it is possible to carry out achieved commercial success.

One of the most effective ways of finding new and useful biocatalysts is by screening. Screening is by no means a new approach, but it has the merits of a successful track-record and capacity to benefit from new complementary technologies such as gene cloning, robotics and information technology. Also the earth's microbial diversity is still substantially under exploited so that there are excellent prospects for finding the biocatalyst one requires. Screening is also particularly valuable because, by the use of relatively simple scientific methods, entirely new and/or substantially improved biological activities with eminently patentable features can be discovered that would not obviously have resulted from other scientific approaches that tend to rely on the rational improvement of already known and available enzymes and microorganisms. Targets for screening can include enzymes and microbial strains that carry out new reactions; have extended substrate specificities and superior reaction selectives, enable the use of preferred raw materials, and that originate from more convenient source microbial strains.

I wish to define a Biocatalyst as an enzyme or cell that is both sufficiently active, and that has enough of the other characteristics necessary for successful and cost-effective use on an industrial scale. For instance, selection of an enzyme or microbial strain that provides little side-products, achieves a high degree of conversion of substrates into products and operates effectively at high substrate concentrations greatly aids downstream processing that can often constitute 50% of overall process costs. Therefore any biocatalyst screen has two types of criteria that have to be met in order to answer the question of 'what exactly should we screen for?' These are firstly The Market Need, such as a lipase that is resistant to protease action for use in enzyme detergent products, and secondly The Performance Criteria, for instance when a new glucose isomerase source was being sought it had to have a much superior productivity to displace existing commercial GI products from the market.

I will emphasise the following key points and try to:-.

- Take an industrial perspective, and therefore touch on patent and engineering aspects that are very important in achieving real success and on some of the economic benefits that can be obtained.

- Demonstrate that much excellent work takes place in areas other than pharmaceuticals, such as agrochemicals and especially my own area of interest of food and personal care ingredients.

- Concentrate on enzyme activities other than simple hydrolases and isomerases; referring to lyases, cyclases, hydrolases and oxido-reductases and including examples of cofactor dependent activities and catabolic pathways.

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- Prove the value of rational selective low-throughput screening as opposed to less selective high-throughput screening.

- Illustrate the types of problems that can be solved, the technical aids required, how to validate screening methods and the benefits that can result, but also mention some conspicuous failures where despite considerable efforts no suitable biocatalyst has yet been found.

- Give examples, including some indication of new developments, ranging from the use of simple commercially available enzymes, through to activities requiring integrated metabolic pathways and involving a good understanding of microbial physiology and the use of inducers to order to design effective screening methods.

- Lastly, and most importantly to convince people that screening involves solving challenging problems, creates interesting new science, and most important plays an important role in increasing the number of valuable and novel biotechnology products so that the real value of screening is in the new enzymes and microbial strains that are made available for study and especially the new industry processes and commercial products that can be created using them.

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Molecular Aspects of Newly Discovered D-Stereospecific Peptide Hydrolases, D-Aminopeptidase and Alkaline D-Peptidase

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Two recent examples of our screening approach for obtaining novel biocatalysts will be presented. We found peptide hydrolases with D-stereospecificity, D-Aminopeptidase and Alkaline D-Peptidase, with synthetic substrates and categorized them as members of the "penicillin-recognizing enzymes" (1). Their uses in organic solvents and other applications will be also discussed.

1. D-Aminopeptidase (2,3). An enrichment culture in a medium containing D-Ala-NH_2 as the sole nitrogen source led to an isolation of a bacterial strain Ochrobactrum anthropi. Since the enzyme exhibited a mode of action typical of aminopeptidases which liberate an *N*-terminal D-amino acid residue with a free amino group, the enzyme was named as "D-aminopeptidase", which was recognized by IUBMB (EC 3.4.11.19) in 1994. There has been no report of an aminopeptidase catalyzing the stereospecific hydrolysis of D-amino acid containing peptides, although the peptides do exist in nature. Similarities in the primary structures among D-aminopeptidase, class C β-lactamase and Streptomyces DD carboxypeptidase were found. The enzyme is a new member of the "penicillin-recognizing enzymes" (1), because of: (i) similarities in the primary structure by gene sequencing, (ii) similarities in the reactions catalyzed in water and organic solvents, and (iii) the findings obtained by kinetic studies of the mutants generated by the site-directed mutagenesis and (iv) the inhibition by β-lactam compounds. The enzyme does not appear to be a selective target of the β-lactam compounds.

2. Alkaline D-Peptidase (4). We characterized a novel extracellular D-stereospecific endopeptidase "Alkaline D-peptidase" (ADP, D-stereospecific peptide hydrolase (EC 3.4.11.-)) from a bacterium Bacillus cereus, which had been isolated with a synthetic substrate $(D\text{-Phe})_4$. The optimal pH for activity was around 10.3. The enzyme (Mr: 37,952, monomer) was strictly D-stereospecific toward oligopeptides composed of D-phe such as $(D\text{-Phe})_3$ and $(D\text{-Phe})_4$. The enzyme had β-lactamase activity toward ampicillin and penicillin G. The enzyme was similar to carboxypeptidase DD from Streptomyces, penicillin-binding proteins from Streptomyces lactamdurans and Bacillus subtilis, and class C β-lactamases. Thus, the enzyme was also categorized as a new "penicillin-recognizing enzyme" (1).

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Chair: Steven A. Benner

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Enzymes as Time Machines

S. L. Bearne, D. C. Carlow, C. W. Carter, A. Radzicka, S. Short and R. Wolfenden
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To appreciate the potential energy barrier faced by an enzyme, or any man-made catalyst, it would be desirable to know the rate of reaction in the absence of catalysis. Uncatalyzed reaction rates are presently known for only a few biological reactions, although it is widely recognized that biological reactions differ substantially in rate, and in the consequent burden that they place on an efficient catalyst. The rate of the uncatalyzed reaction can also be used to estimate the minimal affinity of an existing enzyme for the altered substrate in the transition state, and to compare the potential susceptibilities of enzymes of different types to inhibition by transition state analogue inhibitors (1).

In the absence of enzymes, most biological reactions proceed at rates that are too slow to follow on a human time scale in neutral solution at ambient temperature. Rates of very slow uncatalyzed reactions can be determined by working at high temperatures in sealed quartz tubes (2). By analyzing the contents at timed intervals, and using Arrhenius plots to extrapolate the observed rate constants to ambient temperature, we have examined the uncatalyzed hydrolysis of peptides, nucleosides, glycosides and phosphate esters; the decarboxylation of alanine, proline and orotic acid; H exchange from solvent water into alanine, acetate and mandelate; and the dehydration of malate and mandelate (3-5).

Uncatalyzed rates observed for three reactions, normally catalyzed by enzymes, correspond to half-times of 78,000,000 years for OMP decarboxylation, 70 years for cytidine deamination, and 5 seconds for the dehydration of carbonic acid. In the presence of enzymes, all three reactions proceed by mechanisms that do not involve covalently bound intermediates. Thus, OMP decarboxylase, cytidine deaminase and carbonic anhydrase produce rate enhancements (k_{cat}/k_{non}) of 1.4×10^{17}, 1.2×10^{12} and 1.2×10^{8}, and maximal dissociation constants in the transition state ($k_{non}/[k_{cat}/K_m)$ of 5×10^{-24} M, 1.1×10^{-16} M and 1.1×10^{-9} M, respectively. Substrate K_m values are 7×10^{-7} M, 1.0×10^{-4} M and 8×10^{-3} M, furnishing an indication of the minimal extent to which the formal affinity of each enzyme for its substrate appears to increase in passing from ES to $ES^{\neq}$: at least 1.4×10^{17}-fold, 1.2×10^{12}-fold and 1.2×10^{8}-fold in these three reactions respectively. In each of these cases, the ability of the enzyme to enhance the reaction rate arises from its remarkable powers of binding discrimination between two species that differ only slightly in structure.

To investigate the structural origins of catalytic binding discrimination, and evaluate the apparent contributions of individual groups to transition state affinity, groups on either the enzyme or the substrate can be truncated to determine their apparent contributions to k_{cat}/K_m. In crystal structures of E. coli cytidine deaminase, for example, $ES^{\neq}$ is distinguished from ES and EP by the presence of a hydrogen

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bond between Glu-104 and the 4-OH group of the altered substrate in $S^{\neq}$. This H-bond can be interrupted by mutation of Glu-104 to alanine, or by replacement of the substrate's 4-OH group by a hydrogen atom. These truncations reduce the binding affinity of $ES^{\neq}$ by 4×10^6-fold and 10^7-fold, respectively. Large effects are also observed when single groups are truncated in the enzyme or ligand at other positions. As a result, the sum of the contributions of individual enzyme-substrate interactions appears to surpass the total binding affinity that is generated in the transition state. This apparent dilemma can be addressed by cutting the enzyme or the substrate in two pieces, and then comparing the transition state affinities of the pieces with the affinity of the whole. That procedure, using the uncatalyzed reaction as a standard of reference, furnishes a measure of the binding advantage that is gained when two pieces are joined to form a single molecule. In the interactions between cytidine deaminase and the altered substrate in the transition state, the results of several such "cuts" indicate an advantage of as much as 10^7-fold.

The kinetic barriers to many biological reactions appear to be extremely high in the absence of catalysts, leading one to question how a primitive catalyst could have conferred any selective advantage on the organism that produced it. Little would seem to have been gained by enhancing the rate of a reaction 1000-fold, if its half-time is 78 million years in the absence of catalysts. The slowest of these reactions are found, however, to have extremely large heats of activation. Accordingly, these barriers would have been much lower at the much higher temperatures at which the earliest organisms are presumed to have appeared. A primitive catalyst, if it was able to generate appreciable binding affinity at elevated temperatures, could therefore have allowed a very slow reaction to proceed at a useful rate.

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ENZYME CATALYSIS IN NONAQUEOUS MEDIA: CONTROL OF ENZYMATIC STEREOSELECTIVITY BY THE SOLVENT

Alexander M. Klibanov

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It is now well established that many enzymes can function as catalysts even in anhydrous organic solvents (in addition to their natural, aqueous reaction media) [1]. Moreover, it has been discovered that when placed in such nonaqueous milieus, enzymes exhibit remarkable new properties, including enhanced thermostability, ability to catalyze reactions impossible in water, and radically altered selectivity of action [1]. With respect to the last feature, which is of particular significance for enzymatic catalysis, it has also become feasible to address experimentally the question: "Can enzyme selectivity be substantially altered by switching from one organic solvent to another as the reaction medium?". Recent studies by us and others have answered this question affirmatively [2]. In particular, we have discovered that both prochiral [3] and enantiomeric [4] selectivities, as well as substrate selectivity [2], of several hydrolytic enzymes can be profoundly affected, and even reversed, simply by changing the solvent. In this presentation, representative examples, the mechanistic rationale, and synthetic implications of this phenomenon will be discussed.

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MOLECULAR MODELLING AS A TOOL IN THE UNDERSTANDING OF LIPASE ENANTIOSELECTIVITY

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Hydrolytic enzymes are commonly used as enantioselective catalysts in the resolution of racemic alcohols, acids, esters, amines and amides.[1] Lipases have been shown to be particularly useful with hydrophobic substrates and in applications performed in low water media. Lipase catalysis has become one of the most important methods in organic synthesis for the preparation of optically pure alcohols and acids.

The fast reacting enantiomer of a special transformation can in many cases be predicted from earlier experiments, for example by using some kind of box model.[2] The drawback of such an approach is that they do not contribute to the molecular understanding of the interactions involved in the enantioselectivity.

In recent years a number of lipase structures have been solved. Molecular modelling of substrate enzyme interactions is therefore possible to perform. A molecular modelling procedure to calculate the enantioselectivity of a serine hydrolase was developed using chymotrypsin as a model enzyme.[3] This procedure has subsequently been applied to lipases, which follow the same reaction mechanism as chymotrypsin. Lipase catalysed resolution of two enantiomers of an alcohol will proceed through transition states with different energies. If these transition states could be modelled and their energies are calculated the enantioselectivity can be estimated according to the relationship $\Delta\Delta G^{\#} = -RT\ln E$, where $\Delta\Delta G^{\#}$ is the difference in energy between the transition states and E is the enantiomeric ratio. $\Delta\Delta G^{\#}$ can be obtained from the difference in energy between modelled transition states of the two enantiomers, as the ground-state energies are equal for enantiomers. For chiral alcohols it is easy to define the transition states that are involved in the enantioselective step. On the other hand modelling of the resolution of racemic acids is more complicated as two different acyl enzymes and four transition states are involved.

Enzymes are large molecules, which makes the energy calculations time consuming. It is also difficult to find the lowest energies of the transition states. We have used a combination of molecular dynamics and energy minimisation methods to find representative energies and conformations. The calculations have been performed without taking into account solvent interactions. The energies obtained from the calculations represent enthalpy. The entropic contribution to $\Delta\Delta G^{\#}$ is therefore not included, but it has been regarded as negligible in resolution reactions, a statement that should be re-examined.

The enantioselectivity of an enzyme catalysed reaction depends on how both enantiomers of the substrate bind in the active site. It is therefore important to study both enantiomers and not only the fast reacting species, which often is the case in box models. Molecular modelling calculations suggest binding modes of both enantiomers, which is very important in the molecular understanding of the enantioselectivity. Knowing the binding mode of both enantiomers can be valuable if one wants to go on by increasing the enantioselectivity through substrate or protein engineering.

Molecular modelling of the enantioselectivity of the hydrolysis of 1-phenylethyl hexanoate and 2-octyl hexanoate catalysed by *Rhizomucor miehei* and *Humicola lanuginosa* lipases predicted the right enantiomer and gave a good estimate of the enantioselectivity. In addition the modelling suggested how the enantiomers bound in the

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active sites, showing that the large and the small groups of the substrate change places between the two enantiomers.[4] We have found a similar situation for short chain secondary alcohols in our present work with *Candida antarctica* lipase B. With the latter enzyme the docking of the fast reacting enantiomer of large alcohol moieties can be understood, while the slow reacting enantiomers are not so easy to understand. During this work we learnt that the active site of the *Candida antarctica* lipase is flexible and substrates expressing low reaction rates often need a conformational change of the active site. A situation that would be very difficult to incorporate in a box model.

The enantioselectivity of the esterification of 2-metylalkanoic acids catalysed by *Candida rugosa* lipase was shown to depend on the concentration and chain length of the alcohol used, a result that was not expected from the kinetic models.[5] By molecular modelling it could be shown that the alcohol could bind to the free enzyme and change the binding mode of the acid. Two binding modes for the acid were found which resulted in different enantioselectivity.[6]

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SESSION II
ENGINEERED ENZYMES

Chair: Antonio Ballesteros

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IMPROVING HYDROLASES BY CHEMICAL MODIFICATION

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The *in vitro* preparation of enzymes chemically modified with suitable moieties offers the possibility of obtaining new biocatalysts —semisynthetic enzymes— with better properties (stability, selectivity, new activities, etc.).

The hydrophilic-lipophilic balance (HLB) of an enzyme seems to be essential in many of its properties (recognition of substrates, binding, stability, etc.).

i) In this context, the post-translational modification of proteins by covalent attachment of fatty acids and lipids is a widespread phenomenon in nature but not yet completely understood. Myristic and palmitic acid are the predominant fatty acid residues linked to proteins. Myristic acid is commonly found attached by an amide bond to an N-terminal glycine; palmitic acid is bound to the protein via an alkali-labile ester linkage, usually a thioester.

Several methods of *in vitro* acylation of proteins with fatty acids have been described. Therapeutical applications of these acyl-proteins and acyl-antibodies are very promising, since hydrophobization imparts transmembrane properties to water-soluble proteins or makes possible their insertion into lipid bilayers.

ii) Polyethylene glycol (PEG) is a synthetic polymer with amphipathic properties. The hydrophilic nature of PEG makes possible to modify enzymes in aqueous solution, and its hydrophobic character enables the modified enzymes to function in lipophilic environments. Indeed, PEG-modified enzymes ("pegylated" enzymes) have been reported to be soluble and active in organic solvents. Interestingly, the plasma half-lives of several enzymes of therapeutical use have been notably prolonged by conjugation with PEG.

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We have carried out in our laboratory the chemical modification of a protease from *Bacillus* (subtilisin) and lipases A and B from *Candida rugosa* (formerly *C. cylindracea*) with fatty acids and PEG of different size. The properties of the new biocatalysts (activity in aqueous and organic media, stability toward different denaturants) will be presented.

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Semisynthetic Proteins

R.E. Offord

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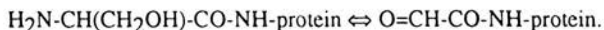
Like several other contributions to this Workshop, this lecture will deal with protein engineering methods which can give access to new macromolecular structures, difficult or impossible to obtain by biosynthetic methods.

Since any given polypeptide chain will have only one α-amino group and one α-carboxyl group, irrespective of the number of side-chain groups, the ability to conduct reactions exclusively at the chain termini permits us to build up novel structures in a completely controlled way.

Methodology

We normally activate the amino terminus of a protein or a fragment for further chemical reaction by extremely mild periodate oxidation. The amino-terminal residue must be threonine or serine since the reaction in question is the oxidation of the 1-amino, 2-ol structure first suggested for protein-chemical use by Dixon (Ref. 1) e.g.

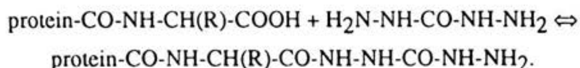


H_2N-CH(CH_2OH)-CO-NH-protein ⇔ O=CH-CO-NH-protein.

Serine and threonine residues that are within the sequence, and therefore lack the necessary free -NH_2 group, do not react in any way. The restriction to Ser and Thr at the N-terminus is not very burdensome since these residues are quite often naturally present in that position, or can be introduced there by genetic manipulation. N-terminal cysteine might also work in this reaction, but we have no experience with it.

The reaction is much faster than the much better known periodate oxidation of the 1,2 diols in carbohydrates and, in the cases that we have studied, the oxidations can be carried out at sufficiently low concentrations of periodate for a sufficiently short length of time that the oxidation of side-chain methionine and tryptophan does not interfere.

For the carboxyl terminus, we use most frequently reverse proteolysis, in the presence of the dihydrazide of carbonic acid, to place a hydrazo group at the carboxyl terminus (e.g. Refs. 2 and 3)



protein-CO-NH-CH(R)-COOH + H_2N-NH-CO-NH-NH_2 ⇔

protein-CO-NH-CH(R)-CO-NH-NH-CO-NH-NH_2.

It will be explained during the lecture why this compound is particularly useful in this context.

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Examples

We have been able to use the above methods, and others related to them, to reconstruct proteins from several large fragments, while replacing an interior segment with a totally non-biological structure (e.g. Ref. 2.). The same methodology permits us to make chimeric fusion constructions between two proteins, head-to-tail, head-to-head, or tail-to-tail (e.g. Refs. 3, 4). Fusion constructions with other categories of macromolecules are also possible (e.g. Ref. 5). Smaller non biological structures can also be placed at one end or other of the polypeptide chain, often giving access to derivatives of practical or experimental value (e.g. Ref. 6).

The above ideas will be illustrated by a description of chimeric proteins in which one segment is an enzyme, but also of chimerae and other analogues taken from the immunoconjugate, cytokine, and chemokine fields. The methodology in these latter examples could just as well be applied to enzymic problems.

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Finding Novel Catalytic Activities of Enzymes: Acylase as a Catalyst for the Selective Synthesis of Esters and Amides

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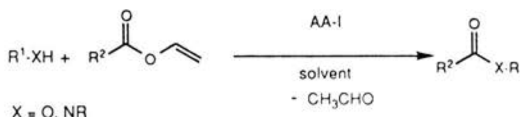
We have recently found [Herradón, B.; Valverde, S. *Synlett* **1995**, 599] that the readily available enzyme Acylase I from *Aspergillus* species (AA-I) is able to catalyze the acylation of alcohols and amines, if a low water-content media and a acylate agent (i. e., a vinyl ester) are provided (Scheme 1)

Scheme 1



This novel catalytic activity of AA-I, which was not previously reported in the literature, is interesting from both synthetic and bioorganic points of view. The synthetic implications (stability, activity and selectivity in organic solvents, the influence of the nature of the acylating agent and the structure of the substrate on the selectivity) will be discussed in detail.

On the other hand, the fact that this enzyme catalyze this "unnantural" reaction opens several questions from a bioorganic point of view: what is the mechanism?, the active centre of this reaction is the same as in the "natural" (amide hydrolysis of N-acylamino acids) reaction or is there a second active centre?, is there any evolutive implication on this fact?.

Work is in progress to try to answer these questions.

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LIPASE-CATALYZED ENANTIOSELECTIVE SYNTHESIS OF METHYL (R) AND (S)-2-TETRADECYLOXIRANECARBOXYLATE THROUGH SEQUENCIAL KINETIC RESOLUTION

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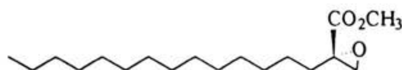
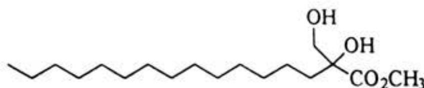
Methyl 2-tetradecyloxiranecarboxylate (**1**) (methyl palmoxirate, methyl 2-tetradecylglycidate) is a potent oral hypoglycemic and antiketogenic agent in mammals including humans [1]. The compound has been reported to be specific inhibitor of long-chain fatty acid oxidation by irreversible active-site directed inactivation of carnitine palmitoyl transferase A, after conversion into the corresponding acyl-coA thioester [2]. Enzyme-catalyzed reactions constitute a great potential for the synthesis of enantiomerically pure compounds through kinetic resolution of the racemic precursors or by asymmetrization of prochiral substrates [3]. Continuing our efforts directed to the enzyme-mediated chiral resolution of secondary alcohols [4,5], we present herein a new, short and straightforward enantioselective synthesis of both enantiomers of **1** by sequential kinetic resolution catalyzed by *Pseudomonas fluorescens* lipase. The procedure involves as the key step transesterification reaction of racemic (*R,S*)-**3** in *tert*-butyl methyl ether as solvent, and affords both enantiomers in good chemical and optical yields. The process offers similar results than those obtained by Sharpless asymmetric dihydroxylation of diol **3**.



(R)-(+)-1



(R,S)-3

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Chair: Donald Hilvert

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Engineering the coenzyme specificity of 2-oxo acid dehydrogenase multienzyme complexes: the role of protein domains

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The three-dimensional structures of many proteins extant today suggest that they have evolved by permutation and combination of a relatively small number of domains or modules that became adapted to perform different functions in different settings. The importance of superfamilies and domain superfolds is increasingly evident (Orengo et al., 1994). Some are self-evident, given the high level of amino acid sequence similarity that they exhibit in different proteins (Brändén & Tooze, 1991). In other instances, however, the relationship may have become obscured by a long period of divergent evolution, and been limited only by a need for certain amino acids to occupy particular sites in order to comply with a set of structural constraints that defines the particular fold. For example, the lipoyl domain (Ricaud et al., 1996) of the dihydrolipoyl acyltransferase (E2) chain of 2-oxo acid dehydrogenase complexes (Perham, 1991) and the biotinyl domain of biotin-dependent carboxylases exhibit only vestigial sequence similarity, yet have similar three-dimensional structures (Brocklehurst & Perham, 1993; Athappilly & Hendrickson, 1995). Likewise, a 30-residue sequence motif predicted (Hawkins et al., 1989) to participate in the binding of the cofactor thiamin diphosphate in the 2-oxo acid decarboxylase (E1) component of the same 2-oxo acid dehydrogenase complexes, has been found to adopt a common fold in the crystal structures of three different and otherwise unrelated enzymes that require this cofactor for catalysis (Muller et al., 1993).

Folding domains are generally at their most obvious in multifunctional proteins, from which they can often be released by limited proteolysis (Bork, 1991; Perham, 1991). The biological function associated, in whole or part, with a given domain is then easy to assess. With many proteins, however, the domains have become intimately incorporated into the three-dimensional structure of the protein, making important contacts with other component parts of the overall structure. In such instances, the domain cannot be released by limited proteolysis and the autonomy of the domain is inferred from the frequency of the occurrence of its characteristic folding topology in different proteins. A typical example is one of the first domains to be described: the dinucleotide-binding domain (Rossmann fold) found in most dehydrogenases (Rossmann et al., 1975; Wierenga et al., 1985). This is a prominent feature in the structures of the flavoprotein disulphide oxidoreductases (Williams, 1992), which include glutathione reductase (Karplus & Schulz, 1987) and dihydrolipoyl dehydrogenase (Mattevi et al., 1993). The latter enzyme is found as the last (E3) of the three enzymes that aggregate in multiple copies to form the 2-oxo acid dehydrogenase multienzyme complexes (Perham, 1991).

In biological systems, reductive endergonic reactions are generally catalysed by enzymes that utilize NADPH as cofactor, whereas oxidative exergonic reactions are almost always catalysed by enzymes that exhibit a marked preference for NAD. The relevant enzymes must therefore discriminate between these structurally similar coenzyme molecules, which differ only by the presence of a phosphate group esterified to the 2'-hydroxyl group of the AMP moiety of NADP. Systematic replacement of a set of amino acids in the βαβ-fold of the NAD-binding domain of *Escherichia coli* dihydrolipoyl dehydrogenase has been used to convert its coenzyme specificity from NAD to NADP. After comparison with the homologous enzyme, glutathione reductase (Scrutton et al., 1990), Glu 203 was replaced with a valine residue, thereby eliminating the potential to form hydrogen bonds with the 2'- and 3'-OH groups of the adenine ribose in NAD. Similarly, Met 204, Pro 210, Phe 205 and Asp 206 were replaced by an arginine, an arginine, a lysine and a histidine residue, respectively, to provide a nest of positive charge to accommodate the 2'-phosphate group of the incoming NADP. In addition,

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Gly 185 and Gly 189 in the βαβ-motif were replaced with alanine residues to facilitate the positioning of the newly introduced Val 203 by allowing a flip of the peptide bond between residues Gly 180 and Gly 181 (Mittl et al., 1993). Wild-type dihydrolipoyl dehydrogenase is inactive with NADP but the mutant enzyme displayed high levels of activity with this coenzyme, the values of K_m, k_{cat} and k_{cat}/K_m comparing favourably with those found for the wild-type enzyme operating with NAD (Bocanegra et al., 1993).

E3 is bound to E2 in 2-oxo acid dehydrogenase complexes by interaction with a small (35-residue) domain in the E2 polypeptide chain (Perham, 1991). The binding site on the E3 dimer is located close to its 2-fold axis, thereby permitting the binding of a single E3 dimer per E2 chain (Hipps et al., 1994; Lessard et al., 1996). The binding site is confined to the well delineated interface domain that provides most of the subunit interface in the E3 dimer, as indicated by the X-ray crystallographic structure of the E3-binding domain complex (Mande et al., 1996). The ability to manipulate the dinucleotide-binding domain without interfering with the other domains in the protein is reflected in the ability of the mutant enzyme to participate *in vitro* in the assembly of an active pyruvate dehydrogenase multienzyme complex, the coenzyme specificity of which reflected that of its dihydrolipoyl dehydrogenase component (Bocanegra et al., 1993). These mutations constitute the basis of a general set of rules for manipulating the coenzyme specificity of NAD(P)-dependent oxidoreductases.

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Engineering Enzymes and Abzymes by Rational and Non-rational Means,
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Subtilisin BPN' is a simple bacterial serine protease which is ideal for protein engineering studies (for review see 1). It can be over-expressed in *Bacillus subtilis*; it is simple to assay; the high resolution structure is known. We have had a long-standing interest in engineering subtilisin as a tool for specific cutting (2) and splicing (3) of proteins.

Here, we present work to design subtilisin as a sequence specific protease (4, 5). Recent studies have uncovered a family of subtilisin-like protease from eukaryotes that recognize poly-basic sequences (for review see 6). Yeast produces a dibasic-specific enzyme, KEX2, involved in processing mating factor; mammalian cells produce tri-basic specific enzymes, furin and PC-1, involved in processing of a number of proteins and small peptide hormones. We wished to impart the specificity properties of these two enzymes into subtilisin BPN', which has broad specificity although preference for sequences containing hydrophobic residues.

Based solely on sequence alignments we were able to identify acidic residues in KEX and furin predicted to be in the specificity pockets that are not present in subtilisin. When some of these from KEX2 were installed we observed a large shift in catalytic efficiency toward di-basic residues (4). An additional acidic residue from furin was added and pushed specificity toward tri-basic sequences (5). These changes in specificity (over 5 orders of magnitude) accumulated in a modular but non-additive fashion. Thus, it is possible to recruit specificity changes even from very distant homologues. These newly engineered subtilisins may be useful for site-specific proteolysis.

Antibodies raised against a variety of transition-state analogs have produced catalysts (7). However, the rate enhancements seldom rival enzymes for the same reactions. We wondered if they could be improved by selecting for tighter transition-state analog binding using phage display (for recent review see 8). For this we focused on a mouse monoclonal antibody raised against a methionyl-phenyl-phosphonate designed to mimic the transition state (ts) for hydrolysis of methionyl-phenyl ester (9). This antibody has good activity and the structure has been solved with the ts analog bound.

Initial recombinant work on this project was hampered by poor expression of the antibody in *E. coli*. We solved this problem by grafting the ts analog binding regions (the CDR's) onto a human antibody scaffold that is well expressed in *E. coli* (10). The "humanized" catalytic antibody bound the ts analog and catalyzed ester hydrolysis just as well as the parent mouse antibody (11). Moreover, the Fab derived from this could be displayed on

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phage at levels >200-times higher than the mouse antibody, making it suitable for affinity selections.

We randomized all residues in the antibody that made direct contacts with the ts hapten (11). After six rounds of sorting for binding to the ts analog, phage were isolated that bound the ts analog 2- to 5-times tighter than the parent Fab. Some of these were combined to create variants that bound up to 8-times tighter. However, all of these variants had activities much lower (some more than 20-times lower) than the parent antibody. We then looked at some isolates with weaker ts analog binding and indeed found one that produced a 2 to 4 fold improvement in activity. Interestingly, this mutant was altered at a site just outside the hapten binding site. Thus, improving ts analog binding did not correlate with increased activity. An activity improvement could be made but at a site not directly contacting the hapten. Given these uncertainties, in the short term it seems more likely that substantial improvements in catalytic antibody function will be made not through rational means but via random mutagenesis and selection.

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Chair: Richard N. Perham

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Engineering of protein inhibitors of metallocarboxypeptidases

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Only a few protein inhibitors of metallo-carboxypeptidases have been described. The potato carboxypeptidase inhibitor (PCI) is the best known. Because of its small size (39 residues), globular character, and the knowledge of its crystal structure in complex with bovine carboxypeptidase A, it is a good model for the development of simple and specific inhibitors of carboxypeptidases through engineering.

With this aim we have developed a very efficient expression system for PCI and side-directed mutants in *E. coli*. This allowed us to systematically analyze the determinants for the folding and function of the inhibitor. Thus, differential mutagenesis of the six cysteine residues of PCI prevent its heterologous expression and indicate that they are essential for the proper folding of the protein. Studies starting from fully reduced PCI indicate that refolding proceeds through a packing and formation of, at least, five defined S-S scrambled species, followed by a slow consolidation by reshuffling. Studies with trimmed molecules indicate that the tails influence folding. Point mutation and deletion studies were performed on the C-terminal tail (Gly35- Pro36- Tyr37- Val38- Gly39), the region that docks with carboxypeptidase. They indicate that Val38 is essential for the inhibitory function and binding, that Tyr37 plays a role in the allocation of the C-tail in the active site, and Pro36 in the folding mechanism of PCI. Molecular dynamics (MD) simulations complemented the view obtained by mutagenesis and clarified the constrains of the C-tail. Deletion experiments at this region further complemented this view.

With the above information in mind, we have tried to "minimize" PCI to smaller molecules keeping the inhibitory capability. These minimized molecules could have biotechnological interest to modulate the activity of pancreatic-like carboxypeptidases, some of them with important physiological action. With this goal, peptide derivatives mimmicking the C-tail of PCI were syntesized and analyzed. In most of these derivatives the N-terminus was capped to mimmic a stopper-like action and to facilitate their productive interaction with the enzyme. Capping groups of very different nature and size were assayed : small to large organic, polimeric organic, branched polypeptidic, oligosaccharide...etc. A few of the derivatives keep a substantial fraction of the inhibitory capability of PCI. They are used as lead compounds for the obtention of more powerful inhibitors

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Analysis and design of ion channel proteins:

W. F. DeGrado

Protein ion channels are essential for the generation and propagation of electrical activity in a variety of cells. Two features important for their activities are ion selectivity and rectification (asymmetry in current/voltage relationships). De novo design is an attractive approach to probe the minimal features required for these functions. Recently, we designed two simple alpha-helical models for ion channel proteins using only Leu and Ser residues. One forms proton-selective channels while the second forms channels selective for cations smaller than 8 Å in diameter. These peptides are believed to form helical bundles with a central core that conducts ions across membranes. Derivatives of these peptides have been prepared to determine how electrostatic and steric interactions affect ion selectivity and rectification.

The principals learned from the study of designed channels have also been applied to the analysis of a proton-selective channel protein (M2) from the influnza A virus. M2 is 97-residues in length and has a single transmembrane helix domain, which forms alpha-helical tetramers in membranes. It is essential for cell infection and is the target of the only approved anti-influenza drugs. Based on a combination of site directed mutagenesis, computer modeling, and spectroscopic studies, the structure of this transmembrane helical bundle has been structurally characterized. The proposed structure predicts a mechanism for the selective translocation of protons through the pore.

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DESIGN OF FUNCTIONALIZED PROTEIN-LIKE STRUCTURES USING SYNTHETIC CONFORMATIONALLY-DEFINED LIBRARIES

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Synthetic combinatorial libraries (SCLs) are broadly recognized as having the capability of greatly accelerating the discovery of new lead compounds. SCL approaches have primarily been focused on the generation of small molecule diversities. The generation of molecular diversities based on defined structural motifs can be expected to broaden the use of SCLs for those applications requiring the presence of a well defined secondary and/or tertiary structure. For example, the *de novo* design of artificial receptors and catalysts in most cases requires the generation of protein-like molecules having the general structural and functional properties found in natural enzymes. Thus, a productive strategy can be seen in the selection-based design of well defined secondary and tertiary structures, which maintain sufficient flexibility to allow the accessibility of the functionalities required for catalysis to occur. An approach will be described for the *de novo* design of protein-like structures in which SCLs were incorporated into an amphipathic α-helical scaffold to generate conformationally defined SCLs. In particular, the SCLs in which the "combinatorialized" positions were on the hydrophilic face permitted the identification of conformation-dependent decarboxylation catalysts.

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Analogues of Dihydrofolate Reductase and Firefly Luciferase Containing Synthetic Amino Acids at Defined Positions

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We have prepared analogues of *E. coli* dihydrofolate reductase and firefly luciferase containing unnatural amino acids at predetermined sites. This was accomplished by the use of genes encoding these enzymes and containing engineered nonsense codons at key positions. Misacylated suppressor tRNAs activated with the unnatural amino acids of interest were prepared and used in *in vitro* translation systems to permit the elaboration of the desired protein analogues by suppression of the nonsense codons. The derived proteins have been purified, and characterized by their mobility on polyacrylamide gels in comparison with the wild-type proteins. Representative proteins containing amino acid analogues have also been analyzed by defined proteolysis to afford peptides containing the unnatural amino acids; the structures of the peptides have been verified through direct comparison with authentic synthetic peptides.

The preparation of analogues of *E. coli* dihydrofolate reductase (DHFR) containing modified amino acids was accomplished by the use of the DHFR gene containing an engineered nonsense codon (TAG) at the positions corresponding to Val-10 or Asp-27. The aspartic acid analogues *erythro*-carboxyproline, cysteic acid, β,β-dimethylaspartic acid, α-methylaspartic acid, *erythro*- and *threo*-β-methylaspartic acid, *N*-methylaspartic acid and phosphonoalanine were incorporated into one or both of the aforementioned positions. Individual DHFR analogues were assayed for their abilities to bind to the substrate analogue methotrexate, and to convert dihydrofolate to tetrahydrofolate. DHFR analogues containing *erythro*- and *threo*-β-methylaspartic acid and β,β-dimethylaspartic acid were all shown to mediate tetrahydrofolate production 74-86% as efficiently as wild-type DHFR under conditions of multiple substrate turnover. Analysis of the rates of tetrahydrofolate production in the presence of NADPH and NADPD at two pH values suggests that this was due to rate-limiting hydride transfer from NADPH bound to DHFR analogues whose active sites had become distorted.

Serine was replaced at position 286 of firefly luciferase (*Luciola mingrelica*) with a series of natural and unnatural amino acids. The effect of these substitutions on the properties of luciferase such as thermostability, pH dependence, and color of light emitted were investigated. Incorporation of Leu, Lys, Tyr, or Gln at position 286 reduced the thermostability of the mutated luciferases and changed the color of emitted light from yellow-green (λ_{max} 582) for the wild-type enzyme to red (λ_{max} 622) for luciferase containing Leu 286. The biologically important amino acid phosphotyrosine and the phosphatase resistant analogue (phosphonomethyl)phenylalanine were prepared using 2-nitrobenzyl as the phosphate protecting group. This new protecting group strategy allowed the incorporation of the phosphorylated amino acids at position 286 of luciferase. Also incorporated was a glucosylated serine derivative. The light emitted by the modified luciferases was measured. While serine phosphonate and glucosylated serine do not change the λ_{max} of emitted light, the incorporation of phosphotyrosine and (phosphonomethyl)phenylalanine do alter the wavelength of emitted light. The dependence of λ_{max} of light emitted from the negatively charged phosphorylated Tyr mutant was analyzed further by assaying the luciferase at different pH values.

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Molecular and Bio-Imprinting - new techniques allowing formation of artificial "binders" and biocatalysts or of enzymes with altered substrate specificity

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The technique of molecular imprinting allows the formation of specific recognition and catalytic sites in macromolecules by the use of templates. Molecularly imprinted polymers have been applied in an increasing number of applications where molecular binding events are of interest. These include (i) the use of molecularly imprinted polymers as tailor-made separation materials, (ii) antibody and receptor binding site mimics in recognition and assay systems, (iii) enzyme mimics for catalytic applications, and (iv) recognition elements in biosensors. The stability and low cost of molecularly imprinted polymers make them advantageous for potential use in analysis as well as in industrial scale production and application.

The concept of molecular imprinting involves that the template or the molecule to be imprinted are first allowed to form bonds with polymerizable entities, which are subsequently crosslinked (see ref. 1-5). Following extraction of the print molecule, specific recognition sites are left in the polymer where the spatial arrangement of the polymer network corresponds to the imprinted molecule. These procedures make use of a high percentage of crosslinker resulting in the formation of rigid and insoluble macroporous polymers. This template-assisted assembly, leading to an artificial recognition matrix, is thus performed in a very direct way.

In the approach developed in our laboratory and found to be extremely versatile as it mimics Nature's kind of interactions occurring in molecular recognition, predominantly noncovalent interactions in the recognition of the imprint species are utilized. The greater the variety of interactions that are available between the imprint species and the functional monomers, the better the artificial binding site becomes. Typical interaction types that have been exploited are ionic interactions, hydrogen bonds, π-π-interactions, and hydrophobic interactions. Since there are strongly dependent on the polarity of the solvent, the best imprints are made in organic solvents such as chloroform or toluene. When these normally weak interactions have been established in solution, polymerization is initiated and a molecular matrix is formed around the imprint species. The formed macromolecular architecture is thus complementary to the shape and function of the imprint species. After polymer formation the imprint molecule can be almost quantitatively recovered by mild extraction from the matrix. Association and dissociation of the original print molecule to the artificial binder takes place without requiring any covalent bond formation or cleavage. The target molecule simply diffuses in and out of the complementary sites.

The following predictions as to future applications of the technology will be discussed:

Separation. There is significant potential for applications in molecular separation and isolation. Since there are presently about 500 optically active drugs on the market racemic resolution of drugs is a major potential application. We and other groups working in molecular imprinting have concentrated on this aspect since the first work on β-blocker drug separation (L. Fischer, R. Müller, B. Ekberg and K. Mosbach, J. Am. Chem. Soc. 113, 9358-9360, 1991 (6)).

Imprinting will allow the preparation of tailor-made supports. A potential drawback is that the imprint material is required in the initial step. The obtained polymers, however, can be reused more than 100, in many cases compensating for initial hardships. However, it has been shown that when using a smaller percentage of cross-linker, one kind of imprint may be used for racemic resolution of related structures making isolation of imprint material unnecessary in each case. Pure material is not required for the first imprint, as long as the material collected from the correct peak is subsequently used.

Instituto Juan March (Madrid)

Antibody/receptor binding mimics. This is an area that we expect will experience rapid development, notably in immunoaffinity chromatography and immunoassays. The stability, reduced cost, obviation of the need for animals, and in cases where it is difficult or impossible to get biological antibodies, are all driving the development of these applications (G. Vlatakis, L.I. Andersson, R. Müller and K. Mosbach, Nature 361, 645-647, 1993 (7); L.I. Andersson, R. Müller, G. Vlatakis and K. Mosbach, Proc. Natl. Acad. Sci. U.S.A. 92, 4788-4792, 1995 (8)). There is also the potential to use small molecularly imprinted polymers in medicine as carriers of (pro)drugs or radioligands.

Biosensor-like devices. The use of molecularly imprinted polymers in biosensor devices seems fairly close to hand. In the short term, efforts should be devoted to obtaining imprinted membrane structures that can be placed in direct contact with the transducer. Biosensor-like devices may eventually be made using imprints that are robust with binding specificities not found in biological molecules (D. Kriz, O. Ramström, A. Svensson and K. Mosbach, Anal. Chem. 67, 2142-2144, 1995 (9)).

In addition to pharmaceutical analysis, other attempts have dealt with the use of molecularly imprinted polymers in environmental analysis. Of special concern in this area is the development of sensitive analytical assays for determination of various hazardous contaminants in water reserves. Very recently, studies concerning the pesticide atrazine, chosen as a model system, have been presented. From these studies, it has become clear that this approach has potential for becoming a powerful tool in environmental analysis and beyond that, for clean-up.

Finally, mention should be made to the intriguing challenge for the use of molecularly imprinted polymers as enzyme mimics (e.g. J. Matsui, I.A. Nicholls, I. Karube and K. Mosbach, J. Org. Chem. 61, 5414-5417, 1996 (10)).

Somewhat related to the above described technique of molecular imprinting is a technique which we like to call bio-imprinting. It has recently been shown that the specificity and activity of enzymes and proteins can be modified when used in anhydrous or nearly anhydrous media. In one case, chymotrypsin was precipitated by addition of 1-propanol in the presence of *N*-acetyl-D-tryptophan (M. Ståhl, M.-O. Månsson and K. Mosbach, Biotechnol. Lett. 12, 161-166, 1990; M. Ståhl, U. Jeppsson-Wistrand, M.-O. Månsson and K. Mosbach, J. Am. Chem. Soc. 113, 9366-9368, 1991; M.-O. Månsson, M. Ståhl and K. Mosbach, In: Biocatalysis in Non-Conventional Media (J. Tramper et al., eds.), Elsevier Science Publishers B.V., pp. 321-327, 1992). After careful drying, the enzyme precipitate was able to catalyze, not only the synthesis of the ethyl ester of *N*-acetyl-L-tryptophan, but also the synthesis of the corresponding D-ester which was not possible with enzyme precipitated in the presence of the corresponding L-derivative of tryptophan or precipitated without ligand present. A possible explanation for this phenomenon is that the conformation of the active site of the enzyme is changed during the precipitation and drying into one that is complementary not only to the L-form but also to the D-form. Addition of water to the conformationally modified enzyme accordingly returned the enzyme to its original conformation active only towards the L-form. It has come to our attention that by subsequent cross-linking it was possible to stabilize the new conformation, such that the enzyme chymotrypsin could subsequently express changed the stereospecificity also in water (L. Fischer, personal communication). In other examples, artificial binding sites could be found after an imprinting procedure in albumin and other natural and synthetic polymers when they were used in organic solvents (L. Braco, K. Dabulis and A.M. Klibanov, Proc. Natl. Acad. Sci. 87, 274-277, 1990; K. Dabulis and A.M. Klibanov, Biotechnol. Bioeng. 39, 176-185, 1992).

More recently we were able to show that the enzyme horse liver alcohol dehydrogenase, when similarly "bio-imprinted" in the presence of $NADP^+$, would subsequently accept not only NAD^+ but also $NADP^+$ as a coenzyme (A. Johansson, K. Mosbach and M.-O. Månsson, Eur. J. Biochem. 227, 551-555, 1995).

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Structural Convergence Seen In The Active Sites Of A Family Of Catalytic Antibodies.

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Abstract: The X-ray structures of three esterase-like catalytic antibodies identified by screening for catalytic activity the entire hybridoma repertoire, elicited in response to a phosphonate transition state analog (TSA) hapten, were analyzed. The high resolution structures of Fab D2.3 account for catalysis by transition state stabilization and in all three antibodies a tyrosine residue participates in the oxyanion hole. Despite significant conformational differences in their combining sites, the three antibodies, which are the most efficient among those elicited, achieve catalysis in essentially the same mode, suggesting that evolution for binding to a single TSA followed by screening for catalysis lead to antibodies with structural convergence.

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SESSION III
OTHER CATALYSTS

Chair: Javier de Mendoza

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Efficiency in Biocatalysis: Lessons from Model Systems

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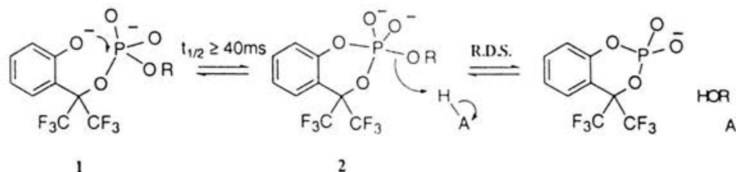
The extraordinary efficiency of enzyme catalysis is the long-term inspiration of our work. We want to understand the principles underlying this phenomenon well enough to be able to "explain" how enzymes make and break covalent bonds, in terms that are intelligible to any properly educated chemist. Understanding in this context means defining and as far as possible quantifying the differences between the transition state in solution and in the enzyme active site. [1] These will be greatest, and thus most interesting and most revealing, for intrinsically very slow reactions, so we have a special interest in the ways enzymes catalyse transfer reactions involving the the extraordinarily stable groups of structural biology - peptides, glycosides and phosphodiesters.

Our chosen experimental tool is the intramolecular reaction: we bring functional groups together on the same molecule to model what goes on when an enzyme brings together the same functional groups in its active site. This procedure has the unique advantage that we can *see* the reactions of interest - we do not need activated and perhaps atypical substrate groups to set up a reaction fast enough to study in the absence of enzyme. For example, we have a special interest in nuclease mechanisms. In the active site of the appropriate nuclease, where the attacking nucleophile is a hydroxyl group, this is reduced to a fraction of a second: the same is true of the simple intramolecular model **1** [2] This phosphodiester was studied in detail for the methyl ester (R = CH_3): the trifluoroethyl ester (R = CH_2CF_3, modelling the better leaving group of a nucleotide OH) has an estimated half life (at 50°C) of 40ms.



The results from this and related work tell us that (in the absence of metals) efficient phosphate transfer from a phosphodiester needs (1) a properly placed OH group, available as the (full or incipient) anion as the nucleophile; (2) a properly placed general acid to protonate the leaving group, and (3) a properly placed ammonium cation to stabilise the phosphorane dianion (a species thought to have only

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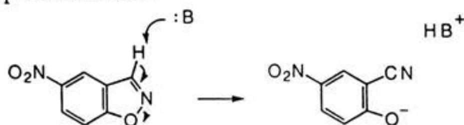
borderline stability ion water). This sort of study provides a firm basis for informed discussion of the mechanisms of enzymes which catalyse phosphate transfer reactions of diesters, in active sites which often display these three features.

A major current interest is in the efficiency of proton transfer catalysis. (Proton transfer being the most common enzyme-catalysed reaction.) Catalysis of intramolecular proton transfer reactions was thought until recently to be intrinsically inefficient, [3] but it turns out that the reaction is simply much more stereoelectronically demanding than group transfers involving heavy atoms. Recent results include the development of small molecules catalysing efficient intramolecular proton transfers reactions, [4] and some relevant results with proteins catalysing the Kemp elimination. [5]



Work in progress is aimed at coupling efficient group and proton transfer reactions.

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GROUND STATE CONFORMATIONS AND ENTROPIC AND ENTHALPIC FACTORS IN THE EFFICIENCY OF INTRAMOLECULAR AND ENZYMATIC REACTIONS

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The studies of intramolecular reactions have had a lasting influence on how the mechanisms of enzymatic reactions are perceived. It is from these studies that enzymologists have accepted the conclusions: (i) that the bringing of reactants together, as in an enzyme-substrate complex, provides a rate enhancement of 10^8 M; and (ii) that enzymatic reactions are entropy driven. The lecture will begin by an analysis of a detailed series of intramolecular reactions. The contribution of ground state conformations to the rate constants will be described by use of molecular mechanics [MM3(92)] which also allows analysis of entropic and enthalpic parameters.[1] Near Attack Conformations (NACs) are identified and, by use of the Boltzman equation, the mole fraction (P) of conformations present as NACs is determined. Values of $\Delta G^‡$ are shown to be a linear function (slope ~1) of log P such that the fraction of conformers present as NACs, in the ground state, determines the rates of reactions. Further, $\Delta H°$ rather than $\Delta S°$, is a function of log P.

Transition state formation from NACs have been studied using *ab initio* calculations at the RHF/6-31+G(d) level.[2] A common transition state (TS) structure is reached for the series of reactions studied whose rates differ by as much as 10^5. Further, vibrational frequency calculations show that the frequencies for the related pairs NAC & TS differ in number only by the required conversion of one frequency of the NAC to a negative frequency in the TS. Comparing substrates, there appear to be virtually no change in $\Delta S^‡$. Thus, there is no support for the proposals that the driving force is entropic; TS stabilization by varying TS structures and the freezing out of low frequency vibrations.

We describe two enzymes which personify different driving forces in their reactions. In the first ground state conformational changes contribute to the lowering of $\Delta G^‡$ [NAD(P)H/NADP$^+$ dehydrogenases].[3] The second enzymatic reaction is catalyzed by the stabilization of the gas phase transition state [an S_N2 displacement of Cl^- from a primary haloalkane by Asp-CO_2^-].[4]

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Chair: Klaus Mosbach

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Supramolecular Reactivity and Catalysis

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Reactivity and catalysis represent major features of the functional properties of supramolecular systems. Molecular receptors bearing appropriate functional groups may bind selectively a substrate, react with it, and release the products. Supramolecular reactivity and catalysis thus involve two main steps: recognition of the substrate followed by transformation of the bound species into products.

Bond cleavage reactions have been extensively studied in this respect. A further step lies in the design of systems capable of inducing bond formation, rather than cleavage, which would thus effect synthetic reactions as compared to degradative ones.

These two aspects of chemical reactivity and catalysis have been realized and will be illustrated mainly through the supramolecular catalysis of phosphoryl transfer by macrocyclic polyamines: – bond cleavage in ATP hydrolysis; – bond formation in pyrophosphate synthesis, ATP synthesis and substrate phosphorylation. The processes are respectively of ATPase and kinase nature. The catalysis of enolisation and proton exchange will also be discussed.

Finally, the coupling of an "artificial enzyme" to a natural enzymatic system has been realized. ATP generated in situ from ADP by the macrocyclic protokinase is used up in a sequence of two enzymatic reactions to produce NADPH along the process.

An approach to the design of enzyme inhibitors based on the newly proposed concept of "virtual combinatorial libraries" will be described.

The design of efficient and selective supramolecular reagents and catalysts may give insight into the elementary mechanistic steps, provide new types of chemical reagents and produce models of reactions effected by enzymes that reveal factors contributing to enzymatic catalysis.

General reference

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DESIGN OF ARTIFICIAL CATALYSTS FROM SUPRAMOLECULAR CHEMISTRY PRINCIPLES

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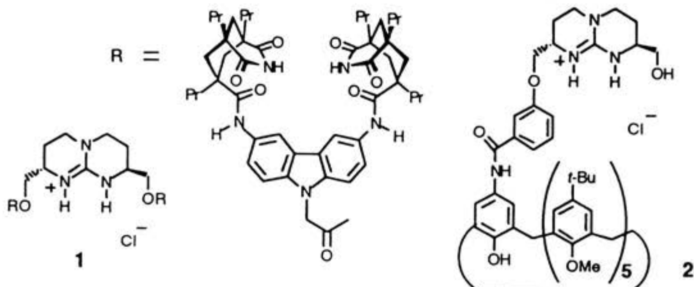
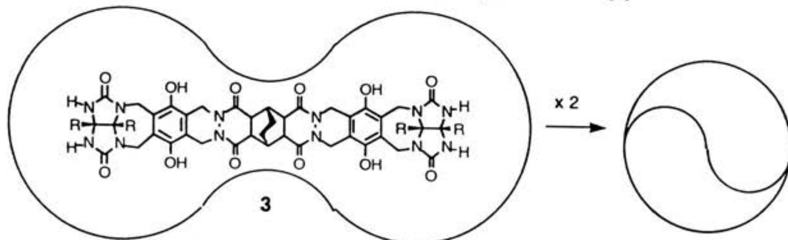
Molecular recognition of biomolecules by synthetic receptors requires modular assembly of various components to complement the molecular characteristics (size, topology, functional groups) of the substrate.



Examples on how molecular shapes and complementary hydrogen bonds can be organized to complement transition states will be provided. Thus, receptor **1**, containing simultaneous aromatic stacking and complementary Hoogsteen and Watson-Crick hydrogen bond patterns, was designed for the molecular recognition of adenine phosphates. It can efficiently transport adenine-containing di- or oligonucleotides across model membranes [1]. Simple guanidiniums (**1**, R = aryl) have demonstrate to catalyze Michael additions to unsaturated lactones. Other derivatives of **1**, carrying reactive groups (imidazoles) at the side arms connecting the guanidinium to the adenine-binding modules, may be useful as phosphodiester cleaving agents. Similarly, receptor **2**, whose design was inspired by the structure of the phosphocholine - antibody McPC603 complex, showed high affinity for phosphatidylcholine derivatives (encapsulation of trimethylammonium cation by the calixarene cavity, and guanidinium-phosphate interaction) [2]. Since the phosphate group is a good model for an ester cleavage transition state, receptor **2** accelerates the aminolysis of acetylcholine.

Reversible self-assembly through hydrogen bonds, may result in formation of stable capsules, such as the dimer of **3**, in whose interiors reactive species can be accomodated. These systems look promising as reaction vessels to accelerate organic reactions [3].



[1] *J. Am. Chem. Soc.* **1994**, *116*, 5501.
[2] *Angew. Chem. Int. Ed. Engl.* **1996**, *35*, 1712.
[3] *Angew. Chem. Int. Ed. Engl.* **1993**, *32*, 1699. *Science* **1995**, *270*, 1409.

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FUSION OF PROTEIN MODULES IN THE GENERATION OF ACTIVE ENZYMES

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The feasibility of creating new enzyme activities from enzymes of known function has precedence in view of protein evolution based on the concepts of molecular recruitment and exon shuffling. The enzymes encoded by the *E. coli* genes *pur*U and *pur*N, N^{10}-formyltetrahydrofolate hydrolase and glycinamide ribonucleotide (GAR) transformylase respectively, catalyse similar yet distinct reactions. N^{10}-formyl-tetrahydrofolate hydrolase uses water to cleave N^{10}-formyltetrahydrofolate into tetrahydrofolate and formate whereas GAR transformylase catalyses the transfer of formyl from N^{10}-formyltetrahydrofolate to GAR to yield formyl-GAR and tetrahydrofolate. The two enzymes show significant homology (~60%) in the carboxy-terminal region which, from the GAR transformylase crystal structure and labeling studies is known to be the site of N^{10}-formyltetrahydrofolate binding. Hybrid proteins were created by joining varying length segments of the N-terminal region of the PurN gene (GAR binding region) and the C-terminal (N^{10}-formyltetrahydrofolate binding) region of PurU. Active PurN/PurU hybrids were then selected for by their ability to complement an auxotrophic *E. coli* strain. Hybrids able to complement the auxotrophs were purified to homogeneity and assayed for activity. The specific activity of two hybrid proteins was within 100-1000 fold of the native *pur*N GAR transformylase validating the approach of constructing an enzyme active site from functional parts of others. A combinatorial approach to improvement of the hybrid enzyme stability and efficiency will be discussed.

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SESSION IV
RIBOZYMES AND ABZYMES

Chair: A.J. Kirby

Instituto Juan March (Madrid)

Biological Catalysis: Lessons from Comparison of RNA and Protein Enzymes

Dan Herschlag, Geeta Narlikar, and Shu-ou Shan, Department of Biochemistry, B400 Beckman Center, Stanford University, Stanford, CA 94305-5307

A classical approach in biology is to compare morphologies in order to glean structural and functional commonalities. The comparative approach has also proven valuable on a molecular level. For example, phylogenetic comparisons of RNA sequences have led to determination of conserved secondary and even tertiary structures, and comparisons of protein structure have led to classifications of families of protein folds. We take this approach in a mechanistic direction, comparing protein and RNA enzymes.

The more recently discovered RNA enzymes, or ribzoymes, provide a distinct perspective on long-standing questions of biological catalysis. The differences observed between RNA and protein enzymes have taught us about aspects of RNA and proteins that are distinct, whereas the common features have helped us understand the aspects that are fundamental to biological catalysis. RNA enzymes, like protein enzymes, are able to use binding interactions away from the site of chemical transformation to facilitate that transformation by positioning substrates and catalytic functional groups and by electrostatic ground state destabilization. These observations allow the concept of 'intrinsic binding energy', put forth by Jencks, to be extended to RNA enzymes, generalized and strengthened.

RNA enzymes appear to be highly amenable to energetic dissection of function. These analyses have general implications for understanding the role of enzyme•substrate interactions in catalysis and for understanding the evolution of catalysts.

The above results will be placed in a conceptual framework for enzymatic catalysis. This framework will further be used to analyze catalytic contributions from enzymatic groups that interact at the site of bond transformation. It is suggested that the enzymatic environment can enhance the ability of hydrogen bonds to preferentially stabilize the transition state, thereby providing rate enhancement relative to the corresponding solution reaction.

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METAL IONS, PHOSPHOROTHIOATES, AND THE HAMMERHEAD RIBOZYME

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When divalent metal ions interact with the hammerhead ribozyme, it is useful to distinguish at least three types of binding: (1) purely electrostatic interactions, which can be replaced by monovalent cations, (2) sites of specific binding needed for proper folding of the RNA, and (3) sites of specific binding needed for the catalytic mechanism. The X-ray crystal structures of several hammerheads performed at high ionic strengths reveal a number of divalent metal ions. Our goal is to develop solution methods to locate functionally important divalent metal ion binding sites and to relate them to the crystal structure.

Three topics will be discussed: (1) The inhibition of the hammerhead ribozyme by Tb^{3+}. (2) The results of systematic single isomer substitution of phosphoromonothioates in the hammerhead ribozyme with an emphasis on the divalent metal ion specificity of cleavage. (3) The properties of hammerhead cleavage with a phosphorodithioate at the cleavage site.

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ENZYMES AND ABZYMES. Stephen J. Benkovic, The Pennsylvania State University, Department of Chemistry, University Park, PA 16802

The development of increasing interactions between the catalytic residues of an enzyme and bound substrate follows from the requirement to diminish the free energy difference between the ground and transition state of the bound substrate. This inference implicates conformational changes in the catalyst throughout the turnover cycle. Since transition states have life times on the order of 10^{-13}-10^{-14} sec., one may imagine elements of the catalyst having matching frequencies not only on this time scale but possibly other frequencies associated with steps in the turnover cycle.

Using dihydrofolate reductase as a paradigm because of the wealth of structural and kinetic data available for the wild-type and mutant forms of the enzyme, we in collaboration with Peter Wright, have determined the dynamics at the amide backbone of the wild-type enzyme·folate complex. After assignment of the resonances of >155 of the 159 amino acid amides, the measurement of longitudinal and transverse relation times by ^{15}N-H NMR found residues in the β-sheet and α-helical structural surrounding the active site moving at frequencies in the millisecond to microsecond time range and residues in various loops resonating at frequencies on the nanosecond to picosecond time scale. Mutagenesis of these latter residues both proximal and distal to the active site, resulted in striking reductions in the rate of hydride transfer as well as in the appearance of steps involving conformational changes not encountered in the kinetic scheme for the wild-type enzyme. One may speculate that there is a causal relationship between these motions and the path chosen to traverse the free energy surface for the reaction.

The nature of the substrate, dihydrofolate, in the ground state E·S complexes was investigated by a Poisson-Boltzman treatment permitting the assignment of the electrostatic potential within the active site cavity. The more favorable electrostatic potential occurred when the enol form of the substrate was bound so that the active site carboxyl derived from Asp27 would be in a neutral form in the hydrophobic environment of this cavity. It appears that in this case the enzyme preorganizes the substrate into a configuration optimal for further reaction.

It is a useful and rewarding exercise to attempt catalyst construction not only for practical purposes but for better understanding of the principles underlying catalysis. We have developed through a unique hapten design a catalytic antibody able to couple a variety of *p*-nitrophenyl amino acid esters and a tryptophan amide to form various dipeptides with all possible stereochemistries at C_{α}. This abzyme also catalyzes tri- and tetrapeptide formation and most recently the production of a four membered amino acid cyclic peptide.

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Chair: Stephen J. Benkovic

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EVOLUTION OF ENZYMES AND ANTIBODIES

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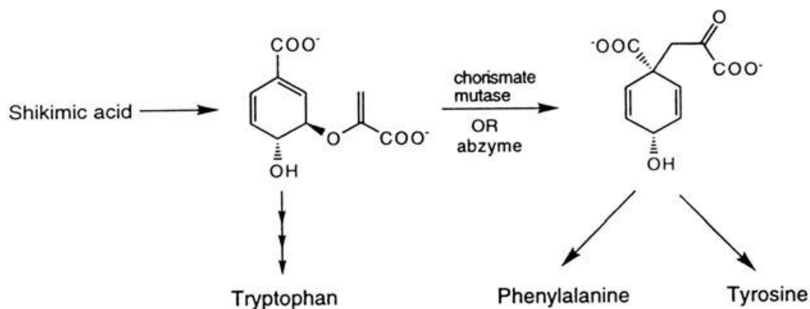
Natural enzymes have evolved over millions of years by a process of Darwinian evolution. Multiple cycles of mutation, selection and amplification can also be exploited by scientists in the laboratory to create and characterize protein catalysts on a human time scale, providing access to macromolecules with tailored activities and selectivities. Recent progress in applying evolutionary approaches to the production, study and optimization of enzymes and antibodies with chorismate mutase activity will be discussed.



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POSTERS

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CONSTRUCTION OF HYBRID GlmS'S BETWEEN *E.coli* AND *Thermus thermophilus*: A TOOL TO ENGINEER PROTEINS.

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GlmS (glucosamine synthase) from *T.thermophilus* and *E.coli* have previously been cloned and purified. These proteins consist of two domains (amino-terminal and carboxy-terminal), each of them having independent catalytic and folding properties. A common restriction site in the coding genes between the two domains, which usage does not alter protein translation frame, was used to construct the hybrid proteins shown in this poster: **GlmSNtCe** (having amino-terminal domain from *Thermus* and carboxy-terminal domain from *E.coli*), and **GlmSNeCt** (having amino-terminal domain from *E.coli* and carboxy-terminal domain from *Thermus*).

To understand the interactions that lead to thermostability, the hybrid proteins wre overexpressed and characterised. Their catalytic activity was measured on the crude extracts at different temperatures, ranging from 30ºC to 60ºC. The activity peak was reached at temperatures intermediate between the growth temperatures of the native organisms. Purification of both hybrids was carried out, and here we present some preliminary results about their biochemical properties.

This represents a particular case of protein engineering, where construction of hybrids to obtain novel proteins with the same enzymatic activity at different temperatures could prove to be useful for a variety of applications.

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Long-distance Control in Stereoselective Reduction of 3-[3-(4'-Bromo [1,1'-biphenyl]-4-yl)-3-keto-1-phenylpropyl]-4-hydroxy-2H-1-benzopyran-2-one; Relative Configuration of Prevailing Diastereomer and Absolute Configuration of its Enantiomers

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Depending on the reducing agent and reaction conditions diastereoselective reduction of 3-[3-(4'-bromo[1,1'-biphenyl]-4-yl)-3-keto-1-phenylpropyl]-4-hydroxy-2H-1-benzopyran-2-one (**2**) proceeds with different stereoselectivity; surprisingly high, ca 90% d.e. of **4A** is achieved with $NaBH_4$ in MeOH at low temperature. Resulting diastereomeric racemates **4A** and **4B** are separated and their respective *syn* and *anti* configurations are assigned on the bases of mechanistic considerations, supported by the ^{1}H-NMR spectra and conformational analysis based on MM2 calculations. The *syn* diastereomer **7A**, 4-OMe derivative of **4A**, was partially resolved by acylation at C(3)-OH with *S*-(-)-camphanic acid to camphanyl ester **12** of (-)-**7A**, leaving (+)-enantiomer **7A**. The assignment of absolute *1S,3R*-configuration to (-)-**7A** is based on comparison of its CD spectrum with those of the model compounds *S*-**14** and *R*-**15**, which represent partial chromophores 4-hydroxy-2H-1-benzopyran-2-one (4-hydroxycoumarin) *A*, and 4'-bromo-1,1'-biphenyl *B*; their excitone coupling is suggested. Preparation of the homochiral form of *R*-**15** is completed by kinetic resolution catalysed by microbial lipases.

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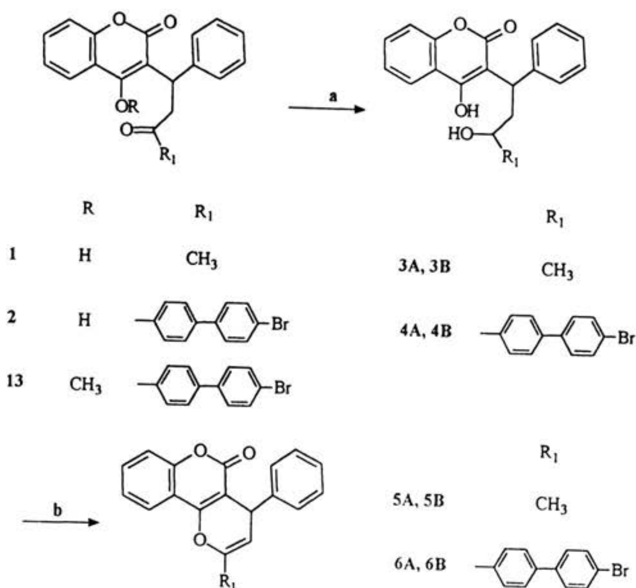
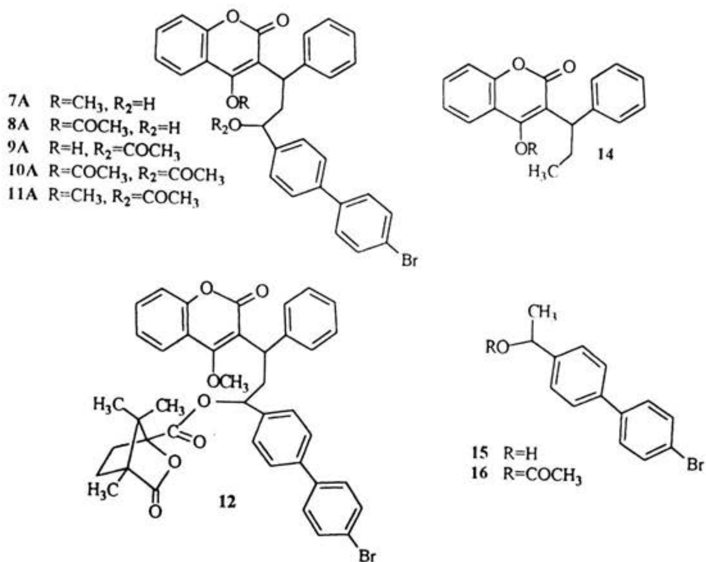


a. $NaBH_4$, solv., b. $BF_3.Et_2O$, 100 ºC



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REGIOSELECTIVITY OF THE TRANSGALACTOSYLATION CATALYZED BY β-GLYCOSIDASES.

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Retaining glycosidases are able to catalyze transglycosylation reactions when acceptors different from water are present in the reaction medium. We studied these processes using *o*-nitrophenyl β-D-galactopyranoside as the donor. The acceptors used were: methyl β-D-glucopyranoside that has primary and secondary hydroxyl groups and D-xylose or L-xylose that only contain secondary hydroxyl groups. Several β-glycosidases of various sources were studied: β-glactosidases from Lamb intestine (Lactase-phlorizin-hydrolase LPH), Bovine testes, Bovine liver, *Aspergillus oryzae*, *Escherichia coli* and *Saccharomyces fragilis*. and a β-glucosidase from *Streptomyces sp*. (gift of Drs. E. Querol and J. Perez-Pons) In all cases, several regioisomeric disaccharides were obtained in variable ratios. Interestingly all the enzymes presented different regioselectivities indicating that the recognition of the acceptor appears to be different for each enzyme. The efficiency of the reaction (transglycosylation versus hydrolysis) was also compared in order to determine the potential use of these enzymes in the synthesis of disaccharides . In paticular β-galactopyranosyl-xylopyranoses are interesting because all the regioisomers are substrates of intestinal LPH and could be used in the diagnosis of Lactase deficiency.

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Baeyer-Villiger oxidation using *Candida antarctica* lipase.

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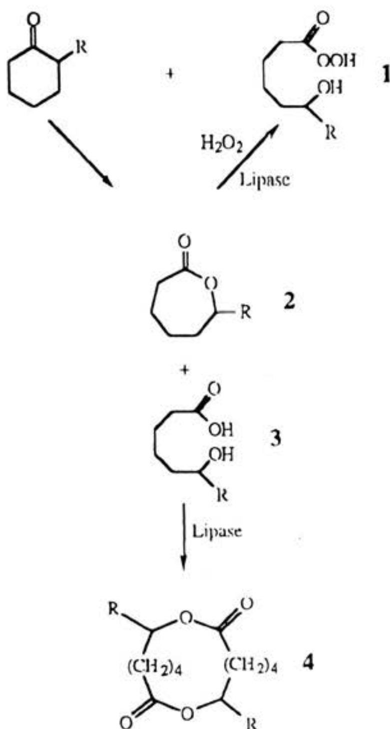

$R = CH_3, C_6H_5, C_6H_{13}$
lactone ee = 40-60%

Baeyer-Villiger chemical oxidation is catalyzed by hydroxyperacid **1** providing from lipase transformation (H_2O_2 acceptor) of lacton **2**. The biotransformation products (lactone **2**, hydroxyacid **3** and bis-lactone **4**) are enantiomerically enriched.

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New formulae for folding catalysts make them multi-purpose enzymes

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Abstract

Whereas protein disulfide isomerase (PDI) and prolyl isomerase (PPI) are considered as efficient protein folding catalysts, very few large scale processes have made use of them, due to economical and technical limitations. Using immobilized enzyme could permit a net cost decrease since post-refolding purification could be omitted and design of biochemical reactors envisionned. To overcome the almost complete PDI inactivation during coupling process, we investigated two different reversible protections of its active site. Specific activity of immobilized PDI reached then 40-64% that of the soluble enzyme. PPI lost most of its activity but was still active enough to be detected. An alternative strategy based on enzyme biotinylation was also studied. Under appropriate conditions, PPI was fully active while 55-66% active PDI was obtained. Biotinylated enzymes proved easy to be removed from refolding mixtures by a single step procedure. In addition to improving enzyme-assisted protein refolding processes, several other applications might be developped taking advantage of these new formulae of PDI and PPI.

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Approach to the Synthesis of a hapten for a [1,7]-sigmatropic hydrogen shift

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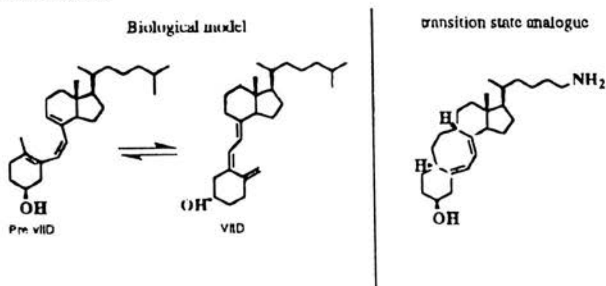
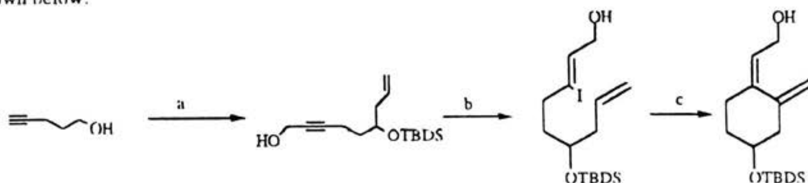
We are very interested in the development of catAbs for the [1,7]-sigmatropic hydrogen shift in a Z-hexatriene. The designed haptens include an 8-membered ring where a methylene group would mimic the in-flight hydrogen.

Due to the important biological equivalent, previtD/vitD, we have designed a hapten for such a reaction resembling the same skeleton.



We have started the synthesis of the transition state analogue by the D ring, using vitD chemistry, as shown below:



a) i. swern oxidation; allylMgBr, THF, -30C, 70%; ii. TBDSCl, DMF, 98%; iii. n-BuLi, THF, (CHO)n, 85%. b) Red-Al, THF, I2, 71%; c) Heck conditions, 80%.

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Antibody Catalyzed Cleavage of an Amide Bond Using an External Nucleophilic Co-Factor

Monoclonal antibodies capable of cleaving an amide bond using phenol as an auxiliary nucleophile were generated. Two haptens were designed and synthesized which are structurally related but display different charged functionalities to induce complementary catalytic amino acid residues in the antibody combining sites. These haptens were used as an immunogen pair in a heterologous immunization protocol, as well as individually in a homologous immunization protocol. One antibody from each immunization protocol was found to affect the hydrolysis of p-nitroaniline propionate *only* in the presence of phenol. On the other hand, the rate of the *uncatalysed* reaction is unaffected by the addition of phenol. Antibody 14-10 which was generated by the heterologous immunization protocol was found to be the most efficient catalyst with k_{cat} = 1.33 x 10-4 min^{-1} at pH 8.0. Its Michaelis Menten parameters were K_m = 136 μM for phenol and K_m = 370 μM for p-nitro-aniline propionate. These results point to a new mode of catalysis for hydrolytic antibodies; as well as, offer further support to the power of heterologous immunization in generating superior antibody catalysts.

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ENZYMATIC REACTIONS UNDER EXTREME CONDITIONS

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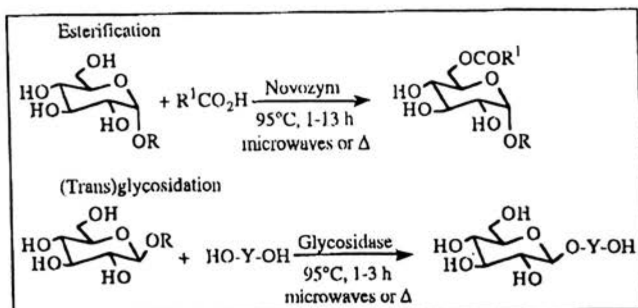
Enzymes are biocatalytic proteins which control almost all biological reactions. Nowadays, they are recognized as useful catalysts for organic synthesis that have valuable industrial applications. The use of enzymes has several advantages over chemical methods: they are substrate, regio and stereoselective. In nature they function in a mild conditions. The discovery of enzyme activity in organic solvents is of great interest as the substrates are highly soluble in non-aqueous media, products are easily recovered, enzymes are more stable and the most important is shifting the equilibrium towards synthesis. Moreover, under these almost unhydrous conditions, they become extremly thermostable.

One of the major limitations of enzymatic synthesis is reversible nature of the reactions which result in low rates and selectivities. A technique to displace the equilibrium toward the desired direction is to continously remove the co-products like water or alcohol(s) by azeotropic destilation, working under vacuum, adding zeolites or the very recent techniques of using microwave irradiation.

Microwaves consist in an electromagnetic radiation whose frequency is imposed at 2450 MHz (λ = 12.2 cm) for industrial, scientific, medicinal and domestic applications. Light polar molecules are strongly interacting with electromagnetic field, which is an alternating current and causes the changes of orientation at each alternation. These caracteristics allow intermolecular friction and subsequent dissipation of energy by heating in the core. Consequently, polar molecules will be very efficiently heated and eliminated from reaction mixture. For the safety reasons, this kind of reactions has to be performed in the absence of organic solvents and adopted to the solvent-free procedures. Enzymes have to be supported, immobilized or dispersed on solid supports of adequate pH. Thus only weak interactions with microwaves will occur preserving the enzyme, and only the substrate or product(s) will be heated. Several types of mineral supports can be used like celite, aluminium oxide, silica, etc. or different polymers. The stability of the biocatalyst is further increased by adsorption on a solid carrier, which also facilitates its separation from reaction mixture and reuse. New thermostable lipases which have been developed for application in laundry detergents tolerate well these conditions. Another group of hydrolases which can be applied in dry medium under microwaves are glycosidases isolated from thermophilic microorganisms.

We have studied the esterification and (trans)glycosidation of some mono and disaccharides (Scheme) under microwave irradiation and compared them with classical heating conditions in terms of yields, reaction rates and purities of the products. Fatty acid esters of carbohydrates as well as glycosides are of interest as biodegradable nonionic emulsifying agents and detergents. They also exhibit antimicrobial activity. Enzymes are able to introduce regioselectively the functionalities into carbohydrates without use of protection-deprotection techniques.

Scheme



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Noumerous supports and different methods for enzyme fixation were tested. The substrates were co-immobilized and dried on different ways followed by addition of fatty acid (esterification) or diol (transglycosidation). Reactions were performed in dry medium without solvent in a monomode reactor with focused microwaves. They were shorter then those performed by classical heating, products were more pure as they were shorter time at high temperature, biocatalitical system was successfully reused.

The advantages of enzymatic catalysis in dry medium using immobilized enzymes or whole cells coupled with microwave activation over classical heated reactions will be discussed.

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Crystal structures of catalytic antibodies

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Since 1986, catalytic antibodies have been obtained as a result of the immune response against the transition state analogs of various reactions. The crystal structure of the liganded antibody brings fundamental informations on the mechanism of action of these antibodies as well as on the relation between the structure of the hapten used to induce the synthesis of catalytic antibodies and the aminoacids present in the combining site and implicated in the catalytic function of the antibody. We recently determined the X-ray structure of antibodies with esterase activity (1) and have shown that the main mechanism of catalysis is the stabilisation of the transition state of the reaction. We shown the poor diversity of the immune system in the amino acids involved in the catalysis of a family of antibodies hydrolysing a p-nitro-benzyle ester and generated against the same hapten.

In order to gain a broader insight on the structure of catalytic antibodies we are now involved in a new approach : catalytic antibodies mimicking cofactor enzymes. These antibodies are designed either to bring into close proximity the cofactor and the substrate or to bind tightly the cofactor to enhance its reactivity. A target of choice for antibody-cofactor catalysis was the modelling of heme proteins such as peroxidases which catalyze the oxidation of various substrates by hydrogen peroxide and alkylperoxides. We obtained crystals of an antibody induced against an iron-porphyrin which increases the reactivity of the cofactor and protects it from degradation allowing it to achieve at least 1000 cycles (2) . We have collected data at 2.8 Å resolution of the crystal infiltrated with the cofactor and are now solving the structure by molecular replacement.

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2 Quilez et al. (1996), F.E.B.S. Letters, 395, 73-76.

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ENZYMATIC SYNTHESIS OF POTENTIAL ANTIPSYCHOTIC AGENTS

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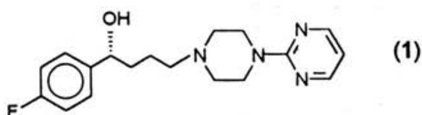
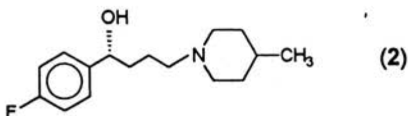
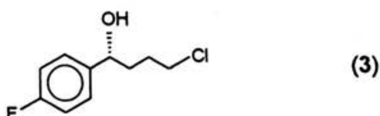
Antipsychotic activity of neuroleptics is exerted by blockade of cerebral dopamine (DA) receptors [1]. However, many dopamine antagonists are also responsible for serious extrapyramidal side effects. Therefore, other neuroleptics containing the 1-(pyrimidin-2-yl)piperazine pharmacophore have been synthesized to avoid such side effects [2]. In this context and in our ongoing program directed to the enzyme-mediated synthesis of pharmacologically active compounds, we present the preparation of both enantiomers of α-(4-fluorophenyl)-4-(2-pyrimidinyl)-1-piperazinebutanol (**1**), a non-fluorinated analogue of BMS 181 100, potent antipsychotic agent *in vivo* with no DA receptor interaction [2], and α-(4-fluorophenyl)-4-methyl-1-piperidinebutanol (**2**) (FG 5155) [3], a metabolite of melperone, known butyrophenone derivative with a wide spectrum of neuroleptic properties. The enantiomers of both compounds have been obtained by two ways, i.e. through enzymatic resolution of α-(4-fluorophenyl)-4-chloro-1-butanol (**3**) with lipases followed by reaction with the appropriate piperazine derivative, and by lipase-mediated resolution of the corresponding racemic compounds **1** and **2**.







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A TAILOR-MADE CATALYST FOR THE DIELS-ALDER REACTION

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Abstract

The molecular imprinting technology (MIT) has been developed rapidly in very recent years and shown considerable potential for applications in chromatography, artificial antibody and as mimetic sensor. One of the most attractive goals of MIT would be to create catalytically active polymers. In this paper, a tailor-made catalytically active polymer catalyzing the bimolecular Diels-Alder reaction is described. The strategy followed was the use of a transition state analogue as an imprinting template, similar to the strategy used successfully for catalytic antibodies, to create specific cavities described as "entropy traps". Kinetic studies carried out in acetonitrile at 82 °C, show a rate acceleration (Kcat/Kuncat) of 270 fold for the Diels-Alder reaction between tetrachlorothiophene and maleic anhydride. The imprinted polymer has an apparent Km of 42.5 mM and an apparent Kcat of 3.82×10^{-2} min^{-1}, respectively. Substrate selectivity studies showed the imprinted polymer had much higher affinities for the print molecules and reactants than the control polymer. Frontal chromatography was performed to determine binding sites and the dissociation constant for print molecules. Inhibition studies showed when the print molecules were added as inhibitors, the reactivity decreased substantially for the imprinted polymer; whereas for the control polymer there is little influence.

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Use of oligonucleotidic probes to isolate dextransucrase encoding genes from *Leuconostoc mesenteroides* NRRL B-1299.

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Glucosyltransferases from various strains of *Leuconostoc mesenteroides* are extracellular enzymes that catalyse the transfer of D-glucopyranosyl units from sucrose onto acceptor molecules. Two different products can be obtained : (i) a high molecular weight polysaccharide or (ii) low molecular weight oligosaccharides when efficient acceptors, like maltose, are added to the reaction medium. Since the structure of dextran and oligosaccharides is highly dependent on dextransucrase producing strain, we initiated a study of the structure of these enzymes, in order to elucidate the structure/function relationships of these glucosyltransferases.
As *E. coli* growth conditions were not compatible with an efficient screening on activity and because all glucosyltransferases of which genes have been already cloned shared numerous conserved domains, screening at nucleotidic level was preferred. Oligonucleotides were designed upon internal conserved sequences or upon conserved sequence in glucosyltransferases signal peptides. After PCR reaction on *L. mesenteroides* NRRL B-1299 genomic DNA using these different primers, probes were obtained and used to isolate parts of dextransucrase genes. In fact, two different dextransucrase encoding genes were isolated. This allowed to isolate a novel enzyme from *L. mesenteroides* NRRL B-1299 producing only $\alpha(1\text{-}6)$ and $\alpha(1\text{-}3)$ linkages and being the first glucosyltransferase described having no signal peptide.

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Studies on Carbon-Carbon Bond Hydrolyzing Enzymes

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Enzymes that hydrolyze carbonyl substrates such as esters and amides have been used for centuries, just as those for the hemiacetals of combined saccharides. Their essential role in organic chemistry for structure determination of natural polymers is well documented. Hydrolysis occurs between carbon and hetero-atom bonds. Interest in these enzymes for regio- and stereoselective synthesis has developed enormously over the last 2-3 decades. Many are produced and used in pharmaceutical, agro-chemical, commodity chemical industries. In contrast C-C bond hydrolases exemplified by β-ketolases have been neglected for biodegraditive and synthetic purposes, yet are very common enzymes for potential exploration. We now describe an acetopyruvate hydrolase from *Pseudomonas putida* which catalyses the hydrolysis of the C_3-C_4 bond to give acetate and pyruvate. The enzyme is induced during growth on orcinol and has been purified to a homogenous protein. The enzyme action has been followed by ^{1}H-NMR and complete kinetic and stoicheiometric relationships of the hydrolysis are revealed with single reaction mixtures.

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Studies of the citric acid production by 2-deoxyglucose-resistant mutant strain of *Aspergillus niger* using cellulose hydrolysate substrate

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Abstract

Cellulose hydrolysate was prepared from cellulose powder by Meicelase, a commercial cellulolytic enzyme of *Trichoderma viride*, in 50 mM acetate or citrate buffer pH 4.5 at 40 ^{0}C. After 72 h incubation, approximately 65% of cellulose powder was hydrolyzed and converted into mainly glucose with a little of cellobiose. Base on the reducing sugar, the yield of citric acid produced was maximally 39% by both *Aspergillus niger* Yang no.2 and C192, a wild type strain and a 2-deoxyglucose-resistant mutant strain, respectively, in semi-solid culture using bagasse as a carrier. Since high level of citric acid production could be obtained at the high sugar concentration, therefore cellulose hydrolysate was concentrated to 150 g/l of the reducing sugars by rotary evaporator. In addition, the types of hydrolysis buffer were shown to affect citric acid production by Yang no.2 and C192. Since acetate buffer seemed to delay citric acid production of *A. niger*, to shorten the time for production, citrate buffer was used instead. Cellulose powder was hydrolyzed in 50 mM citrate buffer and the concentrated cellulose hydrolysate containing 150 g/l of reducing sugars was prepared. When cultivated in a semi-solid culture with the concentrated hydrolysate, within 3 d Yang no.2 and C192 produced 90.5 g/l and 105.5 g/l of citric acid, respectively. It is of interest that C192 exhibited enhanced β-glucosidase production during the entire cultivation periods in comparison with that Yang no.2.

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Workshop on

NOVEL BIOCATALYSTS

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62 **1996 Annual Report**

63 **Workshop on TGF-β Signalling in Development and Cell Cycle Control.**
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