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Principles and Applications

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ISBN 978-1-4020-8360-0 e-ISBN 978-1-4020-8361-7

Library of Congress Control Number: 2008924855

Printed on acid-free paper.

9 8 7 6 5 4 3 2 1

springer.com

Enzyme Biocatalysis

Foreword

This book was written with the purpose of providing a sound basis for the design of enzymatic reactions based on kinetic principles, but also to give an updated vision of the potentials and limitations of biocatalysis, especially with respect to recent applications in processes of organic synthesis. The first five chapters are structured in the form of a textbook, going from the basic principles of enzyme structure and function to reactor design for homogeneous systems with soluble enzymes and heterogeneous systems with immobilized enzymes. The last chapter of the book is divided into six sections that represent illustrative case studies of biocatalytic processes of industrial relevance or potential, written by experts in the respective fields.

We sincerely hope that this book will represent an element in the toolbox of graduate students in applied biology and chemical and biochemical engineering and also of undergraduate students with formal training in organic chemistry, biochemistry, thermodynamics and chemical reaction kinetics. Beyond that, the book pretends also to illustrate the potential of biocatalytic processes with case studies in the field of organic synthesis, which we hope will be of interest for the academia and professionals involved in R&D&I. If some of our young readers are encouraged to engage or persevere in their work in biocatalysis this will certainly be our more precious reward.

Too much has been written about writing. Nobel laureate Gabriel García Márquez wrote one of its most inspired books by writing about writing (*Living to Tell the Tale*). There he wrote "life is not what one lived, but what one remembers and how one remembers it in order to recount it". This hardly applies to a scientific book, but certainly highlights what is applicable to any book: its symbiosis with life. Writing about biocatalysis has given me that privileged feeling, even more so because enzymes are truly the catalysts of life. Biocatalysis is hardly separable from my life and writing this book has been certainly more an ecstasy than an agony.

A book is an object of love so who better than friends to build it. Eleven distinguished professors and researchers have contributed to this endeavor with their knowledge, their commitment and their encouragement. Beyond our common language, I share with all of them a view and a life-lasting friendship. That is what lies behind this book and made its construction an exciting and rewarding experience.

Chapters 3 to 5 were written with the invaluable collaboration of Claudia Altamirano and Lorena Wilson, two of my former students, now my colleagues, and my bosses I am afraid. Chapter 4 also included the experience of José Manuel Guisán, Roberto Fernández-Lafuente and César Mateo, all of them very good friends who were kind enough to join this project and enrich the book with their world known expertise in heterogeneous biocatalysis. Section 6.1 is the result of a cooperation sustained by a CYTED project that brought together Sonia Barberis, also a former graduate student, now a successful professor and permanent collaborator and, beyond that, a dear friend, Fanny Guzmán, a reputed scientist in the field of peptide synthesis who is my partner, support and inspiration, and Josep López, a well-known scientist and engineer but, above all, a friend at heart and a warm host. Section 6.3 was the result of a joint project with Gregorio Alvaro, a dedicated researcher who has been a permanent collaborator with our group and also a very special friend and kind host. Section 6.4 is the result of a collaboration, in a very challenging field of applied biocatalysis, of Dr. Guisan's group with which we have a long-lasting academic connection and strong personal ties. Section 6.5 represents a very challenging project in which Josep López and Gregorio Alvaro have joined Pere Clapés, a prominent researcher in organic synthesis and a friend through the years, to build up an updated review on a very provocative field of enzyme biocatalysis. Finally, section 6.6 is a collaboration of a dear friend and outstanding teacher, Juan Lema, and his research group that widens the scope of biocatalysis to the field of environmental engineering adding a particular flavor to this final chapter.

A substantial part of this book was written in Spain while doing a sabbatical in the Universitat Autònoma de Barcelona, where I was warmly hosted by the Chemical Engineering Department, as I also was during short stays at the Institute of Catalysis and Petroleum Chemistry in Madrid and at the Department of Chemical Engineering in the Universidad de Santiago de Compostela.

My recognition to the persons in my institution, the Pontificia Universidad Católica de Valparaíso, that supported and encouraged this project, particularly to the rector Prof. Alfonso Muga, and professors Atilio Bustos and Graciela Muñoz. Last but not least, my deepest appreciation to the persons at Springer: Marie Johnson, Meran Owen, Tanja van Gaans and Padmaja Sudhakher, who were always delicate, diligent and encouraging.

Dear reader, the judgment about the product is yours, but beyond the product there is a process whose beauty I hope to have been able to transmit. I count on your indulgence with language that, despite the effort of our editor, may still reveal our condition of non-native English speakers.

Andrés Illanes
Valparaíso, May 15, 2008

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Chapter 1
Introduction

Andrés Illanes

1.1 Catalysis and Biocatalysis

Many chemical reactions can occur spontaneously; others require to be catalyzed to proceed at a significant rate. Catalysts are molecules that reduce the magnitude of the energy barrier required to be overcame for a substance to be converted chemically into another. Thermodynamically, the magnitude of this energy barrier can be conveniently expressed in terms of the free-energy change. As depicted in Fig. 1.1, catalysts reduce the magnitude of this barrier by virtue of its interaction with the substrate to form an activated transition complex that delivers the product and frees the catalyst. The catalyst is not consumed or altered during the reaction so, in principle, it can be used indefinitely to convert the substrate into product; in practice, however, this is limited by the stability of the catalyst, that is, its capacity to retain its active structure through time at the conditions of reaction.

Biochemical reactions, this is, the chemical reactions that comprise the metabolism of all living cells, need to be catalyzed to proceed at the pace required to sustain life. Such life catalysts are the enzymes. Each one of the biochemical reactions of the cell metabolism requires to be catalyzed by one specific enzyme. Enzymes are protein molecules that have evolved to perform efficiently under the mild conditions required to preserve the functionality and integrity of the biological systems. Enzymes can be considered then as catalysts that have been optimized through evolution to perform their physiological task upon which all forms of life depend. No wonder why enzymes are capable of performing a wide range of chemical reactions, many of which extremely complex to perform by chemical synthesis. It is not presumptuous to state that any chemical reaction already described might have an enzyme able to catalyze it. In fact, the possible primary structures of an enzyme protein composed of n amino acid residues is 20^n so that for a rather small protein molecule containing 100 amino acid residues, there are 20^{100} or 10^{130} possible

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A. Illanes (ed.), *Enzyme Biocatalysis*.

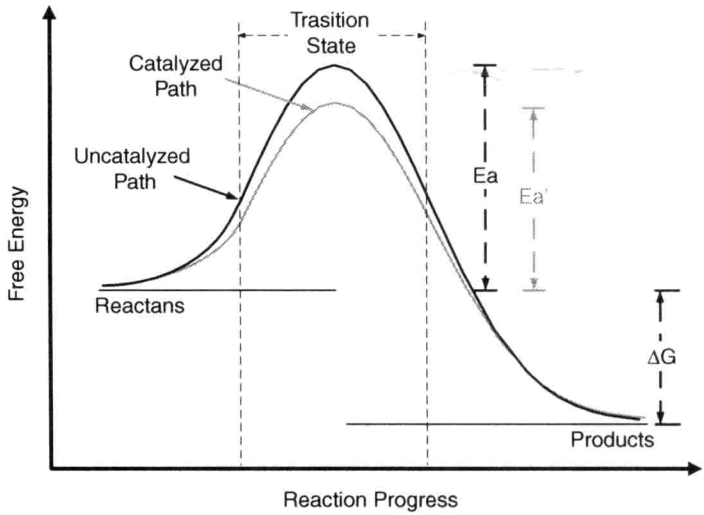

Fig. 1.1 Mechanism of catalysis. Ea and Ea′ are the energies of activation of the uncatalyzed and catalyzed reaction. ΔG is the free energy change of the reaction

amino acid sequences, which is a fabulous number, higher even than the number of molecules in the whole universe. To get the right enzyme for a certain chemical reaction is then a matter of search and this is certainly challenging and exciting if one realizes that a very small fraction of all living forms have been already isolated. It is even more promising when one considers the possibility of obtaining DNA pools from the environment without requiring to know the organism from which it comes and then expressed it into a suitable host organism (Nield et al. 2002), and the opportunities of genetic remodeling of structural genes by site-directed mutagenesis (Abián et al. 2004).

Enzymes have been naturally tailored to perform under physiological conditions. However, biocatalysis refers to the use of enzymes as process catalysts under artificial conditions (in vitro), so that a major challenge in biocatalysis is to transform these physiological catalysts into process catalysts able to perform under the usually tough reaction conditions of an industrial process. Enzyme catalysts (biocatalysts), as any catalyst, act by reducing the energy barrier of the biochemical reactions, without being altered as a consequence of the reaction they promote. However, enzymes display quite distinct properties when compared with chemical catalysts; most of these properties are a consequence of their complex molecular structure and will be analyzed in section 1.2. Potentials and drawbacks of enzymes as process catalysts are summarized in Table 1.1.

Enzymes are highly desirable catalysts when the specificity of the reaction is a major issue (as it occurs in pharmaceutical products and fine chemicals), when the catalysts must be active under mild conditions (because of substrate and/or product instability or to avoid unwanted side-reactions, as it occurs in several reactions of organic synthesis), when environmental restrictions are stringent (which is now a

Table 1.1 Advantages and Drawbacks of Enzymes as Catalysts

Advantages	Drawbacks
High specificity	High molecular complexity
High activity under moderate conditions	High production costs
High turnover number	Intrinsic fragility
Highly biodegradable	
Generally considered as natural products	

rather general situation that gives biocatalysis a distinct advantage over alternative technologies) or when the label of natural product is an issue (as in the case of food and cosmetic applications) (Benkovic and Ballesteros 1997; Wegman et al. 2001). However, enzymes are complex molecular structures that are intrinsically labile and costly to produce, which are definite disadvantages with respect to chemical catalysts (Bommarius and Broering 2005).

While the advantages of biocatalysis are there to stay, most of its present restrictions can be and are being solved through research and development in different areas. In fact, enzyme stabilization under process conditions is a major issue in biocatalysis and several strategies have been developed (Illanes 1999) that include chemical modification (Roig and Kennedy 1992; Özturk et al. 2002; Mislovičová et al. 2006), immobilization to solid matrices (Abián et al. 2001; Mateo et al. 2005; Kim et al. 2006; Wilson et al. 2006), crystallization (Häring and Schreier 1999; Roy and Abraham 2006), aggregation (Cao et al. 2003; Mateo et al. 2004; Schoevaart et al. 2004; Illanes et al. 2006) and the modern techniques of protein engineering (Chen 2001; Declerck et al. 2003; Sylvestre et al. 2006; Leisola and Turunen 2007), namely site-directed mutagenesis (Bhosale et al. 1996; Ogino et al. 2001; Boller et al. 2002; van den Burg and Eijsink 2002; Adamczak and Hari Krishna 2004; Bardy et al. 2005; Morley and Kazlauskas 2005), directed evolution by tandem mutagenesis (Arnold 2001; Brakmann and Johnsson 2002; Alexeeva et al. 2003; Boersma et al. 2007) and gene-shuffling based on polymerase assisted (Stemmer 1994; Zhao et al. 1998; Shibuya et al. 2000; Kaur and Sharma 2006) and, more recently, ligase assisted recombination (Chodorge et al. 2005). Screening for intrinsically stable enzymes is also a prominent area of research in biocatalysis. Extremophiles, that is, organisms able to survive and thrive in extreme environmental conditions are a promising source for highly stable enzymes and research on those organisms is very active at present (Adams and Kelly 1998; Davis 1998; Demirjian et al. 2001; van den Burg 2003; Bommarius and Riebel 2004; Gomes and Steiner 2004). Genes from such extremophiles have been cloned into suitable hosts to develop biological systems more amenable for production (Halldórsdóttir et al. 1998; Haki and Rakshit 2003; Zeikus et al. 2004).

Enzymes are by no means ideal process catalysts, but their extremely high specificity and activity under moderate conditions are prominent characteristics that are being increasingly appreciated by different production sectors, among which the pharmaceutical and fine-chemical industry (Schmid et al. 2001; Thomas et al. 2002; Zhao et al. 2002; Bruggink et al. 2003) have added to the more traditional sectors of food (Hultin 1983) and detergents (Maurer 2004).

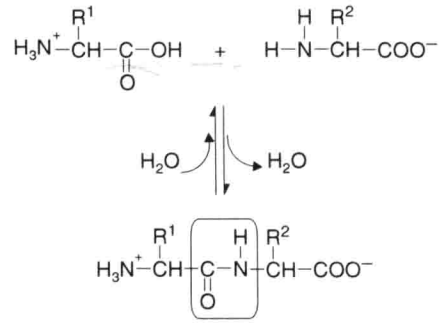

Fig. 1.2 Scheme of peptide bond formation between two adjacent α-amino acids

1.2 Enzymes as Catalysts. Structure–Functionality Relationships

Most of the characteristics of enzymes as catalysts derive from their molecular structure. Enzymes are proteins composed by a number of amino acid residues that range from 100 to several hundreds. These amino acids are covalently bound through the peptide bond (Fig. 1.2) that is formed between the carbon atom of the carboxyl group of one amino acid and the nitrogen atom of the α-amino group of the following. According to the nature of the R group, amino acids can be non-polar (hydrophobic) or polar (charged or uncharged) and their distribution along the protein molecule determines its behavior (Lehninger 1970).

Every protein is conditioned by its amino acid sequence, called *primary structure*, which is genetically determined by the deoxyribonucleotide sequence in the structural gene that codes for it. The DNA sequence is first transcribed into a mRNA molecule which upon reaching the ribosome is translated into an amino acid sequence and finally the synthesized polypeptide chain is transformed into a three-dimensional structure, called *native structure*, which is the one endowed with biological functionality. This transformation may include several post-translational reactions, some of which can be quite relevant for its functionality, like proteolytic cleavage, as it occurs, for instance, with *Escherichia coli* penicillin acylase (Schumacher et al. 1986) and glycosylation, as it occurs for several eukaryotic enzymes (Longo et al. 1995). The three-dimensional structure of a protein is then genetically determined, but environmentally conditioned, since the molecule will interact with the surrounding medium. This is particularly relevant for biocatalysis, where the enzyme acts in a medium quite different from the one in which it was synthesized than can alter its native functional structure. Secondary three-dimensional structure is the result of interactions of amino acid residues proximate in the primary structure, mainly by hydrogen bonding of the amide groups; for the case of globular proteins, like enzymes, these interactions dictate a predominantly ribbon-like coiled configuration termed *α-helix*. Tertiary three-dimensional structure is the result of interactions of amino acid residues located apart in the primary structure that produce a compact and twisted configuration in which the surface is rich in polar amino acid

residues, while the inner part is abundant in hydrophobic amino acid residues. This tertiary structure is essential for the biological functionality of the protein. Some proteins have a quaternary three-dimensional structure, which is common in regulatory proteins, that is the result of the interaction of different polypeptide chains constituting subunits that can display identical or different functions within a protein complex (Dixon and Webb 1979; Creighton 1993).

The main types of interactions responsible for the three-dimensional structure of proteins are (Haschemeyer and Haschemeyer 1973):

- Hydrogen bonds, resulting from the interaction of a proton linked to an electronegative atom with another electronegative atom. A hydrogen bond has approximately one-tenth of the energy stored in a covalent bond. It is the main determinant of the helical secondary structure of globular proteins and it plays a significant role in tertiary structure as well.
- Apolar interactions, as a result of the mutual repulsion of the hydrophobic amino acid residues by a polar solvent, like water. It is a rather weak interaction that does not represent a proper chemical bond (approximation between atoms exceed the van der Waals radius); however, its contribution to the stabilization of the three-dimensional structure of a protein is quite significant.
- Disulphide bridges, produced by oxidation of cysteine residues. They are especially relevant in the stabilization of the three-dimensional structure of low molecular weight extracellular proteins.
- Ionic bonds between charged amino acid residues. They contribute to the stabilization of the three-dimensional structure of a protein, although to a lesser extent, because the ionic strength of the surrounding medium is usually high so that interaction is produced preferentially between amino acid residues and ions in the medium.
- Other weak type interactions, like van der Waals forces, whose contribution to three-dimensional structure is not considered significant.

Proteins can be conjugated, this is, associated with other molecules (*prosthetic groups*). In the case of enzymes which are conjugated proteins (*holoenzymes*), catalysis always occur in the protein portion of the enzyme (*apoenzyme*). Prosthetic groups may be organic macromolecules, like carbohydrates (in the case of glycoproteins), lipids (in the case of lipoproteins) and nucleic acids (in the case of nucleoproteins), or simple inorganic entities, like metal ions. Prosthetic groups are tightly bound (usually covalently) to the apoenzyme and do not dissociate during catalysis. A significant number of enzymes from eukaryotes are glycoproteins, in which case the carbohydrate moiety is covalently linked to the apoenzyme, mainly through serine or threonine residues, and even though the carbohydrate does not participate in catalysis it confers relevant properties to the enzyme.

Catalysis takes place in a small portion of the enzyme called the *active site*, which is usually formed by very few amino acid residues, while the rest of the protein acts as a scaffold. Papain, for instance, has a molecular weight of 23,000 Da with 211 amino acid residues of which only cysteine (Cys 25) and histidine (His 159)

are directly involved in catalysis (Allen and Lowe 1973). Substrate is bound to the enzyme at the active site and doing so, changes in the distribution of electrons in its chemical bonds are produced that cause the reactions that lead to the formation of products. The products are then released from the enzyme which is ready for the next catalytic cycle. According to the early *lock and key* model proposed by Emil Fischer in 1894, the active site has a unique geometric shape that is complementary to the geometric shape of the substrate molecule that fits into it. Even though recent reports provide evidence in favor of this theory (Sonkaria et al. 2004), this rigid model hardly explains many experimental evidences of enzyme biocatalysis. Later on, the *induced-fit* theory was proposed (Koshland 1958) according to which the substrate induces a change in the enzyme conformation after binding, that may orient the catalytic groups in a way prone for the subsequent reaction; this theory has been extensively used to explain enzyme catalysis (Youseff et al. 2003). Based on the transition-state theory, enzyme catalysis has been explained according to the hypothesis of enzyme transition state complementariness, which considers the preferential binding of the transition state rather than the substrate or product (Benković and Hammes-Schiffer 2003).

Many, but not all, enzymes require small molecules to perform as catalysts. These molecules are termed *coenzymes* or *cofactors*. The term *coenzyme* is used to refer to small molecular weight organic molecules that associate reversibly to the enzyme and are not part of its structure; coenzymes bound to enzymes actually take part in the reaction and, therefore, are sometime called *cosubstrates*, since they are stoichiometric in nature (Kula 2002). Coenzymes often function as intermediate carriers of electrons (i.e. NAD^+ or FAD^+ in dehydrogenases), specific atoms (i.e. coenzyme Q in H atom transfer) or functional groups (i.e. coenzyme A in acyl group transfer; pyridoxal phosphate in amino group transfer; biotin in CO_2 transfer) that are transferred in the reaction. The term *cofactor* is commonly used to refer to metal ions that also bind reversibly to enzymes but in general are not chemically altered during the reaction; cofactors usually bind strongly to the enzyme structure so that they are not dissociated from the holoenzyme during the reaction (i.e. Ca^{++} in α-amylase; Co^{++} or Mg^{++} in glucose isomerase; Fe^{+++} in nitrile hydratase). According to these requirements, enzymes can be classified in three groups as depicted in Fig. 1.3:

(i) those that do not require of an additional molecule to perform biocatalysis,
(ii) those that require cofactors that remain unaltered and tightly bound to the enzyme performing in a catalytic fashion, and
(iii) those requiring coenzymes that are chemically modified and dissociated during catalysis, performing in a stoichiometric fashion.

The requirement of cofactors or coenzymes to perform biocatalysis has profound technological implications, as will be analyzed in section 1.4.

Enzyme activity, this is, the capacity of an enzyme to catalyze a chemical reaction, is strictly dependent on its molecular structure. Enzyme activity relies upon the existence of a proper structure of the active site, which is composed by a reduced number of amino acid residues close in the three-dimensional structure of

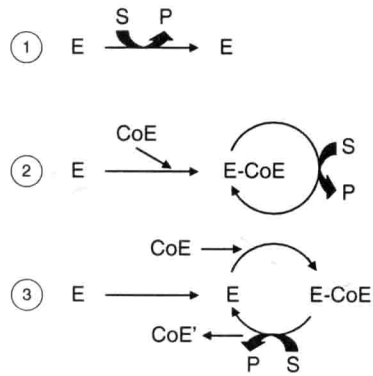

Fig. 1.3 Enzymes according to their cofactor or coenzyme requirements. 1: no requirement; 2: cofactor requiring; 3: coenzyme requiring

the protein but usually far apart in the primary structure. Therefore, any agent that promotes protein unfolding will move apart the residues constituting the active site and will then reduce or destroy its biological activity. Adverse conditions of temperature, pH or solvent and the presence of chaotropic substances, heavy metals and chelating agents can produce this loss of function by distorting the proper active site configuration. Even though a very small portion of the enzyme molecule participates in catalysis, the remaining of the molecule is by no means irrelevant to its performance. Crucial properties, like enzyme stability, are very much dependent on the enzyme three-dimensional structure. Enzyme stability appears to be determined by undefined irreversible processes governed by local unfolding in certain labile regions denoted as *weak spots*. These regions prone to unfolding are the determinants of enzyme stability and are usually located in or close to the surface of the protein molecule, which explains why the surface structure of the enzyme is so important for its catalytic stability (Eijsink et al. 2004). These regions have been the target of site-specific mutations for increasing stability. Though extensively studied, rational engineering of the enzyme molecule for increased stability has been a very complex task. In most cases, these weak spots are not easy to identify so it is not clear to what region of the protein molecule should one be focused on and, even though properly selected, it is not clear what is the right type of mutation to introduce (Gaseidnes et al. 2003). Despite the impressive advances in the field and the existence of some experimentally based rules (Shaw and Bott 1996), rational improvement of the stability is still far from being well established. In fact, the less rational approaches of directed evolution using error-prone PCR and gene shuffling have been more successful in obtaining more stable mutant enzymes (Kaur and Sharma 2006). Both strategies can combine using a set of rationally designed mutants that can then be subjected to gene shuffling (O'Fágáin 2003).

A perfectly structured native enzyme expressing its biological activity can lose it by unfolding of its tertiary structure to a random polypeptide chain in which the amino acids located in the active site are no longer aligned closely enough to perform its catalytic function. This phenomenon is termed *denaturation* and it may be reversible if the denaturing influence is removed since no chemical changes