1
Dirigent Effects in Biocatalysis

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1.1
Introduction

Enzymes are capable of accepting a broad range of substrates and are highly selective, manifested as stereoselectivity, positional selectivity, and functional group selectivity. The nature of the enzyme, the predefined selective molecular recognition of the substrate molecule within its active site, guides selectivity by discriminating between substrate enantiomers converting only one enantiomer. Enzyme engineering is a powerful tool and a widely accepted methodology to optimize and influence enzyme properties, such as the overall activity, selectivity, thermo- and storage stability and the stability toward organic solvents, as it encompasses both directed evolution and rational design. Besides the alteration of enzyme function, enzyme engineering is also capable of directly influencing enzyme-catalyzed reactions, thus controlling their product formation. A proof of concept was presented by the divergent evolution of the promiscuous sesquiterpene synthase γ-humulene synthase. The rational design of residues in the active site of the γ-humulene synthase has shown to possess an additive influence on protein function and promiscuity. By using this approach, a large number of novel specific sesquiterpene synthases has been constructed, each producing one or a few end-products via different reaction pathways including new molecules that do not exist in Nature (Figure 1.1) [1]. This instructive example shows the capability to redesign enzyme function by single amino acid substitutions, and to direct the biocatalytic transformation of diverse substrates via the same mechanism.

This chapter attempts to demonstrate that dirigent effects have been described influencing the outcome of enzyme-catalyzed reactions. It consists of several subchapters that could expand the concept of dirigent properties in biocatalysis by exploiting intrinsic effects which have a considerable impact on the catalytic event of enzyme reactions, but also which extend beyond.

First, a brief report is provided of auxiliary or dirigent proteins which play an important role in free-radical coupling in lignin biosynthesis. These naturally evolved proteins capture the oxidized free-radical substrate, providing a scaffold upon which a selective, radical coupling can occur to yield an optically active product.

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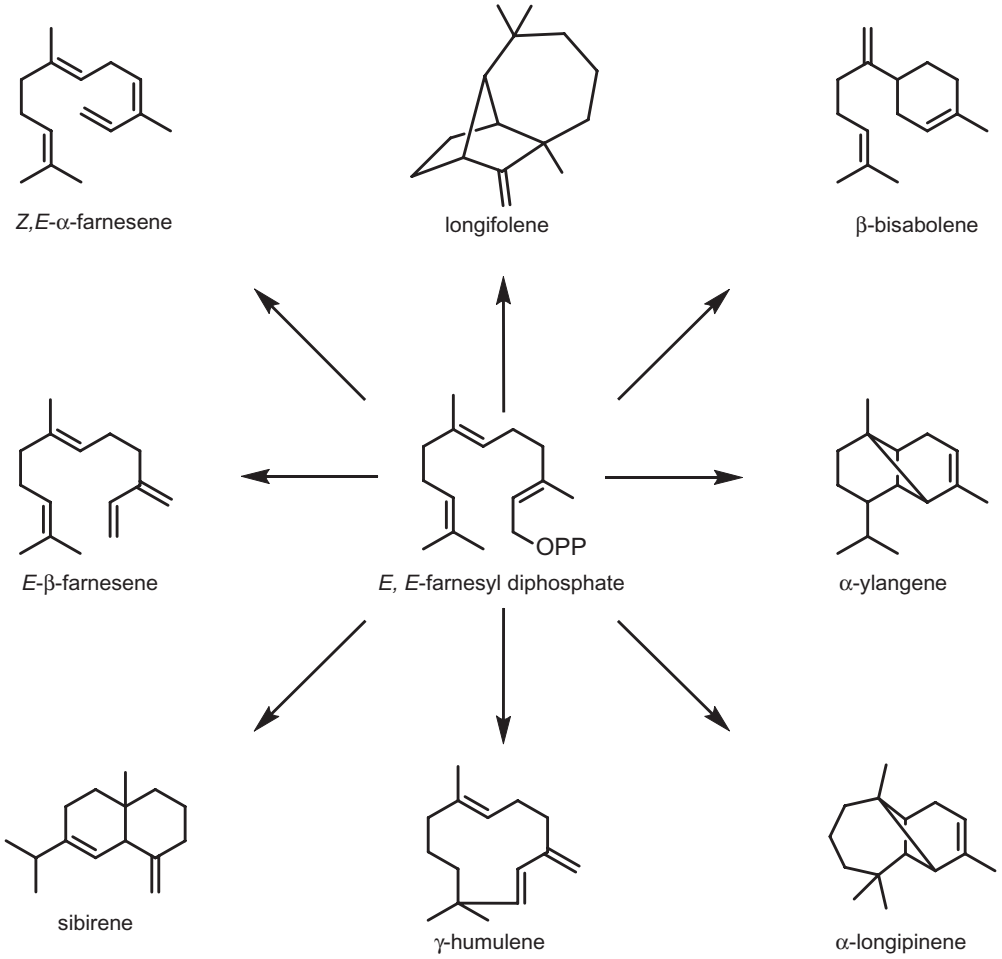


Figure 1.1 Construction of seven specific and active γ-humulene synthases that use different reaction pathways to produce sesquiterpenes. Modified from Ref. [1].

The different behaviors of enzymes in organic solvents and unconventional reaction media, and such behaviors toward enzyme catalysis with respect to activity and selectivity, are also discussed. The active site of an enzyme is determined by the presence or absence of solvent molecules which, in addition to an unconventional reaction medium, may influence not only the size and shape of the active site but also the dielectric constant (and consequently the pK_a values) of the host side chain and electrostatic potential of the site.

In addition, attention is focused on protein structure–function relationships in order to deepen the general understanding of the mechanism, and the folding or motion of proteins of biologically active catalysts. The aim at this point is to

broaden the current understanding of protein folding, which is important for enzyme catalysis as new protein functions can be obtained from existing ones through mutations that alter the amino acid sequence and, hence, the active site architecture.

The intrinsically disordered proteins form a separate class of proteins with specific sequence compositions and functions. Although such proteins fold to form defined structures upon binding, certain parts remain disordered throughout the process. During recent years, these short disordered segments and their function in ordered proteins have undergone extensive investigation and discussion. Indeed, in the case of a small number of intrinsically disordered proteins catalytic activity has been observed; specific examples for which such activity has been identified are described here.

The subject then switches to isozymes, moonlighting proteins and promiscuous enzymes, and their different biological selectivities. More comprehensive overviews on these mechanisms and on catalytic promiscuity have been produced by Hult and Berglund [2] and Bornscheuer and Kazlauskas [3], and by Stefanie Jonas and Florian Hollfelder in volume 1 of this book series. It is suggested that "supertalented" enzymes, defined as the catalysis of multiple reactions versus the catalysis of a single reaction with different substrates, are connected to several conditions (expression, environmental conditions, ligand concentration) and the structural flexibility of the protein.

It should be noted that this chapter does not aim to provide a comprehensive overview on the topics introduced, as detailed information on each subject is available in reviews and articles cited (and in the references therein). Rather, the intention is to present concepts from selected examples that can be put into practice by the reader to help to understand dirigent effects in biocatalysis, and thereby to offer some food for thought concerning the strategies required to engineer enzymes of interest.

1.2 Dirigent Proteins

The discovery of "dirigent proteins" in the mid-1990s, and their abundance in plants, has provided the insight that proteins and enzymes must exist which are able to dictate the stereochemistry of a compound that is synthesized by other proteins. These are enzymes which bind differentially not only various phenylpropanoid (monolignol)-derived substrates (thus guiding the outcome of their coupling yielding in lignin), but also several other compounds such as lignans, flavonolignans, and alkaloids [4]. Hence, specific monolignol (radical)-binding sites have been identified for such proteins, forming the biochemical basis for both regioselective and stereoselective monolignol coupling reactions.

The first dirigent protein was isolated and characterized for the stereoselective bimolecular phenoxy radical coupling in the presence of an oxidase or a one-electron oxidant in 1997 [5]. As a consequence, the term "dirigent" (from the Latin

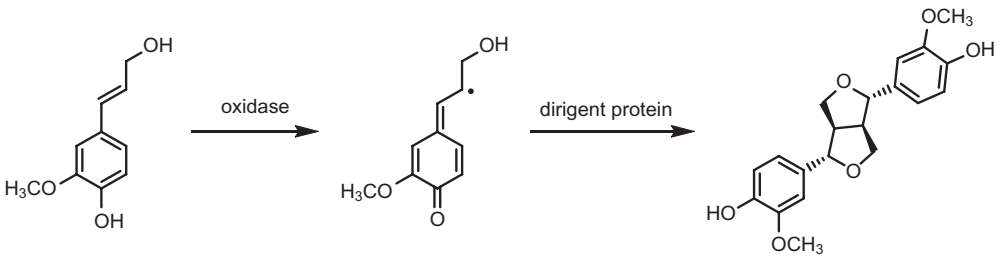


Figure 1.2 Oxidative, enantioselective coupling of coniferyl alcohol results in enantiomerically pure (+)-pinoresinol product formation [6].

dirigere, which when translated means to align or to guide) was chosen to label this new, rather small class of proteins for which about 20 reports have been made to date.

One example of an *in vitro* reaction involving a dirigent protein was the laccase-catalyzed oxidation of coniferyl alcohol, which resulted in the formation of racemic pinoresinol, whereas in the presence of a dirigent protein isolated from *Forsythia intermedia*, enantiomerically pure (+)-pinoresinol was obtained (Figure 1.2) [6]. Recently, an enantiocomplementary dirigent protein from *Arabidopsis thaliana* was characterized which mediates the oxidative phenol coupling to the pure (–)-pinoresinol product [7]. The specificity of the dirigent reaction described above requires two proteins to be localized near to one another, at a concentration that promotes this interaction. Whereas, binding to a specific partner represents one mechanism, another involves the interaction with many partners (multispecificity) that can be important for biological functions. Examples of multispecific, flexible enzymes include the cytochrome P450 monooxygenases, which represent a wide range of different active-site conformations that bind and transform diverse substrates [8].

Unlike dirigent proteins, with their distinct biochemical mechanism and their ability to manage the selective assembly of free radicals formed from monolignol pathways, the establishment of further, more artificial effects appeared to influence the mechanism and the performance of enzyme-catalyzed reactions. This view does not rest solely on experimental evidence, which thus far is incomplete, but instead relies more on the current knowledge of biochemical processes and their reaction conditions, and on considerations of structural, mechanistic and evolutionary implications on the selectivity, specificity and activity of enzymes.

1.3
Solvents and Unconventional Reaction Media

A large number of enzymes show different behaviors in non-aqueous organic media, with effects such as changes in enzyme stability, activity and selectivity being observed by using organic solvents. “Anhydrous” in this context does not mean “no water,” but rather that the amount of water compared to the entire reac-

tion volume is low (often <5%). Furthermore, it should be noted that the effects vary significantly with the solvent used. Although, in general there is no thorough understanding of these solvent effects, the general suggestions can be divided into two categories:

- Organic solvents change the enzyme's conformation and its flexibility, both of which are crucial for enzyme efficiency.
- The use of an organic solvent changes the solubility and desolvation of non-polar substrates and products.

The solubility behavior is often explained as a relationship between enzyme activity and the solvent hydrophobicity log *P*. Thus, log *P* is denoted as the ratio of concentrations of a compound in the two phases of a mixture of two immiscible solvents at equilibrium. In detail, the log *P* value is the partitioning coefficient of a solvent in the two phase system:

$$\log P = \log(c_{\text{solvent1}}/c_{\text{solvent2}})$$

The use of organic solvents can help to suppress unwanted water-dependent side reactions. Solvent effects can also be explained as competitive inhibition, as different types of solvent molecules will have different abilities to bind and block the active site of an enzyme, where there they will act as a competitive inhibitor to the substrate [9, 10]. This ability to affect the properties of enzymes is often summarized in the term "medium engineering" [11, 12]. The solubility of proteins in organic solvents is controlled by parameters, such as: (i) the nature of the solvent used; (ii) the assembly of the enzyme–solvent interface; and (iii) the nature of the protein. Typically, enzymes are very soluble in solvents which are very hydrophilic, polar and protic, such as dimethylsulfoxide (DMSO), ethylene glycol, and formamide, because they do not strip the essential water from the enzymes. A few water molecules bound to charged groups on the surface of enzyme molecules are required in order for enzymatic function to occur. These factors cause different electrostatic and hydrogen interactions at the enzyme–solvent boundary. The different solubility is also reflected in a different activity in the presence of organic solvents [13], with higher catalytic activity being observed in organic solvent if the enzyme is lyophilized from a buffered, aqueous solution at the correct pH value for enzymatic activity [14]. In light of this, the pH of a storage buffer used prior to lyophilization will have an influence on the activity and solubility behavior. The solubility of an enzyme is increased when the pH value of the previously used buffer is far from the isoelectric point of the protein. Due to the fact that the pH concept can only be applied in aqueous systems, the so-called "pH memory effect" was relativized later as a result of the net charge of buffer ions presented in the lyophilizate. The pH memory effect can be overruled by using volatile buffers during the freeze-drying step [15]. Denaturation or inactivation can occur in cases where enzymes are completely dissolved in an anhydrous, homogeneous organic solvent system. In terms of the specificity of active enzymes in anhydrous systems, it should be mentioned that this feature is lower compared to aqueous systems. This type of system has several advantages, especially for up-scaling applications;

notably, due to a lack of any water–organic interface there are no diffusional limitations for substrate and product, while substrate utilization and product formation on the enzyme surface can be controlled much more easily.

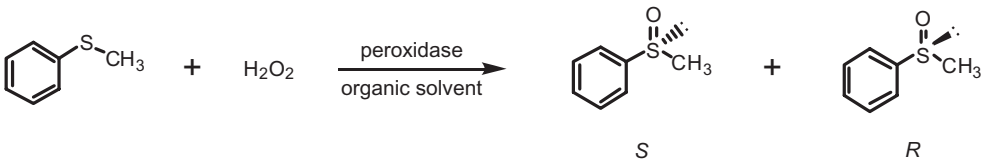
All of the different above-mentioned solvent effects can be further increased and decreased by employing different strategies that include protein engineering, the covalent attachment of amphipathic compounds, entrapment in water-in-oil microemulsions and reversed micelles, immobilization, and "solid" enzymes such as lyophilized or crosslinked enzyme aggregates.

The different effects of organic solvents on enzyme hydration and solvent binding at the active site have been investigated using molecular dynamics (MD) simulations [16]. Over a timescale of few nanoseconds, no significant structural conformation and flexibility changes could be observed on surfactant-solubilized subtilisin BPN′ testing four solvents (water, acetonitrile, tetrahydrofuran, octane). Besides the absence of any significant structural changes, the key factor that causes changes in activity, stability and selectivity is the partitioning of hydration water between the enzyme and the bulk solvent. Depending on the polarity of the bulk solvent used, the important "interface water" is removed from the enzyme to different extents. This behavior of organic solvents can be described as a "water-stripping effect" [16]. The remaining water is very important, however, for the hydration procedure of an enzyme. In short, the activity of an enzyme will increase in line with the amount of water available for the reaction in an anhydrous organic solvent. Is there too little water available, this will lead to an incomplete hydration of the enzyme, accompanied by a loss of flexibility and/or activity. The water concentration can be expressed quantitatively by the thermodynamic water activity a_w. This parameter was developed to account for the intensity with which water associates with various non-aqueous constituents, and is the product of the concentration of water expressed as its molar fraction x_w and the water activity coefficient γ_w [17, 18].

$$a_w = x_w * \gamma_w$$

This equation leads to the rule: with increasing hydrophilicity of a solvent, an increasing amount of water is necessary to achieve optimal activity. For example, a comparatively low water content is necessary when using diethyl ether as solvent, whereas a much higher content of water is required when using 2-butanol [19].

Water can be replaced with an organic solvent without affecting the enzyme in a negative manner, thus demonstrating that enzymes are able to function in a near-anhydrous organic medium. Some experimental confirmation has been provided of the above rationale by the sulfoxidation of thioanisole in different organic solvents. The negative aspects of this reaction in an aqueous system have been problematic because of the poor water solubility of diaryl and alkyl sulfides, the spontaneous uncatalyzed oxidation of sulfides resulting in racemic sulfoxides, and the poor stereoselectivity of the peroxidase used (Scheme 1.1). Each of these three problems can be solved, however, by using organic solvents as the reaction media. Notably, the reaction occurred between 10- and 100-fold faster when using methanol as the reaction media, while the spontaneous auto-oxidation was 100- to 1000-fold slower when using ethyl acetate or acetonitrile [20, 21].



initial rate of product formation:		S	R
	water	1 mol/min	0.5 mol/min
	methanol	68 mol/min	16 mol/min

Scheme 1.1 Peroxidase-catalyzed sulfoxidation of thioanisole with hydrogen peroxide in organic solvents, and initial rates of product formation [21].

1.3.1 Ionic Liquids

Since the beginning of the twentieth century, ionic liquids (ILs) have been used in addition to conventional solvents and aqueous systems for special applications in biocatalysis. Typically, ILs are composed entirely of ions, they are liquid at moderate temperatures, and they are often acclaimed as "designer solvents" due to the fact that it is possible to select different combinations of anions and cations, which significantly change their characteristics such as viscosity, density, hydrophobicity, and solubility.

Changes in selectivity and activity are structural rearrangements which can take place in IL systems. Every protein consists of a specific three-dimensional (3-D) structure, which is maintained by a combination of disulfide bridges, hydrogen bonds, and weak hydrophobic interactions. The cleavage of hydrogen bonds results in an unfolding process, which changes the structure – and thus the activity and/or selectivity – of an enzyme. As the protein unfolds and opens, the hydrophobic groups are able to interact specifically with the solvent or with destabilizing compounds. All unfolding processes proceed through two different steps: (i) a reversible first step, which changes the characteristics and performance of the protein; and (ii) an irreversible, slower step, which mostly results in denaturation of the enzyme. Enzymes in an aqueous system interact with the water molecules which dissolve the enzyme, although the water molecules are not strong enough to dissociate the structural hydrogen bonds. Salts of ILs, as in buffers, increase the energy barrier to unfolding and exposure of the hydrophobic groups. Consequently, ILs can have the same stabilizing slight "salting-out" effect on proteins as do phosphate or citrate buffers [22–24]. However, this effect only occurs when using water-miscible ILs.

Besides the solubility effect of enzymatic reactions in ILs, these "salts" may also have an impact on the enzyme structure. A change in conformation could also explain the advantageous effects of ILs on the reaction, though investigations into

these incidents are at a very early stage. One possible means of studying the conformational change of proteins in ILs would be to employ techniques such as fluorescence spectroscopy, in some cases circular dichroism (CD), tandem mass spectrometry (MS/MS), and Fourier transform infrared spectroscopy (FTIR).

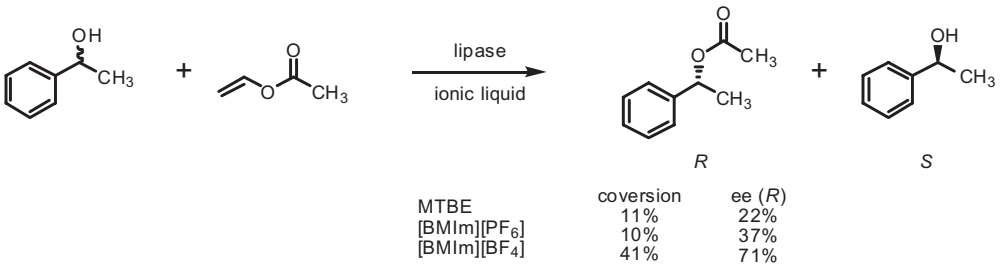
One theory of protein stabilization has been explained as follows: the smaller the cation of an IL (i.e., more chaotropic and a lower B-coefficient), the higher is the stabilizing effect on the enzyme structure. Stabilizing effects have been described as taking place in the water shell of the enzyme that guides the water molecules to interact with the cations. Thus, an increased cation concentration in the water shell would result in an increased stabilizing effect [25].

An opposite effect is seen, however, for behavior in terms of the activity and stability of enzymes in near-anhydrous ILs. While a general rule regarding the compatibility of enzymes in anhydrous ILs has not yet been developed, certain factors must be considered, such as the cation H-bond-donating capability, log *P*, the formation of hydrogen-bonded nanostructures, and the viscosity of the IL. It is well known that the thermal and operational stabilities of enzymes in organic solvents may be much better than in an aqueous system, and a similar behavior can also be recognized when using anhydrous ILs. It has been suggested that the ILs induce a more active and stabile conformation of the enzyme, although many ILs contain very strong coordinating anions, which results in an enzyme–anion interaction. Enzyme deactivation was not observed in non-hydrophobic ILs containing non-coordinating anions such as (PF_6^-) and (Tf_2N^-). One very effective stabilizing effect relates to the high viscosity of ILs, as this alters the speed at which the protein structure migrates from an active to an inactive form. Taken together, these data indicate that enzymes are highly stable in anhydrous ILs, owing to their native conformation in the dehydrated state [26, 27].

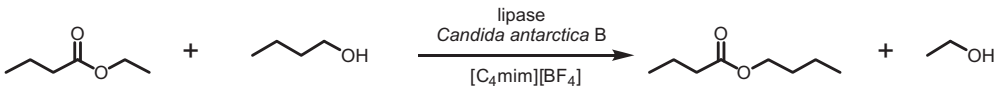
Typically, ILs – due to their unique attractive physico-chemical properties such as low vapor pressure, expected low toxicity, multiple solvation interactions with polar and unpolar substrates, high chemical and thermal stability, and high ionic conductivity – are often used to replace organic and often volatile solvents. A wide range of industrial and scientific applications has been described [26, 28, 29], with ILs being used for extraction processes, in organic and inorganic syntheses, phase separations, nanomaterials sciences, and enzymatic reactions. In these cases, the ILs can be used in a variety of modes:

- as a pure solvent
- in a biphasic system, or
- as a co-solvent in aqueous reaction media

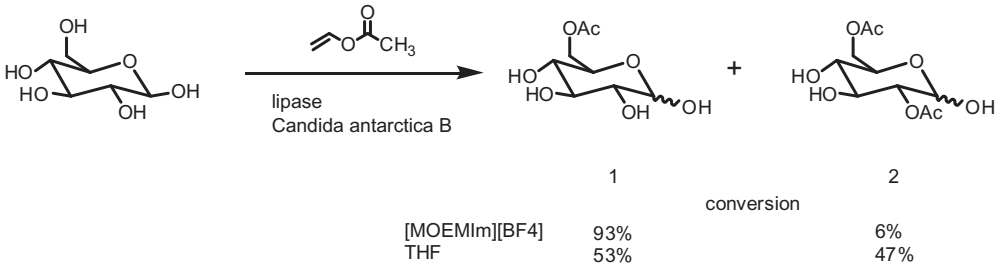
Especially in the field of biocatalysis, ILs have a distinct influence on enzyme thermal stability, storage stability, activity, regioselectivity, and enantioselectivity. Lipases, which are known as to be stable and active in organic solvents, are especially suitable candidates for bioreactions in ILs. For example, a transesterification mediated by *Candida antarctica* lipase A was 10-fold more active in ILs such as [BMIm][PF_6] and [BMIm][BF_4] compared to methyl tert-butyl ether (MTBE) solvent (Scheme 1.2) [30].



Scheme 1.2 Lipase-mediated transesterification of 1-phenylethanol with vinyl acetate in MTBE and ionic liquids [30].



Scheme 1.3 Transesterification of ethyl butanoate in ionic liquid [C_4mim][BF_4] [31].



Scheme 1.4 The regioselective acylation of glucose in an ionic liquid compared to a traditional medium [23].

Due to the insolubility of butyl butanoate in water, transesterification reactions with butanol were tested in two ionic liquids (Scheme 1.3). When 1-butyl-3-methylimidazolium tetrafluoroborate ([C_4mim][BF_4]) and hexafluorophosphate ([C_4mim][PF_6]) were used as media, the product butyl ester was formed in more than 80% yield (83% yield was expected at equilibrium at a molar ratio of 1 : 1). In the presence of 10% (v/v) water, the side product butanoic acid was formed, which lowered the yield to 60%. Although, in addition, butan-1-ol and *tert*-butyl alcohol were tested as solvents, each demonstrated a lower conversion [31].

A further example of the influence of ILs on the overall outcome of a biotransformation included the *Candida antarctica* lipase B-catalyzed esterification of glucose (Scheme 1.4), which is limited by the low solubility of carbohydrates in

organic solvents. In this case, the monoacylated product (1) was more soluble in tetrahydrofuran (THF) than was glucose, with the result that the monoester could undergo a further acylation to form the diester (2). In comparison, the reaction investigated in an IL was much more regioselective and also was much faster as the solubility in [MOEMIm][BF_4] was 100-fold higher. On the other hand, the reaction could be triggered to form only the monoacylated product (1) [23].

Although catalytically active enzymes do not dissolve in ILs, the enzyme powder and the IL form a suspension which remains active. Often, polar solvents are used to solubilize substrates, though the drawback here is that such an environment will decrease the activity or even totally inactivate the enzyme, whereas ILs have no such effect. When using ILs, enzyme-catalyzed reactions can be driven to a polarity range which, previously, was inaccessible; consequently, the solubility of very polar substrates will be higher, which in turn will lead to a better activity and a faster reaction [27].

Enzymes can catalyze unexpected and unusual reactions, depending on the water content in the reaction media. For example, proteases can carry out peptide syntheses, while lipases can catalyze esterification, transesterification, and interesterification reactions. As discussed above, anhydrous ILs can affect not only the initial rates but also regioselectivities and enantioselectivities [32]. Hence, such information should help to provide a better understanding when using ILs in biotransformations. With many different "designer" ILs being available, their correct selection may be problematic but lucrative. On the one hand, ILs help to dissolve substrates and products and can stabilize the biocatalyst, whereas on the other hand they can lead to reactions with high stereo-, regio-, and enantioselectivity. The main disadvantages of ILs, however, are their very high costs and complicated downstream processing or product processing from an environment from which the distillation of an IL is difficult. Hence, while the recycling of ILs after biotransformation is essential, it is not always easy to perform [28].

1.3.2
Microemulsions and Reversed Micelles Systems

During the past few years, the use of microemulsions and micelle-based systems as reaction media has caused much interest, mainly because the self-assembling structures and systems by surfactants generate microenvironments that may have a positive impact on enzymatic conversions [33–37]. The different aspects of biocatalysis in micelles and reversed micelles, with attention focused on the behavior of enzymes compared to neat aqueous phase systems, are discussed in the following subsections.

It is known that anionic, ionic, nonionic and zwitterionic surfactants spontaneously form micelles in aqueous solvents and reversed micelles in organic solvents, while the polar and unpolar regions of the surfactants form structures with different orientations. Typically, micelles appear over a critical surfactant (micelle)

concentration (abbreviated as the CMC). These different systems offer special conditions which may have a certain influence on enzyme catalyst reactions. For example, the number, shape, and properties of the micelles depend on different parameters, such as the nature of the surfactant and its concentration, the water content, pH, temperature, external and internal solvent, and additives. In addition, the different micelle shapes may have a significant influence not only on the enzyme's structure but also on its activity and stability.

The influence of different media systems such as a reversed micelles system on enzyme structure was investigated using techniques such as CD, FTIR, and ^{1}H-nuclear magnetic resonance (NMR). It should be noted that the effect of reversed micelles on a specific enzyme may be very different; for example, according to their CD spectrum, lipases from *Candida rugosa* and *Pseudomonas* sp. loose their native structure at the reversed micelles interface, whereas a spectrometric analysis of human pancreatic lipase showed the peptide conformation to be barely affected. These different structure changes are mostly caused by the nature of the surfactant and water content W_o, which is defined as:

$$W_o = (H_2O)/(\text{surfactant})$$

The activity and stability of an enzyme can be increased and/or decreased by using a reversed micelles system. A reduced water content in the micelles core can lead to a stabilizing effect via a more rigid conformation of the enzyme. For example, *p*-nitrophenylphosphatase was more stable in reversed micelles compared to an aqueous system, and the stability could be further increased by using a smaller water pool (W_o).

For the concept of "superactivity," which was created in connection with reversed micelle solutions, the average substrate concentrations in the water pool may be higher or lower than that in the bulk aqueous solution, depending on its distribution between the water pool and the surfactant layer. Superactivity exists when there is a low partitioning in the surfactant layer. As reverse micelles have certain features similar to those of biomembranes, the enzymatic behavior of superactivity has been explained as being caused by the water in the reversed micelles mimicking the intracellular water in biomembranes. This effect was confirmed in studies using small-angle X-ray scattering (SAXS). As an example, the hydrolysis of *p*-nitrophenol esters catalyzed by α-chymotrypsin in aerosol dioctyl sodium sulfosuccinate (AOT) reversed micelles was enhanced and optimized by creating special interfaces in the micelles. On this basis, it must be considered that the activities of different enzymes depend on the surfactant employed. For example, the wheat-germ acid phosphatase showed a low activity in AOT aqueous micelles, however superactive in lecithin reversed micelles [38–40]. Higher hydrolytic activities of triglycerides with *p*-nitrophenyl alkanoate esters as substrate were observed in AOT compared to cationic, nonionic, or zwitterionic surfactant systems. In this sense, the nature of the surfactant used to create a reversed micelles medium has an important role on the enzyme stability and activity, in contrast to an aqueous buffer medium.

1.4 Structure and Folding

Secondary structure elements, such as α-helices and β-strands, constitute the major building blocks of proteins. The particular structure of a protein defines the unique fold of an amino acid polymer, as well as its specificity and catalytic power [41]. The determination of 3-D structures of proteins at atomic resolution initiated the novel field of protein folding in research. Subsequently, new methods had to be developed to analyze the folding processes, and these led to the introduction of new technologies that included recombinant DNA technology, protein engineering, NMR methods, advanced computer simulations, and bioinformatics [42].

Flexible active site *loops* play important roles in enzyme function, including catalysis, substrate specificity and protein–protein interactions. Consequently, such loops may also provide the basis for functional diversity and the route to the evolutionary divergence of new functions [43]. Sequence changes, such as insertions, deletions or module exchanges, are frequently localized at non-structured specific loop regions. Currently, the methods of protein design being used to study the structure and function of numerous proteins, and to define sequence elements controlling enzyme activity, selectivity and specificity, involve the introduction of mutations into biocatalysts. An analysis is then conducted of possible consequent changes in the biological properties of genetically modified enzymes by altering not only the primary nucleotide and amino acid sequence but also the shape of proteins and their secondary structures (Table 1.1).

The concept of protein design has been studied using lipases which are successfully applied in the preparation of chiral building blocks. All lipases show an α/β hydrolase fold, while many of them also contain a lid that controls access to the active site. The interaction of the enzyme with its substrates induces the motion of the lid, which in turn makes the active site accessible and increases the catalytic activity. The amino acid composition of the lid has also been shown to have an effect on both the specificity and the enantioselectivity of lipases [51]. In order to improve the enantioselectivity of the lipase A from *Bacillus subtilis* (LipA) in the kinetic resolution of 1,2-*O*-isopropylidene-*sn*-glycerol esters, a loop near to the active site entrance was replaced by longer loops of structural homologs that originated from *Fusarium solani* cutinase and *Penicillium purpurogenum* acetylxylan esterase. Loop replacements which involved extending the loop by three or two amino acids yielded LipA variants with improved enantioselectivities. The acetylxylan esterase-derived variant demonstrated an inverted enantioselectivity compared to the wild-type parent enzyme, whereas the cutinase-derived variant showed an improved enantioselectivity, which could be further optimized by directed evolution [52].

Among the most common folds is the $(\beta/\alpha)_8$-barrel, which consists of eight parallel β-sheets connected by eight α-helices and has great functional and structural diversity. Loops link the β-strands with the α-helices, but are considered not to contribute to protein stability [53] (for further details of structural frameworks suitable for engineering, see Birte Höcker's overview in volume 1 of this book

Table 1.1 Examples of protein design through systematic loop exchanges.

Enzyme	Structural fold	Region	Effect
Haloalkane dehalogenase from *Xanthobacter autotrophicus* [44]	α/β hydrolase fold	N-terminal part of cap domain	Substrate specificity
Pyruvate dehydrogenase multienzyme complex from *Escherichia coli* [45]	Flattened β-barrel formed by two four-stranded β-sheets	Surface loop	Structural determinants of post-translational modification and catalytic specificity for the lipoyl domains
Lactate dehydrogenase from *Bacillus stearothermophilus* [46]		Mobile surface loop	Substrate specificity
Blue copper protein amicyanin from *Paracoccus versutus* [47]	Greek key folding motif	Ligand loop	Stable metal binding sites
Halohydrin dehalogenase from *Bradyrhizobium japonicum* [48]	α/β hydrolase fold	Protein surface loop	Enantioselectivity
Glyoxalase II [49]	αβ/βα metallo-hydrolase fold	Several active site loops	Novel β-lactamase activity
Esterase from *Pseudomonas fluorescens* [50]	α/β hydrolase fold	Loop at the entrance to the active site	Esterase into an epoxide hydrolase

series). Due to the fact that the substrate binding and the catalytic residues are located in separate regions, $(\beta/\alpha)_8$-barrels are regarded as ideal targets for reshaping the binding sites for new substrates, or for the grafting of new catalytic functions. For instance, hydantoinases being part of the cyclic amidohydrolase superfamiliy of enzymes share similar structural properties and functions with other members of this superfamily. The D-hydantoinase from *Bacillus stearothermophilus* SD1–a versatile enzyme with a strict enantioselectivity, a high activity, easy overexpression, and thermostability–catalyzed the cleavage of the cyclic amide bond of 5′-monosubstituted hydantoins. Its substrate specificity is, however, limited toward non-substituted hydantoins, and therefore it is not applicable to the synthesis of non-natural D-amino acids with phenyl and hydrophenyl groups. In a previous study, stereochemistry gate loops (SGLs) of enzymes forming the substrate binding pocket were identified, and both the substrate specificity and enantioselectivity were seen to be governed by the conformation and properties of the SGLs in the amidohydrolase superfamily. Rationally, manipulation of the hydrophobic and bulky residues of the SGLs, which interact with the substrates, on the basis of a structural analysis and a comparison of three different

hydantoinases, resulted in altered substrate specificities of the enzyme variants that were generated [54].

Recently, an attempt at loop swapping in the $(\beta/\alpha)_8$-barrel was undertaken using the β/α loop 6 of monofunctional *N*-(5′-phosphoribosyl)anthranilate (PRA) isomerase from *Escherichia coli*, which catalyses the Amadori rearrangement reaction of PRA to 1′-(2′-carboxyphenylamino)-1′-deoxyribulose 5-phosphate (CdRP). This rearrangement is the third step in the synthesis of tryptophan from chorismic acid. The loop is located above the catalytic residue Asp126, and forms a long and flexible lid over the active site pocket. Loop swapping and hinge variability experiments were performed to introduce a range of functionally, structurally, and evolutionarily related loops at the amino and the carboxy ends of the H3 loop. Details regarding the function and stability of some variants were obtained by kinetic studies of PRA isomerase activity, in addition to CD and size-exclusion chromatography [55, 56].

1.5 Structured and Unstructured Domains

For many years, scientists embraced the idea that a folded 3-D structure is necessary for the biological function of proteins. While there is no doubt that the functions of many proteins are directly related to their structure, numerous proteins have been described which contain unfolded or partly folded segments under physiological conditions, and do not fold into stable, precise, rigid configurations [57]. The first proposal for a general mechanism of enzyme action was developed by the chemist Emil Fischer; this was the so-called "lock and key model" [58] of protein action. Subsequently, even the much younger "induced-fit" theory [59] (as developed by Koshland and Neet) failed to explain the function of largely unstructured proteins (see also Chapter 17). Many multifunctional proteins lack, to some extent, a well-defined 3-D structure in the native state, and these are known as intrinsically disordered proteins (IDPs) [60–63]. Such dynamic ensembles of interconverting structures have been given various names, including rheomorphic, intrinsically disordered, natively denatured, natively unfolded, intrinsically unstructured, mostly unstructured, and natively disordered, as well as protein clouds, to name but a few [63]. Some 40% of all human proteins contain at least one intrinsically disordered segment of 30 amino acids or more, and 25% are likely to be disordered from beginning to end [63]. Until now, approximately 600 unstructured proteins – partially or totally – have been identified and described, although it can be assumed that many more of these exist [64].

Fewer than 10 consecutive residues are considered as "just flexible loops" in well-structured proteins, while very long regions (>30 residues) show a different behavior. Certain amino acids have been found to be highly "structure-promoting" (specifically Cys, Trp, Tyr, Ile, Phe, Val, Leu, His, Thr, and Asn), while others are highly "structure-dispromoting" (in particular Asp, Met, Lys, Arg, Ser, Gln, Pro, and Glu). The reduced amount of bulky hydrophobic (Ile, Leu, and Val) and aro-

matic amino acids (Trp, Tyr, and Phe), which would normally form the hydrophobic core of a folded globular protein, are significant for intrinsically disordered proteins. They also possess a low content of Asn and Cys residues, with the latter being known to have a significant contribution to the protein conformation stability via the formation of disulfide bonds or the coordination of different prosthetic groups. The specific feature of their amino acid sequences results in a low mean hydrophobicity (charge–charge repulsion) and a relatively high net charge (low driving force for protein compaction). A combination thereof seems to be particularly important for the absence of compact structure in proteins under physiological conditions [62].

Studies on the correlations between environment and disorder have suggested that proteins with long disordered regions are independent of the phylogenetic branch on the tree of life. Unlike the well-structured proteomes of thermophiles (which might explain the high success rate of these for structure determination), the proteomes of psychrophiles and of Archaea and bacterial halophiles are disordered, which enables these proteins to tolerate structural modifications resulting from frequent mutations [65]. Currently, the design of algorithms for identifying regions with intrinsic order is a very active area of research, and has also been demonstrated by the development of more than 50 predictors of disorder [66]. Although the most common method for obtaining systematic site-specific information on unstructured proteins and their motions in solution is NMR [67, 68], other techniques such as X-ray crystallography, hydrodynamic measurements, CD, fluorescence spectroscopy, vibrational CD spectroscopy, Raman spectroscopy, or other biophysical techniques have revealed more than 200 proteins that contain disordered regions of 30 consecutive residues [61].

Most IDPs undergo transitions to more ordered states, or fold into stable secondary or tertiary structures on binding to their targets. Typically, they undergo coupled folding and binding processes with protein regions that specifically participate in protein–protein interactions termed “molecular recognition features” (MoRFs) or “molecular recognition elements” [69]. Dynamics linked to the configuration of proteins have been described as being critical for a variety of physiological processes, such as transcription and translation regulations, cellular signal reductions, protein phosphorylation, and molecular assemblies. In this sense, a bioinformatics approach was undertaken to study the functional roles of confirmed disordered proteins [70–72]. The results of these comprehensive bioinformatics investigations suggested that proteins with catalytic, biosynthetic, and metabolic functions are enzymes, and therefore are regarded as ordered proteins which are structured for catalysis.

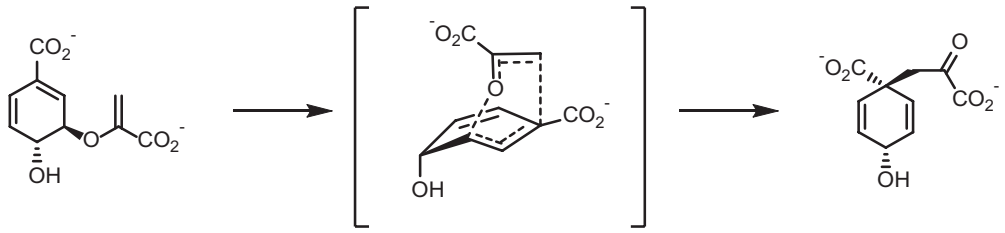
Enzymatic catalysis appears to be less compatible with the presence of structural disorder. The catalytic step in an enzymatic process requires a protein to provide an environment in which the transition state of the chemical reaction can be reached more readily than in the absence of the enzyme. As a very specific organization of the interacting partners within the active site of the enzyme is required to be catalyzed with optimal efficiency (activity, selectivity, and stability), disordered proteins seem – according to their description in the literature – to be poorly

suitable as enzymes. Remarkably enough, evidence suggests that this does not appear to be the case with all IDPs, as there have been reports of enzymatic activity in disordered states [73].

The modulation of mechanical and chemical energy inputs on the conformation and function of PR65, the HEAT-repeat scaffold of the PP2a phosphatase enzyme, has been investigated recently. In this example, HEAT-repeat proteins occur in multicomponent complexes, bringing proteins into contact with other proteins or with other biomolecules. Hence, such HEAT-repeat molecules or protein complexes have a catalytic function. Conformational fluctuations of PP2a were shown to be dominated by PR65 shape changes that opened and closed the substrate binding interface by the motions of a rigid, catalytic, and a more flexible regulatory subunit [74].

In another investigation, the transitions between conformational sub-states and their influence on the overall catalytic cycle or substrate turnover of two phosphotriesterases from *Agrobacterium radiobacter* and *Pseudomonas diminuta*, were addressed. Phosphotriesterases catalyze the hydrolysis of the pesticide paraoxon, which is described to be limited by substrate diffusion or conformational change. Two dominant sub-states, abbreviated as conformational substates (CS) "open" and "closed," have been identified. The "closed" sub-state is preorganized to lower the activation energy of paraoxon hydrolysis, but appears to be incompatible for rapid substrate and product diffusion. Due to the stabilization of a decolalized negative charge, that is formed in the transition state upon nucleophilic attack of the hydroxide ion, through interaction with the positively charged binuclear metal ion, the activation energy is lowered. In contrast, the "open" sub-state enables access to the active site, but is poorly organized for an acceleration of the hydrolysis reaction. Five variants, all distant from the active site, were generated by both natural and laboratory-based evolution approaches and analyzed by structural and kinetic effects. Subsequently, it was shown that variants remote from the active site would affect the turnover rate of paraoxon hydrolysis by a conformational change in the sub-state [75].

A well-characterized example of a structurally disordered protein with a high catalytic activity is chorismate mutase from *Methanococcus jannaschii* (MjCM), which has been converted into a functional monomer (mMjCM) by the insertion of a hinge-loop sequence ARWPWAEK into its long, dimer-spanning N-terminal helix to disrupt the dimer interface. The designed enzyme catalyzes the conversion of chorismate to prephenate (Scheme 1.5), a key step in the biosynthesis of the aromatic acids, with activities similar to that of the native enzyme. Unlike its natural counterpart, the monomer mMjCM unexpectedly possesses all of the characteristics of a molten globule. Molten globules are partially folded proteins that have some native-like secondary structure with a loosely packed hydrophobic core. Although the monomer contains an eight-amino acid insertion and one point mutation relative to the dimer, it still functions and provides the same catalytic power as the native enzyme (Figure 1.3). It has been described that the monomer becomes more ordered than its native-like counterpart upon binding of the transition state analogs (TSAs), but it retains a high flexibility on the millisecond time



Scheme 1.5 Conversion of chorismate to prephenate via a chair-like transition state, catalyzed by chorismate mutase [76, 78, 80].

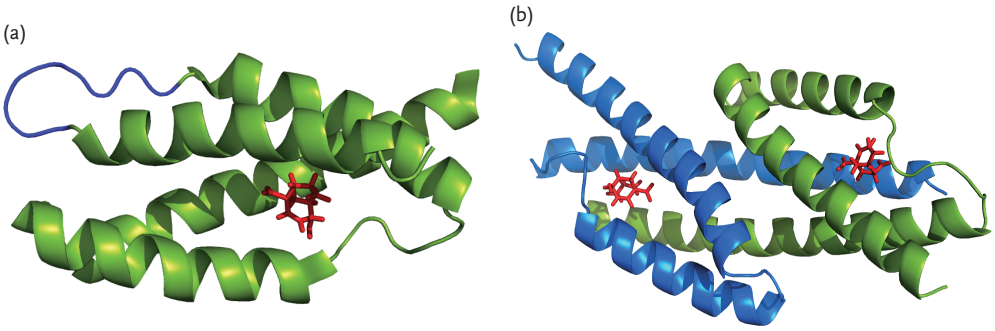
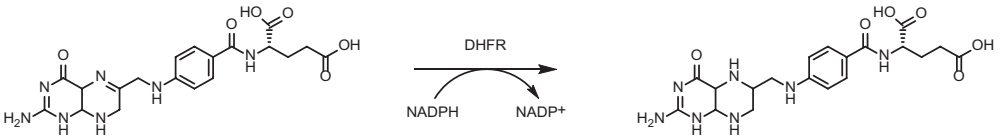


Figure 1.3 Three-dimensional structural representation of (a) monomeric mMjCM and (b) dimeric chorismate mutase. The active site is occupied by the TSA, which is represented in red in both structures [76, 77].

scale and a wider catalytic region. Pre-steady-state kinetics have suggested that the molten globule conformational ensemble undergoes structural changes when bound to the TSA, from a "loose" to a more "tight" catalytically efficient enzyme–ligand complex. A closely related computational study of the relationship between the folding and chemical landscapes of the dimeric and monomeric chorismate mutase and their catalytic power showed that the conformations are broader for the molten globular enzyme than for the native state enzyme, even in the ligand-bound form [76–81].

Another recently studied example explored the correlation between the structure, dynamics, and catalysis of dihydrofolate reductase (DHFR) from *E. coli*. *In vivo*, DHFR catalyzes the stereospecific reduction of dihydrofolate to tetrahydrofolate (Scheme 1.6), which is needed by the cells for the synthesis of DNA, using NADPH as cofactor. An active-site loop – the Met20 loop – has been characterized to be flexible and to adopt two different conformations during the catalytic cycle, namely closed and occluded conformations (Figure 1.4). In the closed conformation, the Met20 loops pack tightly against the nicotinamide ring of $NADP^+$, while in the occluded conformation the nicotinamide ring of $NADP^+$ is sterically hindered from binding in the active site. A comparison of the structures of human



Scheme 1.6 Dihydrofolate reductase-catalyzed stereospecific reduction of dihydrofolate to tetrahydrofolate using reduced nicotinamide adenine dinucleotide phosphate (NADPH) as cofactor [82].

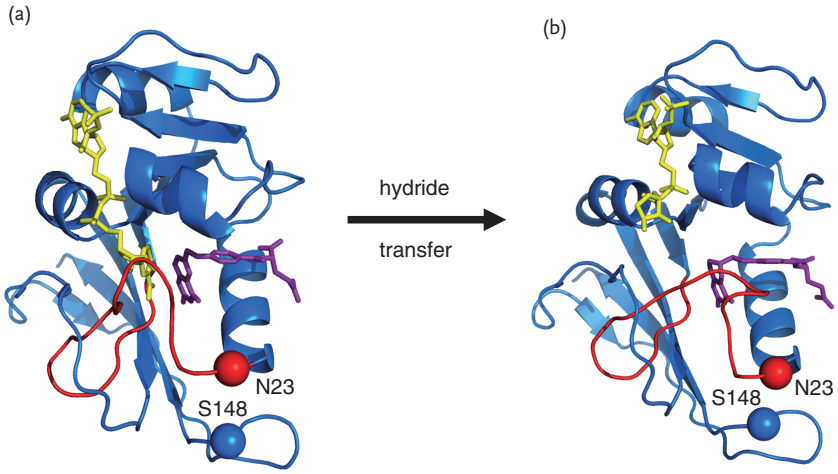


Figure 1.4 Illustration of the closed (a) and occluded (b) conformation of dihydrofolate reductase. $NADP^+$ is shown in yellow, the substrate and product are shown in pink. Red indicates the Met20 loop in the closed and occluded conformation. The sites of mutation N23 (red) and S148 (blue) are shown as spheres [82].

and other vertebrate DHFRs showed that the Met20 loop in human enzymes is more rigid, and that the occluded conformation is destabilized. For this reason, dynamic knockout mutants (N23PP, S148A, and N23PP/S148A) were created to provide an insight into the role of the active site loop in the catalysis by the *E. coli* enzyme preventing the flexible Met20 loop from moving. Pre-steady-state kinetics have shown that the rates of hydride transfer at pH 7 of the N23PP/S148A and N23PP variants were 16-fold lower than that of the wild-type enzyme. Furthermore, by examining the crystal structure of the N23PP/S148A DHFR variant, and comparing it with the wild-type enzyme, it could be seen that the structures were almost identical and that the electrostatic nature of the active site was unchanged by the mutations done. However, NMR studies revealed that the variant was no longer flexible, and that it remained in the closed conformation across the chemical step. It was reasoned that the decreased rate of hydride transfer in the active

site resulted from an impaired flexibility, such that the molecules were not sufficiently close to one another to conduct an efficient catalysis [82].

The role of conformational dynamics controlling the enzymatic activity of proteins remains a subject of great controversy. Studies of DHFR, as described above, have provided an impetus for a computer simulation study generating the catalytic landscape of the enzyme. Warshel and coworkers have elucidated, in their simulation study, that the electrostatic preorganization is different between the variant and the wild-type DHFR enzyme. On this basis, it was concluded that not dynamic effects, but rather changes in the activation free energy of the dynamic knockout mutations, were responsible for the changes in catalysis [83]. Despite some progress having been made in this field, debate persists surrounding the exact role of dynamics in enzyme catalysis. The central law that the function of a protein depends on its fully folded 3-D shape (representing the most energetically stable conformation and the only functional one) began to change with the incompatibility of the lock-and-key model, with the concept of a transient or durable disorder of functional protein segments. The critical response to the idea of dynamical effects contributing to enzyme catalysis – which rapidly gained popularity and has been the subject of significant experimental and theoretical investigation – cannot be ignored. Critics point particularly to the fact that existing studies have still not provided any unique evidence that enzyme dynamics make a significant contribution to the catalysis of the ground-state reactions, and the unclear formulation as to what is actually meant by the contribution of enzyme dynamics to catalysis [84].

1.6
Isozymes, Moonlighting Proteins, and Promiscuity: Supertalented Enzymes

Although, for several decades, the scientific world believed in the paradigm that the structure and function of a protein were linked so tightly that one significant structure only corresponded to one specific enzymatic function, this one gene–one protein–one function idea has been disproved over the past years. In short, enzymes are not always specific for their substrate and the reaction that they catalyze.

It is possible to explain enzyme specificity by describing three different, and very special, phenomena:

- isozymes
- promiscuous enzymes

The first recognition that enzyme structure and function are not so tightly correlated was defined with the term isozyme (or isoenzyme). Isozymes are assigned as different enzyme structures which can catalyze the same type of chemical reaction [32]. Frequently, they are oligomers composed of different polypeptide chains, and so they usually differ in regulatory mechanisms and in their kinetic characteristics.

One example of the advantages of isozymes in adjusting metabolism to different conditions and/or different organs is that of glucokinase and hexokinase, which are typical examples of isozymes. In fact, there are four hexokinases, namely I, II, III, and IV. Hexokinase I is present in all mammalian tissues, while hexokinase IV (also known as glucokinase) is found mainly in the liver, pancreas, and brain. Both enzymes catalyze the phosphorylation of glucose:

$$\text{glucose} + \text{ATP} \rightarrow \text{glucose 6-phosphate} + \text{ADP}$$

Hexokinase I has a low K_m and is inhibited by glucose 6-phosphate, whereas glucokinase is not inhibited by glucose 6-phosphate and its K_m is high. These two facts indicate that the activity of glucokinase depends on the availability of the substrate, and not on the demand of the product.

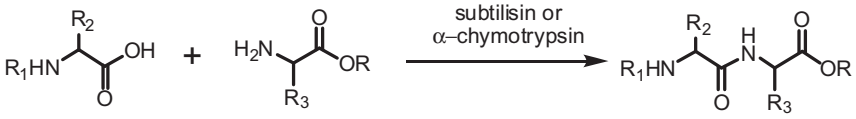
Since glucokinase is not inhibited in conditions of high concentrations of glucose 6-phosphate this enzyme will continue to phosphorylate glucose, which can be used for glycogen synthesis in the liver. Additionally, as glucokinase has a high K_m, its activity does not compromise the supply of glucose to other organs; in other words, if glucokinase had a low K_m, and as it is not inhibited by its product, it would continue converting glucose to glucose 6-phosphate in the liver, making glucose unavailable for other organs.

In contrast, *moonlighting proteins* can have different functions (i.e., to serve additional functions that are general not enzymatic, but rather structural or regulatory) within a single structure/polypeptide chain, and can carry out very different functions at two different locations. Proteins with several functions caused by gene fusion, affiliation to one homologous protein family, or splice variants are not regarded as moonlight proteins. One of the first moonlight proteins to be characterized was an eye lens crystalline protein which can act as a lactate dehydrogenase, enolase, quinone oxidoreductase, and many more. Today, about 50 examples of moonlight proteins are known, many of which are discussed in reviews [85, 86]. Some novel examples of moonlight proteins are listed in Table 1.2.

The term biological *promiscuity* (also known as substrate ambiguity or cross-reactivity) relates to the fact that one enzyme structure can catalyze chemically distinct reactions. In Nature, the phenomenon of enzyme promiscuity is well known and has been established for thousands of years. Changes in the environment play often a significant role in enabling organisms to survive, and this type of selective pressure on enzymes may serve as an advantageous feature for the divergent evolution of new catalytic functions. Normally, in Nature enzymes are developed to a point at which they are "good enough" for their tasks, which means that the cell is satisfied. There are, however, several important exceptions. For example, ribulose 1,5-bisphosphate carboxylase cannot distinguish between carbon dioxide and oxygen, with the result that both substrates are converted and byproducts occur that the cell must cope with. This form of natural promiscuity may be a starting point for new enzyme activity and selectivity. Such behavior in Nature, can be successfully transferred into the laboratory by using techniques such as directed evolution. More recently, due the accelerated progress in enzyme engineering, enzyme promiscuity has become very popular in biocatalysis [2, 87].

Table 1.2 Examples of moonlighting proteins.

Name	First function	Second function
cPrxI	Peroxidase	Chaperone
ARGONAUTE4 (AGO4)	Cleaving RNA transcripts to produce siRNAs	Directing chromatin remodeling factors to a target locus
Ubp6	Deubiquinating cysteine protease enzyme	Delays the degradation of ubiquinated proteins by the proteasome
Lens crystallins	Increasing the refractive index while not obstructing light	Heat shock proteins, lactate dehydrogenase
Mitochondrial aconitase	Enzyme in the citric acid cycle	mtDNA maintenance
Enolase	Enzyme in glycolysis	Assists mitochondrial import of tRNAsLys



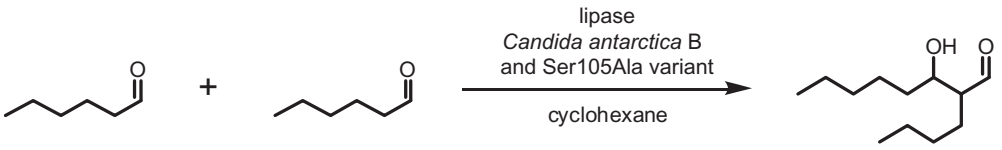
Scheme 1.7 Subtilisin- and α-chymotrypsin-catalyzed synthesis of peptides [88, 89].

Promiscuity can be further subdivided into different forms that cannot always be clearly separated:

- condition promiscuity
- substrate promiscuity
- catalytic promiscuity
  - accidental
  - induced

Both, enzyme condition and substrate promiscuity have been unwittingly employed in many industrial applications, since different reaction conditions such as the use of organic solvents, extreme temperature and pH conditions, the amount of water present, or the choice of reaction medium (ILs or microemulsions) can each lead to enzymes behaving in an abnormal manner by catalyzing reactions that differ from their natural format.

Such *condition promiscuity* may occur if an enzyme carries out unexpected and unusual reactions in low-water-containing organic solvents or in reversed micelles; examples are the serine proteases subtilisin and α-chymotrypsin, which can carry out peptide synthesis (Scheme 1.7) [88, 89]. The carboxyl ester hydrolase lipase B from *Candida antarctica* and its Ser105Ala variant also catalyzes the



Scheme 1.8 Aldol addition of hexanal by lipase *Candida antarctica* B wild-type and Ser105Ala variant in an organic solvent [90].

formation of a carbon–carbon bond in the aldol addition of hexanal in cyclohexane (Scheme 1.8) [90].

Substrate promiscuity is an equally well-known term that describes enzymes which will accept a broad range of chemically different substrates. Simple mutagenesis can lead to substrate promiscuity, or even increase the range of accepted substrates. In this respect, lipases have been used as a biocatalyst for the resolution of racemic acids and alcohols [91, 92].

Catalytic promiscuity can occur as a side reaction using the wild-type enzyme (accidental), or caused by previous mutations (induced). In both cases, the enzyme catalyzes different chemical reactions but with different transition states [2, 32, 93]. The lipase-catalyzed aldol condensation of aromatic aldehydes is an example of catalytic promiscuity, using lipase B from *Candida antarctica* [94].

One great challenge that concerns the above-discussed enzymatic effects is whether moonlighting proteins and the catalytic promiscuity of enzymes can be *predicted*. A very promising tool for this purpose is the further development of bioinformatics molecular docking (MD) simulations that enable the prediction of special functions within an enzyme. Another approach is to use proteomics to identify, characterize, and determine either new or second functions of thousands of proteins, simultaneously. Unfortunately, the development of these methods is at a very early stage, and experience in this area is, at present, minimal.

1.7 Conclusions

Enzyme engineering, leading to increased activity, a broader spectrum of products, altered selectivity and thermostability, is a powerful tool by which the outcome of biochemical reactions can be controlled and directed. The recombination of residues in the active site or second sphere of an enzyme can lead to the construction of newly specific biocatalysts, or even enzymes that produce unnatural products. Although these significant achievements have helped in the discovery and design of novel biocatalysts, many biochemical analyses have indicated that flexible and dynamic regions and domains, as well as the reaction media, play important roles in catalysis. Hence, to summarize, the results of recent investigations have highlighted the need to investigate the structure and function of proteins in greater detail, in order to specifically and selectively convert substrate molecules into the

desired products. Nonetheless, the choice of solvents, ionic liquids and even microemulsions – and their influence on the overall reaction – should not be neglected. Consistent with these points, the capabilities and possibilities of enzyme engineering are by far not yet exhausted, and should lead to improved enzymatic functions.

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