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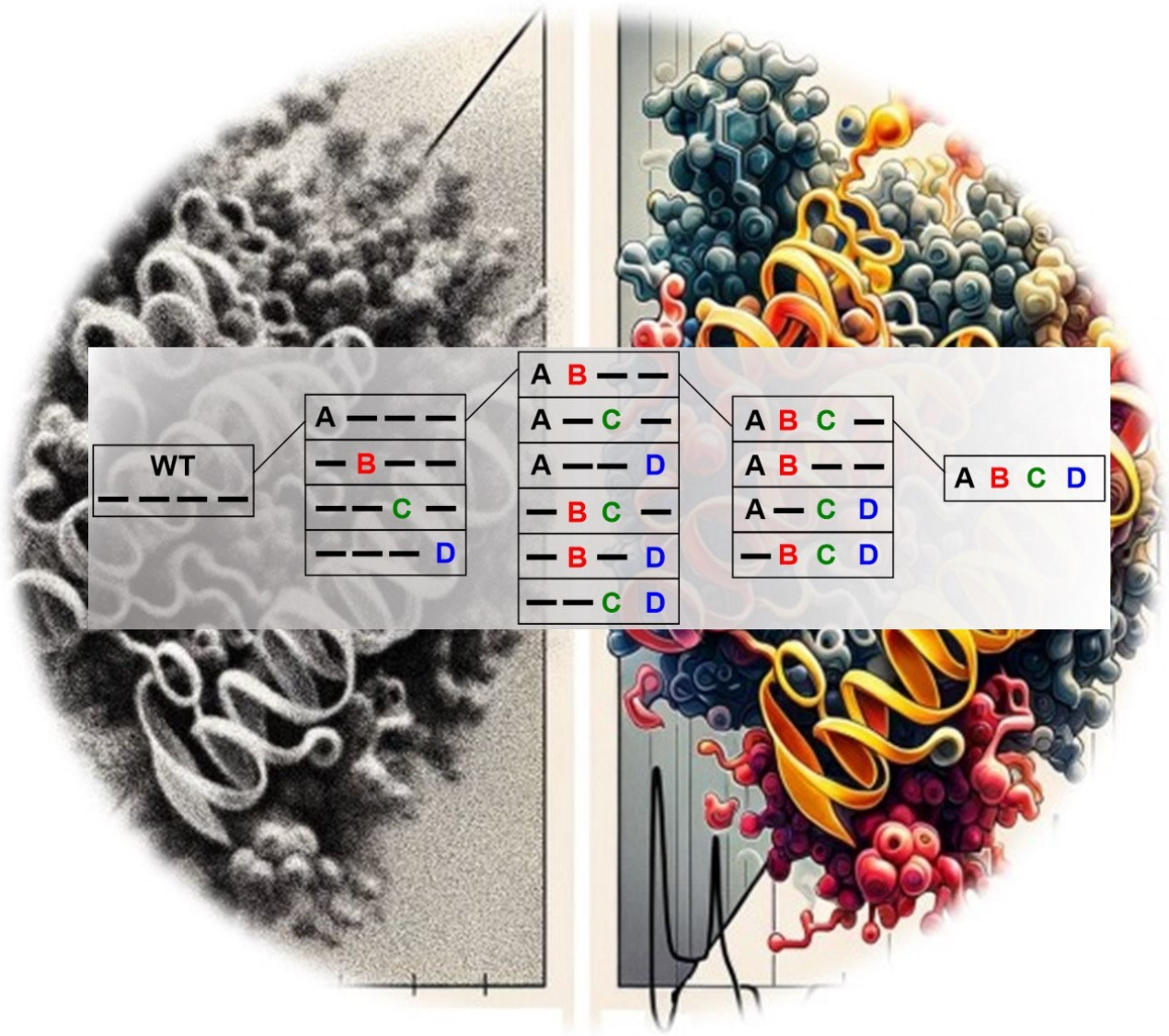
Directed Evolution

Learning from Protein Engineering by Deconvolution of Multi-Mutational Variants

*Frank Hollmann, Joaquin Sanchis, and Manfred T. Reetz**



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Abstract: This review analyzes a development in biochemistry, enzymology and biotechnology that originally came as a surprise. Following the establishment of directed evolution of stereoselective enzymes in organic chemistry, the concept of partial or complete deconvolution of selective multi-mutational variants was introduced. Early deconvolution experiments of stereoselective variants led to the finding that mutations can interact cooperatively or antagonistically with one another, not just additively. During the past decade, this phenomenon was shown to be general. In some studies, molecular dynamics (MD) and quantum mechanics/molecular mechanics (QM/MM) computations were performed in order to shed light on the origin of non-additivity at all stages of an evolutionary upward climb. Data of complete deconvolution can be used to construct unique multi-dimensional rugged fitness pathway landscapes, which provide mechanistic insights different from traditional fitness landscapes. Along a related line, biochemists have long tested the result of introducing two point mutations in an enzyme for mechanistic reasons, followed by a comparison of the respective double mutant in so-called double mutant cycles, which originally showed only additive effects, but more recently also uncovered cooperative and antagonistic non-additive effects. We conclude with suggestions for future work, and call for a unified overall picture of non-additivity and epistasis.

1. Introduction

Directed evolution of enzymes has pervaded all areas of protein engineering, especially evolved stereoselective mutants now playing a notable role in sustainable organic chemistry.[1–4] The ability to evolve multi-mutational variants enabling the catalytic stereoselective formation of chiral pharmaceuticals and other value-added products in an ecologically and economically viable manner has had a major impact on production processes.[1–5] Indeed, without the advent of directed evolution of *stereoselectivity*, enzymes would never have gained a prominent place in the toolbox of organic chemists.[1b,5] In order to understand the origin of enhanced or reversed stereoselectivity on a molecular level, docking and molecular dynamics (MD) simulations and quantum mechanics/molecular mechanics (QM/MM) computations were performed early on,[6] while algorithms for strictly rational enzyme design have been developed.[7–9] Moreover, supported by structural data of wildtype (WT) protein compared to evolved, stereoselective mutants, mechanistic lessons with new insights emerged, work that flourishes to the present day.[1b,5,10–12]

A very different type of lesson concerns the technique of partial or complete deconvolution of multi-mutational stereoselective mutants,[13] *which is the primary focus of the present review.*

Complete deconvolution means that all theoretically possible single, double and triple (and so on) mutations and sets of mutations of a multi-mutational variant, previously obtained by directed evolution, are first produced by site directed mutagenesis, and the respective variants are then assessed for stereoselectivity, activity or thermostability. The early results surprised many protein engineers. Moreover, data derived from complete deconvolution enables the experimental construction of so-called fitness pathway landscapes for increasing activity or thermostability as well as enhancing or reversing stereoselectivity, thereby unlocking unique molecular insights.[13] As will be seen on the basis of numerous studies,[1b] *these fitness landscapes are rugged, because the mutations and sets of mutations interact with one another in a cooperative or antagonistic, non-additive manner, or both.* The present review documents how the technique of partial or complete deconvolution has progressed since the perspective published more than a decade ago.[13] As will be seen, many unexpected insights using different types of enzymes and other proteins have since emerged.

Most traditional protein fitness landscapes rely on evolutionary steps in upward climbs which generally involve successive single point mutations with catalytic concomitant improvement.[14] Many protein engineers then conclude that a smooth (e.g., Fuji mountain-type), not rugged, fitness landscape exists. It would be interesting to see the result of mutational deconvolution in these systems, which has not been done to date.

Following the seminal work of Michael Smith on the genetic introduction of a defined point mutation at any position of a protein,[15] researchers have used this technique to clarify enzyme mechanisms rather than evolving improved biocatalysts for practical applications.[16–19] In most of these studies, two point mutations, designated as X and Y, were introduced separately. Then their individual contributions to the catalytic profile (activity) were ascertained

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experimentally, and subsequently compared to the mutational effect of the respective combined double mutant in a so-called thermodynamic cycle, also called double mutant cycle. Originally, additive effects were generally found according to the Smith/Fersht/Wells equation in which the additional free energy term $\triangle\triangle G_{(I)}$ is zero (Eq. 1).[17,18a,b] In rare cases, the latter term turned out not to be negligible, meaning that non-additivity pertains, which was difficult to explain on a molecular level.[17]

$$\Delta\Delta G_{(X,Y)} = \Delta\Delta G_{(X)} + \Delta\Delta G_{(Y)} + \Delta\Delta G_{(I)} \quad (1)$$

At the time, most biochemists and protein engineers adhered to the credo of additive mutational effects based on experimental observations.[18c–k] Recently, the Smith/Fersht/Wells procedure was applied to different enzyme types, indeed revealing many non-additive mutational effects, accompanied by theoretical treatments.[19,20] These important studies have toppled the earlier conclusion. The technique was also extended to *triple mutant cycles*, an exciting advance.[17o,20] Stereoselectivity was not reported in any of these studies. Again, the aim was to collect data for mechanistic purposes, not for protein engineering of enzyme variants useful in organic chemistry.

As part of our previous work on directed evolution of stereoselective enzymes, the deconvolution procedure was systematized.[13] Strictly additive mutational effects may be operating (Figure 1a), but also non-additivity in terms of cooperative (Figure 1b) or deleterious (antagonistic) effects (Figure 1c).

In the present review, we highlight case studies in which either partial or complete deconvolution of stereoselective multi-mutational enzyme variants was performed. In principle, it is the reverse of the Smith/Fersht/Wells procedure of combining mutations. Originally, we were baffled by our own finding that non-additive cooperative as well as antagonistic mutational effects influencing stereoselectivity occur routinely, not just rarely, especially when applying focused mutational techniques aimed at obtaining efficient catalysts for organic and pharmaceutical chemists.[5] In some of these publications, single mutations from different directed evolution campaigns were combined,[1b] a technique that is similar to the Smith/Fersht/Wells double mutant cycle,[17] except that the aim is to improve catalytic performance for practical applications. However, this procedure does not always guarantee catalytic improvement, which puzzled protein engineer at the time. Following the establishment of complete deconvolution, such failures no longer surprise, because protein engineers now realize that antagonistic mutational effects occur, not just traditionally additive or synergistic influences.

In some new studies reporting individual and multi-mutational variants, the authors do not mention the term non-additive effects, nor are the mutation data analyzed in a way that we believe would provide the highest degree of insight. An alternative and often used term to describe the influence of non-additivity is epistasis.[21,1c] Epistatic effects have also been discussed in evolutionary biology, resulting in many different insights,[23] while the term "non-linear" is seldomly used.[19,23f]

Before we present and analyze selected case studies of partial or complete deconvolution of multi-mutational variants, it is instructive to briefly summarize the most often used mutational methods in directed evolution.[1–4] In the first report of directed evolution of enantioselectivity,[24] a lipase was subjected to four rounds of random mutagenesis

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Joaquin Sanchis, who as a synthetic organic chemist, worked in the Reetz-group at the Mülheim MPI 2005–2008, where he helped to develop the concept of deconvolution of multi-mutational variant as a novel means to learn fundamental lessons from directed enzyme evolution, especially when constructing fitness pathway landscapes.

Manfred T. Reetz (born 1943) obtained his doctoral degree in organic chemistry in 1969 at Göttingen University/Germany. Independent research in Germany followed, including directorship of the Max-Planck-Institut für Kohlenforschung in Mülheim for two decades, where he pioneered the concept of directed evolution of stereoselective enzymes. After his first retirement in 2011, he continued research at Marburg University and at the Tianjin Institute of Industrial Biotechnology/China, and returned to Mülheim in 2019.

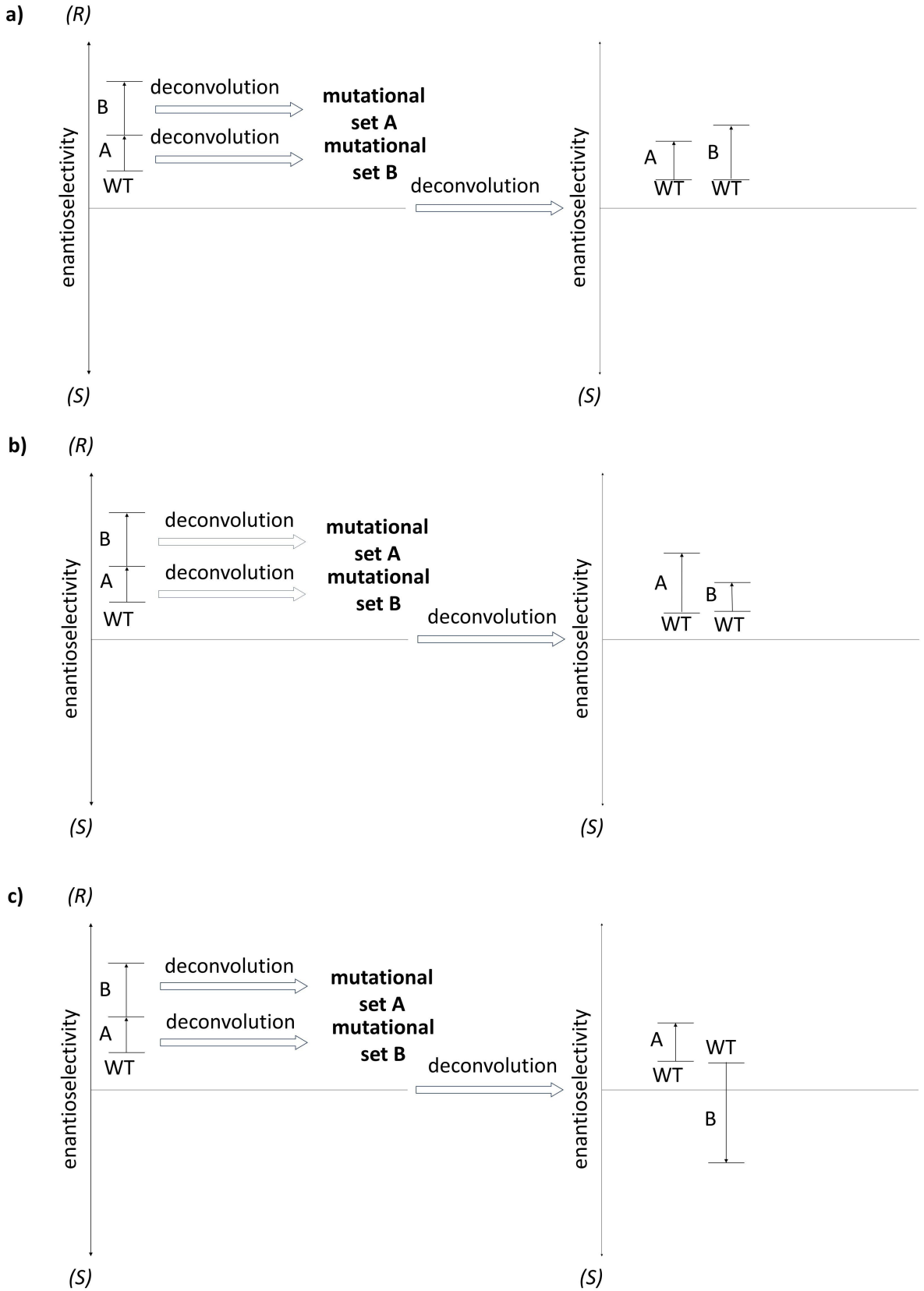

Figure 1. Deconvolution of a multi-mutational enzyme variant obtained by directed evolution process WT→A→B in two steps. a) additive mutational effect. b) cooperative non-additive effect. c) antagonistic non-additive effect. Adapted with permission from an earlier report.[13]

using error-prone polymerase chain reaction (epPCR), a mutagenesis technique that had been reported a decade earlier without any applications.[25] While reversal of enantioselectivity was also possible using the same epPCR-based approach,[26] a subsequent fifth round of epPCR failed to induce significant improvement, the reason being unclear at the time. In order to evolve notable enhancement of stereoselectivity, the combination of DNA shuffling[27] *and focused saturation mutagenesis* at the binding pocket was tested, affording better results.[28] This finally led to the development and systematization of focused saturation mutagenesis at residues surrounding the binding pocket, dubbed *Combinatorial Active-site Saturation Test* (CAST), and *Iterative Saturation Mutagenesis* (ISM).[1c,5b] In contrast to random mutagenesis, these focused approaches proved to be more effective strategies for evolving stereo- and regioselectivity.[1b,5] Subsequently, the purely rational approach was developed, Focused Rational Iterative Site-specific Mutagenesis (FRISM).[1c,22] When applying these techniques in directed evolution of selective enzymes, essential guidelines have been issued in several reviews, which include deep learning techniques and the newest high-throughput screening methods.[1b–c,5,22] They will not be repeated here.

2. Partial Deconvolution of a Triple Mutant Lipase Variant (Case Study 1)

The lipase from *Pseudomonas aeruginosa* (PAL) is the most intensively studied biocatalyst in protein engineering of stereoselective enzymes.[1b] As outlined in the Introduction above, four cycles of epPCR at low mutation rate were originally employed in the hydrolytic kinetic resolution of *rac*-**1** (Figure 2), in each upward step a single point mutation occurring.[24] With the evolved quadruple mutant S149G/S155L/V47G/F259L, the selectivity factor E, reflecting the relative reaction rate of one enantiomer with respect to the mirror image substrate, increased from $E=1.1(S)$ to $E=11.3$ (S). Some protein engineers have interpreted the experimental result of this hydrolytic kinetic resolution shown in Figure 2b as the involvement of strictly traditional additive effects, which suggests that the last step contributes only little to the overall result.[29] However, this conclusion is clearly premature, if not incorrect. In the absence of deconvolution of the quadruple variant, which has not been reported, sound conclusions cannot be made. Unsound would also be a suggestion that a smooth fitness landscape is involved.

The real breakthrough came upon employing the same lipase as the catalyst in the kinetic resolution of *rac*-**1**, used earlier, this time applying CAST/ISM.[5,30] Accordingly, a 2-step CAST/ISM process at sites A and B starting from wildtype (WT→B→A) was designed.[31] This furnished the 3-mutational variant 1B2 (Leu162Asn/Met16Ala/Leu17Phe), characterized by an unprecedented stereoselectivity of $E=594$ in favor of (*S*)-**2**. (Figure 3a). Traditionally, some protein engineers or biochemists would conclude that the

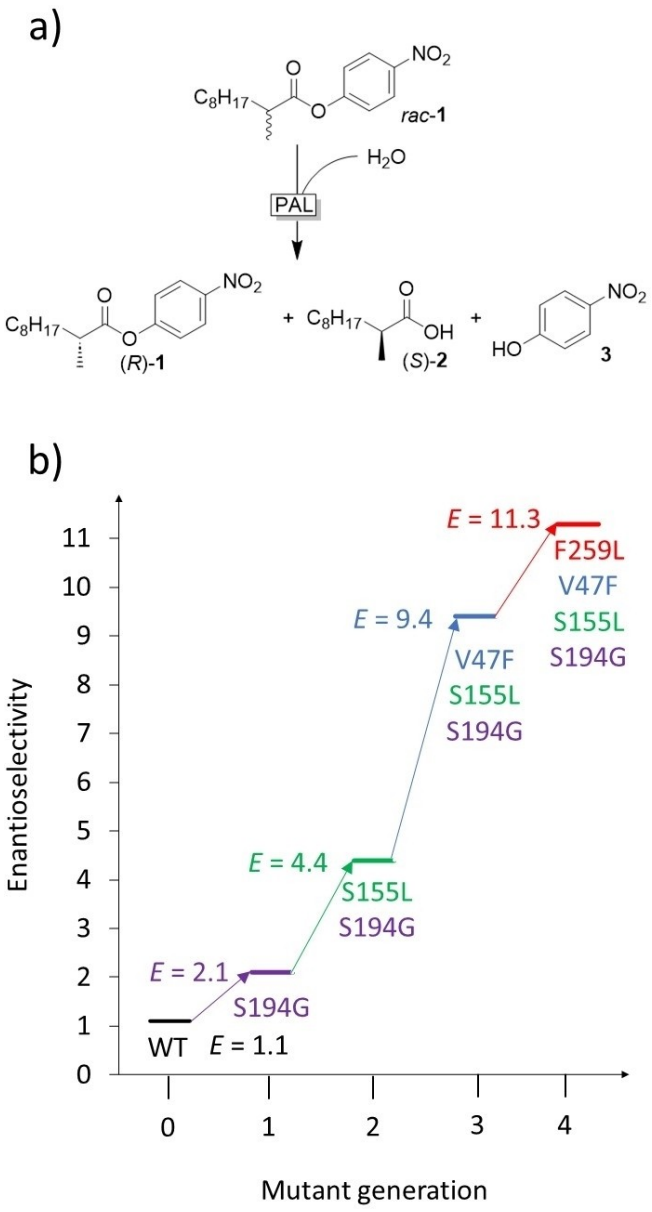

Figure 2. Directed evolution of *Pseudomonas aeruginosa* lipase (PAL) for increasing enantioselectivity in the hydrolytic kinetic resolution of a racemic ester.[24] a) Reaction Scheme b) Result of the 4-Step epPCR-based process, each involving a single amino acid exchange.

second step involving mutations Met16Ala/Leu17Phe is all that is needed for evolving a stereoselectivity of $E>500$, the first small step not really being necessary. However, such a conclusion is untenable, as shown by partial deconvolution: Double mutant Met16Ala/Leu17Phe alone hardly induces any improvement relative to WT, the selectivity factor being only $E=2.6$ (S).[31] If additivity were to operate, the two mutational steps would result in $E=22$, not $E=594$. Therefore, the experimentally determined enormous cooperative non-additive mutational effect corresponds to about 2 kcal/mol, an eye-opening lesson regarding the importance of deconvolution. But how could this startling finding be understood on a molecular level?

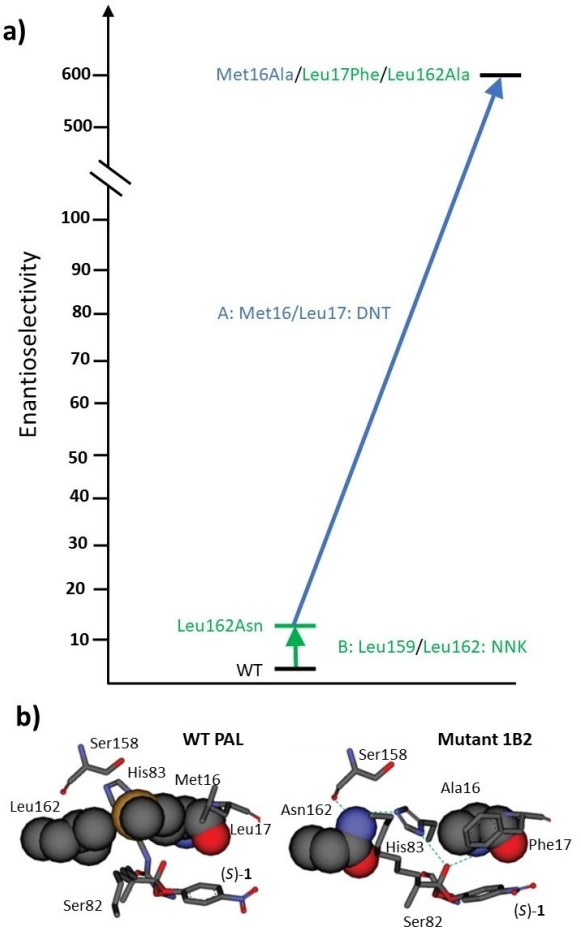

Figure 3. Drastic improvement of stereoselectivity in the kinetic resolution of *rac*-**1** catalyzed by the lipase PAL.[31] a) 2-Step CAST/ISM process. b) Theoretical interpretation of the origin of cooperative (synergistic) non-additivity. With permission from American Chemical Society.

Docking studies and MD simulations, aided by the X-ray structure of WT PAL, revealed the mechanistic origin of the unexpected phenomenon (Figure 3b). Due to space limitations, only part of this learning exercise is summarized here.[31] Substrates (*R*)- and (*S*)-**1** were separately computationally introduced in the PAL binding pocket as the respective oxyanions, covalently bound to catalytically active Ser82 (PAL's catalytic triad consists of Asp229/His251/Ser82). Figure 3b compares the binding of the preferred (*S*)-substrate bound in WT PAL with the triple mutant (Leu162Asn/Met16Ala/Leu17Phe). In WT PAL, the bulky side-chain of Leu162 clashes with the *n*-octyl moiety of the ester *(S)*-**1**, in contrast to the situation in the triple mutant in which the position of Asn162 provides sufficient space for the long alkyl chain of (*S*)-**1**. As also revealed by the MD computations, Asn162 forms a hydrogen bond to Ser158, thereby positioning the asparagine sidechain further away from the bound substrate, thus avoiding steric clashes and enhancing activity. At the same time, the substitution Met16→Ala16 provides more space in a sterically congested part of WT PAL, which allows more sidechain flexibility of His83. The new pose enables additional stabilization of the oxyanion by way of H-bond formation. Finally, the mutation Leu17Phe enables π-stacking between the phenyl sidechain of phenylalanine and the *p*-nitrophenyl moiety of the ester substrate, which is not possible in WT PAL (Figure 3b). Mutation Leu162Asn acting alone exerts little influence, nor does Met16Ala/Leu17Phe by itself, but in concert the three mutations cause drastic cooperative non-additivity. This model, made possible by partial deconvolution, explains the observed high (*S*)-selectivity.[31] Complete deconvolution was not performed, but could probably provide further insight.

Mechanistically related to lipases are esterases, for which cooperative non-additive mutational effects were also found.[32,33] In an impressive study with the aim to invert enantioselectivity of an esterase in the reaction of acetylated tertiary alcohols, the evolved double mutant was deconvoluted with formation of the respective single mutants, one showing (*R*)-selectivity, the other (*S*)-selectivity.[32] In another notable directed evolution study, an efficient dual-channel microfluidic droplet screening system was applied in the stereoselective ester-catalyzed formation of (*R*)- as well as (*S*)-ibuprofen esters and derivatives.[33] Although systematic deconvolution was not strived for in this study, the cooperative non-additive interaction of mutations and sets of mutations was reported. In contrast, mathematically additive mutational effects were believed by some protein engineers at the time to be prevalent.[18c–k,34–36] The question why biopolymers (proteins) "have statistical additivities" was addressed early on and linked to Fuji-type fitness landscapes.[18j]

3. Complete Deconvolution of a Quadruple Mutant Baeyer–Villiger Monooxygenase Variant (Case Study 2)

Baeyer–Villiger (BV) oxidations of ketones using a variety of man-made reagents or catalysts or alternatively evolved Baeyer–Villiger monooxygenases (BWMOs) have been used for a long time to transform ketones into esters or lactones,[37] and enantioselective sulfoxidation of prochiral thioethers is also possible using these enzymes. Here we focus on a single study in which complete deconvolution followed by the experimental construction of a fitness pathway landscape was reported.[38] The WT BVMO, dubbed phenylacetone monooxygenase (PAMO), was known to oxidize prochiral methyl tolyl thioether (**4**) with preferential formation of sulfoxide (*S*)-**5** (ee = 90 %), the goal of the study being complete inversion of enantioselectivity in favor of (*R*)-**5** (Figure 4a).[38] First, the efficacy of CAST/ISM was demonstrated by rationally designing a 2-step process, featuring two focused mutagenesis sites A and B, each having two residues. In the first step, double mutant I67Q/P440F appeared with 73 % ee in favor of the (*R*)-product, followed by the second step leading to quadruple mutant I67Q/P440F/A442N/L443I with 95 % ee(*R*). Complete deconvolution revealed extraordinarily large coopera-

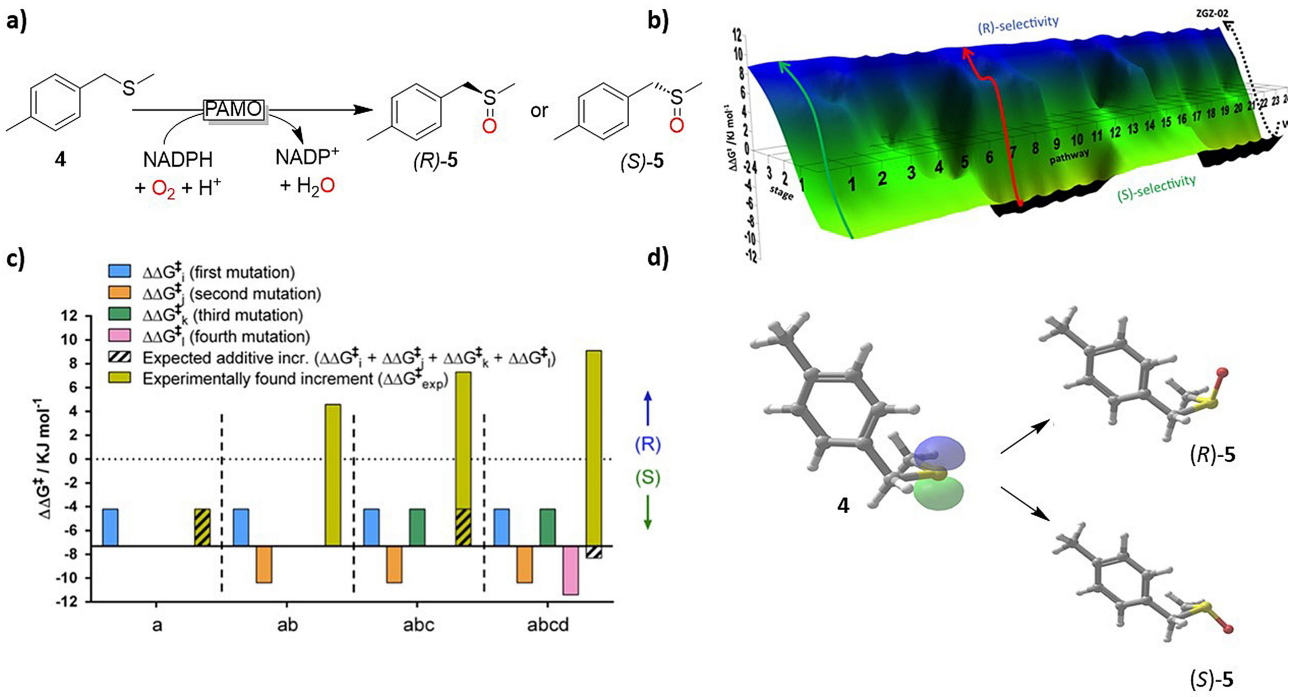

Figure 4. Directed evolution of the Baeyer–Villiger monooxygenase PAMO as the catalyst in the successful quest to invert the sulfoxidation enantioselectivity of a prochiral thioether.[38] a) Reaction of thioether **4** as prochiral substrate. b) Fitness pathway landscape based on mutational data derived from complete deconvolution of the best (*R*)-selective PAMO mutant I67Q/P440F/A442N/L443I. c) Typical deconvolution data showing the effects of the four single mutations. d) Model indicating which lone-electron pair (blue or green) is closest to the active PAMO oxidant FAD-OOH, therefore leading to the (*R*)- or the (*S*)-sulfoxide, respectively. With permission from American Chemical Society.

tive mutational effects (Figure 4b).[38] Only part of the data is highlighted here. For example, all four single mutants derived from the quadruple mutant I67Q/P440F/A442N/L443I proved to be (*S*)-selective! This means that the combination of four (*S*)-selective mutations in concert results in complete reversal of enantioselectivity in favor of (*R*)-**5** (Figure 4a–c), a discovery that theoreticians using the most advanced computational methods would not have predicted:

$$\text{Mutation 1 } (S) + \text{mutation 2 } (S) + \text{mutation 3 } (S) + \text{mutation 4 } (S) \rightarrow \text{combined four mutations } (R)$$

From the respective data and the ee-values of all double and triple combinations, a complete fitness pathway landscape was generated experimentally, in which 4! = 24 pathways lead from WT PAMO to one and the same final (*R*)-selective quadruple mutant (Figure 4a–c). A typical upward climb to (*R*)-selectivity without a local minimum is shown by the green arrow, and one involving a small local minimum is indicated by the red arrow (Figure 4b). The latter is indicative of an antagonistic mutational effect, which is over-compensated in the subsequent step. All 24 pathways in a rugged landscape were analyzed, shedding light on the origin of enantioselectivity at each phase on a molecular level.[38] In an earlier QM/MM study of the related cyclohexanone monooxygenase (CHMO), it was concluded that FAD-OOH is the catalytically active species in stereoselective Baeyer–Villiger reactions.[39] In the sulfoxidation study using PAMO, the same catalytically active species was postulated.[38] A reasonable model based on induced docking calculations was proposed, according to which the closest of the two lone electron pairs of the sulfur atom to FAD-OOH is the one that is oxidized preferentially to the respective sulfoxide. As shown in Figure 4d, oxidation at the blue labeled lone electron pair ensures reversed (*R*)-selectivity, while oxidation at the green labeled lone electron pair leads to (*S*)-selectivity (WT). In conclusion, this deconvolution study demonstrates the existence of a rugged fitness landscape.

4. Complete Deconvolution of a Quintuple Cyclohexyl Amine Oxidase Variant (Case Study 3)

The mechanistic insight gained by complete deconvolution[13,38] was also reported in a directed evolution study of a newly discovered cyclohexyl amine oxidase, dubbed $CHAO_{CCH12\text{-}C2}$, as the catalyst in the asymmetric synthesis of the pharmaceutically significant chiral amine (*S*)-**6** from *rac*-**6** (Figure 5a).[40] Deracemization was achieved by the Turner technique.[41] Upon applying rationally designed CAST/ISM followed by epPCR, a quintuple mutant H68Q/E198G/L200V/I201L/V209S was evolved which showed a 15-fold activity increase relative to WT and >90 % ee in favor of the desired (*S*)-product.[40] Complete deconvolution of the quintuple mutant revealed notable

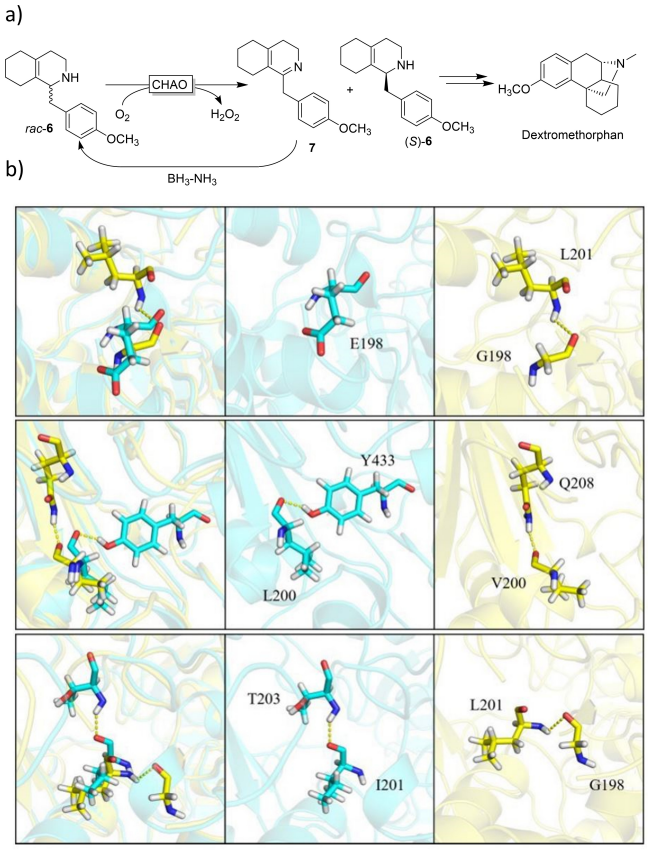

Figure 5. Directed evolution of a cyclohexyl amine oxidase (CHAO) as the catalyst in Turner-type deracemization of a racemic compound with formation of a pharmaceutically significant product.[40] a) Model reaction rac-6→(*S*)-7 enabling the chemoenzymatic production of dextrometherphan. b) Part of the theoretical analysis of the reason for the reshaped binding pocket of the CHAO leading to 97% ee (*S*), is due to H-bonding interactions. With permission from John Wiley and Sons.

cooperative non-additive effects, although a fitness pathway landscape was not generated (which should be possible). For example, the specific activities of single mutant L200V and quadruple mutant H68Q/E198G/I201L/V209S in the formation of (*S*)-**6** amount to 0.191 U/mg and 0.369 U/mg, respectively.[40] If additivity were to pertain, then the final quintuple mutant H68Q/E198G/L200V/I201L/V209S should have a specific activity of only 0.56 U/mg, yet in reality it proved to be twice as high (1.11 U/mg). Similar cooperative effects were demonstrated when comparing the activities of single mutant V209S, double mutant L200V/I201L and triple mutant L200V/I202L/V209S. The theoretical section of this study explains the origin of enhanced enantioselectivity evolved by CAST/ISM.[40] It was discovered that the binding pocket of $CHAO_{CCH12-C2}$ has been significantly reshaped, caused by novel H-bond effects (Figure 5b). On the practical side, application of the Turner deracemization method enabled the gram-scale production of (*S*)-**6**, the precursor of dextromethorphan.[40]

5. Complete Deconvolution of a Cytochrome P450 Triple Mutant and Generation of a Fitness-Pathway Landscape which Exposes Multiple Catalytic Properties (Case Study 4)

To date, multidimensional fitness landscapes, irrespective of the enzyme type, involve the description of a single enzyme trait such as activity, stereoselectivity or thermostability.[1–5,13] This also applies to protein engineering of cytochrome P450 monooxygenases,[42] although a study based on FRISM[1c,22] has elegantly shown that regioselectivity can be evolved at maintained stereoselectivity, accompanied by non-additive mutational effects.[10] Here, we highlight the first case of the construction of a multiparametric fitness landscape, enabled by complete deconvolution of a multi-mutational P450 monooxygenase variant.[43] Three catalytic parameters are involved, namely activity, regioselectivity and diastereoselectivity. It concerns an evolved 2ß-selective triple mutant R47I/T49I/F87 A of P450BM3 monooxygenase as the catalyst in the regio- and diastereoselective hydroxylation of testosterone (**8**) with high activity[43] (Figure 6a). The persisting phenomenon of catalytic tradeoffs[21e,g] became visible in a unique manner, enabled by complete deconvolution of the triple mutant. Moreover, the study identifies the best practical „compromise“ and interprets it by means of QM/MM computations. In this way, cooperative and antagonistic non-additive mutational effects were shown to be modulated by long-range interactions in loops, helices and ß-strands which control the substrate access tunnel.[43] The fitness pathway landscape with 3!=6 upward trajectories for the desired selective formation of 2ß-hydroxy-testosterone (**9**) is shown in Figure 6b. Energetically favored and less favored pathways are marked in green and red, respectively, which illustrates the rugged character of this landscape (Figure 6b, right). The fascinating conformational dynamics of a P450 monooxygenase, that were identified in this study, had not been considered previously in the P450 literature,[42] which adds to the important lessons learned in this area of directed evolution (Figure 6c–f). In order to understand the link between non-additive (epistatic) mutations and conformational dynamics more closely, the so-called Shortest Path Map analysis (SPM) analysis together with the results of MD computations was also performed.[43] A more detailed discussion of the explanations for the non-additive mutational effects can be found in the original paper.[43] Suffice it to say that the multiparametric fitness landscape highlighted here has provided novel insights which can be of use in future P450-based directed evolution in which steroids serve as substrates. Finally, this study notes that non-additive mutational effects and epistatic mutational effects basically mean the same phenomenon.[43]

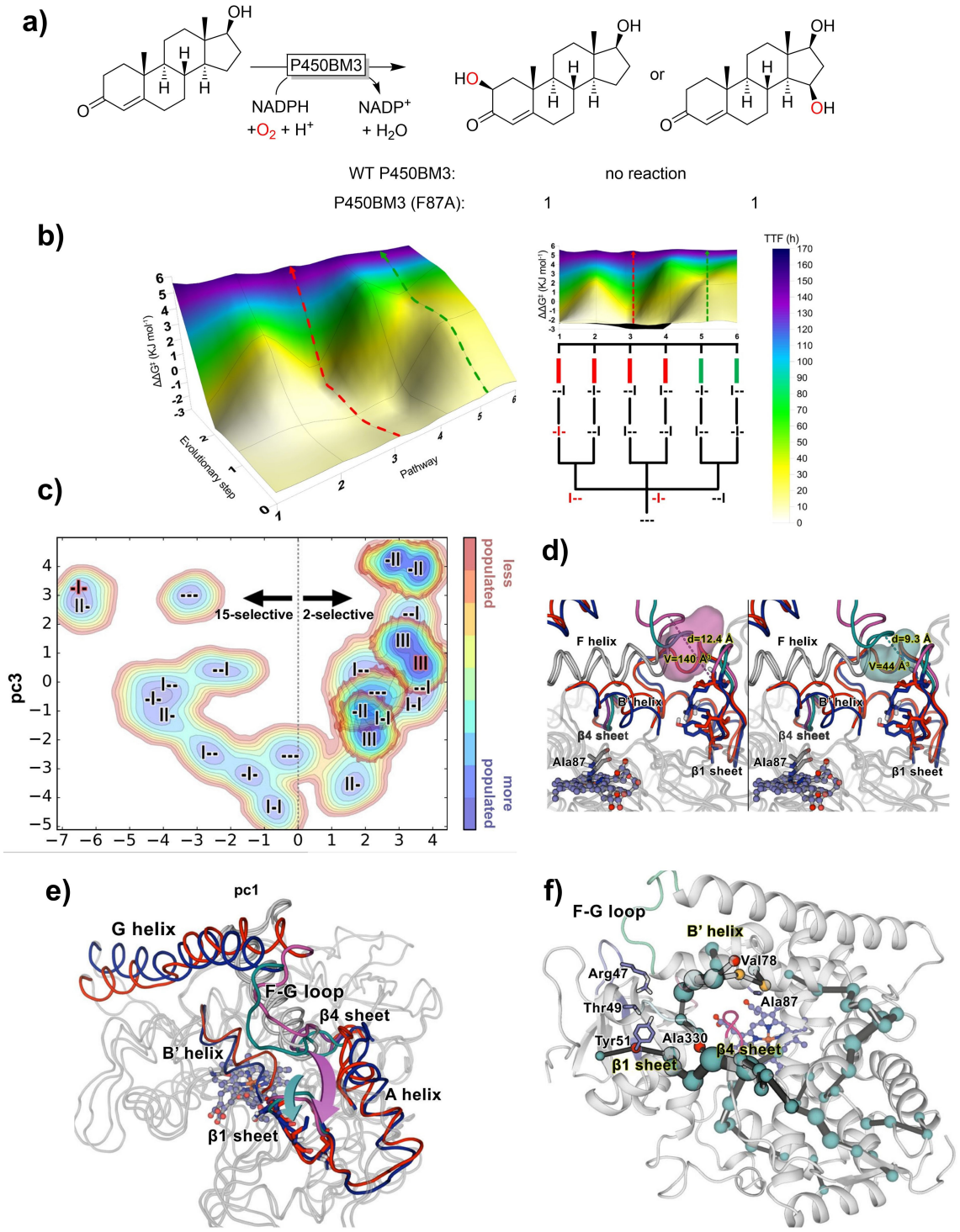

Figure 6. P450BM3 catalyzed oxidative hydroxylation of testosterone (**8**) as the model reaction in a study featuring complete mutational deconvolution with construction of a fitness pathway landscape characterized by three catalytic traits, namely activity, regioselectivity and diastereoselectivity.[43] a) Model reaction of testosterone (**8**). b) Multiparameter fitness pathway landscape of the 3!=6 evolutionary pathways leading selectively from the known unselective parent mutant F87A upward to the final product 2ß-hydroxy-testosterone (**9**), shown in 3D (left) and frontal (right) format (green and red arrows indicate examples of favorable and less favorable pathways, respectively, the latter with a local minimum. c) Analysis of the conformational dynamics of the deconvoluted mutants. Top left: Conformational analysis; top right: Conformational populations of 2ß-selective mutants; d): Overlay of conformational changes; e): Replicas of 15ß selective mutants; f) Essential residues for necessary mutagenesis. Reproduced from ref [43] (CC BY).

6. Partial Deconvolution of a Collection of Lipase Mutants Evolved for Achieving Chemoselectivity in the Reaction of Fatty Acid sters of Different Chain Length (Case Study 5)

Another impressive example of lessons learned by deconvolution of multi-mutational enzyme variants concerns the lipase CalA.[44] This lipase that has been used less often than the famous CalB, but studies utilizing directed evolution based on focused saturation mutagenesis at sites surrounding the CalA binding pocket for enantioselectivity[45] and chemoselectivity of substrates as a function of chain length[46] in reactions of fatty acid esters had been reported earlier. In the new study,[44] highlighted here, amino acid exchange events at the substrate entrance tunnel as well as selected remote mutational sites were examined using region-focused saturation mutagenesis and fatty acid *p*-nitrophenylesters as substrates. This rational protein engineering approach provided a set of selective multi-mutational variants favoring differently sized ester substrates. Partial deconvolution of the most important mutants showing substantial chain length selectivity of fatty acid esters revealed notable synergistic, non-additive mutational effects[44] (Figure 7). Traditionally, this lesson would not have been expected, and again calls for mechanistic explanations. Quadruple variant G237C/L289M/P333S/A369T (SS_12) favoring short-chain esters is a typical example (Figure 7a–b). It was shown that the mutant exhibits a 2-fold activity increase in the hydrolysis of *p*-nitrophenyl butyrate relative to WT CalA, while the C12 and C16 fatty acid esters show no activity (Figure 7b). The authors noted that the point mutations operating in concert in the quadruple variant "alter the local protein morphology" by the creation of less favored binding branches that do not readily accept long-chain substrates.[44] The theoretical analysis of this study identifies the role of some residues remote from the active site causing notable dynamic conformational effects. Partial deconvolution of several other multi-mutational variants in the same study led to similar insights.[44] In this study, selected mutations were also combined, a technique previously applied in other directed evolution campaigns, which not only led to improved enzyme profiles, but sometimes to negative (deleterious) results.[46,47] These studies are related to the Smith/Fersht/Wells technique. Computations of transitions state energies would provide additional mechanistic lessons.[44,46,47]

7. Further Examples of Non-Additive Mutational Effects by Deconvolution or by Combining Sets of Mutations by the Smith/Fersht/Wells Technique

It is well known that combining positive mutations obtained by directed evolution may lead to the anticipated catalyst improvements, but unfortunately not always.[1–5,46,47] The detrimental role of antagonistic non-additive mutational effects is the reason for such disappointments.[46] In addition to the case studies highlighted above, we list here further typical recent publications describing the application of the method of deconvolution[13] and studies utilizing the Smith/Fersht/Wells technique for combining mutations[20] (Table 1). In some (but not all) cases, the underlying phenomena of non-additivity (epistasis) were exposed by QM/MM investigations. In a study reporting the directed evolution of an alcohol dehydrogenase (ADH) as the catalyst in particularly challenging enantioselective ketone reductions, it was demonstrated that the method of deconvolution not only provides mechanistic insights, but also constitutes a novel and efficient technique in protein engineering.[48] More examples are likely to follow. We believe that the deconvolution technique can also be applied in chemoenzymatic approaches aiming to gain access to bioactive natural products.[49a]

8. Summary and Perspectives

The traditional (early) view of enzymologists and biochemists that two or more genetically introduced mutations in an enzyme occur in an additive manner,[17,18] implying the absence of molecular interactions, has steadily changed during the past two decades. In directed evolution of stereoselective enzymes as catalyst in organic chemistry, such a change was originally sparked by performing partial or complete deconvolution of multi-mutational enzyme variants, which brought to light startling results and novel insights.[13,31,38,40,43,44] Parallel to these advances, some researchers joined efforts in applying partial or complete deconvolution, others exploited the original method of combining two or more mutations for gaining mechanistic information. The latter development likewise uncovered drastic non-additive mutational effects, both cooperative and antagonistic, in addition to additive mutational influences.[20]

We do not propose that traditional additive effects never occur, which continue to be observed. Indeed, the occurrence of additive mutational effects does not surprise. Whenever complete deconvolution has been performed, not only in directed evolution but also in evolutionary biology,[23] it is a simple matter to construct a fitness pathway landscape.[13] This enables insights, different from previous reports of fitness landscapes.[14] Little lab work is generally needed when performing complete deconvolution. For example, in the case of a 3-mutational enzyme variant, a mere 6 new mutants have to be formed by site-specific mutagenesis (3 singles, 3 doubles). Fitness pathway landscapes derived thereof unveil at all stages all cooperative and antagonistic mutational effects as well as additive influences. We note once more that these fitness landscapes are based on *experimental* results, not on computations. QM/MM calculations may follow in order to explain the source of non-additivity. When deconvoluting a 4-mutational mutant, only 14 new mutants have to be made (4 singles, 6 doubles, 4 triples).

Hopefully, the advances highlighted in the present review will also serve as an inspiration for future efforts in

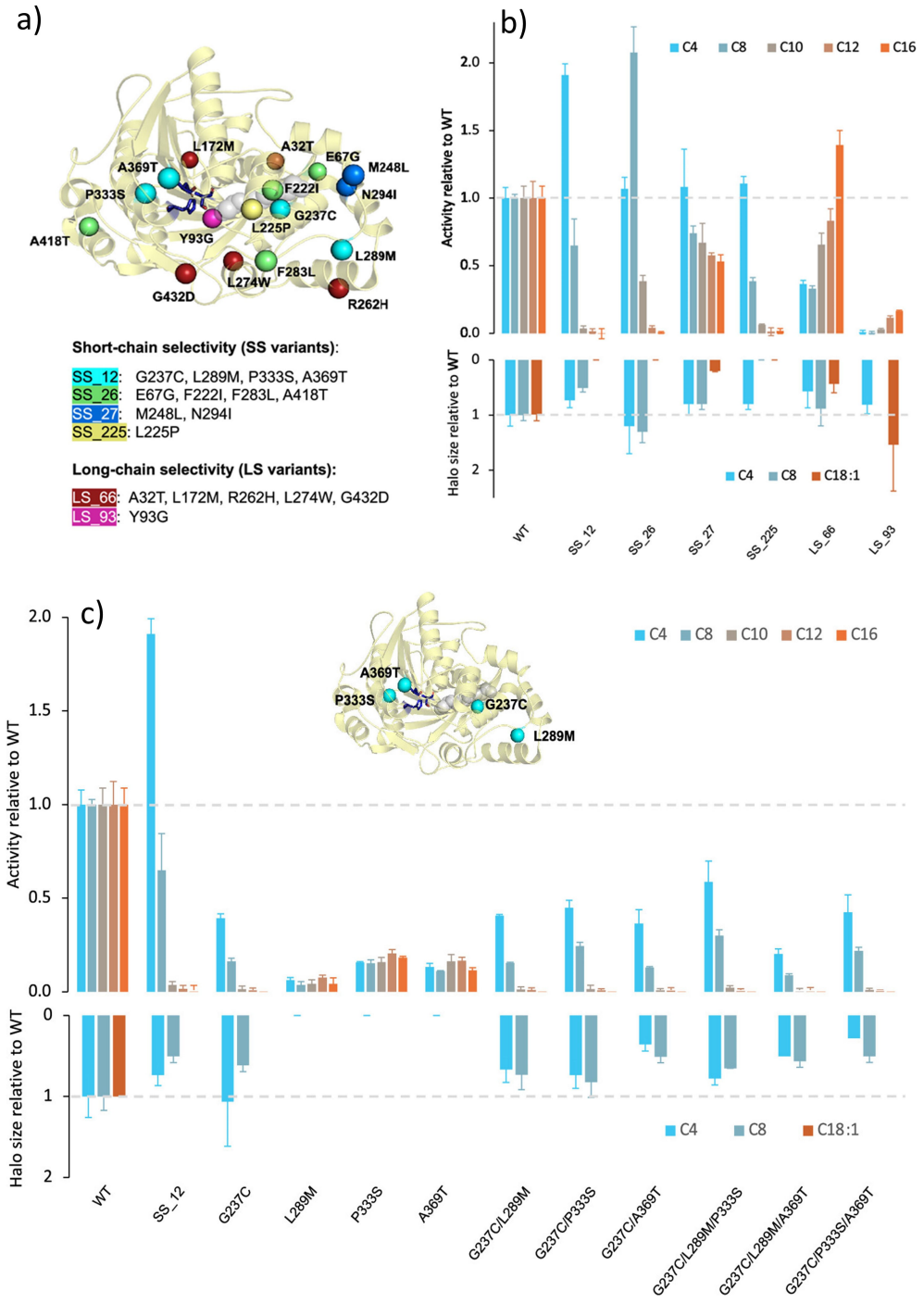

Figure 7. Short- and long-chain selective mutants of Cal-A as the enzyme in the hydrolysis of fatty acid esters.[44] a) Structure of Cal-A (PDB ID: 2VEO with the catalytic triad in purple sticks and key substitutions represented as spheres; point mutations in variants SS_12 (cyan), SS_26 (green) SS_27 (blue), SS_225 (yellow), LS_66 (red), and LS_93 (magenta). A PEG-4 molecule cocrystallized inside the acyl-chain binding tunnel is represented in light gray spheres. b) Hydrolytic activity of chain-length selective variants. Top panel: In vitro assay using *p*-nitro-phenyl esters. Bottom panel: In vivo assays with triglycerides. c) Nearly complete deconvolution of quadruple variant G237C/L289M/P333S/A369T (SS_12). Reproduced from ref [44] (CC BY-NC-ND 4.0).

this exciting research area. The emphasis is on stereo- and regioselectivity as well as activity, but a number of studies cited in Table 1 concern deconvolution of protein mutants having other kinds of important properties such as chemoselectivity, thermostability, binding affinity, sensor properties, pathogenic mutational effects, polymer degradation properties, and resistance to detrimental enzyme aggregation. Protein engineers focusing on artificial intelligence (AI)[109] will realize that their algorithms will greatly profit

Table 1: Further examples of deconvolution of multi-mutational variants obtained by directed evolution for improving catalytic enzyme profiles,[13] and further cases of the Smith/Fersht/Wells technique[17] of combining two or more mutations for mechanistic reasons, non-additivity (epistasis) often prevailing.

Deconvolution type	Enzyme type	Comment	Ref
Partial	Lipase	See Case Study 1	[31]
Partial	Lipase	See Case Study 5	[44]
Partial	Lipase	Activity & stability	[50]
Complete	Esterase	Stereoselectivity	[32]
Partial	Esterase	(*R*)- and (*S*)-Iboprufen	[33]
Partial	Penicillin acylase	Cephradine synthesis	[51]
Complete	Zearalone lactonase	Activity	[52]
Partial	Nitrilase	Activity, triple & hexamutational variants	[53]
Partial	Nitrile hydratase	Activity & thermostability	[54]
Partial	Epoxide hydrolase	Stereoselectivity, enantioconvergence	[55]
Partial	Epoxide hydrolase	Resistance to undesired aggregation	[56]
Partial	Epoxide hydrolase	Enantioselectivity, machine learning	[57]
Partial	Cellulase	Organic solvent resistance, bioethanol	[58]
Partial	Glucosidase	Thermostability, butyl glucoside production	[49]
Patial	α-KG-dependent halogenase	Regio- and stereoselectivity	[59, 60]
Complete	BV-monooxygenase	See Case Study 2	[38]
Complete	BV-monooxygenase	Regioselectivity, chiral Criegee intermediate	[61]
Partial	BV-monooxygenase	Regioselectivity	[62]
Complete	P450 monooxygenase	See Case Study 4	[43]
Partial	P450 monooxygenase	Promiscuous olefin to ketone reaction	[4c]
Partial	P450 monooxygenase	Activity & electron coupling	[63]
Partial	P450 monooxygenase	Regio- & stereoselectivity, natural products	[64]
Partial	P450 monooxygenase	Sulfoxidation, reversed enantioselectivity	[65]
Complete	P450 monooxygenase	Activity & protein expression, taxol derivative	[66]
Partial	P450 peroxygenase	Regio- and stereoselectivity, fatty acid hydroxylation	[67]
Partial	Peroxygenase	Regioselectivity	[68]
Partial	Peroxygenase	Enantioselectivity	[69]
Complete	Cyclohexyl amine oxidase	See Case Study 3	[40]
Partial	Eugenol oxidase	Activity, Selectivity	[70]
Partial	Monoamine oxidase	Enantioselectivity & activity, (*R*)-Praziquantel	[71]
Partial	Phenylalanine ammonia lyase	Bulky phenylalanine analog synthesis / Enantioselectivity	[72]
Partial	Styrene hydratase	Dream reaction, olefins to chiral alcohols	[73]
Partial	Alcohol dehydrogenase	Activity, Thermostability, Enantioselectivity, Substrate Scope	[74]
Complete	Alcohol dehydrogenase	Stereoselectivity, substrate inhibition relief	[75]
Partial & Complete	Alcohol dehydrogenase	Enantioselectivity	[76, 77]
Partial & Complete	Formate dehydrogenase	Cofactor specificity	[78]
Partial	Leucine dehydrogenase	Activity, substrate scope	[79]
Complete	Dihydrofolate reductase	Promoter activity	[80]
Complete	Dihydrofolate reductase	Stability, mutational trade-offs	[81]
Complete	Amine dehydrogenase	Activity, consensus sequencing	[82]
Partial	Ene reductase	Photocatalytic stereoselective alkylation of nitroalkanes	[83]
Partial	Ene reductase	Photocatalytic stereoselective alkene carbohydroxylation	[84]
Partial	Imine reductase	Activity & stereoselectivity	[85]
Partial	Uronate dehydrogenase	Thermostability & activity, D-glucaric acid production	[86]
Partial	Yeast cytosine deaminase	Greater sensitization in *E. coli*/HT1080 cells	[87]
Partiall	L-Threonine aldolase	Diastereo- & Enantioselectivity, Prelog's rule	[88]
Patial	Transketolase	Substrate scope and activity, chiral α-hydroxy ketones	[89]
Partial	Terpene cyclase	Activity and stereoselectivity, cationic cascades	[90]
Partial	Artificial metalloenzyme Sav	Activity and stereoselectivity	[91]
Partial	Purine nucleoside phosphorylase	Activity, 2-halogneated-2′-deoxyadenosines accessed	[92]
Complete	ω-Transaminase	Thermostability and enantioselectivity, machine learning	[93]
Partial & complete	Amine transaminase	Thermostability, B-factor guidance, proline rule	[94]
Partial	Acyltransferase LovD	Activity & stability, simvastatin	[95]
Partial	D-Allulose epimerase	Thermostability, D-allulose bioproduction	[96]
Partial	L-Lysine decarboxylase	Activity, cadaverine production, computation-guided screening	[97]
Partial	α-Keto acid decarboxylase	1,2,4-Butanetriol production from D-xylose	[98]
Partial	Polyphosphate kinase	Adenosine-5'-triphosphate regeneration	[99]
Partial	Methyl transferase	Non-natural terpenoids accessible by evolved mutants	[100]
Partial	O-Methyltransferase	Regioselectivity, Hesperetin dihydrochalcone synthesis	[101]
Complete	Pullunanase	Activity, pH profile	[102]
Complete	Trioephosphate isomerase	Mechanism, activity shutdown	[103]

Table 1: (Continued)

Deconvolution type	Enzyme type	Comment	Ref
Complete	T4-Bacteriophage lysozyme	Thermostability, amino acid network, non-additivity analyzed	[104]
Complete	Barley chymotrypsin inhibitor	Stability, conformational effects	[105]
Complete	Virus-like particles	Enhanced cell uptake properties	[106]
Partial	Corona spike protein	Stability & expression	[107]
Partial	Artificial antimicrobial peptides	Biological activity	[108]
Double mutant cycle technique:			
Double	Green fluorescent protein	Activity; local fitness landscape	[20a]
Double	Review with different proteins	Folding, binding allostery; caveats of double mutant cycle	[20b]
Double	Review with different proteins	New directions in double mutant cycles	[20c]
Double	Proteins in bioelectrical signalling	Toxin-receptor binding model; anti-pain drugs	[20d]
Double	Review with different proteins	High dimensional mutant cycles, molecular switches; allostery	[20e]
Double	Colicin E9 endonuclease	Mass spectrometry-based simplification of double mutant cycles; gas phase	[20f]
Double	Different proteins in cell lysates	MS-based analysis; intermolecular H-bonds identified, *E. coli* extracts	[20g]
Double	Super acid dehydrase	Activity; economic factors, DHIV to KIV conversion	[20h]
Double & Triple	ATP-binding transporter	Binding affinity, positive entropy coupling	[20i]
Double	Kainate receptor	Brain diseases	[20k]
Double	Rieske oxygenase	9,10-Secosteroids accessible	[20l]
Double	Dehaloperoxidase	Chiral cyclopropyl esters	[20j]

from the huge high-quality data which complete deconvolution of multi-mutational enzyme variants provides.

Scientists new to the concept of deconvolution of multi-mutational variants will discover that by performing this simple technique, unique mechanistic lessons can be learned. Crystal structures, MD/QM computations and kinetic investigations should accompany such studies. We expect that chemists and biotechnologists working on the sustainable production of pharmaceuticals and other high-value products by directed evolution of stereo- and regioselective enzymes will profit from such efforts.[1a] Rather than performing site-specific mutagenesis themselves in the lab for accessing the deconvolutants, they can obtain them from companies specializing in massive high-fidelity DNA synthesis on micro-chips, which can then be expressed in the home labs. This leaves industrial and academic protein engineers more time to rationally design mutant libraries.[110] We also anticipate that scientists working in the areas of double mutant cycles for gaining mechanistic information and those active in evolutionary biology will profit from the data and conclusions of studies focused on deconvolution of multi-mutational variants as catalysts in organic chemistry. This also pertains to antibody-based biotherapeutics.[111] Another relevant field in which potentially much can be learned from the deconvolution technique concerns enzymatic and chemoenzymatic cascade processes.[4,112]

It is important to point out once more that the fitness pathway landscapes enabled by complete deconvolution involve evolutionary trajectories that connect the starting enzyme (usually WT) with the final best mutant previously obtained by a directed evolution technique such as ISM. Therefore, it constitutes a so-called "constrained" system, in contrast to a so-called "unconstrained" platform, in which all theoretically possible ISM upward climbs have actually been experimentally tested at all phases. As far as we know, only a single study has been devoted to this issue, which also addresses the question which order of visiting saturation mutagenesis sites (e.g., A, B, C, D) is optimal.[113] Using an epoxide hydrolase as the biocatalyst and a racemic epoxide as the substrate in hydrolytic kinetic resolution, it was clearly demonstrated that all $4! = 24$ pathways reveal good to excellent stereoselectivity in the range of $E = 28$–160, some better than others, and all of them showing non-additive mutational effects. With today's improved versions of CAST/ISM,[1,22] the respective guidelines outlined therein ensure that mainly prolific pathways will be identified. This also applies to Focused Rational Iterative Site-specific Mutagenesis (FRISM).[22]

We hope that this review will effectively inspire the active members of these different disciplines to consider each other's contributions so that a more unified overall picture will emerge in the near future.

Finally, a different, yet pressing, question concerns the Corona pandemic and the continuous occurrence of new mutants which threaten human health.[107,114,115] Understanding how they are formed in spike-proteins calls for more research. Are they formed sequentially as point mutations one by one, or does the simultaneous formation of several mutations occur, these possibly interacting in a cooperative non-additive manner?

Following the submission of this manuscript, many intriguing examples of partial or complete deconvolution of multi-mutational variants using various types of enzymes have appeared. Only a few selected studies are cited here.[116]

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

Keywords: directed evolution · non-additive mutations · stereoselectivity · double mutant cycle · cooperative mutational effects

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Review

Directed Evolution

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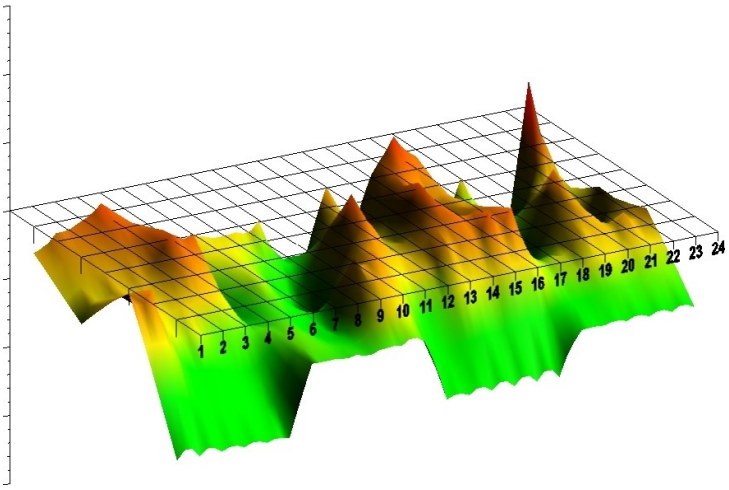
Learning from Protein Engineering by Deconvolution of Multi-Mutational Variants



First derivative of a typical experimental enzyme fitness pathway landscape mapped on the basis of ΔG values, in this case of 24 trajectories in the deconvolution of a 4-mutational variant. Green areas indicate negative slopes and define trajectories involved in energetically favored stages, while red areas indicate positive slopes representing energetically disfavored pathways.