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Santner, Paul; Szabó, László Krisztián; Chanquia, Santiago Nahuel; Merrild, Aske Høj; Hollmann, Frank; Kara, Selin; Eser, Bekir Engin

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Optimization and Engineering of Fatty Acid Photodecarboxylase for Substrate Specificity

Paul Santner,[a] László Krisztián Szabó,[a] Santiago Nahuel Chanquia,[b] Aske Høj Merrild,[a] Frank Hollmann,[c] Selin Kara,*[b] and Bekir Engin Eser*[a]

Fatty acid photodecarboxylase (FAP) is one of the few photoenzymes in nature. The ability of FAP to convert fatty acids into alka(e)nes without the need for reducing equivalents put this enzyme into spotlight for biocatalytic applications. Although it has been discovered only a few years ago, many studies already emerged demonstrating its potential in areas from biofuel production and enzymatic kinetic resolution to being a critical component of multi-enzyme cascades. While there have been few protein engineering studies for modulating activity of FAP towards very short chain fatty acids, no study has yet addressed substrate selectivity within the medium to long chain fatty acid range, where FAP shows great promise for the synthesis of drop-in biofuels from ubiquitous fatty acids with chain lengths from C12 to C18. Here, after determining optimum expression and assay conditions for FAP, we screened 22 rationally designed mutant enzymes towards four naturally abundant fatty acid substrates; C12:0, C16:0, C18:0 and C18:1. Depending on the type of the exchanged amino acid, we observed selectivity shifts towards shorter or longer chains, compared to wild type enzyme. Notably, we obtained two groups of mutants; one group with high selectivity towards only C18:0, and another group that is selective towards C12:0 substrate. Moreover, we measured light and thermal stability of the wild type enzyme as well as the light stability of a mutant engineered for selectivity.

Introduction

Photoenzymes, which require continuous supply of light to catalyze chemical transformations, are rare in nature.[1] Until a few years ago, only three photoenzymes were known, for which continuous and strict dependency on light has been well established; photolyases, protochlorophyllide oxidoreductases and photosystems.[1b] In 2017, the observation of the formation of alka(e)nes in some microalgae species led to the discovery of a new photoenzyme, named fatty acid photodecarboxylase (FAP).[2] This enzyme catalyzes decarboxylation of fatty acids through a flavin adenine dinucleotide (FAD) dependent radical-based mechanism to generate alkanes with one carbon fewer (Scheme 1).[3] Thus, in addition to being a rare photoenzyme, FAP is a promising biocatalyst for the environment-friendly generation of hydrocarbon biofuels, i.e. drop-in biofuels from naturally abundant fatty acids.[1b]

FAP uses blue light for the excitation of its oxidized FAD cofactor, which abstracts an electron from the fatty acid substrate to initiate catalysis. The resulting substrate radical decarboxylates to form an alkyl radical. In the final stage of the catalytic cycle, FAD transfers an electron back to the alkyl radical and a proton transfer renders the alkane product.[2,3] In addition to being one of the few light-dependent enzymes, FAP is the first example of a flavin-dependent decarboxylase that catalyzes fatty acid decarboxylation.

[a] Dr. P. Santner, L. K. Szabó, A. H. Merrild, Dr. B. E. Eser
Enzyme Engineering Group
Department of Biological and Chemical Engineering
Faculty of Technical Sciences
Aarhus University
Gustav Wieds Vej 10
DK 8000, Aarhus (Denmark)
E-mail: bekireser@bce.au.dk
[b] Dr. S. N. Chanquia, Prof. S. Kara
Biocatalysis and Bioprocessing Group
Department of Biological and Chemical Engineering
Faculty of Technical Sciences
Aarhus University
Gustav Wieds Vej 10
DK 8000, Aarhus (Denmark)
E-mail: selin.kara@bce.au.dk
[c] Prof. F. Hollmann
Department of Biotechnology
Delft University of Technology
Van der Maasweg 9
2629HZ, Delft (The Netherlands)

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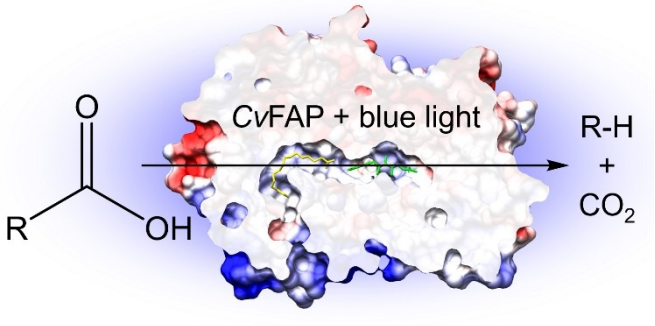

Scheme 1. Representative reaction of *Cv*FAP. A cross section of the structure of FAP is shown to view its substrate tunnel. Under blue light, the enzyme with bound FAD (green) catalyzes the conversion of a fatty acid substrate, depicted here as hexadecanoic acid (C16:0) in yellow, to its alkane counterpart via the removal of CO_2. PDB ID for the *Cv*FAP structure is 5NCC.

In the recent decade, a number of other fatty acid decarboxylases have been extensively studied for sustainable and green drop-in biofuel production.[4] These O_2-dependent enzymes require a continuous supply of reducing equivalents and generally suffer from the inefficiency of electron transfer systems, thus making it challenging to establish a feasible biocatalytic process.[4a] However, the reaction of FAP does not require O_2 and is redox-neutral. The FAD cofactor in FAP does not need any external electrons for regeneration and the enzyme can exhibit total turnover numbers of up to 9000.[5] In other words, FAP simply uses light to generate synthetically powerful alkyl radicals from fatty acids, which are ubiquitous in nature. Thus, FAP is a promising candidate for drop-in biofuel production. In the few years after its discovery, there have been various demonstrations of the catalytic utility of FAP in different synthetic chemistry applications, from biofuel production to enzymatic kinetic resolution.[6]

Recent studies demonstrated that FAP is a promiscuous enzyme and can accept a wide variety of carboxylic acid substrates with different chain-lengths from C5 to C18, where decoy molecule approach further increased efficiency.[6e] However, the enzyme exhibits highest activity towards hexadecanoic acid (C16:0; palmitic acid) and heptadecanoic acid (C17:0), while lower activity levels were observed for longer or shorter chain fatty acids.[2,6a,e] For example, activity towards dodecanoic acid (lauric acid; C12:0) is only about 10% of the activity towards palmitic acid.[2,6a] In addition, the wild type (WT) enzyme also has lower activity towards unsaturated fatty acids of oleic (C18:1) and linoleic acid (C18:2),[6a] which are the major components of vegetable oil and the oil produced by a range of microorganisms. Furthermore, FAP is not quite substrate specific and exhibits similar conversion levels for a number fatty acids with similar chain-lengths (C14–C18).[2] Robust *Cv*FAP variants with distinct selectivities towards shorter or longer chain fatty acids and with high activity are desired from a drop-in biofuel production point of view, since, depending on the chain-length of the resulting alkane, a hydrocarbon component for a different type of biofuel can be obtained, e.g. gasoline (C4–C12 alkanes), jet fuel (C8–C16) or diesel (C10–C22).[7] Although there have been a few enzyme engineering studies for shifting activity towards very short chain fatty acids,[6d,g] the specificity change by mutagenesis within C12–C18 range has been largely unexplored.

In this study, we generated 22 rationally designed mutants of FAP and evaluated them towards medium to long chain fatty acids (C12–C18). We analyzed the active site structure of the enzyme in order to target amino acids that are likely to affect stabilization of a medium or long chain fatty acid at the substrate binding site. As a result, we obtained tailor-made mutants that are more selective towards shorter or longer chain fatty acids. Moreover, some of our mutants exhibited distinct substrate selectivity towards a single type of fatty acid. Practical potential of a C18:0 selective mutant has been demonstrated with a preparative scale reaction comprising of a substrate mixture. We also addressed a few drawbacks of FAP including its instability under blue light exposure, low expression levels and the dependence of activity on assay conditions, in order to render more optimum conditions for protein expression and activity analysis. Lastly, we investigated light stability and thermal stability of the enzyme.

Results and Discussion

Protein expression optimization

In previous literature studies, various expression conditions were used with different gene constructs, leading to variable expression levels. In our study, we aimed at a systematic evaluation of expression conditions and their effect on protein expression levels as well as on enzymatic activity. Thus, we compared the effects of growth media (LB and TB), IPTG concentration (0.2, 0.5 and 1 mM) and ambient light exposure during expression. When comparing LB and TB culture media, TB media resulted in significantly higher expression levels with WT enzyme (Figure S2). Moreover, some of the mutants of our screening library, which were initially found to be inactive upon expression in LB (Table 2A, Figure S8A), were later obtained as active enzymes when expressed in TB media (Table 2B, Figure S8B). A direct comparison between the activities of LB and TB expressed *Cv*FAP on a selection of variants showed significantly higher activity for the TB media expressed mutants (Figure S7, Figure S8A). Notably, we observed that cell density (based on OD_{600}) did not increase with TB over LB, pointing towards higher active protein yield per cell with TB media. This might possibly be due to changes in cell physiology as a result of the metabolism of different media components, as described in the literature.[8] On the other hand, variation of IPTG concentration (0.2, 0.5 and 1 mM) did not affect expression significantly.

According to a previous study,[6c] the expression of soluble *Cv*FAP is negatively affected by high intensity light exposure. This has been attributed to photo-damage caused by FAD-mediated free radical generation in the absence of substrate, as confirmed in a more recent study by EPR spectroscopy.[9] Thus, we compared the expression levels of *Cv*FAP expressed in ambient light vs. darkness. Our results show that there is no detectable difference on the total protein expression levels (Figure S2). However, the activity of the whole cell catalyst protected from light during expression was higher than that exposed to ambient light for C16:0 and C18:1 substrates (Figure 1), indicating deactivation (due to photo-damage) of the protein to some extent, consistent with the earlier study.[6c] Substrate addition to protein expression medium, to avoid deactivation, was analyzed as well. Although addition of substrate did not result in a significant change of protein expression levels (Figure S3), a detectable improvement on enzyme activity was observed towards lauric acid and octadecanoic acid (stearic acid; C18:0) for WT enzyme and towards all substrates, except oleic acid, for Y466F mutant (Figure 1). In line with our results, a recent study showed that administration of medium-chain fatty acids protects purified *Cv*FAP against light inactivation.[10]

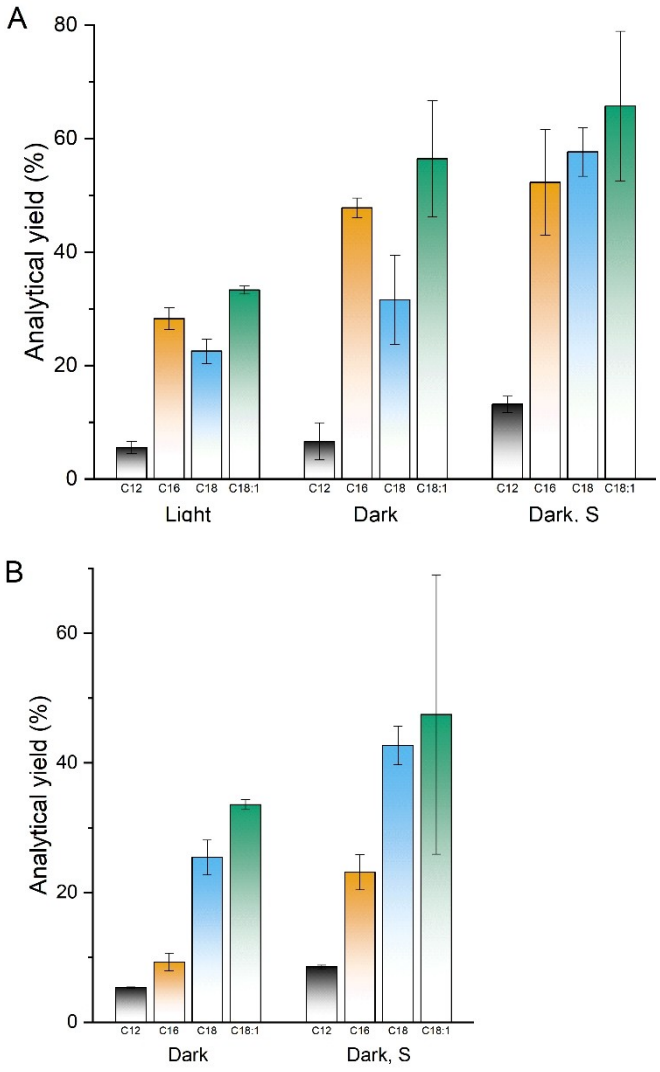

Figure 1. Analytical yield (%) towards target decarboxylation products measured at indicated protein expression conditions. Ambient light protection (Dark); and 50 μM C16:0 substrate addition (S) during LB protein expression were evaluated by assaying (A) WT and (B) Y466F mutant with the following conditions: 50 mg/mL whole cells with *Cv*FAP (LB media), 2 mM substrate, 15% ethanol, 100 mM Tris-HCl buffer pH 8.5, 30 °C, 20 h, 300 rpm stirring and 62 PPFD (Photosynthetic Photon Flux Density, μmol m^{-2} s^{-1}) light. Error bars indicate the standard error of at least duplicate experiments.

Assay optimization

In order to optimize the activity assay used in our mutant screening, we evaluated the effects of co-solvents as well as substrate concentrations. Because fatty acids are poorly soluble in water, a co-solvent is required to increase the availability of the substrate to the enzyme inside the reaction mixture. We evaluated DMSO and ethanol as co-solvents at different concentrations (Figure S5). Within the range of 10–20% (v/v) ethanol and 20–30% (v/v) DMSO, we observed similar activity levels. Since ethanol can be produced sustainably and the standard error of activities was lowest at 15% (v/v) concentration, we chose to use ethanol at this percentage for our assays in this study. We tested active variants derived from our initial screening with 20% (v/v) ethanol as co-solvent in the assays (Figure S8A), instead of 15% (v/v) (Figure S7) and found the resulting activities to be in good agreement.

For the initial activity assay, we used substrates at a concentration of 10 mM. However, only four out of 22 mutants showed activity at this concentration (Figure S8A). Decreasing substrate concentration from 10 mM to 2 mM allowed us to rescue the activity of another ten previously inactive mutants (Figure S8B). We attribute those results to substrate inhibition at high fatty acid concentrations.

Moreover, we monitored the activity of WT enzyme towards C16:0 and C18:0 fatty acids over time (Figure S6). The time-dependent activity pattern (progress curves) for both substrates were similar and the increase in activity leveled off at around 15–20 h. Thus, we performed our assays for a period of 20 h.

Selection of mutation sites

In order to select mutation sites that can render variants with different chain-length selectivity, we analyzed the substrate binding channel of the WT *Cv*FAP structure with C16:0 fatty acid bound (PDB ID: 5NCC), using UCSF Chimera molecular visualization software.[11] Based on structural analysis, we chose 22 mutant candidates by identifying residues close to the bound substrate, but far enough from the FAD cofactor, thereby focusing on modifying only the substrate channel and avoiding disruption of the catalytic activity. Changing the size and hydrophobicity of the residues at specific positions relative to the bound substrate, to accommodate and stabilize/destabilize longer or shorter chain fatty acids, was the main criteria in choosing mutant variants to generate. Such a strategy of modulating substrate specificity has been successfully demonstrated on various fatty acid utilizing enzymes in literature.[12] We also based the selection of some of our mutants on recent studies with FAP, where mutations were generally performed for other purposes than our aim here. The list of mutants and the purpose in their selection are given in Table 1. Mutations aimed at increased selectivity towards medium or long chain fatty acids based on their location with respect to the bound substrate in the channel of the structure (Figure 2) and on the physical property of the replaced amino acid (Table S2). Only for mutation Y466 our aim was to see the general effect since the substrate wraps around the tyrosine and it was also implicated to have a catalytic and/or FAD binding role.[2,3]

Screening of mutants towards varying chain-length fatty acid substrates

We screened our library of 22 rationally designed mutants towards four fatty acids of varying chain-lengths; C12:0, C16:0, C18:0 and (9*Z*)-octadec-9-enoic acid (oleic acid; C18:1), representing the naturally most abundant fatty acids. Substrates and products were analyzed by Gas Chromatography (Figure 3). All of the constructed variants showed visible expression as

Table 1. Mutant library designed and screened in this study.

*Cv*FAP variant	Van Der Waals volume change[a]	Hydrophobicity change[b]	Aimed effect[c]
A384K	68	−64	Medium-chain[6d,i]
Q486R	34	−4	Medium-chain[6i]
Q486Y	27	73	Medium-chain[6i]
G462I	76	99	Medium-chain[6d,g,i]
G462K	87	−23	Medium-chain[6d,g,i]
G462Y	93	63	Medium-chain[6d,g,i]
Y466A	−74	−22	General effect[2,3,6g,i]
Y466F	−6	37	General effect[2,3,6g,i]
Y466L	−17	34	General effect[2,3,6g,i]
386W	39	0	Medium-chain[6d]
T484Y	48	50	Medium-chain[6g]
V453F	30	24	Medium-chain[6g]
A457F	68	59	Medium-chain[6g]
T465R	55	−27	Medium-chain
G455F	87	100	Medium-chain[6g]
G455I	76	99	Medium-chain[6g]
S429A	−6	46	Long-chain
S429G	−25	5	Long-chain
W479A	−96	−56	Long-chain
F469A	−68	−59	Long-chain
T430A	−26	28	Long-chain[6i]
D402A	−24	96	Long-chain

Amino acid property changes due to exchange (positive numbers signify an increase and vice versa). [a] Van der Waals volumes[13] and [b] hydrophobicity indices[14] can be found in Table S2. [c] References refer to studies in which the same amino acid position was also subjected to mutagenesis, in most cases for other purposes than our aim in this study.

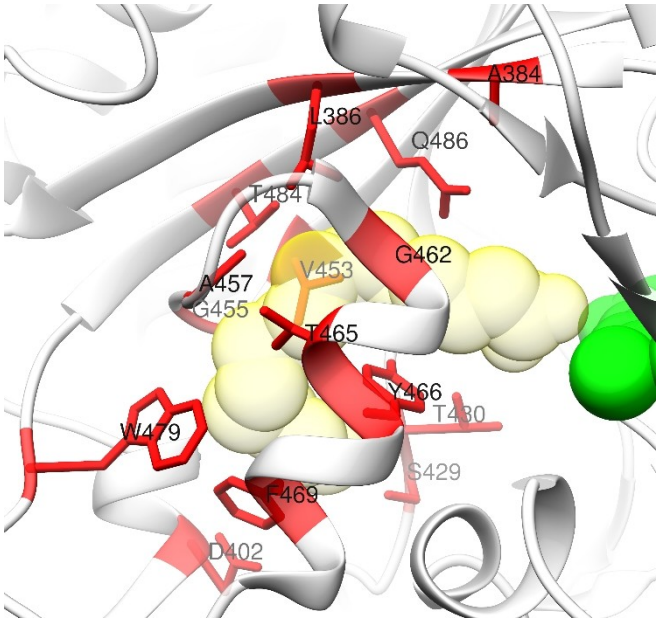

Figure 2. Structure of *Cv*FAP (PDB ID: 5NCC) with (yellow) bound C16:0 substrate, (green) FAD and (red) the 15 mutation positions, screened with 22 variants, highlighted. Structure was generated using UCSF Chimera molecular visualization software.[11]

soluble proteins (Figure S4). Fourteen of these mutants, when assayed as whole cells, displayed measurable activity towards at least one of the substrates (Table 2, Figure S8). Most variants exerted increased selectivity towards certain chain-length fatty

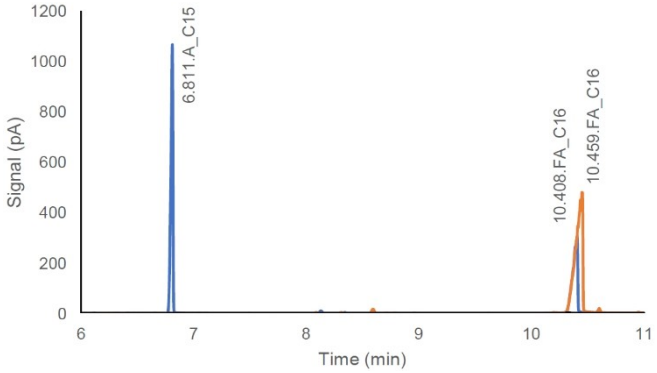

Figure 3. A representative GC chromatogram showing the elution of the C16:0 substrate (FA_C16) and the product hexadecane (A_C15). Two different samples are depicted: (blue) IPTG-induced and (orange) uninduced WT *Cv*FAP whole cells.

Table 2. Screening results.

Analytical yield of alka(e)ne products [%]				
*Cv*FAP variant	A: LB media, 20% (v/v) ethanol, 10 mM substrate C12:0	C16:0	C18:0	C18:1
WT	<1	23±2	28±3	25±6
Y466A	<1	4±1	13	5.2±0.4
Y466F	0	3.0±0.1	26±2	1.7±0.1
Y466L	0	<1	5.1±0.3	<1
F469A	<1	5±1	7±1	1.6±0.2
	B: TB media, 15% (v/v) ethanol, 2 mM substrate			
WT	13±2	39±11	36±2	46±13
G462I	0	0	3.0±0.1	1.7±0.2
G462Y	<1	2±1	2±1	2±1
V453F	5±1	9±3	12±2	16±1
A457F	16±1	4.0±0.4	2.1±0.1	6±1
T465R	12±4	4.66±0.01	2±1	5±1
G455F	4±1	1.2±0.2	<1	<1
S429A	0	1.34±0.04	20.4±0.2	3.4±0.3
S429G	0	0	3±1	0
W479A	3.9±0.2	5±1	24±7	5±1
D402A	0	26±1	55±8	30±3

Analytical product yields in % (Equation 1), obtained from reactions catalyzed by WT or *Cv*FAP variants towards various chain-length substrates. The mutant library was screened with (A) initial and (B) improved conditions outlined in the table. Common assay settings used were: 50 mg/mL whole cells with *Cv*FAP, 100 mM Tris-HCl buffer pH 8.5, 30 °C, 20 h, 300 rpm stirring and 62 PPFD light. Error bars indicate the standard error of at least duplicate experiments.

acids, in agreement with our initial hypothesis when choosing that mutation.

An interesting group of variants that we obtained was those that exhibited significantly higher preference, compared to WT enzyme, for decarboxylation of C18:0 fatty acid. WT *Cv*FAP shows, to a great extent, similar activity levels towards long chain fatty acids, i.e., C16:0, C18:0 and C18:1, as observed in our study as well as in previous studies.[2,6a] However, seven of the mutants, namely Y466A, Y466F, Y466L, S429A, S429G, W479A and D402A, showed moderate to high substrate specificity towards C18:0, albeit with low activity levels in the case of some mutants (Figure 4, Table 2, Figure S8). Most of

these mutants also lost their ability to convert C12:0 fatty acid. The Y466F mutant in particular retained a similar activity level towards C18:0 as the WT enzyme but exhibited a more than 8-fold lower conversion level towards substrates C16:0 and C18:1 (Table 2, Figure S8). Although D402A mutant was not as selective as Y466F towards C18:0, it exhibited the highest analytical yield for the formation of heptadecane (C17) among all variants including WT enzyme (around 50% increase compared to WT) (Table 2).

Moreover, we obtained three mutants; A457F, T465R and G455F, which exhibited higher preference towards C12:0 fatty acid compared to longer chain fatty acids (Table 2, Figure S8). Especially A457F and T465R mutants, while retaining similar analytical yield as the WT enzyme for the formation of undecane (C11), exhibited 10- to 20-fold decreased activity towards formation of C15 and C17 alka(e)nes.

Preparative scale reaction to demonstrate potential practical use of mutants with high substrate specificity

As mentioned above, some mutants displayed very high preference towards only a single fatty acid substrate. We envisioned that such decarboxylases with high substrate specificity could be practically useful for targeted production of a certain chain-length hydrocarbon from a mixture of fatty acids (e.g., waste oil, extracted plant oil). Moreover, it could also be used in the isolation and purification processes of specific target fatty acids, where the contaminating fatty acid(s) could be converted into easily extractable and separable alka(e)nes. Specifically, the Y466F mutant seemed as one of the most ideal candidates for this purpose, with its high activity towards C18:0 but very low activity towards C16:0 (3% vs. 26% product yields), as determined in our screening assays (Table 2, Figure S8A). However, the screening assays were small-scale reactions where only a single fatty acid substrate was analyzed per reaction.

Thus, in order to demonstrate the substrate discriminating potential of Y466F, we set up a large-scale reaction with a substrate mixture of C18:0 and C16:0 fatty acids using the following conditions: 70 mg/mL whole cells with *Cv*FAP (expressed in TB media), 1:1 mixture of C16:0 and C18:0 (200 mg of each), 15% (v/v) ethanol, 100 mM Tris-HCl buffer pH 8.5, 30 °C, 150 rpm shaking and approximately 62 PPFD (Photosynthetic Photon Flux Density, μmol $m^{-2}s^{-1}$) light in a total volume of 100 mL (Figure S10). 92% of the total alkane products were heptadecane (C17) after 44 h reaction time. This result exceeds the substrate selectivity observed in our small-scale screening assays with pure substrates. Samples were taken at various time points to follow the reaction progress (Figure 5). The average percentage ratio of C17 to total product quantity

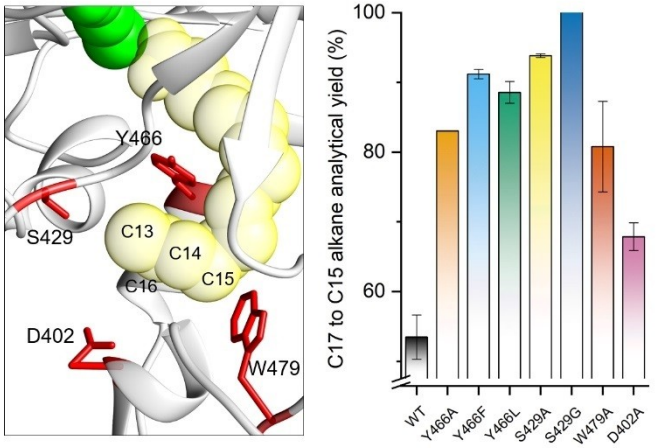

Figure 4. Variants that exhibit significantly higher analytical yield towards C18:0 versus C16:0, compared to WT enzyme. Left panel; structure of *Cv*FAP (PDB ID: 5NCC) with (green) FAD, (yellow) bound C16:0 substrate with the four closest C-atoms to (red) the selectivity variants positions, labelled. Right panel; percent ratio of the C17 product analytical yield to the sum of the analytical yields of the C17 and C15 products, according to the values in Table 2. Error bars indicate the standard error of at least duplicate experiments.

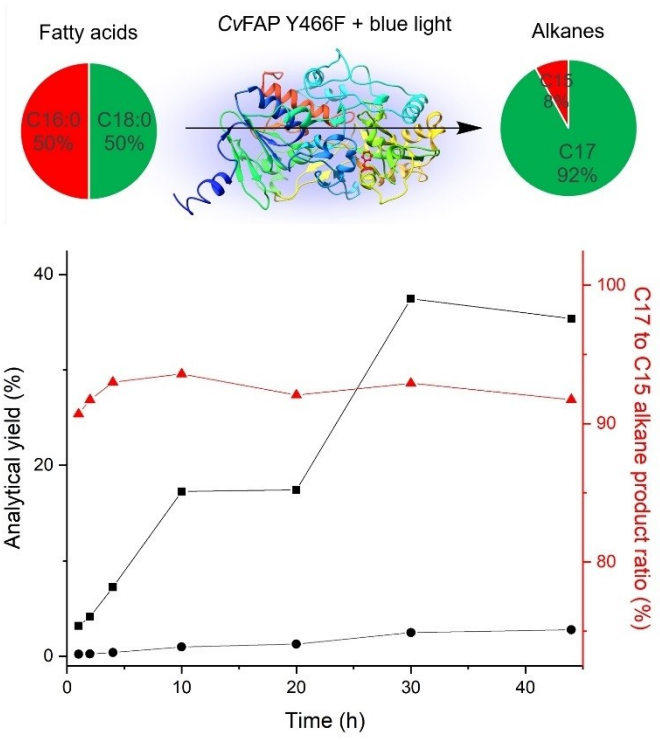

Figure 5. Preparative scale reaction of the selectivity mutant Y466F with a mixture of C16:0 and C18:0 fatty acids. The product ratio was calculated as the percentage of the concentrations of heptadecane (C17) and the combined concentration of both alkane products. Reaction conditions: 70 mg/mL whole cells with *Cv*FAP (TB media), 1:1 mixture of C16:0 and C18:0, 200 mg of each, 15% (v/v) ethanol, 100 mM Tris-HCl buffer pH 8.5, 30 °C, 150 rpm shaking and approximately 62 PPFD light. The standard error indicates at least duplicate experiments.

from seven measured time points was 92 ± 1%. Such a mutant with a high C18:0 over C16:0 selectivity could find application areas, for example in the purification of palmitic acid (C16:0) from palm oil, where around 90% of the total saturated fatty acids are C16:0 and 9% is C18:0.[15] Moreover, expanding the portfolio of such selective mutants can aid in the targeted production of specific chain-length hydrocarbons from various fatty acid mixtures (e.g., waste oil, plant oil) for use as fuel additives.[16]

Light and thermal stability studies of WT and Y466F *Cv*FAP

Various studies have demonstrated that *Cv*FAP activity is highly affected by the presence of light. Especially purified enzyme loses its activity rapidly when exposed to blue light in the absence of fatty acid substrate, due to the formation of radicals inside the protein resulting in its inactivation.[9] Although whole cells and crude lysate have been implicated to be much more stable in previous studies,[6a,9] we wanted to investigate the light stability of whole cells and crude lysate in detail for WT *Cv*FAP as well as for one of our promising mutants, Y466F. Thus, we exposed enzyme preparations to blue light for certain periods of time, after which we assayed their activity by substrate addition. Our results (Figure 6A) show that whole cells of WT enzyme are stable to light exposure at high intensity (452 PPFD) up to two hours, with almost 80% of the activity retained. However, after one hour of high illumination, whole cells of the Y466F mutant started to lose their activity, and over 80% of its activity was lost within two hours. When crude lysate was tested, WT enzyme lost almost 50% of its activity (Figure 6B) but retained its activity upon exposure to light at low intensity also used in our assay (62 PPFD), for the same period (Figure S9). Y466F crude lysate also retained its whole activity after one hour of light exposure at low intensity (Figure S9). It is clear that WT enzyme is more protected from light exposure-based deactivation inside the cell compartment compared to when it is free outside the cell as crude lysate. However, the quick deactivation of Y466F in whole cells might indicate that it is not only the blockage of light by the cell itself that protects the enzyme, but also the presence of endogenous fatty acid substrates in the cell that can bind FAP and avoid formation of off-pathway radicals, as hypothesized recently.[9,10] Possibly, a lower binding affinity of the Y466F mutant towards fatty acids abundant in the cell might lead to its higher deactivation rate.

In addition, we analyzed the thermal stability of WT *Cv*FAP as whole cell catalysts. We incubated the whole cells (expressed in TB media) at specified temperatures for 1 h with 15% (v/v) ethanol in 100 mM Tris-HCl buffer (pH 8.5), without substrate and light. Activity was then assayed by addition of 2 mM C18:0 under the following conditions: 30 °C, 20 h, 300 rpm stirring, blue light at low intensity (62 PPFD). Our results (Figure 7) show that the enzyme activity is within levels of untreated whole cells when incubated at 20 °C. Significant activity losses appear already from 30 °C; the activity is 40% lower than at 20 °C. The activity is completely abolished from 50 °C and higher. This indicates the fragile nature of the enzyme, not only with respect to light but also with respect to temperature.

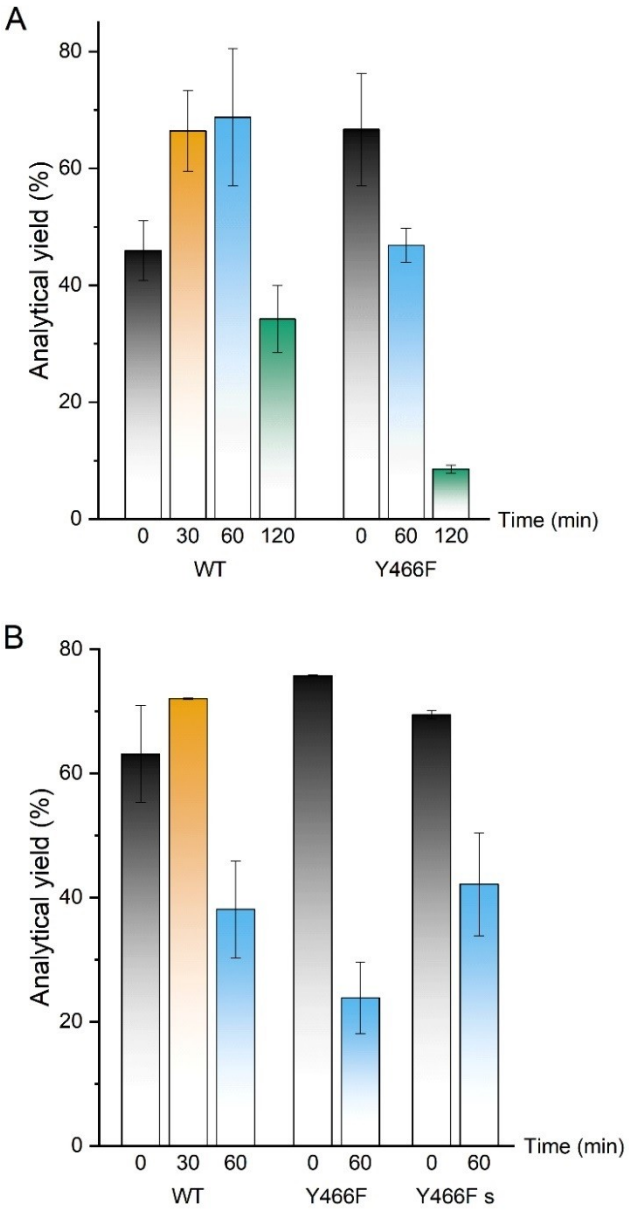

Figure 6. Light stability of WT and Y466F variant was assessed with (A) whole cells, (B) crude lysate and, for Y466F supernatant (Y466F s). Illumination was done at high light intensity of 452 PPFD for different durations. Non-illuminated and already illuminated samples were protected from light. Subsequently, activity was measured after the longest exposure times of (A) 120 and (B) 60 min. with the following conditions: (A) 50 mg/mL whole cells and (B) 20 mg/mL crude extract with *Cv*FAP (TB media), 2 mM C18:0, 15% (v/v) ethanol, 100 mM Tris-HCl buffer pH 8.5, 30 °C, 24 h, 300 rpm stirring and 62 PPFD light. Error bars indicate the standard error of at least duplicate experiments.

Conclusion

Fatty acid photodecarboxylase emerged as one of the most promising enzymes for biocatalysis in the recent years. Here, we optimized expression and assay conditions for *Cv*FAP and screened a library of 22 rationally designed mutants towards four of the naturally most abundant fatty acids (C12:0, C16:0, C18:0 and C18:1) with high potential as biofuel precursors. Our mutant screening demonstrated that the fatty acid chain-length selectivity of the enzyme can be modulated by rationally designing mutations within the substrate binding site. For many of the mutants generated, we obtained specificities that increased either towards shorter chain (C12:0) or towards longer chain (C18:0) fatty acids, in agreement with our initial hypothesis when selecting mutation positions. Moreover, some of the mutants exhibited high preferences only towards C18:0, with very low activities towards C16:0 and C18:1 fatty acids. For the most promising of these selective mutants, Y466F, we demonstrated the increased substrate selectivity with a large-scale reaction containing a 1:1 mixture of C16:0 and C18:0 fatty acids, where an average of 12-fold selectivity for C18:0 over C16:0 was observed. Such selective mutants could open up new application possibilities, e.g., where mixtures of fatty acids are used as substrates or for fatty acid purification. Furthermore, our light stability analysis demonstrated that whole cells are more stable towards light-induced damage compared to crude cell lysate, indicating protection from light of the enzyme inside the cell compartment and stabilization of the enzyme by host-cell derived free fatty acids or fatty acyl-CoAs.[6j,9] The fact that our Y466F mutant has been deactivated by light more rapidly than the WT enzyme in whole cells might signify a lower binding affinity of the mutant towards fatty acids, which have been proposed to act protective on the enzyme when bound.[10] Our future experiments will be dedicated to exploring the practical applicability of the *Cv*FAP mutants in different operation modes, such as fed-batch at larger-scales and optimized light provision using wireless light emitters. Another interesting approach we take will be the immobilization of this enzyme in order to improve its stability, and therefore broaden its applicability for the synthesis of much needed drop-in biofuels.

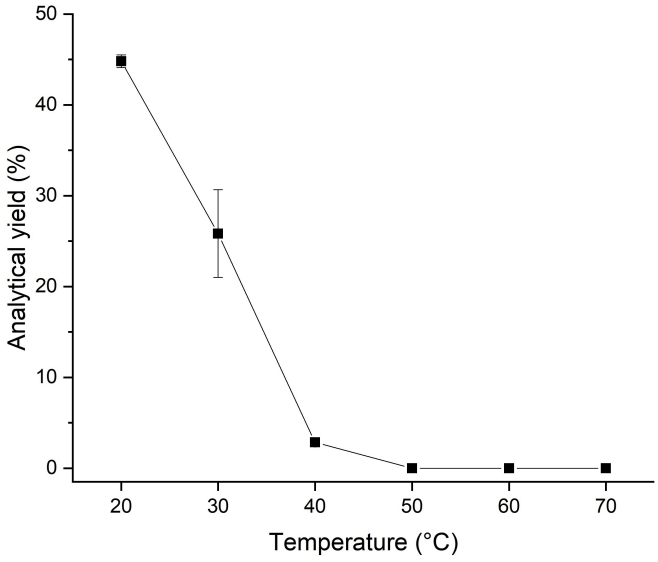

Figure 7. Analytical yield (%) for heptadecane (C17) formation at different temperatures using whole cells of *Cv*FAP WT. Incubation at the specified temperatures was done for one hour with 50 mg/mL whole cells with *Cv*FAP (TB media), 15% ethanol, 100 mM Tris-HCl buffer pH 8.5, 20 h, 300 rpm stirring, no substrate and no light. Subsequently residual activity was measured at 30 °C by addition of 2 mM C18:0 and with 62 PPFD light. Error bars indicate the standard error of at least duplicate experiments.

Experimental Section

Materials

Alkanes C9, 13, and 17 (≥99%), kanamycin disulfate, were purchased from VWR (Radnor, United States). Fatty acids (≥97%), all other alkanes (≥99%), isopropyl β-D-1-thiogalactopyranoside (IPTG, ≥99%, ≤0.1% dioxane), SIGMA*FAST*™ protease Inhibitor, ethanol (≥99.9%), ethyl acetate (≥99.7%), 1-octanol (≥99%), dimethyl sulfoxide (DMSO, ≥99.9%), LB and terrific broth (TB) were purchased from Merck (Darmstadt, Germany). SDS PAGE gels ExpressPlus™ PAGE Gel, 10×8, 12%, 15 wells were purchased from GenScript (Piscataway, United States).

Mutant library generation and protein expression

The *E. coli* codon-optimized pET-28a-*Cv*FAP construct contains a truncated CvFAP gene (62-654) with N-terminal fused His-tag, theoredoxin (TrxA) and tobacco etch virus (TEV) cleavage site.[6a] The nucleotide sequence can be found in the supplementary information. *Cv*FAP variants were generated by PCR with QuickChange II Site-Directed Mutagenesis Kit from Agilent (Santa Clara, United States) according to the supplier's manual and confirmed by sequencing (Eurofins Genomics). Primer sequences can be found in Table S1. The initial protein expression protocol was adapted from previous studies[2,6a,d,e,9,17] and optimized for improved protein yield with respect to ambient light exposure, substrate addition and IPTG concentration as described in the Results and Discussion section. BL21(DE3) *E. coli* cells transformed with plasmid containing the respective *Cv*FAP variants were grown in TB media with 150 rpm shaking at 37 °C to an OD_{600} of 0.6 and induced with 0.5 mM IPTG for 20 h at 17 °C. Cells were harvested by centrifugation (10 min, 4700 g, 4 °C), resuspended to a theoretical OD_{600} of 500 in 0.5 M Tris-HCl buffer with pH = 8.5 and 5% (v/v) glycerol yielding a concentration of 50 mg/mL whole cells. Crude extract was prepared with whole cells, resuspended as above but to an OD_{600} of 200, with the addition of protease inhibitors, and a subsequent sonication with an ultrasonic homogenizer SONOPLUS HD2200 from Bandelin (Berlin, Germany) yielding approximately 20 mg/mL crude extract. To evaluate the solubility of the protein products, crude extract was centrifuged at 17.000 g, 4 °C for 10 min to yield supernatant (soluble fraction) and pellet (insoluble fraction). Protein expression was evaluated by SDS-PAGE analysis. Whole cells and crude extracts were stored in aliquots at −80 °C prior to analysis.

Activity assays and product analysis

Initial assay conditions were adapted from various publications.[2,6a,d,e,9,17] Co-solvents ethanol and DMSO were compared in different concentrations and the substrate concentrations were varied to improve screening results as outlined in the Results and Discussion section. Activity was assayed in 4 mL glass vials, in a final volume of 1 mL, by combining whole cells (50 mg/mL) or clear cell lysate (20 mg/mL) with 2 mM substrate in 15% (v/v) ethanol and 100 mM Tris-HCl buffer at pH = 8.5. The reaction was conducted in self-constructed photobioreactors[18] (Figure S1) at 30 °C with 300 rpm stirring under blue light (62 PPFD) for 20 h, if not otherwise stated. Substrates and products were extracted by vortexing twice for 30 sec with 1:1 ethyl acetate and a defined 1-octanol concentration as internal standard. Analysis of the reactants was performed in an Agilent GC 6850 with a Phenomenex GC column ZB – 1MS 30 m × 0.25 mm × 0.25 μM with an inlet temperature of 250 °C, 1:50 split ratio and 1 mL/min helium flow rate (Table S3, Figure 3). Conversion rates were calculated as indicated in Equation 1:

$$Analytical\ yield\ (\%) = \frac{Measured\ product\ [mM]}{Initial\ substrate\ [mM]} \times 100 \qquad (1)$$

Light and thermal stability

Light stability was assessed by exposing protein expression preparations to different blue light intensities. Samples were irradiated at assay conditions: Whole cells or crude extract, 0.5 or 20 mg/mL *Cv*FAP, respectively, 15% (v/v) ethanol, 100 mM Tris-HCl buffer pH 8.5, 30 °C, 24 h, 300 rpm stirring, but without substrate and either high or low light intensity, 452 and 62 PPFD, respectively. Controls and samples after exposure were light protected until the longest light exposure times passed. Subsequently all samples were assayed simultaneously with 2 mM stearic acid (C18:0) and light.

Thermal stability was evaluated by incubating samples at specified temperatures for one hour with 50 mg/mL whole cells with *Cv*FAP (TB media), 15% ethanol, 100 mM Tris-HCl buffer pH 8.5, 20 h, 300 rpm stirring, no substrate and no light. Subsequently residual activity was measured at 30 °C by addition of 2 mM C18:0 and 62 PPFD light.

Preparative scale reaction

Whole cells with *Cv*FAP (TB media) variant Y466F (70 mg/mL) was used in a reaction scaled up to 100 mL containing a 1:1 mixture of two fatty acid substrates: Palmitic acid (C16:0) and stearic acid (C18:0), 200 mg each. The reaction was conducted in a 250 mL Erlenmeyer flask mounted in a photobioreactor (Figure S10) with vigorous shaking at 150 rpm with following conditions: 15% (v/v) ethanol, 100 mM Tris-HCl buffer pH 8.5, 30 °C, and approximately 62 PPFD light. The standard error indicates at least duplicate experiments. The reaction was carried out by taking samples at different time points with a final Bligh and Dyer liquid extraction step after 44 hours.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: Biocatalysis · Drop-in Biofuels · Fatty Acid Photodecarboxylase · Photoenzyme · Protein Engineering

[1] a) L. O. Björn, *Photochem. Photobiol.* **2018**, *94*, 459–465; b) L.-E. Meyer, B. E. Eser, S. Kara, *Curr. Opin. Green Sustain. Chem.* **2021**, 100496.
[2] D. Sorigué, B. Légeret, S. Cuiné, S. Blangy, S. Moulin, E. Billon, P. Richaud, S. Brugière, Y. Couté, D. Nurizzo, P. Müller, K. Brettel, D. Pignol, P. Arnoux, Y. Li-Beisson, G. Peltier, F. Beisson, *Science* **2017**, *357*, 903–907.
[3] a) D. J. Heyes, B. Lakavath, S. J. O. Hardman, M. Sakuma, T. M. Hedison, N. S. Scrutton, *ACS Catal.* **2020**, *10*, 6691–6696; b) D. Sorigué, K. Hadjidemetriou, S. Blangy, G. Gotthard, A. Bonvalet, N. Coquelle, P. Samire, A. Aleksandrov, L. Antonucci, A. Benachir, S. Boutet, M. Byrdin, M. Cammarata, S. Carbajo, S. Cuiné, R. B. Doak, L. Foucar, A. Gorel, M. Grünbein, E. Hartmann, R. Hienerwadel, M. Hilpert, M. Kloos, T. J. Lane, B. Légeret, P. Legrand, Y. Li-Beisson, S. L. Y. Moulin, D. Nurizzo, G. Peltier, G. Schirò, R. L. Shoeman, M. Sliwa, X. Solinas, B. Zhuang, T. R. M. Barends, J.-P. Colletier, M. Joffre, A. Royant, C. Berthomieu, M. Weik, T. Domratcheva, K. Brettel, M. H. Vos, I. Schlichting, P. Arnoux, P. Müller, F. Beisson, *Science* **2021**, *372*, eabd5687.
[4] a) N. A. Herman, W. Zhang, *Curr. Opin. Chem. Biol.* **2016**, *35*, 22–28; b) C. E. Wise, J. L. Grant, J. A. Amaya, S. C. Ratigan, C. H. Hsieh, O. M. Manley, T. M. Makris, *J. Biol. Inorg. Chem.* **2017**, *22*, 221–235; c) K. Liu, S. Li, *Curr. Opin. Biotechnol.* **2020**, *62*, 7–14.
[5] H. T. Duong, Y. Wu, A. Sutor, B. O. Burek, F. Hollmann, J. Z. Bloh, *ChemSusChem* **2021**, *14*, 1053.
[6] a) M. M. E. Huijbers, W. Zhang, F. Tonin, F. Hollmann, *Angew. Chem. Int. Ed. Engl.* **2018**, *57*, 13648–13651; b) S. Bruder, E. J. Moldenhauer, R. D. Lemke, R. Ledesma-Amaro, J. Kabisch, *Biotechnol. Biofuels* **2019**, *12*, 202; c) S. Moulin, B. Légeret, S. Blangy, D. Sorigué, A. Burlacot, P. Auroy, Y. Li-Beisson, G. Peltier, F. Beisson, *Sci. Rep.* **2019**, *9*, 13713; d) J. Xu, Y. Hu, J. Fan, M. Arkin, D. Li, Y. Peng, W. Xu, X. Lin, Q. Wu, *Angew. Chem. Int. Ed. Engl.* **2019**, *58*, 8474–8478; e) W. Zhang, M. Ma, M. M. E. Huijbers, G. A. Filonenko, E. A. Pidko, M. van Schie, S. de Boer, B. O. Burek, J. Z. Bloh, W. J. H. van Berkel, W. A. Smith, F. Hollmann, *J. Am. Chem. Soc.* **2019**, *141*, 3116–3120; f) M. Amer, R. Hoeven, P. Kelly, M. Faulkner, M. H. Smith, H. S. Toogood, N. S. Scrutton, *Biotechnol. Biofuels* **2020**, *13*, 125; g) M. Amer, E. Z. Wojcik, C. Sun, R. Hoeven, J. M. X. Hughes, M. Faulkner, I. S. Yunus, S. Tait, L. O. Johannissen, S. J. O. Hardman, D. J. Heyes, G.-Q. Chen, M. H. Smith, P. R. Jones, H. S. Toogood, N. S. Scrutton, *Energy Environ. Sci.* **2020**, *13*, 1818–1831; h) H.-J. Cha, S.-Y. Hwang, D.-S. Lee, A. R. Kumar, Y.-U. Kwon, M. Voß, E. Schuiten, U. T. Bornscheuer, F. Hollmann, D.-K. Oh, J.-B. Park, *Angew. Chem. Int. Ed. Engl.* **2020**, *59*, 7024–7028; i) F. Cheng, H. Li, D.-Y. Wu, J.-M. Li, Y. Fan, Y.-P. Xue, Y.-G. Zheng, *Green Chem.* **2020**, *22*, 6815–6818; j) J. Li, Y. Ma, N. Liu, B. E. Eser, Z. Guo, P. R. Jensen, G. Stephanopoulos, *Nat. Commun.* **2020**, *11*, 6198; k) Y. Ma, X. Zhang, W. Zhang, P. Li, Y. Li, F. Hollmann, Y. Wang, *ChemPhotoChem* **2020**, *4*, 39–44; l) W. Zhang, J.-H. Lee, S. H. H. Younes, F. Tonin, P.-L. Hagedoorn, H. Pichler, Y. Baeg, J.-B. Park, R. Kourist, F. Hollmann, *Nat. Commun.* **2020**, *11*, 2258.
[7] S. Karatzos, J. S. van Dyk, J. D. McMillan, J. Saddler, *Biofuels Bioprod. Biorefin.* **2017**, *11*, 344–362.
[8] a) K. E. Kram, S. E. Finkel, *Appl. Environ. Microbiol.* **2015**, *81*, 4442–4450; b) H. Tao, C. Bausch, C. Richmond, F. R. Blattner, T. Conway, *J. Bacteriol.* **1999**, *181*, 6425–6440.
[9] B. Lakavath, T. M. Hedison, D. J. Heyes, M. Shanmugam, M. Sakuma, R. Hoeven, V. Tilakaratna, N. S. Scrutton, *Anal. Biochem.* **2020**, *600*, 113749.
[10] Y. Wu, C. E. Paul, F. Hollmann, *ChemBioChem* **2021**, *22*, 2420–2423.
[11] E. F. Pettersen, T. D. Goddard, C. C. Huang, G. S. Couch, D. M. Greenblatt, E. C. Meng, T. E. Ferrin, *J. Comput. Chem.* **2004**, *25*, 1605–1612.
[12] a) B. Khara, N. Menon, C. Levy, D. Mansell, D. Das, E. N. Marsh, D. Leys, N. S. Scrutton, *ChemBioChem* **2013**, *14*, 1204–1208; b) M. Dietrich, T. A. Do, R. D. Schmid, J. Pleiss, V. B. Urlacher, *J. Biotechnol.* **2009**, *139*, 115–117; c) S. M. Hoffmann, H.-R. Danesh-Azari, C. Spandolf, M. J. Weissenborn, G. Grogan, B. Hauer, *ChemCatChem* **2016**, *8*, 3234–3239; d) S. Meng, R. An, Z. Li, U. Schwaneberg, Y. Ji, M. D. Davari, F. Wang, M. Wang, M. Qin, K. Nie, L. Liu, *Bioresour. Bioprocess.* **2021**, *8*, 26.

[13] N. J. Darby, T. E. Creighton, *Protein structure*, IRL Press at Oxford University Press, Oxford ; New York, **1993**.
[14] O. D. Monera, T. J. Sereda, N. E. Zhou, C. M. Kay, R. S. Hodges, *J. Pept. Sci.* **1995**, *1*, 319–329.
[15] a) J. Jumina, W. Lavendi, T. Singgih, S. Triono, Y. Steven Kurniawan, M. Koketsu, *Sci. Rep.* **2019**, *9*, 10941; b) A. A.-W. Japir, J. Salimon, D. Derawi, B. H. Yahaya, M. Bahadi, S. Al-Shuja'a, M. R. Yusop, *OCL* **2018**, *25*, A203; c) A. A. W. Japir, *Grasas Aceites* **2017**, *68*, e224.
[16] D. D. Bala, D. Chidambaram, *RSC Adv.* **2016**, *6*, 14626–14634.
[17] S. Moulin, B. Légeret, S. Blangy, D. Sorigué, A. Burlacot, P. Auroy, Y. Li-Beisson, G. Peltier, F. Beisson, *Sci. Rep.* **2019**, *9*, 1–8.
[18] M. Hobisch, M. M. C. H. van Schie, J. Kim, K. Røjkjær Andersen, M. Alcalde, R. Kourist, C. B. Park, F. Hollmann, S. Kara, *ChemCatChem* **2020**, *12*, 4009–4013.

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FULL PAPERS

Nice and bright: Fatty acid photodecarboxylase is a light-dependent enzyme that converts fatty acids into alka(e)nes, holding great promise for biocatalytic applications. To alter the substrate specificity of the enzyme, we rationally designed various mutants: One group of our mutants exhibited distinct selectivity towards stearic acid (C18:0), and another group towards lauric acid (C12:0). Light stability of wild type enzyme and a selective mutant was also assessed.

Dr. P. Santner, L. K. Szabó, Dr. S. N. Chanquia, A. H. Merrild, Prof. F. Hollmann, Prof. S. Kara, Dr. B. E. Eser**