Finding the Switch: Turning a Baeyer−Villiger Monooxygenase into a NADPH Oxidase

Patrícia B. Brondani,[†] Hanna M. Dudek,[†] Christian Martinoli,[‡] Andrea Mattevi,*,[‡] and Marco W. Fraaije*,[†]

[†]Molecular Enzymology Group, University of Groningen, Nijenborgh 4, 9747AG Groningen, The Netherlands
[‡]Department of Biology and Biotechnology, University of Pavia, Via Ferrata 1, 27100 Pavia, Italy

Ⓢ *Supporting Information*

ABSTRACT: By a targeted enzyme engineering approach, we were able to create an efficient NADPH oxidase from a monooxygenase. Intriguingly, replacement of only one specific single amino acid was sufficient for such a monooxygenase-to-oxidase switch—a complete transition in enzyme activity. Pre-steady-state kinetic analysis and elucidation of the crystal structure of the C65D PAMO mutant revealed that the mutation introduces small changes near the flavin cofactor, resulting in a rapid decay of the peroxyflavin intermediate. The engineered biocatalyst was shown to be a thermostable, solvent tolerant, and effective cofactor-regenerating biocatalyst. Therefore, it represents a valuable new biocatalytic tool.

Figure 2. Catalytic mechanism of phenylacetone monooxygenase showing the Baeyer−Villiger oxidation and uncoupling reaction (red) routes. Several flavin intermediates are highlighted in color (oxidized flavin in green, reduced flavin in magenta, and peroxyflavin in red) and correlate to the colors used in Figure 3.

Many redox enzymes depend on nicotinamide cofactors. Such biocatalysts promote, for example, reduction of ketones or oxidation of alcohols, including stereo- and regioselective transformations.[1] To avoid the use of equimolar amounts of these expensive reagents, efficient and sustainable strategies for cofactor regeneration are needed, which is one of the current challenges in biocatalysis. The most promising approach is the use of an ancillary enzyme at the expense of a sacrificial cosubstrate.[2]

While the regeneration of the reduced nicotinamide cofactors (NAD[P]H) by enzymatic reactions is well established (for example, by formate or phosphite dehydrogenases), regeneration of the oxidized counterparts (NAD[P]+) still represents a challenge.[3] Some enzymes that regenerate NAD(P)+ by reducing molecular oxygen (O2) have been reported. Such NAD(P)H oxidases are typically flavin-containing enzymes where the flavin prosthetic group acts as an electron mediator, transferring the electrons from NAD(P)H to O2 (Figure 1). Although several NADH oxidases are available, only a few NADPH oxidases are

known, and only some of them can be produced and isolated. Some NADH oxidases can be used with both NADH and NADPH, but the activity for NADPH oxidation is usually low when compared to NADH oxidation, and the respective NAD(P)H oxidases may not be very robust.[4]

Baeyer−Villiger monooxygenases are flavin-containing enzymes that use O2 and NADPH to catalyze, among others, the oxidation of ketones to esters.[5] The oxygenation reactions catalyzed by these biocatalysts proceed via a reactive flavin intermediate, a flavin-peroxide, that is formed after the reduction of the flavin cofactor by NADPH and a subsequent reaction with O2 (Figure 2). In the absence of a suitable substrate, the characteristic oxygen-activating peroxyflavin intermediate can decay to yield oxidized flavin and hydrogen peroxide at the expense of NADPH (Figure 2).[6] The process of oxidizing NADPH without catalyzing substrate oxygenation, referred to as uncoupling, is typically very slow (<0.1 s^{-1}). Albeit slow, this unproductive mode of action of BVMOs effectively corresponds to an NADPH oxidase activity.

Phenylacetone monooxygenase (PAMO) from *Thermobifida fusca* is a thermostable and well-characterized BVMO.[7] Besides the exploration of its biocatalytic properties, several crystal

Figure 1. Example of a biocatalytic process in which a NADPH oxidase catalyzes the regeneration of NADP+.

structures of PAMO have been solved, and its catalytic mechanism has been elucidated in detail (Figure 2).[8] The available structural information and overall robustness of PAMO make this biocatalyst an ideal candidate for enzyme engineering. In fact, we and other groups have succeeded in generating a number of PAMO-based biocatalysts that can be used for a variety of oxygenation reactions.[9] During these studies we observed that some PAMO mutants displayed slightly increased uncoupling rates. For example, the Q152F and Q152F/L153G/M446G PAMO mutants catalyze futile NADPH oxidation with a rate of 0.5−0.6 s^{-1}, while for wild-type PAMO the uncoupling rate is only 0.01−0.02 s^{-1} (Table 1).[7,8a,10] Careful screening of

Table 1. Observed Rate of NADPH Oxidation by PAMO Mutants^a

enzyme variant	activity (s^{-1})	
	in the absence of phenylacetone	in the presence of phenylacetone
wild-type PAMO	0.02	3.0
I67P PAMO	0.10	0.09
M446W PAMO	0.17	0.18
Q152F/L153G/M446G PAMO	n.d.	0.6^b
Q152F	0.5	0.5
Q152F/M446W PAMO	0.38	0.47
I67P/Q152F PAMO	0.13	0.15
C65D/I67V PAMO	2.1	2.2
C65D/I67S PAMO	2.6	2.0
C65D PAMO	4.9	3.8
Q152Y/M446W PAMO	1.3	1.1
C65D/I67P PAMO	0.05	0.05
C65D/Q152F/M446W PAMO	0.43	0.27
C65E/PAMO	0.11	0.43

^{a}Activity was measured by monitoring the rate of absorbance decrease at 340 nm at 25 °C for 3 min. The experiment was performed in the presence (Baeyer−Villiger monooxygenase and/or NADPH oxidase activity) or absence (NADPH oxidase activity) of phenylacetone. The reaction mixture contained 0.1 mM NADPH, (1.0 mM phenylacetone), 0.1−0.5 μM enzyme in 50 mM Tris-HCl pH 7.5 (final volume 1.0 mL). ^{b}Taken from ref 9a.

recently created site-saturation libraries led us to uncover even more enzyme variants with increased NADPH oxidase activities.[10] Among them, M446W and I67P stood out, having higher uncoupling rates (Table 1) and less (M446W) or no (I67P) conversion of phenylacetone when compared with wild-type PAMO (Supporting Information (SI), Table S1). These results prompted us to embark on a dedicated search for PAMO mutants with an enhanced and biocatalytically relevant NADPH oxidase activity. As a first strategy, we decided to combine the previously identified oxidase-promoting single-site mutations into double mutants. However, no considerable increase in NADPH oxidase activity was obtained for all tested double mutants (Table 1). Therefore, we designed and constructed three mutant libraries in which two residues were simultaneously and randomly mutated: Q152&M446, C65&I67, and Q152&L153. I67, Q152, L153, and M446 were chosen because mutating these residues resulted in some increase in uncoupling activity (Table 1 and refs 9 and 10), while C65 and I67 are the residues closest to the locus of the peroxy moiety of the peroxyflavin. At each position, 12 or 13 different amino acids were allowed by choosing specific degenerate primers (details in

SI, Table S2). These specific residue couples form distinct parts of the active site of PAMO. The three libraries were screened using our recently developed assay for detecting (1) NADPH consumption by an enzyme coupled assay and (2) peroxide detection by using the fluorogenic chemical probe Peroxy Green, which specifically reacts with hydrogen peroxide.[10] The Q152/L153 library yielded no active mutants, whereas screening of the two other libraries produced several clear hits with potentially enhanced uncoupling. After sequencing of these clones, the C65D/I67 V, C65D/I67S, C65D, and Q152Y/M446W mutations were identified as the most promising hits. The corresponding proteins were tested for Baeyer−Villiger monooxygenase and NADPH oxidase activities. Although all mutants achieved full substrate conversion after 24 h, uncoupling rates were high, even compared to the previous generation of mutants that displayed uncoupling activity. The best hit was C65D (Table 1), which exhibited a strong NADPH oxidase activity, and this is in line with the fact that this amino acid replacement is shared by three out of the four identified mutants.

Although the single C65D mutation on its own featured interesting properties, we sought to explore the effects of combined mutations. Because we did not observe C65D/I67P as a hit in the screening, this mutant was constructed. In parallel, we constructed a triple mutant combining C65D/Q152F/M446W mutations. Although these mutants did not yield any Baeyer−Villiger oxidation product (SI, Table S1), their NADPH oxidase activities were also low (Table 1). We also introduced a glutamate instead of an aspartate at position 65 because this mutation was not present in the library, but no improvement was observed. In line with these experiments, we also attempted introducing an aspartate residue at homologous positions in other BVMOs. However, the T56D mutant of cyclohexanone monooxygenase showed no NADPH oxidase activity. Mutations F54D and V190D introduced in acetone monooxygenase and 4-hydroxyacetophenone monooxygenase, respectively, resulted in impaired FAD binding. In summary, the C65D mutant of PAMO turned out to be a unique hit, representing a potent NADPH oxidase.

To explore the properties and biocatalytic potential of this engineered NADPH oxidase, further characterization studies were performed. The C65D PAMO mutant could be easily produced and purified using the same protocol as used for wild-type PAMO. By measuring the absorbance spectrum in the range of 250−600 nm, it was confirmed that the FAD cofactor is well bound to the protein (A_{280}/A_{440} = 12).[11] By performing oxygen measurements we confirmed that, in the absence of a ketone, C65D PAMO readily forms H$_2$O$_2$. When using 1.0 mM phenylacetone, we observed that a significant amount (∼50%) of H$_2$O$_2$ was still formed. This is in sharp contrast with wild-type PAMO, which does not show any appreciable uncoupling activity in the presence of phenylacetone. Using different concentrations of NADPH and in the absence of phenylacetone, $K_{M,NADPH}$ = 3.5 μM and k_{cat} = 5.0 s^{-1} were determined (Table 2). Thus, the C65D mutation increases the rate of uncoupling by 2−3 orders of magnitude (5.0 vs 0.01−0.02 s^{-1}).[7,8a] These data also show that this mutant enzyme behaves as a NADPH oxidase by displaying a high oxidase activity and a relatively low Baeyer−Villiger monooxygenase activity. In fact, the kinetic parameters for the C65D PAMO mutant for oxidation of NADPH are in the same range when compared with those of two bacterial NAD(P)H oxidases (Table 2).

To probe the effect of the C65D mutation on biocatalyst stability, we used the ThermoFAD method.[12] This revealed an

Table 2. Steady-State Kinetic Parameters for NAD(P)H Oxidation by Wild-Type PAMO, C65D PAMO, and Two Bacterial NADPH Oxidases

enzyme	k_{cat} (s^{-1})	$K_{M,NADPH}$ (μM)	k_{cat}/K_M (s^{-1} μM^{-1})
PAMO	0.016	0.7	0.02
C65D PAMO	5.0	3.5	1.4
LsNOX[a]	52	6.1	8.5
V193R/V194H SmNOX[b]	20	6	3.3

[a]LsNOX: NAD(P) oxidase from *Lactobacillus sanfranciscus*; kinetic data taken from ref 4b. [b]V193R/V194H SmNOX is a recently engineered mutant of NADH oxidase from *Streptococcus mutant* which accepts NADPH as substrate; kinetic data taken from ref 4d.

apparent melting temperature of C65D PAMO of 53.5 °C, which is somewhat lower than that of the wild-type protein (59 °C).[13] Most importantly, since many alcohol dehydrogenases are more active at temperatures above room temperature, we investigated the thermostability of the C65D mutant by incubation at 40 °C. This revealed that the enzyme retained >80% activity for at least 72 h at this temperature (SI, Table S3). Incubating C65D PAMO in 10% methanol or DMSO for 24 h also did not have a detrimental effect on its activity (>90% activity was retained, SI, Table S4). Clearly, the PAMO-based NADPH oxidase has fundamentally retained the robustness of its parent enzyme.[7,14]

We applied C65D PAMO in reactions either using phosphite and phosphite dehydrogenase, or using an organic substrate and an alcohol dehydrogenase, in which the NADP$^+$ cofactor is regenerated by the engineered NADPH oxidase. The reactions and the results are presented in Table 3, and further details about

Table 3. Using C65D PAMO as NADP$^+$-Regenerating Biocatalyst by a Combined Use with Dehydrogenases[a]

alcohol/phosphite — (alcohol/phosphite dehydrogenase) → ketone/phosphate

NADP$^+$ ⇌ NADPH

H$_2$O$_2$ ← (C65D PAMO) → O$_2$

entry	substrate	dehydrogenase[b]	conversion (%)[c]
1	phosphite	PTDH	100
2	2-butanol	ADH$_T$	100
3	cyclohexanol	ADH$_E$	100
4	*rac*-1-phenylethanol	ADH$_E$	50 (ee >99%)

[a]The reactions were performed for 24 h with 20 mM substrate and 0.5 mM NADP$^+$. See Supporting Information Notes for details. [b]Three different dehydrogenases were used: phosphite dehydrogenase (PTDH), alcohol dehydrogenase from *T. brockii* (ADH$_T$), and alcohol dehydrogenase evo-1.1.270 (ADH$_E$). [c]100% conversion means the substrate is fully converted.

the procedure can be found in the SI (Notes S1–S3). C65D PAMO was effective in regenerating the cofactor, and no side products from Baeyer–Villiger oxidation were observed, even when different types of substrates were used. Moreover, we used the enzyme in a kinetic resolution, leading to an enantiopure chiral alcohol (Table 3, entry 4). The addition of catalase did not affect the outcome of the reactions, and this suggests that the tested biocatalysts are not very H$_2$O$_2$-sensitive. The observation that no Baeyer–Villiger oxidation products were found illustrates that wild-type PAMO is a good starting point to engineer a

NADPH oxidase. Wild-type PAMO has a very narrow substrate acceptance range; therefore, the PAMO-derived NADPH oxidase will be compatible with most reactions that require NADP$^+$ recycling.

To explain the specific and drastic effect of the C65D mutation, we studied the catalytic mechanism of the engineered NADPH oxidase in more detail. By using the stopped-flow technique, we established that the rate of formation of peroxyflavin is similar to that of wild-type PAMO (A→B transition in Figure 3, see also Figure 2).[8a] However, the rate of
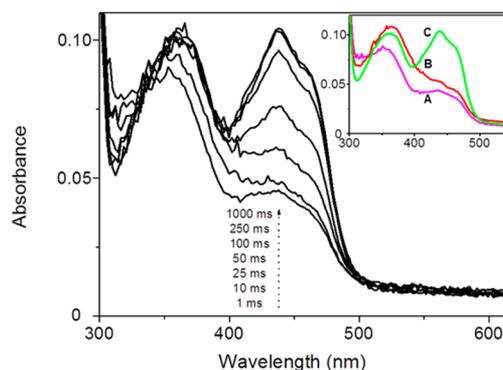
Figure 3. Stopped-flow measurement of the reaction of 10 μM reduced C65D PAMO upon rapid mixing with 120 μM dioxygen. Only seven selected spectral scans are shown of the 1000 collected scans (total reaction time 1.0 s). The inset shows the deconvolution of the spectra and clearly shows, starting for reduced C65D PAMO (A), the initial formation of the peroxyflavin intermediate (B) at a rate of 45 s^{-1}, with its absorbance maximum at 370 nm. The colors of the spectral species comply with the color coding in Figure 2. The peroxyflavin subsequently decays at a rate of 25 s^{-1} to form oxidized flavin (C) and H$_2$O$_2$.

decay of the peroxyflavin has increased tremendously (B→C transition in Figure 3). While this reaction proceeds at a rate of 0.014 s^{-1} in wild-type PAMO, C65D PAMO is extremely rapid in generating hydrogen peroxide (25 s^{-1}).[8a] Such a drastic change is in accordance with the observation that the NADPH oxidase activity of C65D PAMO (5.0 s^{-1}) is even higher when compared to the Baeyer–Villiger monooxygenase activity of wild-type PAMO (3.0 s^{-1}).

The crystal structure of the mutant enzyme was elucidated in order to gain a further understanding of the specific effect of the C65D mutation (crystallographic details in Supporting Information, Table S5). Cys65 belongs to a loop that is in direct contact with the C4a-N5 edge of the flavin (Figure 4). Cys and Asp have almost isosteric side chains, and, indeed, the crystal structures of C65D PAMO in both the oxidized and reduced states are virtually identical to those of the wild-type enzyme (rms deviations of 0.2 Å for all Cα atoms). The only variation can be observed in the crystal structure of the oxidized mutant, where Asp66 rotates toward the flavin as opposed to the conformation observed in both the oxidized and reduced wild-type structures, as well as the reduced C65D enzyme (Figure 4). Interestingly, the same conformation has been observed in the complex of PAMO with APADP$^+$, a NADP(H) analogue which can function in flavin reduction but causes substantial uncoupling due to a reduced stability of the flavin peroxide.[15] The reorientation of Asp66 may favor intermediate decay by the combination of two factors: (1) steric hindrance due to direct contact with the C4a locus of the flavin, and (2) triggering of the protonation of the intermediate proximal oxygen with consequent formation of
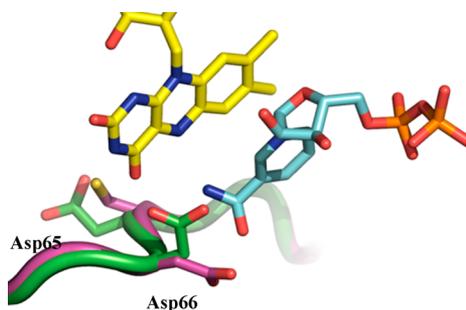
Figure 4. Structural studies of C65D PAMO: oxidized C65D PAMO (green carbons) superposed on the Cys65-Asp66 side chains of wild-type PAMO (magenta carbons). Carbon atoms of the FAD and NADP$^+$ are in yellow and cyan, respectively. Asp65 is engaged in H-bonds with a water molecule and the backbone nitrogen of Ala91, which are not shown for sake of clarity. See also Figure S9 and Table S5 in the SI.

H_2O_2.[15,16] It is very difficult to assign specific causes for the small change in the Asp66 conformation, which likely reflects the altered balance of electrostatic, H-bonding, and van der Waals interactions with nearby protein, cofactor, and solvent atoms caused by the C65D mutation. However, it appears that the properties of the C65D PAMO mutant can be ascribed mainly to stabilization of this uncoupling-promoting conformation of Asp66. Interestingly, introducing an aspartate at the same position in sequence-related monooxygenases did not result in the creation of efficient NADPH oxidases. This indicates that the effect of the C65D mutation in PAMO is subtle and unique.

In this work we report on an enzyme activity switch, converting a thermostable and solvent-tolerant monooxygenase into an oxidase. Intriguingly, it was sufficient to mutate only one amino acid in the parent enzyme to achieve this switch. The observation that a single mutation in a flavoprotein mono-oxygenase results in a drastic boost in uncoupling activity also presents a warning for enzyme engineering approaches that concern flavoproteins. Yet, the observation that similar mutations in sequence-related monooxygenases did not yield NADPH oxidases may suggest that PAMO is rather unique. In fact, we have generated numerous PAMO mutants in previous studies and never observed such high NADPH oxidase activities. The generated PAMO-based robust NADPH oxidase represents a useful biocatalyst for cofactor regeneration, as only very few alternative (bio)catalysts are available.[17] Several specific features (thermostability, solvent tolerance, tight binding of the flavin cofactor, ease of production) make the engineered NADPH oxidase a promising candidate for biocatalytic processes. Furthermore, the biocatalyst may also develop into a useful tool in cell biology research because NADPH oxidase activities are crucial in triggering signal transduction pathways.[18] Moreover, intracellular NADPH levels are crucial regulators of metabolism.[19] The created NADPH oxidase may also develop as a tool in metabolic engineering to manipulate the intracellular NADP$^+$/NADPH ratio.

ASSOCIATED CONTENT

Ⓢ Supporting Information

Primer sequences, steady-state kinetic data of the reported enzymes, crystallographic statistics, and experimental details concerning conversions. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Authors

andrea.mattevi@unipv.it
m.w.fraaije@rug.nl

Notes

The authors declare no competing financial interest.

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