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Functionalization of Oxidases with Peroxidase Activity Creates Oxiperoxidases: A New Breed of Hybrid Enzyme Capable of Cascade Chemistry

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The covalent flavoprotein alditol oxidase (AldO) from *Streptomyces coelicolor* A3(2) was endowed with an extra catalytic functionality by fusing it to a microperoxidase. Purification of the construct resulted in the isolation of a synthetic bifunctional enzyme that was both fully covalently flavinylated and heminylated: an oxiperoxidase. Characterization revealed that both oxidase and peroxidase functionalities were active, with the construct functioning as a single-component xylitol biosensor. In an attempt to reduce the size of the oxidase–peroxidase fusion, we replaced portions of the native AldO sequence with the bacterial cytochrome *c* CXXCH heme-binding motif. By mutating only three residues of the AldO protein we were able to create a functional oxidase–peroxidase hybrid.

Introduction

Oxidases are a versatile class of enzymes capable of performing oxidation reactions with exquisite regio-, chemo- and/or enantioselectivities on a broad range of substrates.[1] Most oxidases belong to the family of flavoproteins and often have their flavin cofactor covalently attached to the protein.[2] One flavoprotein subfamily—the vanillyl alcohol oxidase (VAO) family—is relatively rich in oxidases containing covalently bound flavin cofactors.[3] Recently, members of this specific class of enzymes have been attracting attention as potential industrially relevant biocatalysts in such diverse applications as biosensors,[4,5] dough optimizers (additions that improve the physical characteristics of the final baked product),[6] enantioselective catalysts,[7] and most recently in the production of new antimicrobial scaffolds.[8] Their specificities and their ability to use molecular oxygen as an electron acceptor, thereby circumventing the requirement for an expensive cofactor such as NAD(P)H, make them ideally suited for a myriad of synthetic purposes and biosensing applications.[9] Whereas some oxidases reduce O_2 to H_2O, most produce H_2O_2 as only byproduct.

Like oxidases, peroxidases (EC 1.11.x) belong to the class of oxidative enzymes and are typified by their use of the reduced form of dioxygen: hydrogen peroxide. Also like oxidases, peroxidases are of biotechnological interest.[10] Examples of applications being developed include dye decoloration,[11] enantioselective oxidation of phenols[12] and sulfides,[13] and most recently the degradation of lignin for biofuel generation.[14] The peroxidase family is incredibly diverse and ubiquitous in living organisms.[15] A common feature, however, is a redox cofactor as part of the active site, generally in the form of an iron protoporphyrin IX. This heme cofactor is essential for peroxidase activity; heme itself, indeed, displays intrinsic peroxidase activity in the absence of any protein environment. In light of this, highly minimal peroxidases—so-called microperoxidases—have been artificially created by the digestion of cytochrome *c*.[16] These peptides retain their original peroxidase functionality and have found diverse applications as biosensors,[17] electron carriers,[18] photoreceptors,[19] drugs,[20] and biocatalysts[21] and also in biofuel cells.[22]

Oxidases and peroxidases, although they catalyze vastly different reactions, share hydrogen peroxide as a unifying feature. For one it is a byproduct, for the other a substrate. Nature utilizes this complementarity in, for example, the lignin degradation pathway in white-rot fungi, in which hydrogen peroxide produced by specific oxidases is used by lignin peroxidases to degrade lignin.[23,24] Inspired by this natural example in which peroxidase and oxidase activities are functionally coupled in a cascade reaction, we set out to design a synthetic biocatalyst that would merge both activities in a single protein backbone. Such a construct could open possibilities for novel biosensor development and in the design of novel biochemical pathways while highlighting the power of synthetic biology as a tool to combine various cellular machineries to create an artificial enzyme system. At the same time, such a genetic fusion could possess certain catalytic advantages, such as faster peroxide transfer between the oxidase and peroxidase moiety in a fused variant than in an unfused variant.[25]

The model oxidase we chose to functionalize with peroxidase activity was the recently discovered[26] and extensively characterized[7,27] polyol oxidase alditol oxidase (AldO). This oxidase was chosen because of its amenability to enzyme engineering: it can be expressed in high yields in *E. coli*, its crystal

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structure has been elucidated at high resolution,[28] and—most importantly for this study—it has been engineered for transport to the periplasm of *E. coli* by the twin-arginine translocation (Tat) pathway.[29] Choosing a suitable peroxidase system was confounded by the notoriously difficult expression of such enzymes in bacterial hosts. Most well-studied peroxidases, such as horseradish peroxidase (HRP),[30] are eukaryotic in origin and cannot be expressed in *E. coli*.[31,32] Although several examples of prokaryotic peroxidases belonging to the dye-decolorizing (DyP-type) superfamily[33] have recently appeared,[13,34] these enzymes are only active at low pH values, which is incompatible with flavoprotein oxidases. We therefore turned to the recent work done on minimal bacterial peroxidases, in which it has been shown that short peptide sequences containing the cytochrome *c* heme-binding motif—CXXCH—are sufficient to express functional peroxidases in the periplasm of *E. coli*.[16] Secretion is a prerequisite for these microperoxidases because covalent heme attachment takes place exclusively in the periplasm. In *E. coli*, the eight-protein CcmA–H unit of the cytochrome *c* maturation (Ccm) apparatus is responsible for this process.[35,36] From a synthetic biology viewpoint, such microperoxidases are attractive building blocks because they make it possible to create novel fusion enzymes by decorating the target protein (in this case AldO) with a small, defined hemopeptide exhibiting peroxidase activity.

Here we fused a microperoxidase to the flavoprotein oxidase AldO and expressed the entire bifunctional fusion protein in the periplasm of *E. coli* in an active form. The fusion was shown to exhibit oxidase, peroxidase, and coupled oxidase–peroxidase activities both in vivo and in vitro. Characterization of the purified bifunctional enzyme revealed that it was both completely and covalently flavinylated and completely and covalently heminylated. This combination of cofactors, heme and FAD, with both covalently bound to a single polypeptide chain has never been observed before in a natural protein. Although naturally occurring protein complexes that contain both heme and FAD exist (e.g., cytochrome P450 BM3 and NO synthase), the redox cofactors in these proteins are never found covalently bound to a single polypeptide chain. In order to create even smaller oxidase–peroxidase fusions, we also created AldO variants in which heme-binding segments were introduced in the AldO backbone. This revealed that it was possible to introduce peroxidase activity into AldO by replacing only three amino acid residues. We have hence shown that an artificial enzyme system can be designed and constructed through the fusion of various protein-encoding DNA fragments, resulting in an oxidase that has been functionalized with peroxidase activity to create a new enzyme: an oxiperoxidase.

Results and Discussion

Hybrid design and experimental strategy

In *E. coli*, the covalent coupling of heme to microperoxidases occurs exclusively in the oxidizing periplasm,[36] so the design of our chimeric oxidase–peroxidase required periplasmic transport of the oxidase. In previous studies the model oxidase AldO had been successfully engineered for export to the *E. coli* periplasm by the Tat pathway,[29] making it a logical candidate for peroxidase functionalization. Microperoxidases that can be expressed in the periplasm of *E. coli* with varying yields have been described.[16] Because of its successful fusion to maltose binding protein (MBP) and export by the general type II secretion system (Sec), microperoxidase MP292[37] was chosen to functionalize AldO. The hybrid was designed so that DNA encoding MP292 was cloned at the C terminus of the *aldo* gene equipped with a Tat signal sequence. A Strep-tag was included at the N terminus for purification purposes, creating Tat-Strep-AldO-MP (AldO-MP in Figure 1). A two-plasmid system was used to express the hybrid proteins. A pBAD-derived expression plasmid contained the gene encoding the AldO–peroxidase hybrid and an N-terminal Tat signal sequence for peri-

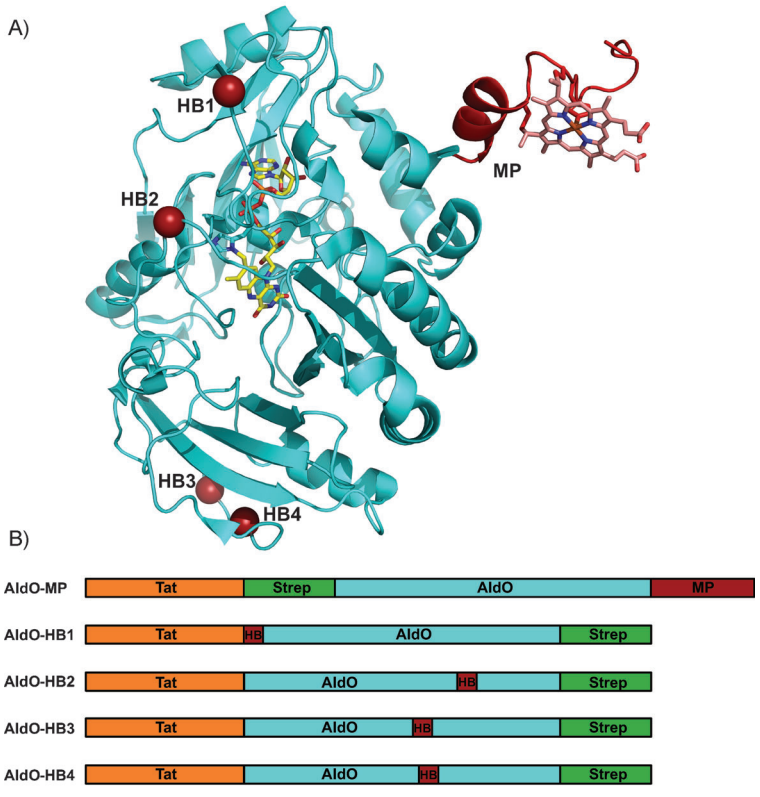

Figure 1. Designing oxidase–peroxidase hybrids. A) Structure of *Streptomyces coelicolor* A3(2) alditol oxidase (AldO, PDB ID: 2vfs) highlighting the locations that were mutated into the CXXCH heme-binding motif (HB1-4, red spheres). Covalently bound FAD is shown as yellow sticks. A model of the microperoxidase (MP) peptide containing a covalently bound heme (pink sticks) that was fused to the C terminus of AldO is shown in red. B) Schematic overview of the prepared constructs. Tat: twin arginine translocation signal sequence. Strep: Strep tag for purification of the constructs with Strep-tactin Sepharose. AldO: alditol oxidase. HB: heme-binding motif CXXCH. MP: microperoxidase MP292.

plasmic export. A second plasmid, pEC86,[38] contained the cytochrome *c* maturation apparatus ccmA–H under the control of a constitutive promoter, required for the transport and covalent attachment of heme to the CXXCH motif under aerobic conditions.[36] SDS-PAGE analysis and activity staining revealed that the construct was expressed in the periplasm of *E. coli* and exhibited both oxidase and peroxidase activities. Through affinity chromatography the oxiperoxidase AldO-MP (Figure 1) was successfully purified from periplasmic extracts (Figure 2).

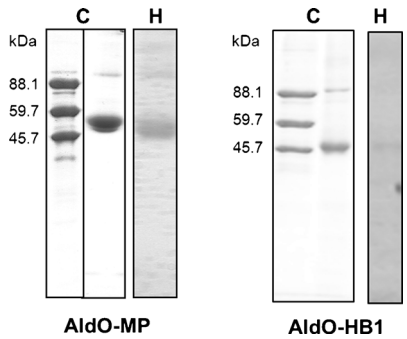

Figure 2. SDS-PAGE analysis of purified oxidase–peroxidase fusions indicates covalently bound heme. Both constructs were purified from periplasmic extracts with Strep-tactin Sepharose. AldO-MP and AldO-HB1 refer to constructs shown in Figure 1. C: total protein stained with Coomassie Brilliant Blue. H: heme stain.

Purification of an oxiperoxidase

The oxiperoxidase AldO-MP (Figure 1) was expressed in *E. coli* BL21(DE3) cells. Because heme attachment to cytochromes occurs in the periplasm of Gram-negative organisms,[36] we anticipated obtaining only fully holo-oxiperoxidase containing both FAD (incorporated in the cytoplasm) and heme (incorporated in the periplasm) by purifying the fusion constructs from periplasmic extracts prepared by the osmotic shock method. After incubation of Strep-tactin Sepharose resin with periplasmic extracts and subsequent washing of the resin, the column material displayed a dark red coloration, indicative of a bound protein containing a heme cofactor. Upon elution, a dark red fraction was collected and was revealed to run as one band on an SDS-PAGE gel stained for total protein (Figure 2), at approximately 50 kDa. This was in good agreement with the 50.6 kDa theoretical mass of the construct (−Tat signal sequence, + FAD and + heme) and in agreement with earlier work in which the Tat signal sequence was cleaved off during transport of AldO to the periplasm.[29] From 1 L of culture, approximately 3 mg of protein was purified from the periplasmic extracts. A duplicate of the SDS-PAGE gel was stained for heme content (Figure 2), which revealed that the purified protein indeed contained heme. Immunoblotting was carried out with antiserum against AldO to confirm periplasmic export; visualization revealed a band in the periplasmic fraction (Figure S1 in the Supporting Information). The efficiency of the fractionation procedure was confirmed by analysis of the levels of DnaK, which serves as a cytoplasmic marker. Together, this shows that AldO-MP is exported to the periplasm as expected.

Because it is known that microperoxidases exist in a variety of oligomeric states at physiological pH,[21] we performed size-exclusion chromatography (SEC) to examine whether the monomeric state of native AldO[26] is altered by the addition of a microperoxidase tag. Analysis on a SEC column revealed that the purified fusion had the same retention time as wild-type AldO. Comparison with a set of molecular weight standards allowed for the calculation of a protein mass consistent with the monomeric form. We have thus successfully cloned, expressed, and purified a Strep-tag-labeled fusion between a flavoprotein oxidase (AldO) and a microperoxidase in the periplasm of *E. coli*: an oxiperoxidase. Characterization of the oxiperoxidase by SDS-PAGE and SEC revealed it to be monomeric and to contain covalently bound heme.

An oxiperoxidase contains both covalent FAD and heme

Having established that the oxiperoxidase AldO-MP (Figure 1) contained both covalent heme and FAD, we set out to determine the ratio of heme and FAD incorporation. The pyridine hemochrome assay was used to determine the heme content of the fusion construct, which yielded an absorption peak at 557 nm. Absorbance at this wavelength is indicative of covalently bound heme *c*[39] and we determined a heme concentration of 20.3 μM. Concentrations of flavoproteins are conventionally determined by examining the characteristic absorption spectrum caused by the flavin cofactor in the region between 350 and 500 nm.[40] Because of the strong absorption of the heme cofactor in this region, however, it was anticipated that this approach would not work. Indeed, as the absorption spectrum (Figure 3) of the fusion construct reveals, it predominantly exhibits the characteristics of a hemoprotein, with a characteristic Soret band at 410 nm and a peak at 280 nm.

The shoulder between 420 and 260 nm (Figure 3) hints at the presence of a FAD cofactor. In order to measure the FAD concentration we specifically reduced the FAD cofactor under anaerobic conditions with xylitol and followed the absorption spectra in a stopped flow spectrophotometer fitted with a diode array detector. The difference spectrum revealed an absorbance maximum at 452 nm, which confirms the presence of the covalently bound FAD.[26] By calculating the difference in extinction coefficients between the oxidized and reduced forms and comparing this to the difference in extinction coefficients for wild-type AldO, we were able to calculate the amount of covalent FAD contained within the oxiperoxidase AldO-MP. The concentration of FAD was 18.5 μM, implying a 1:1 FAD/heme ratio (Table 1), which agrees with the fact that the Tat translocator exhibits quality-control mechanisms, meaning that only correctly flavinylated protein is transported into the periplasm by the Tat pathway.[43] The total protein concentration was also determined, by the Bradford method, and was in good agreement (20 ± 2 μM) with the FAD and heme determinations, indicating that all of the AldO-MP transported to the periplasm is in a fully holo form that contained both covalently bound FAD and covalently bound heme. We have hence produced a true oxiperoxidase: a hybrid enzyme that is fully covalently flavinylated and heminylated.

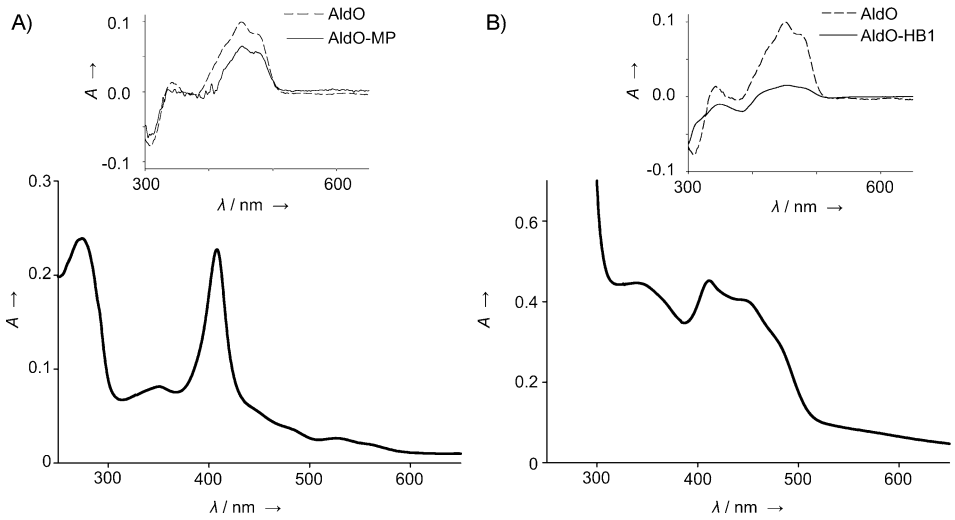

Figure 3. UV/Vis absorption spectra of the oxidase–peroxidase fusion constructs. A) AldO-MP (4.6 μM). B) AldO-HB1 (30 μM). AldO-MP and AldO-HB1 refer to the constructs shown in Figure 1. Inset: difference spectra of the oxidized and reduced forms of wild-type AldO (10.5 μM) and the oxidase–peroxidase fusion constructs (6.9 μM AldO-MP; 2.1 μM AldO-HB1) after reduction of FAD by xylitol.

The designed oxiperoxidase fusion hybrid can be used as a xylitol biosensor

An important aspect of the constructed AldO-MP hybrid (Figure 1) is its ability to act in concert; the peroxidase functionality can thus be used to detect and quantify the oxidase activity. Initial tests showed that coupled oxidase/peroxidase activity could be detected both in periplasmic extracts and with purified protein. A xylitol concentration of 2.3 mg L^{-1} (15 μM) was reliably detected with only 20 nM of AldO-MP in a colorimetric assay with dichloro-2-hydroxybenzenesulfonic acid (DCHBS) and 4-aminoantipyrine (AAP) as peroxidase substrate, indicating that a peroxidase fused to an oxidase can reliably be used to detect low levels of oxidase activity towards target substrates.

We were also interested in whether or not the oxiperoxidase could be used to detect in vivo oxidase activity. With a modification of the plate-based oxidase screening assay developed by Alexeev et al.[42] we showed that xylitol oxidase activity could be detected in whole cells expressing AldO-MP in the periplasm. This shows that the oxiperoxidase is fully functional in *E. coli* cells, endowing the bacterium with a new oxidative catalytic potential. The presence of a PstI cloning site between the *aldo* gene and the DNA encoding the microperoxidase also makes a directed evolution methodology possible, with, for example, subjection of the *aldo* gene to error-prone PCR and then cloning into the MP vector to enable whole-cell screening.

In conclusion, we have successfully created an oxiperoxidase: a hybrid protein that is expressed in the periplasm of *E. coli* and exhibits both oxidase and peroxidase activities. Thanks to the complementary natures of these two activities, our oxiperoxidase can also be employed to perform cascade chemistry, detecting oxidase activity directly with its peroxidase moiety both in vitro and in vivo.

An engineered CXXCH motif in AldO enables covalent heme attachment, merging peroxidase and oxidase activity in a single protein backbone

Bolstered by our success in creating a bifunctional oxidase–peroxidase hybrid by simply linking a short peptide encoding a microperoxidase to an oxidase, we wondered if we could go one step further and incorporate heme-binding motifs within the oxidase sequence. Inspired by landmark studies on the maturation and heme binding of cytochrome *c* peptides, from which it is known that only the short CXXCH motif is required for covalent heme attachment,[16] we hypothesized that it should be possible to mutate a portion of the AldO sequence to encode this motif, thus enabling it to bind heme covalently. In view of the possible mechanistic insights into the maturation of cytochromes and catalytic effects, such as the channeling of H_2O_2 from the reduced flavin to a heme moiety anchored nearby,[25] that we might gain, we decided to mutate a number of regions of the AldO protein into the CXXCH motif and to examine the effects location has on heme anchoring and enzyme activity. The covalent incorporation of FAD in oxidases is an autocatalytic process that occurs in the *E. coli* cytoplasm.[43,44] AldO is thus transported by the Tat pathway in a fully folded form and any heme incorporation at the chosen positions would give an indication of the degree of secondary structure that is tolerated by the Ccm apparatus.

Table 1. Oxidase–peroxidase fusions and their properties. Oxidase activity was determined with xylitol as substrate, peroxidase activity with dimethoxybenzidine as substrate. Numbering of heme-binding (HB) mutants and microperoxidase (MP) fusion refers to overview shown in Figure 1.

Construct	Activity		Kinetic parameters				Covalent binding?	
	oxidase	peroxidase	oxidase		peroxidase		FAD?	heme?
			k_{cat} [s^{-1}]	K_m [mM]	k_{cat} [s^{-1}]	K_m [mM]		
AldO-MP	yes	yes	9.1	0.29	9.1	12	yes (100%)	yes (100%)
AldO-HB1	yes	yes	12.2	0.35	n.d.	n.d.	yes (100%)	yes (15%)

n.d.: not determined.

From the crystal structure of AldO,[28] three regions were identified as candidates for covalent heme attachment. The first was the N terminus, due to its lack of secondary structure; in the crystal structure the first four amino acids are not visible, indicating they have a highly random, undefined structure (Figure 1, AldO-HB1). The second was a loop near the entrance to the active site, due to specific catalytic effects, such as H_2O_2 channeling, that might be observed upon heme binding (Figure 1, AldO-HB2). The last was a loop on the surface of the protein that displays a high degree of flexibility, as evidenced by the high B-factors of these residues. This loop was mutated at two positions to generate the heme-binding variants AldO-HB3 and AldO-HB4 (Figure 1). All four heme-binding mutants were expressed in the periplasm, contained covalently bound FAD, and exhibited xylitol oxidase activity, as was observed by SDS-PAGE analysis, fluorescence staining for the flavin cofactor, and oxidase activity measurements of periplasmic extracts (data not shown). On the basis of heme staining and peroxidase assays, only mutant AldOHB1 displayed peroxidase activity and contained covalently bound heme (Figure 2), thus acting as a true oxiperoxidase. AldOHB1 contained a C-terminal Strep tag to enable facile one-step purification with Streptactin Sepharose; approximately 100 μg of protein was purified from 1 L of culture. This was significantly less than the amount of AldO-MP that could be obtained from the same culture volume. It was clear from heme stains and activity assays that AldOHB1 contained both covalently bound FAD and covalently bound heme, and so we were interested in whether or not the degree of cofactor incorporation was as successful as for the oxidase–microperoxidase fusion AldO-MP. The absorption spectrum of AldOHB1 is very different from that of AldO-MP (Figure 3); the Soret band at 410 nm is much less pronounced and the underlying flavin spectrum is, as a result, much more visible. Pyridine hemochrome determination of the heme content revealed that only 15% of the hybrid, relative to the total protein content, contained heme (Table 1). As expected, because of the quality control carried out by the Tat translocator the protein was completely flavinylated; anaerobic reduction of FAD cofactor by xylitol and comparison of the oxidized and reduced spectra with those of wild-type AldO revealed an FAD content that was equal to the total protein content. The other AldO-HB variants encoding the CXXCH motif within loop regions on the AldO surface, however, do not bind any heme. This is in line with the hypothesis that the Ccm apparatus requires the heme-binding motif to contain no ordered secondary structure for the desired heme incorporation.[45]

Oxiperoxidases retain both oxidase and peroxidase activities

Once it was clear that the engineered oxidase–peroxidase hybrid contained both covalently bound FAD and covalently bound heme and, in the case of AldO-MP, could be used as a xylitol biosensor, we wanted to establish the catalytic competence of their oxidase and peroxidase domains. Steady-state kinetic measurements were performed with xylitol, the preferred AldO substrate.[26] Analysis of both purified oxiperoxidase hybrids, AldO-MP and AldO-HB1, revealed that the kinetic constants for xylitol were essentially unchanged in relation to wild-type AldO (Table 1): $K_m = 0.29$ and 0.35 mM for AldO-MP and AldO-HB1, respectively. The k_{cat} value for AldO-MP (9.1 s^{-1}) was slightly lower than that for AldO-HB1 (12.2 s^{-1}), but both values compared favorably with the k_{cat} value for wild-type AldO (13.0 s^{-1}).[26]

The peroxidase activity of AldO-MP was analyzed with dimethoxybenzidine (DMB, Table 1). Peroxidase activity could be detected and both oxiperoxidase hybrids displayed saturating activity at high H_2O_2 concentrations. The K_m value for H_2O_2 with DMB is relatively high at 12 ± 5 mM. The maximum rate is 9.1 ± 2.2 s^{-1}. These values compare favorably with published values for other microperoxidases,[16,46] which are all in this range. Slow decay of the peroxidase activity of our oxiperoxidases hybrids was observed over time, a feature common to proteins containing such exposed heme moieties.[20] The pH optimum of the peroxidase functionality of AldO-MP was also determined in Britton–Robinson buffer,[47] and as expected from studies performed with microperoxidases[16,46,48] the optimum lies at pH 8.5. The experiments were carried out at the pH optimum of AldO (pH 7.5),[7] at which the peroxidase functionality displayed roughly 60% of it optimal activity. Taken together, these results show that oxidase and peroxidase functionalities can successfully be combined on a single protein scaffold, without negatively influencing the activity of the other functionality, to create a novel and artificial hybrid enzyme that we have named an oxiperoxidase.

Conclusion

We have engineered novel enzyme functionalities through the fusion of genes and protein-encoding DNA fragments. By fusing the gene for an oxidase to a synthetic oligonucleotide encoding the heme-binding CXXCH motif, we generated a novel type of fusion protein with two complementary activities: an oxidase–peroxidase fusion, which we call an oxiperoxidase. Both redox cofactors (heme and FAD) were covalently bound to the protein and were present in a 1:1 ratio to the protein backbone. Furthermore, we have shown that the both the oxidase and peroxidase activities are maintained and that the oxiperoxidase functions as a cascade catalyst both in vivo and in vitro. In an attempt to reduce the size of the oxiperoxidase, we created a truly chimeric enzyme by mutating only three residues of the flavoprotein oxidase AldO. By mutating three residues in the AldO N terminus into the CXXCH heme-binding motif we created a functional oxiperoxidase. After export to the *E. coli* periplasm and co-expression with the Ccm apparatus, heme was successfully incorporated into this construct, albeit with a much lower efficiency: only 15% of the secreted protein contained heme, in comparison with a 100% incorporation rate for the larger fusion variant. These data support current insights into the Ccm apparatus: the heme-binding motif is the only prerequisite for covalent heme incorporation, but this motif cannot be contained in a (partially) folded holo structure. All known proteins that contain this heme-binding motif are transported by the Sec pathway,[49] so this study

is, to the best of our knowledge, the first instance in which the Ccm apparatus has been used in conjunction with a protein exported by the Tat pathway. It has in fact been shown that the speed at which a protein folds after it has been transported to the periplasm is imperative for recognition and covalent coupling of heme. Studies performed with DsbD, a rapidly folding *E. coli* transmembrane oxidoreductase, mutated to contain the CXXCH motif, revealed that only a very small percentage (0.2%) contained covalently bound heme.[45] The speed at which DsbD adopts its native conformation in the *E. coli* periplasm is rapid enough for it to avoid heme incorporation by the Ccm apparatus. This agrees with the findings presented here, in which only a small portion of the AldO-HB1 hybrid contains covalently bound heme. According to the crystal structure, the N terminus is freely accessible and its atoms have very high B-factors, thus indicating that they have a relatively large freedom of movement. Proximity to a folded structure seems to inhibit the incorporation of heme; this provides an explanation of why only 15% of the exported protein contains covalently bound heme. Incorporation of the heme-binding motif in loop regions of the protein, although yielding stable AldO, did not lead to detectable levels of heme-bound protein. It is clear that there are too many secondary-structure elements in these regions that force the CXXCH motif to adopt a detrimental conformation and prevent covalent heme coupling by the Ccm apparatus. Whereas it was known that heme could bind to very short peptides containing only the CXXCH motif,[16] fusing one of these peptides to the AldO backbone to create an oxidase–peroxidase fusion was sufficient to enable complete heminylation; here the short peptide serves as a linker and the folded AldO protein does not inhibit the covalent coupling of heme. Earlier studies in which a microperoxidase was fused to the maltose binding protein MBP showed that the degree of heme incorporation was much lower than the 100% we observe for our oxidase–peroxidase fusion,[37] but this is probably due to the maximum capacity of the Ccm apparatus being reached because the amount of MBP secreted by the Sec pathway is significantly higher than the amount of AldO secreted by the Tat pathway.[29,50]

Molecular biology supplies us with the technology to develop chimeric enzymes with novel and complementary functionalities. The merging of two complementary enzyme activities presented here to create a new breed of enzyme, an oxiperoxidase, is to the best of our knowledge the first time that an oxidase and a peroxidase have been engineered onto a single protein backbone. The oxiperoxidase opens the door to a wide variety of biosensor applications based on such artificial enzyme hybrids.

Experimental Section

Strains, plasmids and growth conditions: *E. coli* strain MC1061 was used for all routine cloning tasks. *E. coli* strain BL21(DE3) harboring plasmid pEC86[38] and the construct under study was used to express the oxidase–peroxidase fusions. pBAD-Tat-AldO was used to construct the various oxidase–peroxidase hybrids.[29] Growth media contained the antibiotics ampicillin (100 $\mu g\,mL^{-1}$) and chloramphenicol (34 $\mu g\,mL^{-1}$). For expression, Terrific Broth (1 L) was inoculated with the appropriate strain (1 mL) grown to saturation at 37 °C, induced by the addition of L-arabinose (final concentration 0.2%, *w/v*) and incubated at 17 °C with orbital shaking for 65 h.

Construction of hybrids: The sequence encoding microperoxidase MP292[37] was obtained from Sigma–Aldrich as two complementary DNA strands with PstI and SalI overhangs. The strands were allowed to dimerize by mixing aliquots of DNA (5 μL, 100 μM), incubating at 72 °C for 2 min, and then cooling to 22 °C over 45 min with use of a PCR thermocycler. Dimerized DNA was ligated into PstI- and SalI-digested pBAD-Tat-AldO, generating pBAD-Tat-AldO-MP. A Strep tag (WSHPQFEK) was introduced at the C terminus of the *aldo* gene in pBAD-Tat-AldO and at the N terminus of the *aldo* gene in pBAD-Tat-AldO-MP by use of PCR, to generate pBAD-Tat-Strep-AldO and pBAD-Tat-Strep-AldO-MP (AldO-MP in Figure 1). Mutants of AldO containing the heme-binding CXXCH motif (AldOHB1-AldOHB4 in Figure 1) were generated by PCR (AldOHB1), and QuikChange PCR (AldOHB2-AldOHB4). For AldOHB1, residues 1–5 were mutated into the heme-binding motif, whereas for AldOHB2, AldOHB3, and AldOHB4, residues 280–284, 232—236, and 235–239, respectively, were mutated into the CXXCH motif by QuikChange PCR with use of plasmids pBAD-Tat-AldO-Strep as template. Primer sequences are available on request. All constructs were confirmed by DNA sequencing (GATC Biotech AG, Konstanz, Germany).

Protein fractionation and purification: Cells expressing oxidase–peroxidase fusions were grown as described above. Periplasmic extracts were prepared by the osmotic shock method. Briefly, cells were harvested (20 OD_{600} units) by centrifugation (5000 *g*, 10 min, 4 °C) and washed with ice-cold Tris·HCl (pH 7.4, 10 mM). Pelleted cells were resuspended in Tris·HCl (pH 8.0, 10 mM, 80 μL) containing sucrose (25%, *w/v*), EDTA (pH 8.0, 20 mM, 10 μL), and lysozyme (10 μL, 5 $mg\,mL^{-1}$) and incubated on ice for 20 min. After centrifugation (12000 *g*, 30 min, 4 °C) the supernatant was collected and designated as the periplasmic extract. For large culture volumes the above periplasmic fractionation protocol was scaled according to the number of OD_{600} units harvested. Strep-tag labeled proteins were purified from the periplasmic extract with use of Strep-Tactin Sepharose (IBA GmbH, Göttingen, Germany) according to the manufacturer's instructions. Eluted fractions were pooled, buffer-exchanged to potassium phosphate buffer (50 mM, pH 7.5), and concentrated.

Analytical methods: Unless stated otherwise, all experiments were performed at 25 °C and in potassium phosphate buffer (pH 7.5, 50 mM). All measurements were carried out in triplicate, and the standard deviation value in the experiments is 5%, unless stated otherwise. Heme binding constructs were detected in SDS-PAGE gels by using the intrinsic peroxidase activity of heme; gels were immersed in water (50 mL) containing 3,3′-dimethoxybenzidine dihydrochloride (DMB, 50 mg, 0.16 mmol) and H_2O_2 (0.7%, *v/v*). Total protein content was calculated by the Bradford method with BSA as standard. Absolute heme concentrations were determined by the pyridine ferro-hemochrome method, with use of the extinction coefficient of pyridine hemochrome *c* ($\varepsilon_{550\,nm} = 30.27\ mM^{-1}\,cm^{-1}$).[39] The oligomeric form of the fusion constructs was investigated by size-exclusion chromatography as described previously.[13] Immunoblotting experiments to verify the fractionation procedure were carried out with use of antisera against AldO and DnaK as described by van Bloois et al.[29]

Determination of FAD content: The FAD content was calculated by measuring the difference in the extinction coefficients of the flavin in its oxidized and reduced forms. In an Applied Photophysics stopped-flow apparatus (model SX17MV), absorption spectra were collected at 2.5 ms intervals with the aid of a diode array detector. The reductive half-reaction of wild-type AldO was followed as described previously[26] by reduction of the flavin cofactor under anoxic conditions with an excess of xylitol. From the resulting absorption spectra the difference in the the extinction coefficients of the FAD in the oxidized and reduced forms ($\Delta\varepsilon_{ox-red}$) at 451 nm was calculated. Subsequently, the reductive half reaction of the relevant hybrids was followed in an identical fashion. The absorbance difference at 451 nm was extracted from the resulting spectra and the FAD content of the hybrids was determined by use of the $\Delta\varepsilon_{ox-red}$ value calculated for wild-type AldO.

Activity assays: Oxidase activity was measured by a HRP-coupled assay described previously.[26] Peroxidase activity was measured either with DMB (1 mM) or with DCHBS and AAP (1 mM and 0.1 mM, respectively) as peroxidase substrate. Product formation was monitored spectrophotometrically at 460 nm (ε_{DMB} = 11.3 $mM^{-1}cm^{-1}$) or 515 nm ($\varepsilon_{DCHBS/AAP}$ = 26 $mM^{-1}cm^{-1}$). Initial reaction rates were calculated and used to determine Michaelis–Menten kinetic parameters. In vivo oxidase activity of cells expressing AldO-MP was measured by a modification of the plate-based assay developed by Alexeev et al.:[42] the lysis step and addition of HRP were omitted and xylitol and DMB (both 1 mM) were used as oxidase and peroxidase substrates, respectively.

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[1] N. J. Turner, *Chem. Rev.* **2011**, *111*, 4073 – 4087.
[2] D. P. H. M. Heuts, N. S. Scrutton, W. S. McIntire, M. W. Fraaije, *FEBS J.* **2009**, *276*, 3405 – 3427.
[3] N. G. Leferink, D. P. Heuts, M. W. Fraaije, W. J. van Berkel, *Arch. Biochem. Biophys.* **2008**, *474*, 292 – 301.
[4] M. R. Romero, F. Ahumada, F. Garay, A. M. Baruzzi, *Anal. Chem.* **2010**, *82*, 5568 – 5572.
[5] G. S. Wilson, R. Gifford, *Biosens. Bioelectron.* **2005**, *20*, 2388 – 2403.
[6] M. W. Cook, H. V. Thygesen, *Food Chem. Toxicol.* **2003**, *41*, 523 – 529.
[7] E. W. van Hellemond, L. Vermote, W. Koolen, T. Sonke, E. Zandvoort, D. P. H. M. Heuts, D. B. Janssen, M. W. Fraaije, *Adv. Synth. Catal.* **2009**, *351*, 1523 – 1530.
[8] Y. C. Liu, Y. S. Li, S. Y. Lyu, L. J. Hsu, Y. H. Chen, Y. T. Huang, H. C. Chan, C. J. Huang, G. H. Chen, C. C. Chou, M. D. Tsai, T. L. Li, *Nat. Chem. Biol.* **2011**, *7*, 304 – 309.
[9] F. Hollmann, I. W. C. E. Arends, K. Buehler, A. Schallmey, B. Buhler, *Green Chem.* **2011**, *13*, 226 – 265.
[10] C. Regalado, B. E. García-Almendárez, M. A. Duarte-Vázquez, *Phytochem. Rev.* **2004**, *3*, 243 – 256.
[11] S. Y. An, S. K. Min, I. H. Cha, Y. L. Choi, Y. S. Cho, C. H. Kim, Y. C. Lee, *Biotechnol. Lett.* **2002**, *24*, 1037 – 1040.
[12] E. Antipov, A. E. Cho, K. D. Wittrup, A. M. Klibanov, *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 17694 – 17699.
[13] E. van Bloois, D. E. T. Pazmino, R. T. Winter, M. W. Fraaije, *Appl. Microbiol. Biotechnol.* **2010**, *86*, 1419 – 1430.
[14] T. D. Bugg, M. Ahmad, E. M. Hardiman, R. Singh, *Curr. Opin. Biotechnol.* **2011**, *22*, 394 – 400.
[15] M. Hofrichter, R. Ullrich, M. J. Pecyna, C. Liers, T. Lundell, *Appl. Microbiol. Biotechnol.* **2010**, *87*, 871 – 897.
[16] M. Braun, L. Thony-Meyer, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 12830 – 12835.
[17] W. M. Huang, J. B. Jia, Z. L. Zhang, X. J. Han, J. L. Tang, J. G. Wang, S. J. Dong, E. K. Wang, *Biosens. Bioelectron.* **2003**, *18*, 1225 – 1230.
[18] R. E. Sharp, C. C. Moser, F. Rabanal, P. L. Dutton, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 10465 – 10470.
[19] H. K. Rau, N. DeJonge, W. Haehnel, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 11526 – 11531.
[20] A. Spector, W. Zhou, W. C. Ma, C. F. Chignell, K. J. Reszka, *Exp. Eye Res.* **2000**, *71*, 183 – 194.
[21] E. N. Kadnikova, N. M. Kostic, *J. Org. Chem.* **2003**, *68*, 2600 – 2608.
[22] I. Willner, E. Katz, F. Patolsky, A. F. Buckmann, *J. Chem. Soc. Perkin Trans. 2* **1998**, 1817 – 1822.
[23] P. Ander, L. Marzullo, *J. Biotechnol.* **1997**, *53*, 115 – 131.
[24] T. K. Kirk, R. L. Farrell, *Annu. Rev. Microbiol.* **1987**, *41*, 465 – 505.
[25] Z. Y.-H. Percival, *Biotechnol. Adv.* **2011**, *29*, 715 – 725.
[26] D. P. Heuts, E. W. van Hellemond, D. B. Janssen, M. W. Fraaije, *J. Biol. Chem.* **2007**, *282*, 20283 – 20291.
[27] R. Baron, C. Riley, P. Chenprakhon, K. Thotsaporn, R. T. Winter, A. Alfieri, F. Forneris, W. J. H. van Berkel, P. Chaiyen, M. W. Fraaije, A. Mattevi, J. A. McCammon, *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 10603 – 10608.
[28] F. Forneris, D. P. Heuts, M. Delvecchio, S. Rovida, M. W. Fraaije, A. Mattevi, *Biochemistry* **2008**, *47*, 978 – 985.
[29] E. van Bloois, R. T. Winter, D. B. Janssen, M. W. Fraaije, *Appl. Microbiol. Biotechnol.* **2009**, *83*, 679 – 687.
[30] N. C. Veitch, *Phytochemistry* **2004**, *65*, 249 – 259.
[31] Z. L. Lin, T. Thorsen, F. H. Arnold, *Biotechnol. Prog.* **1999**, *15*, 467 – 471.
[32] A. T. Smith, N. Santama, S. Dacey, M. Edwards, R. C. Bray, R. N. F. Thorneley, J. F. Burke, *J. Biol. Chem.* **1990**, *265*, 13335 – 13343.
[33] Y. Sugano, *Cell. Mol. Life Sci.* **2009**, *66*, 1387 – 1403.
[34] J. N. Roberts, R. Singh, J. C. Grigg, M. E. P. Murphy, T. D. H. Bugg, L. D. Eltis, *Biochemistry* **2011**, *50*, 5108 – 5119.
[35] J. Grove, S. Tanapongpipat, G. Thomas, L. Griffiths, H. Crooke, J. Cole, *Mol. Microbiol.* **1996**, *19*, 467 – 481.
[36] L. Thony-Meyer, F. Fischer, P. Kunzler, D. Ritz, H. Hennecke, *J. Bacteriol.* **1995**, *177*, 4321 – 4326.
[37] M. Braun, I. G. Rubio, L. Thony-Meyer, *Appl. Microbiol. Biotechnol.* **2005**, *67*, 234 – 239.
[38] E. Arslan, H. Schulz, R. Zufferey, P. Kunzler, L. Thony-Meyer, *Biochem. Biophys. Res. Commun.* **1998**, *251*, 744 – 747.
[39] E. A. Berry, B. L. Trumpower, *Anal. Biochem.* **1987**, *161*, 1 – 15.
[40] P. Macheroux in *Flavoprotein Protocols* (Eds.: S. K. Chapman, G. A. Reid), Humana, Totowa, **1999**, 1 – 7.
[41] M. P. DeLisa, D. Tullman, G. Georgiou, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 6115 – 6120.
[42] M. Alexeeva, A. Enright, M. Dawson, M. Mahmoudian, N. Turner, *Angew. Chem.* **2002**, *114*, 3309 – 3312; *Angew. Chem. Int. Ed.* **2002**, *41*, 3177 – 3180.
[43] J. Jin, H. Mazon, R. H. H. van den Heuvel, A. J. Heck, D. B. Janssen, M. W. Fraaije, *FEBS J.* **2008**, *275*, 5191 – 5200.
[44] A. Hassan-Abdallah, R. C. Bruckner, G. H. Zhao, M. S. Jorns, *Biochemistry* **2005**, *44*, 6452 – 6462.
[45] D. A. L. Mavridou, M. Braun, L. Thony-Meyer, J. M. Stevens, S. J. Ferguson, *Biochem. Soc. Trans.* **2008**, *36*, 1124 – 1128.
[46] J. L. Primus, M. G. Boersma, D. Mandon, S. Boeren, C. Veeger, R. Weiss, I. M. C. M. Rietjens, *J. Biol. Inorg. Chem.* **1999**, *4*, 274 – 283.
[47] H. T. S. Britton, R. A. Robinson, *J. Chem. Soc.* **1931**, 1456 – 1462.
[48] M. T. Wilson, R. J. Ranson, P. Masiakowski, E. Czarnecka, M. Brunori, *Eur. J. Biochem.* **1977**, *77*, 193 – 199.
[49] L. Thony-Meyer, P. Kunzler, *Eur. J. Biochem* **1997**, *246*, 794 – 799.
[50] R. B. Kapust, D. S. Waugh, *Protein Sci.* **1999**, *8*, 1668 – 1674.

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