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Characterization of the Peroxidase Activity of CYP119, a Thermostable P450 From *Sulfolobus acidocaldarius*

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We report the cloning, expression, and purification of CYP119, a thermostable enzyme previously thought to derive from Sulfolobus solfataricus*. Sequence analysis suggested that, in contrast to the conclusions of earlier studies, the enzyme stems from the closely related* Sulfolobus acidocaldarius*, and we were indeed able to clone the gene from the genomic DNA of this organism. For the first time, we report here on the peroxidase activity of this enzyme and the optimization of the associated reaction parameters. The optimized reaction conditions were then applied to the biocatalytic epoxidation of styrene. The values obtained for* k_{cat} *(78.2±20.6 min^{-1}) and* K_M *(9.2±4.3 mM) indicated an approximately 100-fold increased catalytic activity over previously reported results.*

Introduction

Bacterial cytochrome P450 enzymes (P450s) catalyze a wide range of reactions including aliphatic and aromatic hydroxylation, epoxidation, oxidative phenolic coupling, heteroatom oxidations, and dealkylations, often in a regio- and stereoselective manner.[1] This makes them interesting candidates for the production of fine chemicals otherwise difficult to synthesize by standard chemical means. This is particularly true for thermostable P450 enzymes, which reveal high stabilities and activities even under the process conditions necessary for industrial biocatalytic processes. The enzyme CYP119 was one of the first thermostable P450 enzymes to be cloned[2,3] and to have its molecular structure determined by X-ray crystallography.[4–6] This enzyme belongs among the most extensively studied thermostable enzymes—from the cytochrome P450 family in particular—to date.[7–9] Although the endogenous substrate for CYP119 is not known, it has been demonstrated that, for instance, the enzyme binds and catalyzes the epoxidation of styrene[10] and can hydroxylate lauric acid.[11] Furthermore, it has also been used for the electrochemical reduction of nitrite, nitric oxide, and nitrous oxide,[12] as well as for the electrochemical dehalogenation of CCl_4 to yield CH_4.[13] Because the hydroxylation reaction only takes place in the presence of electron acceptor proteins—in particular, putidaredoxin and putidaredoxin reductase—various electron acceptor proteins from two thermophilic microorganism sources (*Sulfolobus tokodaii*[14] and *Sulfolobus solfataricus*[15]) have been explored. Since CYP119 was initially cloned from *S. solfataricus* strain P1,[2] the proteins of this strain were considered to be the native electron donor partners.[15] However, a BLAST search of the complete genome of *S. solfataricus* strain P2[16] does not locate the DNA sequence of the enzyme CYP119 within the deposited database genome of *S. solfataricus* strain P2. Although the genome of the P1 strain has not yet been sequenced, it is unlikely that it contains CYP119 because in the genome of the P2 strain not even a homologous sequence can be found. Instead, the BLAST database search located the gene of CYP119 in the genome of the closely related organism *Sulfolobus acidocaldarius*, recently sequenced by Chen et al.[17]

Here we report the cloning, expression, and purification of CYP119 from the genomic DNA of *S. acidocaldarius*. Furthermore, we have analyzed the peroxidase activity of this enzyme and have carried out an optimization of the associated reaction conditions. The optimized conditions were then used to increase the activity in the peroxide-dependent epoxidation of styrene.

Results and Discussion

To obtain access to the thermostable cytochrome P450 CYP119, we initially tried to amplify the encoding gene from the extremophile organism *Sulfolobus solfataricus* P1 strain (ATCC 35091), as described by Wright et al.[2] To this end, the organism *Sulfolobus solfataricus* P1 strain was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, strain No. DSM 1616), and PCR with the primers reported in the literature[2] was carried out with purified genomic DNA of the *S. solfataricus* P1 strain. No PCR products were obtained in several independent trials, even when different batches of the *S. solfataricus* P1 strain culture were used to produce the genomic DNA (Figure 1). Database research on the complete genome of *S. solfataricus* P2 strain, available from the genome sequencing project reported by She et al.,[16] revealed that this close relative of the *S. solfataricus* P1 strain contained neither the sequence encoding for CYP119, nor any homologous gene. In contrast, however, the genome of the closely related *Sulfolobus acidocaldarius*, the complete genome

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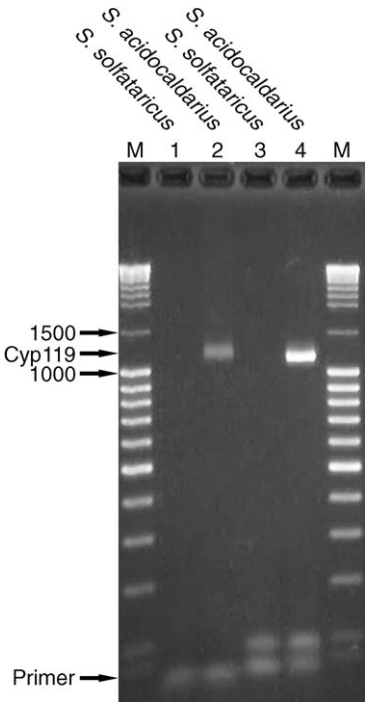

Figure 1. Gel electrophoretic analysis of the PCR reactions amplifying the gene for CYP119 from different sources and with different primers. M) MassRuler ladder from Fermentas. 1) *S. solfataricus* genomic DNA, primers as published.[2] 2) *S. acidocaldarius* genomic DNA, primers as published.[2] 3) *S. solfataricus* genomic DNA, primers as described in Experimental Section. 4) *S. acidocaldarius* genomic DNA, primers as described in Experimental Section. The numbers indicate the numbers of base pairs.

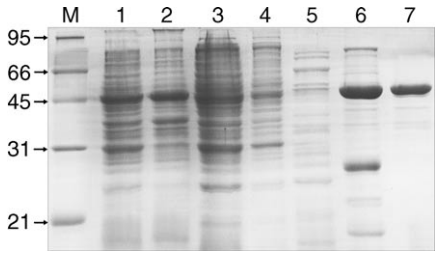

Figure 2. Gel electrophoretic analysis of the purification of CYP119. M) SDS-Broadrange Marker from Bio-rad. 1) Crude cell extract before sonification. 2) Supernatant after cell lysis. 3) Pellet after cell lysis. 4) Supernatant after heat shock. 5) Pellet after heat shock. 6) Eluate from Ni-NTA column. 7) Eluate from MonoQ column. The numbers indicate the corresponding molecular weight in kDa.

sequence of which was recently described by Chen et al.,[17] contains exactly the sequence encoding for the CYP119. This led us to the hypothesis that the *S. solfataricus* P1 strain isolate used for the initial cloning (ATCC 35091)[2] had actually been contaminated with the *S. acidocaldarius* species. This hypothesis was supported by information from the DSMZ, stating that the original stock of DSM 1616 at the DSMZ had been replaced in April 1989 with a pure isolate of *S. solfataricus* P1, due to the contamination of DSM 1616 with *S. acidocaldarius* (Dr. Stefan Spring, DSMZ, personal communication). Thus, the chances were that this might not also have been done for the ATCC 35091. Indeed, when *S. acidocaldarius* (DSM 639) was used as the source of genomic DNA for PCR amplification of the CYP119 gene, the formation of the expected, approx. 1200 bp, PCR product suggested that *S. acidocaldarius* is the real source of the CYP119, rather than the *S. solfataricus* P1 strain (Figure 1). Further confirmation was obtained by DNA sequence analysis in the course of cloning of the PCR product into the recombinational Gateway cloning vector system. Introduction of the PCR product into the Gateway system vector pDONR221 and subsequent transfer of the gene to the pET-DEST42 vector yielded the expression vector pET-EXP42-CYP119, which was then used for overexpression and purification of the desired CYP119 enzyme. SDS-PAGE of the purification product showed a single band for the pooled eluate from the MonoQ column (Figure 2). Therefore, the successful cloning and expression of CYP119 from *S. acidocaldarius* now allowed us to conduct studies on the enzyme's chemical reactivity.

CYP119 is known to oxidize styrene in a H_2O_2-dependent reaction[10] and to hydroxylate lauric acid in the presence of putidaredoxin and putidaredoxin reductase,[11] or otherwise in a reconstituted system comprising 2-Oxoacid:Ferredoxin Oxidoreductase (OFOR) and Ferredoxin (Fdx) from either *S. tokodaii* or *S. solfataricus*, pyruvate, and coenzyme A (CoA).[15] Because CYP119 originates from *S. acidocaldarius* rather than from *S. solfataricus*, as previously assumed,[2,15] we reasoned that both reconstituted systems are in fact nonphysiological. In particular, the fact that no homologous enzyme of CYP119 can be found in the complete genome of *S. solfataricus*[17] suggested that the electron partners used in the reconstituted system had not been optimized to accept electrons from CYP119 by natural evolution, and hence that an optimization of the reaction conditions for this enzyme might prove useful.

Although not yet reported, we initially investigated whether CYP119 has a peroxidase activity, since this reactivity might be induced by using H_2O_2 without the necessity for proteins or other cofactors as oxidants, and also because similar reactivities had been reported for other P450 enzymes previously.[18] Through the choice of AmplexRed as the substrate, any potential peroxidase activity could be conveniently monitored by fluorescence spectroscopy in multiwell plate assays.

Indeed, the CYP119 displayed significant peroxidase activity, and we started to optimize the reaction conditions by initially analyzing its temperature dependence (Figure 3). Previous studies had shown that the enzyme's activity in the hydroxylation of lauric acid was highest at around 70 °C.[14,15] Carrying out a similar assessment of peroxidase activity using AmplexRed, we found a similar peak in activity at around 75 °C, and also the similarly greater reactivity (by about a factor of ten) when the rates at 25 °C and 75 °C are compared, as reported previously for the hydroxylation of lauric acid.[14,15] In contrast to the previously reported data, however, we observed a significantly faster decrease in enzyme activity at higher temperatures. This is likely to have been caused by the high concentration of H_2O_2 (5 mM) in our peroxidase reaction assay, which, at the elevated temperatures, leads to significantly harsher reaction conditions than those in the reported hydroxylation assay without H_2O_2.[14,15]

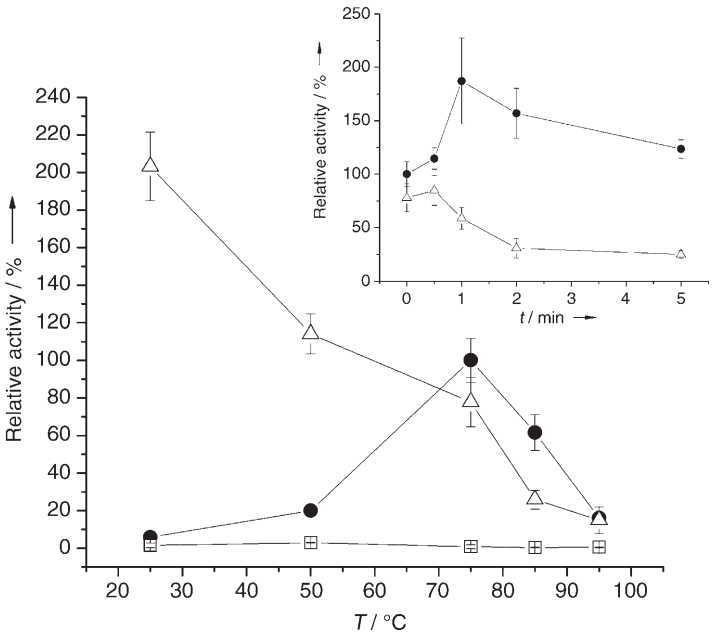

Figure 3. Temperature dependence of the initial rate of peroxidation of AmplexRed in the presence of Cyp152A1 (△), Cyp119 (●), or no enzyme (□). The inset shows the time dependence of peroxidation activity of Cyp152A1 (△) and Cyp119 (●) upon incubation at 75 °C. Error bars each represent the standard deviation of three independent experiments. The v_0/E_0 value for a reaction mixture containing Cyp119 (1.25 μM), H_2O_2 (5 mM), AmplexRed (10 μM), KP_i (50 mM), pH 7.0 at 75 °C was set as 100% relative reactivity.

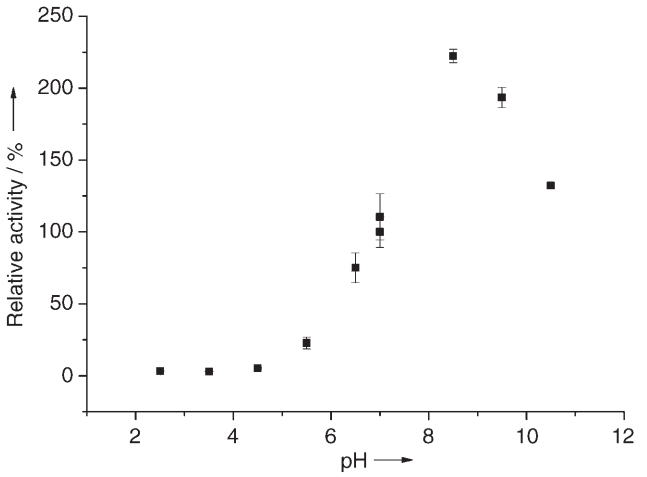

Figure 4. pH dependence of the initial rate of peroxidation of AmplexRed by Cyp119 in the presence of H_2O_2. Error bars each represent the standard deviation of three independent experiments. The v_0/E_0 value for a reaction mixture containing Cyp119 (1.25 μM), H_2O_2 (5 mM), AmplexRed (10 μM), KP_i (50 mM), pH 7.0 at 25 °C was set as 100% relative reactivity.

In our experiments we used CYP152A1 (cytochrome $P450_{BS\beta}$) for comparison, because this enzyme is known for its peroxidase activity.[18] It is shown in Figure 3 that, as expected, the CYP152A1 revealed a constant decrease in peroxidase activity with increasing temperature. This result indicates that the higher reactivity observed for CYP119 at 75 °C is indeed due to the thermostability of CYP119 rather than just to a general acceleration of chemical reactions at higher temperatures. Furthermore, continuous incubation at 75 °C revealed that CYP152A1 is inactived over time, which in contrast does not occur with CYP119 (inset in Figure 3). After 5 min of incubation the difference in relative activities was around 100%, while standard deviation and standard error (not depicted) had values of 7% and 4%, respectively.

To optimize the reaction conditions further, the pH was varied over a wide range (Figure 4). At low pH values CYP119 showed almost no reactivity, but this constantly increased upon raising the pH towards pH 8.5. At this pH value the peroxidase activity was highest, further increases in pH leading to decreasing activities. Because the activity at pH 7.0 in phosphate buffer (50 mM) was similar to that observed in glycine buffer (50 mM), we concluded that the buffering substance did not significantly affect the reactivity of the CYP119. The profile of the dependence of reactivity against the pH significantly differed from the published data for the hydroxylation reaction of lauric acid, in which the enzyme showed highest activity at around pH 4.5.[15] This difference is probably due to the different reaction type (peroxidation vs. hydroxylation) and, in particular, to the fact that the hydroxylation was achieved in a reconstituted system consisting of OFOR-*Ss*, Fdx-*Ss*, pyruvate, and CoA. It is possible that either of the enzymes and/or cofactors involved were the limiting factor/the factors that determined the pH optimum. Because they comprise the essential electron-accepting system, the highest hydroxylation activity observed at pH 4.5 might only reflect the optimal pH value for the entire system, rather than for the isolated CYP119 itself. Our approach, to study the activity of the CYP119 in the absence of other enzymes and cofactors, thus allowed us to perform a more direct assessment of the enzyme's properties.

For further optimization, we also investigated two other electron acceptors: cumene hydroperoxide (CHP) and *tert*-butyl hydroperoxide (TBHP). Both oxidants had already been used with P450 enzymes because they are suitable substitutes of the natural electron acceptors in the enzymatic reaction[19–23] and they are less aggressive towards the enzyme. The reaction conditions used for CHP- or TBHP-peroxidation were similar to those used in the H_2O_2 experiments (1.25 μM CYP119, 10 μM AmplexRed, 5 mM of the electron acceptor in 50 mM KP_i, pH 7.0 at 25°). As shown in Figure 5, both CHP and TBHP were found to be suitable electron acceptors for the CYP119-mediated peroxidation of AmplexRed. In fact, the initial activities of CYP119 in the presence of TBHP and CHP were two- and five-fold higher than that observed with H_2O_2. This is in agreement with earlier studies, in which an increased activity of P450 in the presence of CHP as the electron acceptor was observed.[20]

We also investigated whether the peroxide also has an influence on the stability of the CYP119. To this end, CYP119 was incubated under the same conditions as above in the presence of the corresponding oxidant (5 mM) while the peroxidase substrate was omitted. AmplexRed was added after 0, 1, 2, 4, 8, and 16 min, and v_0 was determined. As shown in Figure 5, the activities decreased significantly within the first few minutes in the cases of all three oxidants. In the cases both of H_2O_2 and of TBHP, the activities dropped to approximately 40% of the

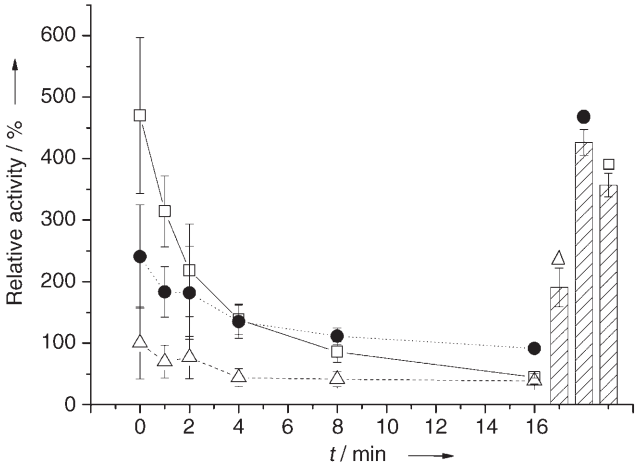

Figure 5. Stability of Cyp119 in buffer containing 5 mM H_2O_2 (△), TBHP (●), and CHP (□) over time. The initial rate of peroxidation is depicted. The bars represent the activity restored by addition of fresh enzyme after 16 min of preincubation. Note that the bars' heights exceed those of the curves at 0 min, due to the addition of fresh enzyme. Subtraction of the remaining activity after 16 min from the corresponding values after the addition of fresh enzyme yields data similar to the initial activity. The error bars each represent the standard deviation of at least three independent experiments. The v_0/E_0 for a reaction mixture containing Cyp119 (1.25 μM), H_2O_2 (5 mM), AmplexRed (10 μM), KP_i (50 mM), pH 7.0 at 25 °C was set as 100% relative reactivity

initial values after 16 min, while in the case of CHP the reactivity was reduced to approximately 10% of the initial activity. Thus, TBHP increased both turnover rate and the stability of the enzyme under the oxidizing reaction conditions. These results are in agreement with recent studies on the influence of peroxides on the reactivity of CYP152A1 towards polycyclic aromatic hydrocarbons and drugs,[24] in which the use of TBHP also led to an increase in reaction rate and enzyme stability. TBHP therefore appears to be highly suitable as an oxidizing agent for screening purposes in which long incubations might be needed to produce sufficient amounts of product for analysis.

We also confirmed that the decrease in enzyme activity was not caused by depletion of the oxidant. This was done by adding fresh enzyme together with Amplex Red after the enzyme had been incubated for 16 min. As indicated by the bars in Figure 5, the remaining peroxide concentration was easily sufficient to restore the initial enzymatic activities.

Because CYP119 is known to oxidize styrene to styrene epoxide in a peroxide-dependent reaction without the assistance of other enzymes,[10] we reasoned that this epoxidation reaction might be improved by applying the optimized reaction conditions determined for the peroxidase reaction. To investigate this hypothesis, we carried out the epoxidation at 70 °C, pH 8.5 in the presence of TBHP as the oxidant. The rate of styrene epoxidation was determined by HPLC analysis with the aid of external calibration curves prepared with pure styrene epoxide. For reasons of comparison we used an enzyme concentration identical to that used in an earlier report.[10] Kinetic constants were determined by Michaelis–Menten analysis with use of variable concentration of the styrene substrate. Values of $k_{cat} = 78.2 \pm 20.6\ min^{-1}$ and $K_M = 9.2 \pm 4.3$ mM were found.[25] On the assumption that the v_{max} value of 0.6 nmol min^{-1} nmol^{-1} reported earlier[10] can be compared with our k_{cat} value, because both values have similar units, our optimized conditions led to about a 100-fold increase in the turnover number. Furthermore, use of amounts of enzyme similar to those used in the previous study[10] led to almost complete conversion of the substrate within only 10 min (Figure 6), while the yields in the earlier study were still increasing after more than 30 min.[10] We believe that this significant increase in turnover is likely due to the optimized reaction conditions, with higher reaction temperature and optimized pH, in addition to the increased stability of the enzyme in the presence of TBHP as the oxidant.

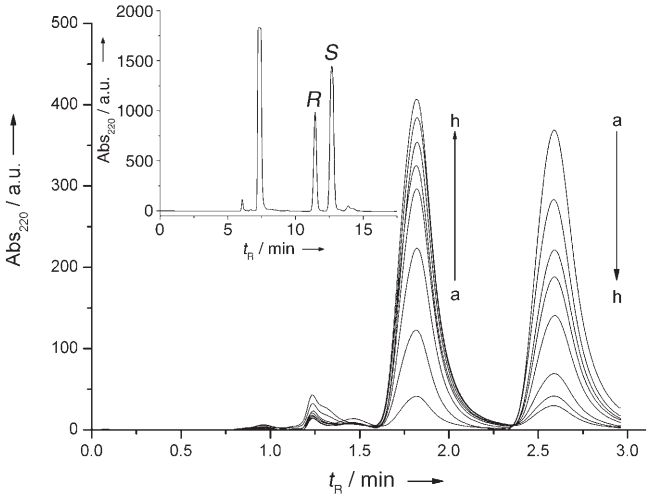

Figure 6. HPLC analysis of the epoxidation of styrene ($t_R = 2.65$ min) to styrene epoxide ($t_R = 1.8$ min) followed at 220 nm at different time points: a) 0 min, b) 15 s, c) 30 s, d) 45 s, e) 1 min, f) 2 min, g) 5 min, h) 10 min. The initial styrene concentration was 6.25 mM. The inset shows the chiral HPLC analysis of the epoxidation of styrene ($t_R = 7$ min) to (*R*)-styrene epoxide ($t_R = 11.7$ min) and (*S*)-styrene epoxide ($t_R = 13$ min).

We also analyzed the enantioselectivity of CYP119 by chiral phase chromatography. An approximately 2:1 preference for the formation of the (*S*) enantiomer was observed (inset in Figure 6), while in earlier studies a ratio of 3:1 (*S*/*R*) had been found.[10] This difference might be attributable to the higher temperature used here, which might induce less tight binding of the substrate in the enzyme's active center.

Conclusions

In conclusion, our results clarify that CYP119 should be considered a protein from *S. acidocaldarius* rather than from *S. solfataricus*. We further report here, for the first time, on the peroxidation activity and the optimization of this reaction. The optimal reaction parameters found were applied in the epoxidation reaction, and gave an approximately 100-fold increase in styrene epoxide production, in comparison to earlier studies. We anticipate that our results might lead to the establishment of new redox chains, as had already been proposed by Puch-

kaev et al.,[15] but based on the incorrect assignment to *S. solfataricus*. Such a setup might additionally enhance the stability of the enzyme, because no peroxide would be needed to facilitate the reaction. Nonetheless, even the nonbiological reaction parameters developed here should open the door to a variety of applications in biotechnology, bioanalytics, and biomolecular-screening processes.

Experimental Section

Cloning, overexpression, and purification of CYP119: The gene encoding for CYP119 was amplified by PCR with use of the forward primer CYP119-UP4 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTT-**TAAGGAGG**ATAGAACC**ATG**TATGACTGGTTTAGTGAGATGAG-3′ and the reverse primer CYP119-DN4 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCTTCATTACTCTTCAACCTGACCA. The primers contained *att*B sites (underlined) to introduce the gene into the Gateway® recombinational cloning system (Invitrogen). Additionally, the forward primer contained a ribosome binding site (bold) and the start codon (bold, underlined), thus enabling the use of the cloned plasmid for in vitro translation. The PCR was carried out with Mol*Taq* (Molzym, Bremen, Germany) under standard reaction conditions according to the supplier's instructions with an annealing temperature of 52 °C and an elongation temperature of 72 °C for 45 s; 30 amplification cycles were performed.

The PCR product was purified by precipitation with PEG (30%)/$MgCl_2$ (30 mM) and cloned by recombination into the pDONR221 vector in a BP reaction, carried out by following the supplier's instructions (Invitrogen). The sequence of the resulting plasmid pENTR221-CYP119 was confirmed by sequence analysis. This so-called entry vector was then recombined with pET-DEST42 in a LR reaction to yield the expression vector pET-EXP42-CYP119, whose sequence was also confirmed by DNA sequence analysis. The plasmid pET-EXP42-CYP119 was then transformed into *E. coli* BL21(DE3) cells. Expression and purification was carried out as previously described,[10] with use of the following modifications: the overnight culture (50 mL) was used to inoculate 2YT (2.5 L). Subsequent to harvesting of the cells and their resuspension in Tris buffer (50 mM), NaCl (150 mM), imidazole (10 mM), pH 8.0, the cells were sonicated and centrifuged. The supernatant was incubated at 65 °C for 1 h with subsequent centrifugation to remove all precipitating proteins. Since the enzyme was hexa-His-tagged by expression from pET-DEST42, the soluble fraction was passed through a Ni-NTA column and washed with Tris buffer (50 mM), NaCl (150 mM), imidazole (20 mM), pH 8.0. CYP119 was eluted from the column with Tris buffer (50 mM), imidazole (250 mM), pH 8.0. The eluted red protein solution was loaded onto a MonoQ column (Pharmacia Biotech) to facilitate further purification by anion-exchange chromatography with an ÄKTA-purifier FPLC system (Amersham Pharmacia Biotech). Bound proteins were eluted from the column by use of a linear NaCl gradient (0–500 mM), and the fractions containing protein were analyzed by SDS-PAGE. The fractions containing a protein of the size corresponding to the hexa-His-tagged CYP119 (47 kDa) were pooled. After the buffer had been changed to KP_i (50 mM), pH 7.0 with a Vivaspin (Vivascience) with a molecular cutoff of 10 kDa, the combined fractions were again analyzed by SDS-PAGE, and were judged to be pure from the appearance of a single band with an apparent molecular weight of 45 kDA. This result was confirmed by UV/Vis spectrophotometry and comparison with previously published spectra of the CYP119.[10,26] From the UV/Vis measurements the concentration of the overexpressed CYP119 was calculated with use of the molar extinction coefficient of $\varepsilon_{415} = 104\ mM^{-1}$.[26]

Recombinant $P450_{BS\beta}$ (CYP152A1) was expressed from *E. coli* M15 (pREP4) by use of the plasmid pQE-30tBSb, kindly donated by Dr. Isamu Matsunaga.[27] The enzyme containing a C-terminal hexahistidine tail was overexpressed in *E. coli* M15 and purified by affinity chromatography, as reported earlier.[24,27,28]

Peroxidase activity: For the determination of the temperature-dependent activity of the cytochrome P450 enzymes, CYP119 or CYP152A1[29] (1.25 μM) in KP_i (50 mM), pH 7.0 was incubated with AmplexRed (10 μM) and H_2O_2 (5 mM). The fluorescence measurements were recorded with a Cary Eclipse (Varian) equipped with a Peltier multicell holder. For control purposes, similar data were recorded with identical concentrations of AmplexRed and H_2O_2 with omission of the enzyme. All other kinetic measurements were carried out with the same enzyme and peroxide concentrations as listed above, and samples were analyzed with the aid of a Synergy HT microplate reader (BIO-TEK) at 25 °C. For the determination of the optimum pH the following buffers were used (at 50 mM concentrations): phosphate/citrate buffer (pH 2.5–7.0), phosphate buffer (pH 7.0–8.5), and glycine buffer (pH 8.5–10.5). In all cases, v_0 was calculated from the linear phase of substrate formation, typically recorded in the initial phase of the reaction diagram, where less than 10% of the maximum signal intensities were reached. All reactions were carried out at least as triplicate independent measurements. For the calculation of the relative activities (Figures 4 and 5), the activity of the reaction mixture containing CYP119 (1.25 μM), H_2O_2 (5 mM), AmplexRed (10 μM), KP_i (50 mM), pH 7.0 at 25 °C was set to 100%. In the case shown in Figure 3 the activity of the reaction containing CYP119 (1.25 μM), H_2O_2 (5 mM), AmplexRed (10 μM), KP_i (50 mM), pH 7.0 at 75 °C was set to 100%.

Styrene epoxidation: Styrene epoxidation experiments were carried out in closed glass vials in total volumes of 400 μL containing CYP119 (1.25 μM), TBHP (5 mM), and variable styrene concentrations in a glycine buffer (50 mM), pH 8.5 at 70 °C. At different time points, aliquots (40 μL) were withdrawn, and the reaction was stopped by addition of acetonitrile (360 μL). This mixture (100 μL) was analyzed by HPLC on an Agilent 1100 system. A Nucleodur C18 column (Macherey–Nagel) was used, with ddH_2O (20%)/acetonitrile (80%) as the mobile phase. Styrene and styrene epoxide were identified by comparison of the retention times with those of the pure substances (styrene from Acros, styrene epoxide from Aldrich) under the same conditions. The retention times found with this setup were 2.65 min for styrene and 1.8 min for styrene epoxide. The absolute concentration of styrene epoxide was determined by use of an external calibration curve prepared from pure styrene epoxide and integration of the respective peak areas.

For the determination of the enantioselectivity, epoxidation reactions were performed as described above with a styrene-saturated buffer (nominal concentration of styrene was 30 mM, but not fully soluble at 70 °C). After 10 min the reaction was stopped by the addition of *n*-hexane (200 μL). After vigorous mixing of the two phases, the *n*-hexane phase was withdrawn and a portion (10 μL) was analyzed by HPLC with the aid of an Agilent 1100 system. A Chiralpak AD column (Daicel) was used, with *n*-hexane/isopropanol (100:1) as the mobile phase. (*R*)-Styrene epoxide, (*S*)-styrene epoxide, and styrene were identified by comparison of the retention times with those of the pure substances (all three from Aldrich) under the same conditions. The retention times found with this setup were 11.7 min for the (*R*)- and 13 min for the (*S*)-styrene epoxide enantiomer. All measurements were carried out in triplicate.

Acknowledgements

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Keywords: cytochromes · epoxidation · monooxygenases · *Sulfolobus acidocaldarius* · thermostability

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