

# Spectroscopic studies on the active site of hydroperoxide lyase; the influence of detergents on its conformation

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**Abstract** Expression of high quantities of alfalfa hydroperoxide lyase in *Escherichia coli* made it possible to study its active site and structure in more detail. Circular dichroism (CD) spectra showed that hydroperoxide lyase consists for about 75% of  $\alpha$ -helices. Electron paramagnetic resonance (EPR) spectra confirmed its classification as a cytochrome P450 enzyme. The positive influence of detergents on the enzyme activity is paralleled by a spin state transition of the heme Fe(III) from low to high spin. EPR and CD spectra showed that detergents induce a subtle conformational change, which might result in improved substrate binding. Because hydroperoxide lyase is thought to be a membrane bound protein and detergents mimic a membrane environment, the more active, high spin form likely represents the *in vivo* conformation. Furthermore, the spin state appeared to be temperature-dependent, with the low spin state favored at low temperature. Point mutants of the highly conserved cysteine in domain D indicated that this residue might be involved in heme binding. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

**Key words:** Allene oxide synthase; Cytochrome P450; Electron paramagnetic resonance; Fatty acid hydroperoxide lyase; Spin state of Fe(III)

## 1. Introduction

Plants continuously fight against life-threatening events such as drought, mechanical damage, temperature stress, and potential pathogens. The most important signal pathway induced by wounding is the octadecanoid or lipoxygenase (LOX) pathway. The first step in the LOX pathway is the dioxygenation of linolenic or linoleic acid by LOX [1]. The formed hydroperoxy fatty acids are further metabolized by enzymes like allene oxide synthase (AOS, CYP74A), peroxxygenase, divinyl ether synthase or hydroperoxide lyase (HPO lyase, CYP74B). HPO lyase cleaves the C–C bond adjacent to the hydroperoxy group, resulting in the formation of  $\omega$ -oxo acids and volatile aldehydes. The (3Z)-aldehydes can be isomerized to their (2E)-isomers and both can be reduced by alcohol dehydrogenase to their corresponding alcohols. The

volatile aldehydes and alcohols, which have a characteristic ‘green, fruity’ smell, are involved in wound healing and pest resistance [2–5], whereas 12-oxo-(10E)-dodecenoic acid (traumatoin) is considered to be a wound hormone [6].

HPO lyases have been purified from a number of organisms, and recently HPO lyases from bell pepper, *Arabidopsis thaliana*, tomato, alfalfa and guava fruit were cloned and expressed in *Escherichia coli* [7–11]. Based on its sequence homology, HPO lyase is supposed to belong to the class of cytochrome P450 enzymes [7]. Similar to other cytochrome P450 enzymes, the heme group in the active site of HPO lyase was identified as heme *b* (protoheme IX) [10,12], and is probably bound by a highly conserved cysteine [7–10]. But in contrast to other cytochrome P450 enzymes, HPO lyases do not show the characteristic absorption maximum at 450 nm after reduction and treatment with CO [12,13]. This behavior is similar to AOSs that also have a low affinity for CO [14]. Furthermore, HPO lyases and AOS show little homology to other cytochrome P450 enzymes in the I-helix region (oxygen binding pocket) and lack the conserved threonine. This can be explained by the fact that HPO lyases and AOS do not require molecular oxygen nor an NADPH-dependent cytochrome P450 reductase for their activity. These enzymes are, therefore, unique within the cytochrome P450 family.

The structure and reaction mechanism of HPO lyase are still unclear, because the amounts of HPO lyase available so far were too low for characterization studies. Unraveling the structure of the active site is a primary step towards the elucidation of the reaction mechanism of HPO lyase. Our previously described expression system [10] made it for the first time possible to obtain enough enzyme to further characterize its active site by electron paramagnetic resonance (EPR), and determine if HPO lyase indeed belongs to the class of cytochrome P450 enzymes. EPR is superior to UV/Vis spectrophotometry in providing information about heme symmetry, identifying heme ligands, and understanding the electronic structure of the heme iron. Point mutants of the conserved cysteine (C<sub>442</sub> of alfalfa HPO lyase) were constructed and studied to determine if this residue is involved in binding of the active site heme. Furthermore, the influence of detergents like Triton X-100, commonly used to solubilize cytochrome P450 enzymes, on HPO lyase was studied.

## 2. Materials and methods

### 2.1. Enzyme preparations

Alfalfa HPO lyase was expressed in *E. coli* M15 cells containing a pQE32 vector (Qiagen) with the *CYP74B4v1* gene (EMBL database,

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**Abbreviations:** AOS, allene oxide synthase; CD, circular dichroism; EPR, electron paramagnetic resonance; HPO lyase, hydroperoxide lyase; LOX, lipoxygenase

accession number AJ249245) without N-terminal sequence encoding the first 22 amino acids, and solubilized from the membrane fraction by 0.2% Triton X-100 [10]. The enzyme was purified to homogeneity by immobilized metal affinity chromatography, as described previously [10]. Triton X-100 was removed from the sample by performing the second wash step and the elution with buffer without Triton X-100.

## 2.2. Construction of HPO lyase point mutants

C<sub>442</sub>A and C<sub>442</sub>S point mutants were constructed by site-directed mutagenesis of the *CYP74B4v1* gene without the N-terminal extension of 22 amino acids. The following primers were used for PCR: N-terminal fragment: 5'-GTTCAAGCATATGGGTACCAG-3' and 5'-TACGGCCGCTGTTTATTGGACACAG-3' (C<sub>442</sub>A) or 5'-TACGGCCGACTGTTTATTGGACACAG-3' (C<sub>442</sub>S), C-terminal fragment: 5'-TACGGCCGTAAGGACATCGTG-3' and Primer-Reverse Sequencing (Qiagen). The N- and C-terminal fragments were restricted with *EagI*, *PstI* and *EagI*, *HindIII*, respectively, and cloned into pBluescript KS+ and pBluescript SK- vectors, respectively. The N-terminal fragments were then cloned into the pBluescript SK- vectors containing the C-terminal fragment, by restrictions with *AflIII* and *EagI*. The combined fragments were isolated by restriction with *PstI*. These were cloned into *PstI*-restricted and dephosphorylated pQE32 vectors (Qiagen) containing the first part of the *CYP74B4v1* gene. The sequences were checked by sequence analyses and the enzymes were expressed and purified as described [10].

## 2.3. Enzyme activity measurements

HPO lyase activity was determined in 50 mM potassium phosphate buffer pH 6.0 containing 100  $\mu$ M of substrate by measuring the decrease of the A<sub>234</sub> due to the cleavage of substrate. Protein concentrations were determined with the bicinchoninic acid method [15]. The substrates, 13-HPOD and 13-HPOT were prepared from linoleic and  $\alpha$ -linolenic acid, respectively (~99%, Fluka), with soybean LOX-1 [16].

## 2.4. Heme analyses

The heme staining procedure was adapted from Thomas et al. [17]. Heme staining of the HPO lyase point mutants was performed as described previously, with wild-type HPO lyase as a positive control [10]. Spectrophotometric analyses of purified HPO lyase were carried out with native and reduced enzyme. Reduction occurred by addition of sodium dithionite to a final concentration of 0.2% (w/v). Pyridine hemoferrochrome was prepared from the purified enzyme by addition of pyridine to a concentration of 20% (v/v) and NaOH to 0.2 M.

## 2.5. Circular dichroism (CD) and EPR analyses

CD spectra were recorded on a Jasco J-600 Spectropolarimeter. Prior to the CD measurements, the HPO lyase samples were dialyzed against 50 mM potassium phosphate buffer, pH 7.5, with or without 0.2% Triton X-100, to remove NaCl. The CD spectrum of this buffer was subtracted from the HPO lyase CD spectra. EPR spectra were recorded on a Bruker ECS-106 EPR spectrometer equipped with a 5350 B Hewlett Packard microwave frequency counter and a cryostat from Oxford Instruments. Liquid He was used to regulate the temperature of the sample. Prior to the EPR measurements, the HPO lyase samples were concentrated to 0.5 mg/ml with a 30 kDa microcon filter (Amicon/Millipore, Bedford, USA).

## 3. Results and discussion

The rhombic EPR spectrum of purified alfalfa HPO lyase in

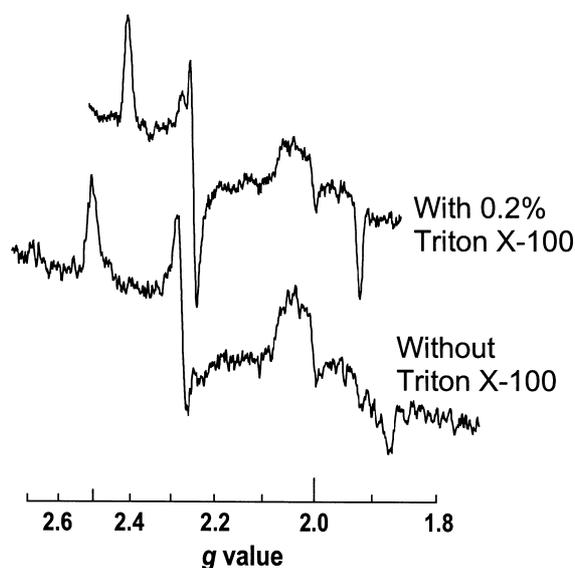


Fig. 1. EPR spectra of alfalfa HPO lyase in the presence or absence of 0.2% Triton X-100. The spectra were recorded at 45 K, 20 dB, 9424.2 MHz and 9421.1 MHz, respectively, with a modulation amplitude of 1.27 mT.  $g$  values of HPO lyase with Triton X-100:  $g_z = 2.40$ ,  $g_y = 2.24$ ,  $g_x = 1.92$ , and  $g$  values of HPO lyase in the absence of Triton X-100:  $g_z = 2.50$ ,  $g_y = 2.27$ ,  $g_x = 1.87$ .

the presence of Triton X-100 at 45 K (Fig. 1) is typical for a heme Fe(III) in the low spin state, bound to an axial thiolate anion (from cysteine) and an OH-group [18–20]. In the EPR spectrum of HPO lyase recorded at 7.3 K, no high spin Fe(III) signal could be detected in the  $g = 8$  region. The  $g$  values,  $g_z = 2.40$ ,  $g_y = 2.24$  and  $g_x = 1.92$  are characteristic for cytochrome P450 enzymes and are clearly distinguishable from the  $g$  values of other heme proteins [18,19]. The EPR spectrum confirms that HPO lyase belongs to the cytochrome P450 class. UV/Vis spectra of HPO lyases and AOS recorded at room temperature indicated that these enzymes contain Fe(III) in the high spin state [10,12,13,21]. This points to temperature dependency of the spin state. The high spin state seems to be favored at room temperature, whereas at the low temperature of the EPR measurements the low spin form is favored. The high spin state was not detectable with EPR at higher temperatures due to fast electron-spin relaxation. Temperature dependency of the spin state of cytochrome P450 enzymes has been observed before and is due to the fact that transitions between the spin states are accompanied by slight energy alterations [19,22].

All HPO lyases and AOS described were solubilized by the use of detergents, mostly Triton X-100. Removal of Triton X-100 leads to a remarkable change in the spectral properties of HPO lyase (Table 1). The Soret maximum shifted from 390 to 418 nm, which indicates that the spin equilibrium moves

Table 1  
Absorption maxima of HPO lyase with or without 0.2% Triton X-100

HPO lyase	+0.2% Triton X-100	-Triton X-100
Native	390 nm	418 nm
Reduced	416 nm	420 nm
Pyridine hemoferrochrome	418 nm	390 nm
Pyridine hemoferrochrome reduced	390 nm	390 nm

Reduction occurred by addition of sodium dithionite to a final concentration of 0.2% (w/v). Pyridine hemoferrochrome was prepared by addition of pyridine to a concentration of 20% (v/v) and NaOH to 0.2 M.

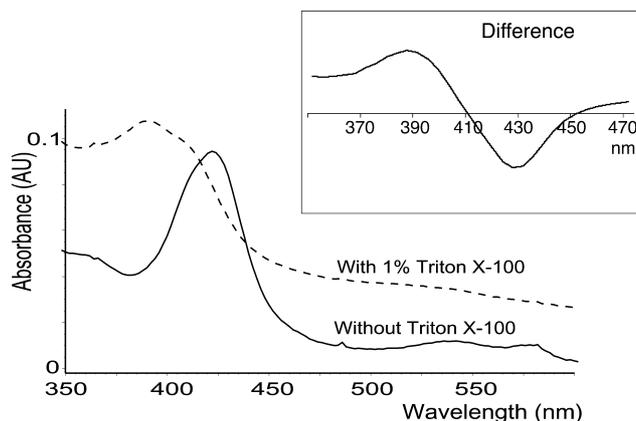


Fig. 2. UV/Vis spectra of alfalfa HPO lyase before and after addition of 1% Triton X-100. The difference spectrum was obtained by subtraction of the spectrum without Triton X-100 from the spectrum of HPO lyase with Triton X-100.

from high towards low spin. By readdition of Triton X-100, the equilibrium shifted back towards the high spin state (Fig. 2). This change in the spin state could also be induced by addition of the detergent *n*-octyl- $\beta$ -D-glucopyranoside (1%), and was thus not specific for Triton X-100. Addition of a mixture of triglycerides to low spin HPO lyase slightly moved the spin equilibrium towards the high spin state as well. The EPR spectrum of Triton-free HPO lyase showed *g* values different from the ones of HPO lyase in the presence of Triton X-100 ( $g_z = 2.50$ ,  $g_y = 2.27$  and  $g_x = 1.87$ ) (Fig. 1). This indicates that Triton X-100 affects the conformation of the heme site in the enzyme. Previously, we observed that the His-tag of recombinant HPO lyase was only exposed in the presence of Triton X-100, which also indicates that Triton X-100 causes a change in the structure of the enzyme [10].

CD spectra of HPO lyase in the presence or absence of 0.2% Triton X-100 showed no obvious structural differences between the two enzyme states (Fig. 3). This indicates that the structure perturbation caused by Triton X-100 is apparently subtle. The CD spectra also indicate that HPO lyase consists for about 75% of  $\alpha$ -helices, which is similar to previously described structures of prokaryotic cytochrome P450 enzymes [23–25].

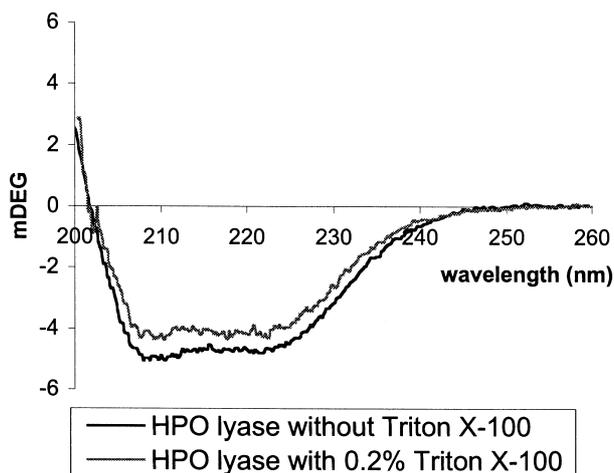


Fig. 3. CD spectra of alfalfa HPO lyase in the presence or absence of 0.2% Triton X-100.

The effect of detergents on the spin state of HPO lyase is similar to a type I interaction. Most cytochrome P450 enzymes are in the low spin state and can be shifted towards high spin by addition of a hydrophobic substrate or a ligand (type I interaction) [26–29]. A substrate molecule probably displaces the water molecule at the sixth, axial, ligand position of the heme iron. This might lead to the movement of iron out of the plane of the porphyrin ring, which makes the high spin configuration favorable [30,31]. Because Triton X-100 is too large to fit into the distal ligand pocket, it can not directly interact with the iron. The different EPR spectra of HPO lyase in the presence or absence of Triton X-100, however, indicate that Triton X-100 does change the conformation of the active site. Detergents probably induce the formation of a high spin complex by indirectly perturbing the heme structure and excluding the original axial heme ligand ( $H_2O$ ), or change the bond length between the heme iron and the sulfur atom. A similar effect was observed when the cytochrome P450 nitric oxide synthase was incubated with a number of large ligands [29,32]. The influence of detergents on the spin state might be typical for enzymes of the CYP74 family, because the cytochrome P450 obtusifoliiol 14 $\alpha$ -demethylase, which was also solubilized by Triton X-100, was still in the low spin state [33].

Interestingly, the enzyme activity of HPO lyase decreased by about 50% due to the removal of Triton X-100 and subsequent shift in the spin state. Previously, it was also observed that the activity of HPO lyase and AOS in crude extracts increased about 2-fold by addition of Triton X-100 or other detergents such as polyvinylpyrrolidone or the non-ionic Emulgen 911 [21,34–36]. This suggests that the spin state of HPO lyase is important for the enzymatic activity of HPO lyase. The functional importance of the spin state of cytochrome P450 enzymes is not yet understood. It has been suggested that the high spin form is more rapidly reduced [37], but on the other hand, the reduction is mostly not the rate-determining step. The spin state of endothelial nitric oxide synthase, which appeared to be dependent on the source of the enzyme, showed no correlation with the enzyme activity [29]. In the case of HPO lyase, the increased activity upon transition from low to high spin state might be caused by a lower binding enthalpy and facilitated substrate binding. Be-

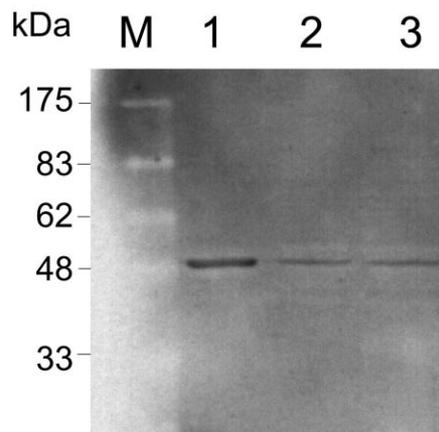


Fig. 4. Western blot of a 10% polyacrylamide SDS-PAGE gel. Lane 1: purified wild-type HPO lyase, lane 2:  $C_{442}A$  mutant, and lane 3:  $C_{442}S$  point mutant, M: molecular weight marker. Detection was performed with Ni-NTA horseradish peroxidase conjugate (Qiagen), as described by the manufacturer.

cause the sixth ligand position in the high spin state is vacant, the heme group might be better accessible for the substrate.

Detergents might mimic a membrane environment. Because HPO lyase is thought to be a membrane protein, it is likely that the more active, high spin state is the *in vivo* conformation of the enzyme. The ability of triglycerides to induce a shift in the spin state as well confirms the essence of a membrane-like environment. It is also possible that HPO lyase activity is partly regulated by the organization of the membrane around the enzyme, as has been suggested before [35].

C<sub>442</sub>A and C<sub>442</sub>S point mutants of alfalfa HPO lyase were constructed, expressed in *E. coli* and purified by immobilized metal affinity chromatography (Fig. 4). The cysteine point mutants appeared to be inactive and no coloring was observed upon heme staining, indicating that these mutants do not contain heme. These results suggest that the highly conserved cysteine (C<sub>442</sub> of alfalfa HPO lyase) is involved in binding of the active site heme.

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