Archives of Biochemistry and Biophysics 514 (2011) 33–43

Contents lists available at SciVerse ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

Regioselective oxygenation of fatty acids, fatty alcohols and other aliphatic compounds by a basidiomycete heme-thiolate peroxidase

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ARTICLE INFO

Article history:
Received 15 June 2011
and in revised form 4 August 2011
Available online 16 August 2011

Keywords:
Peroxidase/peroxygenase
Hydroxylation
Fatty acids
Fatty alcohols
Alkanes
Steroids

ABSTRACT

Reaction of fatty acids, fatty alcohols, alkanes, sterols, sterol esters and triglycerides with the so-called aromatic peroxygenase from *Agrocybe aegerita* was investigated using GC–MS. Regioselective hydroxylation of C_{12}–C_{20} saturated/unsaturated fatty acids was observed at the ω−1 and ω−2 positions (except myristoleic acid only forming the ω−2 derivative). Minor hydroxylation at ω and ω−3 to ω−5 positions was also observed. Further oxidized products were detected, including keto, dihydroxylated, keto-hydroxy and dicarboxylic fatty acids. Fatty alcohols also yielded hydroxy or keto derivatives of the corresponding fatty acid. Finally, alkanes gave, in addition to alcohols at positions 2 or 3, dihydroxylated derivatives at both sides of the molecule; and sterols showed side-chain hydroxylation. No derivatives were found for fatty acids esterified with sterols or forming triglycerides, but methyl esters were ω−1 or ω−2 hydroxylated. Reactions using $H_2{}^{18}O_2$ established that peroxide is the source of the oxygen introduced in aliphatic hydroxylations. These studies also indicated that oxidation of alcohols to carbonyl and carboxyl groups is produced by successive hydroxylations combined with one dehydration step. We conclude that the *A. aegerita* peroxygenase not only oxidizes aromatic compounds but also catalyzes the stepwise oxidation of aliphatic compounds by hydrogen peroxide, with different hydroxylated intermediates.

Introduction

Recently, a new peroxidase type was discovered in the wood-rotting basidiomycete *Agrocybe aegerita* (in order Agaricales, family Bolbitaceae), which turned out to be a true peroxygenase efficiently transferring oxygen from peroxide to various organic substrates including aromatic compounds, among others [1]. The enzyme was first reported as a haloperoxidase [2], related to the chloroperoxidase of *Leptoxyphium fumago* [3] being able to oxidize non-phenolic aromatic compounds. However, due to its unique ability to epoxidize and hydroxylate aromatic rings by means of hydrogen peroxide, and its low halogenase activity, the enzyme is nowadays mostly referred to as an aromatic peroxygenase [4].

This peroxidase/peroxygenase is able to catalyze reactions formerly assigned to intracellular cytochrome P450 monooxygenases (P450s) [5]. However, unlike P450s, which are intracellular enzymes whose activation requires NAD(P)H as electron donor and auxiliary flavin-reductases, or a second flavin domain, for electron transfer [6], the *A. aegerita* enzyme is a secreted protein, therefore far more stable, and only requires H_2O_2 for function [7]. This peroxidase/peroxygenase combines unique capabilities of P450s such as oxygen transfer, and classic properties of peroxidases such as oxidation of phenolic compounds, but its sequence exhibits no homology to classic peroxidases and P450s, and only little homology (∼30%) to ascomycete chloroperoxidase [8]. However, this sequence includes the conserved cysteine residue acting as the fifth heme iron ligand in the two latter enzymes and is, therefore, classified as a heme-thiolate peroxidase [4,9].

The physiological function of *A. aegerita* peroxygenase remains unclear, but its extracellular location and the versatile reactions catalyzed – including peroxygenase, etherase and one-electron abstraction activities, among others – indicate that it could be involved in the unspecific oxidation and detoxification of plant (e.g., methoxylated phytoalexins) or microbial metabolites and also in the degradation of methoxylated compounds deriving from lignin and other aromatic plant sources [4]. In the latter context, it is interesting that the *A. aegerita* peroxygenase is able to oxidize non-phenolic veratryl alcohol, the typical substrate of ligninolytic peroxidases, in a broad pH range, while lignin peroxidase and versatile peroxidase are able to oxidize this and related aromatic compounds only under very acidic conditions (around pH 3).

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0003-9861/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.abb.2011.08.001

The *A. aegerita* peroxygenase has recently been shown to catalyze a high number of interesting oxidation reactions, including among others, the regioselective epoxidation/hydroxylation of naphthalene, the sulfoxidation of dibenzothiophene and thioanisole, the *N*-oxidation of pyridine, the *O*-dealkylation of alkyl–aryl ethers, the oxidation of aryl alcohols and aldehydes and the bromination of phenol [10–13]. Although its real biological function remains uncertain as mentioned above, the *A. aegerita* peroxygenase has an enormous biotechnological potential, since selective oxo-functionalizations are among the most challenging and desired reactions in organic synthesis and, compared with P450s, has the advantage of being a self-sufficient enzyme (i.e. catalyzing oxygenations without the help of intracellular enzymes providing electrons and reducing power) [7,14]. The authors of the current study demonstrate for the first time the action of the *A. aegerita* peroxygenase on fatty acids, fatty alcohols, alkanes and steroids, and provide information on the regioselectivity and oxidation mechanism (by detailed GC–MS analyses and ^{18}O-labeling) expanding the biotechnological interest of the enzyme by including the area of aliphatic hydroxylations and other oxygenation reactions.

Materials and methods

Enzyme preparation

The extracellular peroxygenase of *A. aegerita* (isoform II, 44 kDa) was produced and purified as described previously [2]. The enzyme preparation was homogeneous by sodium dodecylsulfate–polyacrylamide gel electrophoresis, and exhibited an A_{418}/A_{280} ratio of 1.75. Its specific activity was 117 units mg^{-1}, where 1 unit represents the oxidation of 1 μmol of veratryl alcohol to veratraldehyde (ε_{310} 9300 M^{-1} cm^{-1}) in 1 min at 23 °C and pH 7, in the presence of 2.5 mM H_2O_2. The turnover rate of the purified enzyme on veratryl alcohol was estimated as 85 s^{-1} (with a Michaelis–Menten K_m constant ~2.4 mM).

Model substrates

Twenty-four model aliphatic substrates were used including: (i) saturated fatty acids such as lauric (dodecanoic), myristic (tetradecanoic), palmitic (hexadecanoic), stearic (octadecanoic) and arachidic (eicosanoic) acids; (ii) unsaturated fatty acids such as lauroleic (*cis*-9-dodecenoic), myristoleic (*cis*-9-tetradecenoic), palmitoleic (*cis*-9-hexadecenoic), oleic (*cis*-9-octadecenoic), linoleic (*cis*,*cis*-9,12-octadecadienoic) and gondoic (*cis*-11-eicosenoic) acids; (iii) fatty alcohols such as 1-tetradecanol and 1-hexadecanol; (iv) alkanes such as dodecane, tetradecane, hexadecane and octadecane; (v) free sterols such as cholesterol and sitosterol; (vi) sterol esters such as cholesteryl butyrate, cholesteryl caprylate and cholesteryl linoleate; (vii) the triglyceride trilaurin; and (viii) the fatty acid methyl ester methyl laurate. Sitosterol was purchased from Calbiochem, and all the other model substrates were obtained from Sigma–Aldrich.

Enzymatic reactions

Five milliliters reactions of the above model substrates (1 mM) with the *A. aegerita* peroxygenase (1 U) were performed in 50 mM sodium phosphate buffer (pH 7) at 25 °C for 2 h, in the presence of 2.5 mM H_2O_2. The substrates were previously dissolved in acetone and added to the buffer (the acetone concentration in the reaction was 15%). In control experiments, substrates were treated under the same conditions (including 2.5 mM H_2O_2) but without enzyme. Enzymatic reactions with ^{18}O-labeled hydrogen peroxide ($H_2{}^{18}O_2$, 90% isotopic content) from Sigma–Aldrich (2% w:v solution) were also performed under the same conditions described above.

After the enzymatic reactions, water was immediately removed in a rotary evaporator, and the products recovered with chloroform, dried under N_2, and redissolved in chloroform for GC–MS analyses. Bis(trimethylsilyl)trifluoroacetamide (Supelco) in the presence of pyridine was used to prepare trimethylsilyl derivatives.

GC–MS analyses

The GC–MS analyses were performed with a Varian 3800 chromatograph coupled to an ion-trap detector (Varian 4000) using a medium-length fused-silica DB-5HT capillary column (12 m × 0.25 mm internal diameter, 0.1 μm film thickness) from J&W Scientific, enabling simultaneous elution of the different compound classes [15]. The oven was heated from 120 °C (1 min) to 380 °C at 10 °C min^{-1}, and held for 5 min. Other temperature program, from 50 °C to 110 °C (at 30 °C min^{-1}) and then to 320 °C (at 6 °C min^{-1}), was used when necessary. In all GC–MS analyses, the transfer line was kept at 300 °C, the injector was programmed from 120 °C (0.1 min) to 380 °C at 200 °C min^{-1} and held until the end of the analysis, and helium was used as carrier gas at a rate of 2 ml min^{-1}.

Compounds were identified by mass fragmentography, and by comparing their mass spectra with those of the Wiley and NIST libraries and standards, and quantitation was obtained from total-ion peak area, using response factors of the same or similar compounds (those of the saturated substrates and derivatives being in general higher than those of the unsaturated ones). Single-ion chromatographic profiles (of base or other specific ions) were used to estimate compound abundances when two peaks partially overlapped. The relative abundance of products incorporating 1–3 $^{18}O_2$ atoms in the $H_2{}^{18}O_2$ reactions described above was estimated by peak integration using the corresponding ion with 2, 4 or 6 *m/z* increase (with correction from interfering ions in $H_2{}^{16}O_2$ spectra, when required).

Results

Twenty-four model aliphatic substrates, including a series of saturated and unsaturated fatty acids, one fatty acid methyl ester, and several fatty alcohols, alkanes, free and esterified sterols and triglycerides were treated with the *A. aegerita* peroxygenase. All the fatty acids and fatty alcohols showed reactivity towards the enzyme. Among the alkanes, only those of shorter chain length were modified. The free sterols were only slightly modified, and the esterified sterols and triglycerides showed no reactivity. The conversion rate, and the reaction products formed were studied by GC–MS as described below.

Fatty acid oxidation studies

Eleven fatty acids with even carbon number from C_{12} to C_{20} were tested as substrates of the *A. aegerita* peroxygenase (Table 1). All the saturated fatty acids showed reactivity towards the enzyme, although at different extents depending on the chain length, the order of activity as hydroxylation substrates being $C_{12} > C_{14} > C_{16} > C_{18} > C_{20}$. Six unsaturated fatty acids with the same length as the saturated ones were also tested, all of them being more active than their saturated analogs. It should be mentioned that differences in solubility among the several substrates tested, related to chain length and presence of double bonds, could also influence substrate conversion.

For all the fatty acids, with the exception of myristoleic acid, the alkyl chains were monohydroxylated to give predominantly mixtures of the ω−1 and ω−2 isomers (Fig. 1). The position of the hydroxyl group was determined by the mass spectra of their